Tumor Pathology: General Principles

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Introduction

The pediatric tumors are unique in that they recapitulate many of the features shared by normal development – rapid increase in size, increased proliferation rate and brisk mitotic activity. Thus, a multidisciplinary approach is required for the diagnosis and management of these tumors. The success of this approach demands close co-operation between specialists from several disciplines and this is best facilitated by good communication and the development of a clear mutual understanding of the nature of the work of these disciplines. This has resulted in dramatic improvements in outcome for children with cancer.

The purpose of this chapter is to establish the role of the pathologist in this multidisciplinary process, to explain the procedures involved, and to indicate the ways in which the surgeon can facilitate this effort. More detailed consideration of the pathology of individual neoplasms can be found in the relevant chapters of this book.

The role of the pathologist goes beyond providing histological diagnosis and includes provision of prognostic information, facilitation of ancillary studies, audit and research. It is important for the surgeons and oncologists to appreciate that the pathological diagnosis is a clinical opinion based on the interpretation of histological findings in the light of clinical details provided, and that it is not just a 'result'. Just like any informed opinion, its formulation is the product of integration of clinical information, imaging studies and other laboratory investigations, as well as gross and microscopic study. It should be obvious that this may take time and that denial of access to such vital information can only delay the process at best or lead to a diagnosis that may result in inappropriate therapy at worst.

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The Diagnostic Specimen

It should be emphasized that all tumor tissue or suspected tumor tissue, with the sole exception of cytology specimens, should be submitted to the pathology laboratory promptly, unfixed, and in a dry, sterile container. The pathology laboratory should always be alerted in advance, so that they are ready to receive the specimen and process it in an appropriate and timely manner.

There are potentially five types of specimens that might be submitted to the pathologist:

1. Cytology specimen
2. Needle biopsy
3. Incisional biopsy
4. Excisional biopsy
5. Resected specimen

The latter may be either pre- or post treatment and may or may not be an attempt at complete surgical extirpation of the tumor – in which case assessment of margins is important.

The Request Form

In each instance it is essential that a standard request form, paper or electronic, is correctly completed and submitted with a properly labeled and identified specimen. The importance of a correctly completed request cannot be overemphasized. Full patient identification details are necessary if errors of attribution of specimens are to be avoided.

The accurate spelling of a name and also of unique patient identifiers such as date of birth and hospital number are essential. The significance of the site of biopsy is self-evident. Frequently the clinical history is omitted or is inad-

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equate. This omission should be unacceptable in modern practice. The pathologist, as a medical consultant, requires clinical information to assist in the integration of evidence derived from gross and histological examination of a specimen if an accurate and clinically meaningful opinion and diagnosis is to be proffered. It is also very useful for the surgeon to have a dialogue with the pathologist beforehand to indicate any specific features of the case that he particularly needs to be resolved. A good and clear communication with the pathologist in advance of taking the specimen is good practice and in the best centers is routine.

Cytology

In general, cytological techniques have not been much employed in the diagnosis of pediatric neoplasia. This probably reflects the relative rarity of pediatric tumors and may be a hangover from the concept of the "small blue cell tumor of childhood" in which various neoplasms of differing biological potential can appear somewhat similar histologically. In addition, most pediatric lesions are not directly amenable to the surface scraping and fluid aspiration methodologies of classical cytology (Fig. 8.1). However, with the advent of fine needle aspiration cytology (FNAC) that can be guided by radiology, this situation has changed rapidly [1].

The imaging [ultrasound or computed tomography (CT)] guided FNAC allows samples to be obtained from otherwise inaccessible areas and allows for greater use of cytology. The most useful cytological investigation with regard to pediatric neoplasia is FNAC in which both superficial and deeply sited lesions become accessible either by palpation and direct puncture or by means of imaging-guided FNAC [2–5]. The use of cytology for primary diagnosis however is limited by the preference of an institution, the technical skills available for obtaining such samples and most importantly, the confidence and experience of the pathologist.

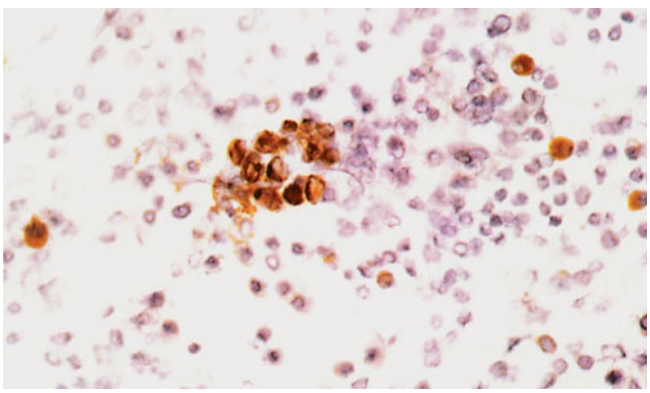

Fig. 8.1 Smear of rhabdomyosarcoma cells stained for desmin, found in ascitic fluid

The question as to who should perform the aspiration is dependent on local circumstances. In the case of palpation and direct puncture, what is more important is that the operator is experienced in the technique of sampling and this can either be a surgeon or a pathologist. Several passages through the lesion with aspiration are required to ensure an adequate sample, and the use of a needle of appropriate gauge (23 or 25) and an aspiration gun to allow single-handed manipulation of needle and syringe are obviously important (Figs. 8.2a, b). In the case of deep-seated lesions, which require imaging guidance, these can be performed by radiologists or other clinicians with expertise in interventional techniques (Fig. 8.3).

A significant feature of a fine needle aspiration is the potential for obtaining "microbiopsies" with preserved histological microanatomy which pathologists frequently find useful in the diagnosis of many pediatric tumors. It is also possible to make cell pellets from an aspiration specimen if pathologists are less experienced in dealing with cytological preparations [6]. These pellets can be then processed and sectioned as histological blocks in the more usual way.

Central to the success of fine needle aspiration is the adequacy of the aspirate and the subsequent production of good smears and cytocentrifuge preparations. It is therefore necessary that the surgeons inform the laboratory well in advance in order for a cytotechnician to be on hand to facilitate the preparation of air-dried and alcohol-fixed smears. A poorly prepared slide can negate the entire procedure and it is frequently difficult to get an adequate smear, particularly if one is not experienced in the preparation of these slides.

Great care is needed if a potentially confusing artifact is to be avoided in the case of alcohol-fixed preparations. Any degree of air-drying in a poorly fixed smear preparation causes artifactual nuclear enlargement and irregularity of chromatin distribution – features seen in malignant cells. It is preferred to have both alcohol-fixed slides stained by the Papanicolaou method and air-dried slides stained by the May-Grun- wald-Giemsa or Diff-Quick methods. These can be either on smears or cytocentrifugation preparations. Cytocentrifugation has the advantage, in fluids and hypocellular samples, of concentrating the cellular component to be studied.

In addition to the standard cellular morphology that can be expected in aspirates of pediatric tumors, it is also possible to apply ancillary techniques to these specimens [7]. Therefore, prior to the aspiration procedure, consideration should be given to the possibility that the diagnosis of a case may benefit from investigations other than morphology. Of particular value are cytogenetics and molecular genetics, immunocytochemistry and electron microscopy. Portions of the aspirate can be allocated to these purposes in order of priority as determined by the clinical presentation of the individual case and presumptive clinical diagnosis.

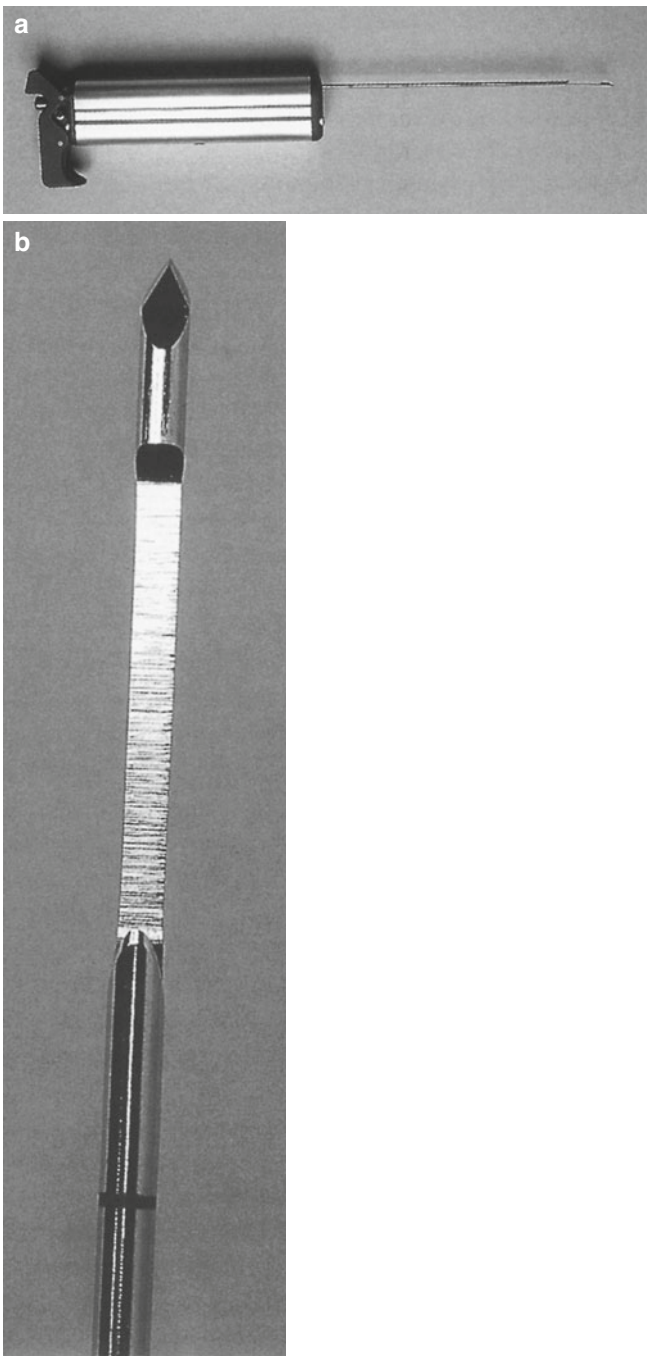

Fig. 8.2 (**a**) Biopsy using an automatic cutting device with a 2.2- cm long needle throw (Pro-Mag 2.2 Manan Medical Products, Inc. Northbrook, USA) and a 14-gauge cutting needle (Manan Medical Products Inc). The biopsy is taken during suspended inspiration. (**b**) Close up of needle tip

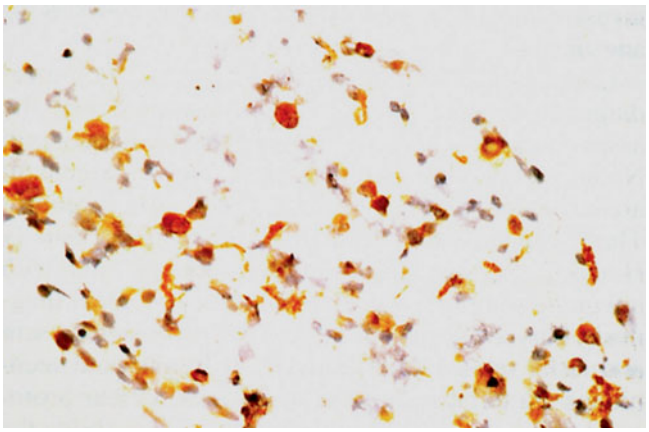

Fig. 8.3 Fine needle aspiration of an embryonal rhabdomyosarcoma stained for desmin

It should be understood that the cytological diagnosis of pediatric neoplasms is a difficult area and expertise is developed over a period of time. It is not possible for a pathologist without experience of this technique to receive a specimen and be expected to make an erudite diagnosis at once. The learning curve is long, and with the paucity of material resulting from small numbers of cases it can be an area fraught with difficulty. It is often sound practice for the pathologist to aspirate resected specimens in the laboratory in order to practice looking at more material than otherwise would be submitted for primary diagnosis.

The complications of fine needle aspiration are minimal. Local bleeding is usually of minor significance even with substantial vascular lesions and needle aspiration of lesions in the lung very seldom lead to pneumothorax.

Cytology has another significant use in pediatric neoplasia and that is to identify infections related to immunocompromise resulting from therapy. Cytological preparations of skin scrapings can identify viral infections such as herpes simplex and infections of the respiratory tract, e.g., Pneumocystis carnii, fungal infections, and viral infections such as Cytomegalovirus (CMV), are also amenable to diagnosis in bronchial lavage specimens by cytological techniques.

It is clear that cytological diagnosis will become an increasingly important part of the pediatric pathologist's workload because it is a relatively noninvasive technique and reduces the use and risk of anesthesia.

Cases which will benefit most from cytological diagnosis involve "neck lumps" and in particular the assessment of cases of lymph node enlargement. Nodal pathologies with persisting node enlargement are a frequent cause of referral to pediatric surgeons. These can be either reactive or neoplastic (usually Hodgkin's or non-Hodgkin's lymphoma) and with adequate sampling are amenable to cytological diagnosis thus avoiding open biopsy with resulting scar and risks related to anesthesia. If cytological techniques are to be applied in such cases, a clear protocol for the pre-biopsy assessment of the child, the obtaining of an adequate sample by a competent experienced operator, the performance of ancillary studies (particularly microbiology) and a plan for follow-up and open biopsy in cases of persistence of the

mass must be in place and followed in all instances if false negative diagnoses are not to be detrimental to the patient. Kardos et al. [8] laid out such a protocol that fulfills these requirements and provides a model of sound practice – it is highly recommended.

It must be emphasized that cytology alone cannot provide all the answers required of a tissue diagnosis and the need for larger biopsy samples will remain with us for the foreseeable future especially when fresh tissue is required for special biological and cytogenetic investigations that may influence therapy.

The Diagnostic Needle Core Biopsy

The core of tissue derived from a needle biopsy either by use of a Tru-cut needle [9] or the more recently available biopsy gun can provide adequate tissue to allow accurate diagnosis of the majority of pediatric neoplasms [10]. More than one core, and preferably at least three, should be taken to allow for tumor heterogeneity and to permit ancillary investigations. With the modern instruments, trauma of the tissue core is usually minimal and although the sample is small, typically 10 × 1 × 1 mm, it is usually possible to obtain material for ancillary studies and for immunohistochemistry. The paucity of material does, however, frequently make it difficult for the pathologist to provide other information, for instance in relation to the presence or absence of anaplasia in nephroblastoma, tumor grading in soft tissue sarcoma or the mitosis/karyorrhexis index (MKI) and other prognostic features in neuroblastoma.

In general, directed biopsies using ultrasound and CT guidance give better samples than a blind biopsy performed either percutaneously or under direct vision at surgery. Drying artifact during transit of fresh samples to the laboratory is a potential problem and rapid transfer in a closed container is essential.

Incisional Biopsy

Incisional biopsies under direct vision provide very adequate tissue samples, which permit all necessary ancillary studies to be performed in the majority of cases. The surgeon will of course have placed his incision to avoid any potential compromise of subsequent resection and to minimize contamination of surrounding structures and tissue compartments. The surgeon should avoid crushing the tissue with forceps during removal. In the archetypical small blue cell tumors of childhood the cells are fragile and injudicious application of force renders the tumor cells into an amorphous smear of nuclear material impervious to diagnosis (Figs. 8.4a, b). The surgeon should also avoid placing the tissue on any surface which might dry out the tissue during transit to the laboratory.

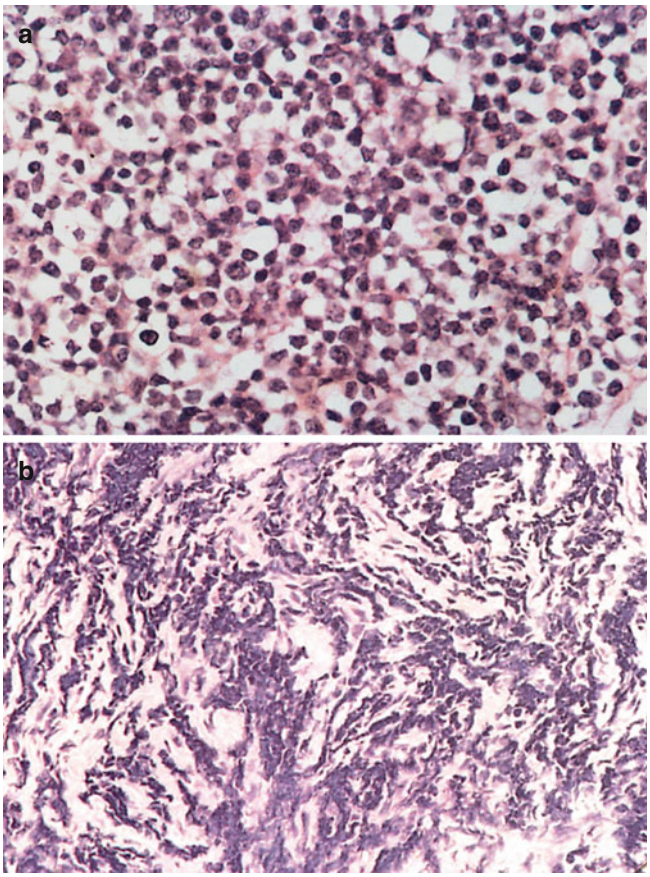

Fig. 8.4 (**a**) Intact biopsy of Ewing's sarcoma. (**b**) Surgical crush artifact of a biopsy of a Ewing's sarcoma

Covering the sample in gauze delays drying artifacts. The skin incision must be placed such that further surgery will include this area, as the biopsy will inevitably have seeded cells into the wound.

Excisional Biopsy

Excisional biopsy entails the apparent complete removal of a small lesion, perhaps up to 5 cm in its greatest dimension. Very frequently this involves the "shelling-out" of a lesion such as lymph node or soft tissue tumors of the limbs. It is usual in the latter situation for tumor to be left behind as the plane of dissection is frequently through the tumor pseudocapsule and not through noninvolved healthy tissue [11]. Once again, this technique provides very adequate material for ancillary studies such as cytogenetics, molecular genetics, and other research activities. The surgeon should consider marking the margins of specific interest if he or she believes that the excisional biopsy represents a clearance of the tumor, and the tumor should not be incised prior to transfer to the pathology department as excision margins will be compromised or contaminated by tumor, giving rise to a risk

of a false diagnosis of incomplete excision resulting in inappropriate further surgery or adjuvant therapy. Again, covering the sample in wet gauze delays drying artifacts.

Surgical Resection

A definitive surgical resection can either be performed as a primary surgical procedure or following pre-operative chemotherapy or radiotherapy. The advent of preoperative therapy allows tumor shrinkage and reduction in vascularity. Many tumors, which were deemed not amenable to resection prior to therapy, may become resectable after chemotherapy (Fig. 8.5) [12, 13].

The margins of the resected specimens should be marked in all cases. It is important that the surgeon should not incise these specimens prior to receipt in the pathology department since this may lead to capsular retraction and render the margins contaminated, making it impossible for the pathologist to be sure that the tumor is completely excised with a margin of clear noninvolved tissue. Any lymph nodes or other tissues removed at the time of primary or post therapy resection should be specifically labeled with their site clearly indicated in the request form and on the specimen containers. It is insufficient to say "lymph node" and not to specify the site from which it is taken because the site of lymph node involvement may determine the stage of the disease and fields for any subsequent radiotherapy.

Specimen Handling in the Pathology Department

Assessment of a specimen submitted for diagnosis involves both gross and microscopic examinations. Even the smallest of biopsy specimens can yield useful information on gross examination [14]. The presence of necrosis, hemorrhage, or a variegated appearance may indicate a heterogeneous histological structure. In the case of larger specimens, the gross examination takes on more importance, particularly with regard to the surgical margins of excision and the vascular and neural margins if appropriate.

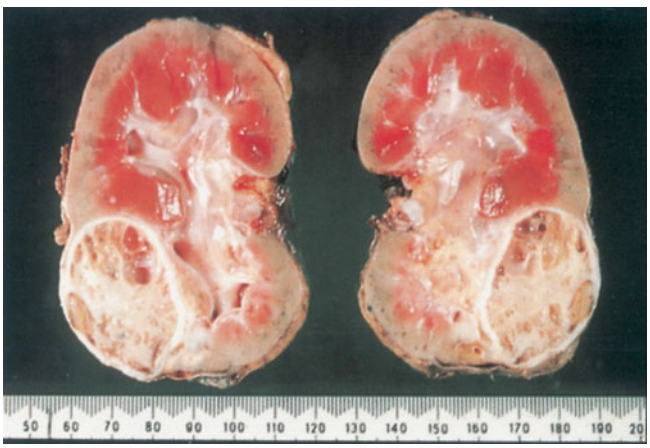

Fig. 8.5 Nephroblastoma after chemotherapy

All tumor specimens, biopsies and resections, should be submitted to the laboratory fresh, i.e., not in fixative. There should be no delay in the receipt of this material in the laboratory and prior notification is essential if appropriate preparations for taking ancillary study samples are to be made in the laboratory. The range of investigations and sampling will obviously be dependent on the size of the sample submitted for diagnosis. In the case of a cytology fine needle aspirate, it is often possible to have some cells submitted for cytogenetic analysis and some for electron microscopy, but often the entire specimen is used for primary cytologic diagnosis. Needle biopsies represent a larger sample, but the volume of material is still extremely small and it may be that only a small piece for cytogenetics can be spared, with the remainder being submitted for histological examination. Incisional biopsies, excisional biopsies, and resected specimens should all provide sufficient material for histological diagnosis, cytogenetics, electron microscopy, and storage of tissue for subsequent molecular studies if required (tumor banking). In addition, it is also often appropriate to submit material for microbiological investigation, particularly in instances of lymphadenopathy. Additional samples may also be taken for ongoing clinical trials if relevant (Fig. 8.6).

The use of intraoperative frozen section for histological diagnosis in pediatric neoplasia should be confined to very specific indications. The desire of the surgeon to be able to tell the parents of the nature of the diagnosis and likely prognosis at the end of the operation is not a sufficient reason for a frozen section to be performed. The only uses for a frozen section during operation are to confirm the presence of tumor, to ascertain that adequate diagnostic material of the native lesion is present in the sample excised, and to assess the need to take wider margins if required. It is often difficult, as a result of artifact related to the frozen section process, to come to a specific histological diagnosis in some of the small, round cell type and spindle cell type pediatric neoplasms. It is wholly inappropriate to use the frozen section diagnosis as a definitive statement for discussions with parents. Such discussions should await the formulation of a definitive paraffin histology-based diagnostic opinion and report.

In the case of excisional biopsy and resection specimens in which complete surgical extirpation of the tumor is intended, evaluation of the surgical margins is important with regard to decisions for further local and systemic therapy. A technique that is widely employed to determine true surgical margins is to paint the entire specimen with Indian

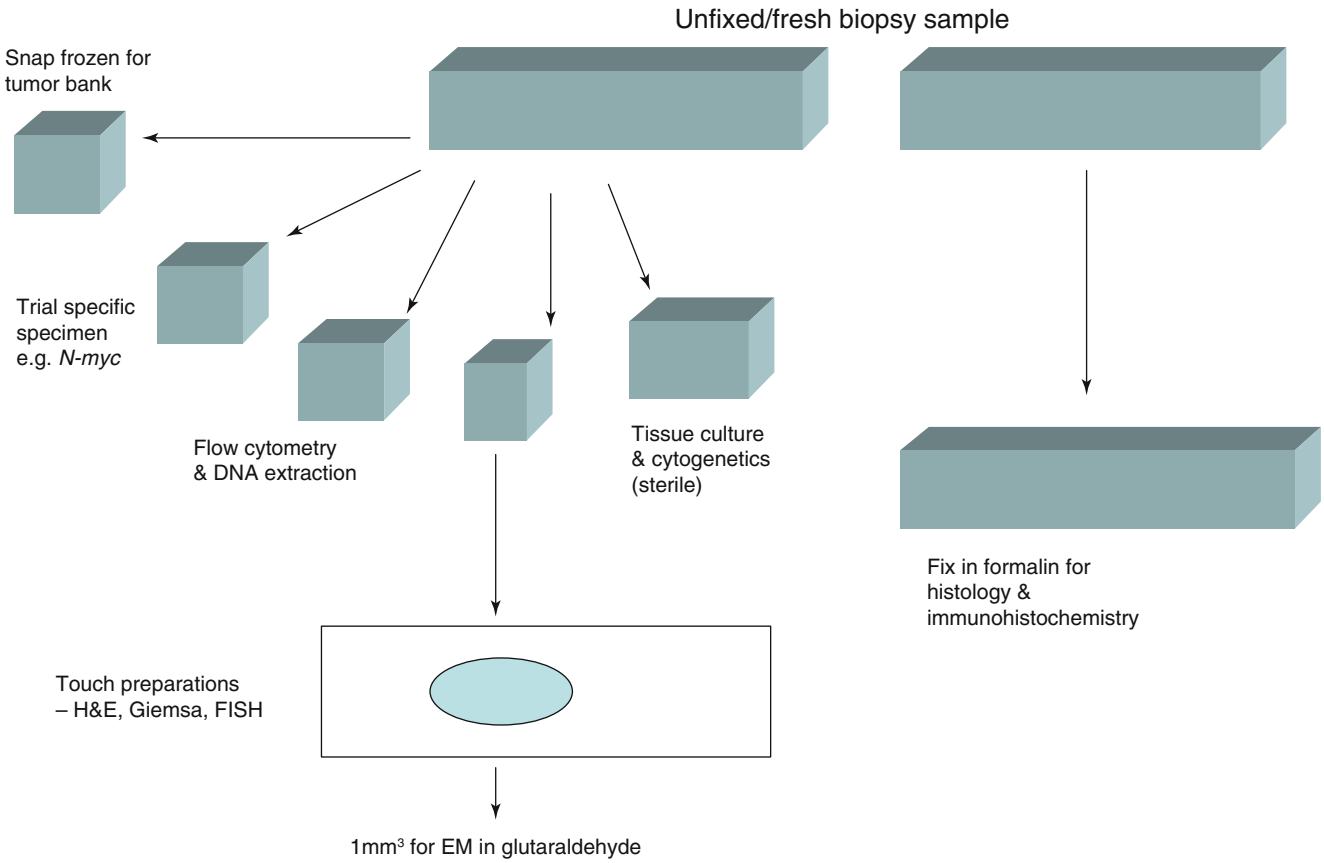

Fig. 8.6 A guide to handling a pediatric tumor biopsy specimen

ink ("inking") or other suitable dyes prior to the incision of a specimen. The dye must be dried onto the surface of the specimen before the specimen is placed in fixative, but if the margin is in question it is a valuable technique. It is axiomatic that the surgeon should not compromise the margins of excision by incising the specimen prior to submission to the pathologist.

Gross examination is vitally important in order that one can be sure that the blocks being sampled from the specimen relate to true surgical margins and not to areas of artifact. It is therefore important that the specimen should be thoroughly examined macroscopically, weighed where appropriate, measured, and photographed, preferably prior to fixation. Where fresh tumor samples must be taken for ancillary studies prior to fixation it is best to incise the specimen through an area in which there is no doubt that excision is complete, i.e., one with a thick intact capsule or covering layer of normal tissue.

Ancillary Studies

Ancillary studies are essential, not optional, in all cases of pediatric neoplasia in which material is submitted for diagnosis. The majority of new SIOP trial protocols include mandatory sampling for biological studies. It is routine practice in our laboratory to take samples in culture medium for cytogenetics as the minimum additional investigation in every case. With more substantial specimens, i.e., open biopsies or resections, a more detailed protocol is applied (Table 8.1). In taking these samples, every effort should be made to use "sterile technique" and sterile disposable instruments, etc. This is particularly important for those samples to be cultured for cytogenetic studies. In the USA, the Children's Cancer Group (CCG) provide kits for specimen procurement in cases of pediatric neoplasia thus facilitating diagnostic studies and ongoing biological research into these complex and fascinating conditions.

Both normal and tumor tissues should be sampled and stored whenever possible. The samples should be taken as promptly as possible after removal of the specimen from the patient. This must be done by the person reporting the specimen, i.e., the pathologist. Therefore, a short delay in transit to the laboratory is acceptable. Our procedure is to leave the photography until after samples have been taken. With a large specimen it is possible to section it and, if homogeneous, take the samples from one half leaving the other for photography. It is always possible to take the samples without compromising assessment of margins. If studies of

Table 8.1 Pediatric neoplasia: ancillary studies (excluding "routine" paraffin section immunohistochemistry)

Cytogenetics	Tumor and normal tissue in cytogenetics medium
Molecular genetics	Tumor and normal tissue snap frozen in liquid nitrogen and stored in liquid nitrogen (gaseous phase) or at –80 °C. Sample held on water ice for mRNA studies to be dealt with without delay
Immunohistochemistry/Fluorescent in situ hybridization (FISH)	Snap frozen in OCT medium, store at –80 °C
Touch imprints (>10) for FISH	Air dry
Electron microscopy	Paper-thin section or 1 mm cubes in 4 % gluteraldehyde
Tissue storage (long term)	Tumor and normal tissue for research, flow cytometry, etc., s stored in liquid nitrogen (gaseous phase) or at –80 °C

mRNA are contemplated, then the tissue sample should be stored in sterile conditions on water ice prior to uplift. The delay in taking the sample should be as short as practicable as mRNA is susceptible to relatively rapid deterioration.

In the case of heterogeneous lesions, the sampling should incorporate multiple areas. Foci of obvious necrosis can be avoided but hemorrhagic areas are often the most viable, and firm fleshy areas may be more fibrous and contain fewer tumor cells. The concept of heterogeneity does not apply to macroscopic appearances only. Within a large tumor mass there is the possibility of clonal heterogeneity and this may be significant if assessment of prognostic features is to have a bearing on the intensity of therapy. Examples would be the identification of N-myc amplification in composite nodular ganglioneuroblastoma or 1p deletion in neuroblastoma [15] – bad prognostic features that can be variably present in different parts/cellular nodules of a tumor, and if only one area is examined a false negative result may be obtained. It is therefore good practice to take tissue from several areas of all substantial tumor specimens in order to minimize this potential problem.

The number of blocks that should be taken for histological examination from a large specimen varies according to the individual case. A useful rule of thumb is to take a minimum of one block for each centimeter of the largest dimension of the lesion, but this should not be regarded as an absolute and in most instances many more blocks are indicated. Points of interest and resection edges of nerves, vessels, and soft tissue margins of questionable clearance which have been indicated by the surgeon by means of marker sutures or in the request form should receive particular attention and will require a larger number of blocks to be taken. Tissue blocks should be taken from normal tissue as well as the lesion. Sectioning of a block of appropriate size is much easier after fixation. The site of origin of individual blocks should be recorded on an appropriate diagram or photograph of the specimen at the time of sampling (Fig. 8.7). This allows the pathologist to return to a specific area of the specimen if initial histological examination identifies additional features requiring further detailed assessment, e.g., focal or diffuse anaplasia in nephroblastoma [16]. It is good practice to take "mirror image" blocks from the tumor with one block frozen down in liquid nitrogen for research purposes while the other is processed for histology. This allows molecular studies, preparations of microarrays, and morphological studies to be conducted on the same potentially clonal areas of an individual tumor.

Fixation and Processing

A number of standard fixatives are available to the pathologist, but the most flexible given the need for speed of fixation, lack of toxicity, etc., is 10 % buffered formalin solution. It is possible to perform electron microscopy on tissues, which have been in 10 % formalin fixative even although ultrastructure is degraded. This fixative is also ideal for most immunohistochemical studies.

In North America, the fixative B5 (sublimate sodium acetate formalin) is widely used for lymph node and renal biopsies. Other commonly used fixatives include Bouin's, Zenker's, and Carnoy's. All have advantages for specific indications but for general use these are outweighed by problems of cost, preparation, and disposal.

Heat fixation by microwave using a standard domestic microwave oven is effective for specimens of substantial size, but we have found a significant frequency of unacceptable cellular artifact and do not use this method as routine.

The volume of fixative is critical. A ratio of 10:1 fixative to specimen is an acceptable minimum. It is important that the specimen should be entirely immersed in the fixative solution. The purpose of these fixatives is to complex the proteins in the tissue, stabilizing tissue and thus stopping the autolytic processes that would degrade tissue structure and ultrastructure.

The penetration of fixative into tissue blocks is one of the most significant rate-limiting factors in determining how long it takes to have material available for the pathologist to study under the microscope. In general terms formalin will penetrate at a rate of 1 mm/h and will go on for a considerable period slowly penetrating into the middle of large tissue specimens. Other fixatives penetrate much more slowly or only penetrate the surface of the tissue sample to any great degree.

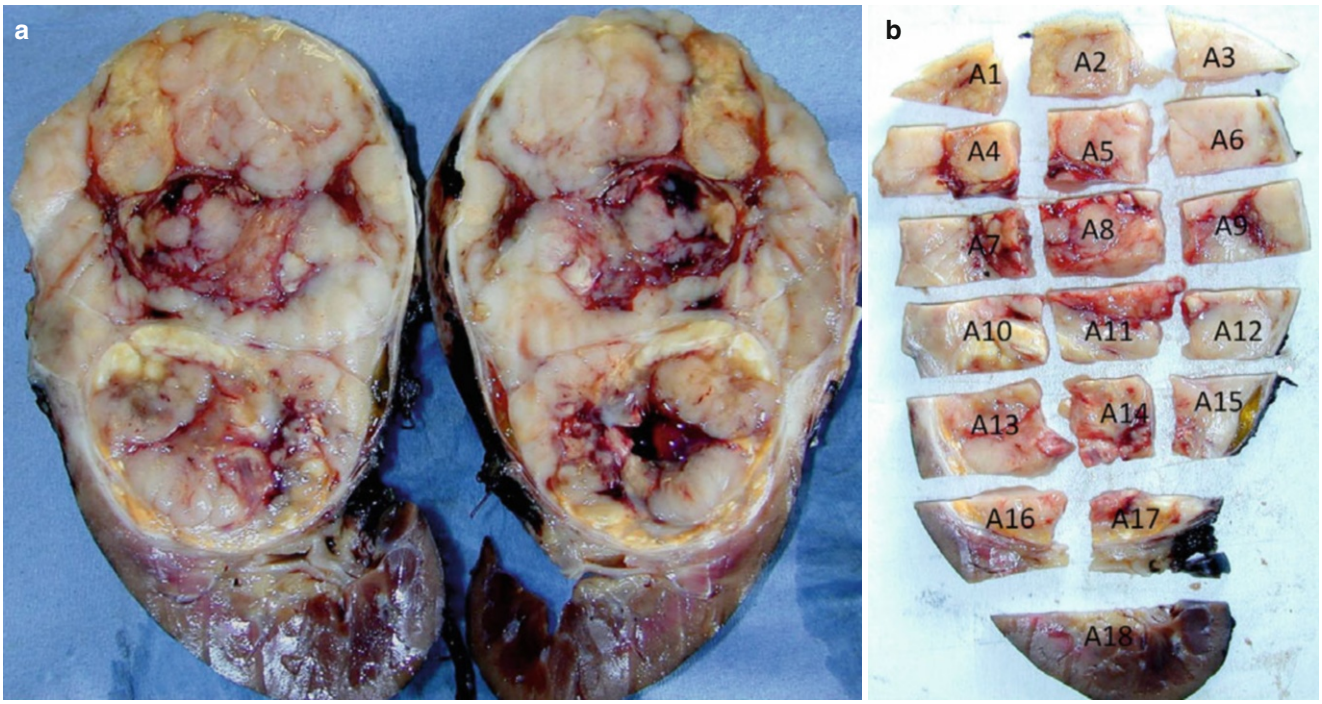

Fig. 8.7 Nephroblastoma after chemotherapy. A photographic block-sheet of the specimen at the time of sampling allows the pathologist to re-visit area of interest

The penetration of formalin is temperature- dependant and while it is entirely satisfactory at as low as 4 °C, it is probably accelerated at higher temperatures and this is one means by which the fixation time can be reduced if there is an urgent requirement for a tissue diagnosis. Standard vacuum embedding processing machines also accelerate both fixation and processing and save time. One can process needle core biopsy samples over a period of only 4 h and have a paraffin section cut and stained for viewing within 5 h of the biopsy having been taken. However, this means that we must accept some degradation in morphology and perhaps also compromise immunohistochemical studies as the fixation may not be as good as it would be with a longer fixation, and processing period and protein linkages which expose or mask antigens are not optimal. More usually, tissue samples are fixed for approximately 24 h before being processed on a cycle that takes between 16 and 18 h to remove tissue water and fat and to replace these with paraffin, providing a paraffin block for sectioning. Dehydration is achieved by use of alcohols; fat is removed by alcohol and xylene (Table 8.2). For bone lesions, decalcification may be necessary and will delay block sampling and subsequent histological examination by several days.

The new generation of tissue processors utilize microwaves to accelerate fixation and this together with use of alternative solvents allows needle biopsies to be processed within 1 h, or a block of a tumor resection (5 mm thickness) to be processed in 3 h. Decalcification of a bone tumor sample can be completed in 12 h.

Table 8.2 Typical tissue processing cycles (vacuum embedding)

Overnight (standard blocks)			Rapid (small biopsies)		
1	120	45	1 Formalin	Passed	
2	30	No heating	2 Water	Passed	
3	60	No heating	3 70 % Spirit	Passed	
4	60	No heating	4 Methylated spirit	25	45
5	60	No heating	5 Methylated spirit	25	45
6	60	No heating	6 Methylated spirit	25	45
7	60	45	7 Methylated spirit	30	45
8	90	45	8 Absolute alcohol	30	45
9	90	No heating	9 Xylene	30	45
10 Xylene	90	No heating	10 Xylene	30	45
11 Wax	45	60	11 Wax	15	60
12 Wax	45	60	12 Wax	15	45
13 Wax	45	60	13 Wax	15	45
14 Wax	45	60	14 Wax	30	45

The Preparation of Histological Material

The histological examination of surgical material is an essential part of the diagnostic process. This requires the cutting of sections from the paraffin block, usually at a thickness of 4–5 µm and these sections are then stained with a variety of dyes, which can demonstrate the various component parts of the tissue sample in question. The standard histological stain for daily use is the hemotoxylin and eosin (H & E) stain, which provides a very good demarcation between nuclei, stained

blue with hematoxylin, and the cytoplasm, stained varying degrees of pink with eosin.

The diagnostic utility of other special stains has been to a considerable extent superseded by the development of immunohistochemistry. However, a limited number of these stains remain useful in specific tumors (Fig. 8.8, Table 8.3).

Formalin-fixed paraffin-embedded tissue sections are also utilized for immunohistochemical studies. Cytology touch preparations and frozen sections are better for in situ hybridization studies and are also essential for immunofluorescence studies when appropriate.

The Diagnostic Process

The development of a final diagnostic opinion is the result of consideration and integration of several sources of information, i.e., clinical presentation, anatomic localization (clinical and imaging), laboratory investigations (e.g., biochemistry) and operative appearances. With this information on hand, the macroscopic examination and sampling of a specimen leads to the final step of histological examination.

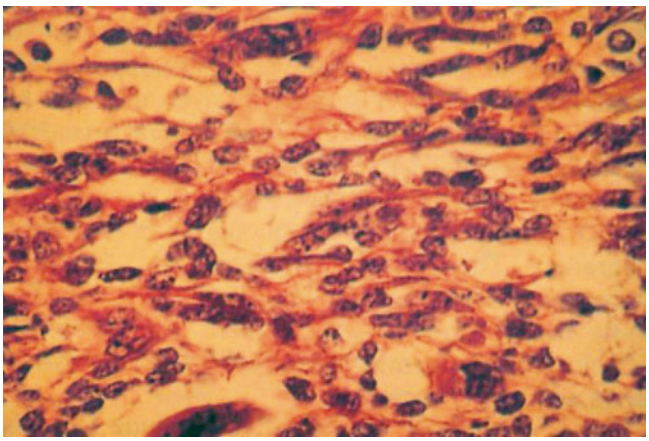

Fig. 8.8 PTAH staining showing cross-striations in a rhabdomyosarcoma

Table 8.3 Special stains in pediatric neoplasia Stain

Stain	
Periodic acid-Schiff (PAS) ± diastase	Glycogen in Ewing's sarcoma
Reticulin	Reticulin fibers (types III and IV collagen) in soft tissue tumors
Phosphotungstic acid-hematoxylin (PTAH)	Cross-striation in rhabdomyoblasts (little used)
Masson-Fontana-melanoma Grimelius	Melanin pigment in clear cell sarcoma and Argyrophilic reaction in paraganglionoma
Perls Prussian Blue pigment von Kossa	Ferric iron Calcium
Alkaline phosphatase	Positive in osteoblasts in osteosarcoma

While it is true that a diagnosis can be inferred from any or all of the steps outlined above, there is no doubt that only histology can provide a definitive diagnostic opinion and this examination will also deliver prognostic information relevant to the individual case.

In making a histological diagnosis the pathologist assesses the presence of features of malignancy and seeks evidence of differentiation, i.e., the development of features indicative of the cell lineage of origin which can be recognized by H & E staining, special stains, and immunohistochemical studies.

The histological diagnosis of malignancy is based on assessment of a lesion with regard to the age of the patient, the site or organ of origin, the nature of the lesion in relation to surrounding structures, the presence of necrosis, degree of organization/differentiation, and cellular morphology. For example, a highly cellular mass in the middle of the kidney is likely to be a neoplasm. Necrosis of a spindle cell proliferative lesion of soft tissue is a strong indicator that one is dealing with a sarcoma. In all instances infiltrative invasive lesional margins as opposed to encapsulated/pseudo-encapsulated expansile margins suggests malignancy. These features and the presence or absence of differentiation do not absolutely predict behavior and the cellular morphology is important. Nuclear enlargement with increased hematoxylin staining density (hyperchromatism) and variation in nuclear and cellular size and shape (pleomorphism) are typical features of neoplasms. An increased mitotic rate with atypical and abnormal mitotic figures is frequently but not invariably seen. Thus, it is the assessment of the lesion both in isolation and in the context of its surroundings that leads to a diagnosis. Frequently, however, the tumor may present as a lesion of small blue cells or an apparently undifferentiated sarcoma. In these instances the search for evidence of differentiation indicating the cell lineage of the tumor requires studies of molecular or ultrastructural differentiation by immunohistochemistry and electron microscopy.

Patterns of regression in childhood tumors can be spontaneous or treatment-induced and may present as either maturation or true regressive changes, e.g., necrosis, fibrosis, cystic degeneration, myxoid degeneration, or calcification [17–19].

Spontaneous regressive features, i.e., not related to therapy, can indicate some prognostic potential. Necrosis is generally regarded as a feature of aggressive, fast-growing malignant tumors but may also indicate the potential for a good response to chemotherapy because of the high cell turnover rate, although this is not true for rhabdoid tumors. The presence of a significant lymphoid cell infiltrate is sometimes an indication of a better prognosis lesion, e.g., inflammatory fibrosarcoma, or a pseudotumor. Myxoid change tends to be a feature of benign or slow-growing tumors of low malignant potential. Myxoid change in botyroid rhabdomyosarcoma is associated with better prognosis.

Maturation of untreated tumors is characterized by increasing differentiation towards mature tissue phenotype. The classical example is neuroblastoma where spontaneous maturation to ganglioneuroblastoma or ganglioneuroma is well recognized. In the case of ovarian teratomas the presence of gliomatosis peritonei is a marker of a good prognosis. Lipoblastomas mature with age into lipomas.

Similar patterns of regression and maturation are seen as an effect of therapy and can pose problems for the pathologist if they are so marked as to preclude most of the prognostic assessment of a tumor (Fig. 8.9). Chemotherapy frequently downstages a tumor – an effect most often seen in nephroblastoma. Post-chemotherapy cystic change in nephroblastoma is common and care must be taken not to mistake this feature and make a diagnosis of cystic partially differentiated nephroblastoma. In osteosarcoma the post- chemotherapy assessment of tumor response is a very accurate predictor of prognosis. If 10 % or more of the tumor cells remain viable after a course of intensive therapy then the prognosis is poor.

It should be remembered that tumors are composed of clones of cells, which as a result of mutation during tumorigenesis may have different patterns and degrees of response to therapy. This may result in a very heterogeneous response with fibrosis of tumor adjacent to viable lesional tissue. Occasionally the therapy seems to select out a particularly "resistant" aggressive clone and the pattern of dedifferentiation is seen. The prognostic implications of maturation under the influence of therapy are not yet clear and similarly metaplasia is not thought to have prognostic significance.

Immunohistochemistry

Immunohistochemistry is a vital tool in diagnostic histopathology and is one of the most useful ancillary investigation in the diagnoses of pediatric tumors. In recent years there has been a massive expansion in the use of antibodies in tissue diagnosis. The principal influence has been in adding a degree of objectivity into the essentially subjective area of histological diagnosis by confirming lineage differentiation in embryonal and undifferentiated neoplasms (Fig. 8.10a–g).

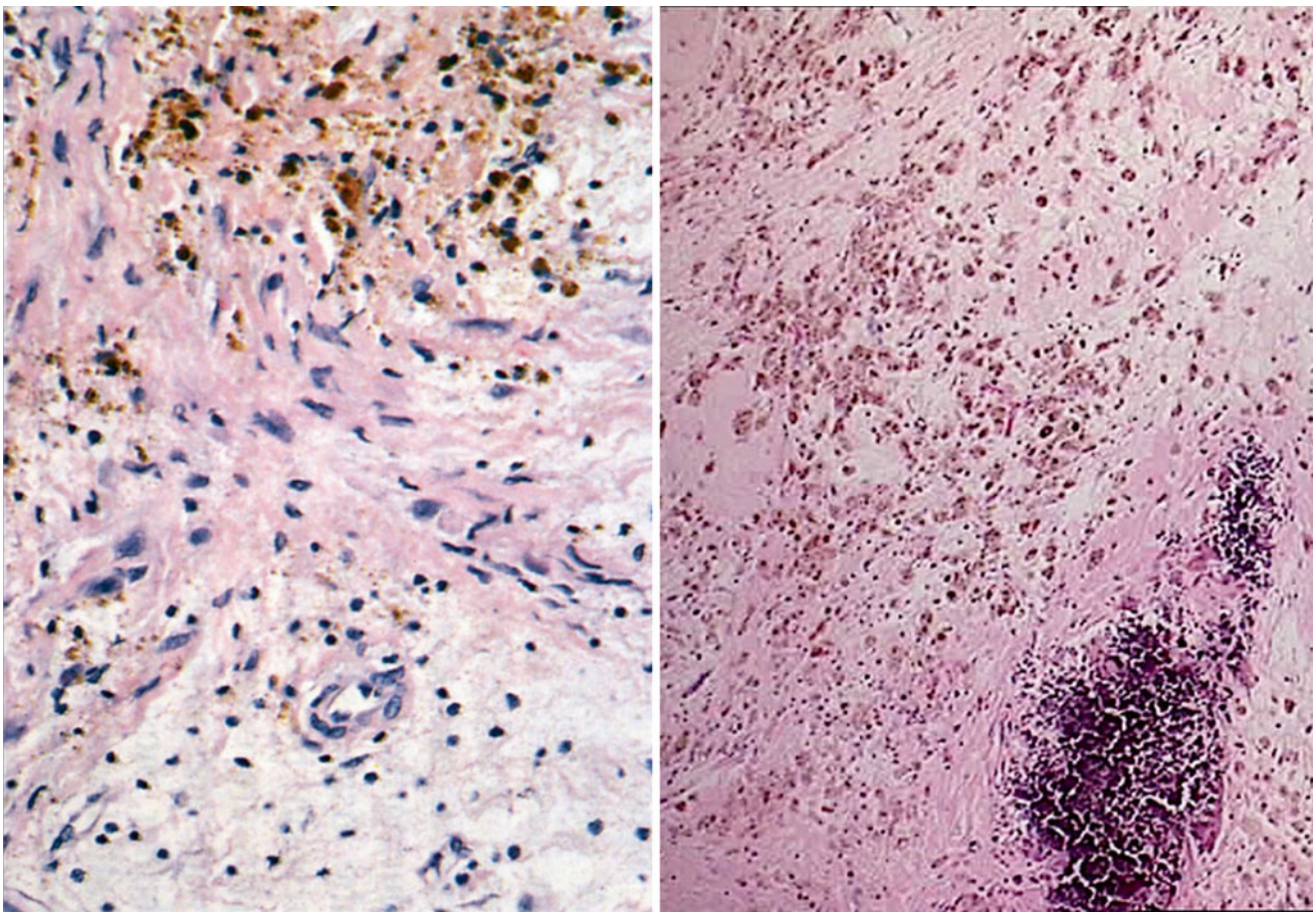

Fig. 8.9 Histology of an embryonal rhabdomyosarcoma showing postchemotherapy changes. Foamy macrophages, hemosiderin-laden macrophages, calcification and fibrosis

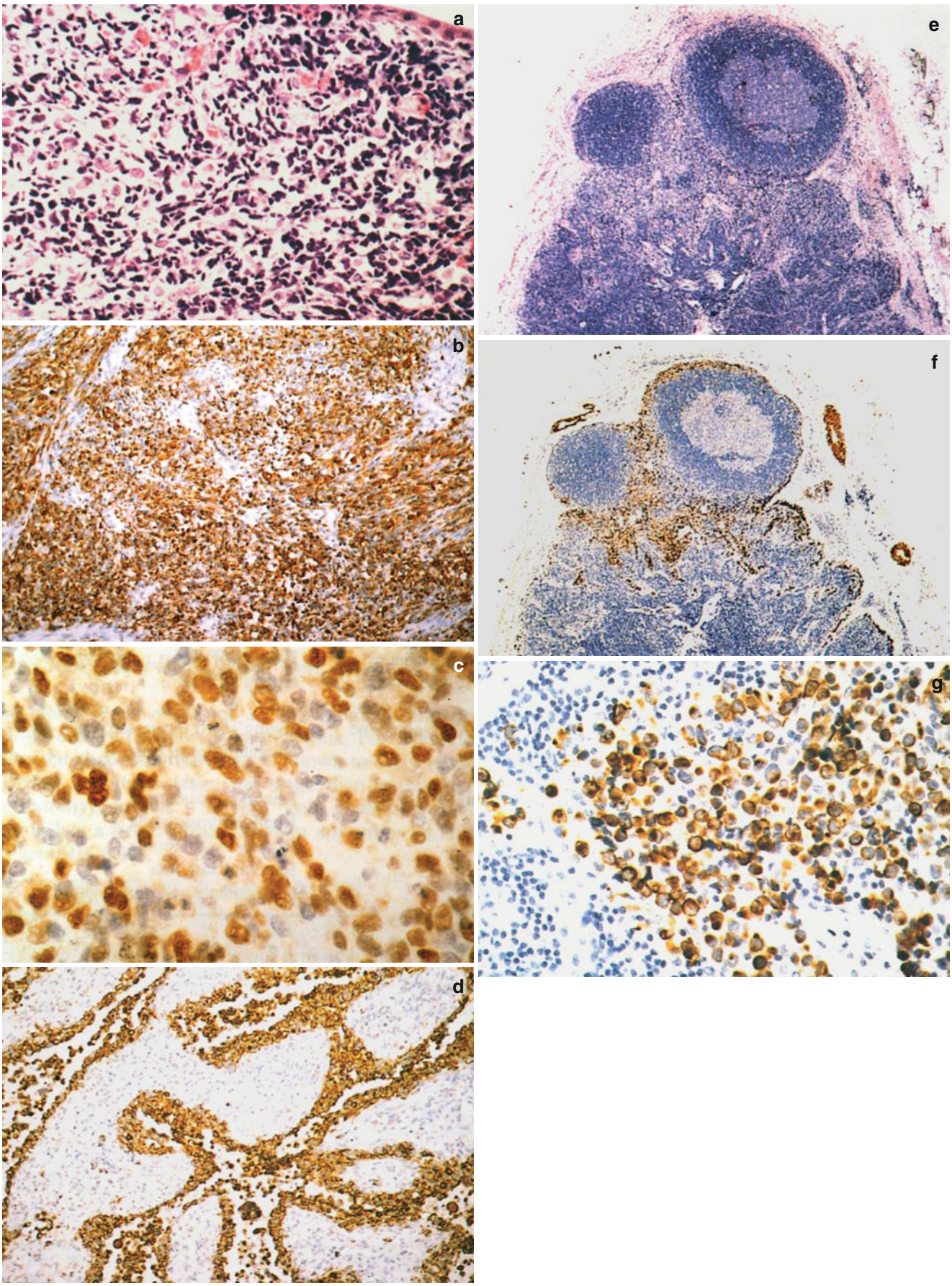

Fig. 8.10 (**a**) Embryonal rhabdomyosarcoma (H & E stain). (**b**) Desmin stain of rhabdomyosarcoma. (**c**) Myo D 1 stain of rhabdomyosarcoma. (**d**) Alveolar rhabdomyosarcoma desmin stain. (**e**) Lymph node (H & E). (**f**) Lymph node with desmin stain. (**g**) Magnification of (**f**)

Immunohistochemistry is based on the premise that a particular component of a tissue, acting as an antigen, can be identified by a specific antibody carrying a label that can be rendered visible. A number of techniques are routinely used, but the underlying philosophy is the same for all. The variation in technique relates to attempts to maximize the intensity of the label signal indicating the presence of a specific antigen of interest.

Two types of antibodies are used. The first are polyclonal and tend to be less specific and sensitive while the second, which are now more commonly used, are monoclonal antibodies which allow for the use of very sensitive and highly specific detection techniques. The most commonly used methods of demonstrating the presence of antibody binding to tissue antigen are the peroxidase-antiperoxidase immune complex method and the avidin-biotin immunoenzymatic method. More detailed consideration of the principles and techniques can be found in a variety of specialist texts [20, 21].

The antibodies are named either by reference to the antigen (protein product/structure) to which they bind or in the case of leukocytes and related antigens, by the cluster differentiation antigen designation (CD) which have been determined at a series of international workshops.

Sensitivity and specificity are of vital importance. A number of techniques have been employed to increase sensitivity and, in general, these attempt to unmask antigens which are hidden during tissue processing presumably by the complexing of proteins during fixation. This can be achieved by digestion of the tissue sections by proteolytic enzymes, e.g., trypsin, by a combination of heat and pressure in a pressure cooker, or by treatment with microwaves with and without the use of additional chemical buffer, most commonly citrate. This process of so-called antigen retrieval using microwaving of sections in citrate or other buffers is now widely used and is extremely successful in allowing low antigen concentrations to be exposed for antibody binding thus increasing the frequency and intensity of positive reactions. Care must be exercised in the use of antigen retrieval as it is possible to produce very convincing and wholly inappropriate false-positive reactions with several antibodies. Meticulous attention to the practical and technical aspects is essential and each laboratory has to establish its own specific methodological conditions, within general principles, for each antibody whichever technique is employed.

Antibodies are used in panels, i.e., several different antisera are individually applied to separate, usually consecutive, sections of a block or blocks of tumor in order to demonstrate evidence of lineage differentiation.

It is vital to avoid false-positive and false-negative staining and to that end standard positive controls and negative controls are always included in staining batches. Pediatric neoplasms commonly exhibit pluripotent differentiation [22] and this potential pitfall is partly negated by the use of multiple antibodies. Example panels of some antibodies commonly used in the diagnosis of pediatric tumors are provided in Table 8.4. The use of more than one antibody specific for

Table 8.4 Examples of antibodies useful in pediatric tumor diagnosis

Leukocyte common antigen (CD45)	B & T Lymphocytes	Lymphomas
CD20 (L26)	B lymphocytes	Lymphomas
CD45RO(UCHL-1)	T lymphocytes	Lymphomas
CD30 (Ber H-2)	Activated lymphocytes/macrophages/ Reed-Sternberg cells	Hodgkin's disease/Anaplastic large cell lymphoma
CD15 (LeuM1)	Reed-Sternberg cells	Hodgkin's disease CD68
(KP1)	Macrophages	Histiocytic neoplasms
Kappa/Lambda	Ig light chains	Lymphoid clonal proliferation
Neuron-specific enolase (NSE)	Neuroectoderm	Neuroblastoma
S100	Glial/Schwann cells/others	Neurofibroma, etc., Langerhan's cells
β2-microglobulin	β2-microglobulin	PNET
Synaptophysin	Neuroectoderm/neuroendocrine	Ewing's/PNET
MIC-2 (CD99)	MIC-2 gene product (glycoprotein P30/32)	Ewing's/PNET
Vimentin	Intermediate filaments/mesenchyme	Ewing's/soft tissue sarcoma
Actin (common, smooth muscle, sarcomeric)	Muscle filaments	Rhabdomyosarcoma
Desmin		Muscle (smooth/striated)
Rhabdomyosarcoma Myoglobin		Striated muscle
Rhabdomyosarcoma Myo D-1		Skeletal muscle
Rhabdomyosarcoma Cytokeratins (AE1-AE3, CAM 5.2, etc.)		Epithelial
Synovial sarcoma		
CD1a histiocytosis	Langerhan's cells	Langerhan's cell

a particular cell lineage is recommended when diagnostic confirmation is sought.

A common problem in immunohistochemistry is the need to recognize and avoid blind overreliance on the presence of a "positive" reaction. There is much cross-reaction and variable expression of antigens in pediatric tumors. For example, in primitive rhabdomyosarcomas it is not uncommon to see positive staining for neuron-specific enolase, which is generally regarded as a marker useful in the diagnosis of neuroblastoma. Similarly, the MIC2 (CD99) Ewing's/primitive neuroectodermal tumor (PNET) marker can be expressed in other pediatric tumors, in particular lymphoma and rhabdomyosarcoma. It is therefore not sufficient merely to expose the section to the antibody, blindly identify a positive labeling signal, and attribute a diagnosis. The positive staining must be in the correct tissue fraction and must correlate with the morphology of the lesion and the clinical presentation of the case. Evaluation of these studies requires an experienced medical practitioner to correlate the data. Blind adherence to immunohistochemical staining may lead to erroneous diagnosis.

Electron Microscopy

The advent of immunohistochemical techniques has been associated with a dramatic reduction in the utilization of electron microscopy in the diagnosis of pediatric neoplasia. There are instances, however, where ultrastructure can indicate lineage specificity of otherwise undifferentiated tumors and clarify its true nature (i.e., neuroendocrine "granules" in neuroblastoma, cytoplasmic glycogen in Ewing's sarcoma, cytoplasmic filaments with Z bands in rhabdomyosarcoma). Electron microscopy is particularly important in the diagnosis of Langerhan's cell histiocytosis where the identification of Birbeck granules is diagnostic (Fig. 8.11).

Tissue submitted for electron microscopy studies must be placed in special fixatives, e.g., gluteraldehyde 4 %. The processing of tissue for electron microscopy requires only very small tissue samples because gluteraldehyde penetrates so poorly. It is labor-intensive and expensive. In tumor diagnosis, we reserve electron microscopy for very specific indications and do not perform these studies as routine in all pediatric neoplasms, although we take appropriate samples in gluteraldehyde fixative from all tumors if sufficient tissues are submitted and additional studies are required.

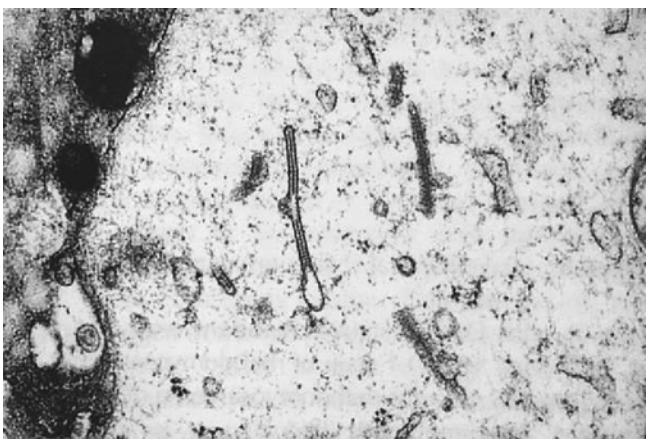

Fig. 8.11 Electron micrographs showing diagnostic Birbeck granules in Langerhan's cells

Cytogenetics

Several pediatric tumors are characterized by specific chromosomal translocations which, in those cases which are diagnostically difficult on light microscopy, can help clarify the diagnosis. Examples include t(2:13) (q35: q14) in alveolar rhabdomyosarcoma [23], t(11:22) (q24:q12) in Ewing's sarcoma, and peripheral PNETs [24], t(x:18) (p11.2:q11.2) in synovial sarcoma [25]. Many other tumors also express consistent chromosomal abnormalities and more examples are being identified almost with every passing week.

Previously these were identified by classical G- banding studies of metaphase spreads of cultured tumor cells, but more recently the use of specific probes and the fluorescent in situ hybridization (FISH) technique has been employed in the rapid identification of chromosomal abnormality such as translocations. FISH can be done on cytological samples and frozen sections thus avoiding the need for expensive and time-consuming tumor cell culture. Interphase FISH studies can also be performed on formalin fixed paraffin embedded sections, including sections from archived tumor cases. The procedure is more technically demanding than that with non-formalin fixed material and requires careful attention to protein digestion and chemical pretreatments to increase cellular permeability and facilitate entry and binding of the DNA probes.

A more detailed review of these cytogenetic lesions and the methodologies employed in their investigation is provided in subsequent chapters of this book.

Molecular Genetics

As diagnostic samples become ever smaller, the provision of adequate tissue for classical cytogenetic analysis becomes problematic. This challenge has been met by the now routine application of polymerase chain reaction (PCR) techniques to amplify tumor DNA or RNA (reverse-transcriptase polymerase chain reaction, RT- PCR) in small tumor samples, thus allowing identification of tumor specific translocations and their fusion transcripts [26–28]. Once again, both standard PCR (DNA) or RT-PCR (RNA) is easier on unfixed samples, fresh or snap frozen in liquid nitrogen, but results can also be achieved on archived tumor paraffin blocks.

The Prognostic Process

Increasingly, in cases of pediatric neoplasia, it is necessary for the pathologist to provide prognostic information as well as a histological diagnosis. This is done by further detailed evaluation of the histology and, in the case of some tumors, by molecular and cytogenetic studies of tumor tissue samples.

Standard Histological Criteria

In many types of tumor the histological subtype alone carries prognostic implications. For example, alveolar rhabdomyosarcoma is known to carry more serious prognosis stage for stage than embryonal rhabdomyo-sarcoma. Spindle cell rhabdomyosarcoma has a better prognosis than any other variant (Figs. 8.12 and 8.13).

It is important to search for prognostic features in individual tumor types, e.g., anaplasia/unfavorable histology in nephroblastoma, the MKI and extent of cellular and stromal differentiation in neuroblastoma [29, 30], because features such as these can influence therapy in individual tumors of a given stage. These and other examples will be discussed in more detail in the chapters relating to individual tumor types.

The more general principles regarding prognostication relate to assessment of tumor stage and, particularly in soft tissue sarcomas, the histological grade. Staging is based on the gross anatomical distribution of disease modified by histological assessment of local excision margins and confirmation/identification of nodal and distal metastases. Specific staging systems apply to several of the organ-specific pediatric tumors, e.g., nephroblastoma, and the National Wilms' Tumor Study (NWTS) definitions of stage are described elsewhere in this book.

An alternative staging system applicable to tumors of all sites is the TNM system: "T" related to the size of the primary tumor, "N" to the presence or absence of nodal metastases, and "M" to the presence or absence of distant metastases [31]. The grading system of Coindre et al. [32] as used by review pathologists in United Kingdom Children Cancer Study Group trials is shown in Table 8.5 for illustration. The reader is directed to references to the grading systems of Markhede, Myhre Jensen, Costa, and Trojani at the end of this chapter for further information [33–36].

Cytogenetics and Molecular Genetics

Vital prognostic information that has a significant bearing on intensity and duration of therapy in certain pediatric neoplasms is obtained from genetic analyses. For example, in rhabdomyosarcoma the confirmation of the alveolar subtype by demonstration of t(2:13) in a lesion previously considered embryonal on light microscopy will result in a more intensive therapeutic regimen. In neuroblastoma the identification of 1p deletion and N-myc amplification [37] are proven indicators of more aggressive tumors with a worse prognosis which require intensive therapy compared with neuroblastomas without these features. High trk-A proto-oncogene expression is associated with a better prognosis and is inversely related to N-myc amplification [38].

An important advance in prognostication has resulted from the capacity of molecular genetic techniques, particularly RT-PCR, to identify previously undetectable tumor cells in peripheral blood or bone marrow samples [39].

Tumors will spread via the blood stream as they metastasize. There is now clear evidence that the presence of this otherwise occult tumor spread is associated with increased incidence of established metastases, reduced disease-free interval, and reduced survival [40]. In those tumors characterized by specific chromosomal translocations and resultant gene fusion transcripts (Table 8.6) the use of RT-PCR can

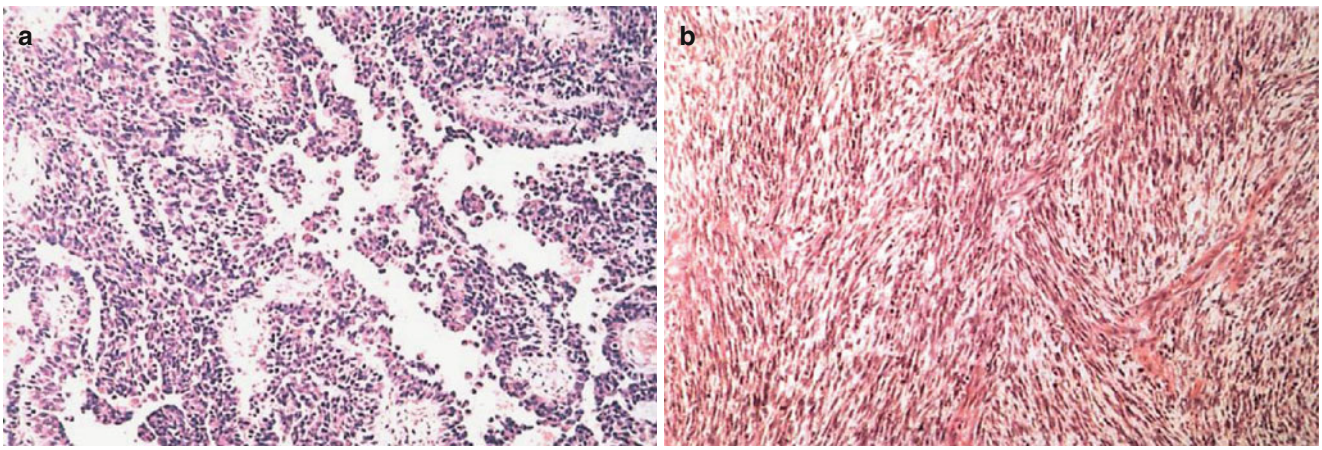

Fig. 8.12 (**a**) Alveolar rhabdomyosarcoma; (**b**) spindle cell variant

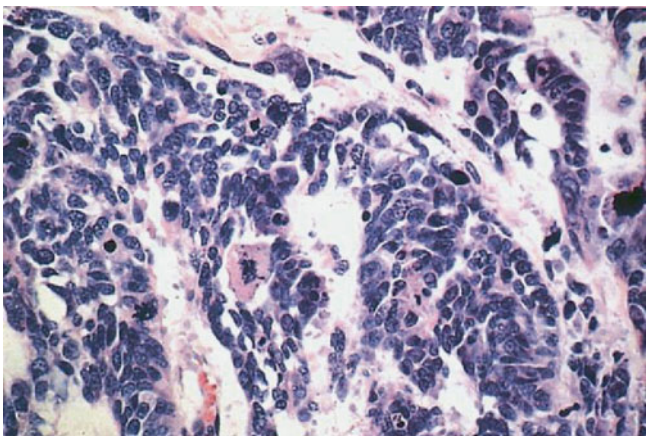

Fig. 8.13 Unfavorable nephroblastoma

Table 8.5 Histological grading in soft tissue sarcoma [32]

Feature		Score
Mitoses	0–9 (per 10 high power fields	1
	10–19 (per 10 high power fields)	2
	>20 (per 10 high power fields)	3
Necrosis	None	1
	<50 % of the tumor	2
	>50 % of the tumor	3
Differentiation	Very highly differentiated	2
	Moderately differentiated but cell type easily recognizable Poorly differentiated or cell type uncertain	3

Grade is determined by aggregate score for all these features, i.e., Grade I, Score 3–4; Grade II, Score 5–6; Grade III, Score 7–9

Table 8.6 Some pediatric tumors with specific, diagnostic chromosomal translocations

Ewing's sarcoma group	t(11;22)(q24:q12) t(21;22)(q22;q12) t(7;22)(p22;q12) t(17;22)q12;q12 t(2;22)q33;q12	EWS-FLI1 EWS-ERG EWS-ETV1 EWS-E1AF EWS-FEV
Desmoplastic small round cell tumor	t(11;22)q13;q12	EWS-WT1
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(2;13)(q335;q14)	PAX3-FKHR PAX7-FKHR
Synovial sarcoma	t(x;18)(p11.2;q11.2)	SYT-SSX1 SYT-SSX2 SYT-SSX4
Congenital fibrosarcoma	t(12;15)(p13;q25)	ETV6-NTRK3

detect this tumor spread in blood and marrow thus providing objective evidence for upstaging or intensification of chemotherapy. Some SIOP tumor protocols now include this investigation as part of patient surveillance.

A more detailed review of this area is given in subsequent chapters of this book.

Additional Techniques

There are a number of techniques, which are nonstandard, but which can provide further valuable diagnostic and prognostic information in pediatric neoplasms.

Flow Cytometry

Flow cytometry is a technique which allows cell suspensions to be analyzed for the presence or absence of a number of features including cell size, DNA content (ploidy), and the presence or absence of cell surface or cytoplasmic antigens [41].

The general principle is that the sample is a suspension of cells stained with dyes or labeled by antibodies to specific cellular antigens, which are passed through a beam of laser light. The cells and their constituent parts reflect back the light, which is picked up by detectors, which count the number of "events" and analyze the different constituent populations of the cell suspension. The data obtained is presented in a digitized form, usually as a scatter curve or as a histogram.

In the case of neoplasia, the features which are most usefully measured using flow cytometry are ploidy and cell surface marker phenotype. Neoplasms can be either diploid (with normal DNA content) or aneuploid (abnormal DNA content). Aneuploidy usually correlates with tumor aggressiveness and a worse prognosis; however, in neuroblastoma hyperdiploid tumors have a better prognosis [42].

The other major use for flow cytometry is in the study of lymphoid tissue enlargement where one cannot be sure if the process is a bizarre reaction or a lymphoma. If the cells are exposed to antisera which bind to cell surface markers it is possible to define the presence of small clones of atypical cells, frequently aneuploid or abnormally large, within a more heterogeneous cell population within the lymph node and also determine the specific lineage, e.g., T or B lymphocyte in non-Hodgkin's lymphoma or CD30 positive Reed-Sternberg cells in Hodgkin's disease. We have used this technique with some success in patients with lymphadenopathy who present with bizarre lymphoproliferative pathology in various inherited immune deficiency disorders.

Flow cytometry with analysis of DNA content/ ploidy can also be performed in paraffin-embedded tissues in which the nuclei are released from the paraffin and rendered in suspension. The DNA is then stained, the nuclei can be counted and the nucleic acid content and therefore ploidy determined.

Indices of Cell Proliferation in Tumors

Growth fraction and other indicators of cell proliferation in tumor samples correlate with prognosis. The S-phase fraction, i.e., number of cells that have committed to mitosis,

indicate the number of dividing cells. Modern techniques of assessment of cell proliferation are based on immunohistochemical principles using antibodies to proteins involved in the mitotic phase of the cell cycle, or in the phase of cell cycle prior to mitoses, or on the identification of features, which correlate with proliferation.

Ki-67 is a nuclear protein expressed in cells in the proliferative phases of the cell, G1, G2, M, and S. It is the most widely used index for the immunohistochemical assessment of growth faction in paraffin sections and appears to correlate with increased tumor aggressiveness [43]. PCNA is another of these proteins but it is less specific, being present in a proportion of cells in the resting phase of the cell. AgNOR proteins were previously used as potential indicators of tumor aggressiveness and proliferation based on their ability to bind with silver stains but now they have little role in paediatric tumors as there are other more robust and reliable prognostic indicators as described previously [44]. Flow cytometry can also measure indices of cell proliferation using real-time PCR very quickly and reliably and are used in hematological malignancies [41].

p53

The p53 tumor suppressor gene product is involved in many cellular pathways including cell cycle control, DNA repair, and programmed cell death (apoptosis) [45, 46]. In human cancers p53 is the most frequently detected mutated gene, and loss of gene product function by mutation or allelic loss is regarded as a central part of the process of tumorigenesis. The Li-Fraumeni familial cancer syndrome is the result of autosomal dominant transmission of germ line abnormalities of the p53 gene [47, 48] and this syndrome is now recognized as having implications for pediatric neoplasia, particularly rhabdomyosarcoma and adrenocortical carcinoma, as well as several different carcinomas and sarcomas in adults.

Given the key role of p53 in cell cycle regulation, p53 immunohistochemistry is used in pediatric adrenocortical tumors [49]. Abnormal p53 protein accumulates in cells bearing p53 gene mutations. Increased p53 immunostaining correlates with more aggressive behavior and poor prognosis [50].

Future Perspectives

The key challenges for pathology in the near future will lie in the need to support more complex and detailed ancillary investigations of pediatric tumors principally for prognostic purposes, which will increasingly select and direct the therapeutic options in any given case. This challenge will be faced in light of increasingly small diagnostic samples and pre-surgical therapy. Cytological diagnosis, particularly the use of fine needle aspiration, will increasingly become the primary diagnostic methodology providing samples for histological diagnosis and genetic studies.

Tumor profiles generated by micro-array based gene expression will enable more accurate tumor diagnosis, classification and prognostication especially in undifferentiated tumors [51–54]. New targets for therapy will be unmasked providing new tools to predict disease recurrence and response to therapy. In a given tumor, genotypes of the different clones that impart tumor heterogeneity, can be analyzed by Laser capture micro-dissection (LCM) where defined population of target cells can be dissected out from tissue sections by a laser gun under direct microscopic visualization. This can then be compared to normal tissues using advanced molecular techniques to generate specific molecular signatures enabling ‘personalized therapy’ for patients [55].

Another major breakthrough in the field of histopathology is the advent of digital pathology. Digital imaging and information communication technology (ICT) can now provide ‘virtual’ interfaces where the entire glass slide can be scanned into an high resolution digital image that can be transmitted immediately and can be viewed remotely. Telepathology using motorized robotic stage even allows the remote user to access and control the digitalized slide in real-time, which can be viewed simultaneously by many pathologists [56]. Thus, expert consultation can be available more readily in difficult cases. As molecular diagnosis finds greater acceptability and applicability in diagnosis and prognostication of pediatric tumors, wider incorporation of advanced molecular techniques will become an integral part of tumor analysis. It is imperative for the pathologist to embrace these novel molecular techniques and diagnose tumors using their knowledge of tumor morphology.

Surgeons will have to appreciate the pressures placed on the pathologist in these circumstances and develop appropriate protocols with their colleagues to ensure that the essential diagnostic and prognostic processes are not compromised to the detriment of the clinical care of patients. Surgeons and pathologists have a responsibility to ensure the supply and retention of tumor and normal tissue samples for research purposes if progress in diagnosis, prognostication, and treatment is to be maintained.

The role of diagnostic histopathology in the management of pediatric neoplasia is greater today than ever. The remarkable and rapidly accruing in-sights into the molecular biology and cytogenetics of tumors and tumorigenesis has dramatically increased the role of pathology where the pathologist is expected to do more with tumor samples submitted for examination. In the face of rapid advances in the field of pediatric oncology, there is an ever increasing need for all specialists to work together in an organized and

coherent multi-disciplinary team approach. Pathologists, as part of this team, have a vital contribution to make, which is at the fulcrum of clinical management.

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