

Chapter 2

Ancillary Studies on Neoplastic Cytologic Specimens

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2.1 Introduction

Fine-needle aspiration (FNA) is a safe, simple, rapid, and cost-effective procedure that is often used as an initial diagnostic modality to work up primary and metastatic tumors at almost any body site. FNA allows acquisition of samples not only from superficial and large lesions, but also from small- or deep-seated lesions, or from multiple lesions during the same biopsy procedure. It provides valuable information that enables oncologists to make optimal therapeutic decisions, including planning preoperative management for patients with operable tumors and choosing adequate medical therapy for patients with hematopoietic malignancies or nonresectable tumors. An informative cytologic diagnosis should address whether the lesion is neoplastic or nonneoplastic, benign or malignant, as well as the histogenesis of the tumor (e.g., epithelial, mesenchymal, melanocytic, or lymphoid), tumor type (e.g., adenocarcinoma, squamous carcinoma, or others), tumor origin (i.e., primary vs. metastatic and if possible the potential primary origin of a metastatic tumor) and information on prognostic and therapeutic markers. Frequently, this information needs to be obtained with the help of ancillary tests. Body cavity fluids may also be utilized for ancillary studies, when indicated.

This chapter outlines the utility of immunocytochemistry (ICC), flow cytometric (FCM), cytogenetic and molecular studies in the cytologic diagnosis of neoplastic lesions including the indications, sample requirements, reliability, and limitations.

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2.2 Immunocytochemistry

ICC combines cytologic, immunological and biochemical techniques for the identification of specific tissue/cell components by means of a specific antigen/antibody reaction tagged with a visible label. Depending on characteristics of the cells, the ICC reaction can be seen in cytoplasm, nucleus or cell membrane, thus the ICC technique allows us to visualize the distribution and localization of specific biomarkers within a cell or tissue. The applications of ICC have increased in parallel with the discovery of novel markers in surgical pathology. It is important to emphasize that ICC should be considered only in the context of a proper differential diagnosis, and appropriate immunomarkers should be selected on the basis of the patient's medical history, current clinical and radiologic presentation, and cytomorphologic features of the lesion. In general, if a lesion exhibits malignant features with poor differentiation and the patient has no known history of malignancy, the initial step in the workup is to identify the histogenesis of the tumor: epithelial (carcinoma), melanocytic (melanoma), hematopoietic (lymphoma), or mesenchymal (sarcoma). Accordingly, the basic markers should include at least keratin, melanoma markers, and leukocyte common antigen (CD45). If a carcinoma of unknown primary origin is encountered, the ICC panel usually starts with cytokeratin 7 (CK7) and CK20 to identify a likely primary site (Table 2.1) [1–3]. If a history of malignancy is known, corresponding markers relatively specific to an organ site (Table 2.2) and other markers that are relevant to cell differentiation may be added to the panel. Notably, the staining patterns and site-specific markers in Tables 2.1 and 2.2 are not absolute and may vary depending on variations in antibody sensitivity and specificity, staining conditions, and tumor differentiation. For example, TTF-1 is a tissue-specific transcription factor expressed in the thyroid and lung, and is one of the most frequently used markers to distinguish between pulmonary and extra pulmonary origin of an adenocarcinoma [4]. It can also be expressed in small-cell carcinomas (pulmonary and extra pulmonary) and in a small subset of squamous cell carcinomas, and carcinoid tumors of lung origin.

FNA specimens are usually prepared as direct smear(s) and cell block. Some laboratories may use liquid-based preparations (ThinPrep, SurePath) in parallel with or to replace direct smears. Air-dried smears are stained with Diff-Quik, and alcohol-fixed smears with Papanicolaou; the air-dried preparations are usually used for immediate on-site assessment for adequacy assessment and triage for ancillary studies. Cells obtained from the needle rinses or tissue fragments are collected in cell preservative medium to make a cell block or for cytospin preparation depending on the size of the pellet after centrifugation. For a cell block, the pellet is fixed in formalin, embedded in paraffin, then sectioned and stained with hematoxylin and eosin, a process virtually identical to that used for surgical tissue specimens. The cell block may partially retain the histologic architecture of a lesion. A cell block, or core biopsy if feasible, is the preferred sample type for ICC. Therefore, when an ICC workup is expected on the basis of immediate assessment,

Table 2.1 Common CK7 and CK20 staining patterns in tumors of various primary sites

CK7+/CK20+	Pancreas: pancreatic adenocarcinoma ^a Biliary duct: adenocarcinoma, including cholangiocarcinoma ^a Genitourinary tract: urothelial carcinoma ^a Stomach: gastric adenocarcinoma ^a Ovary: mucinous adenocarcinoma
CK7+/CK20-	Lung: non-small cell carcinoma, adenocarcinoma Breast: ductal carcinoma, lobular carcinoma Endometrium and uterine cervix: adenocarcinoma Ovary: adenocarcinoma other than mucinous type Thyroid: papillary carcinoma, follicular carcinoma, medullary carcinoma Salivary gland tumor Mesothelium: malignant mesothelioma
CK7-/CK20+	Colon and rectum: colorectal adenocarcinoma Skin: merkel cell carcinoma
CK7-/CK20-	Liver: hepatocellular carcinoma Kidney: renal cell carcinoma Adrenal gland: adrenal cortical tumor Prostate: prostatic adenocarcinoma Testis: germ cell tumor

^a variable expression pattern**Table 2.2** Immunomarkers that are relatively site specific

TTF-1	Lung, thyroid
CDX2	Gastrointestinal organs, pancreas
Thyroglobulin	Thyroid
Calcitonin	Thyroid (medullary carcinoma)
Surfactant, napsin-A	Lung
HepPar-1	Liver (hepatocyte)
RCC	Kidney (renal cell)
PAX-8	Kidney (renal cell), gynecologic organs, thyroid
PSA, PAP	Prostate
PTH	Parathyroid
ER, PR	Breast, gynecologic organs
GCDFP-15, mammaglobin	Breast
Calretinin, mesothelin	Mesothelium
WT-1	Ovary (serous carcinoma), mesothelium
Mart-1, S-100, HMB-45, SOX-10, MITF	Skin (melanoma)
PLAP, AFP	Testis (germ cell tumor)

the on-site cytopathologist usually tries to obtain material for a cell block or core biopsy. Additional needle pass(es) might be obtained if deemed feasible by the aspirator. If a cell block is not available or contains insufficient cells, Papanicolaou-stained direct smear(s), cytopsin preparation, and liquid-based preparation are also feasible for ICC as long as they have reasonable cellularity [5–7]. If the cells of interest are present on only a single or few smears without available cell block

material when a panel of ICC is needed, a cell-transfer technique, in which the original smear material is divided into several pieces and then transferred onto multiple slides, may facilitate multiple ICC studies from the limited material [8–11]. This technique can avoid a repeat biopsy solely for immunophenotyping of tumors.

There are several disadvantages of ICC on the sample types other than cell block. (1) such sample types lack validated control tissue, which should be fixed in the same way as the test specimen at each run of ICC staining. (2) Certain stains such as S-100 cannot be reliably assessed on these preparations. (3) High background staining, often associated with crowding of cells in thick smear and poor cytoplasmic preservation, may be encountered occasionally, and prove problematic for interpretation. (4) A false-positive ICC interpretation may result from the lack of a histologic pattern in the aspirated material and mistaking entrapped benign cells at the biopsy site for tumor cells. For example, an aspirate of a carcinoma metastatic to the lung may contain entrapped benign and reactive bronchial cells that express TTF-1, which may lead to an erroneous conclusion that the tumor is focally TTF-1 positive and thus of lung origin. Last, a limitation that is intrinsic to all types of cytologic samples is scarcity of cells available for ICC due to sampling error, tumor necrosis, or tumor fibrosis. It is conceivable that ICC on a small sample may lead to a false-negative finding in tumors that express some markers only focally and heterogeneously. Therefore, caution should be exercised in the interpretation of ICC results.

The predictive factors commonly tested on cytologic specimens are estrogen receptor (ER), progesterone receptor (PR), HER2 status for breast cancer or gastric cancer, and epidermal growth factor receptor (EGFR), K-RAS and ALK mutations in lung cancer, K-RAS and thymidine synthetase in colorectal cancer. These tests are conducted to assess a patient's eligibility to receive anti-ER, anti-HER2, and anti-EGFR targeted therapies, or responsiveness to 5-FU-based regimens, respectively. Although testing for these biomarkers is usually performed on surgically resected or biopsy specimens of newly diagnosed primary carcinoma and requires standardized fixation conditions (i.e., fix in 10 % neutral buffered formalin for 6–48 h for ER, PR, and HER2), [12, 13] they are frequently determined on cytologic specimens, including aspirated metastatic carcinoma samples when the receptor status needs to be repeated in the relapsed disease, or on primary carcinoma when the cytologic samples are the only material available. ICC of ER and PR can be performed on cell blocks, direct smears, and liquid-based preparations; however, HER2 staining should be performed only on cell block section since ICC of HER2 on direct smears is not standardized and is associated with high variability in sample preparation, fixation, staining, and interpretation [14].

Sometimes, testing of ER, PR, and HER2 is requested by an oncologist after a cytologic diagnosis has been completed and extra slides for these markers are not available. In such case, the Papanicolaou-stained smears that have been used for routine morphologic diagnosis may be used for ER and PR staining [15]. Direct smears can be used for testing HER2 gene copy number via fluorescence in situ hybridization (FISH). The advantage of using archived slides is to enable the

cytopathologist to visualize cytologic features and the amount of tumor cells on the smear prior to the tests, thereby allowing selection of the “most representative” slides for the tests. It is important to note that these markers should be assessed on the invasive component of the breast carcinoma. Since cytologic specimens cannot reliably discriminate invasive from in situ components, interpretation of ER, PR, and HER2 status in a primary setting should be done cautiously. Also, the reliability of ER, PR, and HER2 status determined on cytologic preparations needs to be validated according to the current guidelines [12]. In our experience, ER status in breast carcinoma is generally stable during progression to metastasis with a concordance rate between primary and paired metastatic breast carcinoma to be 92 % [16]. Detection of EGFR and K-RAS mutations is covered in the section on cytogenetic and molecular tests.

2.3 Flow Cytometry

FCM is a powerful tool for determining the phenotype and characteristics of cells. It uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells.

In the past decades, the applications of this technology have evolved substantially. With the wide use of FNA, FCM has become a main ancillary test to evaluate adenopathy and other lymphoid lesions, although the primary diagnosis of lymphoma is usually based on surgical biopsy specimens. However, an accurate cytologic diagnosis relies on correlation of cytologic features with immunophenotypic, genotypic, clinical, and radiologic information in view of the considerable overlap of cytologic features among some types of lymphomas and between low grade lymphomas and reactive lymphoid hyperplasia. FCM is the most commonly used ancillary test, especially in the setting of persistent or relapsed lymphoma. The role of FNA in rendering a primary diagnosis of lymphoma remains controversial.

2.3.1 *Handling and Triaging of the FNA Specimen*

At immediate on-site assessment, a cytopathologist evaluates the cell composition, features, and quantity of the aspirated material and ensures properly triaging of the specimen for ancillary studies. For example, if a lesion shows granulomas or abundant acute inflammatory cells, aspirates should be collected for microbiology culture(s) and special stains; if a lesion shows features of a nonhematopoietic tumor, an effort should be made to obtain a cell block for possible ICC workup; if cells show features suggestive of hematopoietic malignancy, collecting enough cells for FCM is the priority. Specifically, if a lesion is from a patient who has a

history of non-Hodgkin lymphoma or has clinical and/or radiologic findings suggestive of non-Hodgkin lymphoma, even if it exhibits “reactive-like” cytologic appearance, a lymphoma workup by FCM should be performed. When a diagnosis of non-Hodgkin lymphoma is highly suspected in a patient without a history of lymphoma during immediate assessment, a concurrent core needle biopsy should be obtained for further histologic classification and grading. However, if a FNA sample exhibits cytologic features suggesting Hodgkin lymphoma, and/or the patient has a history of Hodgkin lymphoma, the sample should not be sent for FCM since FCM is not helpful in diagnosing Hodgkin lymphoma [17]. The same is true for anaplastic large-cell lymphoma. In such cases, a tissue biopsy is typically required for histologic confirmation and classification since the sensitivity of cytologic diagnosis for these types of lymphoma is relatively low.

At The University of Texas MD Anderson Cancer Center, we usually smear a portion of the aspirates from the first needle pass onto glass slides. If smears show a lymphoid population with features suspicious for non-Hodgkin lymphoma, cells from subsequent aspiration(s) should be collected directly into cell preservative medium (RPMI) and the cells quantified by using an automated counter (Coulter Electronics, Hialeah, FL) to determine if there is a sufficient number of cells for further workup. A minimum of 10 million cells from three or four needle passes is usually required for a standard lymphoma workup. We triage samples for lymphoma workup in the following steps:

- A. Cytomorphologic evaluation on direct smears
- B. Immunophenotyping by FCM (approximately 5 million cells)
- C. Proliferation index (Ki67) assessment by immunostaining on a cytospin preparation; the result correlates strongly with the grade and outcome of lymphoma. To prepare the cytospin, cells in RPMI medium are processed over a Ficoll-Hypaque gradient to enrich mononucleated cells, which are then centrifuged onto glass slides (approximately 1×10^5 cells per slide) [18, 19].
- D. When a specimen is hypercellular, an additional four to six cytospin slides will be kept in a tumor bank (-80°C) for possible ICC or FISH. A panel of ICC including kappa, lambda, CD3, and Ki67 might be informative when FCM fails to demonstrate light chain restriction in a lesion that is clinically suspected for lymphoid neoplasm. FISH analysis may be needed to confirm characteristic chromosomal abnormalities in selected cases.
- E. Aggregates of tissue in the RPMI medium will be salvaged to make a cell block, which can be used for ICC and FISH or in situ hybridization for Epstein-Barr virus infection.

2.3.2 Interpretation of Flow Cytometry

FCM is a highly sensitive technique that can quantify the expression of four or more markers on a single cell and therefore enables identification of aberrant cells within a complex cellular background. It is the preferred method of lineage

determination of hematopoietic neoplasms and subclassification of B-cell lymphomas. The panels of antibodies should be selected on the basis of the cytologic features and clinical information; at first-time diagnosis, FCM should evaluate both B- and T-cell abnormalities.

Mature B-cell neoplasms account for more than 85 % of non-Hodgkin lymphomas, and are the most commonly encountered hematopoietic tumors in cytology practice. Therefore, evaluation of clonality by assessing immunoglobulin light chains (i.e., kappa and lambda light chains) together with CD19 and CD20 expression is important to establish a B-cell neoplastic nature. In non-neoplastic conditions, the specimen comprises a mixture of T- and B- cells. In addition to CD19, CD20, and CD22, mature non-neoplastic B cells typically express polyclonal surface immunoglobulin (i.e., the kappa to lambda light chain ratio is around 1.5:1). B-cell lymphomas, however, express a single clonal light chain (also called light chain restriction), so this ratio is either increased (greater than 3:1 in our experience) or decreased due to a significant lambda excess. B-cell lymphomas are often associated with aberrant expression of other antigens, some of which are of prognostic value. For example, CD38 expression in B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) is often associated with a more aggressive clinical course [20, 21]. Table 2.3 lists key immunophenotypic and molecular features of mature B-cell neoplasms commonly encountered in cytology practice.

Although the interpretation of FCM data in most cases is quite straightforward, potential pitfalls may be encountered and may lead to misinterpretation. A negative FCM result does not necessarily indicate a benign process [22]. Identical immunoprofiles may be seen in different types of lymphoma. It would be a mistake to attempt to interpret a case solely on the basis of FCM results. The common pitfalls include the following:

1. Immunoglobulin light chain may not be detectable due to too few neoplastic cells that may be masked by abundant benign lymphocytes in the background, a situation often seen in T-cell-rich B-cell lymphoma or a partially involved node. Gating on the large-cell population may separate neoplastic large cells from background reactive lymphocytes. In a case of partial involvement by follicular lymphoma, typically CD10-positive B cells demonstrate light chain restriction, whereas CD10-negative B cells express polyclonal light chain consistent with non-neoplastic nature.
2. Since FCM generally evaluates intact viable cells, poor cellular integrity, extensive fibrosis, and necrosis, which occur frequently in large-cell lymphoma, may lead to a false-negative FCM interpretation.
3. In some B-cell lymphomas, FCM shows negative light chain expression caused by aberrant loss of surface immunoglobulin expression. Using different antibodies for each light chain is important to verify true light chain loss or, in some cases, overcome false-negativity resulting from antibody issue. This is because a single pair of light chain antibodies may not successfully identify monotypic light chain expression in each lymphoma cases.

Table 2.3 Immunophenotypic and molecular features of mature B-cell neoplasms commonly encountered in cytology practice

	Immunophenotype	Molecular features
Follicular lymphoma	sIg, CD19+, CD20+, CD10+, BCL6+, BCL2+ CD5-, CD23±	Most common t(14;18)(q32;q21), rarely t(2;8)(p11;q21) and t(18;22)(q21;q11)
Marginal zone lymphoma	sIg, CD19+, CD20+	t(11;18)(q21;q21), t(1;14)(p22;q32), t(14;18)(q32;q21), Trisomy 3, 7, 12, and 18
Mantle cell lymphoma	CD10-, CD5-, CD23-, BCL6- sIg, CD19+, CD20+, CD5+, FMC7+, CD79a+, cyclin D1+ CD10-, CD23-	t(11;14)(q13;q32)
Small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL)	sIg (dim), CD19±, CD20+(dim), CD22+(dim), CD5+, CD23+, CD38+(worse prognosis)	Trisomy 12, 13q14 deletion, 17p and 11q deletions
Lymphoplasmacytic lymphoma	CD10-, FMC7- sIg or cIg, CD19+, CD20+, CD38+, CD43± CD10-, CD5-, CD23-	
Burkitt/atypical Burkitt lymphoma	sIg, CD19+, CD20+, CD10+, BCL6+, Ki67 labeling index ≥ 99 % CD5-, CD23-	most common: t(8;14)(q24;q32), less common t(2;8)(p12;q24) or t(8;22)(q24;q11)
Large B-cell lymphoma	sIg, cIg or Ig undetectable, CD19+, CD20+, high Ki67 labeling index CD10±, CD5±, BCL6± (positive CD10 or BCL6 indicates follicular center cell origin)	t(14;18) (q32;q21)
Plasma cell neoplasm	cIg, CD45±, CD56+, CD38+, CD138+	

Ig immunoglobulin light chain, *sIg* surface Ig restriction, *cIg* cytoplasmic Ig restriction

4. Plasma cell neoplasms and some B-cell lymphomas are negative for surface light chain staining. In these cases, examination of cytoplasmic immunoglobulin light chains is the key to demonstrating clonality.
5. Marginal zone lymphoma, CD10-negative follicular lymphoma and lymphoplasmacytic lymphoma share similar immunophenotypes (i.e., CD5-negative, CD10-negative monoclonal B cells).
6. CD10-positive mature B-cell lymphomas include follicular lymphoma, Burkitt lymphoma, and some large B-cell lymphoma. Based on FCM alone, the distinction among the three may be difficult.
7. Some B-cell SLL/CLL may show an atypical phenotype with a brighter expression of pan-B cell markers and diminished CD23 expression, mimicking the phenotype of mantle cell lymphoma. Similarly, large B-cell lymphomas may show CD5 expression resembling the phenotype of mantle cell lymphoma. Because the prognosis and treatment of these lymphomas are significantly different from one another, it is critical to distinguish clearly among these lymphomas.
8. In some reactive conditions such as Hashimoto thyroiditis, a small population of monotypic B cells may be detected, caution is needed not to over interpret these as lymphoma [23].

Compared with B-cell lymphomas, T-cell lymphomas in general have less predictable patterns of immunophenotypic aberrancy. T-cell lymphomas often show deletion or loss of one or more pan-T-cell markers (i.e., CD2, CD3, CD5, CD7), which can be detected by FCM [24]. In addition, T-cell lymphomas often demonstrate aberrant CD4 and CD8 patterns. T-cell clonality may be detected by FCM using anti-T-cell receptor (TCR) V-beta antibodies, [25, 26] although this technique has not been used for FNA specimens. In difficult cases, polymerase chain reaction (PCR) can aid in the diagnosis by demonstrating *TCR* gene rearrangements that are present in most T-cell lymphomas [27, 28].

While FCM is a valuable adjunct for the diagnosis of lymphoma on cytologic specimens, it is important to view immunophenotyping results in conjunction with cytologic features, and to be alert to potential inconsistencies. When clinical features are not explained by FCM, repeating FNA or obtaining tissue biopsy, with addition of cytogenetic and molecular studies if possible is recommended.

2.4 Cytogenetic and Molecular Tests

Following the rapid advance in molecular and cytogenetic research, it has become clear that genomic alterations are involved in cancer initiation and progression. Two main genetic events are considered to trigger cancer initiation: activation of oncogenes as a consequence of point mutation, amplification, or chromosomal translocation; and/or inactivation of tumor suppressor genes due to chromosomal deletion, mutation, or epigenetic mechanisms.

Identification of specific cytogenetic and molecular abnormalities has several clinical implications.

1. They can help in more accurate diagnosis. As noted earlier, although cytologic features in conjunction with ICC or FCM are usually sufficient to make cytologic diagnosis, there are instances where identification of characteristic abnormalities at the chromosomal and/or molecular levels is necessary to arrive at a definitive cytologic diagnosis. For example, cytologic features of small-to-intermediate cell lymphomas (such as follicular lymphoma, marginal zone lymphoma or mantle cell lymphoma) can considerably overlap with one another as well as with those of reactive lymphoid hyperplasia. Some of these lymphomas, such as CD10-negative follicular lymphoma and marginal zone lymphoma, may share similar immunophenotypic features. A similar situation may be encountered in the cytologic diagnosis of small round blue cell tumors, a group of histogenetically different tumors that share some morphologic and immunophenotypic features. Since lymphomas and these solid tumors frequently carry nonrandom chromosomal aberrations (usually reciprocal chromosomal translocation) (Tables 2.3 and 2.4), a cytogenetic and/or molecular study would be a powerful adjunct for the diagnosis.
2. Demonstration of characteristic chromosomal abnormalities may help in prognostic assessment. For example, the presence of the t(2;5) translocation is associated with relatively good prognosis of anaplastic large-cell lymphoma. In SLL/CLL, trisomy 12, and 11q and 17p deletions are associated with poor prognosis, whereas 13q14 deletion is a marker of good prognosis.
3. Chromosomal abnormalities can be used to predict therapeutic response of some tumors. For example, marginal zone lymphoma of the MALT type with t(11;18) or t(1;14) is unlikely to respond to antibiotic therapy.

Table 2.4 Characteristic chromosomal translocations in some sarcomas

Tumor type	Translocation
Ewing sarcoma/peripheral primitive neuroectodermal tumor	t(11;22)(q24;q12)
	t(21;22)(q22;q12)
	t(7;22)(p22;q12)
	t(17;22)(q21;q12)
	t(2;22)(q33;q12)
Desmoplastic small round cell tumor	t(11;22)(p13;q12)
Synovial sarcoma	t(X;18)(p11;q11)
Myxoid liposarcoma	t(12;16)(q13;p11)
	t(12;22)(q13;q12)
Myxoid chondrosarcoma	t(9;22)(q22-31;q11-12)
	t(9;15)(q22;q21)
	t(9;17)(q22;q11)
Clear cell sarcoma	t(12;22)(q13;q12)
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)
	t(1;13)(p36;q14)
Alveolar soft part sarcoma	t(X;17)(p11;q25)

4. Identification of genetic abnormalities may also be used for identifying minimal residual disease in hematopoietic malignancies.

Cytogenetics is a branch of molecular genetics and is concerned with the study of the number and structure of the chromosomes such as translocations, amplification, deletions, inversions, duplications, or isochromosomes. It includes conventional cytogenetic analysis such as metaphase karyotyping using chromosome-banding techniques, and molecular cytogenetics such as FISH and comparative genomic hybridization. Conventional cytogenetic studies allow complete karyotype analysis and detect most chromosome anomalies; however it is a cumbersome and time-consuming procedure requiring adequate fresh tissue and special cell culture techniques in order to obtain adequate number of proliferating cells. In our experience, approximately 8 million tumor cells in sterile condition are needed for this procedure.

FISH is used widely as an easy and reliable technical substitute to search for well-documented specific chromosomal abnormalities. The method can be used on metaphase or on nondividing interphase cells, and allows the localization of specific genes and DNA segments on specific chromosomes, determines the position and orientation of adjacent genes along a specific chromosome, and can detect the presence of microdeletions or duplications that are not apparent by conventional cytogenetic studies. Furthermore, FISH test has a shorter turnaround time than conventional cytogenetic studies. The interphase FISH (I-FISH) method is particularly advantageous for FNA specimens because it requires only a few cells, and can facilitate a definitive diagnosis on some cases that otherwise fall into indeterminate (i.e., atypical or suspicious) diagnostic category due to scant cellularity [18]. The results of I-FISH are important integral part of cytologic diagnosis, especially for lymphoma and sarcoma. The most common abnormality of these tumors is reciprocal chromosomal translocation (Tables 2.3 and 2.4) [29]. However, in contrast to conventional cytogenetic studies, I-FISH is not informative to identify “unexpected” chromosome abnormalities that are not designed to detect. Cell block, direct smear (air-dried, alcohol-fixed or archived), cytospin preparation, or cellular touch imprint are all suitable for I-FISH. However, direct smear and cytospin preparation appear to be superior to cell block sections in that gene copy number can be enumerated on monolayered and entire tumor nuclei without tissue section-associated truncating artifacts, thereby yielding a more accurate score [30–33]. It is important to ensure that the scoring should be performed on cells of interest such as malignant cells. I-FISH on previously stained archival smears has the advantage of evaluating cytomorphologic features of cells on the same slides before the procedure.

HER2 status is a frequently tested on cytology specimens, using either ICC (on cell block) or FISH (on smear or cell block). Positive HER2 status (i.e., over expression of the HER2 protein with immunostaining or amplification of the HER2 gene with FISH) is associated with a poor clinical outcome, and more importantly, is a prerequisite for anti-HER2 (such as Trastuzumab/Herceptin) treatment. Dual-probe FISH is the preferred method over immunostaining [34].

This was based on the fact that FISH is less affected by preanalytic, analytic, and postanalytic variables and thus more stable and reproducible than immunostaining, as well as the fact that HER2 status determined with FISH is more strongly correlated with responsiveness to anti-HER2 therapy. HER2 status is highly concordant between primary carcinomas and paired metastatic tumors and is quite stable after chemotherapy, [35, 36] or trastuzumab treatment [37].

Bright-field chromogenic in situ hybridization (CISH) has shown potential to replace the I-FISH technique in detection of *HER2* gene amplification in tissue sections since it detects gene copy number using a conventional peroxidase reaction, and allows enumeration of the signals with simultaneous histologic examination by ordinary microscopy [38–41]. This technique is more straightforward than FISH for scoring. The utility of CISH in cytologic specimens is still investigational [42–44].

Molecular tests have now become an important integral part of cytologic diagnosis. PCR is a technique to amplify a specific DNA sequence in an exponential manner. PCR and FISH are complementary for detecting predictable chromosomal abnormalities; however, the overall sensitivity of PCR is lower than that of I-FISH [18, 45–49]. For example, the detection rate of the t(14;18) translocation in follicular lymphoma is less than 70 % with PCR, while it is around 90 % with FISH. This is mainly due to the presence of *BCL2* gene breakpoints outside the known major breakpoint region (*mbr*) and minor cluster region (*mcr*), that are unable to span with primers, whereas FISH circumvents this limitation by using a probe covering the entire *BCL2* region.

In contrast to detection of immunoglobulin heavy-chain (*IgH*) and *TCR* gene rearrangement that are encountered occasionally in cytology practice, detection of *EGFR* and *K-RAS* mutation in non-small cell lung cancer on cytologic specimens has been increasingly requested by oncologists to determine a patient's eligibility for anti-EGFR therapy (such as Gefitinib/Iressa or Erlotinib/Tarceva). Two classes of EGFR mutations, short deletions in exon 19 and the L858R point mutation in exon 21, are the most frequent mutations and account for approximately 90 % of EGFR mutations [50]. The presence of these mutations is associated with response to tyrosine kinase inhibitors. Mutations in *EGFR* and *K-RAS* appear to be mutually exclusive; tumors with a *K-RAS* mutation would not likely be affected by anti-EGFR therapy. Cell block material or cells scraped from direct smears are suitable for the mutation tests [51]. However, the efficacy of the tests is affected to a great extent by the amount of tumor cells available for analysis.

Another common application of molecular tests in cytology practice includes detection of human papillomavirus (HPV) DNA in liquid-based cervical specimens using Hybrid Capture 2 or third Wave (Cervista) technology [52, 53], and diagnosis or surveillance of urothelial neoplasia using multi-probe FISH (UroVysion) [54]. These tests will be discussed in subsequent chapters.

Numerous other molecular tests have been investigated and some have shown substantial promise to aid cytologic diagnosis and optimize personalized management. Gene expression profiling microarray has been used to identify sophisticated multigene prognostic and predictive factors of various tumors. Both core

needle biopsy and FNA are feasible for procurement of fresh samples in the neoadjuvant setting [55–58]. Compared to core needle biopsy sample, FNA samples contain a higher proportion of neoplastic cells and fewer stromal components [59]. Gong et al. have assessed the reliability of testing ER and HER2 status via gene expression profiling and observed close correlation between mRNA levels of ER and HER2 and the routinely determined status via immunostaining and/or FISH, with overall accuracies ranging from 88 to 96 % [60]. In that study, mRNA cutoff values of ER and HER2 were defined using breast carcinomas sampled with FNA and the performance of each cutoff was validated in independent datasets of FNA specimens as well as surgical specimens obtained from seven institutions across 5 countries. These findings indicate that gene expression microarray not only generates large and comprehensive gene expression data from human cancers, this technique also reliably measure ER and HER2. Integration of the individual gene expression with multigene signatures generated from the same microarray data might improve predictive power of tumor response to targeted therapies and therefore optimize clinical decision-making and tailoring the therapeutic regimens on an individual basis.

Management of thyroid nodules largely depends on the FNA diagnosis. Approximately 15–25 % of thyroid FNA diagnoses are indeterminate for malignancy. *RET/PTC* gene rearrangements and mutational analysis of *BRAF* have shown promising potential to refine indeterminate cytologic diagnosis of thyroid lesions [61, 62].

Aberrant CpG methylation at the promoter region of tumor suppressor genes, a main epigenetic mechanism, is associated with transcriptional silencing and has a central role in tumorigenesis of different tissues. The application of promoter hypermethylation has been investigated in archival liquid-based cytologic specimens and showed potential to improve diagnostic certainty [63–65]. However, larger studies are required to validate these findings before clinical application.

2.5 Conclusion

With the rapid advent of sophisticated diagnostic technology and increased understanding of the molecular mechanisms of various tumors, the need to obtain diagnostic, prognostic, and predictive information from cytologic material continues to grow. In addition to adoption and incorporation of the new techniques into routine cytology practice, a major challenge is to standardize specimen processing and validation of testing procedures to ensure optimum results, especially for markers that predict response to targeted therapies. It is critical that ancillary test results be evaluated in concert with cytologic features and clinical and radiologic findings to avoid erroneous interpretation.

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