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Molecular Cytopathology

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Preface

As beautifully articulated by Dr. Richard Demay, cytology is a clinical practice that combines science with art. Cytology allows one to make a diagnosis based on changes of individual cells. The rapid turnaround time, minimum cost and resources needed (often just a microscope is needed), and noninvasive or minimally invasive method of obtaining diagnostic material are the major advantages for cytology. As such, morphology-based cytology has been and will likely continue to be in the forefront of clinical diagnosis and management of various human disease conditions, particularly cancer. While there are many great examples of cytology's instrumental role in patient care and clinical decision-making process, probably the most important one is cytology's contributions to cervical cancer screening. The combination of Pap smear-based diagnosis and colposcopy-based management has helped to drastically decrease cervical cancer incidence and mortality in the screened population.

However, there are indisputable limitations of a morphology-based cytology practice. Morphologic evaluation is not sufficiently capable of determining if a cell harbors the particular genetic or epigenetic changes that are the basis for targeted therapeutic drugs. In an era of precision medicine where more therapies and management schemes are geared toward specific molecular changes in disease processes, additional molecular analysis must be incorporated into a morphology-based cytological diagnostic work-up.

Fortunately, cytologic material has a distinct advantage over formalin-fixed paraffin-embedded tissue for molecular analysis,

such as single cell based next generation sequencing (NGS), quantitative multiplex protein or exosome analysis, or nanomechanical profile analysis. The advantage of cytologic material is that the cells are usually complete whole cells, rather than sections of cells. This in turn enables the precise quantitative determination of the biochemical or molecular changes occurring within a cell. With the advances of techniques such as NGS, microfluidic devices, and nanotechnology, this advantage will likely become more and more significant.

Molecular cytopathology is still in its infancy. This book is not intended to be inclusive of all the progress or publications in the field of molecular cytology to date, but rather to provide a reference or background that may help residents, fellows, cytotechnologists, and cytopathologists who are interested in molecular testing in cytologic specimens. In view of the rapid progress in this area, periodic updates will be necessary to reflect the most current developments.

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1 Development and Validation of Molecular Testing on Cytologic Specimens

Shengle Zhang and Bin Yang

Clinical Utility

Molecular genetic analyses have been increasingly performed on cytologic specimens to facilitate management of cancer patients. Before developing and validating a molecular assay for clinical utility, it is important to evaluate if the assay will significantly change the patient management, e.g., its impacts in diagnosis, risk assessment, prognosis and prediction of therapeutic response. Gene fusions or rearrangements associated with chromosome translocations in neoplasm, mostly in lymphomas and soft tissue tumors, are useful biomarkers for purpose of diagnosis owing to their higher frequency and specificity. For example, detections of gene fusions of *BCR-ABL1* and *EWS-FLI1* have been used for diagnosis and minimal disease monitoring of CML and diagnosis for Ewing's tumor, respectively. Genetic alterations in epithelial or neuroepithelial neoplasms, mostly point mutation, insertion/deletion and amplification, are usually not applied for purpose of

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diagnosis due to their lower frequency (<50 %) and lack of organ/tissue specificity, but they are successfully applied for prediction to therapeutic response and prognosis. For example, N-myc gene amplification and 1p/19q deletions have been used for prognosis/risk assessment of neuroblastoma and oligodendroglioma respectively, and *EGFR*, *KRAS*, and *BRAF* mutations for prediction of response to biomarker-driven (targeted) therapies for lung adenocarcinoma, colon adenocarcinoma and melanoma respectively. Selection and decision of a molecular assay may be affected by many factors, such as official clinical guidelines for patient management including CAP and NCCA guidelines, availability of FDA-approved companion molecular assays for targeted therapy, requests by clinicians for a specific gene or disease, and reimbursable molecular-based assays by insurance company.

Technical Feasibility

Cytologic specimen may contains less amount of target cells compared with formalin-fixed paraffin-embeded (FFPE) surgical specimen. However, cytologic specimens, especially those obtained through fine needle aspiration, are often more suitable for molecular assays due to the high quality nucleic acids by non-formalin fixation and less fragmented genome.

Selection of Molecular Methods

In addition to considering clinical utility as initial step, several factors should be considered before conducting validation testing of a molecular assay. They include: (1) types of genetic alteration, such as amplification, mutation, indels, and gene fusion; (2) clinical sensitivity and specificity; (3) accuracy, precision and detection of low limit; (4) simplicity, associated with shorter turn-around time and lower cost; (5) availability of tissue type, such as fresh tissue, FFPE or cytologic specimen; (6) clinical volume and cost effective issue.

Common genetic alterations in neoplasm include point mutation, indels, gene fusion, amplification, aneuploidy/polysomy and abnormal methylation. Commonly used molecular assays in clinical lab are polymer chain reaction (PCR), reverse transcriptional PCR (RT-PCR), Florescence in situ hybridization (FISH) and conventional (Sanger) DNA sequencing. Recently new highthrough put molecular technologies, such as DNA/RNA microarray, Sequenom's MassARRAY system and next generation sequencing (NGS) have been introduced and increasingly used in clinical laboratories. In addition, conventional cytogenetic lab is employing more and more new molecular technology, such as FISH and microarray comparative genomic hybridization Testing (array-CGH).

PCR-based assays are suitable for detection of point mutation, small indels, gene fusions (RT-PCR), amplification, and methylation. PCR product (amplicon) is also the first step in harvesting targeted DNA fragment for performing DNA sequencing. FISH assays can be used for detection of gene amplification, indels, gene break-apart (surrogate test for gene fusion), and aneuploidy. Sequenom's MassARRAY and next generation sequencing (NGS) are powerful technologies and can be used to detect almost all types of genetic alterations. Table 1.1 summarizes the selection of molecular methods for detection of various genetic alterations (see Fig. 1.1).

Tissue Specimen Type and Cellularity

Another important aspect of setting up molecular test is to select tissue type. Liquid-based cytologic materials are generally good source for both DNA and RNA isolation. Formalin fixed paraffin embedded tissue (FFPE), either needle biopsy or cytologic cell blocks, are generally good for DNA but suboptimal for RNA duo to its degradation during the tissue store and process. However, FFPE tissues frequently are only source available for molecular testing in practice. In this situation, proper primer design for PCR or RT-PCR with small amplicon, usually < 200 bp, is required. In contrast, larger amplicons can be applied with not much difficulty

Table 1.1 Selection of molecular techniques for detection of genetic alterations

Molecular targets	Cytogenetic analysis (metaphase)	PCR (DNA)	RT-PCR (RNA)	FISH	DNA microarray	RNA microarray	Sanger's DNA sequencer	Sequenom MassARRAY	Next generation DNA sequencer
DNA									
Deletion/insertion	X	X		X	X		X	X	X
Amplification	X	X		X	X			X	X
Point mutation/SNP		X			X		X	X	X
Gene fusion (translocation)	X	X	X	X	X		X	X	X
Aneuploidy/polysomy	X	X		X	X				X
Hypermethylation		X						X	X
Genome		X			X		X	X	X
RNA									
mRNA expression			X	X		X		X	X
Gene fusion (translocation)			X	X		X	X	X	X
miRNA/siRNA			X			X		X	X
Transcriptome			X			X	X	X	X

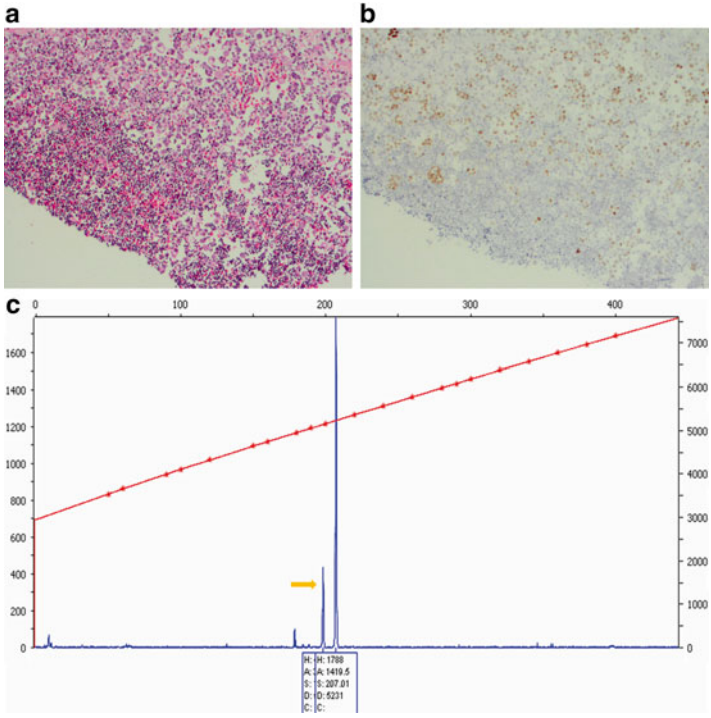

Fig. 1.1 PCR assay for *EGFR* mutation on cytologic cell block of plural fluid. (**a**) section of cell block of plural fluid with lung adenocarcinoma and inflammatory cells, H&E stain; (**b**) immunostain for TTF-1 highlights the cells of lung adenocarcinoma, making it easier to be isolated by microdissection under microscopy; (**c**) PCR product of *EGFR* exon 19 by capillary electrophoreses (Genetic Analyzer 310), showing a 12 bp deletion (*arrow*)

for most enthanol or methanol-fixed cytologic specimens. Cellularity in a specimen is another important factor to considerate, particularly, for testing in cytologic specimens. When assessment of specimen cellularity, there are three factors need to be evaluated before applying to molecular testing: overall cellularity, tumor cell percentage and heterozygosity of a genetic alteration. The overall cellularity is related to the minimal input demanded by a specific molecular method or device. The minimal percentage of targeted tumor cells in a cytologic specimen is determined by the analytical sensitivity of a specific molecular method or device. For example,

in a recent study of analyzing *BRAF* mutation in thyroid FNA specimen, it demonstrated that although a moderate cellularity is needed for generating enough DNA yields for analyzing *BRAF* mutation, 20 % or more tumor cells in thyroid FNA specimens are required for Sanger-based sequencing using ABI device while 5 % of tumor cells is suitable for allelic-specific PCR. The heterozygosity of a specific genetic alteration has also impact on the minimal input of DNA needed for a molecular testing. For example, when using the same device assuming with the same analytical sensitivity to detect two different point mutations, the one with mution in only one allele (heterozygosity) will demand twice more DNA amount/tumor cellularity than that with mutations in both alleles (homozygosity). Given the wide spectrum of analytical sensitivity of each device and method, there is no universal minimal cellularity but just a reference range for any molecular testing. Each laboratory has to test and validate the minimal cellularity and tumor cell percentage for any specific genetic or epigenetic alteration tested and any technical platform utilized in that laboratory. Overall, based on most current publications for reliable and reproduciable testing, >20 % tumor cellularity are required for conventional Sanger-based sequencing, >10 % tumor cellularity are sufficient for most PCR-based assays and >5 % tumor cellularity for next gene sequencing and other more advanced high throughput technical platforms.

In samples with low tumor cellularity, target cell enrichment techniques can be employed pre-analytically or analytically. Pre-analytically, either laser or manual microdissection can be used to enrich % tumor cells and reduce normal tissue component. Manual microdissection with needle or blade, however, works well and can be more practical, efficient and cost-effective than the laser-capture method. Analytically, one may design a nested PCR approach to enrich targeted DNA fragment and sequences to increase analytical sensitivity. For epigenetic testing, using primers targeted for methylated and unmethylated can also specifically distinct promoter methylation in malignant cells from noise of most unmethylated alleles seen in reactive or inflammatory cell background.

Tumor cellularity in FISH-based assay is less critical since FISH is morphology-based and easier to identify target cells among background cells, such as lymphocytes and fibroblasts.

FISH based assay on interphases analysis has been traditionally performed on FFPE tissue, either small needle biopsy or cytologic cell blocks. Recently, it has been shown that cytologic smear with either Papanicolaou stained or diff-quik stained slides can be used for FISH analysis as well after brief destain in acid/alcohol solution (0.5 % HCL in 50 % alcohol, 20 s) or without any destain. For liquid-based slides, membrane-based Thinprep slide is superior to precipitation-based Surepath slide due to its flat single layer cell distribution in Thinprep slides. Truncation of FFPE tissue in FISH could cause signal's artifacts and should be cautions during evaluation. FISH based assay can be performed on smear or thin-prep slides, and even better than FFPE tissue since there is no tissue truncation present. Cytologic smear with diff-quik stain can be used for FISH analysis as well after a brief destain in acid/alcohol solution (0.5 % HCL in 50 % alcohol, 30 s) (see Fig. 1.2 for example). However, H&E stained slides are not suitable for FISH test due to eosin's autofluorescence. Smears and Thinprep slides are common cytologic specimen, and are good source for DNA/RNA isolation too. Since tumor cell population on smear tends to be clustered and separated from adjacent connective tissue, and is therefore easier to be isolated (see Fig. 1.3 for example). In addition, the smears are usually fixed in 90 % alcohol or air dry

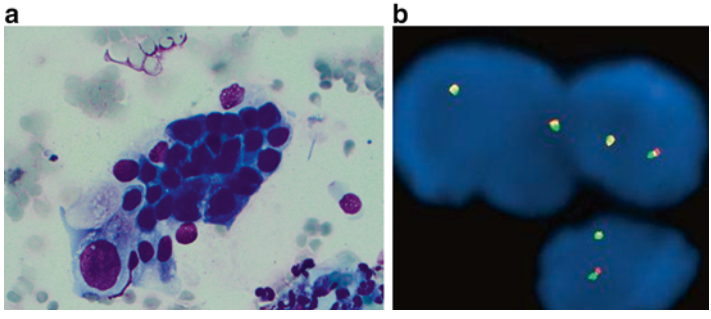

Fig. 1.2 FISH assay for *ALK* rearrangement on smear slides. (**a**) Smear of fine needle aspiration from a lymph node with metastatic lung adenocarcinoma, diff-quick stain; (**b**) FISH with *ALK* probe on the tumor cells after a brief destain, showing bright FISH signals (negative result)

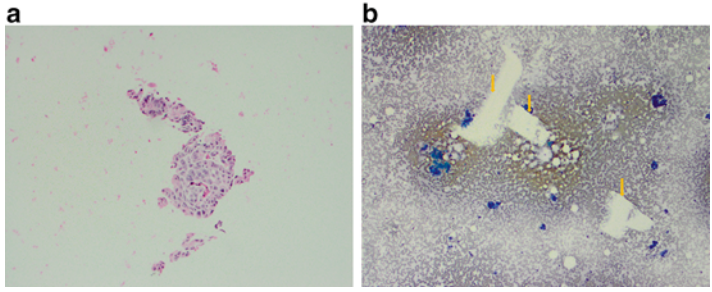

Fig. 1.3 Clusters of target cells in cytologic specimen. (**a**) Isolated tumor cell cluster in FNA cell block. FNA of lymph node with metastatic colon cancer, showing tumor cell group and clean background. A *KRAS* mutation (G12C) was identified (data not shown); (**b**) clusters of metastatic lung adenocarcinoma in cytologic smear from a lymph node FNA, some of them are collected by a needle tip under microscopy (*arrows*). A *EGFR* gene mutation (exon 19 deletion) was identified (data not shown)

immediately after biopsy/aspirate, DNA/RNA are preserved well in general. PCR-based assays usually require minimal 200 tumor cells with at least 10 % tumor cellularity for DNA/RNA isolation. DNA can be isolated from previous H&E- or IHC-stained cells for PCR-based assay.

Guidelines for Validation of Clinical Molecular Genetic Assays

CLIA and CAP require that laboratories validate the performance of tests before clinical application. Federal and state governmental agencies, such as NY State Department of Health (NYSDOH), and professional organizations, such as Collage of American Pathologists (CAP), Clinical Laboratory Standards Institute (CLSI), American College of Medical Genetics (ACMG) and Association for Molecular Pathology (AMP), have issued a variety of guidelines or standards for validating tests for clinical use. Several publications provide overall guideline or recommendation for validation of molecular assays. In general, validation for laboratory developed test (LDT) includes pre-analytic phase, such as

specimen stability, transport and storage condition; analytic phase, such as accuracy, precision, report range, sensitivity, and specificity; and post-analytic phase, such as cutoff value and data interpretation. Molecular Diagnostic Assay Validation by AMP clinical practice committee and Jennings et al. publication (2009) are commonly used guidelines for LDT validation. Although next generation DNA sequencing is a new technology, its validation guideline/standard is now available.

Analytical Validation

Accuracy

Accuracy means the amount of agreement between the test value under evaluation and the reference standard. For assay validation, elements of accuracy that should be addressed include sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Accuracy equals true positive + true negative/total population. For an accurate result, the sequence of primers should be carefully checked with a reliable DNA database, such as National Center for Biotechnology Information (NCBI) and International Nucleotide Sequence Databases (INSD) before initiate the assay validation. During validation, the results of the PCR assay should be confirmed with another type of molecular tests such as cytogenetics, FISH, direct DNA sequencing, or PCR with different primer design. For example, DNA sequencer platform was used to verify the accuracy of PCR based assay on *EGFR* mutations.

Analytical Sensitivity

Analytical sensitivity means the ability of a test to detect a disease or mutation when that disease or mutation is present. Sensitivity = true positive/(true positive + false negative). It has been called "clinical sensitivity" if the reference standard is a disease condition. A reference standard, which is equal or very close to real

target value, should be selected. Frequently, Sanger DNA sequencing or a previous validated PCR assay is used as a reference standard. In addition, the concept of analytical sensitivity has been used as "lower limit of detection" in some assay's validation.

For validation of PCR-based assay, minimal amount of DNA or RNA input may need to be determined. Different types of PCR assay require different amount of DNA/RNA input, ranged from 0.1 to 5 ng per reaction. However, amount of PCR product (amplicon) is not well correlated with input DNA/RNA, partially duo to interfering materials in DNA/RNA samples. Therefore, quantitation of input DNA/RNA is often unnecessary in practice. In fact, enumeration of cells for nucleotide isolation under microscopy is easier and more cost-effective to estimate the DNA/RNA yield. Generally, 200 cells are sufficient for most of PCR or RT-PCR assays, which contain around 1.2 ng DNA (~6 pg/cell) and 4.0 ng RNA (~20 pg/cell). Except for total amount of DNA, % mutant DNA in total DNA is another important factor to considerate. Since tumor cells are always in a background of normal cells such as inflammatory or stromal cells, the genetic alteration with low % of tumor cells may not be detected. As mentioned earlier, different molecular methods/techniques may have different analytic sensitivity. Therefore it is imperative to test and validate in each laboratory and know the testing sensitivity in your own laboratory.

FISH assay has been used to detect gene amplification (e.g., *HER2*, *EGFR*), deletion (e.g., 1p/19q deletion), and gene fusion associated chromosomal translocation (e.g., *SS18, DDIT3, EWSR*). Generally, test sensitivity of FISH-based assay for gene amplification and deletion is compatible with that of PCR-based assay. However, for detection of gene fusions with break-apart FISH probe, the sensitivity is usually higher than PCR based assay duo to the limitation of PCR primer design to cover all variants of gene fusion. For example, FISH with *DDIT3* break-apart probe can detect more *DDIT3* associated gene fusion in myxoid liposarcoma than that by PCR-based assay. FISH is a morphology-based test, in which the target tumor cells can be selectively evaluated even though they are in a small population or low tumor cellularity.

Analytical Specificity

Analytical specificity means ability of a test to give a normal (negative) result in specimens without the mutation or disease. Specificity = True negative/(True negative + False positive). This calculation is also called "clinical specificity" if the reference standard is a disease condition. This concept also refers to the ability of a test to detect the analyte without cross-reaction with other substances containing similar molecular structure.

To set up a PCR-based assay with sound specificity, primers and probes (if real-time PCR) should be carefully designed using a reliable DNA data base and make sure that their sequences have minimal similarity to other non-targeting sequence. The factors that affect specificity also include annealing temperature and Mg^{2+} concentration. Usually, a "gold standard" reference test is used to confirm the specificity, by either a previously validated PCR assay or direct DNA sequencing. By direct sequencing, however, % target mutant in the sample should be above the "low limit of detection," usually 20 %. Analytic specificity should reach or close to 100 % for clinical assay. Any case with "inconsistence" should be investigated for explanation and troubleshooting.

For FISH assay, when using either homemade or commercial FISH probes (ASR), metaphase analysis with the probes should be performed on normal human cells such as lymphocytes. The probe should hybridize to the targeted region or band without cross-hybridization to any other chromosomes. Number of samples and nuclei needed for assay validation should follow relevant guideline. For evaluation of probe's specificity and sensitivity based on NYSDOH guideline, for instance, the FISH probe on metaphase should be examined on at least five samples with 20 nuclei for each (see Table 1.2 for example). The interpretation of FISH assay in validation should be compared with that from a reference lab on selected samples. Alternatively, a second molecular method, such as PCR-based, done in your own lab can be used to demonstrate the accuracy. If there is any discrepancy with that from reference lab or other method, the reason should be searched for troubleshooting.

Table 1.2 Sensitivity and specificity of ROS-1 break-apart probes on metaphase of normal male lymphocytes[a]

Samples	Number of metaphase analyzed	FISH signals present at 6q22 region (%)		FISH signals present at non-6q22 region
		Green (3′) (%)	Orange (5′) (%)	
1	20	100	100	Not identified
2	20	100	100	Not identified
3	20	100	100	Not identified
4	20	100	100	Not identified
5	20	100	100	Not identified

[a]Based on the results, sensitivity and specificity of ROS-1 probes on metaphase analysis are both 100 %

Cutoff Value

For qualitative PCR assay, setting a cutoff value may not be necessary, in which the results, either positive or negative, are simply based on presence or absence of the expected PCR products. However, for a quantitative assay, setting a cutoff value is essential. In real-time PCR validation, cutoff value of end-point fluorescence (EPF) could be generated by testing 20 cases of normal tissue and calculating the mean + 2.58 SD (99 % confidence). Cutoff value of % break-apart in FISH assay can be obtained by testing 20 cases of normal tissue (e.g., lymph node) and calculating mean + 3 SD. Usually, 10–15 % split signals are set as cutoff value for most FISH assays using break-apart probes, such as probes for *ALK, ROS-1, FOXO1, SS18, and DDIT3* genes.

Precision (Reproducibility)

Precision or reproducibility refers to the capability of getting the same results with repetition of the assay, which can be divided into intra-run reproducibility and inter-run reproducibility. Intra-run reproducibility is obtained by duplicates or triplicates on the same sample, while inter-run reproducibility is obtained by repeating

the test on same sample at different days. According to Oncology Molecular Validation Criteria of NYSDOH, a minimum of three negative and three positive patient/clinical samples should be assayed in at least triplicate (intra-assay) and three separate runs (inter-assay) to establish precision/reproducibility, which is applicable for either PCR and FISH based assays.

Quality Accuracy

Quality assurance refers to a comprehensive set of policies, procedures, and practices necessary to assure that laboratory's results are reliable. In general, it consists of (1) competency and training of personnel, including lab director, supervisor and technician, and their credentials or certificates; (2) sufficient facilities and well-maintained laboratory; (3) assay validation and verification according to standards and guidelines, as discussed above; (4) strictly following lab regulation and procedure manuals; (5) participate proficiency tests and/or lab inspection programs, such as from state (NYSDOH), CAP and JCAHO. If proficiency test is not available, an internal check-up with known samples should be performed, usually biannually. Several authors and professional organizations have published excellent guidelines or recommendations with details in quality assurance for molecular laboratories and genetic assays.

Suggested Reading

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Molecular Techniques and Methods Applied in Cytology

2

Gilda da Cunha Santos and Mauro Ajaj Saieg

Introduction

The recent increase in the discovery and clinical implementation of biomarkers has led to the use of a large number of diagnostic molecular assays for identification of individuals eligible for target therapies. Mutations in genes such as *EGFR*, *K-RAS*, and *BRAF* have been investigated in a large number of solid tumors, with the successful use of targeted therapies as alternatives or primary treatment for a great number of patients. Cytological samples stand as an important alternative to tissue specimens, especially in impediment clinical scenarios.

The molecular assays employed for the detection of genomic alterations depend on the alteration to be detected and the type of preparation or specimen used. PCR based methodologies are the most common used for the detection of abnormalities at gene level, such as point mutations. Abnormalities in chromosomal

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structure and number are commonly identified by conventional cytogenetics and/or fluorescence in situ hybridization (FISH). Main challenges for the application of molecular technologies in cytology comprise selecting the appropriate test for that type of specimen and validating a methodology specifically for cytology, even if the method has already been used for histological samples, with the use of negative and positive controls and whenever possible, correlation of cytological and histological results.

In the present chapter, the most common molecular tests currently used in routine clinical practice applied to cytology, the array of cytological specimens that can be potentially used for molecular analysis, and the main issues related to biorepository for cytological materials are discussed.

Polymerase Chain Reaction Based Assays

Polymerase chain reaction (PCR) based assays are the methods of choice for procurement of mutations in several neoplasms. They have well-established protocols and are relatively cheap assays and widely available.

The traditional PCR assay involves DNA denaturation, annealing, and extension, with the use of primers (sequence of nucleotides complementary to the target DNA). A forward and reverse primer flank the designated area containing the desired sequence of DNA to be amplified, and multiple copies (amplicons) of the target DNA (known sequence to verify the presence of mutations) are generated. Variation on the optimal temperatures for annealing, number of cycles and reagents involved vary depending on the primers involved. The amplified DNA can then be sequenced for the verification of any mutations. Direct sequencing is a simple technique that uses chemically modified nucleotides labeled with distinct fluorescent dyes, and the order of incorporation of these fluorescent nucleotides into the amplified DNA will appear as a sequence electropherogram. In case a mutation is present, two different overlapping peaks will be seen (from the wild-type and mutant cells). Similarly, a deletion will be seen as a "truncation" of the peak signals.

The main advantage of classic PCR followed by direct sequencing is that it is an exploratory technique per se, meaning it can not only find mutations known to be commonly present in that specific type of tumor, but also any additional mutation that might be present. It is a robust and popular technique, reproducible in laboratories worldwide. Its main limitation when used in cytological samples is its low sensitivity, usually requiring a high percentage of tumor cells.

Various alternatives to classic PCR followed by direct sequencing have been used for cytological samples, and include, among others, real time-PCR, high-resolution melting analysis (HRMA), restriction fragment length analysis, COLD (co-amplification at lower denaturation temperature)-PCR, scorpion amplification refractory mutation system (S/ARMS), and peptide nucleic acid-locked PCR (PNA-LCA PCR). Real time PCR uses oligonucleotide primers that bind specifically to flank regions of the most common mutations. It is a very sensitive technique and allows a quantification of the percentage of mutated cells, since as the reactions occur a curve shows the number of amplified DNA regions in that sample. HRMA is a rapid and cost-effective method that relies on the combination of real time PCR and evaluation of DNA melting curves to accurately detect mutations, comparing the patterns of obtained curves to preset curves from non-mutated sample. Restriction fragment length analysis, on the other hand, uses mutation-specific restriction endonucleases, and amplification is only possible in the mutated sites. Although more sensitive than conventional PCR followed by direct sequencing, these allele-specific assays only analyze mutations already known and any additional mutations possibly present are not detected. Therefore, they are mostly recommended for small or paucicellular samples where sensitivity is compromised and in clinical instances where finding of additional mutations will not change therapy or prognosis.

More recently high-throughput techniques capable of detecting multiple mutations at the same time have been employed and include multiplex mutation analysis. It consists of multiple primer sets targeting multiple genes at once, saving time and material. It is of especial interest in cytology, since limited specimens may harbor a small number of tumor cells and specific panels of genes

that optimize the use of nucleic acids can increase the usefulness of such specimens. MASS-Array spectrometry using multiplex mutation analysis with preset commercial panels, such as the Oncocarta Panel, have been successfully used in FNA specimens, with reliable results. Customized panels can also be created for analysis of multiple mutations of interest and more commercially available panels specifically for each group of tumors have been recently released. When DNA extracted from fresh cells suspensions retrieved by FNA and stored on FTA cards were used for multiplex mutation analysis, rate of satisfactory results was similar to frozen samples and higher than FFPE tissue, showing that nucleic acids obtained from cytological samples were suitable for this technology.

PCR based assays are the methods of choice for procurement of mutations in *EGFR* in lung cancer, *BRAF* in melanomas and papillary thyroid carcinomas and *KRAS* in colon cancer, for example. All sorts of cytological samples, including fresh cell suspensions, smears (stained and unstained), cytospins and FFPE cell blocks can be used to extract DNA for PCR based assays. Recent CAP and IASLC molecular testing guidelines have recommended that laboratories may use any validated *EGFR* testing method. The minimal number or percentage of tumor cells present in the sample should be established at the discretion of each laboratory at the time of validation, with strong encouragement to use more sensitive tests, with accuracy of detecting mutations in samples with as little as 10 % of tumor cells. Several studies have reported successful results in finding *EGFR* mutations in cytology samples with as little as 1 % of tumor cells, showing that the overall quality of the material is more important than the percentage of tumor cells in the sample, and that very special attention should be paid to the pre-analytical steps of cytology specimens collection and handling.

Fluorescence In Situ Hybridization (FISH)

FISH (fluorescence in situ hybridization) is a popular molecular technique used in routine diagnostic specimens, using fluorochrome-labeled sequences of nucleic acids (called probes), that

hybridize to a complementary sequence in the tested sample, identifying or quantifying the target. It was first described using cytological samples, and the most common types of probe clinically used are chromosome enumeration probes (CEP) and locus-specific identifier (LSI).

CEP probes are employed for copy number enumeration of a given chromosome. LSI probes are applied to detect unique sequences of genes and determine amplifications, deletions, and translocations. Other probes such as break-apart and fusion probes are also used for the detection of translocations.

FISH is a technique that involves the following steps: pre-hybridization, hybridization, pos-hybridization washes, nuclear DNA counterstaining anti-fade, slide examination under fluorescence microscope, and slide storage in the dark. Pre-hybridization is critical and its main objective is to prepare the cells for probe penetration and efficient hybridization. Diverse adjustments in the protocols depend on the type of sample used and will vary on different types of cytological preparations.

The limitations of the technique include its high cost due to the use of specialized equipment (fluorescence microscope) and the limited number of alterations that can be detected at the same time. Alternative techniques such as chromogenic in situ hybridization (CISH) and silver-enhanced in situ hybridization (SISH) use, instead of fluorescent probes, chromogenic and sliver probes and have the advantage of using bright-field microscopes.

Cytological samples are particularly superior to FFPE cell block sections or FFPE tissue sections for in situ hybridization. Different from using tissue specimens, the problem of nuclear truncation, which can lead to inaccuracy in signal visualization and counting, is avoided since the probe hybridizes directly to intact cells on a smear or cytospin. Air-dried unstained and Romanowsky stained smears and then destained are suitable for FISH analysis. Results for the analysis of HER-2 amplification by FISH using breast carcinoma fine needle aspirates have shown to be comparable and sometimes better than using tissue sections. The analysis of HER-2 status for introduction of therapy with trastuzumab in metastatic samples, usually sampled by FNA, have made this application even more popular. FISH has also been

successfully used in sarcomas and hematologic malignancies sampled by FNA, with demonstration of its characteristic chromosomal translocations which allows subtyping of those neoplasms. The use of FISH in cytological samples has also increased recently due to the description of the *ALK* rearrangement in lung adenocarcinomas, with good results on the use of Crizotinib for patients harboring this specific translocation.

Issues Related to Biorepository of Cytological Materials

Most biomarkers studies and original papers describing molecular methodology have been validated with the use of formalin-fixed, paraffin-embedded (FFPE) tissue blocks. For that matter, it's just natural that most of the primary studies involving the use of cytological samples for molecular analysis have relied on FFPE cell blocks. Cell blocks do have this clear advantage of being analogous to paraffin tissue blocks, with minimal need of standardization and prompt reliable results. However, since it usually involves fixation with formaldehyde and paraffin embedding, DNA from the cells tend to be more degraded and multiplex PCR analysis or reactions involving primers resulting in larger amplicons may be hampered.

An extensive array of cytological samples has been successfully used for molecular analysis even including samples classically restricted for diagnosis, such as archival stained smears. High-quality DNA could be obtained from smears more than a decade old, good enough for use in high-throughput techniques such as array-comparative genome hybridization, methylation assays, and genotyping, techniques that require high-quality intact DNA, usually obtained only from fresh cell suspensions submitted to cryopreservation. Classic PCR followed by direct sequencing can be routinely performed in DNA extracted from archived smears, with very good results in the analysis of *BRAF* mutation in melanomas and papillary thyroid carcinomas and *EGFR* mutations in lung carcinomas.

Stained smears have a clear advantage over other specimens for their availability and possibility of retrospective studies. Unstained cytospins and liquid based preparations have also been successfully used. For using archived specimens rapid coverslip removal is an important step. The "freezer method" can significantly speed up the time spent with coverslip removal from old slides.

The use of fresh cells obtained from FNA and body fluids warrants a good preservation of nucleic acids and cryopreservation of cytological samples warrants morphology and nucleic acid integrity. More important than the quantity of cells, however, is the quality of the DNA preserved, and one crucial step for ensuring quality of the stored material is proper pre-analytical handling of the specimens.

A novel option for storage of fresh cell suspensions lies on the use of DNA-preserving paper cards, such as the FTA cards. DNA is trapped in the cards and can be preserved for years, at room temperature, being an inexpensive and practical alternative to cryopreservation. Residual material from the needle rinse of fine needle aspirates stored on FTA cards yielded sufficient quantities of DNA for successful Mass ARRAY spectrometry and the quality of DNA harvested from the cards was better than to nucleic material obtained from FFPE cell blocks and similar to the quality of DNA from frozen samples. Furthermore, the cards are easy to store and transport, being an alternative source for biobanking in remote locations.

For any type of sample used, the pre-analytical control has to be accurately performed, guaranteeing the overall quality of the sample. Accurate and consistent results using all these sorts of samples for molecular analysis will rely on standardized protocols for maximizing DNA yield.

High throughput and multiplex technologies including MassArray spectrometry and next generation sequencing have been applied to cytological specimens for mutational profiling simultaneously several genes on different types of tumors. The use of these technologies is rapidly increasing and promises to address the issue of testing multiple genes in minimal volume of tissue/cells.

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Potential of Next-Generation Sequencing in Cytology

3

Xinmin Li and Bin Yang

Advantages of NGS over Sanger Sequencing

NGS technology has quickly become a dominant sequencing method in life science research and medical practice for several reasons: (1) Speed—NGS is massively parallel, producing 300GB data in a single run on a single flow cell compared to Sanger sequencing, only yielding 0.06 MB data in one run. Illumina HiSeq2500 system can sequence one human genome in a single day while it took 13 years to complete first human genome by using Sanger sequencing method; (2) Cost—The massively parallel nature of NGS reduces sequencing time, manpower and reagents that translate into significant saving. For example, sequencing 1 Mb DNA costs $1500 using 3730XL sequencer while it only costs $0.04 using HiSeq2500, and $0.007 using HiSeq X Ten; (3) Sensitivity—NGS can reliably detect >1 % mutations, whereas Sanger sequencing can only detect mutations that are >20 %. This is critically important for somatic

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Table 3.1 Comparison of Sanger sequencing with next-generation sequencing

Sequencing methods	Sanger sequencing	NGS
Yield (MB/Run)	0.06 MB	600GB–1.8 TB
Cost ($$$/MB)	$1500	$0.04–$0.007
Speed (per human genome)	13 years	2–3 days
Amount of DNA required	500–5000 ng	10–1000 ng
Sequencing sensitivity	>20 % mutation rate	>1 % mutation rate
Multiplexing capability	Single	Multiple

mutations in the heterogeneous tumor samples; (4) Less DNA—With the advance of library construction technology, NGS can perform well with nanogram range of DNA. Both MiSeq and Ion PGM can sequence around 50 targeted genes with 10–50 ng of degraded DNA, and NuGen's Oviation RNASeq System V2 can make good quality RNASeq library for HiSeq2500 sequencing using only 500 pg RNA. Such limited amount of DNA is not feasible for Sanger sequencing. This is particularly important for the most accessible cytology specimens. In many occasions, only available specimen is fine needle biopsy, fine-needle aspiration biopsy or FFPE slides. Those specimens cannot produce enough DNA for classical Sanger sequencing; (5) More targets—NGS technology can sequence multiple genes cheaply and quickly at a high coverage. Genomic research has facilitated the pace of target discovery for disease management. The numbers of genes that are associated with a disease phenotype and need to be assessed are increasing rapidly. This makes Sanger sequencing-based single gene approach impractical.

In summary, NGS can accurately and sensitively sequence more target genes with less DNA cheaply and quickly (Table 3.1). These tasks are sometimes either technically or practically not feasible for Sanger sequencing. For example, Cetuximab (Erbitux®) and panitumumab (Vectibix®) are anti-EGFR antibodies used in the treatment of metastatic colorectal cancer. The patients frequently become resistant to these agents when activating mutations occur in either of *KRAS*, *NRAS*, *PIK3CA*, and *BRAF*. With a highly heterogeneous needle biopsy or a FFPE slide, NGS can simultaneously test all possible mutations at >1 % in these four genes as well as others to guide therapeutic decision-making. This task is both practically (take too long and cost too much) and technically (not enough tissue and low sensitivity) not possible for Sanger sequencing.

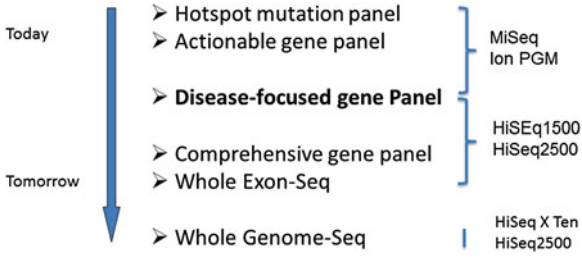

Fig. 3.1 Evolution of the NGS-based clinical testing

Clinical Applications of NGS in Personalized Medicine

NGS technology has revolutionized genomic research and is gradually making its way into clinical laboratories. Today, most clinical applications have been in diagnostic testing for hereditary disorders and, more recently, risk screening for hereditary cancers and therapeutic decision-making for somatic cancers. The testing contents have evolved from hotspot panels, actionable gene panels, disease-focused panels to more comprehensive panels. Although exome and whole-genome sequencing approaches begin emerging, given the incomplete clinical annotation of human genome, the panel-based testing is more practical at present time, and already holds a firm place in clinical applications [1] (Fig. 3.1). Below is a brief summary of the current status of those panels.

Hotspot Panels

The Hotspot panel is a collection of frequently mutated hotspots that are either clinically actionable or with diagnostic/prognostic significance. Over the past several years, there has been a major shift in cancer diagnostics from physical and histological findings to assessment of targetable genomic mutations. A primary example is lung cancer. Since the first approval of targeted drugs, like Tarceva (erlotinib) and Iressa (gefitinib), for non-small-cell lung cancer (NSCLC) with activated *EGFR* mutations a decade ago,

recent approval of Xalkori (crizotinib) for patients with *ALK* gene fusions, routine genetic testing for somatic mutations from lung cancer biopsies is becoming the standard for providing optimal patient care. In fact, the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology issued a joint guideline establishing recommendations for molecular diagnostic testing in 2013 [2]. The new guide suggests that all patients with advanced lung adenocarcinoma—a subset of NSCLC patients—should be tested for *EGFR* and *ALK* abnormalities to determine if tyrosine kinase or *ALK* inhibitor therapy is beneficial, regardless of their clinical variables such as smoking history, gender, or ethnicity. On July 22, 2014, there are 161 FDA-approved targeted therapies (biomarker–drug pairs) listed on the FDA therapeutic biomarker website (http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm). The collection of those targeted gene mutations will provide a powerful tool to improve response to therapies, enhance safety and optimize dosing.

The AmpliSeq cancer panel V1 represents such an example. This first commercially released hotspot cancer panel covers clinically relevant hotspot mutations across 46 cancer genes, including well-established tumor suppressor genes and oncogenes. This RUO product was designed for potential clinical application by including all *EGFR, BRAF, KRAS,* and other clinically actionable hotspot mutations. Given its popularity, Illumina subsequently released a similar product—TruSight Amplicon cancer panel with 48 genes. Today, the Ion AmpliSeq Cancer Panel V2 dominates the Hotspot panel market. This 50-gene hotspot panel maintained compatibility with FFPE samples while expanding mutational content for broader coverage of additional genes and "hot spot" mutations. As expected, this panel has now been clinically validated and offered as clinical testing by several academic institutes and private sectors.

Actionable Gene Panels

These panels evolved from hotspot panels by including all exons of targeted genes (or all clinical relevant regions) so that other pathogenic mutations outside frequently mutated sites can be

interrogated. The common feature of these panels is to focus on actionable genes, particularly targeted therapy-related genes such as *EGFR, BREA, KRAS, PIK3CA, NRAS, KIT,* and *ALK*. These test results complement traditional cancer treatment tools, and often expand treatment options by matching each patient with targeted therapies and clinical trials. These panels are currently offered through commercial vendors, academic institutes as well as private sectors.

The first commercially released, small actionable gene panel is the TruSight Tumor panel, which enables clinical researchers to identify low-frequency mutations across 26 genes that are involved in targeted therapy for lung, colon, gastric, and ovarian cancers and melanoma. This panel has been clinically validated and offered as a clinical testing by several institutes. The V2 Comprehensive Cancer Gene Set offered by Washington University in St. Louis is a medium-sized clinically actionable, customized cancer panel. This panel includes 42 clinically actionable cancer genes (20 for solid tumors, 16 for liquid tumors, and 6 for both) designed for assisting oncologists with stratification of disease subtypes and tailoring of effective personalized therapies. Foundation One developed by Foundation Medicine represents a comprehensive actionable gene panel. It interrogates the entire coding sequence of 236 cancer-related genes plus 47 introns from 19 genes often rearranged or altered in solid tumor cancers. These genes are known to be somatically altered in solid cancers based on recent scientific and clinical literature. This test identifies more potential treatment options from not only FDA-approved targeted therapies, but also clinical trials.

Disease-Focused Panels

The actionable gene panels are collection of well-studied actionable genes that are commonly involved in several diseases. Most of such panels interrogate somatic mutations to aid in therapeutic decision-making. The disease-focused panels are collection of the genes for a particular disease. Those panels largely focus on germ line mutations to screen for the risk of inherited diseases for

preventive medicine. At present, hereditary cancer panels are most popular tests on market. Approximately 5–10 % of all cancers are hereditary. More than 100 cancer susceptibility syndromes have been reported, including hereditary breast and ovarian cancer syndrome (HBOC), Lynch Syndrome, Cowden syndrome (CS), and Li–Fraumeni Syndrome (LFS). Many of these risk genes share molecular pathways and play a role in the repair of DNA damage, such as high risk gene *BRCA1* and *BRCA2*, and modest risk gene *BRIP1* and *PALB2* that are all part of the Fanconi Anemia (FA)-BRCA Molecular Pathway and are associated with increased risk of breast and ovarian cancer [3]. NGS-based screening for all of those genes for a particular cancer provides critical risk information for preventive management. These panels generally have a limited set of genes allowing multiplex and greater depth of coverage for increased analytical sensitivity and specificity, and decreased cost.

As of May 2014, more than 60 clinical laboratories across the world had launched a total of ~1656 NGS-based clinical tests (estimated using Genetic Testing Registry): about one-third in the commercial sector and two-thirds in academically affiliated clinical laboratories. Majority of those are disease-targeted tests. For the risk of inherited breast-Ovarian cancer alone (familial 1), there are ~31 NGS tests available across the world. Although fewer clinical laboratories have launched disease-focused NGS tests for somatic cancers, many laboratories are actively developing such tests. In next few years, this is the area expected to expand quickly. Table 3.2 summarizes a few popular inherited cancer tests.

Comprehensive Panels

Although disease-focused panels have gained popularity, clinical laboratories are facing serious financial and practical challenges associated with (1) development and validation of different disease-focused panels according to ACMG guidelines, (2) the limited number of clinical specimens for a given disease at a given time, (3) requirement to constantly update the content of existing panels. These challenges have made clinicians to wonder whether

Table 3.2 Examples of representative hereditary cancer panels

Gene	Breast panel	Colon panel	Ova panel	Panc panel	Renal panel	Endometrial panel
APC	Δ	• √ Δ	Δ	• √		
ATM	• √	√	• √	• √		
AXIN2		√				
BARD1	• √		• √			
BLM	√	√	√			
BMPR1A	•	• √ Δ	•			
BRCA1	• √ Δ	Δ	• √ Δ	• √		√
BRCA2	• √ Δ	Δ	• √ Δ	• † √		√
BRIP1	• √ Δ		• √ Δ			
CDC73						Δ
CDH1	• √ Δ	• √ Δ	• √ Δ			
CDK4				√		
CDKN1C					Δ	
CDKN2A		Δ		• √		
CHEK2	• √ Δ	• √ Δ	• √ Δ			√
EPCAM	√ Δ	• √ Δ	• √ Δ	• √	•	• √
FAM175A	√		√			
FANCC	√		√	√		
FH					• Δ	
FLCN					• Δ	
GPC3					Δ	
HOXB13	√		√			
MAX						Δ
MEN1						Δ
MET					• Δ	
MIFT					•	
MLH1	√ Δ	• √ Δ	• √ Δ	• √	•	• √
MRE11A	• √		√			
MSH2	√ Δ	• √ Δ	• √ Δ	• √	•	• √
MSH6	√ Δ	• √ Δ	• √ Δ	• √	•	• √
MUTYH	• Δ	• √ Δ	• Δ			√
NBN	• √ Δ		• √ Δ			
NF1	•					Δ
PALB2	• √ Δ		• √ Δ	• √	Δ	
PALLD				√		

(continued)

Table 3.2 (continued)

Gene	Breast panel	Colon panel	Ova panel	Panc panel	Renal panel	Endometrial panel
PMS1	Δ	Δ	Δ			
PMS2	√ Δ	• √ Δ	• √ Δ	• √	•	• √
PRKAR1A						Δ
PTEN	• √ Δ	• √ Δ	• √ Δ		• Δ	• √ Δ
RAD50	• √		• √			
RAD51C	• √ Δ		• √ Δ			
RAD51D	• √ Δ		• √ Δ			
RET						Δ
SDHA					•	
SDHAF2						Δ
SDHB					• Δ	Δ
SDHC					•	Δ
SDHD					• Δ	Δ
SMAD4		• √ Δ				
STK11	• √ Δ	• √ Δ	• √ Δ	• √		
TMEM127						Δ
TSC1					•	
TSC2					•	
TP53	• √ Δ	• √ Δ	• √ Δ	• √	•	√ Δ
VHL				√	• Δ	Δ
WT1					Δ	
XRCC2	√	√	√	√		

• Ambry genetics, √ GeneDx, Δ Baylor

they should move directly to exome or genome sequencing. The question seems to be a relevant one, but laboratories hesitate to make the move when they have to face the hundreds of variants with unknown clinical significance from whole-genome approaches.

A logic compromise is to consider a more comprehensive panel that includes all genes associated with all diseases. By fully taking advantage of high throughput nature of NGS technology, this approach will satisfy the simplicity of disease-targeted testing and also avoid interrogation of genes of unknown clinical relevance. This "one for all" approach will minimize test development and validation efforts for multiple disease focused panels, maximize

the multiplex capability by combining samples with different diseases in one assay, and reduce the frequency of required updates as new genes are identified. In practice, physicians can still order a specific disease focused panel that is relevant to the patient's phenotype first. If the subpanel is negative, they can then request analysis using the full panel for additional information.

Illumina's TruSight One is an example. This comprehensive panel covers 4813 genes that have known association with clinical phenotypes. It was designed to cover the most commonly ordered molecular assays, enabling laboratories to perform all assays with one physical panel. The panel includes all exonic regions harboring disease-causing mutations identified based on information in the Human Gene Mutation Database (HGMD Professional), the Online Mendelian Inheritance in Man (OMIM) catalog, and GeneTests.org, and other commercially available sequencing panels. Thus, the TruSight One panel covers all genes currently reviewed in clinical research settings, and can be used for any disease focused panel testing once it is fully validated.

Whole-Exome Sequencing

While there is ongoing discussion about the readiness of whole-exome sequencing (WES) for clinical application, the ultimate adoption of this approach appears to be inevitable. Numerous studies have illustrated the power of WES in making new discoveries, such as the identification of a germ line mutation in *PALB2*, a gene previously implicated in breast cancer risk, in an individual with familial pancreatic cancer [4], and a germ line mutation in *MAX* in individuals with familial PCC, which was not previously linked to familial PCC [5]. This unique power has made it an ideal tool for testing the patients with undiagnosed disease of suspected hereditary origin.

A preliminary study on 250 people with undiagnosed diseases demonstrated the promise of WES. Data resulted in a genetic diagnosis for 62 of the 250 patients, 20 of whom had autosomal recessive diseases. The diagnosis yield was as high as 25 % in solving these hereditary disease mysteries [6]. A few academic institutes

have already been offering clinical WES, including Baylor College of Medicine, Washington University of St. Louis, and UCLA, with a focus on hereditary disorders. Although WES approach is uniquely suitable for the patients with undiagnosed diseases or the patients with negative result using disease-focused panel, we do not anticipate the full range of clinical use at the present time.

Whole-Genome Sequencing

Whole-genome sequencing (WGE) is the most comprehensive tool for future clinical application. It is expected to provide full coverage of all protein coding regions like WES as well as intronic and other noncoding regions associated with inherited diseases. With the recent release of Illumina HiSeq X Ten, a human genome can be sequenced at 30× coverage at <$1000. The cost of sequencing is not a barrier for clinical WGS anymore. However, some technical issues remain to be addressed. Researchers at the Stanford University School of Medicine have found that significant challenges must be overcome before WGS can be routinely used clinically. In particular, they found that the use of WGS was associated with incomplete coverage of inherited disease genes, low reproducibility of detection of genetic variation with the highest potential clinical effects, and uncertainty about clinically reportable findings [7]. Although the financial cost of the sequencing has made the technology more accessible and the analytical validity of WGS is improving, there are still technical challenges and "considerable" human resource needs in order to interpret and validate the data returned by WGS.

However, it is just a matter of time that WGS will become an ultimate tool for routine clinical practice. As an early exercise, Illumina has already offered Trugenome clinical WGS services to help find the underlying genetic cause of an undiagnosed rare genetic disease, learn about patient carrier status and genetic predisposition towards adult-onset conditions. Customers can also use Illumina's CLIA-certified, CAP-accredited Clinical Services Laboratory to generate whole-genome sequencing data and use their own expertise to make clinical interpretations.

Potential Applications of NGS in Cytology

In clinical practice, cytology specimen, particularly minimally invasive fine needle aspiration (FNA), is typically the first and easiest specimen available for clinical testing. In some instances, cytology specimens are the only material available when tumor size, location or comorbid conditions preclude concurrent core needle or excisional biopsy [8]. In fact, FNA procedures have been included in the recommended guidelines for the diagnosis of thyroid carcinomas, lung carcinomas and sarcomas [9]. It is clear that FAN is emerging as one of the most important tools in pathological diagnosis and molecular analysis for personalized medicine. However, a well-known common challenge for using cytology specimens for Sanger sequencing is the limited amount of, sometimes degraded, DNA. Whether we can effectively use the limited amount of cytology specimens for NGS applications has significant implications in patient care.

Recent improvements of FNA procedures and technological advancement in making DNA library with the small amount of DNA have made NGS technology applicable to cytology specimens in clinical setting. Several studies with FNA and other cytology specimens have established its feasibility in different cancers as briefed below.

Lung Cancer

Lung cancer is the leading cause of cancer-related death worldwide. About 85 % lung cancers are non-small-cell lung cancer (NSCLC). The majority of NSCLC are diagnosed at an advanced stage, and missed the best surgery time. Therefore, the diagnosis and therapeutic decision for lung cancer heavily rely on minimally invasive procedures, either small biopsies or cytology samples.

Lung cancer has the most available targeted therapies. The targeted genes include *EGFR, BRAF, KRAS, ALK,* and *ROS1*. Many more potential targets, such as *PIK3CA, FGFR1,* and *DDR2*, are in clinical trials. Therefore, the number of predictive biomarkers for novel targeted drugs entering into clinical practice is expected to

rapidly increase. This realty poses a significant challenge to Sanger sequencing, which takes too long and cost too much to sequence multiples target genes, and more importantly, Sanger sequencing has technical difficult to work well with limited amount of cytology materials and to detect somatic mutation at low frequency. In contrast, NGS technology is able to interrogate multiple genes and requires limited amounts of DNA, and thus is superior to current standard methodologies.

Ion PGM sequencing technology has been applied to the detection of targeted gene mutations using 38 lung adenocarcinomas cytology specimens [10]. The study simultaneously assessed 504 mutational hotspots from 22 selected lung cancer-associated genes. Of the 38 specimens, 36 cases were successfully sequenced (95 %). 24/36 cases identified at least one mutations. Many of the mutated genes are well known driver genes including *EGFR, KRAS, PIK3CA, BRAF, TP53,* and *PTEN*. Of those, *EGFR* and *KRAS* mutations were found in 6/36 and 10/36 cases, respectively, and were independently confirmed by Sanger sequencing or high resolution melting analysis. Data suggest that NGS can be reliably applied on cytology specimens with high sensitivity, specificity and reproducibility.

Thyroid Cancer

Thyroid cancer is the most common malignancy of endocrine organs. Its incidence is steadily increasing in the USA and worldwide. Thyroid cancer typically occurs in thyroid nodules. FNA followed by cytological examination is an accurate and cost effective diagnostic method for evaluating thyroid nodules. This commonly used approach allows detecting cancer or establishing a diagnosis of a benign nodule in most cases. However, in approximately 25 % of nodules, the diagnosis cannot be established and consequently classified as indeterminate by FNA cytology, hampering clinical management of these patients [11]. Because some molecular markers are highly specific in thyroid cancer, NGS offers the potential to improve the accuracy of cancer diagnosis and prognosis in thyroid nodules.

Nikiforova et al. [12] has recently validated a large series of thyroid neoplastic and nonneoplastic samples using Ion Personal Genome Machine sequencer (Life Technologies). In this study, 228 thyroid frozen, formalin-fixed, and fine-needle aspiration samples were sequenced using the Ion Torrent amplicon-based sequencing approach and a custom-designed ThyroSeq panel. The results showed that thyroSeq delivered overall success rate of 99.6 %. Only 1 out of 51 (2 %) routine FNA samples failed the NGS sequencing, suggesting that vast majority of FNA samples should be amendable to such analysis. They chose the amplicon-based approach for two reasons: (1) it allows using 5–10 ng of input DNA for efficient amplification of genomic regions of interest; (2) it works well with partially degraded DNA due to the small size of amplicons, thus ideal for cytology specimens.

To test if NGS has added value for the diagnosis of thyroid FNA specimens with indeterminate cytology, Le Mercier et al. [13] retrospectively analyzed 34 indeterminate FNA samples using AmpliSeq cancer panel V2 (Life Technologies). Mutations in *BRAF, NRAS, KRAS,* and *PTEN* that are known to be involved in thyroid cancer biology were detected in 5 of the 7 malignant cases, giving a 71 % sensitivity of this molecular test for the diagnosis of malignancy. This study demonstrated that the detection of mutations known to be involved in thyroid cancer can improve the sensitivity of thyroid FNA diagnosis.

Pancreatic Cancer

Pancreatic cancer represents the fourth-highest cause of cancer death in the USA with the lowest survival rate among the most common cancers (~6 %). Many genetic alterations have been associated with the development of pancreatic cancer. The four most frequently mutated genes are oncogene *KRAS* and tumor suppressor gene *CDKN2A/p16, SAMD4,* and *TP53*. Those signature genes have been used as tumor markers for the diagnosis of pancreatic adenocarcinoma. The combination of cytological evaluation and tumor marker mutation analysis, especially for inconclusive cases, can potentially enhance the diagnostic power.

To explore the performance of NGS in the diagnosis of Pancreatic ductal adenocarcinoma (PDAC) using FNA specimen, Dario de Biase et al. [14] analyzed *KRAS* mutation, which has been reported in >95 % PDAC, by using Sanger sequencing (considered as a gold standard technique for DNA sequence analysis), allele specific locked nucleic acid PCR (ASLNAqPCR) and 454 Next-Generation Sequencing (454 GS-Junior platform, Roche). Sixty specimens from endoscopic ultrasonography FNA were analyzed for *KRAS* exon 2 and exon 3 mutations. Sanger sequencing delivered a clinical sensitivity for the detection of the *KRAS* mutation of 42.1 %, ASLNAqPCR of 52.8 % and 454 GS-Junior of 73.7 %. The study not only demonstrated the feasibility of FNA for NGS, but also showed a better accuracy compared to other classical techniques.

The feasibility of NGS testing with cytology specimens has now been established. We anticipate that its applications will increase rapidly in coming years.

Challenges, Solutions, and Future Directions

NGS technology is the driver of genomic medicine, and is having a dramatic impact on the personalized medicine from risk assessment to early diagnosis, prognosis, and treatment. Successful application of NGS technology to cytology specimens can further enhance its power in the disease management. However, there are several key challenges that impede the wide adoption of NGS in clinical laboratories. Addressing the following challenges can pave the way for gene panel, WES, ultimately WGS testing in the daily practice of personalized medicine.

Lack of Evidence Base for NGS Tests

Although there are many examples of the beneficial impact of NGS tests, overall, we have insufficient evidence-based framework to convince the FDA to approve NGS tests, insurance companies to cover them, and physicians to use them. This is perhaps

the biggest challenge for NGS tests to fully penetrate the many facets of clinical care in a timely fashion [15].

In this regard, NGS community may benefit from partnering with public health agencies and private sectors to collectively address this fundamental question. This effort requires data curation from the primary scientific literature, carrying out expensive and time-consuming clinical trials. By forming partnership, we can enrich our knowledge and resources, increase in efficiency and reduce financial burden for a given institute.

The Office of Public Health Genomics at the US Centers for Disease Control and Prevention has developed a framework for evaluating emerging genetic tests. Their evaluation includes four key areas: analytic validity (how accurately and reliably the test measures the genotype of interest), clinical validity (how consistently and accurately the test detects or predicts the intermediate or final outcomes of interest), clinical utility (how likely the test is to significantly improve patient outcomes), and ethical, legal, and social implications that may arise in the context of using the genomic tests. Through partnership and networking, NGS community can use this framework as a basis to further develop a specific and comprehensive plan to provide evidence base for NGS tests.

Lack of Understanding of NGS Tests

There is a growing sentiment that uptake of genomic medicine is slow because health care providers and community in general are lack of understanding of NGS tests. This will directly reduce the number of order, also affect insurance coverage and FDA clearance for these tests. Therefore, enhanced NGS education is the key not only for health care providers but also for other related professions including policy-makers, regulators, lawyers, investors, and insurance underwriters. In addition to classic educational means, such as conferences, publications and media, the NGS education effort should start from the schools, i.e., the universities should train more qualified genomic teachers, have a genomic medicine major, and offer more genomic courses.

A combination of better education in NGS tests as well as better tools for clinical decision support will speed up the NGS adoption. Several efforts are under way to track and make the latest information on genomic tests accessible. The Pharmacogenomics Knowledge Base (PharmGKB) is an online resource that includes information on potentially clinically actionable gene–drug associations and genotype–phenotype relationships. Much of the information is manually curated from the published literature and is used to write evidence summaries and pharmacogenomic-based drug dosing guidelines. Another resource is the National Institutes of Health's (NIH) Genetic Testing Registry, a repository for comprehensive genetic test information that is voluntarily submitted by test providers. Currently, the site lists around 3000 clinical tests, including pharmacogenetic and other types of tests. Table 3.3 listed a few key websites that curate available genomic tests and the evidence to support their use. These tools will help physicians understand and order such tests.

Lack of Clinically Annotated Genetic Variants for Accurate Data Interpretation

Today, the bottleneck of genomic diagnostics has moved from data acquisition to data interpretation. An important challenge for efficiently translating NGS data into actionable information for clinicians is the lack of understanding of the impact of most genetic variants on human health and disease. Understanding these variants will require massive sources of genomic and phenotypic data and shared efforts in studying variants [1]. This will take many years and requires a lot of collective effort. The International Collaboration for Clinical Genomics is working closely with NCBI to develop standards, to assist clinical laboratories in sharing their data and to develop approaches to curate the shared data.

At present, building comprehensive, constantly updated genomic databases is an immediate solution to address current challenge. Progress has been made with the recent launch of the PharmacoGenomic Mutation Database (PGMD. http://www.biobase-international.com/product/pgmd). PGMD is a commercial

Table 3.3 Genomic resources for genomic tests and supporting evidence

Website name	URL address	Brief description
PharmKGB	http://www.pharmgkb.org	A pharmacogenomics knowledge resource that encompasses clinical information including dosing guidelines and drug labels, potentially clinically actionable gene–drug associations and genotype–phenotype relationships
Genetic testing registry	http://www.ncbi.nlm.nih.gov/gtr/	Central location for voluntary submission of genetic test information by providers; includes information on test methodology, validity, evidence of the test's usefulness, and laboratory contacts and credentials; currently includes 19,000 tests for 4500 conditions and 3000 genes as of June 26, 2014
GAPP finder	http://64.29.163.162:8080/GAPPKB/topicStartPage.do	A searchable database of genetic tests and genomic applications in transition from research to clinical and public health practice. The search query can include disease, genes, drug, test, etc. Includes 547 tests as of June 26,2014
FDA pharmacogenomic biomarkers	http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm	A list of FDA-approved drugs with pharmacogenomic information in their labeling. includes 161 biomarker–drug pairs as of June 26, 2014
EGAPP	http://www.egappreviews.org/about.htm	A resource for evidence regarding the validity and utility of genetic tests for clinical practice and recommendations on implementation of genetic tests from professional organizations and advisory committees
Evidence aggregator	http://64.29.163.162:8080/GAPPKB/evidencerStartPage.do	An application that facilitates searching for evidence reports, systematic reviews, guidelines related to the use of genetic tests and other genomic applications

resource for identifying all published genetic variants that have been shown to affect drug response in patients, thus guiding physicians to select appropriate drug and dose for maximum benefit and minimum side effect. CliVar at NCBI (http://www.ncbi.nlm.nih.gov/clinvar/) is a public database specifically focused on relationships among human variations and phenotypes with supporting evidence to help interpretation of clinically relevant mutations. Another resource with a focus of somatic mutations is My Cancer Genome (http://www.mycancergenome.org). This personalized cancer medicine knowledge resource gathers up-to-date, well-established cancer mutation information, related therapeutic implications, and available clinical trials, making a convenient one-stop-shopping tool for physicians. For germ line mutations, The Human Gene Mutation Database Professional (HGMD Pro) is a mature commercial resource providing comprehensive data on human germ line mutations, particularly useful for hereditary disease risk screening and diagnosis.

Given the current challenge on accurate annotation of genetic variants, some third-parties provide genome interpretive services to assist clinicians in understanding the genetic variants and its clinical relevance to treatment. This nascent field currently includes startup companies like Knome (http://www.knome.com), Silicon Valley Biosystems (http://www.svbio.com), and Omicia (http://www.omicia.com). These companies offer software, computer infrastructure, and services required to process, analyze, and produce tailored diagnostic reports.

Lack of Guidelines for Clinical Report

The accurate interpretation of genetic variants identified by NGS is one thing, how to report the finding is another. One of the issues facing laboratories who offer genetic testing is how to report the variants that are unrelated to the indication for testing, such as risk of developing cancer or the risk of developing other genetic diseases or conditions like neurologic or psychiatric illnesses. These findings may have an impact not only on the individual patient but also on immediate family members. This issue is particularly

significant for WES and WGS testing. Although there is some consensus, this topic is likely to remain hotly debated for some time.

It is necessary to develop an ethical and legal framework for reporting incidental findings and returning the data to patients and their families. Some professional societies have been working toward this direction. To guide the development and interpretation of NGS-based tests, the American College of Medical Genetics (ACMG) has developed a position statement for whole-exome and -genome sequencing [16], recommendations for reporting of incidental findings in clinical exome and genome sequencing [17], and recently professional standards and guidelines for more detailed guidelines including validation of NGS methods and platforms, monitoring NGS testing, data interpretation, and reporting [18]. These guidelines can be applied to NGS cancer panels and be used by genetic counselors to guide their cancer risk assessments. The National Comprehensive Cancer Network (NCCN) also addressed the use of gene panels in their 2013 Guidelines for Risk Assessment (National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology, "Genetic/Familial High-Risk Assessment: Breast and Ovarian," 2013). Before comprehensive and consensus guidelines are established, we need to balance privacy issues with the potential advantages and drawbacks of sharing genetic data with patients and their relatives.

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HPV Testing and Molecular Biomarkers in Cervical Cytology

4

Zaibo Li and Chengquan Zhao

Introduction

Cervical cancer is the third most common malignancy in women worldwide and definitive evidence confirms that cervical cancer results from infection of oncogenic types of human papillomavirus (HPV). Cervical cancer screening with cervical Papanicolaou test can detect cervical intraepithelial neoplasias (CINs) and significantly decrease the incidence of cervical cancer and its associated mortality over past 70 years. Although Pap test has high specificity to detect CINs, its sensitivity is relatively low. Recent data from the implementation of high-risk HPV testing (hrHPV) together with Pap test or by hrHPV testing alone suggest hrHPV testing has significantly higher sensitivity in detecting CINs than Pap test. This chapter focuses on discussing current available HPV testing methods and their mechanisms. Besides HPV testing, other cytological molecular markers including p16/Ki67 immunohistochemistry,

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DNA aneuploidy, ProExC assay, methylation markers, TERC and CTNND2 FISH, and HPV L1 detection are also discussed in this chapter.

Human Papillomavirus and Cervical Cancer

Cervical cancer is the third most common malignancy in women worldwide with about 529,000 cases and over 275,000 deaths estimated annually. In the USA, there were 11,000 cervical cancer cases and 3500 deaths reported in 2008 [1]. With implementation of cervical cancer screening protocols, the incidence of cervical cancer has dropped by about 90 % over the past 50 years in the USA. Definitive evidence from a large number of epidemiological, clinicopathological and molecular studies confirms that cervical cancer results from persistent infection of certain high-risk, oncogenic types of human papillomavirus (HPV). Other recognized risk factors are related to acquisition of HPV infection and immune dysfunction, including early sexual activity, multiple sexual partners, human immunodeficiency virus infection and immunosuppressive drug therapy. Social risk factors are related to poor access to cervical cancer screening programs and noncompliance with screening visits, including lower socioeconomic status and lower educational levels. Instead, genetic factors play a small role in the development of cervical cancer.

Human papillomavirus is a member of the papillomaviridae family and is non-enveloped DNA virus. It has circular double-stranded DNA genome with an approximately 7.9 kb in size, which is wrapped around nucleosomes and coated by a 60 nm diameter capsid. Existing data suggest that HPV has evolved along with humans and productively infects only humans. HPVs are phylogenetically divided into alpha, beta, gamma, delta, and mu genera based on their nucleotide sequences. Alpha genus HPVs cause genital and oropharnygeal mucosal infection exclusively and include oncogenic HPV types associated with cervical cancer. Beta genus HPVs cause most cutaneous infections. There are more than 100 types of HPV identified based on the difference of at least 10 % in the nucleotide sequence of L1 open reading frame and

more than 40 types can cause anogenital and upper aerodigestive tract infections. HPV types are classified as high risk (HR) or low-risk (LR) based on their oncogenic potential, which reflects the propensity of HPV-associated lesion to progress to invasive cancer. Infection of hrHPV is the prerequisite for precancerous lesions, such as high-grade cervical intraepithelial neoplasia (CIN), vaginal intraepithelial neoplasia (VAIN), vulvar intraepithelial neoplasia (VIN), penile intraepithelial neoplasia (PIN), and anal intraepithelial neoplasia (AIN), as well as invasive cancers of the cervix, vulva, vagina, anus, penis, and aerodigestive tract. High risk HPVs include carcinogenic types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59); probably carcinogenic type (type 68); and possibly carcinogenic types (types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, and 97). HPV 16 and HPV 18 are associated with the highest rates of carcinogenesis; and HPV 31 and HPV 33 have the next highest risk. On the other hand, lrHPV types cause conditions such as genital warts, but virtually never cause cervical cancer or true precancerous lesions.

HPV genome includes a noncoding upper regulatory region (long control region) and coding sequence. Noncoding upper regulatory region functions as a regulator of transcription, replication, tissue tropism, and host range. The coding sequence includes six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2). E1 and E2 proteins are related to HPV transcription and replication. E5 protein can form complexes with platelet derived growth factor receptor and epidermal growth factor receptor to stimulate cell growth, inhibit apoptosis and maintain epithelial cell proliferation. However, E5 protein expression is absent in malignant cervical cells. E6 and E7 proteins are involved in tumorigenesis by interfering with the normal function of tumor suppressors. L1 and L2 proteins are the major and minor viral capsid proteins, respectively. L1 gene is the most conserved region of HPV genome and is used for phylogenetic classification as mentioned before.

Squamous epithelial trauma exposes basal layer cells to HPV, which allows viral entry by a receptor-mediated mechanism. Once HPV enter cervical cells, the virus replicates by utilizing the normal replicative machinery of the cells and viral protein E1 and E2. Initial HPV infection triggers a burst of viral replication up to

~100 episomal copies per cell. Infected basal cells continue to divide. Some infected daughter cells remain in the basal layer with dividing capacity and serve as a repository for viral replication; while the other infected daughter cells move upward to the suprabasal layer. At this stage, late viral genes (L1 and L2) are transcribed, and then, L1 (major) and L2 (minor) proteins are expressed to form viral capsid for viral particle assembly. The assembled viral particles will be released when infected epithelial cells are shed from surface.

The vast majority of HPV infection will be cleared and only about 10 % of infection will become persistent. During persistent infection, HPV DNA will integrate into host genome by disrupting E2 transcription control region, leading to E6 and E7 mRNA transcription and protein expression. E6 protein can bind and degrade p53 protein, and then inhibit normal apoptosis. E7 protein can bind and inactivate pRB and then allow cell cycle progression through G1/S checkpoint. E6 can also activate catalytic component of telomerase to allow telomere regeneration and immortalize cells and E7 can block cell cycle inhibitors, p21 and p27, to promote cell proliferation. Overwhelming studies indicate E6 and E7 proteins are causative factors for cervical carcinogenesis.

HPV Testing in Cervical Cancer Screening

2001 Bethesda system terminology for reporting cervical cytology results and the availability of findings from randomized trial of strategies for managing minor cervical cytologic abnormalities promoted the American Society for Colposcopy and Cervical Pathology (ASCCP) to develop comprehensive, evidence-based consensus guidelines to aid clinicians in managing women with abnormal cervical cytology, which was last updated in 2012. Current ASCCP guideline recommends reflex HPV testing for the management of ASC-US cytology test and co-testing with cytology and hrHPV testing at 5-year intervals for women aged 30–64 years Co-testing is not only more sensitive for squamous cell dysplasia but also for cervical glandular neoplasias. Recently FDA has approved the Cobas HPV Test for use as a first-line primary screening test for cervical cancer in women 25 or older.

Clinically Utilized HPV Testing Methods

Unlike other DNA viruses, conventional cell cultures cannot detect HPV. Due to limited sensitivity and specificity, classical direct virological diagnostic techniques, such as electron microscopy and immunohistochemistry, are not used routinely to detect HPV. Serological detection of anti-HPV antibodies has not been used routinely for clinical diagnosis because of its limited analytical accuracy. Consequently, all commercially available HPV tests are designed for the detection of HPV nucleic acids in clinical specimens. Although many in-house HPV nucleic acid detection methods have been used successfully in research laboratories worldwide for more than two decades, most of them are not approved by the FDA for clinical use. Current HPV tests can be performed using residual liquid-based cytology samples and are therefore easy to incorporate into screening program. The following considerations are suggested when designing new HPV testing for clinical use: (1) targets should include all 13 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and preferable to include HPV type 66 as well; (2) no low risk HPV types should be included; and (3) no HPV type 53 should be included because of its relatively high prevalence and low association with cervical cancer [2].

Current HPV tests can be classified into the following categories: (1) FDA-approved HR HPV DNA-based assays (Screening and genotyping assays), which don't provide viral integration information; (2) FDA-approved HR HPV E6/E7 mRNA-based assays (screening and genotyping assays), which provide viral integration information; and (3) Assays that have not been FDA approved/cleared; some of these are CE (European) approved.

FDA-approved HR HPV DNA-based assays include: (1) Hybrid Capture 2 HPV DNA test; (2) Cervista HPV HR test, (3) Cervista HPV 16/18 genotyping test, and (4) Cobas 4800 HPV test. FDA-approved HR HPV E6/E7 mRNA-based assays include (1) Aptima HPV assay for the detection of E6 and E7 mRNA, and (2) APTIMA 16 18/45 genotype assay. All of the FDA-approved assays are approved for use with PreservCyt solution (ThinPrep; Hologic Inc., Bedford, MA), but none are FDA approved for

specimens collected in SurePath Preservative Fluid (BD, Franklin Lakes, NJ). Although some laboratories perform HPV testing on samples collected in SurePath Preservative Fluid, it indeed an off-label use of the FDA-approved test.

a. **Hybrid Capture 2 HPV DNA test**: Hybrid Capture 2 (HC2) HPV DNA test was first developed by Digene Corp. (Gaithersburg, MD) in 1997, and it is marketed by Qiagen currently. FDA approved this assay in 1999 for reflex testing of patients with a cytology result of atypical squamous cells of undetermined significance (ASC-US). FDA further approved its use in conjunction with routine Pap testing of women over age of 30 in 2003. As for now, HC2 High-Risk HPV DNA test is the most frequently used diagnostic HPV test worldwide. Newly developed HPV assays are recommended to possess clinical characteristics similar to those of HC2 regarding of the process of clinical validation.

   HC2 HPV DNA test is an in vitro nucleic acid hybridization assay with signal amplification using microplate chemiluminescence for qualitative detection of 13 high-risk types of HPV DNA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). HC2 test detects the presence of high-risk types of HPV, but cannot determine specific HPV genotype. In this assay, a specific high-risk HPV RNA probe cocktail is used to hybridize cervical specimens containing target DNA. The resultant hybrids of RNA probes and targeted high-risk HPV DNAs are captured onto a microplate well coated with specific antibodies against RNA–DNA hybrids. After alkaline phosphatase conjugated antibodies bind with immobilized hybrids, the signals are detected by a chemiluminescent substrate. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification and the subsequent emission light is measured as relative light units (RLUs) on a luminometer. An RLU measurement equal to or greater than the pre-validated cutoff (CO) value indicates the presence of high-risk HPV DNA sequences in a clinical cervical specimen. An RLU measurement less than the cutoff value indicates either absence of targeted high-risk HPV DNA sequences or high-risk HPV DNA levels below the detection limit of HC2 assay. Specimens with

RLU/CO ratios ≥1.0 are considered as positive for those thirteen high-risk HPVs. Specimens with RLU/CO ratios <1.0 are considered as negative. For past several years, HC2 testing Rapid Capture System has been developed for high throughput workload. By using this system, one technologist can process up to 352 specimens in an 8-h shift, with 3.5 h of hands-free operation.

HC2 High-Risk HPV DNA test is designed and validated for use with specimens which are collected and transported using HC2 DNA Collection Device or HC Cervical Sampler (cervical broom) and deposited in either Digene Specimen Transport Medium or a Cytyc PreservCyt vial. Cervical cytology specimens can be stored at room temperature for up to 2 weeks. After 2 weeks, specimens can be stored for an additional week at 2–8 °C. If the assay cannot be performed within 3 weeks of collection, specimens can be stored at −20 °C for up to 3 months prior to testing. Collected specimens placed in Cytyc PreservCyt solution used in preparing Cytyc ThinPrep Pap test slides can be used in HC2 High-Risk HPV DNA test. There must be at least 4 mL of PreservCyt solution remaining for HC2 High-Risk HPV DNA test. Specimens in PreservCyt solution can be stored for up to 3 months at temperatures of 2–30 °C after collection and before processing for HC2 High-Risk HPV DNA test. Specimens in PreservCyt solution cannot be frozen.

Several problems have been notice for current version of HC2 test, including analytical inaccuracy due to the cross-reactivity of its probe cocktail with untargeted HPV types (11, 53, 54, 55, 66, MM4, MM7, MM8, or MM9.39) and lack of an internal control to evaluate specimen adequacy or the presence of potentially interfering substances. It has been shown that HC2 test has an additional false-positive rate of 5 % when no HPV DNA is present in clinical specimen when compared to highly sensitive broad-range PCR tests.

b. **Cervista HPV HR test**: Cervista HPV HR test (Hologic, Bedford, Massachusetts) received FDA approval in 2009 for patients with a cervical cytology result of ASC-US to determine the need for colposcopy, and women age 30 and older to adjunctively screen for the presence or absence of high-risk

HPV types. FDA approved Cervista HPV HR test for use with cervical specimens collected in ThinPrep PreservCyt solution. Similar to HC2 test, Cervista HPV HR test detects the presence of high-risk HPVs, but cannot determine specific HPV genotypes if present. It is an in vitro diagnostic test for qualitative detection of high-risk HPV DNA from 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) in cervical cytology specimens.

Cervista HPV HR test uses Invader chemistry, which is a signal amplification method for detecting specific nucleic acid sequences. Two types of isothermal reactions are used in this assay: a primary reaction that occurs on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal. In the primary reaction, a probe oligonucleotide and an Invader oligonucleotide bind to target DNA sequence. An invasive structure forms when these oligonucleotides overlap by at least one base pair on the target DNA sequence. Cleavase enzyme cleaves the 5′ portion (flap) of the probe at the position of the overlapping sequence. The probes are present in a very large excess. Cycle runs rapidly on and off the target DNA sequence. They produce many cleaved 5′ flaps, which can bind to a universal hairpin fluorescence resonance energy transfer (FRET) oligonucleotide that creates another invasive structure recognized by Cleavase enzyme as a substrate. After enzymes cleave the FRET oligonucleotides between fluorophore and quencher molecule, fluorescence signal is produced as the cleaved flaps cycle on and off (Cervista HPV HR, revision C, 2009). In Cervista HPV HR test, reagents are provided as three oligonucleotide mixtures, which detect high-risk HPV types. Unlike HC test, internal and external controls, including negative and positive controls, are used as quality control in this test. Controls must be run on each assay. Sample results are valid when both positive and negative controls yield correct results. Oligonucleotides that bind to human histone 2 gene (HIST2H2BE) serve as internal controls. The format of HPV HR test makes it possible to detect HPV DNA sequences and HIST2H2BE sequence simultaneously in one single well by using two sets of different 5′-flap sequences on the probes and

two different FRET oligonucleotides, each with a spectrally distinct fluorophore. A positive result indicates the presence of at least one of the 14 high-risk HPV types in cervical cytology specimen. The positive result is represented by a FAM fluorescent signal, which is above an empirically derived cutoff value. For each reaction, a negative result is represented by a FAM fluorescent signal, which is below an empirically derived cutoff value. A final positive or negative or indeterminate result for any particular specimen is generated based on signal-to-noise value. This signal-to-noise value is referred to as FOZ (Fold-Over-Zero). For Cervista HPV test, cervical specimens should be collected in PreservCyt solution of ThinPrep Pap Test preservation system by using a broom-type device or endocervical brush/spatula. Specimens preserved in PreservCyt can be stored at 20–30 °C for up to 18 weeks before performing the test. PreservCyt solution specimens cannot be frozen.

Similar to HC test, Cervista HPV HR test has potential cross-reactivity with other non-high risk HPV types, such as types 67 and 70, which can yield positive results with this assay.

c. **Cervista HPV 16/18 test**: Cervista HPV 16/18 test (Hologic) is an in vitro diagnostic test for qualitative detection of HPV types 16 and/or 18 DNA in cervical specimens. Same Invader chemistry is used in this assay as in Cervista HPV HR test. In the primary reaction, probe oligonucleotides provided for this test bind to target DNA sequence of HPV 16 and/or HPV 18. Cervista HPV 16/18 test received FDA approval for adjunction with Cervista HPV HR test in combination with cervical cytology in women age 30 and older to assess the presence or absence of high-risk HPV types 16 and 18, and for adjunction with Cervista HPV HR test in patients with ASC-US cytology results to assess the presence or absence of high-risk HPV types 16 and 18 (Cervista HPV 16/18, revision B, 2009). The specimen collection and storage requirements are the same as those for HPV HR.

Limitation of Cervista HPV 16/18 test includes cross-reactivity and false negativity. The presence of high levels HPV high-risk type 31 exhibits cross-reactivity in this assay and very low levels of infection may cause a false-negative result.

d. **Cobas 4800 HPV test**: Cobas 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, California) was initially launched in European in 2009 and received FDA approval in 2011. It uses real-time PCR methodology to amplify target HPV sequence and then uses fluorescence signal to detect the amplified nucleic acids. The test is performed with Cobas 4800 system, which consists of two separate instruments: the Cobas z 480 and Cobas x 480 analyzers. FDA approved Cobas 4800 HPV test for use with cervical specimens collected in ThinPrep PreservCyt solution. Cobas 4800 system software integrates sample preparation, amplification and detection, and result management into one process. The software has two different testing options: pooled testing for all 14 targeted high-risk HPV types together, and pooled testing plus separate individual genotyping for HPV 16 and HPV 18. Cobas 4800 HPV test uses PCR amplification of target DNA and subsequent nucleic acid hybridization for detection of 14 high-risk HPV types in a single analysis. The test can specifically identify HPV types 16 and/or 18 while concurrently detecting the other 12 remaining high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) at clinically relevant infection levels (Cobas HPV test, draft package insert, 2011). Cobas 4800 HPV test was approved: (1) to screen patients 21 years and older with ASC-US, (2) to assess the presence or absence of high-risk HPV genotypes 16 and/or 18 in patients 21 years and older with ASC-US cervical cytology results, (3) to adjunctively screen and assess the presence or absence of HR HPV types in women age 30 and older, and (4) to assess the presence or absence of HR HPV genotypes 16 and 18 in women 30 and older. The assay is FDA-approved for use with cervical specimens collected in Cobas PCR Cell Collection Media (Roche) or ThinPrep PreservCyt solution. The FDA has approved Cobas 4800 HPV test for the first HPV test for primary cervical cancer screening for women 25 years and older on April 25, 2014. However, primary screening with HPV test alone is currently not included in the consensus guidelines for cervical cancer screening in the USA.

Advantages of Cobas 4800 HPV test include quality controls (internal control, β-globin, plus positive and negative controls in every run to validate results), high throughput

capacity (up to 280 samples in 1 day), automation (loading and unloading microwell plate is the only manual intervention), and LIS compatibility (Cobas 4800 system can be connected to a laboratory information system—operator's manual software version 1.0, 2011).

However, the data regarding its analytical and clinical validation are limited since it is relative new. When compared to HC2, Cobas 4800 HPV test showed similar clinical sensitivity and specificity. The test has been demonstrated to have sufficient intralaboratory and interlaboratory reproducibility and fulfill all requirements of international guidelines to be considered as clinically validated for screening purposes.

e. **Aptima HPV HR assay**: Aptima HPV HR assay (Gen-Probe, San Diego, Calif.) is the latest FDA-approved HPV test. FDA approved the assay for use with Gen-Probe's Tigris system, which is the only fully automated testing system for molecular diagnostics. Unlike other HPV tests mentioned above, Aptima HPV assay is a transcription-mediated amplification-based assay to detect E6/E7 mRNA transcripts of 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). This assay does not discriminate specific HPV genotypes. Aptima HR HPV assay includes three main steps: target capture by capture oligomers, target amplification by transcription-mediated amplification (TMA), and amplicon detection by Hybridization Protection Assay (HPA). The capture oligomers contain complementary sequences to specific regions of the high-risk HPV mRNA target sequence. After lysis of cells in collected specimens, target mRNA is released and captured by capture oligomers, which bind to specific regions of the high-risk HPV mRNA target molecule and form the capture oligomer/target complex. The capture oligomers are also linked to magnetic microparticles, which can fix the capture oligomer/target complex. Subsequently, the captured high-risk HPV mRNA is amplified by transcription-mediated amplification. The TMA reaction uses two enzymes: MMLV reverse transcriptase and T7 RNA polymerase. The MMLV reverse transcriptase is used to generate a DNA copy of the target high-risk HPV mRNA sequence containing a promoter sequence for T7 RNA polymerase. Subsequently, T7 RNA

polymerase produces multiple copies of RNA amplicon from the DNA copy template. Detection of the amplicon is achieved by Hybridization Protection Assay. The HPA uses chemiluminescent-labeled single-stranded nucleic acid probes that are complementary to the amplicon. Light emitted from the labeled RNA/DNA hybrids is measured as photon signals and reported as relative light units. An internal control is used for quality control. Test results are automatically analyzed by companion software by using signal-to-cutoff (S/CO). A test result may be negative, positive, or invalid (Gen-Probe 502182EN revision A, 2011).

Aptima HPV HR test are used with cervical specimens collected in either ThinPrep Pap test vials containing PreservCyt solution or Aptima Cervical Specimen Collection and Transport Kit. Before transferred to Aptima Specimen Transfer tube, PreservCyt liquid Pap specimens should be stored at 2–8 °C, with no more than 30 days at temperatures up to 30 °C. If a specimen collected in PreservCyt liquid has been transferred to an Aptima Specimen Transfer Tube, it may be stored at 2–30 °C for up to 60 days. If longer storage is needed, the PreservCyt liquid Pap specimen may be stored at −20 °C or colder for up to 24 months. If the specimens are collected and stored in the Aptima collection and transport kit, the specimens can be stored at 2–30 °C for up to 60 days. If long-term storage is needed, the specimens can be stored at −20 °C or colder for up to 24 months (Gen-Probe 502182EN revision A, 2011).

Aptima HPV HR assay detects target mRNA sequence, eliminating cross-reactivity with any tested high-risk HPV types or with normal flora and opportunistic organisms that may be found in cervical samples. Aptima HPV HR test also shows high sensitivity and specificity. At the CIN3+ end point, this assay is equally sensitive (95 %) as HC2, but is more specific than HC2 (Gen-Probe 502182EN revision A, 2011).

f. **APTIMA 16 18/45 genotype assay**: This test is a qualitative assay that specifically targets E6/E7 mRNA from HPV types 16, 18, and 45. The test is designed to distinguish type 16 from types 18 and 45 but cannot distinguish types 18 and 45 from each other. The genotype assay is indicated for management of

two patient populations with APTIMA HR-positive results, those who are 21 years of age or older with ASC-US cytology results and those who are 30 years of age or older. The genotype test also requires 1 mL of sample either taken as an aliquot before cytology processing or taken from the residual sample. Like the APTIMA HPV assay, the genotyping assay utilizes transcription-mediated amplification and chemiluminescent detection. Samples are interpreted as positive if the signal to cutoff ratio is Z1.0. Results may be reported as either positive for HPV 16, positive for HPV 18/45, or positive for HPV types 16 and 18/45. External quality controls are not included in the kit, but positive and negative calibrators are provided. Analytic sensitivity is <100 copies per reaction for each of the three targeted HPV types. No cross-reactivity with other HPV types is reported.

p16/Ki67 Immunostaining in Cervical Cytology

It has been clear that HPV viral oncoprotein E7 can bind and inactivate tumor suppressor pRB and then allow cell cycle progression through G1/S checkpoint [3, 4]. Upon HPV integration into host genome, E7 protein is highly expressed and stimulates enhanced expression of p16, which inhibits cyclin D-dependent kinase 4 and 6 (CDK4/CDK6) complex phosphorylation. Accumulating evidence suggests p16 expression in cervical epithelial cells is a valuable biomarker in detecting high grade cervical intraepithelial neoplasia and cervical cancers [5, 6]. Studies have shown diffuse p16 is seen in the majority of CIN2+ lesions, but not in benign and low grade lesions. In cells with HPV integration, binding E7 with Rb induces E2F release and cell cycle progression. Such active cell proliferation in HPV-related cervical intraepithelial lesions can be labeled and detected by overexpression of Ki-67 [6]. The data from Reuschenbach et al. showed the co-expression of p16 and Ki67 occurs in high grade dysplastic lesions, not in any benign lesions [7]. Several studies have shown a relative high sensitivity and specificity in detecting CIN2+ in both histologic and cytologic specimens by using the combination of p16 and Ki67

immunostains [6, 8, 9]. A recent large cohort study demonstrated p16/Ki67 immunostains has a higher sensitivity than Pap test and a significant higher specificity than hrHPV testing [10]. p16/Ki67 dual immunostains can be potentially to be used to triage those hrHPV positive cytology cases after primary screening, although the more data are needed to confirm its utility and practicality.

Other Molecular Biomarkers Applied in Cervical Cytology

1. **ProExC**: The ProEx™ C immunocytochemical assay is a commercial testing kit (TriPath Imaging, Burlington, NC) utilizing a cocktail of monoclonal antibodies against topoisomerase II (TOP2A) and minichromosome maintenance protein 2 (MCM2), which are overexpressed with aberrant S-phase cell cycle induction. MCM2 functions in DNA replication by linking pre-replication protein complex to DNA and unwinding DNA through helicase activity to permit DNA synthesis. TOP2A enzymatically unlinks DNA strands during DNA replication. Both proteins play roles in the regulation of DNA replication during S-phase and are overexpressed with aberrant S-phase cell cycle induction occurring in cervical dysplasia and cervical cancer [11]. Recent studies have demonstrated the usefulness of MCM2 and TOP2A in the evaluation of cervical biopsy specimens and cytological specimen [12–15] with a sensitivity of 78.6–85.3 % for HSIL detection and a specificity of 71.0–71.7 %.
2. **TERC (3q26) and CTNND2 (5p15.2) FISH**: E6 protein can stimulate the transcription of telomerase reverse transcriptase (TERT), which can repair repeated telomere DNA sequence in order to maintain telomere entireness. Studies have demonstrated gain of chromosome 3q and 5p in cervical cancer, which contains sequence for Telomerase RNA Component (TERC) and TERT, respectively. Specifically, the TERC gene amplification and chromosome polysomy have been associated with progression of low-grade lesions to high-grade lesions and cancer, while their absence has been associated with lack of progres-

sion and even regression [16–18]. A commercial available assay, Cervical BioStrat® Assay (BioVentra LLC), detects copy numbers of TERC (3q26.2) and CTNND2 (5p15.2) regions as well as enumeration of Chromosome 7 (CEP7) via fluorescence in situ hybridization (FISH) in liquid-based cervical cytology samples. A positive result with increased copy number of TERC and CTNND2 genes suggests a higher risk of progressing to high-grade lesions and invasive cancer [18, 19]. Patients with negative result have a lower risk of progression and could be followed up conservatively [17, 18]. A specimen is considered positive if greater than 1 % cells are detected with multiple copies of TERC (3q26) and/or CTNND2 (5p15) gene regions. Used in conjunction with Pap and HPV testing, the Cervical Bio*Strat*® Assay can assist in identifying which LSIL and ASCUS HPV+ patients may be at risk of progressing to severe dysplasia or cancer.

3. **HPV L1 Detection**. HPV L1 protein is the major capsid protein of HPV, which forms an icosahedral capsid with a $T=7$ symmetry and a 50 nm diameter. The capsid is composed of 72 L1 pentamers, linked to each other by disulfide bonds and associated with the minor capsid protein L2. L1 capsid protein is synthesized upon termination of productive phase of viral life cycle. L1 protein is produced within the cytoplasm and translocated into the nucleus of intermediate and superficial squamous epithelial cells, clear visible by strong, homogenous nuclear immunochemical staining. The nuclear staining of L1 protein leads to a very good interobserver reproducibility [20, 21] and the superficial epithelial cells containing L1 protein are easy to obtain by taking a smear. Multiple studies have demonstrated more HPV L1 protein expression in LSIL, but much less expression in HSIL [21–27]. Furthermore, both retrospective studies and prospective studies showed that high risk HPV associated mild and moderate dysplastic squamous lesions (CIN1 and CIN2) without immunochemically detectable HPV L1 protein progressed significantly more likely to CIN3 or invasive cancer than the L1 positive lesions [23–25]. In summary, it has been shown that 75 % of the L1-neagtive LSIL and 94.2 % of L1-negative HSIL progress to CIN3, compared to

only less than 20 % of L1-positive SILs. The high progression rate of L1-negative SILs emphasizes the precancerous nature of these lesions, suggesting a permanent infection with HPV genome integration into host genome. At the same time, low progression rate of L1-positive SILs indicates transient HPV infection without genome integration. The L1 protein detection assay can be easily performed on cervical cytological specimens and the ThinPrep imager can allow the automated examination of L1 stained slides with potential high throughout.

4. **Promoter methylation assays**: Chromosome remodeling (modification of histone and methylation of CpG islands of certain gene promoter regions) can occur in hrHPV infected cells. For example, E7 overexpression causes the modification of histone architecture of p16 locus, which results in p16 expression. Methylation has been evaluated for several host genes in cervical cancer, including cell adhesion molecule 1 (CADM1) gene and T-lymphocyte maturation associated protein (MAL). Recent studies have investigated methylation in these two gene promoter regions in cytologic specimens and found their association to CIN3+ lesions, with a sensitivity of 70 % and a specificity of 78 % [28, 29]. Hesselink A et al. found that CADM1 combined with MAL revealed CIN3+ sensitivities ranging from 100 % (95 % CI: 92.4–100) to 60.5 % (95 % CI: 47.1–74.6), with corresponding specificities ranging from 22.7 % (95 % CI: 20.2–25.2) to 83.3 % (95 % CI: 78.4–87.4) (41). In the other study by Vasiljević N et al. found a panel of six genes, including MAL, CADM1, and other four genes (EPB41L3, EDNRB, LMX1, and DPYS) showed significantly elevated methylation in CIN2 and CIN3 (CIN2/3) versus ≤CIN1. They also found that EPB41L3 methylation was the best single classifier of CIN2/3 in both HR-HPV positive and negative samples [30]. A recent study investigated Cyclin A1 gene promoter methylation status and they demonstrated cyclin A1 gene promoter hypermethylation to be commonly found in cervical cancer and be specific to the invasive phenotype in comparison with precursor lesions. None of the normal cells and low-grade squamous intraepithelial lesions exhibited Cyclin A1 methylation. In contrast, Cyclin A1 methylation was

identified in 36.6 %, 60 %, and 93.3 % of high-grade squamous intraepithelial lesions, microinvasive and invasive cancers, respectively [31].

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Molecular Diagnostics in Thyroid Cytopathology

5

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Key Points

1. Molecular testing of thyroid specimens has become an important adjunct to cytopathology for the management of patients with thyroid nodules.
2. The most widely adopted clinical application of molecular testing in thyroid cytopathology is the evaluation of thyroid FNA specimens with indeterminate cytopathology, which comprise approximately 15–30 % of cases.
3. When cytologically indeterminate thyroid nodules undergo diagnostic surgery, approximately three-quarters prove to be benign, highlighting the need for molecular approaches to identify the benign nodules among this group.
4. Several molecular approaches have been proposed for the evaluation of nodules with indeterminate thyroid cytopathology, including tests that "rule in" thyroid cancer and those that "rule-out" thyroid cancer.

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5. An mRNA expression analysis approach (the Afirma Gene Expression Classifier) demonstrates high sensitivity (90 %) and high negative predictive (NPV) value (≥94 %) and is best utilized to rule out thyroid cancer in cytologically indeterminate nodules.
6. Mutational testing approaches demonstrate high specificity and high positive predictive value (PPV), and are best utilized to rule-in thyroid cancer and inform the choice of surgery.
7. Guidelines, including The NCCN Thyroid Carcinoma Guidelines and UpToDate, suggest that cytologically indeterminate thyroid nodules determined to have a ROM similar to cytologically benign nodules with a molecular test can be clinically observed.
8. Many other applications for molecular testing in thyroid cytopathology are available or under development utilizing a variety of technologies for identification of specific tumor subtypes, prognosis of individual tumors, and prediction of response to targeted therapies.

Introduction

Thyroid Cancer

Thyroid cancer is the most rapidly increasing cancer in the USA, with about 63,000 new cases estimated for 2014. The majority of this increase is thought to be attributed to identification of greater numbers of smaller and nonpalpable thyroid nodules through more widespread use of thyroid ultrasound as well as incidental discovery of thyroid nodules through CT, PET-CT, and MRI imaging studies of the neck for non-thyroid indications. This increased nodule detection rate has led, in turn, to increased rates of surgery and identification of thyroid cancers, including many small tumors. Another factor thought to contribute to this increase in thyroid cancer incidence is the more frequent recognition of certain sub-types of thyroid cancer by pathologists, such as the follicular variant of papillary thyroid carcinoma (FVPTC), which previously was under recognized and therefore underdiagnosed.

Environmental factors, including radiation exposure, are also thought to play a role. Overall, it is likely that a combination of these factors has contributed to the observed increase in thyroid cancer incidence. Interestingly, the increased incidence of thyroid cancer has not led to a corresponding increase in the death rate from thyroid cancer, which has been fairly stable for many years.

For the practicing cytopathologist, the increased detection of thyroid nodules through ultrasound and other imaging modalities has led to a significant increase in the number of thyroid pathology specimens submitted for evaluation. This increase has also led to greater opportunities for the cytopathologist to apply molecular testing for the analysis of these specimens. The following chapter lays out the applications for molecular testing of thyroid nodules, along with the role and appropriate use of these tests in the management of patients with thyroid nodules.

Fine Needle Aspiration (FNA)

The cytopathologic interpretation of Fine Needle Aspiration (FNA) specimens has revolutionized the management of thyroid nodules since its introduction in the USA over 40 years ago. Before thyroid FNA, nodules were typically managed surgically due to the absence of other reliable methods to distinguish benign from malignant lesions. The adoption of FNA, performed with or without ultrasound guidance, in combination with cytopathologic evaluation has enabled definitive and accurate classification of the majority of thyroid nodules (approximately 70–80 %) into benign and malignant categories. Through the use of thyroid FNA, patients with benign cytopathology can be spared unnecessary surgery and managed conservatively while those patients with malignant cytopathology can be triaged for thyroidectomy.

What about the remaining 20–30 % of thyroid nodules that are not clearly benign or malignant? An additional 5–10 % do not contain sufficient cellularity for diagnosis (6 groups of at least 10 follicular cells) and are classified as non-diagnostic/unsatisfactory. Guidelines suggest that patients with non-diagnostic results should undergo repeat FNA after an appropriate period of approximately

three months. The remaining 15–20 % of thyroid nodules fall into a group of "indeterminate" diagnoses that are not clearly benign or malignant cytologically. Historically, this indeterminate group has included nodules carrying a variety of related diagnoses such as "follicular lesion," "cellular follicular lesion," "follicular (or Hurthle cell) neoplasm," "suspicious for follicular (or Hurthle cell) neoplasm," "suspicious for malignancy," and "suspicious for papillary thyroid carcinoma" along with diagnoses mentioning "atypia" or "atypical cells" in some fashion.

In 2008, The Bethesda System for Reporting Thyroid Cytopathology was released in an attempt to standardize the terminology for reporting thyroid FNA specimens and to link the categories with estimated risk of malignancy (ROM) and suggested clinical management. In this system, diagnoses fall into one of six diagnostic categories (Table 5.1): Nondiagnostic (I), Benign (II), Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance (AUS/FLUS) (III), Follicular Neoplasm/Suspicious for Follicular Neoplasm (FN/SFN) (IV), Suspicious for Malignancy (SFM) (V), and Malignant (VI). The so-called indeterminate categories in the Bethesda System include AUS/FLUS (III), FN/SFN (IV), and SFM (V).

The Challenge of Indeterminate Cytopathology

For nodules diagnosed as nondiagnostic (I), benign (II), or malignant (VI) by cytopathology, the clinical management is straightforward. However, for those with indeterminate diagnoses (III, IV, and V), management options are less well-defined and open to interpretation. Some guidelines suggest surgical management of all indeterminate nodules because of the unacceptably high risk of malignancy in these nodules. The Bethesda system, however, suggests a set of management options specific to each indeterminate category: repeat FNA for AUS/FLUS (III), surgical lobectomy for FN/SFN (IV), and lobectomy or total thyroidectomy for SFM (V). These suggestions are driven by an estimated ROM put forth for each of these categories (Table 5.1).

Table 5.1 The Bethesda System for reporting thyroid cytopathology

Diagnostic category	Expected rate of diagnosis	Expected risk of malignancy	Evidence of risk of malignancy	Suggested management
I. Nondiagnostic or Unsatisfactory	<10 %	1–4 %	0–50 %[3,6]	Repeat FNA with ultrasound guidance
II. Benign	60–70 %	0–3 %	0.9–18 %[2,3,5,6]	Clinical follow-up
III. Atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS)	3–7 %	5–15 %	7–48 %[2,3,4,5,6]	Repeat FNA
IV. Suspicious for follicular neoplasm	8–12 %	15–30 %	14–49 %[2,3,4,6]	Surgical lobectomy
V. Suspicious for malignancy	~3 %	60–75 %	53–87 %[2,3,6]	Near-total thyroidectomy or surgical lobectomy
VI. Malignant	3–7 %	97–99 %	93–100 %[2,3,5,6]	Near-total thyroidectomy

The recommended diagnostic categories are listed with the suggested rates of diagnosis, the expected risk of malignancy, and the recommend clinical management for each category. The column listing "evidence of risk of malignancy" summarizes the ranges in ROM seen for each diagnostic category in numerous studies following the publication of TBSRTC. (Modified from Ali and Cibas with kind permission from Springer Science and Business Media)

The suggested approach of repeat FNA for AUS/FLUS (III) has been questioned in several studies reviewing the ROM in nodules with an initial diagnosis of AUS/FLUS. These studies suggest that regardless of the diagnosis of the second FNA, the ROM of the nodule remains about the same as after a single AUS/FLUS result. In other words, even if the second FNA results in a benign cytopathology diagnosis, the ROM remains at or close to the ROM of the initial AUS/FLUS diagnosis and is not reduced to the level of risk of a single benign diagnosis (3–6 %). Furthermore, these and other studies have shown that nodules with AUS/FLUS diagnoses carry a higher risk of malignancy than anticipated in the Bethesda System, very similar to the ROM for FN/SFN nodules (~20–25 %, range 7–48 %; Table 5.1 column 4). Accordingly, reconsideration of the Bethesda recommendation for repeat FNA for most patients following an initial AUS diagnosis has been suggested.

Despite the great strides that TBSRTC has made in standardizing terminology and creating a uniform set of diagnostic criteria for each category, the reproducibility of cytopathology diagnosis remains relatively poor. In a recent prospective study where locally read cytopathology cases were re-read by expert cytopathologists (inter-observer concordance), concordance of diagnostic category, particularly among the indeterminate subtypes (Bethesda III–V) was low—35 % for AUS/FLUS, 66 % for FN/SFN, and 37 % for SFM (Fig. 5.1a, b). Similarly, diagnosis of cytopathology indeterminate subtype was not highly reproducible when cytopathology cases were re-read by the same observer (intra-observer concordance) at least 30 days apart—61 % for AUS/FLUS, 78 % for FN/SFN, and 46 % for SFM (Fig. 5.1a, c). These reproducibility studies suggest that the use of Bethesda subtype diagnoses to drive clinical recommendations may not be reliable, particularly among indeterminate subtypes.

As an alternative to repeat FNA or lobectomy/thyroidectomy for indeterminate nodules, including those with AUS/FLUS (III), FN/SFN (IV), and SFM (V) diagnoses, molecular testing approaches have been developed to assist in clinical management of this challenging group of nodules. These approaches, in their application to indeterminate subtypes as a group, overcome the issue of reproducibility of cytopathology diagnosis. As a group, they have

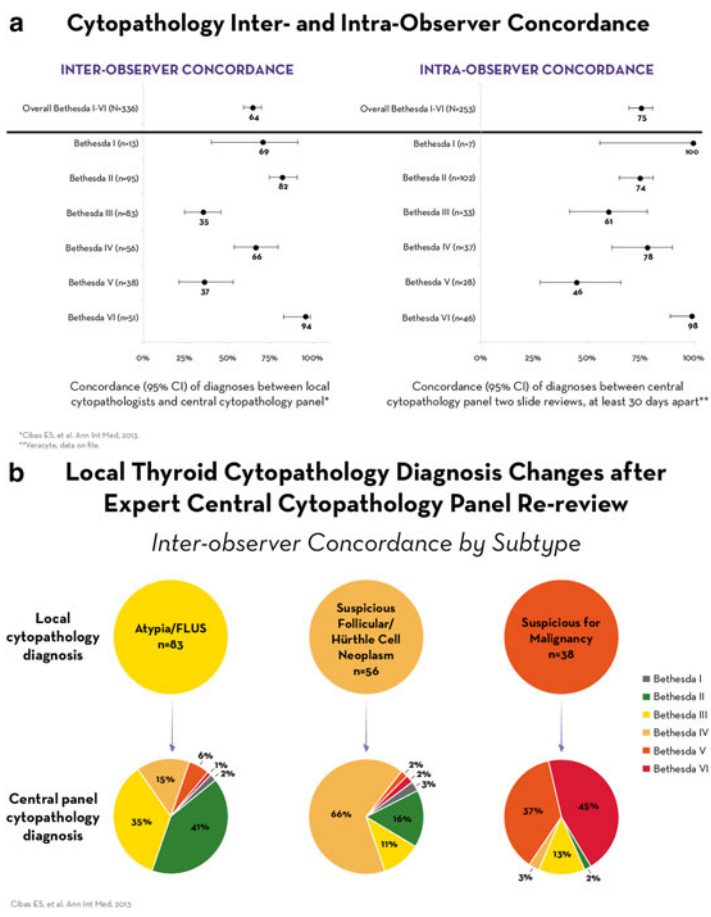

Fig. 5.1 (**a**) Overall cytopathology inter- and intra-observer concordance by Bethesda subtype. (**b**) Cytopathology inter-observer concordance by indeterminate subtype. (**c**) Cytopathology intra-observer concordance by indeterminate subtype

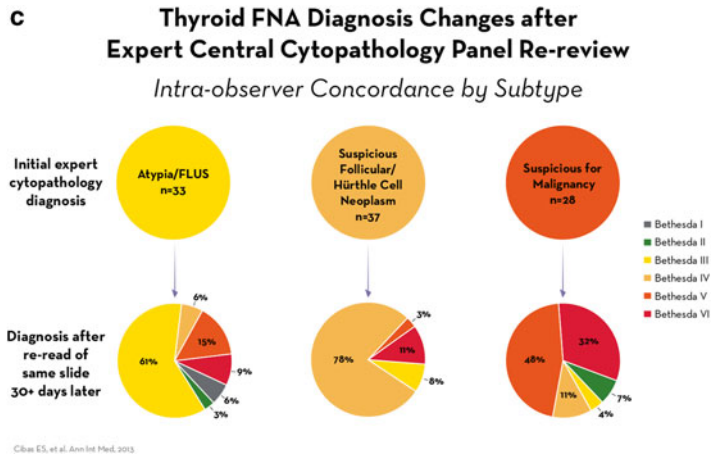

Fig. 5.1 (continued)

demonstrated the potential to identify very low risk nodules among those with indeterminate cytopathology and to thereby prevent many patients from undergoing unnecessary diagnostic thyroid surgery. In addition, some molecular tests have shown the potential to guide the choice of surgery (lobectomy vs. total thyroidectomy) in higher risk nodules, thus preventing fewer repeat/revision surgeries. The following section describes the molecular approaches being employed for the evaluation of indeterminate thyroid nodules and the optimal uses of each approach.

Rule In vs. Rule Out Tests for Evaluation of Thyroid FNAs

Overview of Rule In vs. Rule Out Tests

Historically, molecular testing in oncology has focused on the identification of DNA mutations/alterations in various cancers or cancer syndromes. For example, the presence of the *BCR-ABL* translocation in sampled leukemic cells is virtually pathognomonic for chronic myeloid leukemia, while the presence of specific

germline mutations in the *BRCA1* or *BRCA2* genes is diagnostic of Hereditary Breast and Ovarian Cancer syndrome (HBOC). These tests are examples of "rule in" tests—if positive, they are able to "rule in" a disease or syndrome. However, if negative, these tests do not necessarily "rule out" the possibility of a related disease or condition but rather simply the lack of the mutation being tested.

Rule out tests are much less common in oncology, particularly rule out molecular assays. Screening tests for serum tumor markers such as CEA, CA-125, and PSA are used by some clinicians to rule out specific types of cancer in some populations. Among molecular assays, HPV DNA testing of cervical cytology specimens can serve as a rule out test for cervical dysplasia as it has high sensitivity and high NPV—if HPV testing is negative, there is a very low likelihood of cervical dysplasia.

In thyroid cytopathology, as mentioned, the primary application for molecular testing is the evaluation of cytologically indeterminate nodules. Both rule in and rule out approaches have been developed for the evaluation of these ambiguous nodules. When applied to indeterminate thyroid nodules, the rule in tests look for presence of thyroid cancer by identifying mutations highly correlated with thyroid malignancies. In contrast, the rule out tests utilize an approach that is designed to look for the presence of benign genetic patterns in cytologically indeterminate nodules rather than the absence of specific mutations. This novel approach represents a paradigm shift in molecular oncology testing, from confirmation of malignancy through the identification of specific mutations to confirmation of benignity through the identification of specific benign genetic signatures.

Rule-In Tests

Somatic Mutations and Gene Rearrangements

A number of point mutations and gene rearrangements have been identified in thyroid cancer and can be assessed in thyroid FNA specimens. The presence of some of them conveys a near certainty of thyroid cancer, while the finding of others only raises the probability of thyroid cancer but does not exclude the possibility of a

benign tumor. Therefore, some mutations are better rule in tests than others. The most common mutations in thyroid cancer include those in the *BRAF* and *RAS* genes along with *RET/PTC* and *PAX8/PPAR-γ* rearrangements. A variety of techniques can be used for the identification of specific mutations in *BRAF* and *RAS* genes in genomic DNA purified from thyroid FNA samples, including real-time PCR (RT-PCR) assays, PCR amplification followed by DNA sequencing, and Next Generation Sequencing (NGS) approaches, among others. In the case of gene rearrangements, similar approaches can be employed following isolation of mRNA from FNA samples and conversion to cDNA.

BRAF

BRAF mutations are the most common mutations in thyroid cancer. The vast majority lead to the replacement of a valine amino acid by a glutamic acid residue at position 600 (V600E), a mutation that leads to constitutive activation of the MAPK pathway. This mutation occurs in thyroid cancers of follicular origin including papillary thyroid carcinoma (PTC), poorly differentiated thyroid carcinoma, and anaplastic thyroid carcinoma (ATC); it does not occur in follicular thyroid carcinoma (FTC) or medullary thyroid carcinoma (MTC). Among PTC and ATC, the mutation is present in about 45 % and 25 %, respectively. Among indeterminate thyroid nodules (Bethesda III and IV), the prevalence of *BRAF* mutations is low, ~2–3 %. This low incidence is not unexpected as these diagnostic categories include malignancies that less commonly carry *BRAF* mutations such as FTC and Hurthle cell carcinoma, FVPTC, and MTC. The low incidence is also explained by the finding that *BRAF*-mutated PTCs have cytologic features that cytopathologists typically recognize and classify as suspicious for malignancy or malignant (Bethesda V and VI). As a result of the low incidence of *BRAF* mutations, *BRAF* testing is not a good rule out test for thyroid cancer among Bethesda III and IV nodules. However, when the mutation is present in an indeterminate nodule, it is virtually diagnostic of malignancy as a result of the high PPV and specificity of the assay.

RAS

The *RAS* proto-oncogene encodes three different small GTPase proteins, HRAS, KRAS, and NRAS, involved in several intracellular signal transduction pathways, including the MAP kinase pathway. Mutations in the GTPase domain of the RAS proteins lead to constitutive activation of the proteins. The most common mutations involve codons 12, 13, and 61 for *KRAS* and *HRAS*, and codon 61 for *NRAS*. *RAS* mutations are highly prevalent in FTC and FVPTC (40–50 %), although very rare in PTC (10 %). *RAS* mutations are also relatively common in benign follicular adenomas (20–40 %), although it is unclear whether these *RAS*-positive adenomas are premalignant and have a higher risk of cancer progression. Overall, the prevalence of *RAS* mutations in indeterminate thyroid nodules (Bethesda III and IV) is approximately 12 %. However, as a result of the relatively high rate of *RAS* mutations in benign nodules, *RAS* mutational analysis is not an optimal test to predict malignancy in indeterminate thyroid nodules when performed as a stand-alone assay.

PAX8/PPAR-γ

The *PAX8/PPAR-γ* gene rearrangement resulting from the chromosomal translocation t(2;3)(q13;p25) is the second most common mutation in FTC (23–63 %). It is also found in approximately 5 % of Hurthle cell carcinomas and 2–10 % of follicular adenomas. Tumors with *PAX8/PPAR-γ* mutations rarely harbor concurrent *RAS* mutations, suggesting that FTCs develop through at least two distinct molecular pathways involving either *RAS* or *PAX8/PPAR-γ* mutations. *PAX8/PPAR-γ* mutated tumors tend to present at a younger age, to be smaller in size, to show a solid growth pattern, and to demonstrate vascular invasion as compared to follicular carcinomas that are negative for this mutation. Overall, the prevalence of *PAX8/PPAR-γ* mutations in indeterminate thyroid nodules (Bethesda III and IV) is very low, less than 1 %.

RET/PTC

RET/PTC gene rearrangements result from the fusion of the 3′ end of the *RET* gene and the 5′ end of various unrelated "*PTC*" genes. While there are over 12 types of *RET* rearrangements,

approximately 80 % are represented by *RET/PTC1* and *RET/PTC3*. As a result of *RET/PTC* rearrangements, the portion of the *RET* gene encoding the tyrosine kinase domain is fused in frame with an active promoter of the fusion partner gene. Consequently, the truncated RET receptor tyrosine kinase becomes constitutively expressed and activated, stimulating signaling of the MAP kinase pathway. *RET/PTC* rearrangements are present in approximately 20 % of PTC. They are more common in PTCs of children, young adults, and patients with a history of radiation exposure. Among indeterminate thyroid nodules (Bethesda III and IV), the prevalence of *RET/PTC* rearrangements is quite low, ~1 %. When one of the rearrangements is present, however, it is virtually diagnostic of malignancy.

Other Genes

In addition to the most common mutations in thyroid cancer described above (*BRAF*, *RAS RET/PTC* and *PAX8/PPAR-γ*), a variety of less frequent mutations involving the *TP53, PIK3CA, AKT1, CTNNB1, PTEN, GNAS, RET*, and *TSHR* genes have been identified. A role for *RET* mutations in the development of MTC is well-defined and discussed later in the chapter in the context of the MEN2 syndromes. However, further studies are needed to determine the role of mutations in many of these other genes, if any, in various thyroid cancers, even if they have well-defined roles in other malignancies. As is the case for many *RAS* mutations, other genes including *TSHR, PTEN,* and *GNAS* can be mutated in benign thyroid nodules.

Mutation Panels

Four Mutation Panel Testing

Individual DNA mutations such as those in *BRAF, RAS*, and *RET/PTC* and *PAX8/PPARG*, when performed singly, have relatively high PPVs and specificities—when detected, they accurately predict (rule in) the histological diagnosis of thyroid cancer in most cases. However, when individual mutations are absent, cancer cannot be reliably ruled out because of the low sensitivity and NPV of these markers.

What if these markers are assessed together? Several laboratories have taken this approach and offer this set of molecular markers as a combined four mutation panel for the evaluation of indeterminate thyroid FNA specimens. Furthermore, a number of studies looking at the combined performance of these markers have been conducted. A review of four studies analyzing the four mutation panel showed a mean sensitivity of 64 % for indeterminate thyroid FNAs, indicating a failure of the panel to identify 36 % of thyroid cancers in the indeterminate group. The largest study of the four mutation panel to date involved a retrospective analysis of prospectively collected thyroid FNA samples in which the mutation status was known to the clinicians, including the pathologists. The NPV of the four mutation panel for AUS/FLUS (Bethesda III) and FN/SFN (Bethesda IV) categories was 94 % and 86 %, respectively. However, the prevalence of malignancy in the Bethesda category III group (14 %) was lower than that seen in most other studies (~20–25 %). When a more typical prevalence of malignancy for AUS/FLUS of 24 % is applied, the resultant NPV declines to 89 %. A recent prospective blinded clinical validation study of the four mutation panel, the first of its kind, demonstrated a sensitivity and specificity for detection of thyroid cancer of 47 % and 87.5 % respectively in nodules with indeterminate (Bethesda III/IV) cytopathology. Given this sensitivity, the four mutation panel failed to identify 53 % of thyroid cancers. The NPV of the four mutation panel for Bethesda categories III and IV was 70 % with a prevalence of malignancy of 41 %. When a more typical prevalence of malignancy of 24 % is applied, the resultant NPV increases to 83 %.

Overall, the four mutation panel approach does not provide a sufficiently high NPV to be used as a stand-alone test to rule out cancer in indeterminate thyroid nodules and therefore to allow for conservative management and avoidance of surgery in this patient population. On the other hand, the four mutation panel serves as an excellent rule in test for thyroid cancer as a result of its high specificity and PPV. In cases where the decision has been made for surgery, but the extent of surgery may be influenced by the test result, the four mutation panel can provide valuable guidance. For example, total thyroidectomy, rather than lobectomy, might be

chosen for mutation positive nodules. By performing initial total thyroidectomies in patients with indeterminate cytology when a DNA mutation is present and thereby reducing the number of completion thyroidectomies, one group has reported the possibility of overall cost savings with the four mutation panel approach.

Next Generation Sequencing

NGS is an approach that allows for the simultaneous sequencing of thousands to millions of short nucleic acid sequences in a parallel fashion. Advances in NGS technology have led to dramatic price reductions in recent years to the point where the technology now offers a cost-effective approach relative to conventional sequencing technologies for some applications. NGS permits targeted sequencing of multiple genes or mutations, an approach that is becoming increasingly common for analysis of various tumors, including thyroid cancers. Cancer mutation panels are available on all of the major NGS platforms and include most of the common oncogenes and tumor suppressor genes implicated in the spectrum of human cancers. While the panels are being offered by individual clinical laboratories as laboratory developed tests (LDTs), only a single platform, Illumina's MiSeqDx instrument has been cleared by the FDA for clinical use.

For thyroid FNA analysis, NGS has the ability to detect both the mutations in the four mutation panel as well as other mutations from small amounts of starting material. However, simultaneous identification of both point mutations and gene rearrangements involved in thyroid cancer in a single assay has not yet been demonstrated. Point mutations are best identified through NGS analysis of genomic DNA (DNA sequencing), while translocations are best identified through NGS analysis of mRNA (RNA sequencing through targeted transcriptome analysis) or traditional RT-PCR approaches. An NGS approach called Thyroseq developed at the University of Pittsburgh Medical Center can simultaneously detect 284 mutations in 12 key cancer genes (*AKT1, BRAF, CTNNB1, GNAS, HRAS, KRAS, NRAS, PIK3CA, PTEN, RET, TP53,* and *TSHR*). In a recent study examining DNA from 228 benign and malignant samples from both surgical thyroid specimens as well as thyroid FNAs with follow-up surgical diagnosis, Thyroseq

identified at least one mutation in 68 % of all thyroid tumor types, including 19/27 PTCs (70 %), 25/30 FVPTCs (83 %), 14/18 FCs (78 %), 7/18 (39 %) Hurthle cell carcinomas, 3/10 (30 %) poorly differentiated carcinomas, 20/27 ATCs (74 %) and 11/15 (73 %) MTCs. Notably, 6 % (5/83) of the benign nodules were positive for mutations in the *RAS*, *TSHR, PTEN,* and *GNAS* genes, demonstrating that not all mutations are associated with thyroid cancer. No specific analysis of the performance of Thyroseq on indeterminate thyroid FNA samples was performed. In this regard, a second recent NGS analysis of mutations in 50 genes retrospectively assessed 34 indeterminate FNA samples with surgical follow-up. Mutations in *BRAF*, *NRAS, KRAS,* and *PTEN* were detected in 7/34 indeterminate FNA samples. The NGS test sensitivity and NPV were 71 % and 92 % respectively.

In summary, NGS approaches can be applied successfully to the analysis of thyroid FNA and other thyroid specimens and appear to be an effective rule-in test for thyroid cancer. When combined with assays to detect gene fusions, such as those of *RET/PTC* and *PAX8/PPAR,* through the use of RT-PCR or targeted NGS analysis of mRNA, the sensitivity for detection of thyroid cancer is expected to increase beyond the observed 68–71 % seen in initial studies, possibly to more than 80 %. However, the use of NGS as a rule out approach for indeterminate thyroid nodules requires further investigation to demonstrate clinical validity, including prospective studies of larger numbers of indeterminate FNAs with blinded and therefore unbiased surgical pathology follow-up.

MicroRNA Analyses

MicroRNAs (miRNAs) are small single stranded noncoding RNA sequences (19–25 nucleotides) that function to regulate gene expression. MiRNAs function via sequence-specific interaction with mRNA targets, binding to the 3′untranslated region and thereby suppressing translation and mRNA degradation. In addition to their function in regulation of mRNA, miRNAs have also been shown to function as tumor suppressors or oncogenes in tumor cells. MiRNAs can be detected through various molecular approaches, including microarrays and RT-PCR assays. Working with microRNAs affords several advantages over other nucleic

acids including high-stability, ability to isolate from FFPE tissues, and low input requirements for PCR assays.

In thyroid cytopathology, several studies have suggested that aberrant miRNA expression profiles may separate thyroid cancers from benign thyroid lesions. As the studies have looked at different miRNA panels, it is difficult to directly compare test performance. The most promising study assessed four miRNAs (miR-222, miR-328, miR-197, and miR-21) in 72 indeterminate thyroid FNA specimens. For the differentiation of benign from malignant thyroid nodules, sensitivity was 100 % and PPV was 90 %. These results demonstrate the promise of the miRNA approach in differentiating benign from malignant lesions in indeterminate thyroid FNA specimens. Although promising, the miRNA panels have yet to be tested on indeterminate thyroid FNAs in large, prospective, blinded, multicenter studies, and more clinical data is needed prior to the use of this approach in the clinical management of patients with indeterminate thyroid nodules. At this point, it is unclear whether miRNA panels will be most useful as a rule in or rule out approach.

Rule-Out Tests

Afirma Gene Expression Classifier (GEC)

An alternative to the rule in approach of mutation panels for the evaluation of cytologically indeterminate thyroid nodules is the rule out approach of mRNA expression analysis exemplified by the Afirma Gene Expression Classifier (GEC). Unlike the mutation panels, the GEC utilizes an approach that is designed to look for the presence of benign mRNA expression patterns in cytologically indeterminate nodules rather than the absence of specific mutations.

There are two key advantages to examining mRNA rather than DNA to distinguish benign from malignant lesions in indeterminate thyroid FNAs. First, while there are ~ 23,000 known protein-coding DNA genes, each of these may be transcribed into multiple alternatively spliced variants, with > 240,000 known mRNA isoforms. Disease-causing alterations in the DNA generally exert their effects, at least partially, on transcription, resulting in

downstream changes in the expression levels of multiple mRNA transcripts that can be measured. Second, gene expression may be impacted by lifestyle and environmental factors such that mRNA reflects additional information not discernible from DNA analysis. Thus, mRNA expression analysis has an advantage over mutation analysis in identifying gene signatures that reflect whole patterns of pathway activation resulting from both upstream mutations and environmental factors rather than alterations in a small number of genes.

The GEC was developed and clinically validated to identify benign nodules amongst those with indeterminate cytology preoperatively. Rather than relying on genes previously implicated in thyroid tumorigenesis, the design and development of the test used analysis of the whole exome to identify candidate genes most informative for the prediction of benign signatures. The resulting GEC evaluates the expression levels of 167 genes on an mRNA microarray platform that are then analyzed with a proprietary algorithm to classify indeterminate thyroid FNAs as either "Benign" or "Suspicious." Unlike some of commercially available four mutation panels for the evaluation of indeterminate thyroid nodules, extensive reagent and analytical performance studies of the GEC have been performed and published, demonstrating the reliability, robustness, and reproducibility of the assay under a variety of experimental conditions.

The GEC has been clinically validated in two independent prospective multicenter, double blind studies. The initial clinical validation publication of the GEC, performed on a set of 24 cytologically indeterminate thyroid nodule FNAs, achieved high sensitivity (100 %) and NPV (100 %). The second larger study included the largest ever prospectively collected set of thyroid FNA specimens from 3789 unique patients and 49 sites representing a mix of academic and community practices across the USA. In this study, follow-up surgical pathology was available for 265 cytologically indeterminate nodules (Bethesda III and IV). Performance of the GEC was determined by comparison of the molecular results to the surgical pathology diagnoses for each nodule based on review by a panel of thyroid experts including Dr. Juan Rosai and Dr. Virginia LiVolsi. The study demonstrated a reduction in the

ROM of cytologically indeterminate thyroid nodules (Bethesda III and IV) with "Benign" GEC (negative test) results from ~24 to ~5 %. For "Suspicious" GEC (positive test) results, the ROM of cytologically indeterminate thyroid nodules (Bethesda III and IV) was increased from ~24 to ~40 %.

Overall, the large clinical validation study of the GEC demonstrated the ability of the test to dramatically reduce the ROM for AUS/FLUS and FN/SFN subtypes to a ROM similar to that of a cytology benign diagnosis, about 5 %. In essence, the study showed the effectiveness of the GEC as a rule out test for thyroid cancer in cytologically indeterminate nodules to justify conservative management in lieu of diagnostic surgery. However, the study also concluded that the reduction in ROM for nodules classified as SFM (Bethesda V) from 62 to 15 % with Benign GEC results was insufficient to merit routine use of the test for this indeterminate subtype.

Is the test useful in the clinical setting in identifying benign nodules and preventing unnecessary surgeries? This question has been addressed by several groups who have reported their clinical experience with the GEC in routine clinical practice. In the two largest series, GEC testing of indeterminate nodules (Bethesda III and IV) led to benign results result in just over 50 % of the cases; patients with GEC Benign results were managed conservatively, with observation *in lieu* of operation, 92–94 % of the time. Most GEC benign patients in the clinical series reported to date did not undergo surgery, consistent with the purpose of the test.

An algorithm for the rule out approach of the GEC and potential clinical utility is highlighted in Fig. 5.2. Based on the 2012 estimate of 525,000 annual thyroid nodule FNAs performed in the USA and an indeterminate rate of 15–30 % (~79,000–158,000 nodules), the GEC is predicted to reclassify ~50 % of these cytologically indeterminate nodules as "Benign" (39,500–79,000 nodules). These GEC Benign nodules have a similar ROM (~5 %) as nodules with cytology benign diagnoses and are candidates for conservative management ("watchful waiting"), leading to reduction of a large number of unnecessary thyroidectomies as well as a reduction in overall health care costs. Nodules with "Suspicious" results following GEC testing carry an elevated ROM (~40 %) and are candidates for thyroid surgery, along with nodules carrying cytologic diagnoses of SFM and M (Bethesda V and VI).

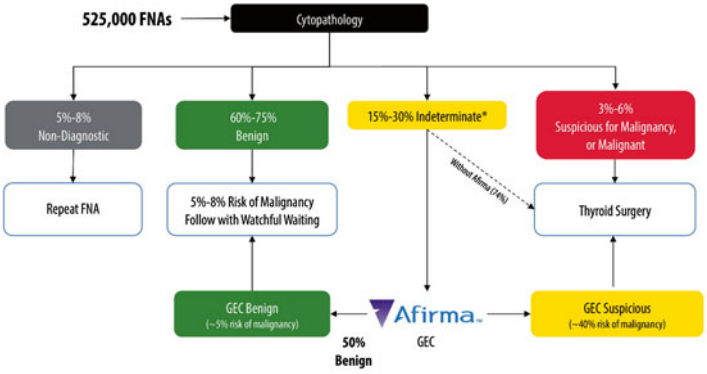

Fig. 5.2 Proposed clinical algorithm for use of the Afirma Gene Expression Classifier

Guidelines and Clinical Applications for Molecular Testing

As the use of molecular testing in thyroid cytopathology has become more widely adopted, particularly for the evaluation of cytologically indeterminate nodules, several organizations and publications including the National Comprehensive Cancer Network (NCCN) and UpToDate have included recommendations for molecular testing in their guidelines for the treatment of patients with thyroid nodules. The American Thyroid Association (ATA) and the American Association of Clinical Endocrinologists (AACE) have also recently commented on molecular testing in the context of thyroid nodule management.

NCCN

In the 2014 NCCN Guidelines (Version 2.2014) for Thyroid Carcinoma-Nodule Evaluation, the authors state: "Molecular diagnostics may be useful to allow reclassification of follicular lesions (i.e., follicular neoplasm, Hurthle cell neoplasm, atypical of undetermined significance (AUS), follicular lesions of undetermined

significance (FLUS) as they are more likely to be benign or more likely to be malignant. If molecular testing predicts a risk of malignancy comparable to the risk of malignancy seen with a benign FNA cytology (approximately 5 % or less), consider observation."

UptoDate

In the 2013 Practice Recommendation for the "Diagnostic Approach to and treatment of thyroid nodules," the authors note that for nodules with AUS/FLUS or FN/SFN cytopathology (Bethesda III and IV), "there are two approaches to the molecular characterization of FNA aspirates that are commercially available in the USA: identification of particular molecular markers of malignancy, such as BRAF and RAS mutational status, and use of high density genomic data for molecular classification (an FNA-trained mRNA classifier). The mRNA classifier measures the activity levels of 167 genes within the nodule (using the FNA aspirate). We favor using an mRNA classifier system (gene expression classifier), when available. Where available, we suggest using this classifier for evaluating patient with FNA cytology showing follicular lesion/atypia of undetermined significance or follicular neoplasm."

ATA

Although new guidelines have not been released since 2008 at the time of publication, the ATA released a draft of its proposed guidelines in June 2014 at the Endocrine Society's 96th Annual Meeting in 2014. Regarding the use of molecular markers to guide decision making in thyroid nodule management, the authors made the distinction between tests with high sensitivity and NPV and those with high specificity and PPV. They further noted that molecular markers were best used for cytological indeterminate nodules (AUS/FLUS and FN/SFN, Bethesda III/IV) in combination with clinical and sonographic features. For patients with a preference of conservative (nonoperative) management, a molecular test with

high sensitivity and NPV was recommended. For patients with a preference for surgical excision, a molecular test with high specificity and PPV was recommended, assuming it would influence the extent of surgery (hemi vs. total).

AACE

While guidelines have not been updated since the 2010 release of the AACE/AME/ETA Thyroid Nodule Guidelines, AACE recently released a "commentary" on molecular testing of thyroid nodules with indeterminate cytopathology. In this commentary, it was noted that "two principal tests are currently marketed for use to improve the malignancy risk assessment of 'indeterminate' thyroid nodules. 'Rule In' and 'Rule Out' tests attempt to confirm or exclude the presence of cancer within a thyroid nodule by means of robust positive (PPV) or negative predictive values (NPV), respectively. The Rule In tests determine the presence of single gene point mutations (*BRAF*V600E or *RAS*) or gene rearrangements (*RET*/*PTC*, *PAX8*/*PPAR*γ) that have been shown to increase the ability to predict cancer, while the Rule Out test (Afirma® gene expression classifier, GEC) utilizes a proprietary gene expression classifier (RNA expression) specifically designed to maximize the ability to define a process as benign. At present, molecular testing is meant to complement and not replace clinical judgment, sonographic assessment, and visual cytopathology interpretation."

Summary and Recommendations for Cytopathologists

These guidelines discuss the two main types of molecular testing for indeterminate thyroid nodules:

1. The "Rule In"/high specificity and PPV tests (the four mutation panel).
2. The "Rule Out"/high sensitivity and NPV tests (the Afirma GEC).

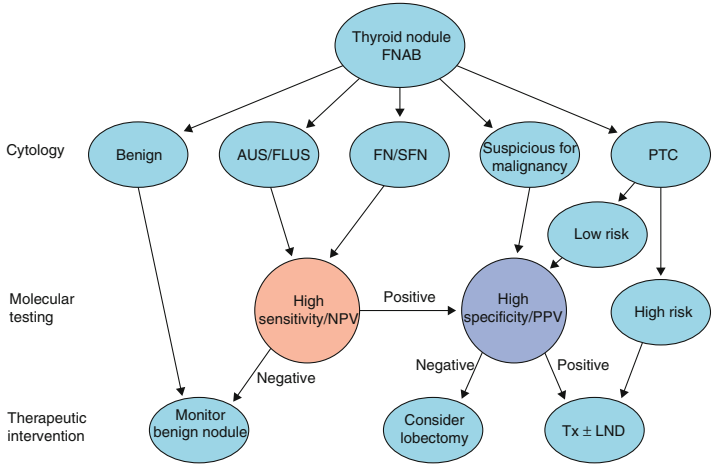

Fig. 5.3 Proposed clinical algorithm for the management of thyroid nodules on the basis of FNA cytopathology and molecular tests (kindly reproduced with permission from Lancet)

As a group, they recommend that the Rule Out approach should be used as a complement to clinical judgment, sonography, and cytopathology for evaluation of cytological indeterminate nodules (AUS/FLUS and FN/SFN, Bethesda III/IV) for patients with a preference of conservative (nonoperative) management and that such management can be considered if the result predicts a ROM comparable to the that of a benign FNA cytology. On the other hand, they suggest that the Rule In approach, as a high specificity and PPV test, is not appropriate for use as a Rule Out Test. They recommend that the Rule In approach be used in the context of patients undergoing surgery to assist in planning the extent of surgery (hemi vs total thyroidectomy).

A recent review elegantly summarizes these approaches in a proposed clinical algorithm for the management of thyroid nodules (Fig. 5.3). In this algorithm, patients with cytologically indeterminate nodules falling in the AUS/FLUS and FN/SFN categories undergo testing with a "high sensitivity/NPV" test (ie, the GEC). Those with negative/benign results proceed to conservative management/monitoring, while those with suspicious/positive results

are then candidates for further testing with a "high specificity/PPV" test (ie, BRAF, four mutation panel) along with those with "Malignant (PTC)" or "Suspicious for Malignancy" cytological diagnoses. If the high specificity/PPV test is negative, lobectomy is recommended; if positive, total thyroidectomy is recommended (with or without lymph node dissection).

Given that the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) was released before these molecular approaches became widely available, the management "suggested" for AUS/FLUS and FN/SFN (Bethesda III/IV categories) should be revised to include molecular testing, particularly rule-out tests such as the GEC. Cytopathologists, in light of their greater understanding of diagnostic testing and molecular diagnostics in general, can assist greatly in educating endocrinologists, radiologists, surgeons, and other physicians managing patients with thyroid nodules in the selection of the appropriate tests for the evaluation of indeterminate thyroid nodules.

Other Applications of Molecular Testing in Thyroid Cancer and Future Directions

Introduction

In addition to the ability to influence treatment decisions in patients with indeterminate nodules, there are several other compelling current and future applications for molecular testing in thyroid cytopathology These include tests to predict tumor response to specific therapies ("companion diagnostics") as well as tests to provide information on prognosis, tumor subtype, and recurrence. Some of the questions molecular testing can address include:

What is the risk of recurrence?
What is the tumor related mortality?
Should radioactive iodine be used? If so, at what dose?
What therapeutic drug targets are present and mutated?
What therapeutic or combination of therapeutics should be given?
What is the type and subtype of thyroid cancer?
What surgery is most appropriate?
Has the tumor recurred? If so, has the tumor changed?

Prognostic Markers

Given that most thyroid cancers are curable, even in the context of metastatic or recurrent disease, a major challenge in thyroid cancer is determining the extent and aggressiveness of therapy. There is a clear need for prognostic markers to guide treating physicians in the type and extent of treatment, including type and extent of surgery and RAI (including dose). The choice of initial surgery can impact whether a patient requires additional surgery or thyroid hormone replacement while the choice to use RAI has consequences relating to side effects and can determine how much can be given in the future, in the case of recurrent disease.

BRAF

In addition to their role as "rule-in" tests in the diagnosis of thyroid cancer, mutational markers have been linked to tumor behavior and prognosis. The *BRAF* V600E mutation in particular has been the subject of numerous studies. Many have shown an association of the V600E mutation with aggressive histopathologic features in papillary carcinomas, such as extrathyroidal extension, lymph node metastasis, tumor size, multifocal disease, and increased tumor stage along with an increased incidence of tumor recurrence of tumor-related mortality. As a result of these findings, many clinicians choose to treat *BRAF* V600E-positive tumors more aggressively. For example, if patients with *BRAF* V600E-positive tumors are detected preoperatively, they may benefit from more extensive initial surgery. Other studies have not shown a definite association of the *BRAF* V600E mutation with a negative prognosis. The reason for the variability in findings relating to prognosis is not known. However, it is possible that rather than the presence of *BRAF* V600E alone it if the coexistence of *BRAF* V600E with other mutations that more accurately determines prognosis.

Other Mutational Markers

The prognostic role of other mutational markers is less clear than for *BRAF*. Controversial data have been reported for the prognostic role of *RET/PTC* rearrangements in PTC. *RET/PTC3* has

been correlated with more aggressive histopathologic features, specifically a larger tumor size, solid variant, and a more advanced stage at diagnosis. In contrast, *RET/PTC1* rearrangement does not appear to correlate with any clinicopathologic characteristics of PTC. Overall, there is no consensus regarding the clinical prognostic value of the presence of *RET/PTC* rearrangements at this time. Similarly, *PAX8/PPAR-γ* mutated tumors have been correlated in some studies with a younger age, a smaller size, a solid growth pattern, and an increased incidence of multifocal capsular invasion or vascular invasion as compared to follicular carcinomas that are negative for this mutation. However, there is no evidence that *PAX8/PPAR-γ* status predicts outcome in follicular thyroid cancer. Larger and more comprehensive outcome analysis will be necessary to better define the prognostic value of both *RET/PTC* and *PAX8/PPAR-γ* rearrangements in thyroid cancer.

Summary

In summary, the only well-established prognostic marker in thyroid cancer is the *BRAF* V600E mutation, which appears to predict more aggressive disease and is being used to inform decisions on the extent of surgery and treatment. In guiding clinical colleagues, the cytopathologist should advise treating physicians that preoperative *BRAF* testing may be indicated if the result would impact the choice of surgery.

Predictive Markers

Response to RAI

First line therapy for differentiated thyroid carcinoma following thyroidectomy is RAI. At this time, there are no molecular markers that predict response to RAI. Rather, response to RAI is predicted by avidity of thyroid tumors to iodine determined through RAI scans. If available, such markers would be useful in guiding decisions on treatment and, for those predicted not to be responsive, sparing unnecessary radiation exposure and its side effects as well as associated costs.

Companion Diagnostics and Therapeutic Targets

Most patients (~85 %) with differentiated thyroid carcinomas are cured with surgery, RAI, and TSH suppression. A small percentage of patients develop or present with metastases and are more difficult to treat. When metastases have RAI avidity, prognosis is better, and further RAI may be used. However, when multiple doses of RAI have been tried or the patient has non-RAI avid disease, other options such as systemic therapy with targeted agents or cytotoxic chemotherapy are needed.

In such situations, drugs targeting tyrosine kinases (tyrosine kinase inhibitors) such as sorafenib, sunitinib, pazopanib, lenvatinib, and vandetanib have shown promise. The targets of these drugs include VEGFR1, VEGFR2, VEGFR3, RET, FGFR1, PDGFR-Beta, c-kit, and BRAF. To this point, only sorafenib has been approved by the FDA for the treatment of patients with locally recurrent or metastatic, progressive differentiated thyroid cancer that no longer responds to RAI treatment. Furthermore, companion diagnostic molecular tests assessing the mutational status of these tyrosine kinases have not been developed for selection of patients likely to respond to sorafenib, other TKIs, or other therapeutic targets in thyroid cancer such as EGFR, histone deactylases, PPARγ, and cyclooxygenase 2. The identification of specific mutations in the genes encoding these proteins that confer either responsiveness or resistance to specific targeted agents promises to advance the effectiveness of these treatments in the future.

Physicians have been especially interested in finding drugs to treat MTC, as thyroid hormone-based treatments (including RAI) are not effective against these cancers. Both vandetanib and cabozantinib are targeted TKIs approved by the FDA for the treatment of patients with advanced MTC. To this point, companion diagnostics have not been developed for stratification of MTC patients into groups of responders vs. non-responders for these therapeutics. However, the existence of these agents for MTC highlights the need for accurate diagnosis.

Tumor Subtyping

Identification of Medullary Thyroid Carcinoma and Other Thyroid Cancer Subtypes

The cytological diagnosis of MTC is challenging as it is uncommon and its cytological features overlap with those of other thyroid neoplasms, including follicular neoplasms and Hurthle cell neoplasms. In approximately 50 % of cases, cytopathology may not make the specific diagnosis of MTC, instead labeling FNAs as indeterminate (AUS/FLUS or FN/SFN) or malignant/suspicious for malignancy without raising the possibility of MTC. A preoperative diagnosis of MTC impacts the patient preoperative evaluation, including evaluation for multiple endocrine neoplasia type 2 (MEN2) and associated *RET* mutation status, concomitant pheochromocytoma and hyperparathyroidism. Additionally, surgical management is altered to include a minimum of total thyroidectomy and central neck dissection. When MTC is not identified preoperatively, inappropriate surgery is often selected with less than half (46 %) of MTC patients receiving the optimal initial surgery. As a consequence, patients with MTC often face potential second surgeries for removal of the remaining thyroid and performance of a central neck dissection, with associated cost, risks, diagnostic delays, and patient anxiety.

As the preoperative identification of MTC is crucial for clinical management, preoperative MTC testing is appropriate in some circumstances where there is a possibility of MTC. Serum calcitonin can be useful but has low specificity for MTC below 500 ng/L. Immunohistochemistry can also be an effective way to rule in or rule out MTC if material is available for a cell block and a small panel of stains including calcitonin and thyroglobulin, at a minimum. However, if serum calcitonin is not sufficiently elevated or if immunohistochemistry testing is not possible or equivocal, an alternative approach is the Afirma MTC Classifier, an mRNA gene expression analysis approach that analyzes expression levels of five genes in parallel with the GEC. Originally, the MTC classifier was one of a series of small gene sets termed “cassettes” designed to assist the GEC in the identification of less commonly encountered lesions that can present clinically and sonographically as

thyroid nodules. In addition to MTC, these cassettes recognize parathyroid tissue and metastatic tumors including renal cell carcinoma, breast carcinoma, and melanoma.

Recently, the MTC classifier became available either in parallel with the GEC for cytologically indeterminate thyroid FNAs or as a stand-alone test for SFM/M thyroid FNAs (Bethesda V/VI). In the context of the original validation study of the GEC, there were 2 MTCs and 263 non-MTCs among histologically confirmed specimens. 0/263 non-MTC specimens and 2/2 MTC specimens were positive for the MTC classifier, suggesting high specificity/PPV and high sensitivity/NPV. A follow-up abstract reporting on 43 patients that were positive for the MTC classifier with clinical follow-up found 42 cases confirmed as MTC (39 with surgical pathology and 3 with elevated serum calcitonin), for an overall PPV of 98 %. The single false positive MTC classifier result was found in a case of an intrathyroidal paraganglioma, a distinct but related neuroendocrine neoplasm with overlapping gene expression. Based on the available data, the MTC classifier therefore appears to be a test with both high PPV and high NPV that can accurately predict the presence of MTC in the context of FNAs that are cytologically indeterminate (Bethesda categories III and IV) as well as those that are suspicious for malignancy or malignant (Bethesda V and VI).

Patients either diagnosed with MTC or determined to have a high suspicion of MTC preoperatively through cytopathology, serum calcitonin, or the MTC classifier should be evaluated for the presence of MEN2 through RET mutation analysis. MEN2 is an inherited, autosomal dominant disorder consisting of three syndromes, MEN2A, MEN2B, and Familial Medullary Thyroid Carcinoma (FMTC), all of which result in a high lifetime risk of developing medullary thyroid carcinoma, due to mutations within the *RET* gene. The identification of patients with one of the MEN2 syndromes preoperatively is important, as previously mentioned, for proper surgical management, including evaluation of associated pheochromocytoma and hyperparathyroidism and handling of unintentionally devascularized parathyroid glands during surgery. *RET* mutation analysis is typically performed by targeted PCR and sequencing approaches and offered on whole blood specimens by the major national reference laboratories.

The identification and subtyping of other non-follicular lesions in thyroid nodule FNA specimens is most commonly approached through immunohistochemical analysis of cell block preparations. IHC allows for the diagnosis of parathyroid as well as metastatic lesions. In the evaluation of some FNAs, as discussed for MTC, in which cytologic material for IHC is not available and additional diagnostic information is needed, the GEC can be helpful in raising suspicion for parathyroid, renal cell carcinoma, breast carcinoma, or melanoma. However, further investigation of the clinical validity of these cassettes is needed to justify their use outside of the context of indeterminate thyroid FNAs.

Future Directions

The future of molecular testing in thyroid cytology holds great promise for continued improvements in the care of patients with thyroid nodules and thyroid cancer. Advances in molecular testing of thyroid nodules with indeterminate cytopathology is expected to improve upon current rule out (high sensitivity and NPV) approaches exemplified by the GEC to provide for concurrent rule in (high specificity and PPV) capabilities.

In addition, molecular testing approaches providing more specific information on tumor behavior and prognosis are likely to be developed, analogous to commercially available molecular tests that predict risk of recurrence and/or aggressive behavior in breast, colon, and prostate cancers, among others. Such tests could be used to predict which thyroid cancers would be the "bad players" meriting aggressive treatment and which would be the "good players" with low probabilities of aggressive behavior or metastasis, possibly candidates for conservative management, similar to the watchful waiting approach employed in prostate cancer.

Finally, future advances in molecular testing in thyroid cytopathology are expected to lead to the development of companion diagnostics that allow for stratification of patients into likely responders and non-responders for various targeted therapies. Such tests could be performed on cytologic specimens obtained from recurrent or metastatic lesions, such as lymph nodes, to assess changes in the mutation status that would impact

therapeutic choices and management. For diagnosis and management of thyroid cancer, as well as other malignancies, the ability to derive information from small samples, such as FNAs or other cytologic specimens, holds great potential for both improving patient care and lowering costs to the health care system.

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Molecular Markers in Head and Neck Cytology

6

Zaibo Li, Huaitao Yang, and Bin Yang

Introduction

A biomarker is an objectively measurable biochemical, molecular, or genetic parameter which can be used not only for discerning the presence and progression of disease but also for addressing early cancer detection, tumor staging, targeting therapies, and post-treatment tumor surveillance. During past several decades, potential biomarkers have been greatly expanded for many tumors with the increased knowledge of molecular mechanisms. In this chapter, we discuss available and potential biomarkers in head and neck cytopathology specimens, which mainly include lymph nodes from head/neck region and salivary glands. The encountered

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malignancies in head/neck lymph node cytology specimen include lymphomas and metastatic malignancies such as thyroid carcinoma, lung carcinoma, breast cancer, and more commonly, head/neck squamous cell carcinoma (HNSCC). We focus on head and neck squamous cell carcinoma and salivary gland malignancies and discuss available or potential biomarkers for these entities.

Head and Neck Squamous Cell Carcinoma

Head and neck cancers refer to malignancies arising in the mucosal surface of oral cavity, pharynx, and larynx and account for approximately 45,000 new cases in the USA each year. Ninety percent of head and neck cancers are squamous cell carcinomas (HNSCCs), which are associated with tobacco and alcohol use, poor diet and human papilloma virus (HPV) infection. The most common available treatments for HNSCC include surgery, chemotherapy, and radiation. Treatment options are limited for patients with recurrent disease or distant metastasis and the prognosis of these patients is poor. In this section, we will discuss the pathogenesis including genetic and molecular alterations, and potential biomarkers for tumor initiation, prognosis, and targeted therapies in both HPV-associated and non-HPV associated HNSCCs.

HPV-Associated HNSCC

Tobacco and alcohol consumption cause the majority of HNSCCs in oral cavity, larynx, and hypopharynx; however, oncogenic HPV causes up to 70 % HNSCCs in oropharynx, the middle part of pharynx including soft palate, base of the tongue, and tonsils. While tobacco and alcohol-related head and neck cancer has been decreasing nearly 50 % in past three decades, the incidence of HPV-associated oropharyngeal cancer has been increasing. It has been estimated that about 1500 women and 5600 men will develop HPV-associated oropharyngeal cancers annually in the USA. Risk factors for HPV-associated HNSCC include certain sexual practices that facilitate repeated viral HPV exposure and impaired immunity conditions such as HIV carriers. The likelihood of

detecting HPV was reported to be 10.0 % in normal oral mucosa, 22.2 % in benign leukoplakia, 26.2 % in intraepithelial neoplasia, and 47 % in oral squamous cell carcinoma.

HPVs are members of the papilloma viridae family and contain ~8 kb double stranded circular DNA genomes encapsidated within 52–55 nm diameter non-enveloped particles. So far, more than 120 types of HPV have been discovered. Based on their characteristic tissue/organ tropism, HPV can be classified as cutaneotropic and mucosotropic. Cutaneotropic papillomavirus (HPV 1, 4, 5, 8, 41, 48, 60, 63, and 65) are isolated frequently in patients with cutaneous plantar warts, verruciform epidermodisplasia, and rarely in some cutaneous tumors. Mucosotropic papillomavirus (HPV 6, 11, 13, 44, 55, 16, 31, 33, 35, 52, 58, 67, 18, 39, 45, 59, 68, 70, 26, 51, 69, 30, 53, 56, 66, 32, 42, 34, 64, 73, 54) are traditionally identified in benign and malignant anogenital tract lesions, but these viral types are also isolated in lesions of the oral cavity, oropharynx, larynx, and esophagus. Mucosotropic HPV is further classified as low-risk and high-risk groups according to their ability to induce malignant transformation. Low-risk HPV types include HPV 6 and 11 as their prototypes and cause non-malignant and non-dysplastic lesions such as condyloma acuminatum. High-risk HPV types include HPV 16, 18, 31, 33, 39, 45, 50, 51, 53, 55, 56, 58, 59, 64, and 68 with HPV 16 and 18 as the most common types in human malignancies and most virulent strains for oncogenic transformation. In the USA, more than half of oropharyngeal cancers are caused by HPV type 16.

The genome of HPV virus contains three regions: the early genes E1–E8; late genes L1 and L2, and a third region called LCR (long control region) or URR (upstream noncoding regulatory region). The early gene products are responsible for replication of the HPV (E1 and E2), DNA transcription (E2), maturation and release of viral particles (E4), cell transformation (E5, E6, E7), and immortalization (E6 and E7). The late gene L1 protein is the most conserved gene among the HPVs and L1 capsid protein represents 80 % of the viral capsid proteins. The L1 capsid protein is highly immunogenic and is used for HPV vaccine production. L2 protein contributes to the incorporation of viral DNA into the virion. The LCR region functions in the gene expression and viral replication.

HPV infection in human tissue can be divided into two biologic stages: episomal stage and integrated stage. In episomal stage, HPV virus forms circular viral particles and is coated with HPV L1 and L2 capsid proteins. Virus particles are produced using host nucleic acid and protein synthesis apparatus. The infection is usually transient and only causes mild squamous dysplasia. During persistent HPV infection, the viral DNA is able to integrate into the host genome to enter the integrated stage. Once integrated into host genome, it will actively transcribe and translate two viral genes, E6 and E7. The E6 protein can form a trimeric complex with p53 and E6-AP to stimulate ubiquitin-dependent degradation of p53 and then promote cell proliferation and tumor growth. The E7 proteins encoded by the high-risk type HPVs can bind to Rb protein at its pocket domains and then disrupt the interaction between pRb and E2F, resulting in the activation of E2F and cell division.

The CDKN2A gene product, p16, is a tumor suppressor protein that inhibits cyclin dependant kinases CDK4 and CDK6. p16 is usually inactivated in many cancers through deletion or promoter hypermethylation, resulting in reduced or absent expression of the p16 protein. However, in cells with persistent HPV infection, Rb protein is reduced through E7-induced ubiquitin-dependent degradation and low level Rb protein decreases its inhibitory effect on p16 expression, resulting increased p16 expression. Furthermore, p16 expression can also be stimulated by the activated E2F in cells with persistent HPV infection. Therefore, overexpression of p16 has been recognized as a surrogate for HPV integration, viral oncoprotein expression and malignant transformation. Since p16 overexpression has been demonstrated extensively in HPV-associated HNSCC, It is now widely accepted that p16 is a sensitive and specific surrogate biomarker of squamous dysplastic cells of oropharyngeal HNSCCs. The sensitivity and specificity of p16 immunohistochemistry in head and neck SCC is greater than 90 % and 74 %, respectively.

HPV status can be determined by either HPV testing in fluid samples or HPV in situ hybridization on cytology smear slides or paraffin-embedded cell block sections. High-risk HPV (especially HPV 16 and 18) and low-risk HPV (HPV 6 and 11) can be detected

by different probes, respectively. HPV testing in liquid materials has been well established in Pap test for cervical cancer or precancerous lesions. It has recently been explored in oral wash material, saliva for patients with oral HNSCCs. Although these strategies have been documented with reasonable feasibility, these oral washing or saliva-based assays have failed to provide a high enough sensitivity and specificity for population-based screening as HPV testing applied in cervical cytology. Currently HPV in situ hybridization with a cocktail of several HR-HPV types is the most used tool in confirming HPV-related HNSCC. However, it should be cautious interpreting the results especially when it is negative because most commercial available HPV ISH methods have relatively lower sensitivity.

HPV status and p16 immunohistochemistry can be used to pinpoint the primary site of metastatic squamous cell carcinoma in cervical lymph nodes, giving the fact that a significant subset HNSCC cases spread to cervical lymph nodes in the absence of a primary tumor clinically and radiologically. It has been recognized that HPV-16 is the most important cause for oropharyngeal HNSCCs, but not for non-oropharyngeal HNSCCs. Positive HPV16 in situ hybridization result from individual cells aspirated from cervical lymph nodes with metastatic squamous cell carcinoma can reliably pinpoint its primary site to oropharynx. Patients with HPV-positive HNSCCs have been shown to have a more favorable prognosis with a lower risk of tumor progression and longer survival than patients with HPV-negative HNSCC. In a multi-institutional retrospective study of 96 HNSCC patients treated with chemotherapy and radiation therapy, HPV-positive HNSCC patients showed 73 % lower risk for progression and 64 % lower death rate than HPV-negative HNSCC patients.

Tobacco and Alcohol-Related HNSCC

Tobacco and alcohol consumption cause the majority of HNSCCs in oral cavity, larynx, and hypopharynx. Tobacco exposure by either tobacco smoking or passive smoking (environmental exposure) is a well-established risk factor for HNSCC and the risk is

correlated with smoking intensity and duration. Nitrosamines and polycyclic hydrocarbons in tobacco smoke are carcinogens with genotoxic effect and account for the increased risk, supported by more frequent p53 mutations in HNSCC patients with smoking history than those without. Alcohol consumption is another independent risk factor for HNSCC, and more importantly, a significant synergistic factor for patients with smoking history based on the fact that the actual risk is much higher than that derived from the additive effects. Since alcohol is not a direct carcinogen, one hypothesis of the underlining mechanism for this synergistic effect is that it might enhance and prolong mucosal exposure to the carcinogens from in tobacco smoke by acting as a chemical solvent.

The most prominent genetic alteration in non-HPV HNSCC is mutation or allelic deletion of the p53 tumor suppressor gene. Other genetic and epigenetic alterations in non-HPV HNSCCs involve epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), Hepatocyte growth factor (HGF) and its receptor c-Met, insulin-like growth factor-1 receptor (IGF-1R), mammalian target of rapamycin (mTOR), and vascular endothelial growth factor receptor (VEGFR). Some are involved in the pathogenesis of HNSCCs and some are related to tumor progression, and some might be molecular targets for personalized therapies.

p53

p53 gene is a tumor suppressor gene located on chromosome 17q13. Up to 50 % of HNSCCs harbor p53 gene mutations, which inactivate p53 tumor suppressor, and lead to uncontrolled cell growth and inability to repair DNA damage. Besides p53 gene mutation itself, mutation or dysregulation of other genes in the p53 pathway can also cause p53 inhibition. The genes in p53 pathway that have been found to be mutated or deregulated in HNSCCs include Ataxia telangiectasia mutated (ATM), $p14^{arf}$, Bcl-2, and Bax. ATM is a kinase that phosphorylates and activates p53. Loss of function of ATM leads to inactivation of p53. $p14^{arf}$ is an alternate reading frame protein product of the CDKN2A locus and it can form stable complex with Mdm2 and then inhibit Mdm2, thus promoting p53. Mutation in the ARF locus controlling $p14^{arf}$

expression has been identified in HNSCC, which leads to elevated levels of Mdm2, therefore, inhibition of p53 and then uncontrolled cell growth. Bcl-2 and Bax are two apoptosis-related proteins in the downstream of p53 pathway, and their mutations have been identified in HNSCCs as well.

Wild-type p53 expression shows patchy weak staining on immunohistochemical studies, and p53 mutants usually show diffuse strong staining due to accumulation of mutated p53 protein or entirely non-staining due to deletion mutation or degraded p53 protein. However, p53 gene mutations in HNSCCs are highly divergent in their effects on p53 protein structure, stability, DNA binding properties depending on where they occur. Although p53 mutations by immunohistochemical assay have been explored to identify high-risk premalignant lesions or undiagnosed HNSCCs in saliva specimens and to study HNSCC prognosis, it has not consistently proved reliable for clinical use.

In contrast, sequencing mutation analysis for specific types of p53 gene mutations is more powerful to predict HNSCC prognosis than p53 protein immunohistochemistry. It has been shown that p53 gene mutations within its DNA binding domain are associated with tumor progression and poor prognosis in HNSCCs.

Epidermal Growth Factor Receptor (EGFR)

EGFR (ErbB-1) is a membranous tyrosine kinase receptor and ubiquitously expressed in head and neck squamous epithelium. In normal cells, EGFR is dimerized and autophosphorylated upon ligand-binding and then activates downstream signaling. In the majority of HNSCCs, EGFR is overexpressed by either gene amplification or transcriptional stimulation and its overexpression can cause downstream signaling activation without ligand-binding. A constitutively active EGFR mutant (EGFRvIII) due to a deletion in exons 2–7 has been identified in other cancers and this truncated form activates EGFR downstream signaling without ligand-binding. However, this mutant was rarely identified in HNSCCs. The downstream targets of EGFR signaling pathway include Ras-MAPK (mitogen activated protein kinase) pathway and PI3K (phosphoinositol-3-kinase)-Akt pathway EGFR is upstream of PI3K and Akt.

EGFR mutation testings are not routinely performed for HNSCCs due to its rarity. EGFR immunohistochemistry testing is occasionally used to confirm EFGR protein overexpression. A chimeric monoclonal antibody directed against the EGFR, cetuximab (™Erbitux) has been approved by FDA as a molecular targeted agent for the treatment of advanced HNSCC in combination with chemotherapy and/or radiation. In addition to cetuximab, panitumumab (™Vectibix), another monoclonal antibody against EGFR, and several EGFR tyrosine kinase inhibitors (TKIs), including gefitinib (™Iressa), erlotinib (™Tarceva), and lapatinib (™Tykerb), are in clinical trials.

Loss of Heterozygosity of 3p and 9p

Loss of heterozygosities at 3p and 9p are common in HNSCC (up to 51 %) and extensive losses at both regions may be related to early process of tumorigenesis in HNSCC. Both LOHs at 3p25 and 9p21 were observed in all stages from squamous metaplasia and squamous dysplasia to invasive SCC and metastatic carcinoma. Other studies also found that 81 % of HNSCCs have allele loss at one or more 3p markers including 66 % with loss for 3p21.3 markers and 56 % with loss at 3p12. LOH at 9p21 has also been shown to be associated with increased risk of local recurrence. Several potential suppressor genes located on chromosome 3p include RASSF1A, *VHL* gene related to von Hippel–Lindau disease and *Fragile Histidine Triad* (*FHIT*) gene. Chromosome 9p21 harbors *p16* gene that encodes p16, a cyclin dependent kinase inhibitor.

Dual LOH at 3p and 9p has been studies to identify high-risk premalignant lesions or undiagnosed HNSCCs in saliva specimens. It has been proven to reliably distinguish those lesions that are likely to progress to invasive carcinoma from those that will not.

Cyclin D1

Amplification of 11q13 region harboring Cyclin D1 gene has been identified in 30–50 % of HNSCCs and is associated with more aggressive tumor behavior and poor prognosis. A recent study found FISH is a simple and sensitive method for detecting cyclin D1 amplification in HNSCCs and Cyclin D1 amplification together

with p16 deletion correlates with a poor prognosis in patients with HNSCCs. Fluorescence in situ hybridization (FISH) for Cyclin D1 gene amplification can be used to predict aggressive behavior and poor prognosis in a portion of HNSCCs.

VEGF, STAT3 and Other Molecules for Targeted Therapy

In addition to EGFR targeted therapy, several of other dysregulated proteins in HNSCCs, such as VEGF and STAT3, are currently being assessed as therapeutic targets. Several studies have illustrated that VEGF overexpression can stimulate angiogenesis by binding to VEGFR2 on endothelial cells. Other studies found that VEGFR2 was also overexpressed in vascular endothelial cells derived from HNSCC samples. STAT3 is a transcription factor which can be phosphorylated and then activated by several kinases including EGFR, Src family kinases and Janus kinase (JAK). Activated STAT3 can stimulate transcription of its downstream targets including Cyclin D1 and VEGF, etc. Elevated level of phosphorylated STAT3 has been found in HNSCCs and this elevation can activate STAT3 downstream signaling.

The antiangiogenic agents including tyrosine kinase inhibitors specific for VEGFR2 [Sunitinib (™Sutent), sorafenib (™Nexavar), and cediranib (™Recentin)], VEGF monoclonal antibody [Bevacizumab (™Avastin)], and a dual TKI targeting for both EGFR and VEGFR2 [Vandetanib (™Zactima)] are being assessed in clinical trials for recurrent or metastatic HNSCCs. STAT3 decoy (an oligonucleotide decoy) and several other targeted therapeutic drugs are also under investigation for recurrent or metastatic HNSCCs.

Salivary Gland Malignancies

Salivary gland cancers (SGC) are uncommon, accounting for only 5 % of head and neck cancers. SGCs are a heterogeneous group of cancers with diverse pathogenesis and molecular alteration, different histology, variable biologic behavior and responsiveness to therapy. Benign lesions including benign cystic lesions, inflammation, degenerative processes and benign neoplasms are much more

common than SGCs; therefore, microscopic evaluation to determine the disease nature in salivary gland is necessary before any treatment. Incisional/excisional or core needle biopsies are not suitable to obtain tissue for diagnostic purposes in salivary glands due to procedure-caused complications. Fine needle aspiration cytology plays a vital role in the diagnosis of salivary gland diseases because of its negligible risk of complications and relatively high accuracy in differentiating benign lesions from malignant lesions or low grade malignant lesions from high grade lesions. However, the diagnoses of SGCs are challenging, even histologically, because of the broad diversities, and more importantly, the overlapping morphology among many SGCs. Surgery and/or radiation therapy are the main treatment modalities for patients with SGCs and systemic chemotherapies are mostly limited to patients with advanced disease or progressive metastatic disease. Therefore, diagnostic, prognostic, and therapeutic molecular biomarkers based on cytology materials are necessary for improving diagnostic accuracy of SGCs, providing prognostic information and developing new therapeutic options for patients with advanced disease. New findings in understanding molecular pathogenesis of many SGCs have been recently revealed, including recurrent chromosome translocations with molecular targets of tyrosine kinase receptors, transcription factors, and transcriptional coactivators. Other biomarkers with potential clinical use have also been discovered by next generation sequencing, genomic and expression profiling methods. In this part, we discuss the most recent developments in the pathogenesis and potential biomarkers of SGCs, and the clinical implications of these biomarkers diagnostically and therapeutically.

Mucoepidermoid Carcinoma (MEC)

MEC is the most common carcinoma in both major and minor salivary glands with a wide spectrum ranging from non-aggressive low-grade to aggressive high-grade. Clinical, histological, and cytological features are different between low grade and high grade MECs. Low grade MECs only need modest surgical excision; however, high grade MECs require aggressive surgical treatment together with lymph node dissection and adjuvant radiotherapy.

t(11;19) *MECT1/MAML2* Translocation

Although low grade and high grade MECs have different histology and clinical courses, both of them harbor same recurrent chromosome translocation t(11;19)(q21–22;p13), which occurs in more than 50 % of all MECs. The translocation t(11;19)(q21–22;p13) results in a fusion involving the MAML2 and MECT1 (or rarely CRTC3) genes. MAML2 (mastermind-like 2) at 11q21 encodes a 125-kDa protein functioning in Notch signaling pathway. MECT1 (mucoepidermoid carcinoma translocated-1, also known as CRTC1, TORC1, and WAMTP1) at 19p13 encodes a 75-kDa protein that acts as a CREB (cAMP response element-binding protein) coactivator. The MECT1-MAML2 fusion protein is comprised of the N-terminal CREB protein-binding domain of MECT1 and the C-terminal transcriptional activation domain of Notch coactivator MAML2. The fusion protein activates cAMP-CERB signaling and Notch signaling, leading to cell cycle dysfunction and differentiation inhibition. Recent studies have shown that MECT1-MAML2 fusion protein can upregulate EGFR-ligand AREG (Amphiregulin), leading to EGFR signaling activation in MEC cells, and then cell proliferation and survival. MEC cells harboring MECT1-MAML2 fusion protein were shown to be highly sensitive to small molecule EGFR tyrosine kinase inhibitors in a xenograft model.

The translocation of t(11;19)(q21–22;p13) can be tested by either FISH with DNA probes or RT-PCR with specific fusion gene primers. MECT1/MAML2 translocation occurs more frequently in low- and intermediate-grade MECs with favorable prognosis than in high-grade MECs with a dismal prognosis, suggesting that MECT1-MAML2 represents a specific prognostic molecular marker in MEC. The median survivals are 10 and 1.6 years in fusion-positive and fusion-negative patients, respectively. Patients with fusion-positive MECs have significantly lower risk of local recurrence and distal metastases.

MECT1/MAML2 translocation can be used a diagnostic approach in fine needle aspiration cytology specimens. FNA cytology has been widely implemented for triage of salivary gland tumors and is accurate in making a diagnosis of high-grade or intermediate-grade MECs. However, it has been shown to be unsatisfactory in making a diagnosis of low-grade MECs. MECT1/MAML2 translocation testing in FNA cytology specimen might be

helpful to make a definitive diagnosis if the MECT1-MAML2 fusion transcript/protein is detected in uncertain cytology cases with low-grade bland-appearing cells. Similarly for high-grade MECs, positive MECT1-MAML2 translocation testing would be helpful to make a definitive diagnosis of MEC and differentiate it from other poorly differentiated carcinoma, although a negative result is not useful. MEC cells harboring MECT1-MAML2 fusion protein were shown to be highly sensitive to small molecule EGFR tyrosine kinase inhibitors (TKIs) in a xenograft model, suggesting that targeting therapy with EGFR TKIs might be a new approach to treat patients with advanced MECT1-MAML2 translocation-positive MECs.

Other Genetic Changes in MECs

The CRTC family includes three genes: CRTC1 (MECT1) at 19p13, CRTC2 at 1q21, and CRTC3 (MECT3) at 15q26. MECT1 shares 32 % sequence homology with the other two genes. A novel translocation between CRTC3 and MAML2 was also identified infrequently in MECs with only about 6 % of cases. So far, there is no CRTC2-MAML2 fusion reported in any MEC cases. CRTC3-MAML2 and MECT1-MAML2 fusions were mutually exclusive and no significant survival difference between patients with CRTC1 and CRTC3 translocation.

Besides t(11;19) MECT-MAML translocation, CDKN2A/p16 gene deletions have been identified in both fusion-positive and fusion-negative MEC cases with poor prognosis, but not in fusion-positive MEC cases with good prognosis, suggesting CDKN2A status is related to the prognosis of patients with MECs.

A t(6;22)(p21;q12) translocation resulting in an *EWSR1–POU5F1* gene fusion was also identified in a high-grade *MECT1–MAML2* negative MEC case. The resulting fusion protein is composed of the N-terminal domain of EWSR1 linked to the DNA-binding domain of POU5F1 and acts as a transcriptional coactivator involving downstream target gene transcription, which was also reported in bone soft tissue tumor.

Similar to MECT1/MAML2 translocation testing, CRTC3/MAML2 translocation and t(6;22)(p21;q12) *EWSR1/POU5F1* translocation testing might be helpful in making a diagnosis of

MEC in FNA cytology specimen, especially in MECT1/MAML2-negative cases. Cases with positive CRTC3/MAML2 translocation also showed better prognosis than translocation negative cases. Array CGH analysis for *CDKN2A* tumor suppressor gene deletion might be another potential prognostic biomarker since it was reported that loss of *CDKN2A* associated with poor prognosis in patients with MECs.

Adenoid Cystic Carcinoma (AdCC)

AdCC is the second most common malignancy in salivary gland and accounts for 10 % of all salivary gland neoplasms. It occurs more frequently in minor salivary glands than in major salivary glands. It may also arise in secretory glands located in other sites including breast, sinonasal tract, tracheobronchial tree, external auditory canal, skin, lung, uterine cervix, prostate, and vulva. AdCC is characterized by slow growth kinetics and less lymph node metastasis. However, local and distant recurrences are quite common after resection of the primary tumor due to its characterized perineural invasion. Some AdCCs can be aggressive with a poor long-term prognosis due to metastatic disease, which can occur in up to 40 % of AdCC patients. Surgery and/or radiation therapy are the primary treatment for AdCC and so far there is no effective targeted therapy for patients with recurrent or advanced disease.

t(6;9)(q22–23;p23–24) *MYB/NFIB* Translocation

The most intriguing molecular alteration in AdCC is a translocation between chromosomes 6q and 9p [t(6;9)(q22–23;p23–24)], which occurs in up to 86 % of AdCCs and results in a fusion involving the *MYB* oncogene and the transcription factor gene *NFIB*. The consequence of this translocation is the overexpression of a fusion transcript with a largely intact MYB oncoprotein. The MYB/NFIB fusion oncoprotein activates transcription of MYB target genes, leading to tumorigenesis. Besides *MYB*/*NFIB* translocation, *MYB* activation is also rarely caused by insertion of the 3′-part of *NFIB* in the vicinity of the *MYB* locus and copy number gain.

The *MYB–NFIB* translocation appears to be specific for AdCC since it has not been detected in other salivary gland malignancies.

Currently, few ancillary testings are available to assist in the differential diagnosis of salivary gland basaloid neoplasms which include AdCC, pleomorphic adenoma (PA), basal cell adenoma and basal cell carcinoma on FNA biopsy. Although CD117 immunostain is a sensitive marker for AdCC, it is positive in up to 20 % of benign PAs. MYB immunohistochemistry has been studied in surgical pathology specimens, revealing overexpression of MYB occurs in up to 89 % of AdCCs, but not in any other salivary gland neoplasms, suggesting that MYB expression may be useful as a diagnostic biomarker for AdCC. A recent study applying MYB immunostain on FNA materials demonstrated a majority of AdCCs (80 %) were immunocytochemically positive for MYB and other salivary gland lesions including pleomorphic adenoma are negative for MYB, suggesting MYB immunocytochemistry may serves as an ancillary test for the cytologic diagnosis of AdCCs.

Similar to MYB immunostain, MYB/NFIB rearrangement FISH may also serve as a diagnostic biomarker for AdCC in FNA cytology specimens since t(6;9)(q22–23;p23–24) translocation occurs in the majority of AdCCs, but not in other salivary gland neoplasms. A recent study used a break-apart FISH approach to investigate its clinical utility in differentiating AdCCs from PAs and revealed that MYB FISH abnormalities showed a 100 % positive predictive value, 50 % sensitivity, and 100 % specificity when differentiating AdCC from PA, suggesting MYB rearrangement FISH in salivary gland FNA cytology specimens has the potential to be a diagnostic biomarker.

Besides being a validated diagnostic biomarker for AdCC, it is also important to know whether MYB immunostain or *MYB–NFIB* rearrangement could be a prognostic biomarker. So far, there is no prognostic difference between MYB-positive AdCCs and MYB-negative AdCCs. However, further studies are warranted to investigate whether different *MYB–NFIB* fusion transcripts may have different oncogenic-transforming capacities and thereby different clinical prognosis.

MYB signaling pathway and its downstream molecules are under investigation. Although targeting transcription factor like MYB has proven to be very difficult, targeting its signaling downstream molecules might be an alternative way to inhibit MYB activation and its oncogenic function. However, MYB regulates the transcription of over 10,000 downstream target genes and it will be very difficult to identify key oncogenic drivers in AdCC tumorigenesis. For example, c-Kit, a MYB downstream target, is ubiquitously overexpressed in AdCCs and has been used as a diagnostic marker for AdCC, but c-Kit inhibitor imatinib has not shown any effects on patients with progressive AdCC. Nevertheless, targeting MYB itself, MYB downstream targets, or the molecules regulating MYB signaling are potential targeting molecular therapies that may improve advanced AdCC patient survival.

Other Molecular Alterations in AdCCs

Gene expression profile analysis by microarray revealed several overexpressed genes including transcription factor Sox4 (a candidate oncogene involved in embryonic development), casein kinase 1-epsilon and frizzled-7 (involved in the Wnt/β-catenin signaling pathway). Variable overexpression of growth factor receptors including fibroblast growth factor receptor 1 (FGFR1), EGFR, and/or HER2 have been identified in AdCCs. However, mutational activation or overexpression due to gene amplification of these growth factor receptor genes is rare in AdCCs. Chromosomal analysis of AdCCs also revealed an AdCC-specific deletion in chromosome 1p35–36, which was also shown to be associated with a poor prognosis. Other molecular alterations in AdCC include activation of TrkC/NTRK3 signaling pathway and mutations in RAS pathway genes including *BRAF* and *HRAS*, suggesting targeted inhibitors for these pathways may be potential therapeutic options for AdCC patients with the molecular alterations.

Mammary Analog Secretory Carcinoma (MASC)

Mammary analogue secretory carcinoma (MASC) is a newly described low grade salivary gland carcinoma that resembles secretory carcinoma of the breast morphologically and molecularly.

Both MASC and secretory carcinoma of breast harbor a t(12;15)(p13;q25) translocation, which results in an *ETV6–NTRK3* gene fusion. MASC is a low-grade salivary gland carcinoma with an overall good prognosis, but high-grade transformation and accelerated clinical course have been reported. Historically, most MASC were categorized as acinic cell carcinoma (AciCC) or adenocarcinoma, not otherwise specified (NOS). However, MASC tends to demonstrate an increased frequency of lymph node metastases comparing to AciCC. Since FNA cytology has been widely used in the diagnosis of salivary gland neoplasms, the ability to make a definitive diagnosis of MASC on FNA cytology can help to guide the following surgical management. For example, the diagnosis of MASC on cytologic materials will alert surgeons to possible lymph node metastases and a possible need for neck dissection in institutions where neck dissection is not routinely performed for patients with AciCC. Although MASC has a characteristic arborizing papillary formation and vacuolated cells with mucin production on FNA specimens, MASC often shows considerable morphology overlapping with other salivary gland tumors including AciCC, mucoepidermoid carcinoma (MEC), salivary duct carcinoma, and oncocytoma.

Translocation of t(12;15)(p13;q25) (*ETV6*/*NTRK3*)

The translocation of t(12;15)(p13;q25), *ETV6*/*NTRK3* fusion, occurs in more than 90 % of MASCs, but not in any other salivary gland tumors, including the most morphologically resembling AciCC. The *ETV6*/*NTRK3* fusion encodes a chimeric tyrosine kinase that activates Ras-MAPK pathway and PI3K-AKT pathway. The *ETV6*/*NTRK3* rearrangements have been identified in neoplasms from other anatomic sites, including acute myeloid leukemia, congenital mesoblastic nephroma and fibrosarcoma. A recent study demonstrated that oncogenic transformation of mammary epithelial cells by *ETV6*/*NTRK3* can be blocked by inhibiting IGF1R/INSR signaling, suggesting an intact IGF1R/insulin receptor (INSR) signaling is necessary for *ETV6*/*NTRK3*-induced transformation. Furthermore, small molecule IGF1R/INSR kinase inhibitors (BMS-754807 and BMS-536924) have been shown to block *ETV6*/*NTRK3*-induced oncogenic transformation in vitro and reduce tumor growth in vivo, suggesting

targeting IGF1R/INSR signaling pathway may benefit patients with *ETV6*/*NTRK3* positive MASCs.

ETV6/NTRK3 FISH analysis has been successfully applied to surgical specimens or blood samples to serve as a diagnostic biomarker. A recent study also implemented this technique on FNA cytology specimen to make a definitive diagnosis of MASC. ETV6/NTRK3 translocation occurs in over 90 % of MASCs. So far, there is no prognostic difference between ETV6/NTRK3-positive and ETV6/NTRK3-negative MASCs. The ability of IGF1R/insulin receptor kinase inhibitors (BMS-536924 and BMS-754807) to block ETV6/NTRK3-induced tumorigenesis may provoke ETV6/NTRK3 translocation as a therapeutic biomarker for IGF1R/insulin receptor kinase inhibitors in treating patients with *ETV6-NTRK3*-positive MASCs.

Others

DOG1, initially described as a marker for gastrointestinal stromal tumors, has been shown as a marker of salivary acinar and intercalated duct differentiation. A recent study reported that MASCs are largely negative for this marker, but the majority of acinic cell carcinomas are positive for DOG1. Therefore, DOG1 immunostain may be used as diagnostic marker to differentiate MASCs from acinic cell carcinomas.

Hyalinizing Type of Clear Cell Carcinoma (HCCC)

HCCC is an uncommon salivary gland malignancy and accounts for 1 % of salivary gland tumors. It usually occurs in women and involves intraoral salivary glands at base of tongue or palate. Morphologically, it shows nests of clear cells surrounded by hyalinized bands with focal myxohyaline stroma and/or mucinous differentiation. HCCC is a low grade malignancy with 15 % nodal metastases and possible late recurrence, but overall HCCC has an excellent prognosis with occasional cases with metastatic spread. The differential diagnosis for HCCC on FNA cytology is broad and includes mucoepidermoid carcinoma, epithelial–myoepithelial carcinoma, clear cell myoepithelial carcinoma and other clear cell-appearing tumors.

A recurrent t(12;22) (q13;q12) translocation which results in *EWSR1/ATF1* gene fusion was recently identified in up to 87 % HCCCs. This translocation was originally identified in clear cell sarcoma and also occurs in a majority of clear cell odontogenic carcinoma (CCOC), which morphologically resembles HCCC, suggesting these entities might be a same disease in different locations. The fact that *EWSR1–ATF1* translocation has not been identified in any other salivary gland tumors suggests *EWSR1–ATF1* translocation could serve as at least a diagnostic biomarker for HCCCs in FNA cytology specimens.

Salivary Duct Carcinoma (SDC)

SDC is one of the most aggressive malignancies in salivary glands and accounts for 10 % of all salivary gland malignancies. It often occurs in males of 50 years or older and occurs either de novo or as SDC ex-pleomorphic adenoma. Treatment for SDC includes surgical excision with lymph node dissection and subsequent radiation therapy with/without chemotherapy. The overall prognosis for patients with SDC is poor since local recurrences, lymph node and distant metastases are common. Morphologically, SDC resembles high-grade invasive ductal carcinoma of the breast.

Like invasive ductal carcinoma of the breast, approximately 30 % of SDCs show HER2 overexpression and *HER2* gene amplification, and anti-HER2 agent trastuzumab has been shown to improve disease-free and overall survival in patients with HER2 overexpression/amplification. Therefore, HER2 testing by either immunohistochemistry or FISH can serve as a therapeutic biomarker for SDCs. SDCs do not express estrogen or progesterone receptors, but androgen receptor (AR) is expressed in the majority of SDCs. Androgen deprivation therapy has shown clinical benefit in patients with AR-positive SDCs. Therefore, AR status determined by immunohistochemistry would be another therapeutic biomarker for SDCs.

Other molecular alterations have also been discovered in SDCs, including *PIK3CA* mutations (20–33 %), *PTEN* deletions (50–59 %) and activating *BRAF* V600E kinase mutations (7 %). Furthermore, PI3K inhibitor temsirolimus and BRAF inhibitor vemurafenib have shown benefits in SDC patients with the specific mutations.

Epithelial–Myoepithelial Carcinoma (EMC)

EMC is a rare low-grade salivary gland tumor with both epithelial and myoepithelial components and accounts for less than 0.5 % of salivary gland tumors. Eighty percent of MECs arise in parotid gland and 60 % of them involve women. Recently, *H-RAS* gene mutations have been identified in up to 25 % of EMC cases, suggesting *H-RAS* mutation analysis may be used as a biomarker to select a subset of EMC cases which might benefit from RAS downstream signaling MEK-specific inhibitors.

Carcinoma Ex Pleomorphic Adenoma (Ca-ex-PA)

Ca-ex-PA is carcinoma arising from a benign pleomorphic adenoma and can manifest as an adenocarcinoma-not otherwise specified (NOS), an undifferentiated carcinoma, or any other histologic types including salivary duct carcinoma, MEC and AdCC. Several molecular alterations have been discovered in Ca-ex-PA including *PLAG1*/*HMGA2* gene fusions, amplification of *MDM2* and *HMGA2* in 12q13–15, mutations of *TP53*, and/or amplification of *HER2*. Most Ca-ex-PAs with *HER2* amplification are identified in patients with salivary duct carcinoma histologic type arising in pleomorphic adenoma, who may benefit from HER2 antibody trastuzumab treatment.

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Molecular Biomarkers in Pulmonary Cytology

7

Qing Kay Li and Bin Yang

Introduction

Lung cancer is the leading cause of cancer-related deaths in the USA and worldwide [1]. Non-small-cell lung cancer (NSCLC) accounts for approximately 85 % of all lung cancers [1, 2]. Among them, adenocarcinoma (ADC) and squamous cell carcinoma (SqCC) account for 40 % and 30 % of cases, respectively. Only a small portion of NSCLC patients are diagnosed at early stage (stage I and/or II), when the tumor can be treated by surgical resection [2]. The 5-year survival rate for patients with NSCLC is approximately 16 %. Over the past decade, numerous efforts have been focused on the identification of candidate biomarkers in order to improve the survival of lung cancer patients. The efforts lead to the development of the targeted therapy for lung cancers. For example, the discovery of *EGFR* (epidermal growth factor receptor) mutations leads to the development of gefitinib and erlotinib therapies [3]; the

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discovery of *EML4-ALK* (echinoderm microtubule-associated protein-like 4 (*EML4*) and the anaplastic lymphoma kinase (*ALK*)) gene fusion [4] has led to the development of crizotinib therapy. These targeted therapies have improved the survival of lung cancer patients. Currently, the molecular characterization of the tumor is performed routinely in the clinical management of lung cancer patients.

Lung cancer development and progression are a multistep process, which is characterized by aberrant genetic and protein expressions [3–7]. Tumor cells are entirely dependent on the activity of specific genetic alterations and protein expression for their growth and survival. It is also clear that lung cancer is the result of progressive aberrant genetic alterations, including both inactivation of tumor suppressor genes and/or activation of pro-oncogenes [3–6]. In addition, posttranslational modifications of proteins are also played crucial role. For molecular characterization of the tumor, a variety of clinical techniques have been used to obtain biological material [8, 9]. Particularly, in recent years studies using fluid-based specimen or the so-called "fluid-biopsy" specimen have progressed rapidly [8].

Among all cytological materials, fine needle aspiration (FNA) cytology is an important approach to obtain specimens, particularly in patients with advanced and/or metastatic lung cancers. FNA procedures can also be performed repeatedly during the disease progression. In this chapter, we summarize recent achievements in the clinical application of biomarkers in the diagnosis and treatment of lung cancers, with particular focus on the discussion of the utility of cytological material and recent advances in the field of biomarkers in lung cancers.

Type of Cytological Specimen in Lung Cancer: Specimen Collection and Limitations

It is well known that candidate biomarkers may be differentially expressed during tumor progression; and certain biomarkers may not be expressed in the early stage of the tumor [5, 6, 8, 10]. Several types of cytological samples are used in the molecular

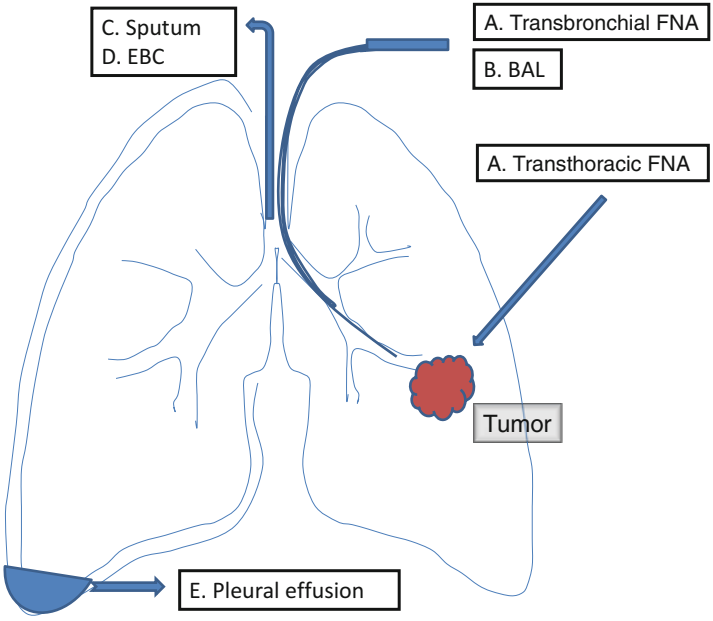

Fig. 7.1 Anatomical structures of the lung and types of cytological specimens. (*A*) Fine needle aspiration (FNA) is the most used technique to obtain tumor tissue. It can be performed by bronchoscopically (transbronchial with or without ultrasound guidance, TBNA) or transthoracically (with CT and/or ultrasound guidance). (*B*) Numerous proteins are secreted or leaked into the alveolar space from lung parenchymal cells. Proteins in the alveolar space can be recovered by bronchoalveolar lavage (BAL) using endobronchoscopy. (*C*) Sputum is a fluid secreted by bronchial epithelial cells from lower airways. It is usually collected as spontaneous and/or induced sputum. (*D*) Exhaled breath condensate (EBC). EBC is easy to collect from mouth and nose of the patient via a special colleting instrument. (*E*) The pleural cavity is a space between the lung and chest wall. A large amount of fluid, known as pleural effusion, can accumulate in the pleural cavity in a variety of diseases including lung cancers, particularly when the tumor metastasizes to the pleural cavity

analysis of lung cancers (Fig. 7.1). Among them, tumor tissues, fluids from lung airway (bronchoalveolar lavage and sputum) and/or body cavity (pleural effusion) are the most used material for the clinical characterization of lung cancers.

Tumor Tissue from Core Biopsy and Cell Block Preparation

Tumor tissues can be obtained from all NSCLC patients by several techniques such as endobronchial ultrasound-guided (EBUS) fine needle aspiration (FNA), transbronchial fine needle aspiration biopsy (TBNA), transthoracic FNA and transbronchial/transthoracic core needle biopsy of the tumor. Among these techniques, TBNA and core biopsy of the tumor tissue are preferred approachs [11] for their ability to provide large volume of tumor samples. However, in past 5 years, EBUS-TBNA has become the most popular approach for clinical staging and obtaining cytologic materials for diagnosis and molecular testing. Both core biopsy and cell block preparation can provide tumor tissue for molecular testing. Recent studies also demonstrated that cell block preparations from FNA and other specimens, such as BAL and pleural effusions, are superb for molecular testing. A NIH pathology group showed that as a few as 50 tumor cells microdissected from cell block can be used for the detection of *EGFR* and *K-RAS* mutational testings. Powrozek et al. recently showed that tumor cells from cytological smear were adequate for mutational analysis [12]. The percentage of tumor cells, DNA concentration, and percentage of mutated DNA as well as ΔCt values have been reported to be similar in cytology slides and histology materials. Recent studies/data from the Cleveland Clinic Foundation have demonstrated that leftover cytolyte from the Thinprep preparation maybe an alternative choice for both conventional and next-generationsequencing-based mutational assays. Thinprep slide can be successfully used for *ALK* gene rearrangement assay as well.

When extracting nuclear material using a tumor tissue and/or cell block lysis, contamination from blood cells, inflammatory cells, plasma, and stromal cells is a major concern. Therefore, microdissection of cancer cells from tumor tissue should be considered to eliminate these potential contaminations during analytic process. The most effective method for isolating tumor cells is laser capture microdissection (LCM) [13]. Tumor cells can be isolated using LCM with high purity; however, the technique requires special instrumentation and is time-consuming. LCM-assisted microdissection yields only a limited quantity of tumor

sample. Another alternation is to microdissect tumor tissue using microscope and analyses potential biomarkers from selective areas of the tumor. The technique of microdissection of tumor tissue is more convenient and provides a relatively large quantity of tumor samples. Currently, the microdissection of tumor tissues is the technique used by most of molecular studies.

Bronchoalveolar Lavage (BAL)

In addition to using tumor tissue, the other promising biological material for the study of biomarkers in lung disease and cancer is bronchoalveolar lavage fluid (BAL) [14]. Proteins and DNA molecules in BAL are derived from secretion or leakage from lung parenchymal cells. The level of these biomarkers directly reflects the physiological or pathological status of the lung. The analysis of BAL can characterize the complex alveolar microenvironment and provide profiles in the discovery of potential biomarkers of lung diseases and cancers. BAL specimen is much easier to obtain than tumor tissue, and is collected by bronchoscopy. The proteomic analysis of BAL specimen has been applied to the study of a variety of benign lung diseases such as asthma and interstitial lung disease. Recent study from the Johns Hopkins Medical Institutions also shows that proteomic analyses can lead to the identification of tumor-associated protein biomarkers [15]. By using this approach, airway proteins and DNA can be recovered from a large area of lung parenchyma. This is especially important in the study of preinvasive and early cancer, since these lesions may not have visible histological changes under bronchoscopy. It is also an important method to study peripheral located lung cancers (particularly adenocarcinomas), since adenocarcinomas arise from lung parenchyma away from main bronchus and may not be reached by bronchoscopic biopsy needles.

Sputum

Sputum is a fluid secreted from lower airways. Two types of sputum, spontaneous and induced sputum, are usually collected for study of lung diseases. Spontaneous expectorated sputum is easy

to collect; however, it may contain saliva from oral cavity. In order to reduce oral and up respiratory airway contamination, induced sputum is preferentially used for the study of lung diseases/cancers. Induced sputum can be obtained after saline inhalation with a nebulizer; the procedure is usually performed in a clinic setting.

More than 250 proteins have been identified in sputum. Gray et al. showed that several proteins such as calgranulins and Clara cell secretory protein in the induced sputum were differentially expressed in patients with cystic fibrosis, asthma, COPD (chronic obstructive pulmonary disease) and bronchiectasis [16]. More recently, Nicholas et al. found that lipocalin and alipoprotein A1 were significantly reduced in patients with COPD when compared with healthy smokers [17]. Terracciano et al. studied the peptide profile in the induced sputum and found that several proteins were differentially present among patients with asthma, chronic obstructive pulmonary disease and healthy controls, such as human α-defensins (human neutrophil peptide (HNP)1, HNP2, HNP3) and three C-terminal amidated peptides, one of which was phosphorylated on serine [18].

While a number of protein biomarkers have been identified in CF (cystic fibrosis), COPD and other inflammatory airway diseases, it is quite evident that the low-abundance protein components of induced sputum could be used as biomarkers in lung diseases/cancer. However, the study of protein profile in lung cancer patients is still at its early stage. The presence of abundant, high-molecular-weight and highly charged mucin proteins may interfere with the analysis unless such disturbing proteins are removed from the sample. Other limitations of using sputum include: (1) difficulty in obtaining healthy control samples, and (2) lack of general standardization of sample collection. Despite all these apparent drawbacks, the profile of proteome may provide important information in this field.

Pleural Effusion

The surface of the lung is covered by a thin layer of pleura, which is frequently involved by lung cancer during tumor progression. The pleura is lined by a single layer of mesothelial cells, which

covers the surface of lung (visceral surface) and inner surface of the chest wall (parietal surface). The pleural cavity is formed between these two layers of cells. Normally, the pleural cavity contains only a small amount of fluid to lubricate the visceral and parietal surface when they move against each other during respiration. The protein composition in the pleural effusion is similar to that of the plasma. In the presence of variety of diseases, particularly when lung cancer metastasizes to the pleura, a larger amount of fluid, known as pleural effusion, can accumulate in the pleural cavity, due to the increased leakage of protein and/or decreased reabsorption of the fluid. Pleural effusion has been considered as a biological specimen with an enrichment of tumor-derived biomarkers in lung cancers [19].

Cell block preparation can provide tumor material for molecular characterization of the cancer. Proteomic analysis may also provide an important insight into tumor-related biomarkers during lung cancer progression [8, 19]. Other advantages of using pleural effusion are: it (a) is easy to obtain by thoracentesis, (b) has a minimal risk to the patient, (c) provides a large quantity of samples, and (d) can be performed repeatedly during disease progression for study progression markers.

Potential Clinical Application of Biomarkers

By definition, biomarker is an indicator of normal biological or pathological processes, disease progression, or pharmacological responses to a therapeutic intervention. From a biochemical point of view, a biomarker may be defined as an objectively measured biomolecule and its level changes significantly in a specific disease and during the disease progression. Potential biomarkers should be able to predict the biological behavior of the disease or cancer and the probability of the disease in response to the chemotherapy. Biomarker analysis using cytological specimens can define potential therapeutic targets, molecular signature related to early detection (cancer versus benign disease), prognosis (likelihood of cure or risk of progression and metastasis), and prediction (probability of response to therapy) in lung cancers.

Discovery of Targeted Therapy

Lung cancer development is the accumulative result of inactive tumor suppressor genes, activation of pro-oncogenes, and aberrant protein expressions. During the process, certain genetic alterations are the driving force for tumor initiation and progression. Receptor tyrosine kinases are fundamental regulators in many cellular signaling pathways; and their aberrant activations play a crucial role and are the driving force in the oncogenesis and tumor initiation. However, these genetic and proteomic abnormalities does not occur simultaneously, and they play different roles in the disease process. The concept of personalized medicine emphasizes that the tumor which arises in a patient is unique. Therefore, the clinical goal is to identify the right patient for the right therapy (targeted therapy).

All aforementioned cytological material can be used for molecular analysis, except sputum. The current therapeutic target and its related agents are summarized in Table 7.1 [20]. Taken together, the profile of biomarkers in lung cancer plays an important role in this new era of personalized medicine and targeted cancer treatment. Although molecular profiling of the tumor and targeted therapies have significantly improved the lung cancer patients' survival, unfortunately, large numbers of genetic and protein alterations of lung cancers are still unknown [5, 6].

Monitoring the Therapy Response and Drug Resistance

In lung cancers, the *EGFR* mutation is associated with a 70–80 % response rate to tyrosine-kinase inhibitors (TKIs) therapy and a longer progression free survival rate in patients [3, 5, 21]. However, the primary resistance to the TKI therapy occurs when the tumor has certain genetic alterations, such as tumors with *KRAS* mutations, *PIK3CA* mutations, loss of *PTEN* expression, and altered IGFR (insulin like growth factor receptor) signaling pathways. The acquired resistance of tumor cells also occurs during targeted therapy, such as *EGFR* T790M alteration. This alteration causes an increased binding of EGFR to ATP, which reduces the efficacy of TKIs. These genetic alterations can be detected by using cytological specimens.

Table 7.1 Major somatic alterations in lung cancers (adopted from ref. [20])

Gene	Pathway	Aberration	%ADC	%SqCC	%SCLC	Drugs, approved and investigational
EGFR	RTK	Mutation Amplification	20–30 % >20 %	Rare 7 %		Erlotinib, Gefitinib Afatinib (approved), Dacomitinib, Cetuximab, Necitumumab, Neratinib
ALK		Fusion with EML4 and other rare partners	3–13 %			Crizotinib (approved), X-396, LDK378; Ganetispib, AUY922, AT13387
MET		Mutation Amplification post-treatment with EGFR inhibitor	5 % 20 %			Tivantinib, Cabozantinib, Ornatuzumab, Tivantinib
ERBB2		Mutation Amplification	2–4 % 5–10 %			Trastuzumab, Afatinib, Neratinib, MGHA22
ERBB3		Mutation		2 %		MM-121
ROS		Mutation	1.5 %			Crizotinib, AT13387 (HSP90)
RET		Translocation with KIF5B and other genes	1–2 %			Vandetanib, Cabozantinib ?
FGFR1		Amplification	1–3 %	22 %	6 %	AZD4547, BGJ 398, BIBF 1120/ nintedanib, dovitinib, HGS1036
DDR2		Mutation		3.8 %		Dasatinib
IGF1R		Overexpression	ND	ND	95 %	AXL1717, OSI-906

(continued)

Table 7.1 (continued)

Gene	Pathway	Aberration	%ADC	%SqCC	%SCLC	Drugs, approved and investigational
KRAS	RAS	Mutation	30 %	5 %		Selumetinib, Trametinib, MEK162, and BKM120, everolimus, sirolimus AUY922, BYL719, Reolysin MEK162
NF1			8–10 %	11 %		
HRAS				3 %		
NRAS			<1 %	<1 %		
RASA1				4 %		
BRAF	RAF	Mutations	6 %	4 %		Vemurafenib (only for V600E)
PIK3CA	PI3K	Mutation	Rare	16 %		BKM120, PX-866, GDC-0941
PTEN		Deletion	Rare	8 %		BKM120, PX-866, GDC-0941 (PI3K), MK-2206 (AKT)
AKT1,2,3			Rare	16 %(AKT3) 20 % all		MK-2206
TSC1,2				6 %		Everolimus, sirolimus, temsirolimus
LKB1	LKB1/AMPK	Mutation	15–30 %	2 %		Biguanide compounds
MDM2	TP53	Amplification	20 %			Inhibitors of TP53–MDM2 interaction
CDKNA2/ p16INK4	RB1/CDK	Deletions, silencing, mutation	>20 %	72 %		CDK inhibitors PD0332991, BAY1000394
MYC MYCN MYCL	Transcriptional regulators	Amplification	31 %	rare	16 %	Aurora kinase inhibitors BH3 mimetics

ADC Adenocarcinoma, *SqCC* Squamous cell carcinoma, *SCLC* Small cell lung carcinoma

Furthermore, the growth factor receptors are *N*-glycosylated transmembrane proteins, their biological functions are in part regulated by intracellular endogenous lectins, such as galectins. Galectins can cross-link growth factor receptor glycoproteins and regulate the distribution of receptors on the cell surface. Galecin-3 has been shown to bind to *N*-glycans of EGFR and limits its distribution on the plasma membrane [22]. Furthermore, the mutation with the deletion of extracellular domain of EGFR can cause a loss of 4 out of 12 *N*-glycan sites on the receptor in tumor cells. This deletional mutation induces receptor dimerization and signaling. These data indicate that the interaction of *N*-glycan and receptor protein may regulate the distribution and residency of the growth receptor on the cell membrane in addition to receptor mutations (Fig. 7.2).

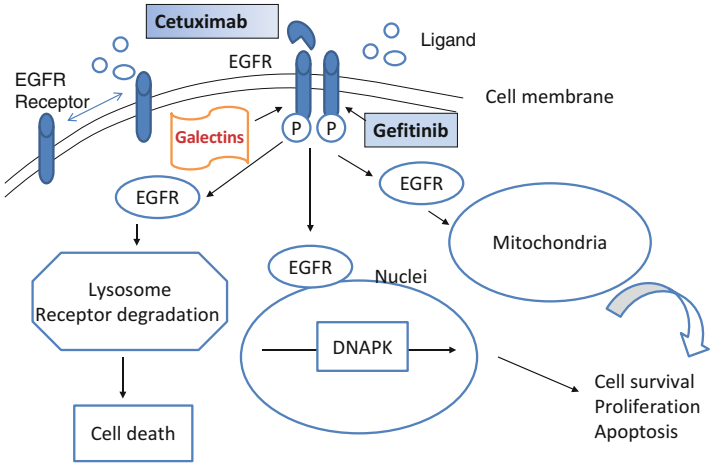

Fig. 7.2 EGFR signaling pathways. In lung cancers, the *EGFR* mutation is associated with a 70–80% response rate to tyrosine-kinase inhibitors (TKIs) therapy, such as cetuximab and gefitinib. Intracellular endogenous lectins, such as galectin-3, have been shown to bind to *N*-glycans of EGFR and limit its distribution on the plasma membrane. Furthermore, the deletion of extracellular domain of EGFR can cause a loss of 4 out of 12 *N*-glycan sites on the receptor in tumor cells. This deletional mutation induces receptor dimerization and signaling. The interaction of lectin and receptor protein may regulate the distribution and residency of the growth receptor on the cell membrane. It may involve in the development of TKI drug resistance in lung cancer. *DNAPK:* DNA-activated protein kinase

This is a poorly understood area. The study of functional role of glycoprotein has the potential of understanding the mechanism of drug resistance in lung cancer, particularly among patients who are treated with *EGFR* inhibitors.

Monitoring Cancer Progression and Prognosis

The progression of lung cancer is a multistep process. It is believed that tumor at a later clinical stage is more aggressive than a tumor at an early stage; therefore, tumor at different stages may express a unique subset of biomarkers that can be used in monitoring tumor progression. The most commonly used strategy to identify such potential biomarkers is to compare biomarker expression from different stage of tumor tissue as well as from patients responding or not responding to a certain treatment [23]. An alternative approach is to study biomarker expression within different stages of tumors and to correlate them with patients' clinical survival rates.

For example, *BRAF* V600E mutations are often detected in women with or without smoking history; and it is associated with more aggressive clinical course and micropapillary morphological features. Our recent study has shown that a subtype of lung adenocarcinoma, papillary adenocarcinoma of the lung, has a high level of NRF2 (nuclear erythroid-2 related factor 2) and p53 protein expression, and presents a poor clinical prognosis, in comparison to the conventional lung adenocarcinomas [24]. NRF2 is a transcription factor that regulates the expression of genes encoding antioxidants and xenobiotic detoxification enzymes. The elevated levels of NRF2 have been shown to relate to the cancer cell survival and potential protections against chemotherapeutic agents. The mechanism that regulates this increased NRF2 expression is complex. In some cases, it is related to mutations of the *KEAP1* (Kelch-like ECH-associated protein) gene, which prevents binding of the KEAP1 protein to NRF2 and causing NRF2 accumulation in the nucleus; where it can affect another gene, *ARE* (antioxidant response element) gene expression. In this complex oxidative stress signaling pathway, *MGAT5* (Mannosyl (alpha-1,6-)-glycoprotein beta-1,6,-*N*-acetyl-glucosaminyltransferase) gene also

plays an important role. This gene encodes mannosyl (alpha-1,6-)-glycoprotein beta-1,6-*N*-acetyl-glucosaminyltransferase, which regulates the synthesis of protein-bound oligosaccharides on cell surface. In the mouse model, cell-cycle progression (p53 pathway) is dependent on Mgat5/*N*-glycan interaction. The alteration of cellular *N*-glycoproteins causes significant changes of cellular adhesion and migration. These data indicate that oxidative stress and MGAT5 pathways may interactive in the regulation of cell growth.

Detection of Precancerous Lesion and Early Cancers

Clinical studies have shown that only a minority of preinvasive lung lesions progress to invasive cancer [25, 26]. Thus, the analysis of biomarker expression during the process may identify potential tumor-associated biomarkers. The identification of preinvasive lesion with a high risk of progression can also improve the early detection of lung cancers.

In a study of fresh frozen lung adenocarcinoma and patient-matched normal lung tissue, Rho et al. have used a comprehensive glycoproteomic enrichment by lectins of ConA, WGA (wheat germ agglutinin), and AIL (amylase inhibitor-like protein), then analyzed glycoproteins by 2-D PAGE and MS/MS approaches [27]. They have found that eight glycoproteins are upregulated, including alpha1-antitrypsin, fructose-bisphosphate aldolase A, annexin A1, calreticulin, alpha-enolase, protein disulfide isomerase A1, proteasome subunit beta type1, and mitochondrial superoxide dismutase. In comparison, seven glycoproteins are downregulated including annexin A3, carbonic anhydrase 2, fetuin A, hemoglobin subunit beta, peroxiredoxin-2, receptor for advanced glycosylation end products and vimentin. In addition, they have also identified that transgelin is overexpressed in stromal compartment whereas transgelin-2 is overexpressed in lung cancer tissue.

Recently, we studied the protein profile in the BAL fluid from lung cancer patients [15]. Among identified proteins, we also identified a subset of glycoproteins which was differentially expressed in BAL samples of lung cancer patients, compared to benign lung disease controls. Our study demonstrates a highly specific identification

of glycoprotein biomarkers. This finding is particularly important because it provides initial evidence that profiling biomarkers in BAL could lead to the discovery of cancer-specific protein biomarkers.

Chemoprevention of Lung Cancer

In the USA, 94 million current or former smokers are at a high risk for developing lung cancers [1]. The estimated new cases of lung cancer have already reached 222,520 in the USA in 2010, and the lung cancer-related death is the major cause of cancer-related death in both man and woman [1]. In order to decrease the mortality in lung cancer patients, the treatment of the early precancerous lesion in high risk population is urgently needed. In smokers, bronchial epithelium may have aberrant genetic and protein expression long before the development of cancer. These preinvasive lesions can be subtyped into the mild, moderate, server dysplasia and carcinoma in situ particularly in squamous cell lung cancer. Studies using serial bronchoscopic biopsy have suggested that 3.5 % of mild or moderate dysplasias might progress to severe dysplasia, 37 % of severe dysplasias might progress to carcinomas in situ, and 50 % of carcinomas in situ might progress to invasive carcinoma within a 2- to 3-year period [25, 26]. Currently, several clinical trials of chemoprevention to treat these patients with bronchial epithelium dysplasia have shown the regression of the lesions. Thus, the quantitative measurement of probability of having lung cancer based on the biomarker analysis of the bronchial epithelium in high risk population may potentially reduce the mortality of lung cancers.

Potential Limitations

In biomarker discovery and potential clinical application, it is important to use carefully selected clinical materials in both discovery process and subsequent validation phase. Despite the rapid progress in biomarker field, the workflow in analysis of clinical samples hinders the necessary throughput in the large scale study. Most current biomarker analyses are performed using a limited

number of clinical samples. Therefore, the improvement of analytic throughput ability is needed for study of a large scale of patient cohort. In addition, technologies still need to be further improved in terms of accuracy and sensitivity in measurements of clinical material. The analysis of dynamic range and molecular coverage need to be more sufficient, particularly in large scale of patient cohorts. Finally, the potential success of biomarker discovery and application largely depends on the quality and availability of patient samples. This requires large number of carefully selected patient cohorts to determine the potential utility of the biomarker. Clinical validation of potential biomarkers must be conducted in a fashion to avoid the occurrence of false positive and/or false negative results. For each candidate biomarker, robust and reproducible assays need to be developed and used in the validation phase.

Perspectives

The most common clinical samples available to molecular biomarkers analysis include, but not limited to, lung tumor tissue, sputum, bronchoalveolar lavage (BAL), and pleural effusions. It is absolutely essential to use clinically relevant samples from well characterized patients to further understand the biology of lung cancer and to discovery biomarkers. The recent advances in cellular and molecular technology clearly facilitate the discovery of novel biomarkers in lung cancers. These advances have significantly improved our knowledge in the field of lung cancer biology, and also promote the potential clinical utility of these biomarkers in the targeted therapy of lung cancers. Currently, many biomarkers are identified with the continued exponential growth rate in the research field, but full validations of these potential biomarkers for the diagnosis and monitoring progression of lung cancer are lacking. Despite years of extensive research attempting to identify and validate candidate protein biomarkers, the number of FDA approved biomarkers is still limited; and there is still a lack of effective diagnostic and prognostic biomarkers in lung cancers. Further improvement of the workflow and validations are both needed in the discovery of lung cancer molecular biomarkers.

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Molecular Analysis of Breast Cancer in Cytology Samples

8

Yun Gong

Introduction

Breast cancer is the most common cancer in women worldwide. Molecular studies have been increasingly used as adjuncts to histopathologic and cytopathologic features in the diagnosis, prognosis, and treatment of breast cancer. At present, the molecular biomarkers that have the most important prognostic and predictive significance for breast carcinoma are hormone receptors including estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Positive expression of hormone receptor occurs in about 70 % of invasive breast carcinoma cases and is associated with better tumor differentiation and a more indolent natural history. The *HER2* gene is located on chromosome 17q12-21 and encodes a transmembrane tyrosine kinase receptor. Amplification of the *HER2* gene or overexpression of HER2 protein has been identified in 15–20 % of invasive breast carcinoma cases and is associated with adverse clinical outcome. Knowledge of the status of these biomarkers is important for therapeutic decision-making and especially for assessment of a patient's eligibility for endocrine therapy and anti-HER2 targeted therapies, respectively.

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Patients with tumors that are negative for hormone receptors and HER2 need to be treated essentially with chemotherapy alone.

These biomarkers are typically tested in surgically resected or core needle biopsy (CNB) specimens of newly diagnosed primary breast carcinoma that are formalin-fixed and paraffin-embedded (FFPE), and require standardized preanalytical and analytical conditions. For example, the fixation conditions should be 10 % neutral buffered formalin for 6–48 h. However, testing of these biomarkers in metastatic breast carcinoma is often requested by clinicians even though the biomarker status of the patient's primary tumor is known, because metastatic carcinoma may show loss or gain of the expression of these receptors during disease progression and demonstrate a receptor status different from that in the corresponding primary tumors. Assessment of these biomarkers in a metastatic setting therefore has a direct effect on the management of metastatic disease.

Fine needle aspiration (FNA) is the most commonly used technique to sample metastatic tumors because it is a safe, simple, fast, and cost-effective procedure and is often used as an initial diagnostic modality to work up mass/nodule lesions at almost any body site. This procedure is especially suitable for low-resource settings. Compared with more invasive sampling procedures such as CNB and open biopsy, FNA can minimize associated anxiety, pain and treatment delay. However, because preanalytical and analytical conditions for FNA samples are different from those for surgical or CNB specimens, selection of an appropriate and validated method is crucial. This chapter addresses the current and evolving roles of breast cytology both in molecular testing such as hormone receptor and HER2 status as well as in personalized medicine and future directions. Pitfalls of cytology samples in different testing methods and alternative strategies are also discussed.

Hormone Receptor and HER2 Testing on FNA Samples

When an adequate cell block is available, ER, PR, and HER2 status should be determined immunohistochemically. HER2 testing can also be performed using in situ hybridization (ISH)

methods including fluorescence ISH (FISH), chromogenic ISH (CISH), silver ISH (SISH), and RNA ISH (RISH). If an adequate cell block is not available, direct smear and liquid-based preparations may be used for immunostaining (for ER and PR) as long as the sample is reasonably cellular.

ER and PR immunostaining on direct smears require technical validation before use in routine cytology practice. In the past, unstained direct smears prepared during on-site immediate assessment were used. However, there were disadvantages associated with this preparation because cellular constitution and the number of tumor cells cannot be assessed prior to immunostaining and the smear may contain scant or no tumor cells. In addition, the preprepared smears have to be made prospectively at the time of FNA, whereas in routine practice a biomarker is often retrospectively requested by the treating physician after a cytomorphologic diagnosis has been completed when cell block tissue or preprepared smears are not available. Under such circumstances, the existing Papanicolaou-stained smears that have been used for routine cytomorphologic diagnosis may be used for ER and PR immunostaining. A decade ago, researchers at MD Anderson Cancer Center performed a validation study. They compared ER staining results between direct smears fixed under different conditions and the corresponding FFPE tumor sections and found that ER staining can be reliably performed on previously Papanicolaou-stained smears (without destaining) and that the use of antigen retrieval procedure greatly improved ER detectability and staining intensity without introducing false positivity. This technique has advantages because it allows for using archival Papanicolaou-stained smears for retrospective analyses of hormone receptor, and for evaluating cytologic features and numbers of tumor cells on the slides prior to immunostaining, and thereby allows for selecting the "most representative" slide for the test (Fig. 8.1). Since this validation study, this technique has been used in daily practice at MD Anderson Cancer Center. In some laboratories, a liquid-based monolayer preparation is used for ER and PR staining.

On the rare occasion where tumor cells of interest are present on a single smear but two or more immunostains are needed, a cell-transfer technique may be used. In brief, the original smear material is peeled, lifted, divided into several pieces, and then

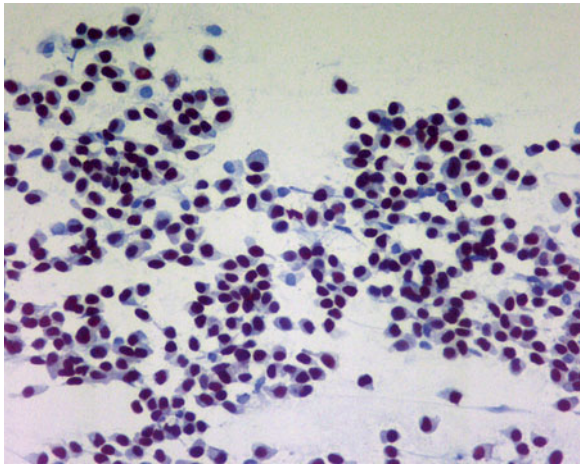

Fig. 8.1 Estrogen receptor was determined with immunocytochemical staining on a direct smear of a breast ductal carcinoma and was positive in approximately 95 % of tumor cells

transferred onto multiple new slides, thereby allowing for multiple immunomarker stains on different slides. This technique can avoid a repeat biopsy solely for immunophenotyping of the lesion.

Unlike hormone receptor testing, immunostaining of HER2 on direct smear or liquid-based preparations is not reliable for clinical use because tumor cells in these preparations may show distorted morphology and may not have intact cell membranes, preventing accurate evaluation of HER2 staining in the cell membrane. High variability in sample handling, fixation, staining, and interpretation has been reported (Dietel et al. 2007; Papouchado et al. 2010; Gong et al. 2011). However, FISH is an optimal method to test HER2 status in such preparations. Studies have showed that HER2 status determined using FISH can be reliably evaluated in cytologic slides with a significant correlation (91–100 %) between cytologic samples and paired FFPE tissue sections. The use of cytologic smears for FISH testing has an advantage over the use of FFPE section in that tumor cells on smears are mostly monolayered, which facilitates enumerating all the HER2 signals within an entire nucleus without a truncating artifact (Fig. 8.2).

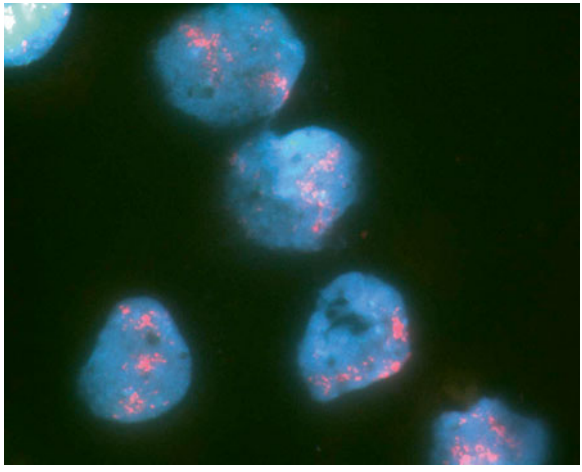

Fig. 8.2 HER2 testing using fluorescence in situ hybridization (FISH) was performed on a direct smear of a breast ductal carcinoma and showed HER2 gene amplification

Other in situ hybridization methods such as CISH and SISH can be evaluated using bright field microscopy and appear comparable to FISH in the determination of HER2 gene amplification on tissue sections. These methods detect HER2 copy number with a conventional peroxidase reaction and allow enumeration of gene copy number using a regular microscope in conjunction with histologic evaluation. A few studies have demonstrating that demonstrated that CISH can be performed with moderate accuracy on FNA sample, including cell block section, direct smear, or cytospin. However, large validation studies are required before this technique can be used for routine cytology practice.

There are several challenges associated with immunostaining on direct smear and liquid-based preparations. First, such preparations do not have proper control tissue, which should be processed and fixed in the same manner as the test sample for each run of immunostaining. Second, high background staining, which is usually associated with crowding of cells in a thick smear, may lead to misinterpretation of the staining result. Third, hormone receptor and HER2 status should be assessed only on the invasive component of

the breast carcinoma. The lack of reliable histologic architecture associated with aspirated material may lead to difficulty in distinguishing ductal carcinoma in situ from invasive carcinoma and therefore cause misinterpretation of the staining result. Therefore, caution should be taken in the interpretation of these biomarker results in primary breast carcinoma, especially if the tumor is small. Finally, sampling error or a small number of cells may lead to a false-negative interpretation in tumors that express a marker only focally and heterogeneously. Therefore, interpretation of immunostaining results on a sample with low cellularity should be very cautious especially when it is a negative result.

Overall, the decision regarding which test to perform on which sample type should be made on the basis of preparation type and expertise available in the laboratory. If a laboratory chooses to perform prognostic and predictive marker studies on cytology specimens, the preanalytic and analytic conditions should be validated according to the current guidelines. For laboratories that do not have specific experience with immunostaining on smear and liquid-based preparations, an effort should be made to obtain adequate cell block tissue for these tests.

Stability of Hormone Receptor and HER2

The status of hormone receptor and HER2 can be changed during disease progression, or altered by chemotherapy or targeted therapies. Therefore evaluating the stability of these biomarkers in a metastatic breast carcinoma is necessary. In large comparison studies at MD Anderson Cancer Center between primary and paired metastatic breast carcinomas, primary carcinomas were mostly FFPE sections whereas metastatic carcinomas were sampled mostly via FNA, and the biomarker status of metastatic tumors was usually tested in cytology direct smears. For ER testing, a high concordance rate (92.5 %) was observed for ER status in primary breast carcinomas and the paired metastatic breast carcinomas. When evaluating the effects of intervening endocrine therapy and chemotherapy, metastatic site (locoregional vs. distant), intervals between the two ER assays (<5 years vs. ≥5 years),

and sample type for the metastatic carcinoma (direct smear vs. cell block vs. CNB), researchers found that these factors did not significantly affect the ER concordance. For HER2 testing, the FISH technique was the sole method used for both primary and paired metastatic breast carcinomas in a study, and a high concordance rate (97 %) was observed for 60 patients with paired primary and metastatic breast carcinoma. It agreed with many other studies, indicating that HER2 status is generally stable during disease progression. Interestingly, several studies including ours showed that if the HER2 status does change, loss of HER2 protein overexpression and/or gene amplification seems to be more common than gain of it. However, the loss of HER2-positive status was seemly unrelated to intervening trastuzumab-based therapy. In addition, discordant ER, PR, and HER2 status between primary and paired metastatic breast carcinomas is associated with poorer clinical outcome compared with those with concordant status. The underlying mechanisms responsible for biomarker discordance could be multifactorial, including biologic evolution, intratumoral heterogeneity, technical (preanalytical and analytical) inconsistency, and inter-laboratory and inter-observer variability.

To standardize hormone receptor and HER2 testing and improve accuracy, reproducibility, and predictive power regarding response to targeted therapies, the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) have published guidelines to unify preanalytic (tissue processing and fixation), analytic (assay validation and standardization), and postanalytic (interpretation and reporting criteria) factors. FFPE tissue obtained via CNB or surgical resection is the typical sample type for primary breast carcinoma, whereas FNA is commonly used to sample metastatic tumors. The processing and fixation conditions for FFPE sections and for FNA smears are quite different. It is not uncommon that, in routine practice, a primary carcinoma is sampled and tested at a local hospital and a metastatic carcinoma from the same patient is biopsied at a tertiary referral hospital. In some patients whose primary carcinoma were diagnosed decades ago when biomarker status may have been tested by an old method (e.g., ligand-binding assay for ER) but the biomarkers for recently developed metastatic carcinoma may be tested

using immunostaining. Differences in preanalytic and analytic factors or in testing methods may account for biomarker discordance between primary and metastatic carcinomas.

Similarly, the discordance for HER2 status could be caused by different testing methods used in primary and metastatic carcinoma. The common scenarios are using immunostaining for primary tumor and FISH for metastatic tumor and vise versa. Although both methods are generally comparable in reliability, FISH seems to have higher sensitivity and specificity and better reproducibility than immunostaining, because FISH is less susceptible to variations in tissue processing and fixation. Notably, FISH measures the ratio of HER2 to CEP17 copy number; tumor cells with extra copies of chromosome 17, called polysomy 17, may appear to be HER2 negative according to FISH but may express high level of HER2 protein, leading to positive immunostaining result. A high-quality technique for HER2 testing in a laboratory requires experience. In a study that evaluated breast carcinomas showing 3+ HER2 immunostaining and/or HER2 gene amplification as determined by local laboratories, only 74% of the cases were confirmed to be HER2 positive by a central laboratory.

Even if primary and metastatic carcinomas are both sampled and tested using the same method at the same laboratory, interobserver variability in the interpretation may cause biomarker discordance, especially for tumors with borderline results (such as approximately 10% positive tumor cells for ER or 2+ immunostaining for HER2), as these cases are often associated with difficulty and subjectivity in interpretation. Furthermore, discordance may occur when different cutoffs are used for the tumor pairs. Cutoffs for positive ER and HER2 status as redefined in the latest ASCO/CAP guidelines are different from those used previously. Therefore, discordance of ER or HER2 status might be seen in some patients whose metastatic tumors were tested and interpreted following the current ASCO/CAP guidelines but whose primary tumors were tested before application of the guidelines. HER2 expression with a 2+ immunostaining score or a HER2/CEP17 ratio of 2.1 may have been classified previously as positive status, but according to the latest guidelines this is not the case without further confirmation.

Overall, given the importance of biomarker status in the management of breast cancer patients, hormone receptor and HER2 status should be tested in metastatic breast carcinoma if feasible.

The Evolving Role of Molecular Study in Breast Cancer Treatment and Research

To date, the common approach in identifying prognostic and predictive variables is to test one or a few biomarkers in a cohort of patients, usually retrospectively. The resulting information may not fully capture the biologic heterogeneity in tumor growth, invasion, and metastasis and cannot accurately determine the risk of relapse for individual patients. Consequently, some patients may be overtreated with systemic therapy, whereas others may not receive necessary therapy. Because each treatment modality has risks and benefits, managing breast cancer patients on an individual basis has become increasingly important.

As a high-throughput technique, gene profiling microarray facilitates personalized medicine because it allows for simultaneously measuring thousands of gene products from a single tumor sample. Gene combinations (i.e., gene signatures) provide more accurate prognostic and predictive information than any single gene measurement alone and therefore enable a better understanding of the complexity of breast cancer.

Gene profiling microarrays have been used widely in breast cancer research and treatment in three major ways. (1) Microarrays have been used in identifying intrinsic subtypes of breast carcinoma. The molecular classification is based on the similarity of gene expression patterns. At least five molecular subtypes have been described: luminal A, luminal B, normal-breast like, HER2-positive, and basal-like. Tumors that show similar clinicopathologic features may have different molecular phenotypes. In addition, molecular subtypes are of prognostic value. In ER-positive tumors, the luminal B subtype is more aggressive and more frequently develops resistance to endocrine therapy than does the luminal A subtype and therefore should also be treated with chemotherapy. (2) Microarrays are used in identifying gene signatures of prognostic variables, such as the 70-gene signature

(MammaPrint), 21-gene recurrence score (Oncotype DX), 76-gene signature (Veridex 76-gene panel), 97-gene signature (MapQuant DX). (3) Microarrays are used in identifying gene signatures that predict tumor response to neoadjuvant chemotherapy, endocrine therapy, and other targeted therapies.

Both FNA and CNB samples yield adequate amounts of total RNA for gene profiling microarray in experienced hands. According to a study from MD Anderson Cancer Center, the success rate of gene expression profiling with FNA samples began at 70–75 % and increased with practice to 97 %. There is a difference in cellular composition between FNA and CNB samples, with a high proportion of carcinoma cells in FNA samples and more stromal cells in CNB samples. The suitability of FNA samples for gene profiling microarray has been shown in a number of studies that sought to identify prognostic variables and to develop or refine genomic predictors of sensitivity to preoperative chemotherapy and a genomic index of sensitivity for endocrine therapy as well as to determine drug resistance mechanism. With comprehensive expression microarray data available, there has been interest in whether hormone receptor and HER2 status can be reliably generated from these data. A large multi-institutional study found a significant correlation between mRNA expression of ER and HER2 and the routinely determined status, with overall accuracies around 90 %.

It is promising that integration of ER and HER2 mRNA expression data with multigene signatures from the same microarray data may refine and improve their predictive power for tumor response to targeted therapies and therefore optimize clinical decision-making and tailoring of therapeutic regimens on an individual basis.

Breast Cancer Risk Assessment

Identification of women at high risk for developing breast cancer is an important step in cancer prevention, because these women may benefit from preventive intervention such as antiestrogen agents or surgical treatment. The risk stratification is assessed on the basis of Gail risk score and pathologic findings. Nipple fluid aspiration, ductal lavage, random periareolar FNA, and CNB have been used for cell acquisition. Some researchers performed nipple

aspiration followed by ductal lavage; however, these two methods were both associated with low diagnostic yield and some discomfort to the patients. In addition, to date there are no data available regarding the efficacy or mortality reduction for ductal lavage used as a screening or diagnostic tool. Periareolar FNA seems a better option for obtaining ductal and lobular cells and a better accepted method by study participants. The aspirated cells can be evaluated morphologically as well as for expression of biomarkers (epidermal growth factor receptor, ER, p53 protein, HER2, insulin-like growth factor 1, and others). A diagnosis of ductal cell hyperplasia with atypia is associated with an increased risk of developing breast cancer. Using FISH to screen for aneusomy in periareolar samples, researchers found that aberrations of chromosomal number were common in women at high risk for breast cancer. High-risk patients had significantly more monosomy of chromosomes 1, 11, and 17 and significantly more polysomy of chromosome 8 compared with low-risk patients.

Conclusions

In addition to the use in cytomorphologic diagnosis, FNA samples have been increasingly used for the molecular study of breast cancer to facilitate research sample procurement and, more importantly, to provide prognostic and predictive information and thereby guide clinical management. When FNA tissue is used to test biomarkers for patient care, it is important to standardize or validate the method for the steps of sampling, processing, staining, and interpretation.

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Molecular Pathology of Urine Cytology

9

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Urine Cytology and Urothelial Cell Carcinoma (UCC)

It is projected that close to 75,000 new cases and over 15,000 deaths of urinary bladder cancer in the USA in year 2014, most of which are urothelial cell carcinoma (UCC) [1]. The male to female ratio is about 3 to 1. The cost per patient is the highest of all the cancer types, reaching approximately 200,000 US dollars per

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patient from diagnosis to death [2]. Majority urothelial cell carcinomas (75 %) present with superficial disease (Stage Ta and T1), while 20 % present with Stage T2 or greater disease.

There are basically two types of UCC, low grade and high grade, which follow different molecular tracks for development and progression, and thus have different clinical presentation and behavior [3]. The low grade tumor, typically initiated with FGFR3 mutation, presents as papillary lesions that have minimal to mild cytological abnormalities, are usually superficial, non-muscle invasive, and not-metastatic. The high grade tumor, on the contrary, is usually associated with p53 abnormality, can be either flat or papillary lesions that have more prominent cytological and nuclear atypia, and has much higher risk for muscle invasion and metastasis. Carcinoma in situ, which is considered a precancerous lesion in other organ systems, is a high grade malignant condition that typically precedes the development of high grade papillary UCC or invasive UCC. However, about 30 % low grade UCC may progress to high grade UCC with time [4].

Traditionally UCC is detected and monitored by the combination of cystoscopy and urine cytology tests. These two tests are complementary as cystoscopy is highly effective in identifying papillary lesions while urine cytology is effective in detecting high grade, especially flat lesions such as carcinoma in situ. Unfortunately, cystoscopy is an expensive and invasive procedure and often may miss a flat lesion, whereas urinary cytology, though noninvasive has very low sensitivity (between 20 and 50 %) for low-grade papillary tumors [5]. Therefore, adjunct molecular markers with high accuracy for the detection of both low and high grades of urothelial carcinoma will significantly reduce patient cost, anxiety, and morbidity.

Whereas numerous adjunct markers have been studied thus far, and there are many excellent reviews for this topic in the past, this chapter will discuss mostly the two commercially available cellular-based tests, the more widely used UroVysion or fluorescence in situ hybridization (FISH) test and the less well known ImmunoCyt or uCyt test. Other tests that are either have great potential (e.g., ProEx c) or commercially available but non-cellular based (e.g., NMP 22) are also briefly discussed.

Fluorescence In Situ Hybridization (FISH) UroVysion™

Cytogenetically, urothelial cell carcinoma, especially high grade UCC is an aneuploid cancer, and contains multiple copies of chromosomes [6]. Many of these findings were confirmed using with Feulgen nuclear staining with digital imaging analysis of DNA density in cells. Subsequent to this, FISH utilized molecular probes to detect the chromosome abnormalities of urothelial cells. Centromere enumeration probes for chromosome 3, 7, and 17 label the centromere of each respective chromosome. Presence of more than two signals within a cell would indicate an abnormal DNA content and increase the suspicion for malignancy.

The DAPI stain (4′6-diamidino-2-phenylindole) is used to stain the nucleus blue under fluorescence microscopy [7]. Benign urothelial cells will show a homogeneous staining pattern, demonstrating an even chromatin distribution within a cell with normal DNA content. Malignant cells show large nuclei and a clumped, heterogeneous chromatin pattern. This reflects an aneuploid cell with dark, coarse chromatin distribution and nuclear irregularity, characteristic of the findings of urothelial carcinoma cytology. These cells can be detected of fluorescence microscopy either manually by a molecular technologist or cytotechnologist. Automated screening systems may also be used.

After detection of a morphologically abnormal cell on DAPI staining, various filters can be used to detect the fluorescent probes. Centromere enumeration probes (CEP) directed toward the chromocenter of chromosomes 3 (red), 7 (green), and 17 (aqua) reflect the number of copies of chromosomes. Gold detects the locus specific 9p21. Interpretation is performed by screening for large abnormal cells on DAPI, then examining them with each filter. In many laboratories cytotechnologists, who are already trained to screen for abnormal cells on bright field microscopy, can be trained to detect probes through the various filters and perform a count.

In regard to specimen adequacy, while the number of urothelial cells varies by lab, the presence of 25 urothelial cells may be accepted in most cases. Different labs have various cutoffs for abnormal cases. Generally, when one cell with an abnormal

number of probes is detected, a search for at least 4 or more abnormal cells should be performed. Once four cells are found, the case may be signed out as "Positive for aneusomy." These patients are at increased risk for cancer, even when the cytology is negative. An abnormal cell shows more than 2 signals in 2 or more probes. For instance, 3 signals in chromosome 7, and 6 in chromosome 17 would be considered an abnormal cell. Special cases to consider are when all probes show 4 signals (tetrasomy). These cells may represent malignancy; however, they may also represent a dividing urothelial cell, which may be 2N. Tetrasomic cells are found more frequently in the upper urothelial tract and should be interpreted with caution. Once 4 abnormal cells are found, some laboratories may count 100 consecutive urothelial cells and provide a percentage of abnormal cells. Higher percentages of abnormal cells would indicate a higher tumor load. True 9p21 (gold) loss occurs in clusters of urothelial cells which may represent low grade papillary lesions of the bladder.

FISH in the upper urothelial tract should be interpreted with caution, because of the possibility of false positive cases [8]. Tetrasomic cells in the upper tract are more frequently found due to more mitotically active cells present in the upper tract and pelvis. Tetrasomic cases should be interpreted as suspicious for malignancy, but not as positive. In addition, concomitant urothelial carcinoma of the bladder may lead to a false positive result.

According to the FDA criteria, the normal diploid urothelial cells have two signals for each chromosome. An abnormal FISH assay for a suspected urothelial neoplasia would require a minimum of four cells with polysomy of at least two of the four chromosomes (Fig. 9.1a) or a minimum of 12 cells with homozygous loss of P16 genes when a minimum of 25 large atypical cells were examined.

The sensitivity of this test is estimated at 30–86 % and the specificity is estimated at 75–100 %. The sensitivity increases in higher grade tumors. Low grade and early stage tumors are more difficult to detect since the test depends on the amount of tumor cells on the slide. Additionally studies have shown up to 50 % of false positive cases developed bladder cancer recurrence within months ("false" false positive or anticipatory positive), suggesting Urovysion may be utilized as an adjunct to predict recurrence.

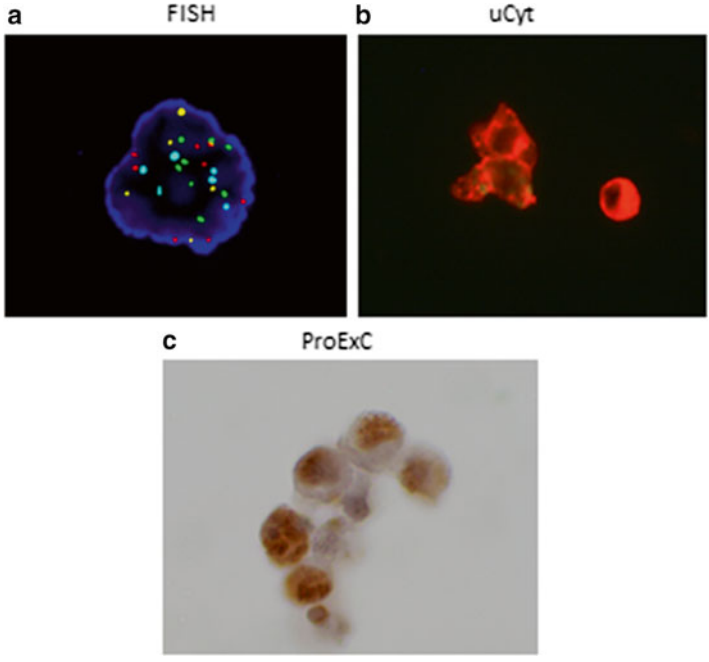

Fig. 9.1 (**a**) UroVysion fluorescent labeling in an abnormal cell with polysomy of chromosomes 3, 7, and 17. FISH analysis with probe panel (4 probes) which includes alpha satellite (CEP) probes specific for chromosomes 3, 7, and 17, and locus specific probe (LSI) at the 9p21 region was performed. (Courtesy of Dr. Oscar Lin, Memorial Sloan Kettering Cancer Center). (**b**) The uCyt immunofluorescence assay from a malignant urine cytology specimen demonstrating positive staining. One antibody is directed against glycosylated carcinoembryonic antigen labeled with Texas Red (*red*). The other antibodies are against mucin glycoproteins, LD10 and M344, labeled with fluorescein (*green*). The presence of at least one atypical cell with either red or green fluorescence was considered positive for malignancy (60× objective). (**c**) ProEx C immunostain shows positive nuclear staining of the cellular cluster (60× objective)

The advantages of the test are the high specificity for detecting bladder cancer and ability to predict bladder tumor recurrence prior to clinical detection. The disadvantage of the assay is not all bladder tumors demonstrate these chromosomal abnormalities, especially the low grade tumors. Thus the test has relatively low

sensitivity to other tests such as uCyt (see below) for detecting low grade tumors, which is the area that really need help in urine cytology. The difficulty in defining abnormality or positive sample as showing above is also one of the limiting factors.

ImmunoCyt (uCyt+)

The uCyt+ test is a quantitative test that detects tumor-related antigens, commonly mucinglycoproteins, using monoclonal antibodies [9]. Three antibodies are used: fluorescein labeled M344 and LQ10, both of which are directed against sulfated mucinglycoproteins and Texas Red linked antibody 19A211 directed against glycosylated forms of high molecular carcinomaembryonic antigens (CEA). A positive score is assigned when 1 of the 3 biomarkers (2 for the mucin glycoprotein and 1 for the glycosylated form of carcinoembryonic antigen) is detected in a cell with red or green fluorescence. Both green and red fluorescent cells may be seen in a single positive sample (Fig. 9.1b). Though manufacture instruction suggests any sample with one cell positive with either red or green fluoresce is scored as positive, in practice this cutoff may produce high false positive rate thus, a borderline positive is introduced in some laboratories.

In our practice, uCyt test is used as reflex test for atypical urine cytology. If no positive cell (either green or red fluoresce) is found, the specimen is scored as negative, and routine follow-up is recommended. If a specimen has 1–4 cell positive, it is scored as borderline or equivocal for the test. In such a case, repeat urine cytology within 3 months is recommended. A sample is scored as positive if 5 or more cells are positive, in such as case immediate cystoscopic follow-up is recommended.

The uCyt+ test has many advantages including high sensitivity, simple technical procedure, and relative less expensive comparing to UroVysion. Unlike many of the other molecular tests offered for urine cytology, uCyt is more sensitive in detecting low grade urothelial tumors. The M344 antibody is most sensitive for these lesions. The estimated sensitivity ranges from 67 to 100 % and the estimated specificity ranges from 62 to 84 %. As compared with cytology and Urovysion, uCyt+ outperforms both tests [3,10].

One disadvantage of uCyt is the low specificity as compared with cytology and Urovysion. However, just like FISH, studies have shown that patients with a positive uCyt but negative cystoscopy have an increased rate of bladder cancer detection at 12-month follow-up. These findings may represent early lesions that are not visible on cystoscopy. Potential benign conditions that lead to false positive uCyt results include urinary tract obstruction caused by renal stones and benign prostatic hypertrophy. Another potential disadvantage is interpretative difficulty due to lack of experience of dealing with fluorescence microscopy. Also, uCyt is not indicated in patients with loop or ileal conduit urine specimens since bowel mucosal cells are positive for glycosylated CEA, one of the tested antibodies.

Other Potential Cellular Markers

ProEx C

ProEx C is an antibody cocktail targeting the expression of topoisomerase IIa and minichromosome maintenance protein-2 (MCM2). ProEx C staining originally was used to assist in diagnoses of the gynecological specimens [11,12]. Moatamed et al. have shown that ProEx C stain is a useful adjunct test to urine cytologic analysis. In urine smears, this test is most useful in stratification of the "atypical" diagnoses into benign and malignant subsets [13]. In their studies, they scored ProEx C as positive in urine cytology samples when nuclear staining was seen in at least one morphologically atypical urothelial cell (Fig. 9.1c). ProEx C stain has an overall sensitivity of 78.4 % and specificity of 95.7 %. Subsequently the same group evaluated and compared the assay performances of ProEx C immunostain with uCyt [14]. Their comparative study of ProEx C and uCyt assays in atypical urine cytology demonstrated that ProEx C has superior specificity to uCyt. The combination of the two tests yielded high sensitivity not only for high-grade urothelial carcinoma but also for low grade papillary urothelial carcinoma. ProEx C displayed a lower sensitivity in detecting low grade urothelial carcinoma (72 %) than in detecting high grade urothelial carcinoma (92 %). They have also used histologic sections of the

urothelial neoplasia, which demonstrated that the ProEx C staining involves the full thickness of the cancerous epithelium in high grade urothelial carcinoma, whereas the reaction is focal in low grade papillary urothelial carcinoma and the positive cells may not reach the surface for exfoliation into the urine samples. This observation may account for the lower sensitivity of the assay in low grade papillary urothelial carcinoma [15]. Chang et al. have compared the assay performance of ProEx C immunostain and UroVysion FISH on urine cytology specimens. This study showed that ProEx C immunocytochemistry has a more favorable performance than FISH [16].

Telomerase

Telomerase is a polymerase chain reaction based test that quantitatively assesses telomerase levels in urine. Telomerase is a ribonucleoprotein that adds telomeres to the ends of chromosomes; telomeres are segments on the ends of chromosomes that maintain the integrity of DNA and regulate cell death. Increased levels of telomerase lead to immortality of tumor cells. Telomerase can be measured by PCR amplification (telomeric repeat amplification protocol (TRAP) assay) or by real time PCR. It has a sensitivity of 7–100 % and a specificity of 60–70 % [17]. Lymphocytes and other benign cells may explain the lower specificity. TRAP has a low sensitivity of approximately 35 % in detecting recurrent tumors. Urine must be processed within 24 h for the telomerase test and at least 50 cells must express telomerase for the test to be reliable, making telomerase testing more difficult to implement as compared with other molecular ancillary tests. Recently an antibody based Telomerase test has been developed by Sienna Inc, Australia. However, the test remains to be validated.

Cytokeratins

Molecular methods to test for various cytokeratin expressions have been used as a potential evaluation tool for bladder cancer. Cytokeratins compose the majority of intermediate filaments in

epithelial cells. There are 20 cytokeratins that have been identified in epithelial cells, of which cytokeratins 8, 18, 19, and 20 have been linked to bladder cancer.

Presence of cytokeratin 8 and 18 in urine of bladder cancer patients can be detected by UBC-Rapid and UBC-ELISA tests. The UBC-Rapid is a point of care test, while the UBC-ELISA is a 2 h sandwich ELISA test. The sensitivity ranges from 12 to 79 % and the specificity ranges from 63 to 97 % for both primary and recurrent bladder cancers. The sensitivity decreases in lower grade and lower stage tumors; the sensitivity of stage Ta tumors and in situ lesions is estimated at 21–25 %, making this test an unreliable diagnostic tool [18].

Cytokeratin 20 expression can be detected by reverse transcription PCR assays. Normal expression of cytokeratin 20 is usually seen in the superficial and occasional intermediate cells of the bladder, without expression in the basal cells. Aberrant expression is seen in bladder cancer cells. The reported sensitivity ranges from 78 to 87 % and the specificity ranges from 55 to 98 % [3].

Cytokeratin 20 immunocytochemistry can be a useful adjunct marker in atypical urine cytology cases and used to stratify cases into low and high risk categories for clinical follow-up. The sensitivity has been reported as 65–86 %, while the specificity is 86–100 % [19]. False positive results were seen in patients with pre-malignant conditions, while completely healthy patients have negative results. As with other keratin testing, sensitivity varies based on tumor grade and stage, with sensitivity being much higher for grade 2 and 3 tumors.

Cytokeratin 19 can be measured in the urine by a solid phase sandwich immunoradiometric assay or an electrochemiluminescent immunoassay. CK 19 is expressed in normal urothelium and a soluble fragment CYFRA 21-1 can be measured in urine when the cells are exfoliated and lysed. Levels of CYFRA 21-1 are increased in patients with bladder cancer. The average quantitative levels in patients with bladder carcinoma are 154 ng/mL; in patients with other urologic conditions including urolithiasis, urinary tract infection, and benign prostatic hypertrophy, the average level is 22 ng/mL; and in normal patients the average level is 2.4 ng/mL. When a cutoff of 4 ng/mL is used, average sensitivity ranges from 43 to 79 % and specificity ranges from 68 to 88 % [20].

BLCA-4 and BLCA-1

There are specific nuclear matrix proteins which are present in patients with bladder cancer (BLCA1-6) and those present in normal bladder tissue (BLNL1-3). The specific BLCA-4 marker is expressed in the normal bladder and malignant areas of the bladder in patients with urothelial carcinoma, likely representing a "field effect" from the tumor to non-tumor regions. An indirect ELISA shows BLCA-4 levels to be significantly higher in patients with bladder cancer as compared with normal controls [21]. Similarly, BLCA-1 is another potential tumor marker; however, it is only seen in tumor areas and not expressed in normal adjacent tissue.

Hyaluronic Acid/Hyaluronidase

Hyaluronic acid (HA) and hyaluronidase (HAase) levels are increased in patients with bladder cancer. HA is a glycosaminoglycan that promotes tumor cell adhesion and angiogenesis. HAase is the enzyme that cleaves hyaluronic acid into fragments, which promotes angiogenesis and causes tumor growth and invasion. It is an ELISA-like test that combines the analysis of both HA and HAase. Regardless of tumor grade, there is a two to sixfold increase in HA-HAase activity in patients with urothelial carcinoma. The estimated sensitivity is 83 % and the estimated specificity is 90 % [22]. HA-HAase testing can be used for detecting both primary and recurrent bladder tumors and has high sensitivity to detect low grade/low stage tumors and high grade/high stage tumors.

Survivan

Survivan is an anitoapoptotic protein. Survivan mRNA has been shown to be expressed in bladder tumors. Its presence in patients with various tumors, including urothelial carcinoma and colorectal cancer, has been associated with unfavorable prognosis. A polyclonal antibody and real time PCR for survivan has been helpful in detecting new onset and recurrent bladder cancer [23].

Additionally, survivan levels appear to be higher in patients with recurrent tumors as compared with those in remission after BCG or mitomycin C therapy [24]. The sensitivity of using RT-PCR to detect survivan ranges from 53 to 94 % and specificity ranges from 88 to 100 % [23].

DNA Ploidy and S-Phase Fraction

DNA ploidy is probably the most widely studied urine adjunct biomarkers for UCC. In addition to FISH testing, DNA ploidy and S-phase fractions can be assessed by flow cytometry, image cytometry and laser scanning cytometry to identify malignant cells with increased nuclear size and increased chromatin ratios. Studies have shown the DNA ploidy analysis, especially if detected using Quantitative Fluorescence Image Analysis (QFIA), can be quite effective in detecting and monitoring UCC [25]. In a longitudinal prospective study of high risk occupational, DNA ploidy abnormality, as indicated by DNA 5c exceeding rate analyzed by QFIA analysis, was a rather sensitive and specific marker for detecting UCC [26]. Together with M344, the bladder tumor antigen that is included in the ImmunoCyt or uCyt test, over 95 % of UCC were detected. However, technical limitations prohibited the widespread application of these techniques. Flow cytometry analysis requires a large number of cells. Image based methods, including QFIA or laser scanning cytometry, though has advantage of analyzing these markers on single cell basis, require careful quality control measures and appropriate training of laboratory personal.

DD23

DD23 is a monoclonal antibody that recognizes an antigen present in the majority of bladder tumors, up to 81 %. It is derived from the immunization of mice with bladder cancer. The antigen can be tested for by Quantitative Fluorescence Image analysis. The estimated sensitivity is 85 % when used alone and 94 % when used in

combination with cytology. The estimated specificity is 95 % when used alone and 85 % when used in combination with cytology [27]. Another modality for testing the antigen is immunocytochemistry. The estimated sensitivity is 81 % when used alone and 85 % when used in combination with cytology. The estimated specificity is 60 % when used alone and 55 % when used in combination with cytology. The fluorescent assays have better overall detection of bladder cancer and the specificity decreases when used in combination with cytology

Quanticyt Nuclear Karyometry

Quanticyt is an automated quantitative karyometric cytology system that interprets nuclear shape and DNA content based on microscopic images. Light microscopy nuclear images of cytospin preparations are transferred to a computerized image analysis system, where an internal lymphocyte standard measure nuclear shape and DNA content. The results are then used to risk stratify the sample into low, intermediate, or high risk. Sensitivity is estimated at 59–69 % with sensitivity being highest for higher grade tumors. The estimated specificity is 70 % [28]. The test has limited clinical utility due to low sensitivity, complicated instrumentation and necessary expertise, and potential to overestimate the risk of bladder cancer.

Prostate Stem Cell Antigen

Prostate stem cell antigen is a glycosylphosphatidylinositol anchored cell surface antigen that has increased expression in bladder cancers. It is expressed in more the majority of local and metastatic tumors. The PSCA immunocytochemistry test is used on voided urines. The estimated sensitivity is 80 % alone and 83 % with cytology. The estimated specificity is 85 % alone and with cytology [29]. Limitations include false positivity in patients with interstitial cystitis and hematuria.

Dips-Tick Based Markers

Nuclear Matrix Protein 22 (NMP-22)

Nuclear matrix protein 22 is a molecular marker that is currently used as an adjunct screening tool for urothelial carcinomas [30]. Nuclear matrix proteins are a web of RNA and proteins that make up the framework of the nucleus and are associated with DNA replication and RNA synthesis. NMP-22 is a 238-kDA protein that is released from the nuclei of urothelial tumor cells during apoptosis into urine. There is a 25-fold greater concentration of NMP-22 released by urothelial carcinoma cells as compared with normal urothelium. The enzyme linked immunoassay uses 2 monoclonal antibodies to measure mitotic activity in urine. The estimated sensitivity ranges from 32 to 100 % and the specificity ranges from 56 to 95 % when used in conjunction with cytology [31]. False positivity was most commonly attributed to benign inflammatory conditions, stents, renal calculi, bowel interposition, and instrumentation. Overall, NMP-22 has a higher sensitivity that cytology alone, especially in assessing for low grade neoplasms [32]. The test is best used to detect low grade bladder cancer and to monitor for recurrence after transurethral resection [33].

Bladder Tumor Antigen (BTA)

Bladder tumor antigen (BTA) encompasses three tests that are used for detecting invasive urothelial carcinoma: BTA, BTA Stat, and BTA TRAK. The original BTA is a latex agglutination test that measures quantity of basement protein antigen released into urine. The estimated sensitivity of the test is 52 % and the specificity is 85 %. The BTA Stat and BTA TRAK detect human complement factor H protein (hCFH). The BTA Stat is an immunoassay, and the BTA TRAK is a standard enzyme linked immunosorbent assay ELISA. The sensitivity ranges from 9 % to 89 %, and is dependent on tumor grade, stage and size. The specificity has been reported up to 90 % in healthy individuals, but is reduced to only 50 % in patients with hematuria, proteinuria, renal calculi, nephritis, and cystitis. A recent meta-analysis of 13 publications with total 3462

patients showed that BTA test though has greater sensitivity than the urine cytology test, has lower specificity, area under curve (AUC), and the Q index compared to the urine cytology test [34].

Fibrin–Fibrinogen Degradation Products (FDP) [35]

Fibrin degradation products (FDP) is a quantitative test that measures fibrin–fibrinogen products, which are increased in patients with bladder carcinoma. Bladder tumor cells cause increased vascular permeability and proteins including fibrinogen are passed into the urine. Urokinase converts the fibrinogen to fibrin–fibrinogen degradation products. The estimated sensitivity ranges from 68 to 83 % and the specificity ranges from 68 to 100 %. The test is best used in detecting high grade urothelial tumors. As with the other molecular tests discussed, the disadvantages include poor sensitivity in low grade tumors and poor specificities in patients with inflammatory conditions, renal calculi, cystitis, and hematuria.

Summary

Adjunct markers for urine cytology of detecting and monitoring UCC have been an area of intensive research in the past few decades. However, there is still no single marker that can replace the standard practice of urine cytology combined with cystoscopic evaluation. Much may be due to the fact that UCC has two distinctive types of diseases with different clinical and molecular features as well as cytological presentations. Nevertheless, some of the markers, such as FISH and uCyt, do provide clinical value that may help cytological evaluation of urine sample for either detecting or monitoring UCC.

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10 Molecular Biomarkers of Pancreatobiliary and Gastrointestinal Tract Neoplasms

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Introduction

Molecular genetic alterations in gastrointestinal tract neoplasms can be classified into four categories: (1) Chromosomal alterations, including numerical and or structural anomalies of chromosome; (2) Gene-level somatic alterations, including point mutation, deletion, insertion, and amplification; (3) Epigenetic alteration, including promoter methylation of specific genes and microRNA deregulation; (4) Protein level alteration, including increased or

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decreased protein expression and posttranslational modification. Chromosomal numerical alterations are changes in copy number of various genetic regions. Chromosomal structural alterations are gains, losses, translocations, or rearrangement of chromosomes, resulting in alterations of DNA copy number at the chromosome locus level. Genetic mutation is permanent alteration of DNA sequence of a gene and can be either germ line, i.e., present in all cells of the body, or somatic in tumor cells only. It is true that not every mutation has clear biological significance. Mutations that have functional consequence and confer selectively growth for tumor development are "driver" mutation. Conversely, genetic mutations without functional consequence of tumor growth are "passenger mutation." Mutations can be classified as "Small-scale mutations" and "large-scale mutations." Point mutations, insertions, deletions are small-scale mutations, whereas amplification, deletion of large regions, gene fusion such as chromosomal translocations, inversions, interstitial deletions, and LOH are large-scale mutations. Amplification is an increase in the number of copies of a gene without increasing copy numbers of a chromosome. Epigenetics generally modifies and regulates transcriptional activity by adding methylation to CpG islands at the promoter regions of a specific gene, or regulates chromosomal structure by methylating or demethylating certain histone proteins.

Molecular Alterations in Pancreatic Ductal Adenocarcinoma

Several core signaling pathways have been reported involving in carcinogenesis of the pancreatic ductal adenocarcinoma, including K-RAS, TGFβ, JNK, Integrin, Wnt/Notch, Hedgehog, small GTPase pathways, control of G1/S phase transition, apoptosis, DNA damage control, invasion, and hemophilic cell adhesion. Mutations in KRAS, P16/CDKN2A, TP53, and SMAD4/DPC4 are commonly reported in pancreatic ductal adenocarcinoma. KRAS is the most frequently identified oncogene (>90 %) in ductal adenocarcinoma. Somatic mutations in KRAS have been linked to the reduced survival, especially when combined with other

genetic alterations (such as p16). The frequency of tumor suppressor genes alterations are p16 (95 %), p53 (75 %), and SMAD4 (55 %). Somatic inactivation of SMAD4 occurs via homozygous deletion or intragenic mutation coupled with loss of the wild-type allele. SMAD4 loss is associated with metastasis and poor prognosis. MicroRNAs including miR-21, miR-155, and miR-221, have been reported to overexpress in ductal adenocarcinoma, but not in benign tissues.

Despite substantial molecular research progress in pancreatic ductal carcinoma, very limited molecular biomarkers are available for clinical use in the aspects of diagnostic, prognostic, and predictive points. To date, there are no established tissue markers, gene signatures, or genomic targets in pancreatic ductal adenocarcinoma

Molecular Cytologic Assays in Detection of Pancreatobiliary Carcinoma

Data generated from multigene next-generation sequencing revealed that at least one mutated gene was observed in 70 % cholangiocarcinomas, Among them KRAS is the most frequently mutated gene (13.6–28 %). Although these techniques seem to have a high sensitivity, they suffer from low specificity. In 2014, the Papanicolaou Society of Cytopathology published guidelines for pancreatobiliary cytology utilization of ancillary studies in the cytologic diagnosis of biliary and pancreatic lesions. In this guideline, KRAS mutation tests are belong to "insufficient specificity for malignancy to warrant usage" for pancreatobiliary strictures/lesions. Other mutated genes in pancreatobiliary carcinoma include IDH1/2, ARID1A, BAP1, PBRM1, and SMARCB1, and less commonly BRAF, APC, PIK3CA, CDKN2A, and PTEN. Novel gene fusions involving the tyrosine kinases FGFR2 and NTRK1 have been reported recently. Cells harboring FGFR fusions showed enhanced sensitivity to the FGFR inhibitors, suggesting patients with FGFR fusions may benefit from targeted FGFR kinase inhibition. On technical aspect, there are several platforms, such as FISH, LOH, and NGS assays that have either tested or utilized clinically.

FISH assay is "diagnostically useful" and "is the preferred test to complement routine cytology" per the recently published Papanicolaou Society of Cytopathology guideline. Kipp et al. at Mayo Clinic evaluated chromosomal gain/loss in pancreatobiliary tract brushings and aspirate specimens by using commercially available FISH assay Vysis® UroVysion (CEP 3, 7, and 17 and LSI 9p21). They observed that FISH had higher sensitivity than cytology alone without scarifying significantly clinical specificity. Several other independent studies also showed similar findings by using the FISH assay with UroVysion probe. Fritcher et al. reported by far the largest cohort study assessing 498 patients comparing cytology and FISH assay with clinicopathologic follow-up. The sensitivity of FISH was significantly higher than cytology for detecting malignancy (43 % vs. 20 %; $P<0.001$). Patient with a polysomy was 77.6 times more likely to have malignancy than a patient with a negative FISH result. Of all the ancillary molecular techniques currently available for analysis of cytology specimens obtained by brushings from pancreatobiliary strictures, FISH appears to improve diagnostic sensitivity the most over that achievable by routine cytology.

Loss of heterozygosity (LOH) analysis has been well studied in biliary duct brushing cytology specimens. Ohori et al. studied LOHs in bile duct brushings paired with surgical cases by using PCR amplification with microsatellite markers associated with tumor suppressor genes, including CMM, MYCL 1 (1p36–1p34), VHL (3p26–3p25), APC (5q23–5q23), MCC, CDKN2A (9p21–9p23), PTEN (10q23–10q23), MXI 1, and p53 (17p13–17p13). They found LOH frequency of each focus ranged from 25–71.4 % in carcinoma cases. Khalid et al. developed a panel of microsatellite markers of RIZ (1p36–1p34), VHL, APC, CDKN2A, PTEN, and p53 to detect LOH of pancreatobiliary malignancy in brushing cytology samples. They found that pancreatobiliary malignancy has abundant LOHs defined by fractional mutation rate while brushings from cases without cancer carried no LOH. Finkelstein et al. performed LOH analysis in centrifugation supernatant fluid from pancreatobiliary duct samples. A panel of 16 microsatellite markers was targeting common sites of

tumor suppressor genes associated with pancreatobiliary cancer, including 1p, 3p, 5q, 9p, 10q, 17p, 17q, 21q, and 22q. Out of 33 specimens with outcome tested, mutations defined by LOH markers were detected in 25/28 malignant specimens, while no mutations were found in 5/5 of benign specimens. Silverman's group examined LOH and KRAS mutation in 40 pancreatic duct brushings and 21 bile duct brushing specimens. The descending frequency of detectable mutational involvement in pancreatic cytology was KRAS point mutation (58 %), LOH at 3p25–26 and 17q21 (35 %), LOH at 5q23 (33 %), and LOH at 1p36 (28 %). These studies demonstrate that incorporation of molecular studies in cytologic specimens can be very useful in making a more definitive and accurate diagnosis of malignancy in pancreatobiliary cytology specimens.

RedPath Integrated Pathology, a Pittsburgh based commercial company, offers a non-FDA approved PathFinderTG® biliary testing, which assesses biliary stricture profile using oncogene mutations and LOH markers. Studies have shown an added value of PathFinderTG in detecting malignancy of biliary tract. PathFinderTG is especially valuable in cases where no malignant cells are obtained for testing, since it works for cell-free supernatant fluid collected from brushing biliary strictures procedure.

Additional techniques for determining malignancy in pancreatobiliary lesions include microRNA (miRNA) analysis. MicroRNAs (miRNAs) are short, noncoding 18–25 nucleotides RNAs that targeting specific mRNA moieties for translational repression or degradation. Nearly 100 miRNAs are differentially expressed in pancreatic ductal adenocarcinoma, including upregulation of miR-21, 23a, 27a, 31, 100, 143, 146a, 55, 181b, 200a, and 221 and downregulation of miR-148a, 217, and 375. In addition, high expression of miR-21 and miR-31 and low expression of miR-375 are associated with poor overall survival. The miRNA profile analysis has been successfully applied in fine needle aspiration specimens and may serve as potential diagnostic and prognostic biomarkers for pancreatic ductal adenocarcinoma. Presence of miRNA including miR-21 and mi-155 supports a diagnosis of adenocarcinoma. However, its clinical utility needs to be determined.

Molecular Alterations in Pancreatic Cystic Neoplasms

Pancreatic neoplastic cystic neoplasms encompass intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms, and serous cystadenomas. The first two cystic neoplasms have the potential to progress to pancreatic adenocarcinoma. Cyst fluids can be obtained by EUS endoscopic aspiration and are often acellular, making it difficult to evaluate cytomorphologically. However, these cystic fluids are valuable and can be analyzed for biochemical and molecular markers.

IPMNs are the most common type of neoplastic cysts (25–35 %), which can be divided into main duct, branch duct, and mixed type based on location. Histologically, IPMNs can show intestinal, gastric, pancreatobiliary, and oncocytic types. The intestinal-, pancreatobiliary-, and oncocytic-type IPMNs occur predominantly in the main duct and possess higher risk than gastric type in progression to invasive carcinoma. GNAS gene is a well-known oncogene functioning as a signal transducer between hormonal receptors and adenylyl cyclase. Using massively parallel next-generation sequencing, Wu et al. recently sequenced 169 genes in cyst fluids of 19 IPMNs GNAS and KRAS mutations were detected in 61 and 82 % of the IPMN fluids, respectively. More than 96 % of IPMNs had either a GNAS or a KRAS mutation and more than half have both. All GNAS mutations occurred at codon 201, resulting in a G12D, G12V, or G12R amino acid change. Combination of GNAS and KRAS mutation detection provides high sensitivity and specificity for IPMNs. GNAS or KRAS mutations were not identified in any cases of SCAs. RNF43 gene, encoding a protein with intrinsic E3 ubiquitin ligase activity, was the third most commonly mutated gene identified in IPMNs. Mutations in p53 and BRAF genes seem to be late event since they were only observed in high-grade IPMNs. Different subtypes of IPMN appear to have different pathways of neoplastic progression. Gastric- and pancreatobiliary-type IPMNs show higher rates of KRAS mutation than intestinal-type IPMNs, whereas GNAS mutations are most prevalent in the intestinal-type IPMNs.

In mucinous cystic neoplasm, KRAS is the most frequently mutated gene and correlates with the degree of neoplastic progression: 26 % of low-grade dysplasia, 38 % of intermediate-grade dysplasia, and 89 % of high-grade dysplasia or carcinoma. p53 mutation is a relatively late event of MCN and occurring only in areas with high-grade dysplasia or invasive carcinoma.

The most common genetic abnormality in serous cystadenoma is the VHL gene mutation.

In the Papanicolaou Society of Cytopathology recently published guidelines for pancreatobiliary cytology utilization of ancillary studies, the proposed clinical helpful molecular biomarkers for cystic pancreatic lesions include KRAS, GNAS, RNF43, and VHL mutations. KRAS mutational testing is used to distinguish mucinous from non-mucinous cysts. GNAS mutations support the diagnosis of IPMN. RNF43 mutations assessment is used to distinguish mucinous from non-mucinous cysts. Presence of VHL gene mutation supports the diagnosis of SCA. Recently Wu et al. demonstrated that analysis of five genes (KRAS, GNAS, RNF43, VHL, and CTNNB1) was able to define types of pancreatic neoplastic cystic lesions. IPMNs had alterations of RNF43, GNAS or KRAS and never had VHL or CTNNB1 mutations. MCNs always harbored KRAS or RNF43 mutations but never contained GNAS, CTNNB1or VHL mutations. SCAs had intragenic mutations of VHL or LOH in or adjacent to VHL and did not contain mutations of the other four genes.

Molecular Alterations in Pancreatic Neuroendocrine Tumor

Patients with MEN1 syndrome have germ line mutations in MEN1 tumor suppressor gene, and 60–70 % of them develop pancreatic neuroendocrine tumors. Up to 45 % of sporadic pancreatic neuroendocrine tumors have inactivating somatic mutations of the death domain-associated protein (DAXX) and alpha-thalassemia/mental retardation syndrome X-linked (ATRX) genes, which are involved in chromatin remodeling. Mammalian target of rapamycin (mTOR)

gene mutations are reported in 15 % of pancreatic neuroendocrine tumors. Other genes with somatic mutations in pancreatic neuroendocrine tumors include PIK3CA, PTEN, and TSC2. About 25 % of sporadic pancreatic neuroendocrine tumors have deletion of VHL gene.

Molecular Alterations in Pancreatic Acinar Cell Carcinoma

Molecular alterations including microsatellite instability, large chromosomal gains and losses, and somatic mutations involving the Wnt signaling pathway (including APC and CTNNB1) as well as BRAF have been reported in pancreatic acinar cell carcinoma. Whole-exome sequencing of pancreatic neoplasms with acinar differentiation found that genes altered in other neoplasms of the pancreas were occasionally targeted in carcinomas with acinar differentiation, including SMAD4 (26 %), TP53 (13 %), GNAS (9 %), RNF43 (4 %), and MEN1 (4 %).

Molecular Alterations in Solid Pseudopapillary Neoplasm

Solid pseudopapillary neoplasm contains remarkably few genetic alterations but shows activating somatic mutations of beta-catenin gene (CTNNB1, Catenin (Cadherin-Associated Protein), Beta 1) in 95 % of cases. All these mutations of CTNNB1 are missense mutations which occur at codon 32, 33, 34, or 37. Mutations of CTNNB1 within these regions inhibit phosphorylation and consequent degradation of beta-catenin protein. CTNNB1 mutational analysis was reported in endoscopic ultrasound-guided FNA cytological specimens of solid pseudopapillary neoplasms by using next-generation deep sequencing and was shown to be feasible for clinical cytology diagnosis. Currently immunostaining with antibody to beta-catenin on cell block perhaps is the most useful tool when encountering challenging cases.

Molecular Alterations in Barrett's Esophagus and Esophageal Adenocarcinoma

Barrett's esophagus (BE) is the main risk factors for esophageal adenocarcinoma (EAC), which has a poor prognosis, with a 5-year survival rate of less than 15 %. For patients with BE, endoscopic surveillance to detect dysplasia is the primary strategy recommended to decrease morbidity and mortality from esophageal adenocarcinoma. Several tumor suppressor genes, oncogenes as well as growth factors are involved in EAC carcinogenesis.

p53 is the most important cell cycle regulators and control the cell growth and apoptosis. Disruption of the p53 pathway gives cells the ability to escape from the growth inhibitory control. It has been shown that p53 alteration has been immunohistochemically detected in Barrett's esophagus and its frequency gradually increased in dysplasia and adenocarcinoma. Evidently p53 mutation and deletion has been found in the majority of EAC. Increased p53 expression is correlated well with mutation status and may be a valuable biomarker predicting increased risk of disease progression in patients with BE and dysplasia.

Retinoblastoma protein (pRb) is master control of major point of regulation for cell proliferation occurs in the transition from G1 into S phase of the cell cycle. The ability of cells to bypass this key regulatory point allows them to avoid growth inhibitory signals and to replicate without limit. p16 (CDKN2A gene, cyclin-dependent kinase inhibitor 2A) is a member of the INK4 family of cell cycle inhibitors and regulates the synthesis of proteins that alter the function of pRb. Protein encoding by p16 functions as inhibitor of CDK4 kinase. Recent studies indicated that inactivation of pRb is found in the late stages of Barrett's carcinogenesis (i.e., dysplasia and carcinoma), but not in non-dysplastic Barrett's metaplasia. However, p16 inactivation was found to the earliest and most common genetic alteration in non-dysplastic Barrett's metaplasia. About 73–87 % of biopsy specimens from patients with non-dysplastic Barrett's esophagus harbors p16 inactivation. Methylation of p16 promoter is the predominant mechanism for p16 inactivation in esophageal carcinogenesis.

Evidence for chromosome instability including copy gain, loss and loss of heterozygosity (LOH) has shed light on the understanding of esophageal carcinogenesis. A recent large cohort study with 10 year follow up on 243 patients with BE were evaluated for p53 and p16 alterations, tetraploidy, and aneuploidy. The relative risk of developing EAC at 5 years in those with baseline 9p LOH (p16) and 17p LOH (p53) and a DNA content tetraploidy and aneuploidy was as high as 79 %. Therefore, a SNP-based 9p and 17p LOH approach could be incorporated in esophageal brushing cytology to provide biomarkers for cancer risk prediction and early detection of dysplasia and EAC.

Ras/Raf/MAPK pathway plays the central role in regulation of cell growth and amny MAPK pathway components were reported to be upregulated in EAC. It has shown that Ras and BRAF mutations is rarely seen in non-dysplastic BE, but is detected in 40 % and 10 % of dysplasia and EAC resepectively. Epidermal growth factor (EGF) has a stimulatory effect on epithelial cell proliferation via activation of EGFR. Amplification of EGFR is present in high grade dysplasia and about 30 % of EAC. EGFR expression in esophageal adenocarcinomas was correlated with advanced pathologic tumor classification and lymph node metastasis. The protein encoded by *c-myc* is a multifunctional nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. The c-myc protein functions as a transcription factor that regulates transcription of specific target genes. Upregulated c-myc expression increases in the progression from BE to EAC. Amplification of *MYC* is found in 25 % of high grade dysplasia and 44 % of EACs and may be a late event in esophageal carcinogenesis.

Recently Halling et al. reported a FISH assay using 12 FISH probes (CEP 7, 9, 17, Y and LSI 5p15, 5q21–22 (APC), LSI 7p12 (EGFR), 8q24.12–13 (C-MYC), 9p21 (P16), 17p13.1 (P53), 17q11.2–12 (HER-2/NEU), 20q13.2 (ZNF217)) for the detection of genetic abnormalities in cytology specimens in 138 patients with Barrett's esophagus. Gains of 5p15, CEP 7, 7p21, 8q24.12-13, CEP 17, 17q11.2, and 20q13.2 were detected in patients with progression from benign squamous epithelium to high-grade dysplasia and to EAC. They found that this FISH assay had the sensitivity and specificity for detection of 84 % and 93 % for detection of high

grade dysplasia, and 94 % and 93 % for esophageal adenocarcinoma, respectively. Using FISH assay Falk et al. studied archival cytology slides from 40 patients with biopsy-proven BE using FISH probes of 9p21, 17p13.1, and CEP for 6, 7, 11, 12. Aneusomy of chromosomes 6, 7, 11, and 12 or a loss of 17p13.1 was identified in 95 % of the high grade dysplasia/carcinoma cases, including all five cases with cytologic diagnosis as "indefinite for dysplasia." The sensitivity and specificity of the FISH assay mentioned above for the detection of high grade dysplasia/carcinoma was 95 % and 100 %, respectively (95 % CI: 74–99.8 % and 79.1–100 %).

Halling et al. also compared relative sensitivity and specificity of conventional cytology, DNA ploidy, and FISH analysis for the detection of dysplasia and adenocarcinoma in patients with Barrett's esophagus of cytologic brushing specimens. They found polysomy associated with significantly higher and earlier progress from BE to high grade dysplasia/EAC. The finding of 9p21 loss or gain of a single locus appears to be associated with a risk for progression that is less than patients with polysomy but significantly greater than those with a negative FISH result. Their data suggests that FISH assay has higher sensitivity than DNA ploidy analysis and much higher sensitivity than cytology for the detection of dysplasia and EAC in patients with BE. Therefore, FISH assay in brushing cytology may serve as a reliable ancillary tool in facilitating accurately identifying patients with high-grade dysplasia or esophageal adenocarcinoma.

Molecular Alterations in Gastrointestinal Stromal Tumors (GIST)

GISTs are the most common mesenchymal neoplasms of the GI tract occurring in different sites. The most common site of GIST is stomach (60 %). Other sites include small intestine (30 %), duodenum (5 %), rectum (2–3 %), colon (1–2 %), and esophagus (<1 %). GISTs originate from interstitial cells of Cajal or their precursors and GISTs from different anatomic sites share a similar genetic profile with KIT or PDGFRA (platelet-derived growth factor receptor, alpha polypeptide) gain-of-function mutations.

Both KIT and PDGFRA genes are located on chromosome 4q12 and encode highly homologous transmembrane glycoproteins which belong to the type III receptor tyrosine kinase family. The members of this tyrosine kinase family consist of three conserved domains: an extracellular (EC) domain with five Ig-like loops; a cytoplasmic domain with juxtamembrane (JM) region and a cytoplasmic split tyrosine kinase (TK) domain. In KIT gene, EC domain contains exons 8 and 9; JM domain contains exon 11, and cytoplasmic domain contains exons 13, 14, 15, 16, and 17. For PDGFRA gene, exons 12, 14 and 18 are in cytoplasmic domain. Activation of receptor tyrosine kinase by gain-of-function mutation of KIT or PDGFRA stimulates their downstream signaling pathways, including MAP kinase (RAF, MEK, and ERK), PI3K/AKT and STAT3 pathways.

There are two types of KIT and PDGFRA mutations in GIST: (1) Primary mutation is detected in primary tumors before tyrosine kinase (TK) inhibitor therapy such as imatinib and mainly affects exons 11, 9, 13, and 17 of KIT gene, and exons 18, 12 and rarely exon 14 of PDGFRA gene; (2) secondary mutation occurs during TK inhibitor treatment, is responsible for TK inhibitors resistance, and is usually detected in exons 13, 14, and 17 of KIT and exon 18 of PDGFRA. In-frame deletions are the most common KIT primary mutations in GISTs. They are virtually all identified in exon 11 of KIT gene. Point mutations are the second most common KIT primary mutations, and most of them affect exon 11 as well. Point mutations of KIT gene occasionally occur in exons 13, 17, and rarely exon 9. Duplications are the third most common KIT primary mutations in GISTs and are identified in exon 9 and 11. Insertions and complex mutations in KIT are not common. KIT receptor activating mutations occur in 60–85 % of all GISTs. A great majority (>80 %) of KIT exon 11 duplications are identified in gastric GISTs. Most KIT exon 9 duplications occur in intestinal GISTs. PDGFRA mutations occur almost exclusively in gastric and omentum GISTs, most commonly in exon 18. A small portion of GISTS (10–15 %) do not have detectable mutations in any of these two genes.

Successful management of GIST requires a multidisciplinary approach. Guidelines of management of GISTs published by

different organizations such as NCCN, ESMO, FNFCC, and GEIS have emphasized the importance of molecular systematic analysis of KIT and PDGFR. These molecular biomarkers will provide important predictive and prognostic information on management of GISTs' patients. Compared to those with exon 9 mutations or wild type, patients with an exon 11 KIT mutation have better response to Imatinib treatment, and a longer time to progression, progression-free survival as well as overall survival. Mutations in KIT exon 9 are the only predictive factor for imatinib response in patients who received high doses of imatinib (800 mg/day). For sunitinib as second-line therapy, patients with either KIT exon 9 mutations or wild-type KIT gene are associated with a more favorable outcome compared with those with KIT exon 11 mutations. For high risk patients, it is recommended adjuvant treatment with imatinib. However, adjuvant imatinib is not recommended in patients with D842 V PDGFRα mutation based on its known resistance. Compared to those with PDGFR exon 17 and 18 mutations, patients benefit most from sunitinib second-line treatment for those with secondary KIT mutations in exon 13 and 14.

Successful mutation analysis of KIT and PDGFRA in EUS-FNA cytology materials has been reported. Recently, KIT and PDGFRA genotype analysis in materials obtained from cytology smears using targeted next-generation sequencing has also been reported by investigators from Mayo Clinic. DNAs can be extracted from stained cytology slides after coverslips have been removed. It has been proven that mutational analysis of KIT and PDGFRA in cytology samples (cell blocks from EUS-FNA or stained cytology slides) is feasible and will provide informative clinically relevant data regarding their mutation status, which helps guide individualized therapy.

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Molecular Biomarkers in Hematopoietic Neoplasms

11

Serge Alexanian, Zicheng Mo, and Jianyu Rao

Hematologic malignancies are increasingly recognized as genetic diseases, with precise entity-defining molecular alterations progressively splitting diagnostic categories into narrower subsets. While traditional clinical and morphologic evaluation still underpins the initial approach to hematological disease, newer immunologic, genetic, and molecular modalities are currently critical for accurate categorization.

Specimens acquired through conventional cytologic methods such as fine needle aspiration (FNA), as well as cerebrospinal fluid (CSF) and body fluid analysis provide a convenient and noninvasive source of diagnostic material given that many of these studies

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such as flow cytometry and subsets of genetic analyses require fresh, unfixed tissue. Though naysayers abound in the medical literature, FNA analysis of lymph nodes remains a generally accepted procedure in the initial evaluation of lymphadenopathy and numerous investigations have corroborated the stability and suitability of this approach for such analyses, opening an entirely new avenue for advanced diagnostic modalities prior to invasive excisions [1–6].

As in most areas of cytology, the primary diagnostic bottleneck revolves around logical triaging of limited quantities of tissue; needless to say, one's ability to maximize the amount of input material obtained for analytic purposes—whether by performing multiple dedicated passes or obtaining buy-in from clinicians who may procure more voluminous samples to avoid a vague diagnosis—will invariably dictate the extent of ancillary studies available and ultimately the ability to render an appropriate diagnosis.

Here, we review some of the techniques available for analysis of hematopoietic lesions, briefly review key findings in the more common lesions which may be encountered in general practice, and propose a triaged approach for different specimen types. This chapter is not intended to provide an exhaustive treatise of all genetic changes in hematopoietic lesions but rather to guide generalists and cytopathologists in their first steps towards a diagnostic odyssey.

Section 1: A Brief Overview of Ancillary Methods in Cytologic Analysis of Hematopoietic Lesions

Broadly speaking, the approach to triaging specimens with potential hematopoietic lesions for ancillary studies depends on history related factors, such as patient presentation or determination of recurrence of a known lesion, and history unrelated factors, such as the type of specimen (e.g., lymph node FNA, CSF, body fluid) and the quantity available.

For patients with no known prior history, one must prioritize assays which are capable of providing maximal information while taking into consideration their unique characteristics.

Techniques which are available in the many laboratories include: immunocytochemical/immunohistochemical phenotyping and in situ hybridization; multiparametric flow cytometric immunophenotyping (FCI); cytogenetic analysis, including karyotyping, fluorescence in situ hybridization, and microarray analysis; molecular diagnostic methods including amplification/sequencing via a variety of modalities and automated or gel interpretation, polymerase chain reaction (PCR) and so-called "next generation sequencing" (NGS) techniques. A number of method variants exist but are beyond the scope of this chapter.

Immunocyto/Histochemistry

Immunocyto/histochemistry, while arguably not a "genetic" test, does provide details on expression patterns within cells and remains the mainstay of much of diagnostic pathology, including hematopathology. We discuss it briefly here in comparison to other modalities. Such techniques are particularly well suited for cell surface marker analysis and require a lower complexity lab for technical preparations. Compared with other modalities, it offers the benefit of preservation of architecture and cytologic detail superimposed on immunologic pattern analysis and material can easily be referred for second opinion or for the addition of rare markers which are typically unavailable by FCI. However its preparation to analysis time typically takes 24–48 h, it offers only semi-quantitative (barring image analysis methods) results, it is ill suited to situations of high background, and significant amounts of material are needed to prepare sufficient slides as most labs can only prepare a single antibody at once. While such techniques are typically limited to paraffin blocks, cytologic specimens may be processed either using immunocytochemical techniques on smears or immunohistochemical techniques on cell buttons—both markedly limited by the quantity of source material. Furthermore, some immunohistochemical stains such as NPM1 and ALK protein, discussed below, may truly provide genetic information depending on staining patterns.

Multiparametric Flow Cytometric Immunophenotyping

Clinical FCI has transformed the field of hematopathology and remains one of the most widely utilized and robust modalities available. It is used in nearly all facets of hematopathology covering diagnosis of B-, T-, and NK-cell lymphoproliferative disorders, myeloproliferative/myelodysplastic disorders, and leukemic disorders, paroxysmal nocturnal hemoglobinuria clone assessment, and, in some labs, minimal residual disease assessment [7–9].

Briefly, FCI involves preparing single-cell suspensions of interest incubated with targeted antibodies covalently bound to a variety of fluorescent molecules. The bound cell–antibody–fluorescent molecule trio is processed via single cell laminar flow across a variety of excitation lasers while scattered as well as emitted fluorescent light is collected and analyzed. Scattered light provides information regarding cell size (forward scatter) and cytoplasmic complexity (side scatter) while fluorescence intensity provides information regarding the availability of antigens on/in the cell of interest. Using combinations of multiple fluorochromes with defined emission spectra, modern clinical FCI allows anywhere from 1 to 12 antigens (or "colors") to be compared simultaneously on a single cell.

FCI offers distinct advantages and disadvantages when compared with traditional immunohistochemistry. Turnaround time is typically on the order of hours and results are quantitative (both numerically and with regards to intensity), less plagued by background, and can be presented in a matrix of multiple simultaneous antibodies allowing immunologic distinction of cellular subsets within polymorphous backgrounds. Multicolor FCI enables detection of more antigens in a single tube, thus requiring fewer cells to perform a complete panel [8]. However this modality lacks the ability to superimpose immunologic data on morphologic and cytologic considerations aside from cell size, cannot be used for absolute enumeration where specific numeric cutoffs are required due to subpopulation enrichment and differential losses of populations during processing, is exquisitely dependent on quality control preparation/processing factors, requires operator dependent gating and color compensation strategies, may require a significant

number of cells as opposed to immunocytochemistry on a paucicellular smear, and is notoriously difficult to send out as part of a consultation given the customized nature of most in-house histograms. Furthermore, the material on which FCI can be provided is limited to unfixed tissue and addition of markers from an outside lab is all but impossible. Lastly, this technique does not obviate the need for other ancillary modalities and one must consider how/when to use it within an immunophenotyping algorithm.

In cytologic specimens, FCI is particularly well suited to CSF, body fluid, and lymph node analysis. Review of cerebrospinal fluid (CSF) from patients with unexplained neurologic symptoms and/or staging of patients with a concurrent or prior history of leukemia may frustrate diagnosticians given that certain blasts may be nearly indistinguishable from reactive lymphocytes, thereby leading to false-positive results, particularly on suboptimal preparations [10–14]. FCI of CSF greatly enhances both sensitivity and specificity in this scenario but may be technically challenging to perform due to a paucity of targets given both the typical cellularity and volume of CSF samples [15–17]. In such cases, one must take care to select a panel of antibodies which returns maximal information as hypocellularity may allow for performance of only a single simultaneous tube reaction.

Traditional Cytogenetic Analyses

Karyotype analyses are not generally performed on cytologic specimens given the quantity of input material but will be briefly discussed here as a foil for molecular genetic methods. Generally speaking, conventional karyotypes are performed in most laboratories and offer the benefit of providing non-biased analyses by probing the entirety of the genome for both expected and unexpected findings. However they require fresh material, dividing, viable cells, and suffer from a 1–2 week turnaround time and an inability to detect submicroscopic abnormalities. They are also relatively insensitive compared with molecular methods with an estimated 5–10 % minimal number of clonal cells required for a lesion to be identified.

Fluorescence in situ hybridization (FISH) studies provide a greater degree of resolution by using fluorescent probes to target known or presumed DNA sequences and may be used on interphase cells from air dried or paraffin embedded tissue. This technique is already used extensively throughout cytology and has been discussed in greater detail elsewhere. However it is worth pointing out that compared with molecular methods such as PCR, FISH possesses distinct advantages in certain diseases such as the ability to detect numeric abnormalities and probes which bind over very large regions, preventing some false negative PCR reactions when mutations occur at small binding sites.

Molecular Genetic Studies

Once the realm of the most subspecialized clinical laboratories, molecular genetic pathology has emerged as a critical element in hematopathology's armamentarium. These studies have striking generation-on-generation improvements in sensitivity and specificity and possess the ability to determine cryptic submicroscopic changes well beyond the ability of standard cytogenetic analyses. However these improvements are coupled with challenges not the least of which include contamination, subclinical molecular anomalies, and incorporation of vast troves of data within diagnostic algorithms. Here we endeavor to discuss some of the more relevant modalities that a generalist or cytopathologist may need to be familiar with in practice and divide them into studies to determine clonality, qualitative and quantitative translocations, and other disease modifying biomarkers. These may be critical to monitor the therapeutic response and early relapse in treated patients and their identification is now considered standard practice in certain hematologic cancer diagnoses [18].

Clonality

Clonality studies are among the most important tools for lymphoid neoplasia and function under the assumption that a reactive conglomerate of lymphoid cells will demonstrate a wide range of

physiologic antigen/antibody specificities characterized by a heterogeneous genetic signature while a neoplastic clone typically demonstrates a homogeneous narrow band of one to a few signatures. In this regard, mature B-cell neoplasia, with its better defined natural history of B-cell immunoglobulin (IG) antigen selection and antibody refinement, lends itself more easily to most modalities while T-cell neoplasia is more often limited to molecular studies in the majority of labs—a discussion of T-cell receptor (TCR) "V-Beta" analysis by FCI is beyond the scope of this chapter though we briefly discuss FCI below in comparison to true clonality techniques.

For B-cell/plasma cell neoplasms many consider FCI the first line modality. While the finding of a *monotypic* population of B- or plasma cells showing either kappa or lambda light chain restriction appears synonymous with clonality in the community, most hematopathologists agree that they are *not* synonymous and that monotypia by FCI (or even immunohistochemistry) may *suggest* monoclonality but does not provide the same degree of rigor. True clonality studies require genetic studies and are significantly more involved.

Historically, the method of choice was southern blot hybridization, a highly sensitive but laborious and costly procedure in which non-amplified DNA samples are digested with restriction enzymes, separated by mass using agarose-gel electrophoresis, transferred onto a nitrocellulose membrane, and then labeled either using a radioactive probe or, more recently, using chemiluminescent methods [19]. Based on the analyzed pattern of banding one could infer the status of rearrangements of either *IG* or *TCR* genes. While this method can theoretically detect clonality at levels down to 5 % of a sample if a clean background is present, it often takes up to a week or more to complete, requires a significant quantity of DNA from fresh tissue, and is quite expensive. Furthermore, reagent for many of these assays are in short supply nationally given reduced general usage.

As a result, PCR DNA methods have progressively replaced southern blotting and may be used both on paraffin-embedded tissue such as cell blocks as well as on fresh tissue. These techniques use PCR to amplify input DNA in an automated cyclical fashion and

subsequently employ capillary electrophoresis using fluorescently labeled primers for detection. Such combined techniques are faster, cheaper, require far less input tissue, can be automated, and are not limited by radioactivity concerns. However PCR for *IGH* rearrangement suffers from a high false negative rate of up to 20 %, particularly in lymphomas showing a high incidence of primary or ongoing somatic hypermutation such as diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and plasma cell myeloma; sequence amplification may fail in these entities due to mutations in regions typically targeted by primers [20]. Furthermore, some lymphomas are characterized by *IGH* deletions which can lead to complete failure of this assay. Although concurrently detecting *IGK* gene rearrangements can improve the sensitivity, the false negative rate remains a concern and steps must be taken to reduce their incidence such as targeting Framework 3 and Framework 2 regions of Vh genes and the use of BIOMED-2 multiplex PCR consensus primers [21–24]. At our institution, negative cases by PCR for which sufficient fresh DNA remains have traditionally been reflexed to southern blot analysis to address such issues.

With PCR, a small clonal population may be missed if mixed within a polyclonal background, with sensitivity exquisitely tied to the proportion of neoplastic cells present [25]. Samples containing highly degraded DNA or a paucity of lesional cells may lead to amplification of non-representative populations with false "pseudoclonality" [25]. These pseudoclones may be revealed upon duplicate testing. Contrariwise, some immunologic disorders produce oligoclonal false positives which may remain constant with duplicate testing; frequent offenders include Sjögren's disease, rheumatoid arthritis, hepatitis C, and H. pylori gastritis. Immune system reconstitution after bone marrow transplantation as well as the normal immune response to tumors may also lead to false positives. Lastly, *IGH* gene rearrangement is not specific for mature B-cell neoplasia and may be seen in up to 90 % of B-cell acute lymphoblastic leukemia (B-ALL), many T-cell acute lymphoblastic leukemia, and even myeloid leukemia [22]. Thus, the result of PCR analysis must be interpreted in consideration of patient history, cell morphology and other ancillary studies (see Table 11.1).

Table 11.1 Causes of false positive and false negative results in PCR clonality (IGH/TCR) studies

False positive	False negative
Lineage infidelity (TCR gene rearrangements in B-ALL, etc.)	Sample integrity: DNA degradation, sampling, fixation
Oligoclonal immune reaction due to inflammatory diseases	Use of non-optimized primers
Immune reconstitution post marrow transplant	Incompletely rearranged VDJ segments in some B-ALL
Immune response to tumors (ex, T-cell clones in CML)	Lymphomas with primary or ongoing somatic hypermutation (FL, DLBCL, myeloma)
Contamination	IgH deletions
Pseudoclonality due to small biopsies	

Translocation Assessment

In addition to providing clonality determination, DNA based PCR assays detect "quantitative" translocations often seen in lymphoid malignancies which lead to upregulation/overexpression of proto-oncogenes by transposition next to a transcriptionally active gene, such as t(14;18) in most follicular lymphomas. RNA based reverse-transcriptase PCR (RT-PCR), on the other hand, detects "qualitative" translocations often seen in myeloid malignancies which result in the production of novel chimeric genes such as *BCR-ABL* fusions and subsequent production of a new protein. However the marked sensitivity of PCR and especially "nested" PCR assays may backfire as many translocations which were once thought to be disease defining have been found in the circulating blood of normal patients who may never develop illness; examples include the classic translocations t(9;22), t(14;18), t(11;14), t(2;5)/inv(2), t(12;21), and t(8;14) *typically* seen in chronic myelogenous leukemia, follicular lymphoma, mantle cell lymphoma, anaplastic large cell lymphoma, B-cell acute lymphoblastic leukemia, and Burkitt lymphoma, respectively. Such considerations must temper the incessant quest for ever greater sensitivity and lower limits of detection by reminding diagnosticians of the importance of synthesizing multiple sources of information.

Non-translocation Mutations and Next Generation Sequencing

Molecular genetic methods are also employed in determining the presence or absence of specific mutations of clinical significance in hematopathology, such as *BIRC3* mutations in chronic lymphocytic leukemia (CLL) and *FLT3*, *NMP1*, and *CEBPA* mutations in acute myeloid leukemia (AML), discussed below. A variety of techniques may be employed to detect such mutations and are well beyond the scope of this discussion. However we briefly mention the evolving platform of NGS. This methodology, which is discussed in greater detail elsewhere in this book, essentially consists of simultaneous parallel sequencing of millions of short nucleic acid sequences and utilizes a complex informatics pipeline to overlay sequenced fragments to reconstruct a full region of interest. While still growing in popularity, the method shows considerable promise where small samples are concerned: whereas classical molecular genetic methods would require numerous single or multiple gene assays and a large amount of cumulative input material to complete an extended panel, an optimized up front NGS panel can provide information on all relevant lesions with a relatively scant starting specimen. To date NGS platforms in hematopathology have been primarily used for determination of translocations and non-translocation mutations, though some laboratories have begun investigating their use in clonality assessment given the even lower amount of input DNA required compared with PCR methods.

Other Techniques

Ploidy studies, while falling out of favor, are still used by some practices in pediatric populations to estimate DNA content in B-ALL given that 50 % of children may show numerical chromosomal changes (see B-ALL section below). However they cannot resolve chromosomally defined prognostic subgroups with gains/lacks of *specific* chromosomes, such as the particularly favorable high-hyperdiploidy with duplications of chromosomes 4, 10, and 18. Furthermore, use of a DNA index in lieu of karyotype or FISH cannot account for alterations in prognosis based on structural

chromosomal changes present superimposed on numerical chromosomal changes and must thus be interpreted with caution. Such techniques realistically play no significant role on cytologic specimens of hematopoietic lesions.

Section 2: The Role of Ancillary Diagnostics in Lymphoid Neoplasia

In the past 30 years, numerous recurrent genetic abnormalities associated with hematopoietic neoplasms have been discovered, many of which also play a pivotal role in pathogenesis. Below we briefly discuss the application of molecular/genetic diagnostics in the more common lesions which generalists/cytopathologists may encounter.

Precursor Lymphoid Neoplasm: B-Lymphoblastic Leukemia/Lymphoma

B acute lymphoblastic leukemia (B-ALL) is a malignant disease of the lymphoid cell line occurring more frequently in children. Approximately 30 % of B-ALL cases carry recurrent genetic alterations including balanced translocations and other chromosomal abnormalities. Genetic studies are required at diagnosis not only for identifying specific genetic abnormalities but also for monitoring disease progression and response to therapy.

The most common recurrent genetic abnormalities include hyperdiploidy, t(9;22)(q34;q11.2); *BCR-ABL*, t(12;21)(p13;q22); *ETV6-RUNX1* (*TEL-AML1*), and t(v;11q23) with *KMT2D/MLL* (Mixed Lineage Leukemia gene) rearrangements, though numerous other WHO and non-WHO groups exist and are beyond the scope of this chapter. Given the frequent recurrence and extramedullary relapse of some subtypes of B-ALL, these lesions are commonly seen even in generalist and cytopathologist practices.

The *BCR-ABL1* abnormality is more often seen in adult B-ALL (25 %). In contrast, it is only identified in 3 % of childhood B-ALL. This translocation is associated with the worst prognosis,

common CNS involvement, and frequent relapse, thereby leading most treatment regimens to suggest transplantation after first remission. While not pathognomonic by any stretch, this variant of B-ALL may demonstrate cytoplasmic granularity and larger blasts more commonly than other variants. FISH with dual color fusion probes (D-FISH) detects this lesion at a minor p190 breakpoint in 85 % of children and half of adults while a p210 major breakpoint is seen in the remainder of children and adults.

ETV6-RUNX1 is typically seen in children, accounting for up to 35 % of pediatric B-ALL. This translocation confers an excellent prognosis and FISH or RT-PCR is generally required for detection given its subtlety on classic karyotype.

B-ALL with translocations of 11q23 involving the *MLL* gene tend to occur in infants. These cases are characterized by the highest presenting white blood cell count and poor outcomes, with many treatment regimens also recommending transplantation upon first remission. The most common abnormalities involving *MLL* are the t(4;11)/*MLL-AF4* and t(11;19)/*MLL-ENL. MLL* gene rearrangements can be readily identified by FISH using break-apart probes.

Generally speaking, of all genetic diagnostic modalities, FISH studies provide the greatest yield for B-ALL cases. However specific patterns by FCI may suggest underlying genetic changes: a "pro-B" (or "pre-pre-B") phenotype with expression of TdT, CD34, and HLA-DR, but absence of CD10 is often seen with high risk *MLL* rearranged cases; a "pre-B" phenotype with cytoplasmic immunoglobulin and occasional CD34 negativity is often seen in B-ALL with *BCR-ABL1;* a "common-B" immunophenotype with expression of CD10 and frequent myeloid aberrancies is often seen in well-behaving B-ALL with *TEL-AML1*. While none of these patterns is inviolate, the presence of the former two findings may be sufficient to warrant a phone call warning clinicians to monitor patients more carefully. PCR for clonality may be attempted although caution must be exercised as 60 % of B-ALL cases also have T-cell receptor gene rearrangements and many B-ALL cases may not have fully rearranged VDJ segments, requiring the use of an alternate DJ primer over the typical VJ primer.

Mature B-Lineage Neoplasms

Follicular Lymphoma (FL)

FL is not infrequently discovered initially on lymph node FNA and poses morphologic challenges in the absence of architecture given constituents such as centrocytic and centroblastic cells that may be seen in normal germinal centers.

Approximately 85 % of cases of FL carry t(14;18)(q32;q21) resulting in juxtaposition of the *BCL2* gene to the *IGH* locus with subsequent overexpression of the BCL2 antiapoptotic protein [26, 27]. A BCL2 immunohistochemical stain may be performed on cell blocks though mutations in *BCL2* may lead to loss of reactivity in many commercial clones. Furthermore, antigen expression is not specific for translocation and may be seen in other disease entities including 20 % of de novo DLBCL and in unaffected patients as well.

PCR methods may also detect the *BCL2-IGH* fusion gene in approximately 75 % of cases with a cytogenetically demonstrable fusion. However, due the presence of additional break sites 3′ to the *BCL2* gene, D-FISH provides superior sensitivity and is more commonly employed for the detection of *BCL2* gene rearrangements [28].

D-FISH is applicable to cytospin specimens as well as cell blocks. The identification of the t(14;18)/*BCL2-IGH* abnormality together with appropriate morphology supports the diagnosis of FL, though higher grade lesions often lack both t(14;18) rearrangements and BCL2 antigen expression: negative ancillary studies cannot rule out FL if other pathologic findings are supportive. Additionally, pediatric follicular lymphomas tend to be negative for *BCL2-IGH* translocations and may instead demonstrate *IGH-IRF4* translocations.

Clonality studies by PCR or Southern Blot may demonstrate false negative results in FL cases due to the high incidence of somatic mutations involving binding sites. Additional gene abnormalities are seen in greater than 20 % of FL with 1p− being the most common.

Newer techniques include NGS for recurrent mutations such as *KMT2D*/*MLL* and specific microRNAs such as miR330,

miR17-5p, miR106a, and miR210, though their identification has not yet become standard of care.

Mantle Cell Lymphoma (MCL)

MCL, despite typically being lumped in the "small B-cell lymphoma" category, behaves more aggressively and must be identified rapidly to prevent poor outcomes. Cytomorphology alone may be suggestive with the characteristic small crumpled appearance and absence of prolymphocytes/paraimmunoblasts seen in its mimic chronic lymphocytic leukemia/small lymphocytic lymphoma; however, blastoid variants may be misdiagnosed and behave far more aggressively.

Genetically, 95% of MCL typically demonstrate t(11;14)(q13;q32) which juxtaposes *IGH* sequences with the *BCL1/CCND1* locus, leading to upregulation of the *CCND1* gene and consequently overexpression of cyclin D1 [29]. Cyclin D1 tightly controls the transition from G0-G1 to S phase of the cell cycle but is not usually expressed in lymphoid cells [30].

PCR techniques can only detect 30–40% of cases of MCL given that chromosome 11q13 breakpoints aside from those involving the major translocation cluster are notoriously heterogeneous [28, 29, 31]. Immunohistochemical stains for cyclin D1 are diagnostically more sensitive for MCL than DNA PCR and may be performed on cell blocks though they may be negative in the so-called "cyclin D1 negative MCL". In this scenario, a monoclonal version of the Sox11 antibody is recommended, though false positives may be seen in Burkitt lymphoma, T-prolymphocytic leukemia, acute lymphoblastic leukemia, and hairy cell leukemia. Given that cyclin D1 mRNA is seen in up to 95% of cases of mantle cell lymphoma, some laboratories use quantitative reverse transcriptase PCR as a diagnostic assay. However FISH studies on either cell blocks or cytospin specimens appear superior with sensitivity reported up to 99%; currently they remain the method of choice for diagnosis of MCL [32].

Additional molecular targets which may be probed include *ATM, TP53, NOTCH1, UBR5, TET2*, and others, though of these *TP53* mutations are currently the most clinically relevant and seen in up to 25% of cases with poor prognosis blastoid morphology.

Extranodal Marginal Zone B-Cell Lymphoma (MZL)

Extranodal MZL is less often seen in cytologic practice due to its particular distribution but may be recognized by the combination of centrocyte-like cells and monocytoid B-cells with plasmacytic components. Splenic marginal zone lymphomas are considered too rare in this practice setting to merit consideration.

The most common recurrent gene rearrangement in extranodal MZL of mucosa-associated lymphoid tissue (MALT lymphomas) is t(11;18)(q21;q21) resulting in poor prognosis from translocation of *BIRC3/API2* on chromosome 11 to *MALT1* on chromosome 18. Other changes include t(3;14)(p14;q32);*FOXP1-IGH*, t(1;14)(p22;q32);*BCL10-IGH*, t(14;18)(q21;q32);*MALT1-IGH*, and trisomies 3, 12, and 18 [33, 34]. These chromosomal lesions show characteristic anatomic preference: t(11;18) and t(1;14) are more commonly seen in pulmonary (50%) and gastric (30%) lymphomas; t(14;18) is more commonly seen in ocular, parotid, hepatic, and cutaneous sites; t(3;14) can be detected in thyroid, ocular, and cutaneous sites [33–37].

Due to variability in breakpoints for *BIRC3/API2-MALT1* fusions, DNA PCR may suffer from false negative results and reverse-transcriptase PCR amplification of the fusion mRNA provides superior sensitivity. Alternatively, FISH, either on cytospin or cell blocks, remains the best choice for confirming recurrent translocations in MALT lymphomas. Furthermore, the finding of t(11;18) or t(1;14) also guides clinical management in cases with underlying *H. pylori* by predicting antibiotic resistance [38].

Some laboratories additionally offer BCL10 protein immunohistochemical staining with nuclear positivity correlated with t(11;18) and t(1;14) though it may be seen in other lymphomas and is not considered standard of practice. Numerous additional mutations have been noted but do not currently enter into standard practice guidelines either.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL)

CLL/SLL, one of the most common lymphoproliferative disorders in the elderly, may be encountered both on nodal FNAs and in body fluids as an unexpected finding. Cytologically it demonstrates a monotonous population of small relatively round

lymphocytes with scattered prolymphocytes, paraimmunoblasts, and transformed large cells.

Genetically, CLL/SLL shows significant heterogeneity with a relatively large number of recurrent mutations as the disease derives from memory B-cells which may or may not have undergone *IGH* somatic hypermutation. Cases with unmutated *IGH* genes (>98% homology with germline sequence) behave more aggressively while hypermutated cases often show a relatively better prognosis [39–41]. *IGH* mutation status is not routinely assessed in most laboratories and NGS use has not yet become widespread.

FCI can detect the presence of surface CD38 expression and intracellular ZAP-70 protein kinase, both of which have been used as surrogate markers for unmutated *IGH* status though they appear to additionally show independent prognostic significance [39, 42, 43]. However, ZAP-70 by FCI shows poor inter-laboratory correlation with many labs either unwilling or unable to perform this assay.

In addition to *IGH* mutational status, other recurrent genetic abnormalities are well described to date and play a role both in diagnosis and prognosis. The most common chromosomal lesions include del 13q14, trisomy 12, del 11q22–23, and 17p− [44–46]. The deletion of 13q14, seen in approximately 50% of overall cases, was classically thought to confer a favorable prognosis though more recent findings suggest that deletions in greater than 70% of cells confer a poor prognosis and further stratify these deletions into favorable type I deletions without involvement of *RB1* and unfavorable type II deletions involving *RB1*. In contrast, 11q− and 17p− are seen in aggressive cases of CLL [47–49]. The majority of these recurring cytogenetic changes are readily detected by FISH, whereas PCR studies are not recommended given their inability to assess for the balanced translocations typically seen above. Traditional karyotype typically fails due to the infrequent presence of metaphases.

More recently defined genetic alterations include mutations in *TP53*, *NOTCH1, BIRC3,* and *SF3B1* as well as 2 microRNA genes (miR-15 and miR-16) which may be implicated in CLL pathogenesis associated with 13q deletions [50]. Most are still not considered standard of care though some risk-assessment algorithms may include *TP53* and *BIRC3* in high risk groups and *SF3B1* and *NOTCH1* in intermediate risk groups.

Plasma Cell Myeloma

While most plasma cell dyscrasias are not typically within the realm of cytologists, soft tissue plasmacytomas may occasionally be subject to fine needle aspiration.

Immunocytochemistry and immunohistochemistry provide excellent, rapid probes for determination of a predominant light chain restricted plasma cell population and, combined with cytologic assessment for immaturity and atypia, may be sufficient to provide a strong suspicion for a plasma cell dyscrasia. FCI may provide additional diagnostic information but can markedly underestimate the underlying plasma cell population given this cell type's frequent destruction during processing.

Regarding genetic studies, plasma cell neoplasms show characteristically low proliferative activity not amenable to classic karyotypic analysis. FISH, on the other hand, provides ample diagnostic and prognostic information in up to 90 % of cases. The most common findings, translocations of *IGH* and hyperdiploidy, each account for approximately half of cases. The most frequent translocation partners for *IGH* include *CCND1*, *CCND3*, *CMAF, MAFB,* and *FGFR3* with the former two entities portending a favorable prognosis and the latter three portending a poor prognosis. Translocations involving *CMAF* and *FGFR3* are also curiously overrepresented in younger female patients with IgA-lambda dyscrasias. Additional changes include del 13q, del 17p, and aneuploidy or hypodiploid genotypes.

Molecular studies in plasma cell neoplasms are more limited with PCR clonality studies frequently failing due to ongoing somatic hypermutation. Additional studies are limited to the identification of late stage changes seen with tumor progression, typically involving *MYC, RAS*, and *TP53* as well as alterations of the NF-kB pathway.

Burkitt Leukemia/Lymphoma

Burkitt leukemia/lymphoma is a highly aggressive B-cell lesion which may be seen both in pediatric and adult populations. 90–95 % of cases are characterized by the presence of *MYC* gene rearrangements, typically with juxtaposition adjacent to the *IGH* gene t(8;14)(q24;q32) or, less commonly, the *IGK* t(2;8)(p11;q24) or *IGL* t(8;22)(q24;q11) genes [51, 52].

Breakpoints vary significantly depending on epidemiology and render this entity a poor choice for PCR. FISH using a break-apart probe flanking the *MYC* breakpoint is a highly sensitive screen for *MYC* gene rearrangements and the specific translocation should be confirmed using D-FISH [53]. Nuclear expression of MYC protein by immunohistochemistry on a cell block may also be used for screening and has high sensitivity but low specificity; a negative MYC immunohistochemical stain makes translocation unlikely. Newer molecular targets have been identified but are not currently included in standard practice panels.

Given the rapidly progressive nature of this disease as well as the 90 % rate of cure for early lesions treated with intensive combination chemotherapy regimens, cytologists and generalists alike must be able to diagnose or triage specimens expediently [54].

Diffuse Large B-Cell Lymphoma (DLBCL)

DLBCL remains the most common large cell lymphoma and is not uncommonly encountered on initial cytologic specimens, though a diagnosis on morphology alone is troublesome given that any lymphoma may harbor scattered large centroblastic to immunoblastic cells. Regardless, given additional clinical features a diagnosis may be suggested and warrants first line ancillary studies which some may opt to perform prior to excision.

Flow cytometry has traditionally been considered suboptimal in large cell lymphomas due to cellular destruction during processing, though procedural optimization may still allow for the demonstration of light chain restriction and select immunophenotypic aberrancies in most cases.

Genetically, DLBCL shows a heterogeneous signature with some noteworthy recurring abnormalities including translocations of *BCL6*, *BCL2*, and *MYC* seen in 30 %, 20 %, and up to 10 % of cases, respectively [55]. MYC protein expression by immunohistochemistry may be used as a screening test but portends a poor prognosis regardless of underlying translocation status.

Some overlap exists among DLBCL, Burkitt lymphoma, and the so-called “double hit” lymphomas with *MYC* translocations as well as *BCL2* and/or *BCL6* gene rearrangements occurring in the poorly behaving latter entity [56–59].

In practice, FISH testing for the changes above is more widespread than PCR testing. Gene expression profiling, which has provided evidence for distinction between the so-called "germinal center B-cell like" and "activated B-cell like" signatures, remains a research modality. Some limited gene models may be probed using quantitative real time PCR but are still not employed in clinical algorithms, though immunohistochemistry provides a surrogate modality in the form of the Hans and Choi algorithms. NGS studies have also demonstrated numerous mutations which may provide future prognostic and therapeutic targets and attempts are underway to standardize algorithms to deal with the massive influx of new data.

Classical Hodgkin Lymphoma (cHL)

The importance of cytology in cHL diagnosis cannot be overstated, with the finding of the entity defining Reed-Sternberg (RS) cell and variants often limited to smears and touch preparations even on excisional specimens. In the context of initial screening diagnosis, however, ancillary methods on fine needle aspirates are often focused on ruling out other entities as much as ruling in cHL.

Immunocyto/histo chemistry on cytospins, smears, or cell blocks may be most useful when RS cells or variants are identified, though given their scarcity and the inability of most labs to perform multi-antibody staining, such an approach may be limited. For the vast majority of laboratories, FCI is most useful in demonstrating the absence of light chain restriction and B- or T-cell abnormalities. Though it is commonly stated that lesional cells of cHL cannot be assessed by flow, research modalities have been used in limited practices to directly probe RS cells and variants but are not considered here.

Other molecular and gene expression profiling studies remain in the research domain at the moment.

Mature T-Cell Neoplasms

T-cell neoplasms are distinctly uncommon in most generalist and cytologist practices and we will only discuss one group in this section.

As previously described, *TCR* clonality studies by southern blot or PCR methods may identify the presence of T-cell clonal population. If a T-cell undergoes neoplastic transformation with a resultant clonal expansion, its TCR may be used as a tumor marker specific to that cell lineage for monitoring recurrence [60]. The same pitfalls which afflict *IGH* clonality studies apply to *TCR* studies.

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is a morphologically and genetically distinct subtype of mature T-cell lymphoma. Aside from the ironically named "small cell variant," lesional cells are usually large with myriad cytologic characteristics. The so-called "hallmark" cells with kidney shaped nuclei and an eosinophilic paranuclear clearing are characteristic, but not pathognomonic.

ALCL is broadly split into anaplastic lymphoma kinase (ALK) positive subtypes with better prognosis and a younger age at diagnosis and ALK-negative subtypes with poor prognosis and a later age of onset. ALK-positive cases demonstrate translocation of the *ALK* gene located on 2p23 with a variety of fusion partners: t(2;5)(p23;q35); *NPM1-ALK* occurs most commonly in 75 % of cases; t(1;2)(q25;p23); *TPM3-ALK* in 15 % of cases; t(2;3)(p23;q12)/*ALK-TFG*, inv(2)(p23q35) and other rare abnormalities make up the remainder [61, 62]. FISH using a BAP probe is most commonly used to detect *ALK* rearrangements though RT-PCR may be used for NPM1-ALK transcripts.

Interestingly, immunohistochemistry for the ALK protein may be the most useful screening test as it not only correlates well with FISH studies but also predicts translocation partners based on the cytoplasmic, nuclear, or nucleolar localization of staining. Caution should be exercised, however, as ALK may be expressed in some B-cell lymphomas showing plasmablastic morphology and should not be considered diagnostic of ALCL [63, 64].

Additional genetic studies are currently not utilized in the diagnosis of non-cutaneous ALCL aside from research based gene expression profiling studies to characterize the genetic signatures of ALK-positive ALCL in comparison to ALK-negative ALCL and peripheral T-cell lymphoma, not otherwise specified.

Section 3: The Role of Ancillary Diagnostics in Myeloid Neoplasia

While most myeloid neoplasia is seen in the context of bone marrow analysis, for the purposes of this chapter we will be discussing scenarios where disease may be seen in body fluids, particularly bronchoalveolar lavages, FNA of extramedullary "myeloid sarcomas," and CSF interpretation of CNS leukemia.

Myeloproliferative Neoplasms

The prototypical myeloproliferative neoplasm, chronic myelogenous leukemia (CML) results from an overactive tyrosine kinase created by the t(9;22)(q34;q11); *BCR-ABL1* fusion which may be identified cytogenetically in up to 95 % of cases, with the remainder requiring molecular techniques for identification [65–67]. Cases lacking this translocation are currently grouped elsewhere in the 2008 WHO classification.

Depending on the phase of involvement, CML demonstrates a cellular myeloid preponderance with variable numbers of immature cells/blasts which may be morphologically indistinguishable from a leukemoid reaction in cytologic specimens, particularly body fluid; thus, ancillary studies are mandatory when clinical suspicion exists. FCI finds limited use in such settings aside from identification of increased blasts. Both conventional cytogenetics and molecular techniques are employed in diagnosis and therapeutic monitoring of CML after targeted tyrosine kinase inhibition (TKI), though in different capacities.

Conventional cytogenetic studies are invaluable at both diagnosis and to assess for clonal evolution seen in longstanding disease such as +8, +19, +i(17q), +Ph. Some translocations may be cryptic but still amenable to FISH.

RT-PCR may also be employed for diagnosis though primers must be selected to account for rare intronic breaks. It may be useful to subtype fusion proteins in the context of minimal residual disease testing though this method does not employ cytologic specimens. However this method cannot detect t(9;22) negative

clonal evolution or progression to accelerated or blast phase. Newer modalities such as NGS are not currently widely utilized but this may change given the increased emphasis on testing for mutations such as T315I which may confer TKI resistance.

The non-CML myeloproliferative neoplasms including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are uncommon in the practice of cytopathology. They are mentioned here in the context of discovery of underlying molecular mechanisms including alterations of the Janus kinase 2 (JAK2) gene at V617F or exon 12 and *MLL* [68–71].

Acute Myeloid Leukemia

AML has increasingly been subdivided into narrower categories with the discovery of newer genetic abnormalities. A complete discussion of the heterogeneous signature of each entity far exceeds the scope of this work and we will instead focus on three of the more common translocation-defined leukemias which may be seen in extramedullary locations as well as some newer molecular biomarkers given that 45 % of AML cases demonstrate a normal karyotype.

AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*

The most common subtype of AML, typically affecting younger patients, this entity demonstrates classic cytomorphology with blasts possessing "salmon pink" large granules, neutrophils with Auer rods, and increased numbers of eosinophils. Such findings may raise the specter of this entity and ancillary immunophenotyping by flow often shows aberrant expression of CD19, CD79a, CD15, and CD56 on blasts.

Either FISH or RT-PCR assays may be used for genetic characterization given the limited number of closely clustered breakpoints. The translocation fuses the *RUNX1* gene (formerly known as AML1 or CBPA2) with *RUNX1T1* (formerly ETO), subsequently inhibiting transcription of numerous hematopoietic genes [72].

In addition to the molecular tests listed below, all cases of presumed *RUNX1-RUNX1T1* AML should be probed for *KIT* mutations which, while conferring a poor prognosis, may allow for additional targeted therapy. In the absence of *KIT* mutations, this entity confers a favorable prognosis compared with other AML subtypes.

AML with inv(16)(p13.1;q22); *CBFB-MYH11*

Another AML subtype commonly affecting younger patients, this entity demonstrates excess blasts with myelomonocytic morphology accompanied by atypical eosinophils with excess abnormal purple-violet granules. The defining translocation results in juxtaposition of the *CBFB* gene to myosin heavy chain gene *MYH11* and may be missed by karyotype [73]. FISH studies best demonstrate this cryptic lesion.

Aside from demonstrating excess blasts, FCI does not typically reveal a noteworthy immunophenotype. *KIT* mutations may be detected and portend a poor prognosis without additional targeted therapy. Other molecular findings aside from *FLT3* TKD (see below) are not currently employed in most diagnostic/prognostic algorithms.

Acute Promyelocytic Leukemia (APL) with t(15;17)(q22;q21); *PML-RARA*

A true hematologic emergency, APL accounts for 5–10 % of de novo AML and demonstrates a spectrum of morphologic appearances ranging from excess atypical promyelocytes with reniform to bilobed nuclei with tremendous cytoplasmic granularity to the so-called "microgranular" forms with bilobed nuclei and submicroscopic granules.

The characteristic t(15;17)(q22;q21) results in the fusion gene *PML-RARA* and blockage of cellular differentiation, though this may be bypassed using relatively nontoxic cell differentiation therapy in the form of all-*trans* retinoic acid (ATRA) [74, 75]. While FCI showing reduction of HLA-DR and CD34 with increased CD33 along with morphology may suggest APL, final diagnosis relies on genetic confirmation of the fusion gene by

either D-FISH or by RT-PCR analysis. Properly designed FISH studies may provide additional information in rare cases of APL with variant fusions such as (11;17)(q23;q21); *ZBTB16–RARA* which are resistant to ATRA therapy.

Recurrent Non-translocation Mutations

Cryptic mutations may be present in up to 95 % of cytogenetically normal cases of AML and their detection plays an increasingly important role in diagnosis on hematologic specimens, though their utility in cytologic specimens remains uncertain. The majority are currently detected using PCR techniques though given the necessity for broad genotyping panels in leukemia some laboratories are moving towards NGS panels. Other findings such as epigenetic changes, microRNA alterations, and copy number variation will not be covered.

NPM1

Mutations in the gene nucleophosmin (*NPM1*) are among the most common, occurring in up to 50–60 % of cytogenetically normal AML [76]. Mutations lead to abnormal mislocalization of the nucleophosmin protein into the cytoplasm and characteristic cytology with blasts showing "cup-like" nuclear invaginations. Immunohistochemistry with probes against the nucleophosmin protein may be used as a surrogate for PCR testing if staining localizes to the cytoplasm instead of normal nuclear staining.

While *NPM1* mutation generally confers a favorable prognosis, concurrent *FLT3* internal tandem duplications (ITD) are seen in 40 % of *NPM1*-mutated patients and essentially remove any protective effect, highlighting the importance of performing both tests simultaneously [77].

FLT3

FLT3 encodes a tyrosine kinase involved in induction and cell survival. Mutations, which occur more frequently as ITD or less commonly as missense mutations (TKD), lead to constitutive activation and subsequent growth induction and apoptosis inhibition of

hematopoietic progenitors. The former finding confers a particularly poor prognosis independent of other cytogenetic findings. While overall *FLT3* mutation rates are seen in approximately 30 % of cytogenetically normal AML, the more clinically relevant ITD subtype is seen in 23 %.

Along with *NPM1* mutations, some cases of *FLT3* mutated AML demonstrate "cup-like" nuclear invaginations on morphology. No immunohistochemical surrogate is currently in wide usage and detection of both *FLT3*-ITD and *FLT3*-TKD is best achieved by PCR methods.

CEBPA

The *CEBPA* gene encodes a transcription factor involved in myeloid differentiation and proliferation with mutations seen in approximately 10 % of de novo AMLs [78]. This finding confers a favorable prognosis and may be seen in concert with the previously described mutations. While no characteristic morphologic changes are noted in AML with *CEBPA* mutations, blasts show a tendency to co-express T-cell antigens. PCR remains the best modality for detection.

Section 4: Specimen/Disease Specific Algorithmic Approach

Given the vast genetic changes seen in hematopoietic neoplasms and the often limited source material on cytologic specimens, protocols should be designed to maximize diagnostic/prognostic yield while preserving as much sample as possible for follow-up analysis; we propose the following triaging algorithms below:

Specimens Without No Known or Limited History

Most commonly cytologists and generalists receive specimens with either no or limited history and are asked to perform a variety of screening tests. This is less often the case with FNA where one

may be asked to assess for causes of lymphadenopathy or other mass lesions, but frequently occurs in the setting of CSF analysis in patients with or without neurologic symptoms; body fluids are almost invariably sent without a specific finding in mind.

Nodal or Soft Tissue FNA (See Fig. 11.1)

Of all cytologic specimens for hematologic neoplasms, FNAs provide the greatest amount of material with the least background contamination. Attempts should always be made to procure the greatest amount of specimen with dedicated passes in RPMI or similar preservative for ancillary studies. Smears and cytospin specimens should be reviewed as a matter of course and depending on the findings next steps should involve screening FCI on a portion of the sample if a predominant lymphoid population (typical or atypical) or immature myeloid cells are seen. For general purposes, a B-cell FCI screen should include antibodies against surface kappa and lambda light chains within a panel while a T-cell FCI screen should include antibodies against CD2, CD5, and CD7; one should never order a FCI panel without prior slide review as this practice sacrifices too much material and increases cost in favor of saving a few hours. Furthermore, the presence of any significant number of RS or variant cells should lead to cell button prioritization though one may opt still to perform very limited FCI studies for B-cell monotypia and T-cell abnormalities given the presence of "RS-like" cells in some non-Hodgkin lymphomas.

For the majority of lymphoid neoplasms, any additional material should be sent for targeted FISH studies depending on the morphologic and immunophenotypic suspicion, particularly with mantle cell lymphoma given its possibly banal appearance and aggressive clinical course. In the absence of a diagnosis or sufficient information to lean towards a B- or T-cell lymphoproliferative disorder, molecular studies may be a last line for B- or T-cell gene rearrangement studies by PCR as southern blot analysis requires far too much tissue. Given that most FNAs are performed on superficial lesions though, a follow-up procedure to obtain additional fresh tissue for molecular studies prior to an excision may be considered. A recent study has shown that fine needle

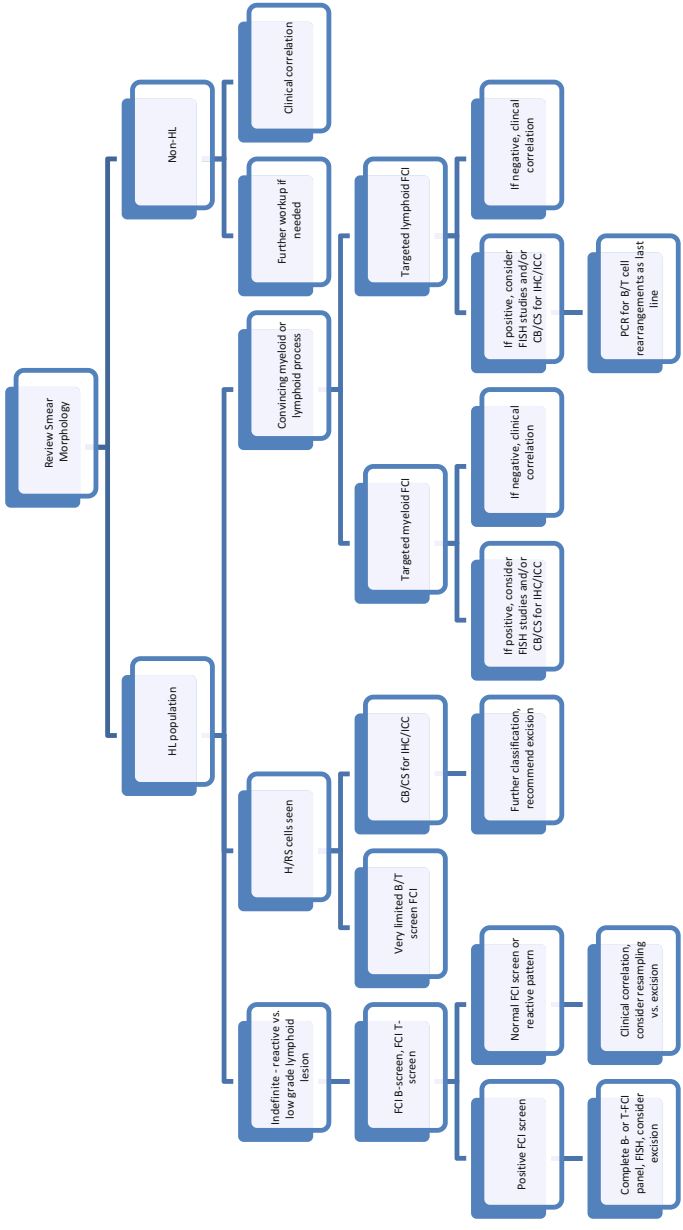

Fig. 11.1 No prior history—FNA of mass. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping, *H/RS* Hodgkin/Reed Sternberg, *CB/CS* cell block/cytospin, *IHC/ICC* immunohistochemistry/cytochemistry

aspiration samples may yield sufficient DNA for next-generation sequencing for multi-gene mutation profiling of solid tumors and consensus panels for hematologic malignancies are likely to follow [79].

CSF (See Fig. 11.2)

The least voluminous of all cytologic specimens, CSF material poses quantity-specific challenges to diagnostic interpretation. A few cytospin slides should be prepared and reviewed and, as described above, a limited FCI panel may be selected to assess for the presence of B-lineage monotypia by kappa or lambda light chain restriction or for blasts of acute leukemia. It may be impossible to detect indolent lymphoproliferative disorders with limited ancillary studies though one may at times be left with a differential of reactive lymphoid cells vs. possible blasts. At our institution we have occasionally opted to etch cells of interest with a diamond pen and then attempt a destaining and restaining procedure with a single targeted immunocytochemical probe though such approaches are far from full proof. FISH and molecular methodologies cannot realistically be applied given cellularity.

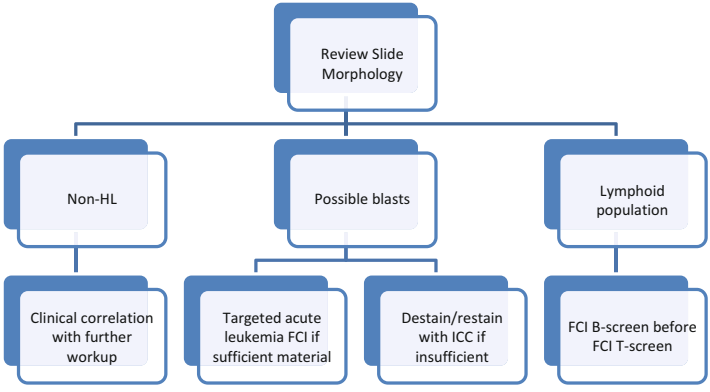

Fig. 11.2 No prior history—CSF. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping, *CB/CS* cell block/cytospin, *IHC/ICC* immunohistochemistry/cytochemistry

Body Fluid (See Fig. 11.3)

Given the background contamination and poor cellular viability, body fluid poses a challenge more from quality than quantity considerations. In the case of a lymphocytosis it can be nearly impossible to discern reactive lymphoid constituents from involvement by a lymphoproliferative disorder, and screening panels by FCI are essentially the sole consideration. Contamination rules out the ability to use either cytogenetic or molecular methodologies on most samples.

Specimens with Prior History of Hematologic Malignancy

In patients with known prior history, triaging for recurrence or progression may be far more targeted. For most small B-cell lymphomas (see Fig. 11.4), a selective flow panel can identify B-cell monotypia and specific immunophenotypic aberrancies, and FISH should follow to assess for characteristic molecular changes given

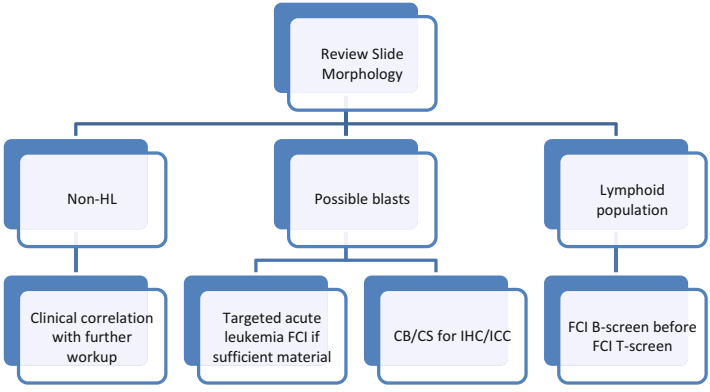

Fig. 11.3 No prior history—body fluid. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping, *CB/CS* cell block/cytospin, *IHC/ICC* immunohistochemistry/cytochemistry

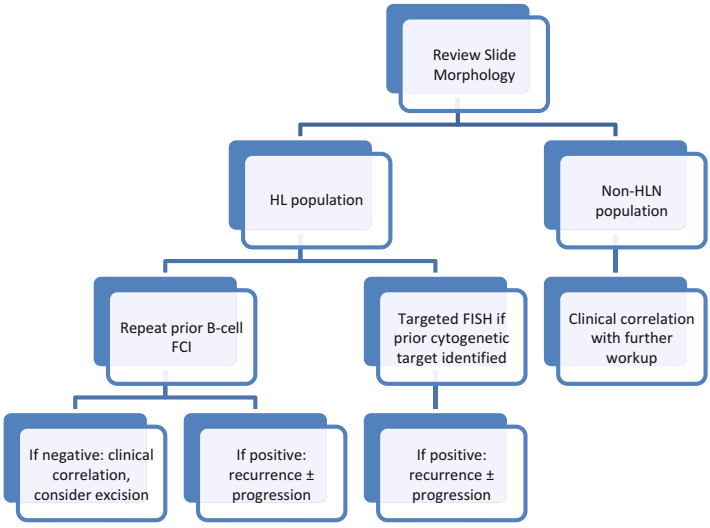

Fig. 11.4 Prior history of HLN—small B-cell lymphomas. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping

its superior sensitivity over PCR for most of these entities. More aggressive lymphomas such as DLBCL, ALCL, and Burkitt lymphoma follow a similar pattern with FISH providing advantages over PCR. Patients with a history of cHL should have material prioritized for cell block analysis and additional smears with FCI acting only to "rule-out" other concurrent hematologic malignancies. Plasma cell dyscrasias (see Fig. 11.5) are best assessed by demonstration of cytoplasmic light chain restriction by flow or IHC; while FISH is particularly useful at first diagnosis, most do not consider it useful for monitoring progression though the presence of a peculiar cytogenetic abnormality may allow for low-level detection on subsequent specimens.

Acute leukemias (see Fig. 11.6) are often easier to diagnose upon recurrence on FCI alone, but occasionally ancillary studies are necessary. While FISH studies are effective in all of the AML variants previously described, RT-PCR may also be used in the case of AML with t(8;21)(q22;q22) and APL. If sufficient material

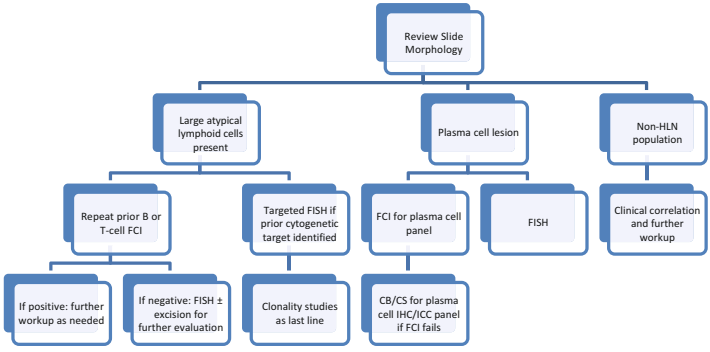

Fig. 11.5 Prior history of HLN—large cell lymphomas and plasma cell lesions. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping, *CB/CS* cell block/cytospin, *IHC/ICC* immunohistochemistry/cytochemistry

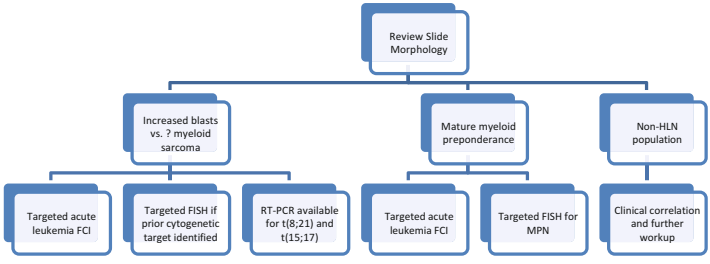

Fig. 11.6 Prior history of HLN—myeloproliferative neoplasm (MPN)/leukemic lesion. *HL* hematolymphoid, *FCI* flow cytometric immunophenotyping, *IHC/ICC* immunohistochemistry/cytochemistry

from a myeloid sarcoma FNA source exists and clinicians are not planning a bone marrow biopsy, then targeted molecular studies for the more common mutations *FLT3*, *CEBPA*, and *NPM1* may also be added. For extramedullary involvement by CML, FISH studies may provide the benefit of diagnostic as well as prognostic information by identifying cytogenetic changes characteristic of transforming disease.

Section 5: Summary

The field of hematopathology marches forward at the vanguard of the molecular genetic revolution with disconcerting speed, and it is difficult for specialists, let alone generalists in other areas, to keep pace. As previously indicated, we have attempted to provide a snapshot of the more common lesions which may be encountered in generalist practice herein, but in reality far greater complexity exists.

Regardless of one's practice setting, we always recommend that cytologists maintain professional contacts with cytogeneticists, molecular geneticists, and hematopathologists to keep abreast of any recent changes as well as to consult regarding options available given a specific diagnostic scenario. Additional precaution up front may prevent incorrect usage of a specimen which could be irreplaceable, or at the very least incur additional unwarranted charges for a patient. And as always, it is of paramount importance to maintain strong relationships with clinicians and to educate them regarding the importance of accurate, thorough histories as well as the necessity for high quality, large volume specimens; given the myriad techniques available and necessary in this area of pathology, one can almost never have too much starting material.

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Molecular Markers in Soft Tissue and Bone Tumors

12

Yaxia Zhang

Introduction

Bone and soft tissue tumors encompass a remarkably diverse spectrum of benign and malignant entities. Over the last couple decades, molecular genetic abnormalities have demonstrated to be an important, sometimes necessary adjunct for diagnosis, prognostication, and therapeutics of bone and soft tissue tumors. In general, bone and soft tissue tumors can be classified broadly into two groups: those neoplasms with complex, nonspecific cytogenetic profiles (Table 12.1) and those with relatively simple, consistent, and recurrent cytogenetic and molecular aberrations (Tables 12.2 and 12.3). The methods used to detect those molecular genetic aberrations include fluorescence in situ hybridization (FISH), array-based comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) array, polymerase chain reaction, and sequencing. Those tests are frequently performed on formalin-fixed, paraffin-embedded (FFPE) tissues in the clinical laboratories. However, with the popularity of new technologies such as interventional radiology and ultrasound-guided fine needle aspiration, clinicians often request that specific molecular genetic tests

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Table 12.1 Bone and soft tissue tumors with complex and nonspecific cytogenetic and molecular alterations

Tumor	Cytogenetic alteration	Molecular alteration
Osteosarcoma	Complex	p53..??
Chondrosarcoma	Complex	*IDH* mutation
Angiosarcoma	Complex	Myc amplification?
Leiomyosarcoma	Complex with frequent deletion/rearrangement of 1p	??
Pleomorphic liposarcoma	Complex	??
Pleomorphic rhabdomyosarcoma	Complex	??
Malignant peripheral nerve sheath tumor	Complex	*NF1* mutation?
Undifferentiated pleomorphic sarcoma	Complex	*??*

Table 12.2 Bone tumors with simple cytogenetic and molecular alteration

Tumor	Cytogenetic alteration	Molecular alteration
Osteochondroma	8q24 and 11p11/13	*EXT1/2*
Enchondroma		*IDH1/2*
Chondromyxoid fibroma	t(1;5)(p13;p13), 6q	??
Synovial chondromatosis	6p	??
Bizarre parosteal osteochondromatous proliferation	t(1;17)(q32;q12)	??
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12) many other translocations with 9q22	*EWSR1-NR4A3*
Mesenchymal chondrosarcoma	Inv(8)(q13q21) t(1;5)(q42;q32)	*HEY1-NCOA2* *IRF2BP2-CDX1*
Fibrous dysplasia		*GNAS1* mutation
Subungual exostosis	t(x;6)(q24;q15)	??
Well-differentiated osteosarcoma	Ring form of chromosome 12	*MDM2*, *CDK4* amplification
Ewing sarcoma/PNET	t(11;22)(q24;q12) many other translocations with 22q12	*EWSR1-FLI1* *EWSR1 with many partners*

(continued)

Table 12.2 (continued)

Tumor	Cytogenetic alteration	Molecular alteration
Tenosynovial giant cell tumor	Rearrangement of 1p11-13	*CSF1* overexpression
Giant cell tumor of bone	Telomeric association (tas)	*RANKL* *H3F3B mutation*
Chondroblastoma		*H3F3A* mutation
Aneurysmal bone cyst	t(16;17)(q22;p13) many other translocations with 17p13	*USP6-CDH11 fusion* *USP6 with other partners*

Table 12.3 Soft tissue tumors with simple cytogenetic and molecular alteration

Tumor	Cytogenetic alteration	Molecular alteration
Lipoma, chondroid subtype	t(11;16)(q13;p12-13)	*??*
Lipoma, spindle cell/ pleomorphic	13q or 16p rearrangement	*??*
Lipoblastoma	8q11-13 rearrangement	*PLAG1 fusion*
Hibernoma	11q13 rearrangement	??
Atypical lipomatous tumor Well-differentiated/ dedifferentiated liposarcoma	Ring form of chromosome 12	*MDM2*, *CDK4* amplification
Myxoid liposarcoma	t(12;16)(q13;p11) t(12;22)(q13;q12)	*FUS-DDIT3* *EWSR1-DDIT3*
Nodular fasciitis	t(17;22)(p13;q13)	*MYH9-USP6*
Desmoid fibromatosis	Trisomies 8, 20	*APC* or *CTNNB1* mutation
Dermatofibrosarcoma protuberans	Ring form of chromosomes 17,22, t(17;22)(q21;q13)	*COL1A1-PDGF-B*
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23) many other translocations with 2p23	*TPM3-ALK*

(continued)

Table 12.3 (continued)

Tumor	Cytogenetic alteration	Molecular alteration
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	*FUS-CREB3L2*
Myxoinflammatory fibroblastic sarcoma	t(1;10)(p22;q24)	*FGF8-TGFBR3*
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(q35;q14)	*PAX3-FOXO1A* *PAX7-FOXO1A*
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11) t(12;22)(p36;q12)	*FUS-ATF1* *EWSR1-ATF1*
Alveolar soft part sarcoma	t(x;17)(p11;q25)	*TFR3-ASPL*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWSR1-ATF1*
Infantile fibrosarcoma	t(12;15)(p13;q25)	*ETV6-NTRK3*
Synovial sarcoma	t(x;18)(p11;q11)	*SS18-SSX1*
Gastrointestinal stromal tumor		*KIT, PDGFRA, BRAF mutation*
Endometrial stromal sarcoma	t(7;17)(p15;q21)	*JAZF1-JJAZ1*
Epithelioid hemangioendothelioma	t(1;3)(p36;q25) t(x;11)(p11;q22)	*WWTR1-CAMTA1* *YAP1-TFE3*
Neurofibroma	Normal	*NF1 mutation*
Schwannoma	Monosomy 22	*NF2 mutation*
Myopericytoma	t(7;12)(p22;q13)	*ACTB-GLI*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1-WT1*

be performed on the limited material. It is feasible to have some molecular tests done on cytology specimens, such as touch prep, thin-prep, cell block, or fluid. Some tests are actually more easily performed on cytology specimens and can provide much better results (such as FISH performed on thin-prep slide). This chapter is divided into two major sections: (1) summary of recurrent or tumor-specific genetic events in bone and soft tissue tumors, and (2) overview of the molecular approaches commonly used in clinical practice. A brief discussion of some molecular tests for bone and soft tumor performed on cytology samples is also provided.

Bone and Soft Tissue Tumor with Tumor-Specific Alterations

A major group of high grade bone and soft tissue sarcomas are associated with complex nonspecific genetic abnormalities (Table 12.1). For this group, because of the high degree of genomic complexity and intratumoral heterogeneity, clinical genetic tests are not generally helpful for diagnosis, except to exclude other differential diagnostic possibilities. Thus, there is no further discussion of this group in this chapter.

Bone and soft tissue tumors with relative simple, specific cytogenetic and molecular genetic alterations can be divided into two groups. About one-third of soft tissue sarcomas exhibit a non-random chromosomal translocation, which leads to the production of a chimeric gene, such as the *EWSR1-FLI1* fusion of Ewing sarcoma and *ETV6-NTRK3* in infantile fibrosarcoma. Those fusion genes often encode for abnormal oncogenic proteins that are presumed to be the initiating sarcomagenic event. Another subset of bone and soft tissue tumors are characterized by recurrent aberration in isolated genes, such as *IDH* mutations in cartilaginous tumors and the mutations of *KIT* or *PDGFR* in gastrointestinal stromal tumor (GIST).

Bone and Soft Tissue Tumors with Specific Translocation

Translocation is a chromosome abnormality caused by rearrangement of nonhomologous chromosomal materials. A chimeric gene is created by juxtapositioning two otherwise-separated genes, which results in the production of an abnormal often oncogenic protein. Functionally, the majority of those chimeric proteins are aberrant transcription factors that cause transcriptional deregulation. Ewing sarcoma, synovial sarcoma, alveolar rhabdomyosarcoma, myxoid liposarcoma and many others are in this category. Other chimeric proteins are aberrant tyrosine kinases, such as those from infantile fibrosarcoma, inflammatory

myofibroblastic tumor, which deregulate kinase signaling pathway. Dermatofibrosarcoma protuberans, which harbors the fusion gene PDGFB-COL1A1 resulting in an activated transmembrane tyrosine kinase, responds to targeted therapy with tyrosine kinase inhibitors such as imatinib.

A soft tissue tumor translocation is often present as the only cytogenetic alteration and is presumed to be the initiating oncogenic event. Furthermore, these tumor-specific alterations are retained throughout the clinical course, such as in the tumor metastasis or dedifferentiated transformation. Thus, it is also important to identify those aberrations in poorly differentiated sarcomas, which may provide aid in the management of those tumors. While the majority of translocations in bone and soft tissue tumors are specific, the same translocation has been identified in the unrelated neoplasms, for example, *ETV6-NTRK3* in infantile fibrosarcoma is also present in mammary analogue secretory carcinoma of salivary glands, acute myeloid leukemia and cellular mesoblastic nephroma of kidney. Thus, it is important for pathologists to use the morphologic-molecular integrated approach to render an accurate diagnosis.

One of the facts is that many translocation variants exist in the same tumors and more variants will continue to be discovered. Most cytogenetic variants arise from a rearrangement of one consistent gene with different partner genes, for example, in aneurysmal bone cyst, the translocation t(16;17) with *CDH11-USP6* fusion gene was first discovered. Afterwards, four more *USP6* fusion genes with different partner genes *COL1A1*, *osteomodulin*, *ZNF9*, and *TRAP150* were cloned. On the other hand, the fusion subtypes are often the result of genomic breakpoint differences that produce distinct chimeric transcripts. For example, clear cell sarcoma of soft tissue is characterized by a t(12;22)(q13;q12) translocation, which results in fusion of EWSR1 to the transcription factor ATF1. The fusion protein was demonstrated to bind to the promoter of the microphthalmia-associated transcription factor and stimulate the activity of the melanocyte-stimulating hormone, which leads to melanocytic differentiation. At least four different fusion transcripts have been described. The type of fusion transcript does not appear to affect clinical outcome. It should be emphasized that fluorescence in situ hybridization analysis may

possibly detect rare or undiscovered variants that are not detected by the more specific RT-PCR primer sets; however, FISH analysis does not distinguish between these variant types.

Bone and Soft Tissue Tumors with Recurrent Aberrations in Single Genes

A subset of BST tumors are characterized by activating oncogenic mutations. For example, mutations of KIT or PDGFRA in gastrointestinal stroma tumor (GIST) (Fig. 12.1) lead to constitutive activation of tyrosine kinases, which stimulates the downstream signal pathway. The type of mutations includes in-frame deletions, internal tandem duplications, and point mutations. KIT and PDGFRA, located to 4q12, encode for the same class of receptor tyrosine kinases.

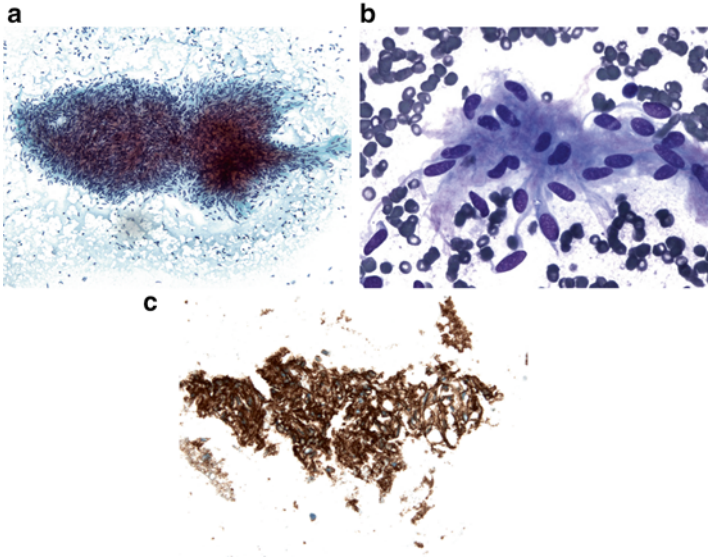

Fig. 12.1 Endoscopic ultrasound guided fine needle aspiration (EUS-FNA) of gastrointestinal stromal tumor (GIST). (**a** and **b**) PAP and Diff-Quick stains show clusters of bland spindle cells in short fascicle. (**c**) The tumor cells are positive for C-Kit on cell block

Cytogenetic and Molecular Tests Commonly Used in the Clinical Practice for Bone and Soft Tissue Tumors

Fluorescence In Situ Hybridization (FISH)

Hybridization is used to detect and localize the presence or absence of specific DNA sequences on chromosomes by using fluorescent labeled complementary DNA or RNA probes. FISH testing with bicolor break-apart or dual fusion probe sets are most commonly used for the detection of translocation, locus specific probes for evaluation of amplification, or loss of tumor suppressor gene locus. The overall resolution of interphase FISH is 50–100 kb. The advantage of FISH testing is that it can be performed on non-dividing (interphase) cells from fresh (smears, touch prep, cytospin preparation) or formalin fixed, paraffin embedded (FFPE) tissue. Cytologic preparations are usually air-dried and subsequently fixed in a 3:1 ratio of methanol to glacial acetic acid. The advantage of performing FISH on cytology preparations, compared to FFPE tissue, is that the nuclei are intact, which provides the most accurate assessment of subtle aneuploidy changes (Figs. 12.2, 12.3, and 12.4). In contrast, portions of most nuclei are lost during sectioning of FFPE tissue and this may lead to false-positive results in the evaluation of chromosomal deletions or losses.

Reverse Transcription PCR

Reverse transcription polymerase chain reaction is a technique whereby RNA is first reverse transcribed into complementary DNA (cDNA) using the enzyme reverse transcriptase, and the resulting cDNA amplified in nested PCR reaction. It is particularly useful for detecting tumor-specific fusion genes created by chromosomal translocations. It can detect transcript variants produced by different breakpoints, such as in Ewing's sarcoma. It is also a very sensitive test and may be used to monitor minimal residual disease. It is more sensitive and specific compared to FISH. However, one pitfall to be aware of is that it may not be able to detect uncommon cytogenetic variant translocations because of primer design.

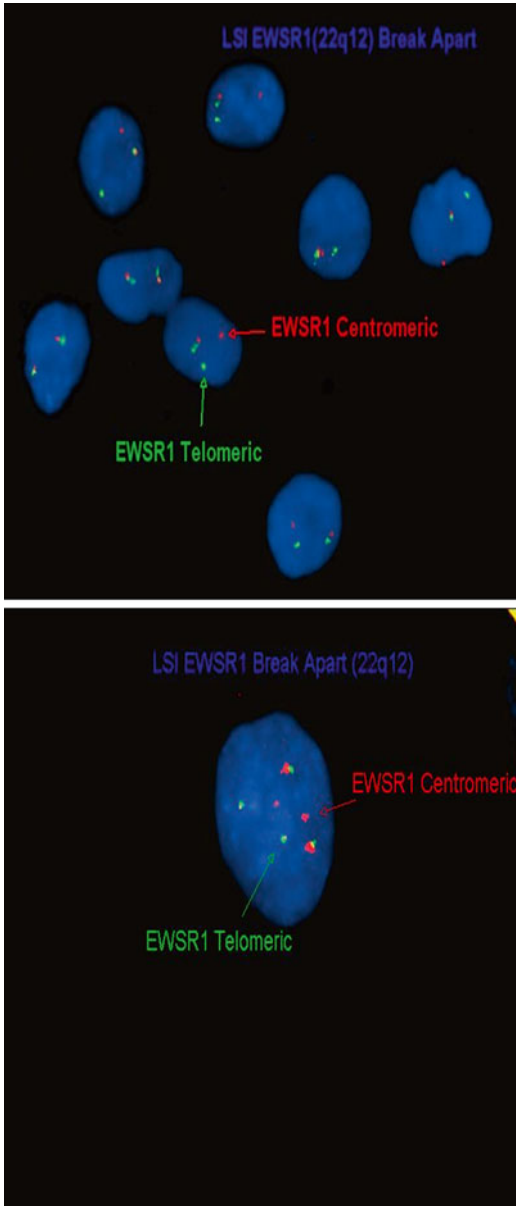

Fig. 12.2 FISH positive *EWSR1* break-apart on touch prep slide

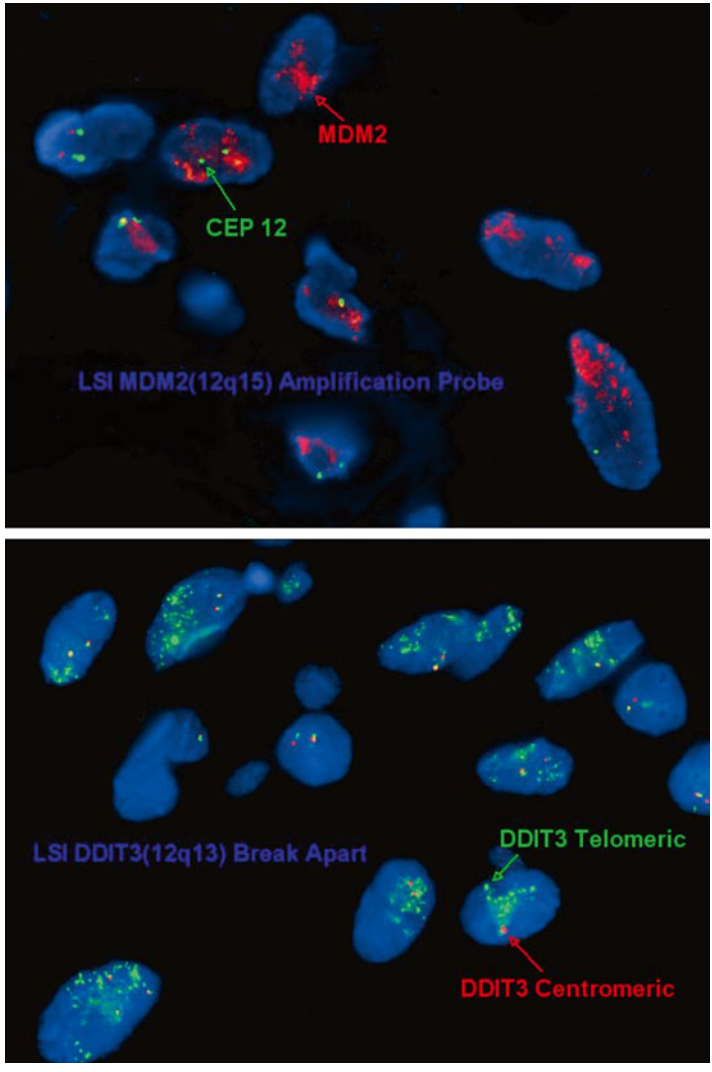

Fig. 12.3 Amplification of MDM2 and DDIT3 in differentiated liposarcoma

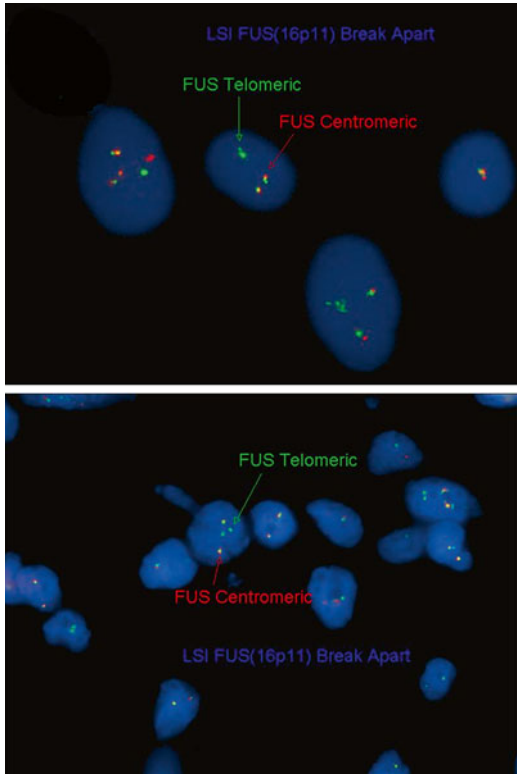

Fig. 12.4 Positive FISH test for FUS translocation on touch-prep slide and FFPT in myxoid liposarcoma (round cell component)

Sequencing Analysis

DNA sequencing is used to precisely characterize the sequence variants at the single base level. Sanger sequencing is still very commonly used in the clinical lab to detect single point mutation. Next-generation sequencing includes whole-genome, whole-exome, and transcriptome approaches.

Conclusion

Over the last couple of decades, great advances in genomic technologies have furthered our understanding of bone and soft tissue tumorigenesis. The current WHO classification of bone and soft tissue tumors have been based on the distinct histopathologic and cytogenetic molecular aberrations. More importantly, personalized therapy based on specific aberrations within a given tumor has the potential to improve the disease outcome. Pathologists should recognize the limitations of different assays and different tissue preparations, including cytologic preparations.

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Suggested Reading

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Molecular Biomarkers in Body Fluid Cytology

13

Rachel Conrad, Christine Chow,
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Introduction

The pericardial, pleural, and peritoneal cavities are lined by mesothelial cells and typically contain a small amount of fluid. In pathologic conditions, excessive fluid may accumulate and can be examined cytologically to help determine the cause of the effusion, which has important clinical management implications. A malignant effusion, for example metastatic adenocarcinoma, indicates a late stage process; therefore aggressive treatment such as systemic chemotherapy may be needed.

Removal of pleural or peritoneal fluid is a relatively simple and safe procedure; it is less invasive than a biopsy and samples a much larger area. The procedure is both diagnostic and therapeutic, relieving symptoms such as dyspnea, cough, and pressure that are secondary to excess fluid.

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Although the majority of effusions are due to circulatory disorders, problems with plasma oncotic pressure, infection, pulmonary embolism, autoimmune disease, or other causes, up to 20 % are associated with malignancy [1, 11].

Cytologic examination of effusions involves collection of preferably fresh fluid, refrigerated for storage, with fixation in 50 % ethanol if processing is delayed. A minimum of 30–50 mL is recommended [13]. Subsequently, cytospins, monolayer smears, or liquid-based preparations are made and stained with Papanicolaou stain and Romanowsky stains. Paraffin cell blocks may also be prepared and stained with hematoxylin and eosin as well as stains for microorganisms, mucin or immunocytochemical stains. Additional studies can be simultaneously performed, including microbiology cultures, chemical analysis, and flow cytometry for lymphoid proliferations.

Cytologic examination of effusions for malignancy has a sensitivity of 50–60 % [1] or 32–76 % [20], specificity of 97 %, positive predictive value of 95–100 %, and negative predictive value of 86–88 % [1]. Limitations to cytologic evaluation of effusions include a lack of architecture, abundance of background elements, paucity of malignant cells, and the altered appearance of neoplastic cells after prolonged suspension in fluid (rounding up, vacuolation). Specific challenges include distinguishing reactive mesothelial cells from metastatic epithelial cells, and (even more difficult) determining if mesothelial cells are reactive or malignant.

Numerous different techniques have been used to study molecular markers in effusions. The presence of a mixed population of cells (mesothelials, histiocytes, inflammatory cells, degenerating cells, blood cells, and occasionally malignant cells) in effusions can complicate the interpretation of these test results; however, with a good sample, most techniques can be performed just as effectively on cytologic samples as on tissue samples. DNA-based tests appear to be very effective on cytology slides, even when the material is over 10 years old. Prior staining does not seem to inhibit these tests, although some studies have shown a better performance in smears previously stained with Romanowsky rather than Papanicolaou methods [24]. PCR was able to detect loss of FHIT,

p16 mRNA, and K-ras mutations and thus identify malignant pleural effusions with an overall sensitivity of 74 %; it was also able to identify malignant cells in some cases that were morphologically negative for disease [26].

Additional RNA based and protein based tests are being studied. Telomerase activity can theoretically distinguish between benign mesothelial cells and malignant cells, but has limited practical use due to its high false negative and false positive rates [40]. Epigenetic tests assessing DNA methylation and histone modification have begun to show promise in distinguishing malignant and benign effusions, and Lin et al [27] have described a new fluorescence probe for BMVC [3,6-Bis(1-methyl-4-vinylpyridinium) carbazole diiodide] that binds to DNA or mitochondria in malignant cells.

Recently, a novel class of biomarker, cellular biomechanical or biophysical markers has been developed to detect malignant cells from body fluids. The markers comprise a host of biophysical properties such as cellular elasticity, adhesion force, deformability, and permeability, where the underlying biological basis is the cellular cytoskeletal remodeling associated with malignant transformation process, especially tumor invasion and metastasis. It utilizes variety of technological platforms such as atomic force microscope, microfluidics, and membrane filtration techniques; the main advantage of such an approach is that it is label free [8, 14, 38]. Since cytomorphologic analysis of effusions with or without costly immunohistochemical confirmatory staining is a time consuming and labor-intensive process, the development of a simple, quick, and label free prescreening tool such as deformability cytometry to identify high-risk patients may be able to maximize the use of available resources, reduce sample processing burdens, reduce chances of cross-contamination, and decrease the time to diagnosis.

As the main function of cytological analysis of body fluid is to detect malignancy (either primary or metastatic), and the molecular markers for each setting are largely different, we will discuss the markers for detecting primary mesothelioma and metastatic processes separately in this chapter.

Markers for Mesothelioma

Malignant mesothelioma is a rare cancer that is associated with asbestos exposure, smoking, high dose radiation, chronic irritation, or infection with simian virus 40. Separation of reactive mesothelial cells and malignant mesothelial cells can be challenging, especially if one relies exclusively on cytomorphologic findings. Generally, malignant mesothelioma produces hypercellular smears with numerous clustered and single mesothelial cells in the absence of a foreign cell population. The cytology may be bland, and malignant cytologic features such as irregular nuclear contours, atypical mitoses, or coarse chromatin are not always prominent.

Ancillary tests may be useful in challenging cases. In general, malignant effusions tend to have a lower pH (<7.2), elevated amylase, low glucose (<60–80 mg/dL), high lactate dehydrogenase, and high total protein concentration. Levels of CEA tend to be <30 ng/dL in mesothelioma, but are usually elevated in adenocarcinoma (>150 ng/dL) [11]. Elevated pleural fluid hyaluronate levels >225 mg/L and hyaluronate immunostaining (membranous and cytoplasmic) [9] have been noted in some mesotheliomas, although these tests are not routinely used. Mucicarmine and Alcian Blue can stain hyaluronic acid in mesothelial cells just like they stain mucin in adenocarcinomas; thus these stains are not as useful as the more specific PASD, which does not highlight hyaluronate.

Electron microscopy is another ancillary test that is seldom utilized since it is cumbersome and not readily available. It displays long slender microvilli in well-differentiated epithelioid mesothelioma, in contrast to the short and stubby microvilli of adenocarcinoma.

Immunohistochemical markers, especially when performed on cell block sections, have helped tremendously in body fluid cytology especially in distinguishing reactive mesothelial cells from a metastatic process. The International Mesothelioma Interest Group recommends using a panel of at least 2 mesothelial markers and 2 carcinoma markers, choosing immunostains that have >80 % sensitivity or specificity, and defining negative staining as <10 % of cells highlighted for cytoplasmic and membranous stains [20]. The

choice of immunostains will vary depending on the morphologic appearance (spindloid vs epithelioid), location (pleural vs peritoneal), and the patient's clinical history of other malignancies. A typical panel of stains may include Calretinin (for mesothelial cells), D2-40 (for mesothelial cells), MOC31 (for metastatic columnar cells), and BerEP4 (for metastatic epithelial cells), as show in Fig. 13.1.

Calretinin, mesothelin, and pankeratin are highly sensitive, and are seen at least focally in almost all mesotheliomas. Negative

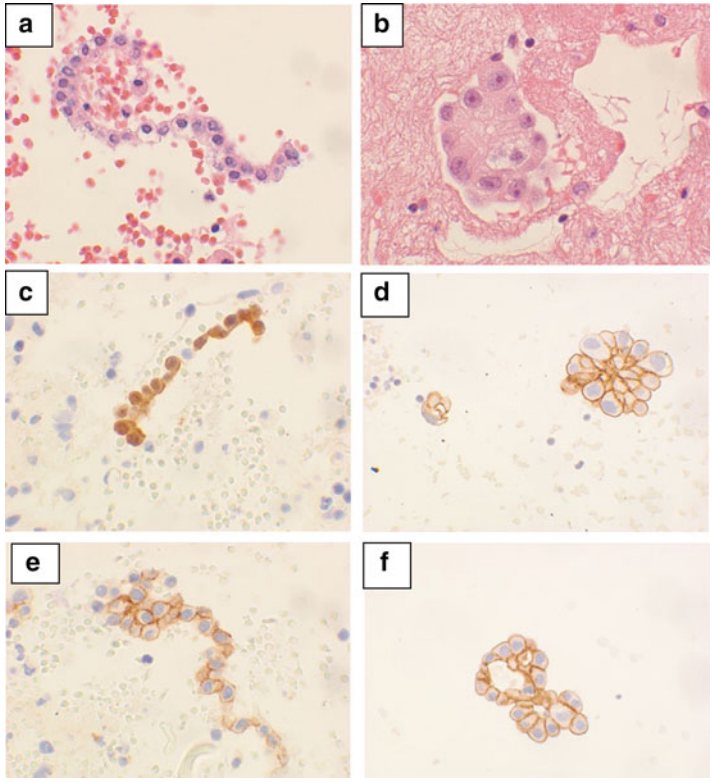

Fig. 13.1 Immunohistochemical stains of pleural fluid cell block sections. (**a**) Reactive mesothelial cells show positive staining for calretinin (**c**) and D2-40 (**e**). (**b**) Metastatic lung adenocarcinoma cells show positive staining for BerEP4 (**d**) and MOC31 (**f**). All photos were taken at 60×

results in otherwise morphologically straightforward cases should prompt reevaluation of a mesothelioma origin. Mesothelin, WT1, and CK5/6 are less sensitive in sarcomatoid cases, but calretinin remains useful. Podoplanin (D2-40) is also highly sensitive and useful in sarcomatoid subtypes, but it can be expressed by some sarcomas and squamous cell carcinomas. In differentiating between benign or reactive mesothelial cells and malignant mesothelioma, desmin tends to highlight benign proliferations.

One of the best stains for separating mesothelial cells from carcinomas is MOC31, which is nonreactive in benign mesothelial proliferations, slightly reactive in mesotheliomas, and strongly reactive in almost all other carcinomas, including pulmonary adenocarcinomas and squamous cell carcinomas. BG8, BerEP4, monoclonal CEA, and B72.3 are also useful. For identifying lung carcinoma, TTF1 and Napsin A useful because they are seldom positive in mesothelial cells. P63 can assist in identifying squamous origin. ER, GCDFP-15, and mammoglobin are also seldom expressed by mesothelial cells but are often seen in breast malignancies. Some stains (Ki67, HBME-1, and caldesmon) have fallen out of favor and are no longer considered useful in evaluating mesotheliomas.

Unfortunately, there is no specific immunohistochemical marker that can be used to distinguish reactive mesothelial cells from mesothelioma. EMA, GLUT-1, p53, IMP3, and XIAP may be more positive in malignant proliferations, but are not unique to mesothelioma. Additional molecular markers may be used to assist in separating malignant and reactive effusions, although no specific mutations have been described that are exclusively characteristic of mesothelioma. Deletion of 9p21 p16/CDKN2A (cyclin-dependent kinase inhibitor 2A and adjacent genes CDKN2B and methylthioadenosine phosphorylase MTAP) is one of the most common mutations seen in mesothelioma. It is the only molecular test recommended currently by the International Mesothelioma Interest Group [20]. It has a high positive predictive value when evaluated by FISH and is negative in benign and reactive conditions, so it can be useful for confirming malignancy in cases with challenging cytomorphologic findings. False negatives may result from admixed reactive mesothelial cells in the cell block. Unfortunately, deletions of chromosome 9p are not

distinctive for mesothelioma and have been seen in numerous other tumors. Thus, it cannot separate malignant mesothelioma from lung adenocarcinoma, melanoma, or sarcoma. Homozygous deletion can also be a prognostic marker, correlating to a shorter survival.

The only other molecular test that is regularly utilized in mesothelial evaluation is PCR for detection of t(X;18). This mutation is characteristic for synovial sarcoma and, when present, can exclude a diagnosis of sarcomatoid mesothelioma. This is seldom used in cytologic specimens since sarcomatoid mesotheliomas rarely present in effusions.

Additional mutations have been noted in mesotheliomas and are currently being assessed as therapeutic targets. The neurofibromatosis 2 gene on chromosome 22q11 produces a tumor suppressor protein (merlin). Inactiviating mutations are found in 35–40 % of pleural mesotheliomas, as well as some cutaneous melanomas, renal cell carcinomas, meningiomas, ependymomas, and schwannomas. Loss of NF2 leads to mTORC1 activation, a pathway which can be targeted by kinase inhibitors, rapamycin, and everolimus. A Phase II trial of second-line chemotherapy with everolimus for mesothelioma (Southwest Oncology Group SWOG study) failed, but these patients were not evaluated for NF2 deletion. A Phase I study with mTOR inhibitor GDC-0980 showed some promise [36].

BRCA-associated protein 1 (BAP1) on chromosome 3p21 is mutated or lost in 25 % of malignant pleural mesotheliomas. Nonspecific mutations are also seen in some uveal and cutaneous melanomas, as well as clear cell renal cell carcinomas, and are associated with more aggressive tumors in these patients. This mutation may also be seen in lung and breast carcinomas. Its prognostic significance in mesotheliomas has not yet been fully studied, but it may be associated with increased risk. BAP1 is a nuclear-localized deubiquitinase and modulates gene transcription. Histone deacetylase inhibitors (Vorinostat SAHA, trichostatin A, valproic acid VPA) have shown some use in uveal melanomas, but failed to demonstrate usefulness in a second-line chemotherapy Phase III trial for mesothelioma (VANTAGE trial) [18]. An immunohistochemical stain has been developed to evaluate loss of BAP1, and appears most useful in epithelioid mesotheliomas [7].

Clonal abnormalities such as deletion of 1p, 3p, or 22q [15] and polysomy of chromosome 7 or 9 by FISH [1] have also been observed in malignant mesothelioma on destained Diff-Quik smears. Chromosome 7 polysomy has been reported in 88 % of malignant mesotheliomas, and polysomy of chromosome 9 has been observed in 69 %. Aneuploidy of chromosomes 11 or 17 has also been reportedly helpful in discriminating between benign and malignant effusions. Additional nonspecific mutations have also been noted in Large Tumor Suppressor 2 (LATS2) on chromosome 13q12 [32]. The combination of fragile histidine triad (FHIT), p16 mRNA loss, and K-ras gene mutation detected by PCR demonstrated usefulness in separating malignant pleural effusions from benign ones [26].

Several other studies have assessed DNA methylation profiles, microRNA regulation, and gene expression arrays to locate prognostic associations and therapy targets. Higher levels of Aurora kinases A and B, VEGF, and fibroblast growth factor 2 as well as loss of PTEN and FAS-ligand negativity have been associated with a poor prognosis. Additional adverse prognostic indicators include cMet receptor tyrosine kinase and PKCbeta2 expression, which may also serve as therapeutic targets. Improved survival rates were seen with Aquaporin-1 and mir-29c microRNA overexpression [18], as well as lack of osteopontin and HIF-1 [9]. Matrix Metalloproteinase-7 (MMP-7), XIAP, KOC/IMP3, and ProExC show increased IHC expression in mesotheliomas and may serve as chemotherapeutic targets. The importance of finding new targets for therapy cannot be underestimated, since chemotherapy resistance in mesothelioma patients is a major problem, with a median progression-free survival time of 5.7 months with the standard first-line therapy of cisplatin plus pemetrexed [35].

Markers for Metastatic Malignancy

Metastatic involvement of pleural fluid is far more common than primary tumors. Adenocarcinoma comprises over two-thirds of these malignant effusions. Among men, lung cancer is the most frequent etiology, followed by lymphoproliferative lesions and gastrointestinal tract malignancies. Breast, lung, ovary, and lymphoid neoplasms make up the majority of malignant effusions in

women. Pediatric malignant effusions are uncommon but typically involve small round blue cell tumors, which may be hard to distinguish from normal background lymphocytes. In general, malignant involvement of the serous cavities (especially the pericardial cavities) is a poor prognostic sign with an average survival time of 3–12 months [1].

Occasionally, an effusion can be the presenting symptom for an occult malignancy. Often patients who present at this stage are poor surgical candidates and cannot tolerate invasive biopsies or excisions. In these cases, effusion fluid is usually the only material available for diagnosis and molecular testing to determine drug targets for the best therapeutic course.

The general cytologic appearance of metastatic disease involving an effusion is that of a foreign cell population admixed with normal mesothelial cells, histiocytes, and lymphocytes. The malignant metastatic cells may be arranged in three-dimensional clusters, papillary fragments, glandular or acinar structures, or as single atypical cells. Immunohistochemical stains such as mucicarmine, MOC31, BerEP4, and B72.3 can be useful to identify these cells.

As discussed above, since the application of a panel of immunohistochemical markers including calretinin, MOC31, BerEP4, and D2-40 usually allows effective diagnosis of metastatic malignancy, the current interest has shifted to utilizing the body fluid samples to perform molecular analysis for the purpose of detecting drug targets for therapeutic purposes. While the complete discussion of the topic can be found in relevant chapters, we will briefly outline molecular testing that may be performed in body fluid for some of the most common malignancies:

1. Lung: The CAP guidelines recommend testing for EGFR and ALK mutations in pulmonary adenocarcinoma [28]. These molecular tests have been successfully performed on pleural effusion specimens [6, 29], although CAP guidelines prefer the use of cell blocks over smears if possible.
   (a) EGFR (epidermal growth factor receptor) mutation is seen in approximately 10–35 % of lung cancers and is typically not associated with smoking. It confers sensitivity to geftinib and erlotinib therapy. Both immunohistochemical staining as well as the CAP-preferred PCR DNA-based

testing for EGFR mutations have been effectively performed on a variety of pleural effusion specimens, including alcohol-fixed smears, spray fixed smears, Pap or Diff-Quik stained smears, SurePath liquid-based preparations [23], paraffin cell blocks [10], and even cell-free supernatant [25]. Although the concentration of tumor cells can affect results, next generation sequencing showed superior performance compared to Sanger sequencing on BAL and pleural fluid samples in a 48-case study that included specimens with less than 10 % tumor cells [4, 12].

(b) EML4-ALK fusion has been seen in approximately 5 % of lung cancers, typically in younger nonsmoking patients. Sensitivity to crizotinib. ALK rearrangement analysis has performed adequately on routine Papanicolaou cytology slides (immunohistochemical methods) and on Diff-Quik stained smears (FISH) [3, 37].

(c) K-ras testing has also been successfully performed on cytology specimens from effusions [6].

2. Hematopoietic malignancy: Approximately 25 % of all lymphomas have associated pleural effusions, although pericardial and peritoneal effusions are much less common [1]. Flow cytometry is an important adjunct test in effusions with increased leukocytes, since low-grade lymphomas can be very difficult to distinguish from reactive lymphocytes. Flow cytometry is not useful for detecting Hodgkin lymphoma, in which case immunohistochemical stains may prove helpful. Caution must be advised in interpretation of CD15, since occasional staining may be seen in mesothelial cells with high concentrations of hyaluronic acid.

   Primary effusion lymphoma is a rare process that presents in HIV patients in the absence of any tumor mass. The effusion fluid is filled with large high grade cells with immunoblastic, anaplastic, or plasmacytoid morphology. The cells also display HHV8 positivity, high levels of IL-6, a null-phenotype and high mitotic activity.

3. Breast: Breast carcinoma is a common cause of malignant effusions. Useful immunohistochemical stains include ER, GCDFP-15, and mammoglobin, all of which are typically positive in

breast cancer and negative in mesothelials. PR has a lower sensitivity and thus is not as commonly applied. E-cadherin loss or p120 catenin expression may also be used. CAP guidelines recommend HER2 testing on primary invasive tumors and metastatic sites [39], including serous cavity effusions.

4. Gynecologic tumors: Gynecological tumors including ovarian and endometrial carcinomas often involve peritoneal fluid. Serous ovarian malignancies are especially difficult to distinguish from mesothelial cells, since they share several markers (WT1, calretinin). MOC31 and BG8 are some of the most helpful immunostains, since they are positive in serous carcinomas and negative in mesothelial cells. PAX2 and PAX8 also appear to be positive markers for serous carcinomas, although only a limited number of studies have been performed so far. D2-40 is typically negative in serous carcinomas and positive in mesothelial cells.
5. Gastrointestinal tumors: Malignancies from the gastrointestinal occasionally present with effusions. CDX2 and CK20 are negative in most mesothelial cells and positive in several gastrointestinal malignancies. For colon cancer MSI, KRAS, BRAF, and PTEN may be tested whereas for gastric cancer Her-2/neu may be evaluated using FISH.
6. Melanoma: Melanoma can rarely appear in effusions, and the metastatic cells can be very challenging to separate from mesothelial cells. BRAF (V600E, V600K) is mutated in 30–60 % of melanomas, and this mutation confers a sensitivity to vemurafenib therapy [19].
7. Others: While the above malignancies comprise the majority of malignant effusions, any tumor can metastasize to the pleural, peritoneal, and pericardial cavities. Pleural fluid involvement has been observed in squamous cell carcinoma, sarcomas, Merkel cell carcinoma, thyroid papillary carcinoma, and even salivary duct carcinoma [21]. Fortunately, these unusual metastases seldom present as occult malignancies. Instead, patients typically have a well-established history of disease at a primary site with multiple recurrences. Ancillary studies can be helpful in confirming the diagnosis.

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Molecular Biomarkers in Prognostication of Uveal Melanoma

14

Charles V. Biscotti

Introduction

Uveal melanoma arises from melanocytes of the uveal tract (iris, ciliary body, and choroid) and represents the most common primary intraocular malignancy in adults. The mean age-adjusted incidence of uveal melanoma in the USA is approximately 4.3 new cases per million people, with no clear variation by latitude. Males have a higher incidence than females (4.9 vs. 3.7 per million). Uveal melanoma is diagnosed mostly at older ages, with a progressively rising, age-specific, incidence rate that peaks near the age of 70 years. Diagnosis often occurs late in the course of disease, and prognosis is generally poor.

Surprisingly to most cytologists, definitive uveal melanoma treatment rarely requires cellular or tissue diagnosis. Rather, experienced ophthalmologists can directly visualize ocular tumors and make accurate diagnoses with noninvasive tests. For example, only approximately 2.5 % of patients with ocular tumors required diagnostic fine needle aspiration in the large series reported by Shields and colleagues. However, ocular oncologists are increasingly aspirating uveal melanomas for prognostic testing. With regard to

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prognosis, uveal melanomas stratify into two approximately equal groups. Local treatment (radiation plaque therapy or enucleation) cures one group, while the other half succumbs to metastatic melanoma almost always involving the liver, this despite the fact that only about 2 % of uveal melanoma patients manifest clinically evident metastases at initial diagnosis. Unfortunately, most experts agree that the die is cast at presentation with the poor prognosis patients having occult metastases. To date, systemic chemotherapy has been ineffective in treating metastatic disease.

Despite systemic chemotherapy's ineffectiveness, uveal melanoma management requires accurate prognostication. Firstly, published experience confirms that patients want to know their prognosis. As a result, ocular oncologists recommend prognostic testing to all of their uveal melanoma patients. Secondly, eligibility for emerging clinical trials requires prognostic testing.

Clinical and Morphologic Prognostic Variables

Until recently uveal melanoma prognostication relied on assessment of clinical and morphologic variables. Significant clinical variables most notably include patient age, tumor location, and tumor size/thickness. Specifically, increased patient age, increased tumor size, and ciliary body location confer increased risk for metastases. In contrast, iris lesions tend to follow a benign clinical course. In a large series of iris lesions, reported by Shields and colleagues, only 3 % of patients had metastases at 5 years and 10 % of patients had metastases at 20 years, in contrast to the approximately 50 % metastatic rate for uveal melanomas overall.

Iris lesions deserve additional comment because they differ from choroidal and ciliary body melanomas in multiple respects. Iris melanomas account for only 2–5 % of uveal melanomas. Understandably, due to their location iris melanomas present earlier in their natural history and, as a result, the lesions are smaller. Further, iris lesions are associated with more favorable morphologic features, most notably spindle cell type. Interestingly, Shields and colleagues reported that chromosome 3 abnormalities, including complete or partial loss of one copy, appear similar to those identified in small ciliary body or small choroidal melanomas.

Morphologic variables correlate with uveal melanoma prognosis. These include cell type, nucleolar size, necrosis, mitoses, tumor infiltrating lymphocytes and macrophages, vascular density, extravascular matrix patterns, extent of pigmentation, and scleral invasion. Notably, the epithelioid cell type, which tends to have more nuclear pleomorphism, coarse chromatin, and prominent nucleoli, associates with more aggressive behavior. In contrast, the pure spindle cell melanomas have a more favorable prognosis. High lymphocyte counts have been linked to decreased survival; however, only about 5–12 % of uveal melanomas contain lymphocytes. Other adverse morphologic prognostic variables include increased nucleolar size, increased mitotic counts, and complete vascular loops. Hamadeh and colleagues analyzed a series of aspirate samples and linked cellular features, including nuclear grade and cell type, to prognosis. Specifically, tumors with low grade nuclear atypia and a pure spindle cell pattern had a favorable prognosis while high grade nuclear atypia associated with an adverse outcome (Fig. 14.1).

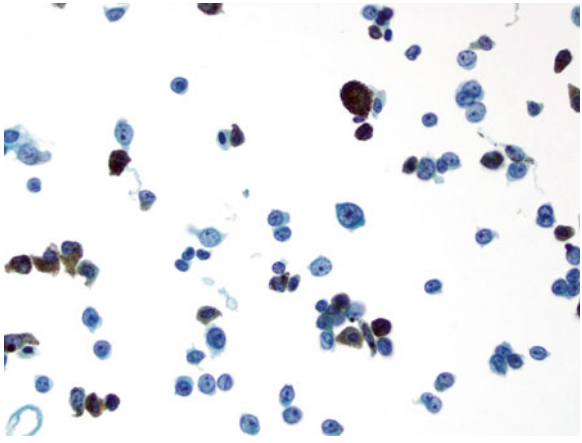

Fig. 14.1 This aspirate sample of a choroidal melanoma illustrates high-grade nuclear atypia characterized by nuclear pleomorphism, prominent nucleoli and some hyperchromasia and coarsening of the chromatin. These nuclear features associate with the epithelioid cell type, as illustrated in this case. This specimen was processed by the ThinPrep method, Hologic Corp, and stained with a Papanicolaou stain

Uveal Melanoma Cytogenetics

Clinical and morphologic prognostic variables are imperfect, especially in an individual patient. Currently, a variety of molecular analyses including cytogenetics, gene expression profiling, multiplex ligation-dependent probe amplification, and mutational analysis supersede in uveal melanoma prognostication.

In contrast to most solid tumors, and more in keeping with hematolymphoid tumors and some soft tissue tumors, uveal melanomas have relatively simple karyotypes. Consistent karyotype abnormalities in uveal melanoma involve gains and/or losses in chromosomes 1, 3, 6, and 8. Further, these abnormalities are not random but rather linked to uveal melanoma's pathogenesis and behavior. Monosomy 3 is the single best cytogenetic prognostic variable. Partial loss of chromosome 3 has also been linked to an adverse outcome (Fig. 14.2). The prognostic effect of monosomy 3 is greatly enhanced when paired with other independent

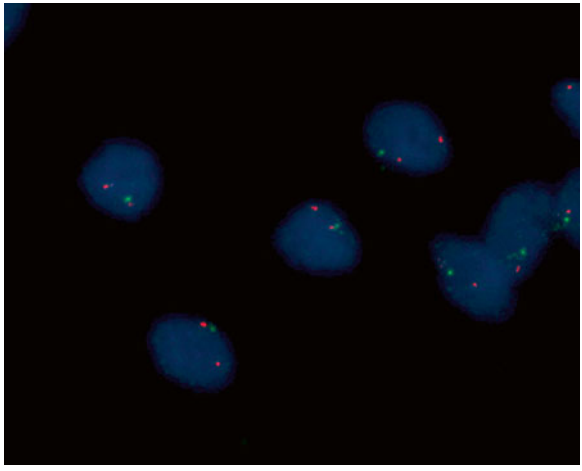

Fig. 14.2 Fluorescence in situ hybridization analysis demonstrates chromosome 3 eusomy (disomy) (2 *red signals*) and deletion of the 3p25 locus (1 *green signal*) in this uveal melanoma (reprinted with permission of S. Karger AG, Basel, from FNA Cytology of Ophthalmic Tumors. Biscotti and Singh eds. *Monographs in Clinical Cytology*, Volume 21

cytogenetic abnormalities. Monosomy 3 affects about 50 % of choroidal and ciliary body tumors combined. Monosomy 3 occurs more often with increasing tumor size, ciliary body location, and epithelioid appearance. Monosomy 3 frequently associates with chromosome 8q gain. In fact, 82 % of uveal melanomas with monosomy 3 also had 8q gain and 45 % of choroidal and ciliary body tumors have both monosomy 3 and 8q gain. Monosomy 3 appears to be an early event in uveal melanoma pathogenesis while 8q gain occurs later. Increasing dosage of 8q amplification adversely affects prognosis. Similarly Van de Bosch and colleagues found that an increasing percentage of melanoma cells with monosomy 3 had an adverse prognostic effect. Other independent cytogenetic abnormalities that greatly affect the adverse prognostic effect of monosomy 3 include 1p deletion, 6q deletion and 8p deletion. Ewens and colleagues reported that other than monosomy 3, 8p deletion was the only independent chromosomal prognostic variable. Some studies have linked amplification of chromosome 6p to the spindle cell type and a favorable prognosis.

Cytogenetic analysis using liquid based cytology methods has proven practical and effective in the prognostic analysis of uveal melanoma. Liquid based cytology obviates the need to account for the partial nuclei that affect tissue sections. Specimen adequacy is not an issue with enucleation specimens but adequacy issues affect aspirate samples obtained at the time of radiation plaque placement. Not surprisingly, aspirate sample cellularity varies directly with tumor thickness. Cohen and colleagues reported a 90 % adequacy rate for cellular diagnosis using fine needle aspiration of lesions between 2 and 4 mm in thickness and a 98 % adequacy rate for lesions greater than 4 mm in thickness but only 40 % adequacy for lesions less than 2 mm in thickness. Though reported adequacy rates for FISH analysis, of aspirate samples, have varied, overall adequacy rates up to 81 % have been reported with much better results for thicker lesions. For example McCannel and colleagues reported that 91 % of aspirates from melanomas more than 5 mm thick had sufficient cells for FISH analysis. In contrast, these investigators reported FISH adequacy rates, with in vivo aspirates, 53 % and 68 % for tumors less than 3 mm and 3–5 mm thick, respectively. Interestingly,

sampling does not appear to be an issue for FISH analysis of aspirate samples despite the often limited cellularity. Specifically, published data suggest that aspirate samples are representative of the entire tumor with regard to monosomy 3 status.

Methods for analyzing uveal melanoma aspirates by FISH vary. Specifically adequacy and diagnostic thresholds vary. Regarding monosomy 3, we use a threshold of 20 % monosomic tumor cells per 200 tumor cells counted. Others have reported a 15 % threshold for monosomic cells per 100 tumor cells counted. Importantly, when present, monosomy 3 usually affects most of the melanoma cells. For example, Van de Bosch and colleagues, using a 15 % threshold for monosomy 3, found 134 (61 %) of 220 tumors had monosomy 3. Of these, 109 (81 %) had monosomy 3 in at least two thirds of the tumor cells and only 9 (7 %) of tumors had monosomy 3 in only 15–33 % of tumor cells. Thus using our threshold of 20 % monosomy 3 per 200 tumor cells counted, a cell yield of as little as 60 melanoma cells would have detected approximately 80 % of the tumors with monosomy 3 in their study. Thus, relatively limited cell samples, which are often the case in these specimens, can provide prognostic information. A multiprobe cocktail prioritizing the detection of monosomy 3 (centromeric and/or locus specific probes) combined with probes to detect 8q amplification, 8p deletion, 1p deletion, and 6q deletion as dictated by specimen cellularity is recommended.

Gene Expression Profiling

Gene expression profiling using aspirate samples effectively stratifies uveal melanoma patients into risk groups. A proprietary technique stratifies uveal melanoma patients into class 1 (favorable prognosis) and class 2 (unfavorable prognosis) utilizing a PCR-based microfluidics platform that measures RNA expression of 12 discriminating genes and 3 control genes (Decision Dx-UM, Castle Biosciences). Class 1 tumors are further divided into 1A and 1B with 2 and 21 % 5-year metastatic rates, respectively. In contrast, Class 2 tumors have a reported 72 % 5-year metastatic rate. PCR creates a sensitive assay effective at low melanoma cell

levels. Onken and colleagues reported that only 1 of 51 cytologically insufficient samples had an inadequate result with this gene expression profiling method. The authors report a 5.2 % technical failure rate, almost always due to RNA quality issues. Importantly, Oken and colleagues conclude that intratumoral heterogeneity uncommonly affects gene expression profiling results, despite the small sample size.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

Similar to Gene expression Profiling, MLPA utilizes techniques including polymerase chain reaction (PCR) that permit successful analysis of limited samples thus addressing the specimen adequacy issue that limits cytogenetic analysis by FISH. MLPA utilizes probes to target DNA sequences and reference probes within the patient sample and reference samples comparing relative signal strengths to identify changes in copy number and even detect point mutations. Not surprisingly, increased sensitivity for monosomy 3 detection in uveal melanoma has been reported using the MLPA technique.

Mutational Analysis

Evolving methods for uveal melanoma prognostic testing include mutational analysis to find driver mutations that can identify molecular therapeutic targets. For example, G-alpha protein mutations in the genes GNAQ or GNA11 occur early in uveal melanoma pathogenesis. These mutations activate cellular proliferation pathways which can be therapeutic targets because these mutations are sensitive to MAPK kinase, PKC, and AKT inhibitors. In clinical trials, the MEK pathway inhibitor, selumetnib, has increased progression free survival and overall survival in uveal melanoma patients with metastatic disease. Mutations in BAP1, SF3B1, and EIF1AX are later events that are largely mutually exclusive. Mutations in BAP1 are strongly associated with metastasis, whereas those in SF3B1 and EIF1AX are associated with good prognosis.

Conclusions

Ocular oncologists are increasingly aspirating uveal melanomas for prognostication rather than diagnosis. Morphologic prognostic variables have merit but lack predictive value sufficient for optimal clinical application. Prognostication using cytogenetic analysis or other molecular testing supersedes. Gene expression profiling and multiplex ligation-dependent probe amplification are currently favored, in part, because these PCR-based methodologies minimize the adequacy issues that can affect cytogenetic analyses. Mutational analysis has the added potential benefit of identifying molecular therapeutic targets.

Suggested Reading

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15 Current Status of Microfluidics-Assisted Cytology: The Application in Molecular Cytology

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Introduction: What Is Microfluidics?

Born out of several fields, including the fields of molecular analysis and microelectronics, microfluidics emerged as a discipline in the early 1990s. Since that time developers have been designing and testing small scale devices to perform primarily sample analysis, using techniques that have their origin in the principles of microfabrication used in the semiconductor industry. Microfluidics is the science and technology of platforms containing elements constructed within the μm scale, that operate on and process fluid, where the

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fluid amount is often also within the μm scale. This essentially provides a miniaturized laboratory system and analysis approach. There are several advantages associated with using the miniaturized laboratory analysis approach that is offered with microfluidic techniques. A few rather obvious advantages are the potential for developing low cost tests that are portable and require little maintenance. As well, consider that in many clinical instances only a small volume of a patient's specimen is available for multiple needed analyses; here, techniques that can achieve the same level of accuracy using a fraction of the sample in question would be extremely valuable. Nanofluidics, an emerging field, focuses on platforms with features constructed on the nm scale! In the microfluidic field's infancy the focus had been on chemical analyte analysis. However, developers are quickly realizing the potential for platforms in this area to address additional needs within medical diagnostics and clinical pathology in particular. With regard to cellular diagnostics, microfluidics has much to offer. Given the similar length scales to human cells (tens of μm) it is not surprising that these systems have been widely used to manipulate, separate, and interface with cells.

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In this chapter we discuss the steps involving device formation, how microfluidics is poised to meet the needs of molecular cytopathology, example technologies that are near prime time for clinical use, and what future advances may be required in this field.

Device Formation

An attractive feature of the field of microfluidics is the ease with which devices may be formed using simple fabrication techniques that were adopted from the field of microfabrication and the semiconductor industry. Microfluidic devices are frequently manufactured using a combination of photolithography and soft lithography. Lithography, or, the formation of an object by etching shapes into a substrate, is the mainstay by which many microfluidic devices are created. Though much of the work of device creation is done bench top in an academic setting, commercial grade microfluidic devices have been developed and are positioned to make a remarkable impact in medical science. As an introduction to their use, it is instructive to consider the steps by which such devices are commonly manufactured.

1. *Process flow:* A common process flow for microfluidic device development includes these series of steps: (a) creation of a device mold using photolithography, (b) formation of an elastomer from the device mold using soft lithography, and (c) final device fabrication. Figure 15.1 illustrates this process conceptually. Note that the features displayed here are not drawn to scale and are exaggerated so as to demonstrate the techniques discussed. Typical device features are on the micrometer scale, whereas the silicon wafers used are usually 4″ in diameter.

   To start with, to make a device mold, a developer will first deposit a thin film of a chemical called photoresist (PR) onto a silicon wafer. Photoresist chemicals are usually polymer compounds that are photoactive; that is, the compound will undergo physical property changes once exposed to light. In this example, the photoresist depicted is a negative photoresist: one that will harden when exposed to light. After this thin film of photoresist

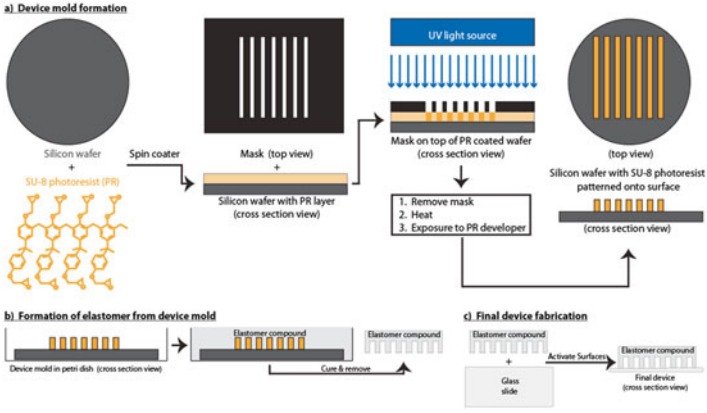

Fig. 15.1 Device creation process. (**a**) After spinning photoresist (PR) onto the surface of a silicon wafer, a mask is placed on top of the PR covered wafer. Then, the PR covered wafer is exposed to UV light, followed by PR developer. This results in a silicon wafer mold with PR patterned on the top layer. (**b**) The PR pattered silicon wafer is now used as a mold; it is covered with an elastomer compound, such as PDMS, which is then cured and removed. (**c**) The cured elastomer and a glass slide are now bonded together, creating a device prototype

is deposited, the developer will place a mask over the wafer, and then expose the mask covered wafer to UV light. Here, the area corresponding to the planned device features is translucent, so that light can shine through and cause the photoresist in the exposed area on the wafer to react. The rest of the mask is opaque, preventing the unexposed photoresist from reacting. After this, the mask is removed from the exposed wafer, heated to further harden the photoresist layer, and then placed in photoresist developer, which will dissolve unexposed photoresist (Fig. 15.1a).

After creation of a device mold, at a prototyping stage, we usually use an elastomer compound for device formation. Very commonly, the compound used is poly(dimethylsiloxane) or PDMS. PDMS allows for rapid prototyping, is optically translucent, which allows for easy microscopic evaluation of the device and cells within and, is biocompatible, and gas permeable (thus compatible with living cells) [1]. In this case, the developer pours PDMS (the example elastomer compound) over the device mold, to form one part of the final device.

Fig. 15.2 Clean room microengineer. *Left*: An example silicon wafer. *Right*: Substrates undergoing surface activation using clean room instrumentation

Finally, the elastomer compound mold is bonded to a glass slide to fashion the final device. Note that both surfaces are usually activated, so that they will bond to one another. The act of bonding the molded elastomer to a glass slide, in this case, allows for the formation of microchannels for sample fluid flow. Fluid can be introduced into the device via tubing connected through holes punched through the elastomer mold prior to bonding onto a glass slide. Mold creation using photolithography in general occurs in a clean room (Fig. 15.2); after which fabrication of device prototypes can be done on the bench top in a laboratory setting. The entire process can be performed over a course of 1–2 days.

2. *Device design:* Device designs are usually created using a software package used for drawing, such as AutoCAD. Once created, designs are then transferred onto a mask. The created mask is then used for the process described above.
3. *Device elements:* Typical device components include channels, valves, filters, mixers, pumps, and reservoirs for reagents [2]. These components are designed using a drawing software package as described above. The final device drawing is converted to a photomask, which is used for photolithography (Fig. 15.1), to make a device mold. The device mold is then used for soft lithography to create the final device, as described above.

Microfluidics and Molecular Cytopathology: Addressing the Need to Generate Pure Populations of Specific Cell Types for Molecular Analysis

As it relates to medical diagnostics, thus far much of the work in the microfluidics community has focused on miniaturization in the area of chemical analyte analysis, flow cytometry, and differential blood cell counts[3–6]. However, the community has now recognized that additional needs are present, and that these needs are ideally addressed with microfluidic tools. Particularly in the area of the analysis of cytology-based diagnostics, the focus within the field is shifting into one where developers are working to create microfluidic chips that can simplify and enhance the tools already used here. For example, in cytopathology, there is a pressing need for improved methods to enrich and purify cells from cytological samples, after which, these samples may be then reviewed by a pathologist for evidence of malignancy. Current sample preparation methods are most often completed on a laboratory bench top manually with macroscale instruments [7]. This practice likely reduces the consistency between operators and labs. One can imagine several clinical scenarios where a diagnosis may be potentially missed due to sampling error or inadequate sample preparation.

Many microfluidic approaches employed to address sample preparation have aimed to scale down macroscale techniques. For example, with one macroscale technique, cells can be isolated in a conical tube by incubating cells with specific immunomagnetic beads, followed by placing a strong magnet in proximity to the tube, and then performing multiple rinse steps to wash isolated cells. Microscale technologies followed suit, by integrating with on-chip and off-chip magnets for separating cancer cells, bacteria, and fungi from blood [8]. Other concepts were borrowed from analytical chemistry, like affinity and size-based chromatography, using instead "columns" sized for cells and affinity approaches specific to cell biomarkers like size or surface proteins. Approaches that make use of unique physics accessible in microfluidic systems are also poised to make an impact [9–12]. We refer the reader to several comprehensive reviews that discuss the physical operating mechanisms of various microfluidic cell separation and concentration approaches [13–15].

Pathologists continue to refine the art and science of accurately identifying abnormal morphological characteristics that denote malignancy. Microfluidics can be an important partner in this endeavor by engineering devices whereby quality samples are prepared in a high throughput, robust manner and delivered to the pathologist for review, and more importantly for molecular analysis. Retrieving target cell populations in solutions containing mixed cell populations and preparing them for analysis with minimal perturbation is a complex sample preparation task. In this regard, there are some challenges facing the microfluidics community; we will review these challenges here.

1. *Concentrating rare cells from patient specimens with large volumes:* Such patient specimens where a large volume could be submitted for analysis include but are not limited to blood, urine, pleural, peritoneal, and bronchial-alveolar lavage fluid samples. For example, 50 mL of pleural fluid is adequate to gather enough material for a cell smear in malignant pleural fluid analysis. In another case, the presence of >5 cancer cells within 7.5 mL of blood was shown to be an independent predictor of overall patient survival in metastatic breast cancer [16]. A sample with a low cellularity combined with inadequate sampling and the presence of rare cells can lead to unsatisfactory results, prompting further diagnostic testing. Concentrating in the presence of a large number of background cells is also challenging and requires combined separation and concentration approaches.

   In order to achieve large volume processing, the goal is to process samples at flow rates in the mL•min^{-1} scale instead of the μl•min^{-1} scale to satisfy the workload requirements of a clinic with a single machine or achieve rapid turnaround to aid in quicker clinical decisions. Scaling up from μl•min^{-1} to mL•min^{-1} represent a 10^3 order of magnitude increase. An increase such as this raises concerns that cells in a device using these higher flow rates can be exposed to high shear stress in the microchannels, leading to cell damage. To mitigate this concern, developers can scale up flow rates by creating massively parallel arrays of devices, or by increasing the channel dimensions of the device, enabling more volume throughput while maintaining acceptable forces on cells.

To that end, it is important to design systems that can be expanded in a parallel fashion and still retain the fundamental fluid flow mechanics [11, 17, 18]. These mechanics typically take advantage of particle, or, in this case, cell, size, shape, deformability, and density using hydrodynamic forces induced by microchannel features. For example, Deterministic Lateral Displacement (DLD) and inertial microfluidics uses the physical phenomena unique to these systems in various channel shapes and orientations to guide particles above certain size cutoffs into specified outlets for collection. Since these channels can easily be arrayed, there is no limit to the amount of parallelization that can take place, offering macroscale regime flow rates in a compact microfluidic device. In another technique, acoustic waves maneuver cells to defined pressure nodes [19–21]. While able to process at high flow rates, the technology requires an external piezo or patterned interdigitated transducer (IDT) to initiate fluid flow, which may present a challenge for parallelization. Still, forces from these systems are large compared to other types of forces, such as dielectric and magnetic forces. Therefore device systems that incorporate these physical phenomena can operate with appreciable flow rates (0.05–0.5 mL•min^{-1}) with only a single channel. The ideal technology would be one where microchannels can be stacked indefinitely to obtain rapid processing times.

2. *Efficiently preparing small volume samples for multiple assays:* For the majority of clinical circumstances where the sample of volume available for testing is limited, efficient sample preparation is paramount. Factors that may reduce the sample available for further analysis include dead volume in a chip or external tubing, parallel assays, and loss of cells due to an inefficient on-chip sample preparation process. Innovative approaches to load and pump fluids without significant dead volume will lead to a reduction in the effective volume of sample needed for analysis, while sequential assays can make the most of a sample volume. Traditionally, the microfluidic field has touted the small volume processing capabilities as an inherent advantage of the small scales of operation. For the purpose of small volume processing, various methods have been developed that take advantage of microchannel dimensions as well as

the materials used to create the microchannels. Example methods include capillary-driven and vacuum-driven fluid flow.

In capillary-driven flow, fluid flow is initiated by surface tension, in which the flow can be driven by differences in surface energy upon the wetting of a channel, network of channels, or porous structure such as paper [22–24]. Contrast this with a technique using vacuum-driven flow, where fluid flow is initiated by using the porous structure of normal polydimethylsiloxane (PDMS) as a negative pressure source after the device is taken out of a vacuum chamber [25, 26] While both techniques offer a standalone system for working with small liquid volumes and concentrating cells with no moving parts and external components, these approaches potentially have long processing times, which may be prohibitive for certain point-of-care diagnostic applications.

Another technique that requires additional external components makes use of centrifugation of a small volume of biofluid on a microfluidic chip to achieve pumping. As with traditional centrifugation—but with much smaller volumes—this approach allows for separation of plasma from whole blood and the subsequent mixing of the plasma with lyophilized reagents for detection [27]. A recent innovation that works most effectively with microscale volumes separates cells specifically bound to immunogenic magnetic micro beads by, instead of removing background fluid, pulling the bead-bound cells themselves through an immiscible fluid phase into a second wash volume [28].

The above described techniques in general apply for continuous-flow microfluidic systems, where fluid flow occurs in one continuous fluid stream. However, there is significant interest in developing platforms on which fluid samples are analyzed after conversion into a series of droplets. This is the main focus of the area in microfluidics called digital microfluidics. There are some advantages gained by using the approach of converting fluid samples into droplets for further downstream testing, such as reduced risk for contamination and improved mixing efficiency. In this example, investigators combine two methodologies, electrowetting-on-dielectric (EWOD) and optoelectronic tweezers (OET), into a single microfluidic device for the purpose of converting a suspension of HeLa cells into HeLa cell

containing droplets that can then be manipulated using electrical signals [29]. The EWOD technique is attractive in that samples are converted to droplets with electrical energy, and not active mechanical chip components, such as pumps and valves [30]. It will be important to see how this technology can be further adapted for use in cytological analysis.

3. *Preparing samples with high cellularity:* Patient specimens that are highly cellular, such as blood or bone marrow aspirates will contain diverse and large populations of cells. These high cellularity samples can be challenging for the cytopathologist and for microfluidic processing for many reasons, given a large background of healthy cells. To start with, cells within these samples can interact within fluid flow to prevent accurate separation. Also, cells have a higher likelihood of aggregating, and clusters of cells can clog flow within microfluidic channels. Of course, there is the need to achieve a high purity of a specific population of cells as they are separated from a diverse background population of cells. Finally, it can be difficult to retrieve specific cells from within a highly cellular solution, particularly if these specific cells are present in rare numbers. Despite these challenges, there has been much progress in processing samples that have high cellularity, especially for isolating specific cell populations from complex fluids like blood. There are several mechanisms that can be used for the purposes of cell separation and many approaches that can be used to address these challenges, some of which we will now discuss.

   For example a simple solution to address aggregation would be to include a dilution step, which would decrease the potential for cell aggregation and microchannel clogging. However, adding a dilution step increases sample volume and will then increase the time required for sample processing. Another approach to reduce cell aggregation involves designing assays where cell separation occurs at the microchannel surface, rather than within the crowded fluid phase. An example that demonstrates this technique would be an affinity capture-based approach [31, 32]. Here, the microchannels are coated with antibodies that are specific to receptors of target cell populations so that cells are captured on-chip. These affinity based-platforms have been developed for isolating circulating tumor cells from blood [31–34].

Alternatively the crowded fluid phase may be used to aid in separation through the margination of particular cell populations to preferred locations within a fluid flow stream [35, 36]. This can be done using a mechanism that exploits cell size to assist in separating cells within a sample. Consider DLD, where the physical phenomenon at work directs particles to move at an angle with respect to flow through an array of microposts. Larger cells undergo a bumping mode whereby cells are deflected from the normal trajectory, while smaller particles follow streamlines in a zigzag mode. This approach operates effectively even with samples that have high cellularity. Tuning the shape of microstructures in the flow or creating mixing flows to allow surface contact even in highly cellular solutions is a challenge that is being addressed [31, 33, 37].

Downstream sample analysis may also be facilitated by segmenting cells within a highly cellular solution into more easily analyzed single cells. This would be beneficial in many clinical scenarios. One such scenario involves developing diagnostics for mutation-targeted drug therapies. Here, it would be important to isolate and analyze single cells with particular genetic mutations so that these specific mutations can be accurately identified. A possible solution is to segment highly cellular solutions into droplets containing single cells using droplet generators [38, 39]. One main advantage is that single cells can be detected using fluorescent imaging and separated into different zones for further analysis.

While these systems offer rapid throughput and high efficiency of cell capture, a successful technology should be able to balance these aforementioned criteria, in addition to achieving high purity and having the ability to retrieve cells in solution.

4. *Automating multistep sample preparation:* To standardize the sample preparation process the steps involved in sample preparation should be automated. These steps include centrifugation, pipetting, and cell staining, all operations usually manually performed in clinical labs. For example, when working with a large volume patient specimen (e.g., peritoneal fluid obtained after paracentesis), technicians must aliquot, centrifuge and manually pipette the mixed sample to make cell smears. However, mishandling and user error in processing, such as neglecting a step to

homogenize the sample before performing a decanting step, may lead to misleading results. A standardized fluid plumbing system in which an entire volume is mixed and processed through a device would address this issue by removing user sampling bias; it can also allow for processing large liquid volumes. Automation of these steps to minimize sample handling will preserve sample integrity and lead to less error overall, ultimately leading to cost reduction and increase in the diagnostic accuracy.

Some microfluidic technologies already offer multiple steps that are integrated onto a single platform. Centrifugal microfluidics is a technology that can prepare blood samples through a series of pumping, valving, volume metering and mixing [40]. Abaxis currently sells this sample-to-answer technology in the Piccolo clinical blood analyzer system for both medical and veterinary diagnostics. Other technologies like the Vortex (or Centrifuge) Chip, a method that recreates the functions of a benchtop centrifuge in a microfluidic format, combines cell concentration, separation, and staining. This technology is particularly useful for samples with low cellularity [18]. An ideal technology would automate cell staining techniques in a microfluidic platform to enable efficient uniform labeling using traditional and immunocytochemical stains as well as to enhance the performance of cytogenetic analysis, e.g., FISH.

5. *Obtaining high purity for molecular assays* (Table 15.1, Fig. 15.3): Achieving high purity of specific cell populations from heterogeneous solutions presents a critical challenge in clinical sample preparation. Purity is important in preparing cellular samples for nucleic acid analysis, for cell counting of specific selected subpopulations, and for reducing the presence of background cells and other noncellular particulates that can mask the evaluation of cells of interest. For example, bloody specimens containing leukocytes can contaminate molecular analysis results when attempting to detect gene mutations or perform nucleic acid sequencing in a target cell population [41]. Strategies directed toward the removal of contaminating cells can also aid in the development of platforms for cell counting and sorting of specific subpopulations. The removal of blood cellular components and the concentration of target cells into a small field of view may expedite and increase the accuracy of cytology examinations [13, 18, 42].

Table 15.1 The grand challenge in biofluid sample preparation

Challenges	Requirements	Methods	Throughput	Max volume	Operational cellularity	Integration ability	Average purity	References
Concentrating rare cells from large volumes	1. High volumetric flow rate (mL/min) 2. Ability to parallelize channels 3. Continuous flow	DLD	0.35 mL/h, ~10^7 cells/min	None	Diluted blood (50 %)	Detection	99 %	[17]
		Inertial	~10^6 cells/min	None	Diluted blood (~2 %)	Detection	80–99.71 %	[9, 11, 18, 51]
		Acoustics	80 μL/min, ~10^8 cells/min	None	Whole blood with sheath	Detection	100 %	[19–21]
Preparing small volume samples for multiple assays	1. Reduced dead volume 2. Sequential assays	Capillary	0.8–20 nL/s	300 nL	NA	Assays, detection	NA	[22]
		Vacuum	0.5–2 nL/s	5 μL	Whole blood	Assays, detection	99 %	[25, 26]
		Centrifugal	5 nL/s–0.1 mL/s	2 mL	Whole blood	Assays, detection	>99 %	[27]
		Mechanical filters	0.75 mL/min, ~10^9 cells/min	None	Whole blood	Assays, detection	90 %	[52]
		Immiscible Phase	1 cm/s	20 μL	While blood	Assays, detection	>80 %	[28]
Preparing samples with high cellularity	1. Cell separation at channel surface 2. Segment highly cellular populations into single cells	Affinity-based	1 mL/h	None	Whole blood	Assays, detection	9.2–68 %	[31–34]
		Cell margination	NA	15–70 μL	Whole blood	Assays	NA	[35, 36]
		Droplet	8 μL/h	NA	Diluted blood (10 %)	Assays, detection	99 %	[38, 39]
		DLD	0.35 mL/h, ~10^7 cells/min	None	Diluted blood (50 %)	Detection	99 %	[17]
Automating multistep sample preparation	1. Standardize fluid plumbing system 2. Minimize sample handling 3. Ability to stain, concentration, and lyse cells	Inertial solution Exchange	NA	None	Diluted blood (10 %)	Assays	NA	[53]
		Centrifugal	5 nL/s–0.1 mL/s	2 mL	Whole blood	Assays, detection	>99 %	[27]
		Droplet	8 μL/h	NA	Diluted blood (10 %)	Assays, detection	99 %	[38, 39]
		Digital	NA	>1 mL	NA	Assays, detection	NA	[54]
Achieving high purity cell populations	1. Reduced background of cells 2. Concentrate cells into small field of view 3. Make cells readily available in solution	Biopolymer System	2 μL/min	NA	Whole blood	Assays, detection	NA	[44]
		Centrifuge Chip	0.1 mL/h	None	Diluted blood (5 %)	Assays, detection	~40 %	[18]

A few microfluidic approaches address these issues and are capable of extracting cells at high purity [33, 34, 43]. However, it is worth noting that some methods lack the ability to make cells readily available in solution after sample preparation, limiting the ability to integrate these techniques with downstream cytological or flow cytometry. Some technologies have been successful in recovering cells after on-chip processing [18, 44]. In the "Centrifuge-on-a-Chip," target cancer cells are selected by size from a bloody sample, collected in a concentrated solution, and imaged in a small field of view [18]. Specifically, this technology has been shown to improve the detection of KRAS gene mutations in lung cancer cells processed on this chip, as compared with lung cancer cells processed with standard bench top centrifugation [45]. This device has been further optimized for high-purity extraction of circulating tumor cells (CTCs), which can be performed on high liquid volumes with a rapidity that highlights the capability of Vortex technology use in clinical sample preparation [46]. In another technique, viable cells are recovered from an affinity-based cell isolation chip using a hydrogel coating layer which can be subsequently degraded to release cells for downstream molecular assays [44]. The microfluidic methods discussed here are critically addressing this challenge of obtaining high purity cell separation. Methods such as these are poised to open up new opportunities for the cytopathologist to analyze blood and other body fluids for rare cells of diagnostic importance that were previously not accessible given the background of healthy cells.

Case Studies of Microfluidic-Assisted Cytopathology

The introduction of new technology and tools into a discipline is sometimes met with inherent skepticism from those within the discipline regarding whether these tools are as helpful as they claim to be. In some instances, by proposing to altogether eliminate current methodologies upon which standards of practice have been

built, the introduction of new tools can be met with frank antagonism. However, the very nature of work within the field of microfluidics should hopefully lessen the likelihood of this negative response. Successful work within the field requires an interdisciplinary approach. This means that all stake holders need to be involved, including microengineers, industry, laboratory technicians, research assistants, and physicians, even at the early inception stage. Within this framework innovators can more likely develop effective tools that will meet the needs of pathologists who practice medicine in the twenty first century. Thus far we have endeavored to give context regarding how microfluidics and pathology can be partners in addressing unmet needs in medical diagnostics. In this section, we highlight specific examples where microfluidic technology has been shown to assist in making important pathologic diagnoses, and where this multidisciplinary framework has proven to be successful.

1. *Purification of Body Fluids with Vortex Technology:* Previously, in the "Centrifuge-on-a-Chip" system, designers fashioned a microfluidic chip that performs the functions of a bench top centrifuge, such as concentration of rare cells and solution exchange, with high throughput volume processing [18]. This device concentrates and removes rare cells of interest (such as circulating tumor cells) from a larger background of cells in diluted blood by using physical phenomena unique to fluid flowing through microchannels and additional microstructures. This technology has been shown to rapidly enrich cells of interest based on their size from body fluids like pleural effusions and blood. Using this technology, malignant and mesothelial cells of interest to a cytopathologist were purified and concentrated from background erythrocytes and leukocytes from clinical samples. Besides potentially improving the accuracy of cytomorphological reads, such an approach was shown to improve molecular detection accuracy of clinically relevant gene mutations (see Fig. 15.3) [45]. The Vortex Technology has also been shown to have the capability of performing high purity separation of circulating tumor cells from whole blood with a short amount of processing time (20 min for 7.5 mL of blood) [46]. This high purity separation of cells from within a

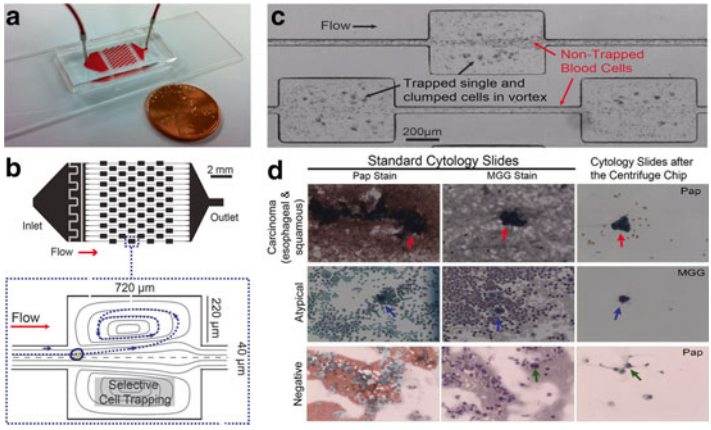

Fig. 15.3 The centrifuge chip. (**a**) Actual device, (**b**) Device schematic, (**c**) Microscopic image of enriched cells as they are captured by the device. (**d**) Centrifuge chip processed sample cytology slides have reduced cellular background. Patient samples were prepared for cytology review with traditional cytological methods (e.g., centrifugation) and the Centrifuge Chip. Note that slides prepared with the Centrifuge Chip have a reduction in background material, as compared with cytology slides prepared with standard methods. This results in a clearer view of diagnostically important cells. *Pap* = Papanicolaou. *MGG* = May-Grunwald-Giemsa

small volume enables cytopathologists to have access to rare cells from highly cellular samples like blood and conduct any desired downstream staining or molecular testing. In this way, it also facilitates molecular analysis by cytogenetics, given that there are less non-target cells to screen. In addition, cells purified in this manner are now available for gene mutation analysis for personalized medicine.

2. *Automated and Quantitative Analysis of Pleural Effusions:* In this example, the authors developed and used a microfluidic based technology to aid cancer diagnosis by using the platform in a prescreening role to identify malignant cells in pleural fluids. The technique, called deformability cytometry (DC), allows one to robustly perform rapid and label-free measurements of the mechanical properties of cells, properties which appear to have origins in traditionally analyzed cytomorphological features, such as nuclear to cytoplasmic ratio, chromatin structure,

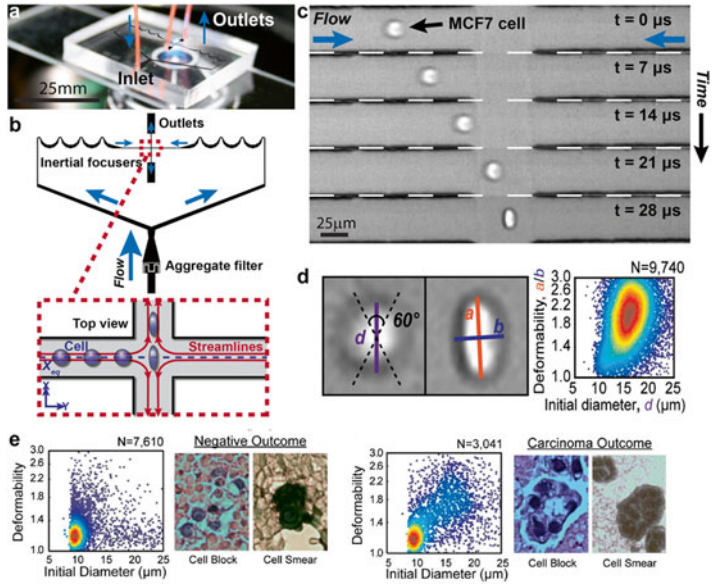

Fig. 15.4 The deformability cytometry (DC) Chip. (**a**) Actual device. (**b**) Device schematic with an enlarged view of a cell undergoing deformation as it flows through the device microchannel. (**c**) High speed microscopy images of a cell undergoing deformation as it flows through the device microchannel. (**d**) Definition of the cell shape parameters used to obtain the deformability measurement. (**e**) DC measurements of patient pleural samples displayed with cell block and cell smear preparation of the same samples. DC measurement scatter plots show a different pattern of deformation for each clinical outcome: no malignancy as compared with carcinoma

and cytoskeletal arrangement [47]. This automated technology is capable of evaluating the mechanical properties of cells with significantly higher throughput than other platforms, approximately 2000 cells per second in a flow through format (see Fig. 15.4). Using this platform, investigators analyzed pleural effusions from 119 patients [48] to identify mechanical markers associated malignancy and inflammation. An advantage of the approach is that only red blood cell lysis is required, followed by a 5 min quick processing, and automated analysis to yield a result. In the analysis, the authors used an algorithmic diagnostic scoring system that incorporates information on cell size,

deformability, and the distribution of these characteristics in the cell population. With this technique, 63 % of the samples could be correctly classified as malignant or benign, with 100 % positive predictive value and 100 % negative predictive value. In this way, the platform could potentially be used to prescreen and classify a large number of samples that are clearly negative or positive for malignancy. This prescreening step would save time and effort for cytopathologists, who could then focus on the diagnosis of more challenging samples. In addition, in the same study, using this technology improved the diagnosis of challenging samples (approximately half of the samples identified as containing atypical cells were definitively diagnosed using the DC approach). Deformability cytometry can then provide assistance with the analysis of atypical cells, and could potentially be combined with other technologies to analyze cells and perform downstream purification. Future work using such technologies are exploring the ability to detect specific sites of origin for cells disseminated into pleural or other body fluids using deformability and other label-free physical markers of cells.

The Future

Looking ahead, there are many opportunities for collaboration between microfluidic developers and molecular cytopathologists. This chapter has largely focused on microfluidic platforms designed to efficiently prepare body fluid samples for cytological tests and molecular analysis. Note, however, that there has also been progress made in developing microfluidic systems to allow for tissue staining and preparation to be done on chip [49]. One such system evaluates the presence of four important breast cancer cell biomarkers: estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and Ki67 on human breast needle biopsy samples [50]. We have a grand challenge before us; to create devices that allow high purity isolation of clinically relevant cells that can also be done in a high throughput manner (Fig. 15.5), and provide these cells obtained for not only morphological but more importantly molecular-based analysis. We are very nearly realizing this, as evident by devices

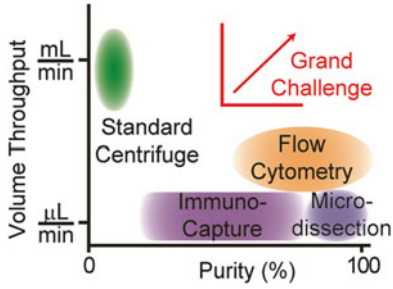

Fig. 15.5 The grand challenge in biofluid sample preparation. The ideal microfluidic device in cytological sample preparation should be able to process large fluid volumes quickly while also achieving high purity separation for the cells of interest

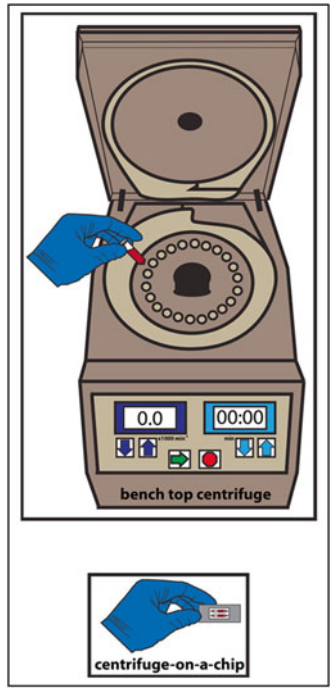

Fig. 15.6 Next generation pathology. As depicted here, next generation pathology techniques will include the use of microfluidic devices so that macroscale laboratory methods can be performed on chip

currently nearing the final stages of development (Fig. 15.6). To further address this challenge, we need intense collaboration between developers and end users, so that we correctly address the problems at hand. This area allows for interdisciplinary work, and a team approach, with all partners contributing their expertise for the common goal of improving our methodology for not only diagnosing illness but also defining the correct targeted therapy.

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