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Introduction

Appropriate diagnosis of pediatric tumors requires an integrative approach utilizing several clinical and diagnostic resources, including a comprehensive clinical exam, diagnostic imaging studies, and a variety of laboratory techniques. The role of the latter cannot be emphasized enough. Routine laboratory techniques include microscopic evaluation, immunohistochemistry, flow cytometry, conventional cytogenetics, and molecular diagnostic studies.

Fine-Needle Aspiration and Core Biopsy

In clinical practice, the initial approach to the diagnosis of a newly discovered tumor often involves fine-needle aspiration (FNA) and a concurrent core needle biopsy. While the use of FNA in the pediatric population is less widespread than core needle biopsy sampling, cytologic evaluation may be helpful in particular situations as long as diagnostic pitfalls that are specific to the pediatric population are recognized [1]. Usually, these samples are obtained under imaging guidance, including ultrasound for more superficial and accessible lesions and computed tomography (CT) and magnetic resonance imaging (MRI) for lesions involving visceral organs or those that are deeply situated and less accessible percutaneously. The advantage of FNA and core needle biopsy samples is that they are of limited invasiveness and offer a

balance between adequate sampling and potential morbidities associated with surgical sampling [2]. It should be noted that minimally invasive sampling approaches of pediatric tumors may occasionally present specific issues that should be kept in mind when sampling options are considered. For example, percutaneous sampling approaches for bone tumors should avoid contamination of fascial compartments through tumor seeding. Additionally, sampling of localized renal tumors in young children who are ultimately diagnosed when Wilms tumor might result in the patient being upstaged due to iatrogenic breach of the tumor capsule.

Intraoperative Evaluation

The main indications for frozen section evaluation in pediatric tumors include assessment for malignancy, evaluation of tissue adequacy, margin assessment, and allocation of tissue to appropriate ancillary studies on the basis of the preliminary working diagnosis [3]. Intraoperative consultations also offer an opportunity to perform touch imprints and scrape preparations, both of which help in assessing sampling adequacy and offer superior cytologic details compared to frozen section tissue samples while largely preserving the specimen for permanent histologic processing. In addition both methods are rapid and simple, and can be performed on site. In addition air-dried touch preparations without subsequent processing can be archived at 4 °C for days or weeks, or they can be frozen at -70 °C and utilized much later for additional ancillary studies such as fluorescence in situ hybridization (FISH) and molecular diagnostics.

From the standpoint of triaging freshly acquired tissue samples, it is important to note that the only techniques with an absolute requirement for viable tissue include flow cytometry and conventional cytogenetics. Most other techniques, including FISH and molecular diagnostics, can currently be performed reliably on formalin-fixed paraffin-embedded (FFPE) material. Accordingly, fresh tissue should be procured in cases where such techniques are needed. In cases

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where tissue is limited, prioritization should be based on differential diagnostic considerations and should be communicated between the pathologist and surgeon or interventional radiologist. For example, flow cytometric evaluation is of less significance in a patient with suspected sarcoma or Hodgkin lymphoma.

Immunohistochemistry

Immunohistochemistry (IHC) is an integral part of diagnostic pathology. Immunohistochemistry combines histological, immunological, and biochemical techniques for the identification of specific antigens by means of antigen-antibody complex formation tagged with a chromogen (Fig. 1.1). Among the many advantages of IHC is its ability to permit visualization of antigen distribution within tissues. In addition to providing a qualitative assessment of tissue composition, IHC is amenable for semiquantitative and fully quantitative approaches for cell enumeration.

Techniques to produce quality antibodies for clinical immunohistochemistry have improved dramatically over the past few decades. Antibodies against a specific antigen can be monoclonal or polyclonal, and they may be produced in a variety of hosts (commonly mouse or rabbit) against a wide array of epitopes. In comparison with the nascent years of IHC technology a few decades ago when frozen tissue was required and manual staining methods were predominant, immunostaining techniques are currently much more robust, automated, and amenable to being performed on a variety of tissue fixatives and tissue processing techniques. Nonetheless, IHC quality remains a function of a broad range of factors that include antibody specificity, antibody dilution and incubation conditions, antigen retrieval, tissue fixation, decalcification methods, and histologic processing [4]. For example, the length of tissue fixation and type of fixative might significantly alter a target epitope and thus impact IHC quality [5, 6]. Tissue processing techniques may similarly impact IHC particularly when novel techniques such as microwave are introduced.

More recently, colorimetric in situ hybridization (ISH) stains have become widely available. These stains are typically performed on the same automated platforms on which IHC is done. Instead of an antigen-antibody design, ISH entails the use of chromogen-tagged nucleic acids complementary to target DNA or RNA sequences [7]. Like IHC, ISH permits the identification of target sequences in a tissue-specific context. Commonly used ISH stains include those for the detection of human papillomavirus DNA, Epstein-Barr virus RNA, and immunoglobulin light-chain mRNA transcripts.

Interpretation of IHC requires a thorough knowledge of histology, antigen distribution in tissue, and antigen distribution in cells (membranous, cytoplasmic, and/or nuclear), and

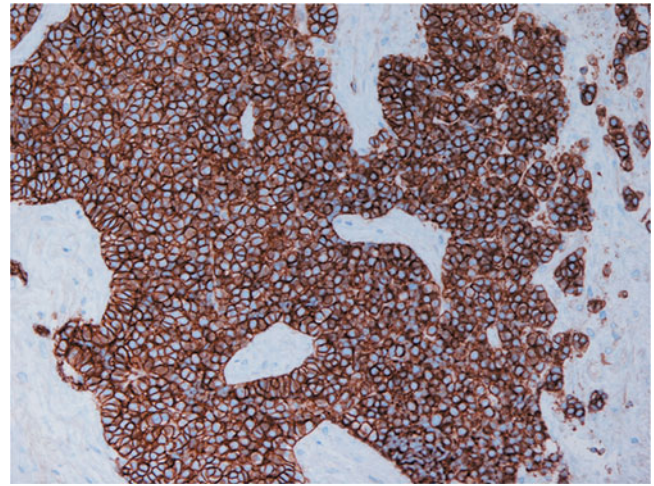


Fig. 1.1 CD99 immunohistochemistry in a case of Ewing sarcoma family tumor demonstrates diffuse strong expression of the CD99 antigen by tumor cells

knowledge of potential artifacts that may impact staining quality. Accordingly, it is necessary to distinguish true staining from nonspecific cross-reactivity or background “noise.” Required elements to ensure adequate IHC quality include the use of positive and negative controls as well as systematic validation processes to ensure that critical components of IHC staining (e.g., buffers, color development kits) are performing optimally.

Flow Cytometry Analysis

Multicolor flow cytometry analysis (FCA) is an invaluable laboratory tool for the characterization of hematolymphoid malignancies. It permits multiparametric measurement of cellular properties that include size, cytoplasmic complexity, and antigen expression. A typical flow cytometer is composed of a laminar flow cell transport system, one to several laser lights, photodetectors, and a computer-based data management system. The intricate design of flow cytometers ensures that cells flowing in a fluid sheath are hydrodynamically focused to intercept laser light at a specific frequency. The interaction of the laser light with the cell results in light scatter and, in the presence of bound fluorochrome-tagged antibodies, excitation and resultant emission of light at a different wavelength. These events are captured by sensitive photodetectors and converted to measurable parameters. Scattered light captured by a detector positioned at a right angle (90°) from the laser source measures cytoplasmic complexity whereas scattered light captured by detectors along the original trajectory of the laser beam (180°) measures cell size.

Flow cytometry analysis is a robust tool to simultaneously assess coexpression of multiple antigens expressed by cells

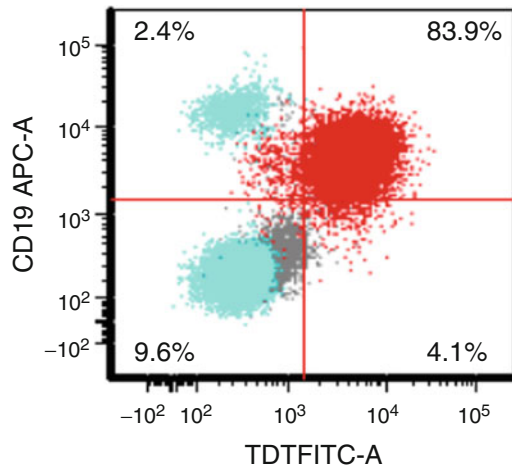


Fig. 1.2 Flow cytometry analysis of a case of B lymphoblastic leukemia/lymphoma demonstrating TdT expression by CD19-positive blasts (*red*). In this plot, the lymphocyte gate is highlighted in *turquoise*. Note the presence of a small population of normal CD19-positive B cells (*upper left-hand quadrant*) as well as a population of normal T cells (CD19 negative) (*lower left-hand quadrant*)

(Fig. 1.2). This is useful to many clinical assays including cell lineage determination, biomarker detection, minimal residual disease assessment, enumeration of cell subsets (e.g., stem cells, T-cell subsets), and measurement of proliferation and apoptosis.

For such applications, antibodies with covalently linked fluorescent molecules (fluorochromes) are used to identify target antigens and provide a means for qualitative and quantitative assessment of antigen expression. This ability to perform multiparametric analysis on an individual cell offers FCA a distinct advantage over immunohistochemistry particularly in hematolymphoid disorders [8]. On the other hand, the use of FCA to evaluate solid tumors remains technically limited.

Cytogenetics

Conventional cytogenetic analysis (cytogenetics) is a laboratory discipline that involves the study of chromosomes, also known as karyotyping. Chromosomal alterations are common in cancer and are broadly categorized into recurrent and nonrecurrent abnormalities. Tumors arising in the pediatric age group are more likely than those arising in adults to harbor recurrent cytogenetic abnormalities. Frequently, such recurrent cytogenetic abnormalities are integral elements of pediatric cancer pathogenesis and their detection has emerged as an important adjunct for diagnostic evaluation.

For conventional cytogenetic analysis of tissue samples viable fresh cells are required for analysis. The average viable human cell divides once every 24 h and certain cell types,

such as lymphocytes, do not divide at all, which mandates special culture techniques and growth stimulation of the cell of interest to increase the yield of analyzable material. Bone marrow is typically cultured for 24–48 h whereas lymphocytes from tissue may require 3–4 days in culture medium containing proliferation inducers for maximum yield. Cultured cells are then subjected to metaphase arrest before being processed to prepare chromosome spreads. Chromosomes are then stained, most commonly with Giemsa or Wright stains, for visualization of the characteristic banding patterns. Positively charged dyes in stains bind to the negatively charged DNA in chromosomes.

Conventional cytogenetic analysis begins with the identification of chromosomes typically by analyzing 20 metaphases. Chromosomes are aligned in pairs sequentially from chromosome 1 to 22 followed by the pair of sex chromosomes. Chromosomal abnormalities are broadly divided into numerical and structural. Numerical abnormalities (aneuploidy) result in deviation from the usual diploid complement of 46 chromosomes and result either in hyperploidy or hypoploidy. The spectrum of structural chromosomal abnormalities is broad. Most common alterations in pediatric cancers are balanced translocations resulting in pathologic juxtaposition of genes that normally belong on different chromosomes. The first step is to assess the number of chromosomes, a total of 46 in a normal diploid human cell.

While providing important information in the laboratory work-up of pediatric tumors, conventional cytogenetics in tumors has some disadvantages. Among the salient disadvantages is the absolute requirement for viable tumor tissue and the intensive time and labor requirements that are inherent in cytogenetic techniques. Furthermore, subtle cytogenetic alterations such as cryptic translocations or inversions are often impossible to recognize due to the typically low resolution of routine cancer cytogenetics methods. These limitations have led laboratories to rely on FISH, which generally bypasses most of the limitations of conventional cytogenetics. Other cytogenetic techniques such as array comparative genomic hybridization (CGH) have also made their way into diagnostic laboratories, but their clinical use remains limited particularly for pediatric tumors.

Fluorescence In Situ Hybridization

The use of FISH has grown exponentially over the past decade and plays a critical role in the laboratory work-up of many pediatric cancers [9]. In FISH, fluorochrome-tagged DNA probes designed to be complementary to a specific area of a chromosome (locus) are used to make qualitative and quantitative assessments regarding the targeted locus. Staining can be performed on a broad range of sample types, including touch preparations, smears, and FFPE tissue

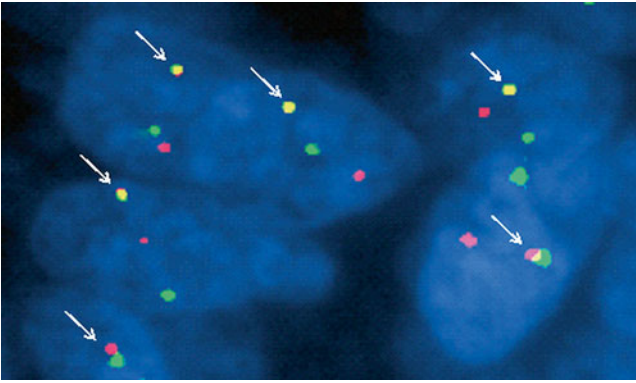


Fig. 1.3 Fluorescence in situ hybridization signals may be juxtaposed signals (*arrows*) or separate. Depending on the design of the assay probes, these patterns might represent fusion or rearrangement at a particular locus. In this example of an *EWSR1* breakapart probe demonstrating gene rearrangement (separate signals) in a case of Ewing sarcoma family tumor. Fused signals (*arrows*) represent the intact *EWSR1* allele

sections. Probes are incubated and allowed to hybridize to target DNA and then, after applying a background nuclear stain, signals are visualized on a fluorescent microscope. The availability of fluorochromes with different light emission characteristics allows simultaneous application of probes of different colors and thereby permits a wider range of data to be obtained.

Probes used in FISH provide important cytogenetic data and this depends to a large extent on the design of the probe and, as applicable, the composition of probes that occasionally comprise a FISH assay. Information about a specific locus obtained from FISH may be quantitative or qualitative. For instance, while a probe might indicate rearrangement involving a specific locus (e.g., *MYC* gene), it could also demonstrate copy number changes (gains/losses) at that particular locus. Probes can be designed to provide information if detected signals are juxtaposed (fusion probes) or located farther apart than they should (breakapart probes) (Fig. 1.3). In addition, by combining FISH probes designed to be complementary to a particular gene with other probes targeting the centromeric portion of a chromosome, a FISH assay can distinguish between copy number alterations resulting from focal chromosomal deletions and those that are secondary to the loss of an entire chromosome.

The many advantages of FISH have positioned it as an indispensable laboratory technique particularly in cancer. Advancements in FISH techniques now allow testing to be reliably performed on FFPE and have largely mitigated many of the limitations of conventional cytogenetics. In addition, FISH assays can be performed rapidly and their interpretation is less intricate than that involved in conventional cytogenetic analysis.

Molecular Diagnostics

Mutations are an integral component of cancer at the molecular level. Common molecular aberrations include point mutations, insertions/deletions (indels), amplifications, translocations, and DNA methylation variations. Characterization of these aberrancies is a critical component of the pathologic evaluation of tumors at diagnosis and during follow-up particularly for pediatric tumors since many harbor characteristic nonrandom molecular alterations [10–12]. Some of the more commonly used assays in the practice of diagnostic molecular pathology include polymerase chain reaction (PCR), DNA sequencing methods, array CGH, gene expression profiling, and microRNA profiling. PCR and DNA sequencing are the most widely used methods in routine laboratory practice.

All molecular techniques start with DNA or RNA extraction from a sample that could be fresh and unfixed or from FFPE material. In PCR-based methods, a limited segment of DNA or RNA (cDNA) is amplified and usually subsequently sequenced to identify a mutation or detect a pathogenic fusion resulting from a chromosomal translocation/inversion. Such methods are generally sensitive and specific, especially when amplification products are subsequently sequenced or otherwise confirmed, with a reasonably quick turnaround time frame. Automation of a sizeable component of the technical aspect of molecular testing has become widely adopted particularly in laboratories with high volumes. Interpretation of results is generally straightforward and is less time consuming than cytogenetic analysis. High-throughput, or next-generation, sequencing technologies parallelize DNA sequencing producing thousands or millions of sequences concurrently. Although these nascent methods are gradually being adopted in clinical molecular diagnostics their clinical role in pediatric oncology has not been established yet [13].

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