Cytology Techniques

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Contents

List of Frequently Asked Questions

1. What types of stains are the routinely and commonly used for diagnostic cytology?
Two types of stains are typically performed: Romanowsky stains using air-dried, methanol postfixed smears and Papanicolaou stains using smears fixed in 95% ethanol or Carnoy's solution. Both stains are complementary for cytologic diagnosis.

Romanowsky stains allow better estimation of relative cell and nuclear sizes, preferably highlight cytoplasmic details, smear background elements, and intercellular matrix components. Romanowsky stains are also useful for detecting microorganisms and diagnosing hematolymphoid neoplasms.

Papanicolaou stains allow better visualization of nuclear characteristics such as the nuclear membrane, chromatin, and nucleoli. Papanicolaou stains also yield well-stained cytoplasmic transparency and differential cytoplasmic counterstaining. Many modifications of the original Papanicolaou stains staining method have been made for the Papanicolaou stains [1].

2. What is regressive Papanicolaou staining method?
Papanicolaou Technique I uses Harris hematoxylin regressively. The cells are intentionally overstained and then excess hematoxylin is removed by differential extraction in HCl. Running water bath is required during the staining process.

3. What is progressive Papanicolaou staining method?
Papanicolaou Technique II uses hematoxylin progressively. Since the reduction in staining time prevents overstaining of the cytoplasm, differential extraction in HCl is not necessary. Mayer hematoxylin and Gill hematoxylin are always used progressively because they rarely overstain nuclei, regardless of staining time. Progressive staining is usually recommended for cell samples that do not adhere well to glass slides, since the running water step used for regressive staining can be avoided.

4. What are the dyes used in Papanicolaou stain?
Papanicolaou stain includes both acidic and basic dyes. Acidic dye stains the basic components of the cell and basic dye stains the acidic components of the cell. The multichromatic Papanicolaou stain involves the following dyes:

- Hematoxylin (e.g., Harris hematoxylin, Gill hematoxylin, hematoxylin S) is the nuclear stain which stains cell nuclei blue. It has affinity for chromatin, attaching to sulfate groups on the DNA molecule.
- Orange green (OG) is an acidic counterstain (cytoplasmic stain) which stains matured and keratinized cells. The target structures are stained orange in different intensities.

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- Eosin azure (EA) is the second counterstain which is a polychrome mixture of eosin Y, light green SF, and Bismarck brown. Eosin Y gives a pink color to cytoplasm of superficial epithelial squamous cells, nucleoli, red blood cells, and cilia. Light green SF stains blue to cytoplasm of other cells such as nonkeratinized squamous cells. Staining solutions commonly used in cytology are EA-36, EA-50, and EA-65. The number denotes the proportion of the dyes. EA-50 and EA-36 contain twice the amount of light green used in EA-65. EA-65 is preferred for the thick nongynecologic smears since EA-50 and EA-36 stain the background intensely. Bismarck brown Y stains nothing and is often omitted.

5. What is the hydration step used in Papanicolaou stain?

The series of graded alcohols (50%, 70%, 80%, and 95%) are used for hydration and dehydration which helps to minimize cell distortion.

6. What is the fixative used for Papanicolaou method? How long should the fixation time be?

- Ethanol (95% ethyl alcohol) fixative demonstrates excellent results and is employed by most laboratories. This fixative can be applied for all smears, including nongynecologic specimens such as fine-needle aspiration, as well as gynecologic specimens, such as cervical or vagina smears. This fixative can also be used for the final fixation of all smears of either fresh fluids or those initially collected in 50% alcohol or other preservatives.
- Prior to staining, the smears should be kept in the 95% ethyl alcohol fixative for a few minutes. However, the appearance of the smear will not be altered if the specimen is left in the fixative for several days or even weeks.

7. What is the optimal time between collection and preparation of the cytology samples?

- Urine and CSF need to be processed as soon as possible. These specimens can endure only 1 or 2 hours delay. Refrigeration does not protect cells.
- Unfixed cells from samples containing thick mucus, such as bronchial aspiration and sputum, remain intact longer because mucus coating helps preserve cell structure. Refrigeration can slow growth of bacteria that can damage the cells and break down the mucus. Respiratory samples can be preserved in the refrigerator for 12–24 hours.
- Body cavity effusions, including pleural, peritoneal, and pericardial fluids, may be preserved for 24–48 hours without refrigeration. Body cavity effusions have a high protein content which serves as a tissue culture medium and helps preserve cell morphology.
- Fixation of smears made from FNA specimens varies with the type of stain being utilized. Slides for Romanowsky staining can be left at room temperature until the time for postfixation and staining. Those to be Papanicolaou stained should be placed in 95% ethanol (or equivalent fixative) within a few seconds of preparation. The cell block can be made from the remaining specimen in the fixative.

8. What are the advantages and disadvantages of prefixation?

- Prefixation is recommended when the specimen has to be sent to the distant laboratory.
- Prefixative or preservative is added to the fresh sample until the time of slide preparation. Prefixation can better preserve cellular morphology which is essential for making accurate diagnosis. Some specimens can be preserved for days without deterioration of cells. But the length of preservation time depends on the PH, protein content, and enzymatic activity of the specimens.
- There are disadvantages of using prefixatives. Prefixation can harden the cells in spherical shapes, condense chromatin, and precipitate protein. If the specimen can be processed immediately, no prefixatives are needed.

9. What solutions are used for prefixation?

- Ethyl alcohol (50% solution) is commonly used as prefixative for fluid and can be added in equal volume to the fluids. Higher concentration (>50%) of ethyl alcohol should not be used in fluids rich in protein since the sediment becomes hardened and difficult to be spread on slides. However, 95% ethyl alcohol can be used in collection of gastric washing.
- Methanol can improve preservation of cellular details in specimens with PH higher than 4.5.
- Saccomanno's fixative can be used as prefixative or fixative. Equal volume is added to the specimen.
- Mucoliquefying preservative is designed for use in the collection of mucoid and fluid specimens.
- Cytospin Collection Fluid (Thermo Electron Corporation) is available.
- CytoRich Red was developed for use with automatic cytology system. There is a marked reduction of erythrocytes and background material, when compared to slides prepared from the same specimens collected in Cytospin Collection Fluid.

10. What are the methods used to lyse erythrocytes prior to slide preparation?

Bloody smears can be difficult to interpret. Prior to slide preparation, lysing erythrocytes helps for better recovery of epithelial cells. Carnoy's fixative has been used for hemolyz-

ing erythrocytes. A newer method has been developed for erythrocyte hemolysis. Commercial agents are now available to lyse red blood cells and fix the cellular elements. Two commonly used fixatives are CytoRich Red and CytoLyt Solution. The fixative (1 ml) is added to the sample (per 25–50 ml). The supernatant is poured off after centrifugation. An alternative way is to add solution to the bloody sediment after centrifugation and the sample recentrifuged when the erythrocytes are lysed.

11. What are the components of Carnoy's fixative? How do we use this fixative? What are the advantage and limitation of using this fixative?

- Carnoy's fixative contains 95% ethanol (60%), chloroform (30%), and glacial acetic acid (10%). Carnoy's fixative can hemolyze red blood cells and is thus useful for processing bloody specimens.
- The bloody smears are placed in Carnoy's fixative for 3–5 minutes, until the sediment becomes colorless. Then the smears are transferred to 95% alcohol.
- Carnoy's fixative must be prepared fresh when needed and discarded after each use. This fixative loses its effectiveness on standing, and the chloroform can react with acetic acid to form hydrochloric acid.
- Carnoy's fixative is rapid acting and gives good nuclear preservation and retains glycogen. However, it lyzes red blood cells and dissolves lipids and can produce excessive hardening and shrinkage. Shrinkage of the epithelial cells is greater in Carnoy's fixative than those specimens fixed in 95% ethanol.

12. How are the solutions maintained and how often does it need to be replaced?

- The solutions used for Papanicolaou stain need to be replaced in order to ensure a crisp and well-delineated staining quality. The frequency of replacement depends on the volume and nature of the materials processed.
  - Alcohols for rehydrating and dehydrating need to be changed weekly or discarded each day to avoid filtering these solutions.
  - Hematoxylin solution may be used for a longer period of time if it is stored in a dark bottle. Only a small amount of fresh solution needs to be added every day to replace the solution lost due to evaporation.
  - OG-EA needs to be replaced weekly, or as soon as the cells appear dull and lose crisp contrasting.
  - Bluing solution needs to be changed at least once a day.
  - Xylene should be replaced as soon as it appears tinted with any of the cytoplasmic stains.
  - Water rinse should be replaced after each use.

13. What is the routinely used method for urine or CSF specimen?

For the low cellular and low protein content cytology specimens such as urine and cerebrospinal fluid, various methods have been developed. The goals are prevention of cell loss and satisfactory preservation of morphologic details. Nowadays, cytocentrifugation combined with direct smears of the sediments have been widely used. The cytocentrifuge preparation method results in cell-rich monolayer slides with excellent cytomorphology. Specimens are sent to the cytology laboratory either fresh or having an equal volume of 50% alcohol added to help preserve the specimen. Refrigeration is recommended if transport to the lab or processing could be delayed. However, sample fixed in alcohol can cause cell loss up to 74–98% [2, 3].

14. What is Diff-Quik stain?

Romanowsky stains are neutral stains composed of a mixture of oxidized methylene blue (azure) dyes and eosin Y. The azures are basic dyes that bind acid nuclei and result in a blue to purple color. The eosins are acidic dyes that are attracted to the alkaline cytoplasm, producing red coloration. There are many commercially available Romanowsky stains modified for rapid performance.

Diff-Quik is a commercial Romanowsky stain variant, commonly used in cytological staining. The results produced with Diff-Quik are similar to those obtained with Wright-Giemsa. The three-step staining procedure (methanol, Hema-Diff solution 1, Hema-Diff solution 2) produces excellent staining results in just over 1 minute. So the Diff-Quik-stained smear is processed much quicker than the routine Papanicolaou stain.

15. What are the differences between conventional smear, SurePath, and ThinPrep?

- The conventional manner of collecting sample is spreading the sample on the glass slides with brush instruments.
- SurePath (Becton, Dickinson and Company, Franklin Lakes, NJ) and ThinPrep 2000 System (Hologic, Marlborough, MA) are two such liquid-based test systems currently approved by the FDA for cervicovaginal testing. By using the SurePath and ThinPrep methods, the sample is directly transferred to a vial of fixative after sample collection.
- Both ThinPrep and SurePath methods show a clean background without air-drying artifact. The ThinPrep procedure takes about 70 seconds per slide and results in a thin deposit of cells in a circle 20 mm in diameter (contrast with cytospin: diameter = 6 mm). SurePath prepares an evenly distributed deposit of cells in a circle 13 mm in diameter. In most cases, only a fraction of the sample is

used to prepare the slide used for diagnosis. If needed, the residual sample is available for additional ThinPrep slide preparation, cell block preparation, or molecular diagnostic testing (e.g., high-risk HPV, chlamydia, gonorrhea).

16. What is a cell block and what are the advantages and disadvantages of cell block?

- Cell block technique is an old method for preparing cytological materials. The basic steps of cell block preparation include fixation, centrifugation, and transferring a cell pellet for paraffin embedding. This technique is recommended for processing all residual cytological materials following cytological preparations. The materials suitable for cell block processing include materials obtained from fine-needle aspiration/biopsy or other sample types such as effusions, urine, bronchial brushing, and sputum.
- The cell block method uses histologic techniques for processing and thus offers the following advantages:
  - In cell block preparations, fresh cellular materials are fixed in formalin and processed via the identical procedures for regular paraffin-embedded tissues. Thus, the results are equivalent to other formalin-fixed and paraffin-embedded surgical specimens.
  - The cell block materials often contain tissue fragments which show the histologic patterns besides cytologic features, whereas cytosmears usually lack this useful information and may preclude a definitive diagnosis in some cases.
  - Multiple sections can be obtained from the cell block and can be utilized to perform different studies such as immunohistochemical stains, special stains, ultrastructural studies, and molecular studies. The control slides can be readily found from other paraffin-embedded pathology tissue. Compared to direct smears, immunostaining on cell block sections is less likely to have background staining (Fig. 1.1).
  - Both cell block material and smears yield comparable result for molecular testing.
- The disadvantages of the cell block technique are as follows:
  - Because of suboptimal cellular recovery, multiple needle or brushing passes may be needed in order to obtain adequate material for a good-quality cell pellet.
  - The turnaround time is increased, since the processing time for a cell block is longer than direct smears.
  - Cell block technique is more labor-intensive and thus increases medical expense.

17. What are the methods used in cell block preparation?

A good cell block preparation depends on the presence of adequate cellular aggregates or tissue fragments. The challenging step is to harden the cell pellet so that it can be easily transferred without losing diagnostic material. A variety of technical modifications have been reported and are still being improved [4].

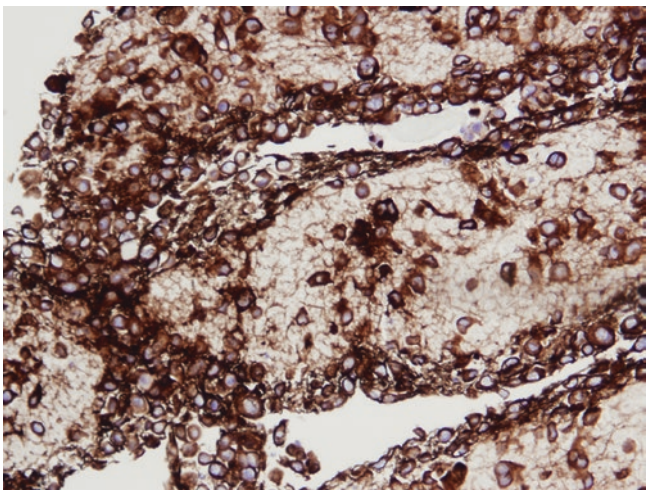

Fig. 1.1 Case 1. Tumor cells on cell block tissue sections are positive for CK-7 by immunohistochemical stain

- Normal saline needle rinse method:
  - This method is utilized to rinse the fine-needle aspiration needle in 20–30 ml of normal saline, followed by centrifugation and collection. Alternatively, formalin (10 ml) or paraformaldehyde or ethanol (50%) can be used to rinse the materials for cell block [5], or an equal amount of 95% ethanol and 10% formalin can be used as fixative.
- Tissue coagulum clot (TCC) method:
  - The TCC method allows the clot of tissue and blood mixture to form in the lumen of the needle. It is used to obtain better recovery of cellular materials in cell block sections. As the coagulum streams out from the needle tip, it is collected onto a piece of filter paper and slightly air-dried. The tissue coagulum is then transferred into a formalin container and subsequently processed in the histology laboratory [5–7].
- Plasma thrombin or thrombin clot method:
  - Plasma and thrombin are added to the cellular pellet to enmesh the cellular material. Although this method may cause an uneven concentration of cells, the problems can be avoided by continuous agitation to disperse the cell population evenly throughout the fibrin mesh [8, 9].
- Agar embedding, collodion bag methods:
  - Due to the difficulties in the recovery and processing of small tissue fragments, manual cell block methods have been alternatively utilized. Basically, the concentrated sediments are supported by a substance such as

agar or a collodion bag. Since agar solidifies below 50 °C, this property can be used to form the cell pellet. The collodion bag effectively coats the tube before the cell pellet is added. This collodion bag method is recommended for scant cellular materials. Collodion is a nitrocellulose substance which can be used for friable tissue such as brain. Other alternative methods have been used including HistoGelTM, gelatin albumin, pre-gelatinized starch, sodium alginate, gelatin foam, and polyvinyl alcohol foam [10–13].

- ShandonTM CytoblockTM method:
  - This cell block preparation system can concentrate cells by cytocentrifugation in a Thermo Shandon Cytospin. The Cytoblock cassettes and reagents are available in the kit [14].
- Rapid cell block method and automatic cell block system:
  - A tissue cassette and filter assembly are designed to deposit the needle rinse in one plane for microtomy. This technique allows to yield a better tissue fragment. The automated cell block preparation system has been developed to recover the small tissue fragments and rapidly deliver the tissue in paraffin for histological sectioning in less than an hour. Cellient automated cell block system is a newly introduced CB technique, and more studies are required to validate the results of this system [15–18].

18. What are the advantages and disadvantages of using direct smears for the immunostains?

- Direct smears are sometimes the only available materials for diagnosis. In the absence of cell block, immunoperoxidase studies can be performed on the direct smears and provide useful information for diagnosis (Fig. 1.2). If the smears are cellular, the cells can be peeled off and transferred to multiple slides for a panel of immunostains. The previously Papanicolaou-stained slides can be used for immunostains.
- However, there are some disadvantages of direct smears:
  - It is more difficult than cell block to interpret the results due to thick smears, overlapping cells, poor cytoplasmic preservation, and the higher background staining that are frequently associated with staining on direct smears.
  - Cells can get lost during staining.
  - Cells might be disrupted during the mechanical process of making direct smears, which may cause leakage of antigens.
  - In contrast to cell blocks, staining on smears lack validated control tissue. This might cause difficulty in interpreting the staining result. Some immunostains cannot be reliably interpreted on smears fixed with alcohol-based fixative, such as S-100.
  - Three-dimensional groups of cells may trap antibodies leading to nonspecific staining. Because of lacking histologic pattern, the benign entrapped cells could be erroneously interpreted as tumor cells, leading to false-positive interpretation. False negative can also potentially occur on a small specimen if the tumor cells express some markers only focally or heterogeneously.

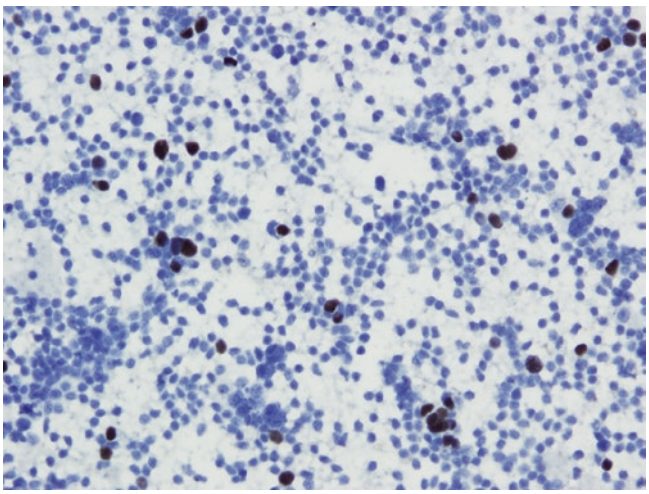

Fig. 1.2 Case 2. Tumor cells on a Papanicolaou-stained smear are positive for Sox-10 by immunohistochemical stain

19. What is a cytocentrifuge? What are the advantages and disadvantages of using cytocentrifuge for immunostains?

- A cytocentrifuge (cytospin) is a device that spins cells in a fluid suspension directly onto a glass slide. Multiple cytocentrifuge slides can be prepared at the same time.
- The advantages of using cytocentrifuges for immunostains are listed as follows:
  - Since cells are concentrated in a small area, only small amounts of antibodies need to be used.
  - There is less background staining in cytocentrifuge slides than in smears.
- The disadvantages of cytocentrifuges are as follows:
  - Loss of cells may occur during cytospin.
  - Lack of good control tissue for staining, similar to staining on smears.

20. What is PAS stain and PASD stain? What is GMS stain and how to interpret the stain? What methods are used to stain pigments, such as melanin and iron (hemosiderin)?

- Periodic acid-Schiff (PAS) is a commonly performed special staining method, which can detect polysaccharides and mucosubstances. Cells rich in glycogen and microor-

ganisms such as fungi are PAS-positive (magenta red) in standard PAS-stained slide. Extracellular mucin with glycoproteins and glycolipids are also PAS-positive.
- PASD stands for periodic acid-Schiff (PAS) with diastase digestion. Cells with glycogen can be determined if they are unstained in the diastase-treated slide. Therefore, PASD can be used to detect both intracellular and extracellular mucin as well as discriminate between mucinous and glycogen-rich tumors.
- Grocott-Gomori's (or Gömöri) methenamine is a silver stain, abbreviated GMS. Fungi and *Pneumocystis jiroveci* stain black sharply delineated. Glycogen and mucin stain rose to gray.
- Most common pigments are iron, hemosiderin, or melanin. Iron stain (Prussian blue) is used for staining iron or hemosiderin. It stains iron-containing pigments (hemosiderin) blue and stains nuclei red. Currently immunostains such as melan A and HMB45 are used for identifying melanoma cells with melanin pigment.

21. What is Oil Red O stain?

Oil Red O stain is a type of lipid stain. It stains fat orange to bright red and stains nuclei blue. Since histiocytes phagocytize fat in lipoid pneumonia, Oil Red O stain can be used to stain fat in sputum or bronchial washing specimens to determine if there is lipoid pneumonia. Sometimes it is also used to stain smears for fat.

22. What are universal precautions?

Universal precautions refer to disease prevention measures that should be utilized by all health care workers who may be exposed to blood or body fluids. Universal precautions are recommended by the *Centers for Disease Control* and Prevention (*CDC)* for all the patients because infection with HIV or other blood-borne pathogens cannot be reliably predicted by clinical findings alone.

23. What is the artifact called "cornflakes" and how does it solve the problem?

Cornflakes, also called "brown artifact," is a common artifact seen on coverslipped glass slides. It appears as a brown refractive pigment-like substance on the surface of the cells. It occurs when the mounting medium and coverslips are applied too slowly. This can result in evaporation of xylene before coverslipping since air is trapped and deposited on the surface of the cells, causing distinct appearance so called "cornflake." If this happens, the slide needs to be recoverslipped. The slides may be soaked in xylene and alcohol. Then the slides are rinsed in running water and restained.

24. Why do we need fixation before immunostaining?

Delayed in fixation may cause diffusion of antigens from the intracellular sites leading to reduced immunoreactivity. Autolysis can also cause nonspecific binding of antibodies. Thus, appropriate fixation is necessary for preservation of antigens and preserving morphological integrity. Buffered neutral formalin (10% formaldehyde) has been commonly used as fixative for cell block samples because of its preservation of morphological details, as well as its low cost and ease of preparation. The principle of formalin is the formation of hydroxylmethylene-type linkages between protein end groups and formation of coordinate bonds for calcium ions [19].

25. What are the antigen retrieval methods used for cytology specimen?

- Prolonged fixation in formalin may lead to reduced immunoreactivity of many antigens due to extensive crosslinking. Crosslinks may either directly affect the epitope itself or form between two or more different molecules masking the epitope. Antigen retrieval methods have been used to unmask the antibody-binding site, restore the epitope reactivity, and significantly enhance the immunostaining signal of the antigen.
- There are two main antigen retrieval techniques. One method is called proteolytic-induced epitope retrieval (PIER). Proteolytic enzyme digestion can break the crosslinks in the antigen and expose the masked epitopes. Optimization of proteolysis time, enzyme concentration, pH, and temperature are important to obtain optimal results. Over-digestion may result in false-positive staining; in contrast, under-digestion may cause a false-negative result.
- The other method, which is a major advance in antigen retrieval, is called heat-induced epitope retrieval (HIER). It is developed by using moist microwave heating of sections and has already been applied to cytological samples. The possible mechanism of this method is that heating can lead to disruption of formalin-induced bonds between calcium ions and proteins. However, heat retrieval methods could potentially expose unwanted epitopes of sequence in other antigens which could lead to unexpected cross-reactions and false-positive results. HIER and PIER can be combined in some cases [20–23].

26. Why is it important to use controls in immunohistochemistry stain?

It is important to use the positive and negative controls for immunohistochemistry stains for tissue sections as well as for cytology preparations. The positive controls can be obtained from the tissues with the antigens of interest. The negative controls can be acquired from tissue with absence of that antigen. If there is only a single slide available, internal control can be used as the negative control. The best positive and negative control reactions are those tissues present within the same patient. The controls should be processed in

the same manner as the test case. As part of the quality control process, positive and negative controls need to be validated before they could be used in the clinical assays. The validation of ambiguous results should be evaluated by using antibodies to different epitopes of the same molecule and by the use of antibodies to related markers [24–32].

Controls for non-formalin-fixed cytology smears ideally should be the cells that are prepared and stained in the same manner. However, in reality, it is difficult to obtain such controls and often histology samples are used for the controls in such situation. In addition, the antibody concentrations are not always customized for cytology specimens. Therefore, a disclaimer should appear in the bottom of the cytology report [33, 34].

27. What is immunofluorescence and its application in pathology?

Immunofluorescence techniques were first developed in the 1940s. The basis of immunofluorescence is labeling of antibodies with a fluorescent substance. The antigen-antibody product therefore can be visualized in fluorescence microscope in cells or tissues. This technique has been commonly used as a diagnostic tool in renal pathology as well as other immunologically related pathology. However, this technique has relatively low sensitivity, lacks morphological details, results in short storage time of the stained slides, and requires fresh frozen tissue, which limits its use in surgical pathology. In contrast, fluorescence in situ hybridization (FISH) depends on the formation of a hybrid between a fluorescently labeled DNA probe and its target chromosomal DNA, therefore permitting localization of a particular DNA sequence to a specific chromosome region [35, 36]. UroVysion, which is used in upper urinary tract surveillance, is a successful example of applying fluorescence in situ hybridization (FISH) technique in cytology.

28. What are TMA and CMA and what are their utilities?

Tissue microarrays (TMA) are assembled by taking preexisting formalin-fixed paraffin-embedded tissue blocks and re-embedded in a single recipient paraffin block. TMA blocks can hold up to several hundred tissue samples per slide which can contain a variety number of tissues including normal and tumor tissues [37–39]. Since a variety of samples are embedded on a single block, only a small amount of antibody is required for hundreds of samples examined. The results for these samples are more comparable because the conditions in the staining process are identical for all the samples, including incubation time, antibody concentration, and antigen retrieval conditions. A potential limitation of TMA is that only a relatively small tissue volume is represented in each core.

TMA may be used for quality control/quality assurance in the immunohistochemical lab. It can be used to analyze sensitivity and specificity of new antibodies, establish positive and negative control tissues, and discover new biomarkers. Molecular profiles can also be analyzed on the TMA blocks, and the results can correlate with diagnosis, prognosis, and management for tumors or disease. New genes and molecules may be potentially discovered on the samples. TMA also preserves archived tissues from rare tumors.

Cytology microarrays (CMA) have been constructed by some labs [37–39]. CMA is assembled by depositing cell suspensions on a glass slide in an array pattern. The process can apply to a wide range of cell suspension materials. CMA has a great potential in clinical research and practice.

29. What is flow cytometry?

Cytometry is the science of analyzing and classifying cells based on cell measurements. Flow cytometry technique plays an important role in defining cells and their functional status. Cells flow in single files through a measuring sensor, and cells in suspension are measured. Flow cytometry is able to make measurement and record a large number of cells in a short period of time and precisely control the stain conditions for all cells. It can not only measure the cell features but also quantify the cells' constitutions and functional characteristics.

30. What is the clinical application of flow cytometry technique? What is the minimum number of cells in fluid or FNA sample sufficient for flow cytometry test?

- Flow cytometry analysis has many applications in research and clinical laboratories. This technique has been carried out on any cell suspensions, including peripheral blood, bone marrow, effusions, and spinal fluid.
- The most common clinical application of flow cytometry is in the diagnosis of lymphoproliferative disorder. Flow cytometry plays a variety of roles in the field of lymphoma/leukemia, including diagnosis, classification, staging, minimal residual disease detection, evaluation of prognostic markers, detection of target molecules for therapies, molecular studies, and evaluation of multidrug-resistance markers. Flow cytometry has been proved to be an important diagnostic tool in cases of lymphoma from which the tissue provided was heavily involved with B cells and had monoclonal light chain restriction [40].
- The second application is DNA flow cytometry of solid tumors. Many solid malignant tumors are composed of cells with abnormal DNA contents, corresponding to abnormal chromosomes. Flow cytometry can analyze cellular DNA content and distinguish aneuploidy from diploidy in tumor cells. It can also measure proliferative activity of tumor cells (as expressed by S-phase fraction) by displaying tumor cell cycle distribution. The well-

differentiated neoplasms often show diploidy DNA modal pattern and demonstrate a more favorable clinical course. But it remains unclear if DNA contents are independent prognostic factor in poorly differentiated tumors.
- Only little sample preparation is required for flow cytometry analysis. A total of five million cells is usually sufficient for analysis.

31. What is in situ hybridization?

In situ hybridization (ISH) technique combines molecular and histochemical approaches to detect and localize DNA/RNA which may be present in the nucleus of a cell or on a chromosome. ISH can identify specific chromosomal abnormalities. It is a sensitive method since it has the ability to detect low copy gene expression on single cells and chromosome. It is also a specific tool because it can identify individual abnormal cells in a variety mixture of cell population [41]. ISH has been applied in a number of diagnostic situations, for example, in the determination of the presence of various types of HPV in precancerous cervical lesions and cancer.

32. What is the principle of FISH?

Fluorescence in situ hybridization (FISH) is a powerful molecular cytogenetic technique and is applicable to almost all types of cytological specimens. FISH technique depends on the formation of a hybrid between a fluorescently labeled DNA probe and its target chromosomal DNA sequences of interest, therefore permitting localization of a particular DNA sequence to a specific chromosome region [35, 42]. FISH has been widely used to detect chromosomal abnormalities. FISH can be applied in quantitation of chromosomes and genes, including chromosomal amplifications, deletions, translocations, and aneusomy in nuclei [35, 41–43].

The specific DNA probe is chosen to detect target DNA sequence (size ranging from less than 1 kb to several mega bases) on a specific chromosome site. The DNA probe is first labeled with an immunofluorescent compound such as biotin-11-dUTP or digoxigenin-11-dUTP. The targeted DNA and the probe are denatured. Then the labeled probe is hybridized to the target overnight either to metaphase chromosome or nondividing interphase preparations. The unbound probe is removed by washing. The resultant fluorescent signal is detected under fluorescence microscopy.

33. What is the application of FISH in cytology specimens? What are the requirements for the cytology preparation for FISH?

- FISH technique has many applications in cytology specimens. The initial application was the diagnosis of urothelial cancer in urinary sediment by UroVysion system [44, 45]. FISH techniques have currently been applied to a variety of cytology specimens (e.g., urine, effusion, sputum or bronchial washing, and lymph node aspirate) (Case 2, Fig. 1.3) [46, 47].
- FISH is applicable to almost all types of cytologic specimens including formalin-fixed paraffin-embedded cell block, air-dried or alcohol-fixed direct smear, cytospin preparation, cellular touch imprint, or liquid-based specimen (e.g., ThinPrep or SurePath).
- Direct smear and cytospin preparation appear to be superior to cell block sections because the gene copy number can be enumerated on monolayered tumor cells with entire nuclei without tissue section-associated truncating artifacts, thus yielding a more accurate score. Although formalin can be used for fixation, the best results are generally obtained with methanol/glacial acetic acid (Carnoy's solution) or acetone.
- The hybridized FISH specimens can be washed to remove the first set of FISH probes, followed by rehybridization with another set of FISH probes. If hybridization is performed within a few weeks after cytological preparation, greater than 95% the success rate can be achieved.

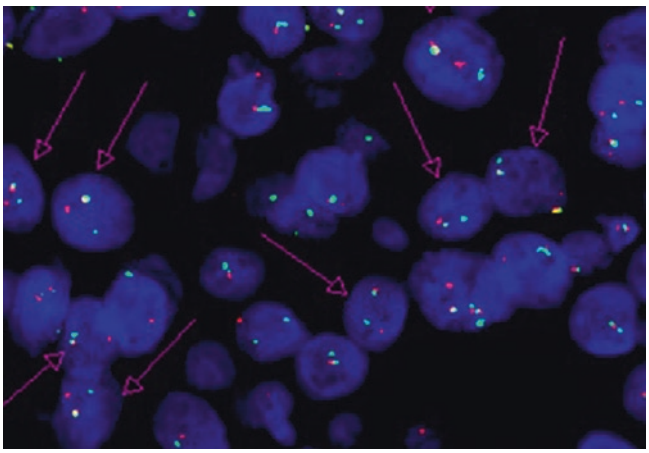

Fig. 1.3 Case 3. FISH study on the tumor cells within a cytospin shows t (11: 14) translocation

34. How many cells are required for FISH scoring?

There are many ways to score the FISH signals on a slide. In a research setting, the scoring of at least 100 or 200 consecutive cells is often considered as adequate. While in the standardized diagnostic analysis, the number of scored cells is usually lower. In the multitarget FISH assays, not more than 25 cells are required for scoring. It may be sufficient to score as few as 20 cells in amplification detection (e.g., HER2 FISH). The scoring should be performed on cells of interest such as malignant cells [48]. The current applications in cytology are scoring of HER2 gene amplification in breast cancer and aneusomy detection in urinary specimens in conjunction with UroVysion multitarget FISH testing.

35. What types of probes are used for FISH?

Two types of probes are used for FISH. The first type is locus-specific indicator (LSI), which hybridized to specific loci or genes of target. The LSI probe can detect translocation and rearrangement of genes or chromosomes. The abnormal signal patterns (e.g., fusion, split) in the nucleus can be visualized by using fluorescence microscope. The second type of probe is centromere enumeration probe (CEP). CEP can detect gain or loss of a specific gene region or chromosome. The numbers of copies of targeted chromosomes can be identified. FISH can detect the presence of microdeletions or duplications that are not apparent by conventional cytogenetic studies. Classical satellite, alpha-satellite centromeric, or beta-satellite centromeric probes are useful for the detection of copy number aberrations in interphase nuclei.

36. What is I-FISH?

Interphase FISH (I-FISH) allows assessment of chromosomal abnormalities in cells independent of their proliferative capacity. The I-FISH method is particularly useful for cytology specimens since it required only a few cells and it can facilitate a definitive diagnosis for the indeterminate cases due to scanty cellularity [49]. Cell block, direct smear (air-dried, alcohol-fixed, or archived), cytospin preparation, or cellular touch imprint are all suitable for I-FISH [50–53]. However, I-FISH is unable to identify "unexpected" chromosome abnormalities which are not designed.

37. What is the clinical significance of testing HER2 status?

Determining HER2 expression status is important for prognosis prediction and treatment decision in breast cancer. Overexpression of the HER2 protein (via immunostaining) or HER2 gene amplification (via FISH) is associated with a poor clinical outcome, and more importantly, is a prerequisite for anti-HER2 (e.g., trastuzumab/Herceptin) treatment. Dual-probe FISH is the preferred method over immunostaining [54]. HER2 status determined with FISH is more stable and reproducible and more strongly correlated with responsiveness to anti-HER2 therapy.

38. What are the differences between FISH, CISH, and SISH for HER2 test?

- Most of the HER2 gene tests are performed by immunohistochemistry (on cell block or tissue section) and FISH (on smears or cell block). However, there are some disadvantages of using FISH. Fluorescence microscope has to be used in the dark working area to visualize the cellular morphology, and the fluorescence signals fade with time.
- A new developed technique is called bright-field chromogenic in situ hybridization (CISH). CISH detects gene copy number by using a conventional peroxidase reaction and allows enumeration of the signals with simultaneous histologic examination under light-field microscopy. This technique is more straightforward than FISH for scoring. Signal counting is much easier than FISH because cellular morphology and histology are easier to be recognized by using counterstains in CISH. Although CISH has shown potential to replace the I-FISH technique in detection of HER2 gene amplification in tissue sections, its application in cytological specimens is still under investigation [48].
- Another technique is called silver in situ hybridization (SISH) which also uses bright-field technology. SISH can produce better contrasted signals than CISH.
- However, both CISH and SISH are required to have two separate slides, with one for HER2 counting and the other for chromosome 17 counting. In contrast, two-color FISH can count both on one slide. Two dual-color HER2 CISH/SISH assays have been developed recently [35, 42–48].

39. What is the principle of DNA microarray?

DNA microarray technology has emerged as a high-throughput approach for analysis of gene expression profiles and patterns. Thousands of oligonucleotides can be arrayed on a square centimeter chip, a solid supporting system. The mRNA is obtained from fresh tissue and serves as a template to create cDNA. Cancer cell cDNA is labeled with red fluorescence, while normal cell cDNA is labeled with green fluorescence. Labeled mRNA from the cells of interest are hybridized to their corresponding sequence on the array. Data is analyzed. The red signals indicate higher expression of the genes in cancer cells. While the green signals indicate higher expression of the genes in normal cells. The yellow signals indicate an equal expression of that gene in both normal and cancer cells. The matching genes are identified and the data analysis requires a computer analysis [55–57].

In contrast to the previous molecular methods, such as PCR, northern blot analysis, which can only study a specific gene expression level at a time, the microarray technology can study thousands of genes and a wide variety of tumor types which are contained in a single chip and detect which genes are overexpressed or active in cancer cell. This technique provides a new system for tumor classification and offers new information about which genes are involved in different forms of cancer and provides therapy-based molecular profiles.

40. What is the principle of polymerase chain reaction (PCR)? What are the applications of PCR in cytology?

Polymerase chain reaction, or PCR, is a molecular technique used to make multiple copies of DNA segment. The template DNA is heated to 94 °C, which separates the two strands of the double helix. When the temperature is lowered to 55 °C,

the primers anneal to the ends of the target sequence. Once the primers have annealed to the template, the temperature is increased to 72 °C. This is the optimum temperature for the Taq polymerase to elongate DNA using the primers as starting point. The newly synthesized DNA strands serve as template for the next PCR cycle. PCR is now often used in the cytology laboratory. The applications include identifying infectious organisms, detection of gene mutations in solid tumors, and diagnosis of lymphoma by B- and T-cell clonal gene rearrangement tests.

PCR-based methods have been successful in diagnosing viral infections in cytological samples, such as high-risk type of HPV infection and herpes virus infection in cervicovaginal cytological specimens. In *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection, PCR methods are also available for liquid-based gynecology cytological specimens. In addition, applications of PCR-based assays have been done on fine-needle aspiration specimen, such as identifying oncogene mutations or amplifications or activation, tumor suppressor gene deletions or mutations, and other genetic abnormalities, such as *BRAF* mutation in papillary thyroid carcinoma and unique translocations in sarcoma. Detecting gene mutations by PCR and other sequencing methods in a variety of cytopathologic samples has shown promise to increase the sensitivity in cytopathologic diagnoses. In addition, B- and T-cell clonality in malignant lymphomas can be identified by using DNA from fine-needle aspirates and other cytopathologic specimens.

41. How are the high-risk types of HPV tested by hybrid capture 2 (HC2) assay?

Hybrid capture 2 (HC2) assay is performed on cervical samples. Nucleic acids of samples are released and denatured, followed by HPV target DNA hybridization with a mixture of HPV-type-specific labeled RNA probes. The hybrids of DNA-RNA are captured on to the surface of a microtiter well coated by specific antibodies. Immobilized hybrids are then reacted with alkaline phosphatase-conjugated antibodies specific for RNA-DNA hybrids and detected with a chemiluminescent substrate. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted that is measured by a luminometer.

42. What is telecytology? What are the different types of digital cytology imaging?

Telepathology has been defined as "the practice of transmitting digital pathology images of microscopic or gross findings through telecommunication networks to remote viewing locations for diagnosis, storage or education." Telecytology is a branch of telepathology. Telecytology refers to "diagnostic cytopathology performed on digital images." Telecytology was first used with mainly cervical smears, and its application in cytology has been expanded [58, 59].

- There are three types of microscopic digital imaging:
  - The first is static (still images). The pathologist selects the areas, and the images are captured with a camera and are digitized. The image data is transmitted to a remote personnel through the Internet [60–62].
  - The second type is dynamic (real-time/live microscopy). The microscopic live images are directly transmitted to the recipient by live telecommunication. It finds application mainly in frozen section telepathology and rapid on-site evaluation for fine-needle aspiration [63–65].
  - The third type is virtual microscopy. The glass slides are scanned and converted to digital images. The specialized software allows simulation of panning around and zooming in or out using a conventional microscope. As whole slide imaging (WSI) technology has developed over the past years and it provides us with virtual microscopy that can be accessed anywhere in the world by using the Internet any time. WSI and virtual microscopy are increasingly being utilized in cytopathology.

43. What are the clinical applications of telecytology? What are the advantages and limitations of telecytology?

- Digital pathology has been applied to many fields, including remote frozen section diagnosis, rapid on-site evaluation (ROSE) of fine-needle aspiration, consultation, education, and slide archiving [66–72]. With the integration of LIS and the change of workflow, digital pathology may change the traditional practice of cytopathology in the future.
- The advantages of digital cytopathology are as follows:
  - In contrast to the tissue block in histology, every cytology slide is irreplaceable and unique. Implementation of digital cytology has a helpful aspect especially when the slides are damaged or defective. The permanent digital copy can be kept for archiving.
  - Digital data establishs a permanent reproducible record that can readily be communicated and displayed at variable magnifications. Digital imaging has been employed in evaluation of gynecologic material obtained by cytobrush technique (PAP tests) [73, 74] and nongynecologic material [75–77]. They are ideally suited for teleconsultation. Telecytology would diminish the need to physically send slides over long distances which could increase the chance of damaging the slides. Case consultation by transmitting images would reduce the time and cost.
  - Telepathology is an excellent teaching tool. Learning cytopathology with virtual microscopy is more efficient than from glass slides. A universal database with

digital cytology images of a variety of cases can be accessible to cytopathologists from everywhere. It can also be used in distance-based continuing education at teleconferences by using images accompanied by lectures, real-time microscopy sessions.
  - Telecytology has also been used as a tool for quality assessment and improvement in the evaluation of cytologic samples. It also finds application in cytology proficiency testing and various research purposes [78–80].
- The limitations of telecytology are as follows:
  - Cytology specimens offer unique challenges, particularly for direct smear preparations where three-dimensional groups are commonly encountered. Since cytological material tends to be distributed throughout the glass slides, intensive screening is required. Cytology relies on individual cell features evaluated at a higher magnification and good-quality images which telepathology may not fulfill. These factors become the barrier to the adoption of whole slide imaging for routine diagnostic cytopathology [81].

44. What are the circulating tumor cells and what is their clinical application?

The presence of circulating tumor cells in blood from metastatic carcinoma patients have been reported to be associated with short survival. Technical advances have facilitated the detection of rare circulating tumor cells. The CellSearch technique (Veridex) and CellSpotter assay systems were designed to detect rare tumor cells in whole blood. The principle of this system is based on the immunostaining method. The epithelial tumor cells are separated from the blood by antibody-coated magnetic beads and identified with the use of fluorescently labeled antibodies against cytokeratin. Then the epithelial tumor cells are enumerated. Circulating tumor cell detection for breast cancer patients have been reported to be an independent prognostic factor for early relapse [82–95].

45. What is next-generation sequencing (NGS)? What is the application of NGS in cytology?

Next-generation sequencing (NGS) is a DNA sequencing technology. Using NGS, an entire human genome (three billion base pair) can be sequenced within a short period of time. The NGS platforms perform sequencing of millions of small fragments of DNA in parallel. Bioinformatics analyses can piece together these fragments by mapping the individual reads to the human reference genome. Each of the three billion bases in the human genome is sequenced multiple times, providing high depth to deliver accurate data and an insight into unexpected DNA variation. NGS can be used to sequence entire genomes or constrained to specific areas of interest, including all 22,000 coding genes (a whole exome) or small numbers of individual genes.

Traditional Sanger sequencing is restricted to the discovery of substitutions, small insertions, and deletions. In contrast to Sanger sequencing, NGS captures a broader spectrum of mutations. The spectrum of variation comprises substitutions, insertions and deletions of DNA, large genomic deletions of exons or whole genes, and rearrangements such as inversions and translocations.

Rapid on-site evaluation (ROSE) for adequacy assessment is performed on the FNA materials to allow immediate triaging of materials for molecular testing such as NGS. The FNA sample tends to contain a purer population of tumor cells than tissue biopsy since cells are more easily aspirated than stromal components. In addition, there are better-quality nucleic acids from non-formalin-fixed direct smears. Therefore, aspiration cytology specimens provide an excellent source of cellular material for molecular studies. However, additional validation studies for a variety of cytology specimens, fixatives, and stains need to be performed on the cytology specimen for molecular study [96–105].

Case Presentations

Case 1

Case history

A 78-year-old female who has no prior history of malignancy and presented with abdominal discomfort. Abdominal CT showed a 12 × 8 cm necrotic mass in the right hepatic lobe in a background of cirrhosis and intrahepatic biliary dilatation and splenomegaly. The mass encases the right portal vein branches, and there is evidence of portal hypertension. The patient had elevated AST (49 IU/L; reference range: 15–46 IU/L) and Alkaline phosphatase (190 IU/L; reference range: 38–126 IU/L).

Specimen source

Ultrasound-guided fine-needle aspiration was performed. A Romanowsky-stained smear, a Papanicolaou-stained smear, and a cell block were made from the aspiration.

Cytologic findings

- Smears are cellular and contain well-preserved cells in loosely cohesive groups and singly dispersed forms. The tumor cells are polygonal with hepatoid appearance. The cells are uniform and have round to oval nuclei, coarsely granular chromatin, and small nucleoli. Cytoplasm is scant to abundant and appears granular. The neoplastic cells have a plasmacytoid appearance (Figs. 1.4 and 1.5).

- Cell block contains loosely cohesive neoplastic cells in a background of necrosis. The tumor cells have pleomorphic hyperchromatic nuclei, irregular nuclear membrane, prominent nucleoli, and pink cytoplasm. Apoptotic bodies are noted (Fig. 1.6).
- Needle core biopsy contains cohesive tumor cells forming glands. The tumor cells are round to polygonal shape with hepatoid appearance. The background shows desmoplastic stroma. Mitotic figures are seen (Fig. 1.7).

Differential diagnosis

- Cholangiocarcinoma
- Hepatocellular carcinoma
- Metastatic carcinoma

IHC and other ancillary studies

- Pan-CK positive
- CK-7 positive (Fig. 1.1)
- CK20 negative
- CDX-2 negative
- HepPar1, arginase, and glypican3 negative (Fig. 1.8)

Final diagnosis

- **Poorly differentiated adenocarcinoma**

Take-home messages

- Clinical and radiologic impression was hepatocellular carcinoma (HCC) versus cholangiocarcinoma; nonetheless, HCC is favored based on cytologic features.
- In well-differentiated hepatocellular carcinoma (HCC), the thickened cords of neoplastic hepatocytes are often surrounded by spindle-shaped endothelial cells. However, poorly differentiated HCC is difficult to distinguish from cholangiocarcinoma and metastatic carcinoma by cytomorphological features alone. In addition, there are rare cases of combined hepatocellular-cholangiocarcinoma (cHCC-CC) that present a special diagnostic challenge. Immunohistochemical studies are helpful in the challenging cases by using cytological material such as cell block or smears.
- Subsequent immunostains performed on cell block sections show tumor cells to be positive for PanCK and CK7 and negative for HepPar1, arginase, glypican3, CK20, and CDX2. Since the immunohistochemical profile was not specific for any primary origin, a differential diagnosis of poorly differentiated adenocarcinoma from the pancreaticobiliary tree, including primary intrahepatic cholangiocarcinoma, is rendered.
- This case demonstrates a well-established truth. Sometimes morphology and clinical findings are more useful than magic markers. This is the kind of case that is all too common and frustrates clinicians who want a definitive answer and pathologists who know that many times only a differential diagnosis can be rendered based on clinical judgement and morphology.

Reference: [105]

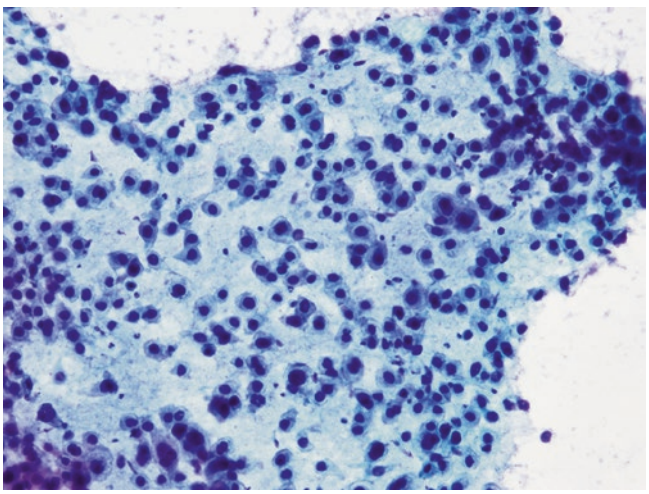

Fig. 1.4 Case 1. Cellular smears show well-preserved cells in loosely cohesive groups and singly dispersed forms. The tumor cells are polygonal with hepatoid appearance. The cells are uniform and have round to oval nuclei, coarsely granular chromatin, and small nucleoli. Cytoplasm is scant to abundant and appears granular. The isolated cells have plasmacytoid appearance (Papanicolaou stain)

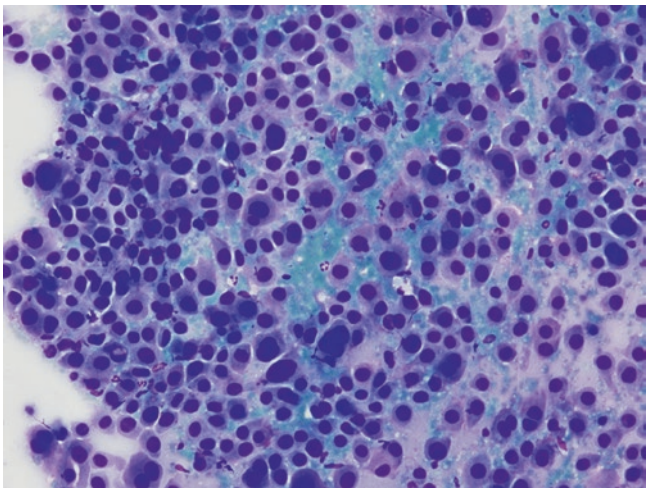

Fig. 1.5 Case 1. Cellular smears show well-preserved cells in loosely cohesive groups and singly dispersed forms. The tumor cells are relatively uniform round to polygonal nuclei and hepatoid appearance, coarsely granular chromatin, and small nucleoli. Cytoplasm is scant to abundant and appears granular. The isolated cells have a plasmacytoid appearance (DQ stain)

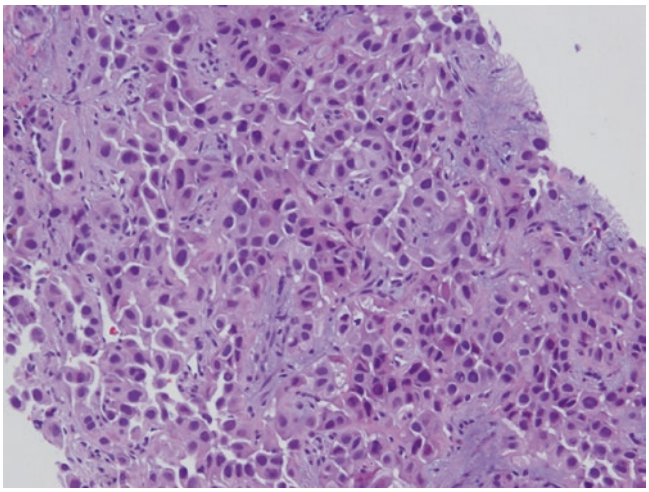

Fig. 1.7 Case 1. Needle core biopsy shows cohesive tumor cells forming glands. The tumor cells are round to polygonal shape with hepatoid appearance. The background shows desmoplastic stroma. Mitotic figures are seen

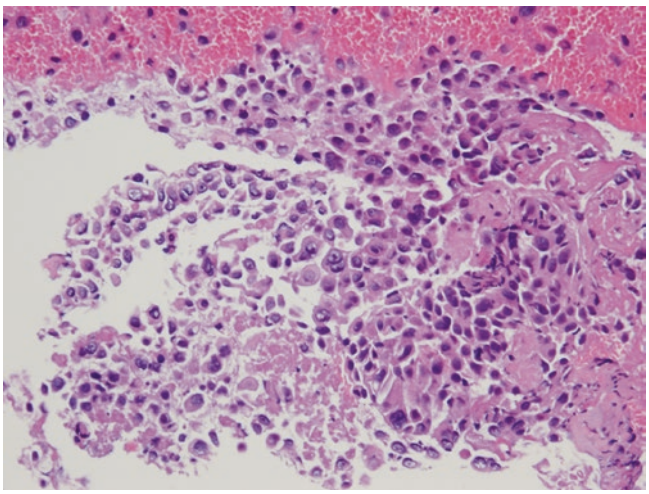

Fig. 1.6 Case 1. Cell block shows loosely cohesive neoplastic cells in a background of necrosis. The tumor cells show pleomorphic hyperchromatic nuclei, irregular nuclear membrane, prominent nucleoli, and pink cytoplasm. Apoptotic bodies are noted

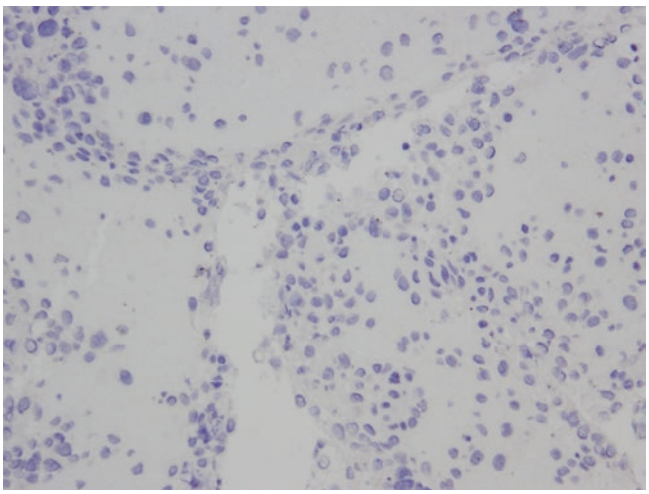

Fig. 1.8 Case 1. Tumor cells are negative for HepPar1 by immunohistochemical stain

Case 2

Case history

- A 71-year-old female had a history of melanoma of the vagina two years ago. Ultrasound showed an enlarged inguinal lymph node (1.8 cm).

Specimen source

Ultrasound-guided fine-needle aspiration was performed. A Romanowsky-stained smear and a Papanicolaou-stained smear were made from the aspiration.

Cytological findings

Pap-stained smears showed scattered slightly large cells present in a lymphoid tissue background. The atypical cells were scant and the degree of atypia was so subtle that they could be easily overlooked (Fig. 1.9).

Differential diagnosis

- Lymphoid proliferative disorder
- Metastatic carcinoma
- Metastatic melanoma

IHC and other ancillary studies

- Since cell block was not available, immunostaining for SOX10 was performed on a Pap-stained smear and the tumor cells were highlighted (Fig. 1.2).

Final diagnosis

- **Metastatic melanoma**

Take-home messages

- Clinical and radiologic impression was metastatic melanoma.
- Few atypical cells may be overlooked in a background of abundant lymphocytes.

Immunohistochemical study helps not only to highlight easily missed neoplastic cells but also to confirm the clinical suspicion of metastatic melanoma.

Reference: [106]

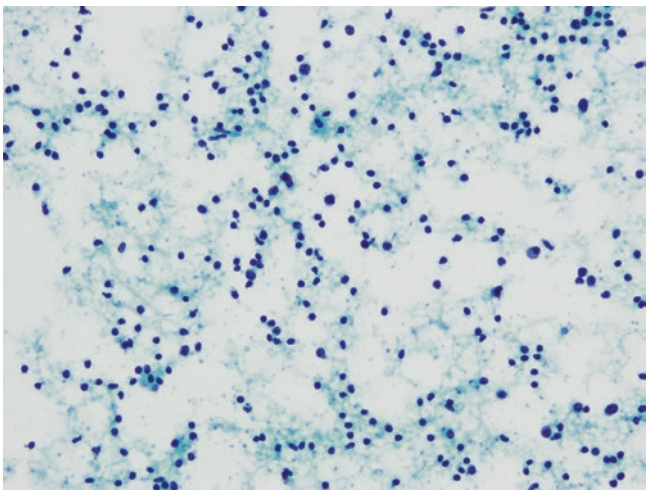

Fig. 1.9 Case 2. Smears showed lymphoid tissue with scattered slightly large cells. The atypical cells were so few and the degree of atypia was so subtle that they could be easily overlooked (Papanicolaou stain)

Case 3

Case history

A 77-year-old male with a history of mantle cell lymphoma two years ago was found to have a 1 cm new nodule in the soft tissue of the chest wall.

Specimen source

Fine-needle aspiration was performed. A Romanowsky-stained smear and a Papanicolaou-stained smear were made from the aspiration.

Cytologic findings

Smears contained a monotonous small- to medium-sized lymphoid population.

Differential diagnosis

- Lymphoma
- Soft tissue tumor

IHC and other ancillary studies

Flow cytometric immunophenotyping demonstrated a monotypic B-cell population with kappa light chain restriction (Fig. 1.10).

Immunohistochemical studies were performed. These cells were positive for CD19, CD20, CD22, CD79b, and CD5, while negative for CD10, CD11c, and CD30. SOX11 staining, which is a biomarker for

cyclin D1-negative mantle cell lymphoma, was positive on a Pap-stained cytospin smear (Fig. 1.11).

FISH study shows t (11: 14) translocation (Fig. 1.3).

Final diagnosis

- **Mantle cell lymphoma**

Take-home messages

Ancillary studies such as flow cytometry, immunohistochemical stains, and FISH help to establish the diagnosis.

Reference: [107]

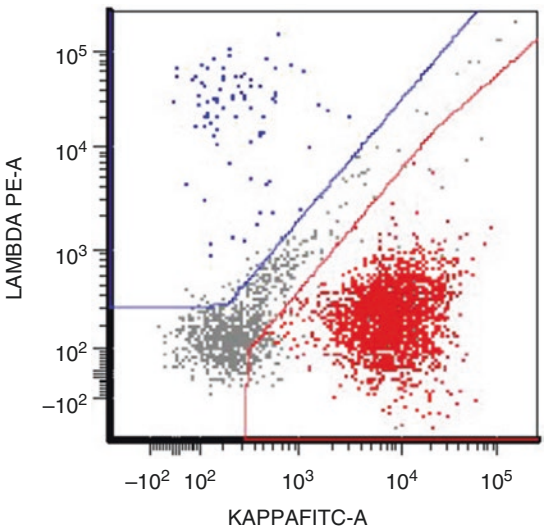

Fig. 1.10 Case 3. Flow cytometric immunophenotyping demonstrated a monotypic B-cell population with kappa light chain restriction

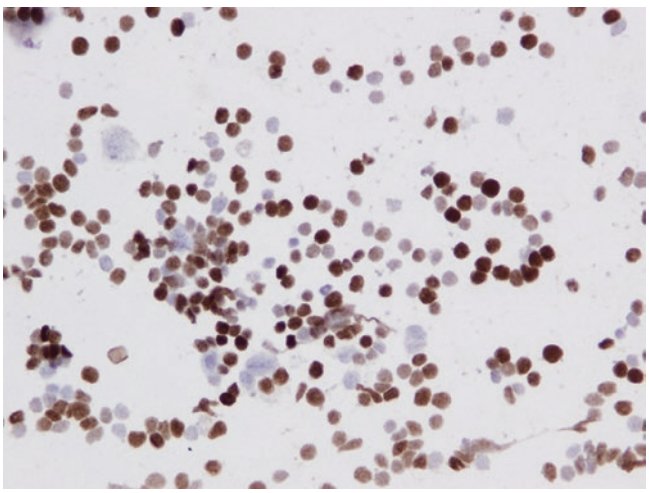

Fig. 1.11 Case 3. Immunohistochemical stain for Sox11 performed on the tumor cells within a Pap-stained cytospin was positive

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