The 4th EFCS Annual Tutorial

Ospedale Universitario di Cattinara, Strada di Fiume, Trieste

Handouts for lectures and workshops II

Non-gynaecological cytopathology II

- Mrs Rietje Salet-van-de Pol
  - Preparatory techniques, special stains, cell blocks, ICC, ISH *etc*.

- Professor Lukas Bubendorf, University Hospital Basel, Basel, Switzerland
  - Respiratory tract cytology
  - Urinary cytology with ancillary tests

- Dr Laszlo Vass, Pest County Hospital, Budapest, Hungary
  - Salivary gland FNAC

- Professor Pio Zeppa, University degli Studi di Salerno, Italy
  - Lymph nodes: non-neoplastic, lymphoma, metastatic malignancy, with ICC and flow cytometry

- Professor Luigi Di Bonito, Ospedale Universitario di Cattinara, Trieste
  - Breast FNAC

- Dr Arrigo Bondi, Ospedale Maggiore, Bologna
  - Immunocytochemistry

- Professor Ales Ryska, Charles University, Prague, Czech Republic
  - Thyroid FNAC

- Professor Torill Sauer, Olso University Hospital, Ullevaal, Norway
  - Spinal fluids
  - Serous fluids

- Dr Leena Krogerus University Central Hospital, Helsinki, Finland
  - Biliary cytology and EUS pancreatic cytology

NON-GYNAECOLOGICAL CYTOPATHOLOGY

Methods and preparatory techniques in non-gynecological cytology

Rietje Salet-van de Pol

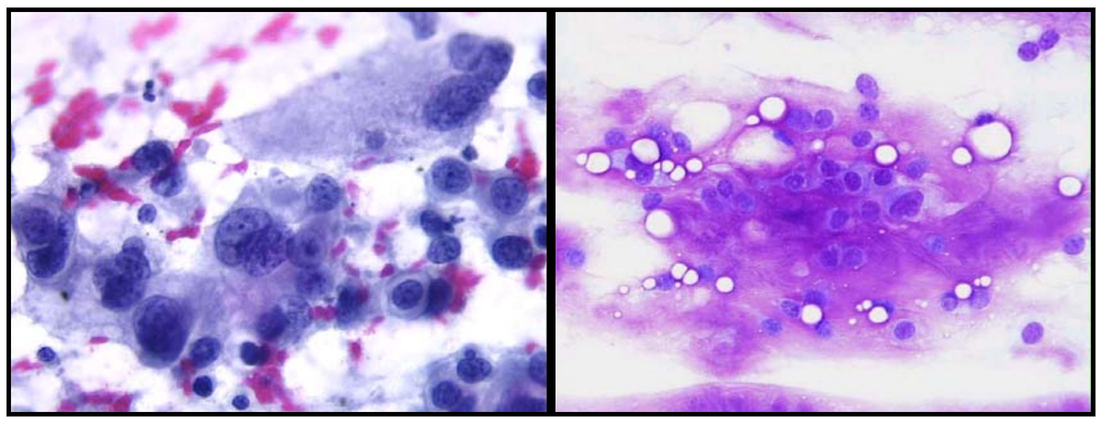
The diagnostic accuracy of cytology starts with the cytopreparation of the specimens. The goal is to obtain an optimal diagnostic cell sample. The preparation techniques vary between laboratories and depend on preference and logistic situations. To receive good material from your clinicians it is important to give them good guidelines on how to deliver the material to the laboratory.

For Papanicolau staining you use fixed material and for the Romanowsky methods (May-Grunwald Giemsa, Giemsa Wright's stain) you need air-dried smears.

When you use a fixative for fixation of the cell sample choose one that penetrates the cells rapidly, minimizes the cell shrinkage, de-activates autolytic enzymes and stops cellular and microbiological growth. Whatever fixative is used, immediate fixation (within seconds) is necessary to preserve cellular features. Most of the fixatives used in cytology are ethanol- or methanol-based.

The **Papanicolaou stain** is better for nuclear detail, nucleoli and squamous differentiation. The stain is accepted worldwide and there are numerous modifications: staining times vary, use of tap or distilled water, use of progressive or regressive method. (See also "Gynecologic cytology: technical aspects"). Whatever method you use it is important to standardize the staining to achieve reproducible results.

The **Romanowsky stain** consists of methylene blue/azure B and eosin, dissolved in acetone-free methanol, and include Jenner, Giemsa, May Grunwald and Leishman stains. The Romanowsky stain is better for cytoplasmic detail, stromal component, mucin, colloid and bare bipolar nuclei. For the Romanowsky stain you need air-dried smears with a thin layer of cells, take care of quickly drying of the cells with a fohn, fan or on a hot plate (max.50 ° C).



Papanicolaou stain *Romanowsky stain*

Fixation

Body fluids need no prefixation: they can stay overnight in a refrigerator, to make smears or cytospins for Papanicoalou or Romanowsky stain. Remains of sediments can be fixed with unifix for cell block if additional (immuno)histochemistry is needed.

For fine needle aspirations we use direct smears (1 air dried for Romanowsky and 1 fixed for Papanicolaou) for morphology and residual material is immediately fixed in unifix for AgarCyto cell block.

Sputum is fixed in ethanol 50%-carbowax for the Saccomanno method and for bronchial brushing we use 1 air dried smear for Romanowsky stain and 1 fixed smear for Papanicoloau stain.

Cerebrospinal fluid needs no prefixation, because ethanol may precipitate proteins.

Urine and bladder washings are received fresh or in ethanol 50%-carbowax fixative.

Lysing blood cells

There are several methods for lysing blood cells:

In fixative: Carnoy's or Clarke's fixative both contain glacial acetic acid, or commercially-available lysing fixatives such as Cyto Rich Red.

After fixation: by placing slides in a lysing solution: methanol-glacial-acetic acid 10%.

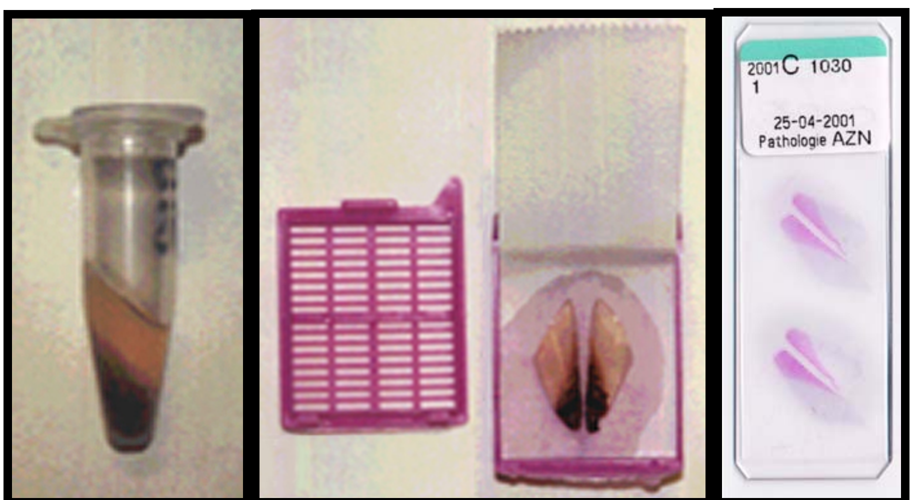
Don't forget to stop the lysing action before staining: in 95% ethanol.

AgarCyto cell block preparation

For additional morphological, cytochemical, immunocytochemical and molecular techniques we developed a cell block method for cytological rest material. After making 2 slides, 1 for Papanicolaou and 1 for Romanowsky stain, the rest material is immediately fixed in unifix.

- Cells are fixed in unifix
- Fixed cells are pelleted by centrifugation (10 min/2000 rpm)
- Supernatant is carefully loosened and transferred into a 1.5 ml Eppendorf reaction tube
- Spun down for 10 minutes at 2000 rpm
- Pellet is resuspended in 1 ml 2% liquid agarose at 65 °C
- Reaction tube is centrifugated for 5 minutes at 1000 rpm to concentrate the cells in the agar
- Agar-cell pellet is solidified at 4 °C for at least 1 hour
- The agar cone is carefully taken out of the tube and laterally divided in 2 halves
- The 2 agar pieces are wrapped in filter paper for biopsy preparation and embedded in paraffin
- 4 µm sections are cut, mounted on glass slides and air dried overnight at 56 °C

- For cytomorphological examination AgarCyto sections are stained with hematoxylin-eosin (H&E)



The AgarCyto method

With the cell block method many slides can be made for additional techniques. The techniques can be performed identical to routine histological techniques with the use of the same control tissues. The results are very good and reliable. Another great advantage of the cell block method is that you can look with different cytochemical and immunocytochemical stainings to the same cells or group of cells. This method is labor-intensive and therefore only performed when indicated.

Closing remarks

- Body cavity fluids have high risk for contamination: stain separately
- Every specimen should be handled as though it were infectious
- For better cell adhesion to glass slide use Mayer's albumin, polylysine or coated slides
- All methods must be described and standardized to achieve reproducible results and this is mandatory for good quality and accreditation purposes

References

Comprehensive Cytopathology, ed. by Marluce Bibbo and David Wilbur, 3th edn. Saunders Elsevier, 2008

Theory and practice of histological techniques, ed. by John D. Bancroft and Marilyn Gamble, 5th edn. London, Churchill Livingstone, 2002

Kerstens H.M., Robben J.C., Poddighe P.J et al: AgarCyto: a novel cell-processing method for multiple molecular diagnostic analyses of the uterine cervix. J.Histochem Cytochem.2000: 48: 709-718.

Grefte J.M., de Wilde P.C., Salet-van de Pol M.R.J. et al.: Improved identification of malignant cells in serous effusions using a small, robust panel of antibodies on paraffin-embedded cell suspensions.Acta Cytol. 2008: 52: 35-44.

Van Aspert-van Erp A.J., Heijnen-Wijnen H.H., van Zuylen I.B., Vooijs G.P.: GROPAP- a new staining method. Cytopathology 1994: 5: 171-176.

Respiratory cytology, EBUS and ancillary tests

Professor Lukas Bubendorf

Workshop cases

Each workshop case provides a brief case history on the front of the packet, slides at the time of diagnosis and in some cases histology sections. Examine the slides and make up your own mind as to how they should have been reported. The correct diagnosis and a slide review are given on the back.

1. Introduction

Respiratory cytology is an important field of non-gynecological cytology as it covers a broad range of different pulmonary disorders including tumors, interstitial lung diseases, infections and drug-related changes. It is important to approach these in an interdisciplinary manner by considering histo- and cytopathological findings together with clinical information and radiological findings. This handout will provide a summary of important technical aspects, indications and diagnostic criteria of respiratory cytology in neoplastic and non-neoplastic diseases.

2. Laboratory techniques

Here you will find some information on the methods that we use in our laboratory. The cytological slides should be immediately fixed with Delaunay solution or fixation spray. We use Papanicolaou as the standard coloration, as this staining preserves a high transparency of the specimens even in case of a high mucous content. It also guarantees a clear distinction of the chromatin structure. The May-Grünwald-Giemsa-coloration (MGG) is applied to broncho-alveolar lavage specimens in order to facilitate the analysis of inflammatory cells.

Preparation of smears from secretions

Sputum: Appropriate instruction of the patient is crucial in order to obtain representative sputum specimens: teeth brushing and washing of the mouth with water followed by deep coughing, preferentially in the morning. The yield of sputum can be increased by inhalation with a mucolytic substance and by palpitating the back.

Bronchial secretion: Aspiration of the secretions or bronchial washing.

Bronchial brushings are prepared like bronchial secretions.

Number of smears for a probe for these preparations: 2 – 4.

Preparation of bronchoalveolar lavages (BAL)

Indication: interstitial or disseminated alveolar diseases or search for opportunistic infections. In most cases, the interpretation of the analyses must be made in consideration of the clinical context.

The following specimens and/or analyses are performed from BAL:

- One smear stained by MGG after dry fixation for cell differentiation.
- Two smears stained according to Papanicolaou (after immediate fixation in fixative) for the search of tumor cells.

Depending on the clinical picture and specific requests:

- 3-6 cytospin specimens (air-dried with post-fixation using acetone) for immunocytochemical analysis of lymphocyte subpopulations (e.g. CD2, CD4 and CD8). Alternatively, residual sediment can be submitted for fluorescence-assisted cell sorting (FACS).
- 2-8 extra cytospin specimens for fluorescence stains or immunofluorescence and/or immunofluorescence (e.g. Fungiqual A (fungi), Auramin/Rodamin (acid-phase bacilli) or immunofluorescence antibody assays (Pneumocystis Jirovecii, CMV, RSV or Legionella pneumophila). These techniques provide a result within 1 to 2 hours.

Fine needle aspirates

Transbronchial fine needle aspiration (TBNA):
Peripheral lesions and mediastinal lymph node staging.

Endobronchial ultrasound (EBUS) TBNA

This technique allows a US-guided, precise punction of mediastinal lymph nodes for staging and initial diagnosis of lung cancer. The yield is typically higher than in TBNAs without US guidance.

CT-guided transthoracic FNA:
In case of peripheral lesions and negative bronchoscopy.

Preparation (2 alternatives):

- Direct smear of the aspirate made by clinician with immediate fixation. The result depends on the skills of the operator!
- Collecting the aspirate in cell culture medium or physiological solution of sodium chloride with subsequent preparation of cytospin specimens in the lab.

Cell blocks

Many laboratories prepare cell block specimens, especially from transbronchial fine needle aspirates. Cell block specimens can be routinely processed in the general immunohistochemistry lab together with histological specimens. However, cell blocks require a certain minimal amount of tumor cell material, which is not always available in these specimens. We routinely prepare cell blocks from malignant effusions, where the amount of material is usually not an issue.

3. Normal findings

Table 1: Normal cytological findings

The cells	Characteristics cytological features
Ciliated bronchial cells	Cylindrical cells with cilia, which are fixed to the cell membrane and cytoplasm by ciliosomes. The ciliosomes form a terminal bar.
Goblet cells	These cells contain brownish or red mucous granula in the cytoplasm (Papanicolaou staining).
Basal cells	Small cubic cells with narrow cytoplasm, mostly arranged in small regular plates.
Metaplastic squamous epithelial cells	Similar to basal cells, but with broad cubic or polygonal cytoplasm.
Macrophages	Vacuolated cytoplasm loaded with anthracotic pigment (smokers). Cave misinterpretation of activated macrophages as adenocarcinoma.
Lymphocytes	Should be easy enough to recognize.
Polymorph nuclear granulocytes	Typically segmented nuclei.
Eosinophiles	Two nuclear segments with dense chromatin. The eosinophilic granules are difficult to recognize in Papanicolaou, but apparent in MGG.

Table 2: Normal values of different cell types in bronchoalveolar lavage (mast cells in number/10 high-power fields).

Cells/cell type	**%**	**x 10^6/L**
Total number		
Non-smoker		50
Smoker		to 300
Macrophages		
Non-smoker	95%	40-100
Smoker	80-90%	100-300
Lymphocytes	<10%	<10
Neutrophilic granulocytes	<10%	<10
Eosinophilic granulocytes	<0.5%	<0.5
Mast cells		3/10 HPF

4. Non-neoplastic diseases of the bronchial system

Table 3a: Non-neoplastic changes

Type of change	Cytological features / comments
Ciliated cells with enlarged nuclei	Increased nuclear size due to irritation, infection or deficiency of vitamin-B
Giant ciliated cells	Up to 20 or even more unsuspicious nuclei. Cause: Irritations, secondary upstream bronchitis due to tumors or tuberculosis.
Creola bodies	Ciliated cells and goblet cells. The presence of ciliae is a key feature to avoid misdiagnoses of adenocarcinoma.
Ciliocytophtoria	Degenerative alterations of ciliated cells: Ciliate ragged off the cytoplasm. The cytoplasmic fragments show eosinophilic granulations. The nuclei are pycnotic.
Goblet cell hyperplasia	Large number of hypertrophic cells with intracellular mucous. The ratio goblet cells / ciliated cells is > 1:5
Regeneration	Highly activated epithelial cells with enlarged nuclei, coarse chromatin and several nucleoles. „Streaming" cell pattern.
Parakeratotic squamous epithelial cells	Small squamous cells, strongly keratinized (eosinophilic). Avoid making a diagnosis of squamous cell carcinoma because of a few atypical parakeratotic cells!
Cellular damage by cytostatic drugs	Ciliated cells with sometimes highly enlarged and hyperchromatic nuclei. Important findings to avoid misdiagnosis of adenocarcinoma: Similar changes across different cell types (continuum). Presence of ciliae.
Hemosiderin-laden macrophages	Alveolar bleedings: Congestion, vasculitis, and hemosiderosis.
Epitheloid cells, Langhans giant cells	Epitheloid cells: Elongated cells, often with folded nuclear membranes, fine pale chromatin. Giant cells: Abundant cytoplasm, eccentrically located nuclei; may contain asteroid bodies.

Table 3b: Extracellular corpuscular elements

Element	Description
Curschmann spirals	Present in conditions with excess mucous production (e.g. asthma or smoking). Darkly staining center with a lighter staining periphery, spiral like a corkscrew.
Charcot-Leyden cristals	Bipyramidal red crystals composed of condensed granules derived from eosinophiles, associated with asthma and other allergic conditions.
Corpora amylacea	Concentrically laminated, non-calcified alveolar casts. Unspecific finding with no clinical relevance.
Ferruginous bodies	Iron salts precipitates onto tiny inhaled fibers (often asbestos). Typically golden-brown and frequently engulfed by macrophages.

5. Non-neoplastic diseases of the lung

Eosinophilia

Pronounced eosinophilia (> 20-50%): Idiopathic eosinophilic pneumonia, parasitosis (e.g. ascaris), vasculitis (CHURG-STRAUSS), allergic bronchopulmonal aspergillosis (ABPA), allergic asthma (clinical history).

Less pronounced (< 5-10%): Drug reaction, pulmonary Langerhans cell histiocytosis (typically >6%). Idiopathic pulmonary fibrosis (< 5%).

Drug-related lung disorders (bronchoalveolar lavage):

There are few drugs with characteristic BAL-findings: often, there is a mixture of cytotoxic effects (changes of epithelial cells with enlarged and atypical nuclei) and interstitial inflammation in conjunction with mild to moderate lymphocytosis (e.g. methotrexate or cyclophosphamid). The commonly used anti-arrhythmic drug amiodarone causes a typical foamy change of the alveolar macrophages. Electron microscopy reveals “lamellar bodies”. CAVE: foam cells and lamellar bodies in patients receiving amiodarone prove previous intake of amiodarone but not lung disease caused by this drug. Correlation with clinical and radiological findings is needed to make this diagnosis. For more detailed information on pulmonary side effects of different drugs we refer to www.pneumotox.com

Table 4: Cytological differential diagnosis of interstitial lung diseases in BAL, characteristic findings in bold.

Numbers of macrophages x 1 million/L

	MPH	**L**	**T4/T8**	**NG**	**EG**	**Mast cells**	**Others**
Sarcoidosis	=	>/>>	**>2.0**	=	=	=	
Hypersensitivity pneumonitis	Foamy cells	>>/>>>	**<0.5**	>	>	**>/>>**	CD8>
Langerhans cell histiocytosis	**>1000**	>	=	>	>	=	CD1a>6% Birbeck granules
Organizing pneumonia	300-500	**>/>>**	<0m5-1	**>/>>**	>	**>**	
Respiratory bronchiolitis	500-1000	=	=	=	=	=	=
Pulmonary fibrosis	**>500**	=/>	=	+/++	(>)	>	CD1a >6%
Pneumokoniosis	Like pulmonary fibrosis but with birefringent crystals in macrophages and/or ferruginous bodies.						
Alveolar proteinosis (rare)	Macroscopical diagnosis: milky white fluid. Cytology: coarsely granular PAS-positive debris. EM: Surfactant material.						

6. Neoplastic lung diseases

The WHO-classification of lung cancers separates small cell lung cancer (SCLC, approximately 15%) from the non-small cell lung cancers (NSCLC 85%). NSCLC mainly consist of 3 major subtypes:

Adenocarcinoma (AC): 50-60%.

Squamous cell carcinoma (SCC): 30-35%.

Large cell carcinoma (LCLC): 10%.

Less common subtypes: Sarcomatoid carcinoma, typical and atypical carcinoid, and salivary gland carcinomas, other very rare subtypes.

The WHO-classification is based on histological resection specimens, which allow the analysis of the whole tumor. Since the majority of the lung cancers are not eligible for resection because of an advanced stage, diagnostic reality consists mostly of small biopsy and/or cytological specimens, which make a definitive subclassification of NSCLC subtypes often difficult. Thus, approximately 30% of such specimens are diagnosed as NSCLC not otherwise classified (NOS). Although subclassification of NSCLC is typically based on histology, it can be achieved by cytological specimens alone with similar precision.

Cytological criteria for the diagnosis of the major lung cancer types:

Small cell lung cancer

Numerous isolated cells or syncytial aggregates of small cells. At low power, they appear relatively uniform; however at high power, the cells are quite pleomorphic. Spindle cells also may be seen. The nuclei are hyperchromatic. Nucleoli are invisible or small. The cytoplasm is delicate and the cells have a very high nuclear/cytoplasmic ratio. Nuclear moulding and crush artifacts is another characteristic feature.

Adenocarcinoma

Defining key features of adenocarcinoma are definite glandular differentiation or mucin production by malignant cells. In addition, the cells are often cylindrical and/or show a nuclear cytoplasmic polarity (basal nuclei, apical cytoplasm). The cytoplasm is often finely vacuolated. The nuclei typically have a vesicular appearance with a more open chromatin and more prominent nucleoli than SCC. Bronchiolar alveolar carcinoma (or: adenocarcinoma with “lepidic” growth pattern) growths along pre-existing alveolar walls. This adenocarcinoma subtype can be suspected by cytology. The nuclei are quite uniform, round or oval and tend to crowd. The nuclear membrane is sometimes wrinkled and intranuclear cytoplasmic invaginations may be seen. There is a non-mucinous (more common) and a mucinous type of BAC.

Squamous cell carcinoma

The most characteristic features of keratinizing SCC are marked pleomorphism and keratinization. The nuclei are irregular and hyperchromatic with coarse chromatin, often reminiscent of volcano stones. Nucleoli often have a dense chromatin. The background is often necrotic. Diagnosis of non-keratinizing SCC is more difficult, as it lacks the squamous pearls. Poorly differentiated or basal cell like SCC may be difficult to distinguish from small cell carcinoma. Nuclear moulding is usually more prominent in small cell carcinoma, however, while the chromatin is usually coarser and nucleoli more prominent in SCC than in small cell carcinoma.

Large cell carcinoma

Large cell carcinoma is a "waste basket" category of poorly differentiated NSCLCs that are difficult to classify. Immunohistochemistry can help to refine categorization. Cytology reveals obviously malignant cells without specific differentiation by morphology.

Carcinoid tumors

Carcinoids are often clinically suspected by their presentation as endobronchial polyps or sharply demarcated opacities by radiology. Cytology reveals relatively small and monotonously uniform cells. The nuclei have smooth nuclear membranes, "salt and pepper" chromatin, which is typical for a neuroendocrine differentiation, and inconspicuous nucleoli. Despite some similarities with a small cell carcinoma, the cells of carcinoids are much less polymorphic. In addition, there is no necrosis, and crush artifact is uncommon. Peripherally located carcinoids typically have spindle cell morphology. Since the separation of typical from atypical carcinoids is defined by histological features alone (more than one mitosis/10 HPF and/or necrosis), this distinction cannot be made by cytology alone.

Large cell neuroendocrine carcinoma

These carcinomas have cytological similarities with small cell carcinomas (e.g. "pepper and salt chromatin"), but the cells have the size of cells from a NSCLC, and often prominent nucleoli. Since their prognosis is similarly poor as the prognosis of small cell carcinoma, is has been proposed to lump these two tumor types in a category of "poorly differentiated neuroendocrine carcinoma of the lung".

7. Molecular analysis in respiratory cytology

Immunocytochemistry

Immunocytochemistry is routinely used for a more precise cancer classification on small biopsies, cell block preparations or cytological specimens, depending on the local expertise and preference. There are two main indications for immunocytochemistry:

- Distinction between primary pulmonary tumor and metastases of an extrapulmonary primary tumor: depending on the clinical suspicion, there are a

growing number of different antibodies to allow these distinctions. The most commonly used in our lab are TTF1, CK7, CK20 and CDX2.

- Subclassification of NSCLC-NOS: as mentioned earlier, the distinction between AC and SCC has become most important for therapeutic reasons. The immunophenotype is most helpful in further stratifying NSCLC-NOS by immunophenotype. This can be usually achieved by 1-2 SCC markers (e.g. CK5/6 and p63) and 1-2 AC markers (e.g. TTF1 and CK7).

- Confirming a diagnosis of SCC: it is possible to make a definite diagnosis of SCC by typical cytological morphology alone. However, we use ICC liberally in case of any doubt (pancytokeratin and one or two of the neuroendocrine markers CD56, Synaptophysin and Chromogranin A).

Fluorescence in-situ hybridization (FISH)

FISH is an established and technically robust method to visualize changes in chromosome copy number and gains or deletions of specific chromosomal sites. Since chromosomal aberrations are a hallmark of cancer but usually not present in benign cells, FISH can be used to improve the sensitivity of cytology. A multiprobe FISH test (LA Vysion, Abbott Molecular Inc.) has been designed to increase the sensitivity of cytology for the detection of non-small cell lung cancer. We found that FISH-analysis is especially very helpful in equivocal cases where a distinction between reactive changes and carcinoma is impossible by cytology alone. This FISH analysis allows to reliably stratifying benign from malignant epithelial cells. The positive predictive value is up to 100%, while the sensitivity is 70%. Automatic relocation of the individual cells or small groups of atypical cells is a precondition for such a good diagnostic performance.

Predictive marker analyses

The need for molecular analyses to determine the status of specific therapeutic target is rapidly growing. Since 30 to 40% of all NSCLC are diagnosed by cytology alone, such analyses are not anymore optional, but have become a necessity. Currently, EGFR-mutation analysis is required to select patients with advanced adenocarcinoma for therapy with EGFR tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib. Clinicians will soon ask for other mutations (e.g. BRAF and HER2 mutation status). The enrichment of tumor cells from heterogeneous cytological specimens with a variable amount of benign admixed cells is a technical challenge. However, this can be achieved by laser capture microdissection that allows to specifically “harvest” cancer cells while leaving the benign cells behind on the slide. In specimens, which are mainly composed of tumor cells (e.g. TBNA specimens with > 50% of tumor cells), these tumor cells can be mechanically scratched off the specimens for subsequent DNA-extraction and sequence analysis.

EML4-AALK gene fusion is another genetic alteration that can be specifically targeted by a drug (crizotinib). This gene fusion is found in approximately 5% of all NSCLC. FISH with a break-apart probe is the current gold standard to identify this molecular target and can be easily applied to cytological specimens.

Suggested Reading

DeMay: Practical Principles of Cytopathology (Revised Edition 2007), American Society for Clinical Pathology Press, Chicago IL

Johnston WT and Elson CE: Respiratory Tract (Chapter 13); in Comprehensive Cytopathology, 3rd Edition, edited by M. Bibbo and D.C. Wilbur, Saunders Elsevier, 2008

Glatz K et al.: An online quiz uncovers limitations of morphology in equivocal lung cytology. Cancer. 2006 Dec 25;108(6):480-7.

Urine cytology with ancillary tests

Professor Lukas Bubendorf

Workshop cases

Each workshop case provides a brief case history on the front of the packet, slides at the time of diagnosis and in some cases histology sections. Examine the slides and make up your own mind as to how they should have been reported. The correct diagnosis and a slide review are given on the back.

Introduction and aims

Cytology of the urinary tract has successfully been used for initial diagnosis and surveillance of urothelial neoplasias for decades. Nevertheless, the value of urinary cytology has repeatedly been questioned. The lack of a uniform classification system of urinary cytology has been a main problem, which has led to innumerable local classification systems and major problems in interlaboratory communication. This is further complicated by complex clinical pathological associations due to the fundamentally different clinical significance of the various types of urothelial tumors and the different algorithms for treatment and surveillance. Diagnosis and the interpretation have also constantly been adapted to the changing concept in histopathological classification of urothelial tumors. A reasonable and useful application of ancillary methods for an improved diagnosis of urothelial tumors is another challenge. In this workshop we will discuss the cytomorphological criteria for the diagnosis of urothelial neoplasia, their interpretation and classification systems. In addition there will be an opportunity to learn about the utility of fluorescence in-situ hybridization in urinary cytology. Besides urothelial neoplasia there will also be urinary specimens with non-neoplastic alterations such as viral infections.

Transport and processing of voided urines and urinary tract washings

Voided urine, bladder washings and washings of the upper urinary tract are the most common materials. A correct processing of the materials is a pre-condition for a meaningful cytological diagnosis. Immediately after obtaining the urine, the whole fluid should be diluted with an equal volume of 50% ethanol for conservation (but not fixation) of the urinary cells during the transport. With this simple method the cells remain well evaluable for up to 3 days even at room temperature. We prefer preparing cytospin specimens from the sediments. Two cytospin specimens are considered as representative. The preparation of regular smears is reasonable in case of abundant sediment, but not recommended otherwise. Fluid based cytology preparations (e.g. ThinPrep®, UroCyte® or SurePath®) lead to increased costs but do not improve the quality as compared to cytospins.

Classification and reporting of urinary tract cytology

There is no uniform classification of cytological findings of the urinary tract. An international consensus classification system, as it exists for gynecological or thyroid cytology has not yet been established. All existing classification systems share the effort

to separate the truly dangerous high-grade urothelial carcinomas (UC) from the other lesions.

The following points can be considered as a basis for the classification of urinary cytology:

1. The categorization of a cytomorphological continuum inevitably leads to some unavoidable degree of interobserver variability.
2. The cytological diagnosis of high-grade UC and carcinoma in situ is straightforward. However, it is important to be familiar with certain knowing changes that can mislead an untrained observer to a false-positive high-grade urothelial carcinoma (e.g. Decoy cells, degenerative atypia and radiogenic alterations).
3. A clear cytological diagnosis of flat or papillary low-grade urothelial neoplasia is difficult or and often impossible, especially in case of papilloma, PUNLMP and well-differentiated non-invasive urothelial carcinoma (G1 according to WHO 73). The diagnosis of these lesions has low clinical importance, however, since they are quite harmless. Therefore it has previously been suggested to avoid the term “carcinoma” for such lesions and lump them in a category of low-grade urothelial neoplasia, instead.
4. It is practically impossible to decide with certainty whether an urothelial carcinoma is invasive or not by cytology alone.
5. There is a grey zone between high-grade and low-grade UC. This grey zone includes a subgroup of the low-grade UC that has previously assigned to the WHO 1973 grade 2 and now partly to low-grade UC and partly to high-grade UC. The better-differentiated low-grade tumors of the former G2 UC are sometimes difficult to diagnose by cytology. A definitive diagnosis of urothelial neoplasia is possible in some of these cases, however.
6. There is an area of non-definitive diagnoses between the unequivocal diagnostic categories of “negative” and “positive for high-grade UC”. In this area of uncertainty, the main goal is to give a good estimate on the probability of low-grade or high-grade urothelial neoplasia.

Suggestion of a classification system

Based on our own experience and the dates from the literature we suggest the following classification system.

Non-representative

Provide the reasons: Poor cell preservation, no cell material or too little cell material. We require at least 50 urothelial cells per cytospin. For washing cytologies, a cell number of 100 to 400 per cytospin are ideal.

Negative

Normal urothelial cells or reactive changes. In case of reactive changes there are usually abundant umbrella cells with enlarged and often multiple round nuclei with variable size. The nuclear cytoplasmic ratio is normal. The cytoplasm is often finely vacuolated, especially around the nuclei.

Cytological atypia, not further classified

Although such atypia is mostly mild and reactive in nature, it is not possible to definitely rule out (low-grade) urothelial neoplasia. The uniformity and monotony of the cells is the most important feature low-grade urothelial neoplasia, while the nuclear atypia is barely visible. The nuclei are still central in the cytoplasm, the contour of the nuclei is regular and the chromatin structure is unremarkable. The nuclear cytoplasmic ratio can be mildly increased. In case of unsuspicious cytsoscopy and radiology of the upper urinary tract this diagnosis has no immediate clinical relevance.

Suspicious

Such findings are suspicious of urothelial neoplasia and do not allow for a definite diagnosis. Nevertheless, depending on the degree of the atypia and the experience of the cytopathologist, an urothelial neoplasia (at least low-grade) can be made with high likelihood. The cytological criteria that are described on the positive category are partly present.

Positive

In case of a high-grade urothelial neoplasia this is usually a straightforward diagnosis that can be made within a few seconds. There is immediately apparent severe nuclear atypia as evidenced by polymorphy, increased size, hyperchromasy, irregular contour and chromatin distribution of the nuclei. The nuclear cytoplasmic ratio is high. In case of low-grade neoplasia the alterations are more subtle, but can sometimes still be unequivocal and diagnostic. Important criteria include the eccentric location of the nuclei, the high nuclear cytoplasmic ratio, the dark and irregular chromatin as well as irregularities of the nuclear membrane.

Commentary in addition to the cytological diagnosis

The diagnostic category alone does not reflect the complexity of the clinical-cytological associations. Therefore, an explanatory note should always accompany the diagnosis. We also recommend narrowing down the categories when ever possible. Examples:

Positive, at least carcinoma in situ.

Positive, diagnostic for high-grade UC.

Positive, favor high-grade UC.

Positive, at least low-grade UC.

Positive, consistent with low-grade UC.

Multi-target Fluorescence in-situ hybridization (UroVysion®)

In contrast to benign urothelial cells, UC cells often show chromosomal aberrations. Such chromosomal aberrations can easily be detected by fluorescence in situ hybridization (FISH). Centromeric probes are used to diagnose aneusomy (increased or decreased whole chromosome copy numbers) while local specific probes identify copy number losses and gains of selected genes. The UroVysion FISH assay (Abbott Molecular Inc.) increases the sensitivity of cytology for the detection of urothelial

neoplasia at a high specificity. This probe cocktail contains centromeric probes for the chromosomes 3, 7 and 17 and a local specific probe for 9p21. This test has a sensitivity of 90 – 100% for the detection of invasive bladder cancer (pT1-4) and a specificity of >95%. In clinically far less relevant category of low-grade non-invasive bladder cancer, FISH-increase is the sensitivity of cytology from 25% to 60 to 75%. Recent reviews provide a comprehensive overview on the indications, performance and technical issues of this FISH assay. One of the most rewarding applications is the category of atypical urinary cytology that cannot be further classified. This category is especially relevant in the upper urinary tract, where repetitive clinical controls are technically difficult.

Indications for UroVysion FISH-analyses in urinary cytology
Atypical urinary cytology
Control after intravesical BCG treatment
Upper urinary tract cytology
Surveillance after transurethral resection
Hematuria in patients with an increased risk of UC

Conclusion

Urinary cytology has a well-established role in the detection and monitoring of urothelial carcinoma. The main strength of cytology is the high specificity for high-grade urothelial carcinoma and carcinoma in situ, while it has a low sensitivity for low-grade, non-invasive tumors. In addition there is a poorly defined, but commonly used category of a typical cytology of uncertain significance. Multiple-probe FISH is a helpful tool to address this diagnostic problem. Due to a superior sensitivity of cytology in different situations, FISH can help in patient management.

<u>Suggested Reading</u>

Rosenthal and Raab SS: Cytologic Detection of Urothelial Lesions, Essentials in Cytopathology, Series Editor: Dorothy L. Rosenthal; 2006 Springer

DeMay: Practical Principles of Cytopathology (Revised Edition 2007), American Society for Clinical Pathology Press, Chicago IL

Renshaw AA: Compassionate conservatism in urinary cytology. Diagn Cytopathol 2000;22:137-8.

Bubendorf L. Multiprobe fluorescence in situ hybridization (UroVysion) for the detection of urothelial carcinoma - FISHing for the right catch. Acta Cytol.2011;55(2):113-9.

Caraway NP, Katz RL: A review on the current state of urine cytology emphasizing the role of fluorescence in situ hybridization as an adjunct to diagnosis. Cancer Cytopathol 2010;118:175-83.

Brimo F, Vollmer RT, Case B, Aprikian A, Kassouf W, Auger M: Accuracy of urine cytology and the significance of an atypical category. Am J Clin Pathol 2009;132:785-93.

Salivary gland FNAC: guide to the workshop

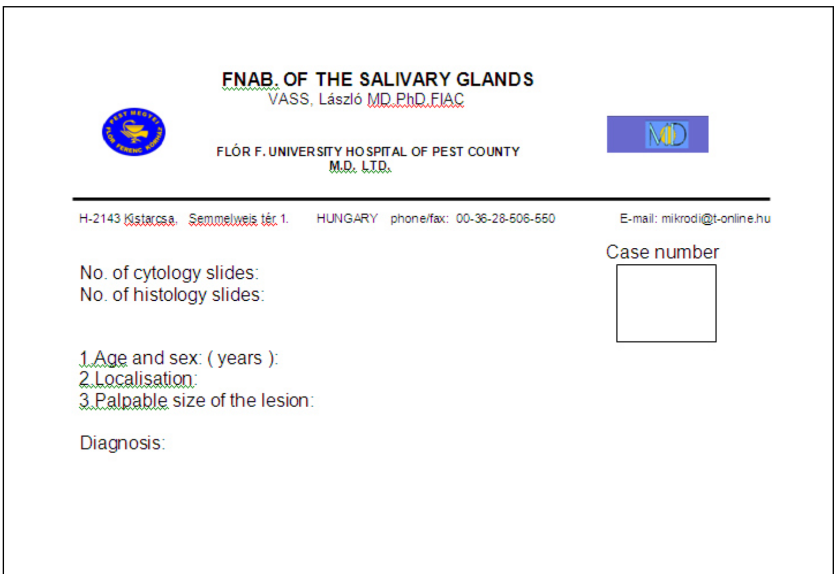
Dr László Vass PhD FIAC

PRACTICAL INTRODUCTION

You will have approximately 150 cases: these are packages (holders) with slides. There are more than one slide in each holder; in most of them you will have both aspiration smears and histology sections of the same case. The total number of smears and slides will be put on the information side (the back side) of the package. Please, check the number of slides in each case both before starting and after finishing your work with the package: the material belongs to the patients and to our laboratory; I do have responsibility for not losing "one single cell" during the course! Thank you for your continuous attention in this matter in advance!

The information side of the holder contains the following data:

- Personal data of the patient
- Data concerning the lesion
- Diagnosis



FNAB. OF THE SALIVARY GLANDS
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Case number

No. of cytology slides:
No. of histology slides:

1.Age and sex: (years):
2.Localisation:
3.Palpable size of the lesion:

Diagnosis:

The head and neck region shows the presence of many organs, many different kinds of tissues, many different cell types in each organ and tissue:

- Nasal, paranasal, nasopharyngeal, hypopharyngeal, laryngo-tracheal, oral cavity, oropharyneal, salivary gland-related, lacrimal gland pathology, odontogenic, ear lesions, etc.

- Organs and lesions which are to be found in many different parts of the body, still they occurrence are significant in this anatomic localization: lymph nodes, vascular tumors, skin tumors, adnexal tumors, etc..

Main diagnostic questions to be answered

What kind of tissue was aspirated? In all cases we need to know all of the important clinical data, image analysis data, symptoms and the way of the sampling. The more clinical and anatomic data are available the easier the diagnosis will be.

What does the general cellular characteristic of the smear tell to us?

Quite a lot but not enough:

- cystic lesion and fluid containing squamous cells may equally represent squamous (lateral) cyst content or colliquated necrotic squamous cancer.
- Cellular pleiomorphism is characteristic in mixed salivary gland tumors, which are nearly always benign, slightly atypical homogeneous cell population may be characteristic in acinic cell cancer, monomorphic but hardly atypical basaloid population will be found both in benign, monomorphic basal cell tumor and in the highly malignant adenoid cystic carcinoma.

Sampling methods

Freehand FNAB is useful in superficial lesions since they must be localized and fixed using the fingers of the sampler. Superficial, subcutaneous or submucosus lesions do not need US guidance; most of the US heads are too large for these lesions.

US guidance is an absolute must in deep seating or larger cystic lesions. Small lesions localized next to the large vessels also need guidance to avoid unnecessary bleeding complications.

CT guidance is not appropriate, but we may use the CT scan images for localization before a freehand or US-guided puncture.

Staining methods

Papanicolaou, Giemsa or H&E staining will equally be used.

Papanicolaou and H&E are informative in squamous cell lesions while Giemsa-stained specimens do have the advantage of metachromasia: extra cellular matrix and the cytoplasm of the myoepithelial and connective tissue cells show metachromatic purple staining. Since many salivary gland lesions and tumours are related to myoepithelial cells and these tumors may have a characteristic stroma the Giemsa (Romanowsky-type) stains) are probably the most important in this organ.

In complicated cases immunocytochemical reactions may help the diagnosis:

Most useful immuno-markers in salivary gland pathology:

- Nonspecific markers of luminal/acinar epithelial differentiation: epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA) low molecular weight keratins (CK 8, 18, 19)

- Nonspecific markers of myoepithelium: (also seen in some ductal epithelial phenotypes) S-100 GFAP, high molecular weight keratins (CK5, 14), vimentin, maspin
- Markers of muscle differentiation (myoepithelium): alpha-smooth muscle actin, smooth muscle myosin heavy chain, Calponin, Caldesmon, p63
- Markers of cell organelles/secretions: mitochondria (oncocytic metaplasia and oncocytic tumors), amylase, lactoferrin, lysozyme, secretory component (acinar differentiation), Type IV collagen, laminin,
- Markers used in given differential diagnostical problems (non-specific!): AR (ductal cc.), CD-117 (C-Kit) (adenoid cystic cc.), P 63 (basaloid squamous cell cc. vs. adenoid cystic cc.)

(based upon, with additional information: Zarbo, RJ. :Salivary Gland Neoplasia: A Review for the Practicing Pathologist, Mod Pathol 2002;15(3):298–323)

ANATOMY AND BASIC CYTOLOGY OF THE SALIVARY GLANDS

There are three pairs of main salivary glands: the sublingual, submandibular and the parotid gland. The number of the small salivary glands is more than 1 000. The structure is serous, mucinous or mixed. The glands have ductal system, nerves, vessels, connective tissue and especially the parotid does contain 30-50 intraglandular lymph nodes. The pathology-cytopathology of the salivary glands is very similar to the pathology of the lachrymal glands, the sweat glands of the skin, the small submucosal glands of the trachea and the bronchi and the breast.

Main cell types in the salivary glands:

- <u>Myoepithelial cells</u>: they are usually aspirated together with the acinic cells but also as single cells or as main cell type of tumors does play a significant role in salivary gland cytopathology.
- <u>Basal cells</u>: small cells with round nucleus, the cytoplasm are almost always euchromatic. We may find them in true tissue fragments or in three dimensional cell clusters showing sometimes the palisading arrangement of the most "outer" cell lines. The background stroma is important in differentiating basal cell, basal cell like or small cell lesions.
- <u>Acinic cells</u>: large cells with serous or mucinous cytoplasmic content and small nuclei. Zymogen granules are often seen in the cytoplasm.
- <u>Ductal cells</u>: are usually in small honeycombed structures; the nucleus is centrally located, the cytoplasm is homogeneous. Myoepithelial cells may be attached to ductal cell fragments.
- <u>Lymphoid cells</u> (any types) usually represent intraparotid lymph nodes.

TUMOUR-LIKE LESIONS OF THE SALIVARY GLANDS

Inflammations:

- <u>Acute inflammation</u>: swollen, painful enlarged gland. There are cells of the acute inflammation (PNL), histiocytes, plasma cells present. The acinic and

ductal elements are vulnerable. Severe acute purulent parotitis in elderly patients is sign of a life- threatening general status!

- Chronic inflammation: detritic ductal content as background material is usually present. The acinic cells are extremely vulnerable, the naked cell nuclei in the background usually represent nuclei of "dead" acinic cells. The round cellular background (in nearly all other organs characteristic) is not a typical sign for chronic inflammation in the parotid gland!
- Chronic sclerosing sialoadenitis (Küttner tumour): special type of chronic, destructive inflammation mainly of the submandibular gland. Both the reactive metaplastic squamous cells of the inflamed ducts and the enlarged reactive connective tissue cells may mimic malignant lesion!
- Sialoadenosis: is a form of salivary gland swelling characterized by persistent, asymptomatic, bilateral, diffuse, non-inflammatory, non-neoplastic parotid swelling. It can be mixed up with acinic cell carcinoma cytologically.
- Benign lymphoepithelial lesion/Sjögren disease: it is an autoimmine inflammation of the salivary gland. The smear is similar to that of the lymph nodes. It may be cystic; hence the cells may show osmotic change. In case of follicular lymphoid proliferation the differentiation from malignant lymphoma may be difficult.
- Cysts in the parotid gland: there are typical benign cysts in the organ: retentional salivary cast, lymphoepithelial cyst, etc. Furthermore nearly all kind of tumors may undergo cystic change, which does indeed make the diagnosis of any cystic lesions important and sometimes very difficult in the same time

BENIGN TUMOURS

Pleomorphic adenoma (Mixed tumor): the most common tumor of the parotid, app. 60-70% of all parotid tumours does belong to this group. It consists of different types of myoepithelial cells, in some cases differentiated to squamous cells, keratinizing cells, glandular cells, etc. The myoepithelial may be plasmocytoid or epithelIoid. The stroma is chrondroid, hyaline, fibrotic or even osseous. The stroma is almost always metachromatic with Romanowsky-type staining. Some extremely atypical looking, enlarged cells may be part of a benign lesion in case they are many, furthermore mitotic figures occurred, an in situ malignant pleomorphic adenoma is encountered.

8- 9% of all pleomorphic adenomas may undergo invasive malignant transformation; it is usually squamous cell epithelial, undifferentiated or even sarcomatoid spindle cell tumor.

Warthin tumor: occurs equally in males and females, strongly "smoking-related", it is frequently bilateral. It has cystic, oncocytic lymphoepithelial structure. The aspirated material usually looks like pus. The background is the cystic content, embedded in this are oncocytic cells, lymphocytes and histiocytes. Following necrotic change squamous metaplasia may occur, mimicking squamous cancer.

Oncocytoma: monomorph middle sized oncocytic epithelial cells are in the smear.

Myoepithelial adenoma: monomorph epitheloid or plasmocytoid cells, clean background, some cells representing normal salivary gland epithelium. The chondromyxoid stroma is not present.

Basal cell adenoma: small basaloid cells in homogeneous population. Sometimes basal membrane like fragmented background is seen.

MALIGNANT TUMOURS

Acinic cell carcinoma

Epithelial-myoepithelial carcinoma

Mucoepidermoid carcinoma

Malignant myoepithelioma

Squamous cell carcinoma

Undifferentiated carcinoma

Acinic cell carcinoma: four patterns (solid, microcystic, papillary-cystic, follicular) and four cell types (acinar cell, clear cell, intercalated duct-like cell, and vacuolated cell) may characterize the tumor. The tumor is more common in women, it occurs in children as well. The tumor usually is heavily infiltrated by lymphocytes; in this case the cytology picture is similar to a well-differentiated adenocarcinoma metastasis to a lymph node! The cytodiagnosis is based upon the presence of acinic tumor without any ductal elements! Smear of sialodenosis may mimic acinic cell carcinoma.

Adenoid cystic carcinoma: Infiltrative malignant "basaloid" tumor of the salivary gland. The smears are rich in basaloid, monomorphic cells (the cyto-morphology does not correspond to a highly malignant tumor!) closely connected to or embedded into metachromatic, cylindriform hyaline globules. The basaloid cell population is partly of myoepithelial in origin.

Mucoepidermoid carcinoma: the tumor consists of squamous, mucus producing and intermediate epithelial cells embedded in mucinous background. Since it is nearly always multifocal, normal salivary gland elements may hide the real nature of the lesion – this is one of the most difficult cytology diagnoses in the salivary gland.

Epithelial-myoepithelial carcinoma: the tumor is composed of two cell types, which form duct like structures: one of them is luminal - inner layer - the other is abluminal (clear, myoepithelial cell), myoepithelial – outer layer. The cytology is that of a malignant clear cell tumor, with different degrees of cellular and nuclear atypia. There are both true tissue fragments and isolated cells present; the stromal component is metachromatic, hyaline like and elongated in appearance.

Malignant myoepithelioma: it is composed of malignant myoepithelial cells in an aggressively growing pattern showing high mitotic activity. The cyto-morphology is that of a monomorphic, clear cell or epitheloid-plasmocytoid cell malignant tumor.

Squamous cell carcinoma: is similar to the same type of tumors found in other anatomical sites: keratinizing or non-keratinizing atypical squamous cells are found in true tissue fragments, cell clusters as well. Since the lesion is frequently liquified and necrotic, the result of the puncture may be dirty, purulent-like fluid – only the microscopic appearance may distinguish these lesions from inflammatory content of any kinds of epithelial cysts!

Undifferentiated carcinoma: this tumor is clinically already malignant: firm lesion, facial nerve palsy is usually present. The aspirate contains large, highly atypical cells with large, atypical nuclei.

OTHERS

Haemangioma of infants: congenital, large painless lesion during the first 6 month of life. The punctate contains of peripheral blood in which the proliferative endothelial cells may be seen. It is always benign!

Lymphangioma: developmental disorder, the punctate is yellowish slightly bloody fluid which contains mainly lymphocytes. The cystic lesion refills usually in a couple of minutes after the needling.

Peripheral nerve sheath tumor: the tumor is connected to the facial nerve in the parotid region hence the puncture is painless. The cytology pattern resembles to the histology: Verocay body like structures of mesenchymal cells are found.

Literature:

1. Seifert G, Donath K. On, the pathogenesis of the Kuttner tumor of the submandibular gland—analysis of 349 cases with chronic sialadenitis of the submandibular glands. HNO 25: 81–92. 1997
2. Klijanienko J, Vielh P.: Fine-needle sampling of salivary gland lesions. VII. Cytology and histology correlation of five cases of epithelial-myoepithelial carcinoma. Diagn Cytopathol. Dec;19(6):405-9. 1998
3. Klijanienko J, Lagacé R, Servois V, Lussier C, El-Naggar AK, Vielh P.: Fine needle sampling of primary neuroendocrine carcinomas of salivary glands: cytohistological correlations and clinical analysis Diagn Cytopathol. Mar;24(3):163-6. 2001.
4. Klijanienko, J., Vielh, P.: Salivary Gland Tumours. Karger 2001
5. M.Tiemann, A. Teymoortash, C. Schrader, J. A. Werner, R. Parwaresch, G. Seifert, G. Klöppel:Chronic Sclerosing Sialadenitis of the Submandibular Gland is Mainly Due to a T Lymphocyte Immune Reaction. Mod Pathol 15(8):845–852, 2002.
6. Zarbo, R.J.: Salivary Gland Neoplasia: A Review for the Practicing Pathologist Mod Pathol;15(3):298–323, 2002
7. J.W. Eveson, J.W., Auclair, P.,Gnepp, D.R.,El-Naggar, A.K.: Tumors of the salvary glands In.: Pathology and Genetics of Head and Neck Tumours, World Health Organization Classification of Tumours, IARCPress Lyon, 211-281, 2005
8. Emanuel, P., Wang, B., Wu, M., Burstein, D.E.: p63 Immunohistochemistry in the distinction of adenoid cystic carcinoma from basaloid squamous cell carcinoma . Modern Pathology 18, 645–650, 2005
9. Klijanienko J, Caillaud JM, Lagacé R.: Cytohistologic correlations in schwannomas (neurilemmomas), including "ancient," cellular, and epithelioid variants. Diagn Cytopathol. Aug;34(8):517-22. 2006

Lymph node cytopathology

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INTRODUCTION

Lymph nodes have probably been the first organs to be investigated by fine needle cytology (FNC) and last in which this technique has been accepted as a useful diagnostic procedure. Risk of seeding, false negatives, impossibility to discriminate between reactive hyperplasia and lymphoma, delay in definite pathological diagnosis ad even disturbance in microscopic evaluation of histological sections have been put forward to maintain the limitation or useless of the FNC. Nonetheless because this technique is fast, cheap and effective, lymph nodes FNC is often required by general practitioners as well as specialists and surgeons as first diagnostic approach; moreover ancillary techniques such as immuocytochemistry, flow cytometry, fluorescence in situ hybridization and molecular techniques are successfully applied to cytological samples, therefore FNC of lymph nodes have gained credibility and a definite role in the diagnosis of lymphoadanopathies. Exhaustive books and excellent articles are available for lymph nodal cytopathologists, which are required for knowledge of the pathology, awareness of limitations of the technique and a little bit of recklessness to practice this field of cytology.

PRELIMINARY SUGGESTIONS

► Perform yourself FNC of lymph nodes: clinical evaluation, correct FNC, management of the diagnostic material, perfect smears and their immediate evaluation are mandatory for a correct diagnosis.

► Distrust smears prepared by others and do not try to diagnose on sub-optimal or technically poor smears.

► Use ancillary techniques according to the cytological features and clinical indication.

► Do not delay, if possible, essential information to the clinicians (benign-malignant) while waiting for further microscopic evaluation, special techniques results or bibliographic researches.

► Report your diagnosis in a regular and consequential manner, reporting, if possible, 1) the anatomical identification of the target as a lymph node, 2) the diagnostic category: negative, positive, suspicious, inadequate, 3) a brief and pertinent cytological description, 4) the final diagnosis considering what may be asserted, what may be only suggested and what can not be maintained.

TECHNICAL ASPECTS

- FNC and Smear preparing
- Basic staining
- Cytospin
- Cell block

- Immunocytochemistry
- Flow cytometry
- Fluorescence in situ hybridization
- Molecular biology

- **FNC and smear preparing**

► Palpable lymph nodes FNC should be performed using 23 or 25 gauge needle; aspiration may be used or not. Small lymph nodes (1 cm or less) may be easier approached with short needle, without aspiration equipment, getting closer to the target.

► Block the lymph node between two fingers trying to shorten the distance between the skin and the target and drive the needle proportionally to the distance to pass through.

► FNC of impalpable or deep located lymph nodes have to be performed under ultrasound control. In this case chose the shortest and more direct way: the longer and more oblique the way, the higher the risk of deviation of the needle. Movements of the needle are limited in the driver, if lymph node is sufficiently large try to address the most significant part.

► Follow the needling perceptions: skin offers the first resistance, lesser the capsule; getting in the lymph node you may have the needling sensation of getting in an "empty" space; if so you have reached the target.

► Move gently the needle up and down and in the three directions with a width proportional to the size of the target.

► When you have inserted the needle, ask the patient if he feels pain; if so you might be out of the target.

► Evaluate the needling: soft, hard, fibrous; crushing it might hel you in the evaluation of the smears.

► Control the hub of the needle during aspiration; when some material appears, release the piston and prepare the smears. If blood appears, interrupt immediately the pass; blood hampers your smear and haemorrhage compromises a possible second pass.

► Aspirations should not exceed 20-30; if a node feels "fibrous" at needling, further aspirations may be useless or cause micro-haemorrhages.

► Flush very gently the aspirated material; if you get it in small drops you may prepare additional smears from the same pass or may use the material for ancillary techniques.

► Smearing should be light enough to obtain monolayer smears which immediately air dry and gently enough to preserve the morphology of lymphocytes.

► Examine immediately a Diff Quik stained smear for adequacy and to decide the management of material.

- **Smears basic staining**

Diff Quik and Papanicolaou are complementary and the most utilized staining. Diff Quik allows an immediate evaluation of the smears, keeps the background, highlights the fibrous connective tissue metachromasia and the orange granules of eosinophils. Papanicolaou is informative of nuclear and cytoplasm details; if necessary may be distained and used for immunocytochemical stains.

- **Cytospin**

Remainder material in the hub of the needle and or second pass may be suspended in buffered solution at pH 7.4. One or two millions of cells, which are easily obtained from one pass, are needed to prepare six or more cytospins to be used for immunocytochemistry. Air-dried cytospins can be stored at room temperature for up to 1 week or may be stored at -20° for longer periods (1-2 years). Both immunoalkaline phosphatase and immunoperoxidase can be used on cytospin.

- **Cell block**

Cell blocks can be prepared including in paraffin centrifuged cell suspensions; Cytoblock cassettes for formalin-fixed cell suspension may be used. Numerous sections, long term storing and the same antigenic conditions of formalin-fixed histological sections are the main advantages of these cell blocks.

- **Immunocytochemistry**

May be performed on alcohol-fixed smears or on sections obtained by cell block. Biotin-Streptavidin or two steps

- **Flow cytometry**

Residual material in the hub of the needle and additional passes may be suspended in buffered solution at pH 7.4 or RMPI. One or two millions of cells, which can be collected by one pass, are distributed in six tubes for a basic panel of fluoresceinated antibodies. These latter are used in triplet or even four antibodies conjugated with different fluorochromes using double laser instruments. If possible, keeping one tube for possible further evaluation would be useful. Fluoresceinated antibodies conjugation has to be performed promptly because vital cells may swell in suspension. After conjugation cell swelling may prevented by fixation, adding a drop of buffered formalin, and analysis may be postponed or repeated. Advantages of FC are: direct reaction antigen-antibody, exact quantification of antigen expression, possible co-expression of two different antibodies on the same cells. Disadvantages are absence of morphology, loss of large cells, difficulty to identify numerically scanty cell populations.

- **Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) may be performed on smears or cytospins. (Jang F, Caraway NP). The advantages of using cytospins are: cell concentration, probe saving, and reducing times of analysis. Detection of specific translocations of NHLs is the main advantage of FISH.

- **Polymerase chain reaction**

Aspirated cell may be suspended in triazol or DNA later for RNA and DNA extraction respectively. Cells may be stored at room temperature until DNA extraction. Specific enzymes and are used to cut specific DNA segments which are amplified using oligonucleotide probes and polymerase enzymes in repetitive cycles of denaturation and

annealing. The products of amplification are analysed by gel electrophoresis. The Jh locus of Ig heavy chains or the Tc receptor are amplified and evaluated to assess monoclonality or polyclonality.

NORMAL LYMPH NODE HISTOLOGY AND CYTOLOGY

- Normal lymph node histology
- FNC evaluation
- Cytotypes
- Diagnostic material management and ancillary techniques
- Cytological patterns

• Normal lymph node histology

Lymph nodes are surrounded by a fibrous capsule and divided by septa. Thin reticular fibers and reticular fibers are the support of the internal components.

Capsule is reached by afferent lymphatics ducts carrying the lymph that flows in the sub capsular and toward peripheral sinus.

Lymph nodes are anatomically and functionally composed of three distinct areas: cortex, paracortex and medulla. Cortex is a B-cell dependent area, which contains primary and secondary follicles. Primary follicles are spherical aggregates of small lymphocytes. Secondary follicles are the place of antigen exposition and B-lymphocyte recruitment and rearrangement and are formed by a “pale” germinal centre encircled by a dark, thick border of small B-lymphocytes. Paracortex surrounds follicles and is interposed between cortex and medulla; it is a T-cell dependant area, formed by small lymphocytes. Medulla is the place of the sinus where lymph is collected towards the efferent lymphatic duct and is a B-dependent area; medulla contains macrophages, plasma cells and lymphocytes. Each of these areas is far from static can expand in different pathological conditions reflecting in the smears the corresponding cell composition prevalence.

• FNC evaluation

► Look at the smear at low magnification: is the smear cellular or scantly cellular? monomorphous or polymorphous? aggregated or dispersed?

► Identify isolated cells, lymphoid cells in the different stages of development and their eventual numerical prevalence, granulocytes and histiocytes.

► Look the cell groups: are there immature lymphoid cells?, macrophages-lymphoid complex?, epithelioid cells?, metastatic cells?

► Identify stroma and vascular structures.

► Evaluate nuclear atypia but be aware that most of NHL cells are just the clonal expansion of the corresponding non-lymphomatous cytotypes.

► Compare cytological findings with ICC and/or FC, FISH and molecular data if available.

- **Cytotypes and their prevalence in different pathological entities**

► Mature lymphocytes have small and dark nuclei with coarse chromatin and a thin rim of cytoplasm. B or T phenotype is indistinguishable by cytological features whereas small cytoplasmic “tales” seem typical of T-lymphocytes. T-lymphocytes may be excessively numerous in lymphocyte-predominance Hodgkin lymphoma or in T-rich B-cell lymphoma.

► Plasma cells have eccentrically located nuclei with cartwheel chromatin and large, well defined cytoplasm with pale rim in paranuclear area. Plasma cells can predominant in syphilis, in lymphplasmacytoid lymphoma and in primary plasmacytoma.

► Centrocytes are medium-sized centrofollicular cells with pale irregular nuclei and scanty cytoplasm. Deep nuclear cleavages are better appreciated in histological sections than in the smears in which centrocytes shows irregularities in shape sometimes vaguely polygonal instead of roundish. Centrocytes can be significantly present in follicular hyperplasia, follicular lymphoma and mantle cell lymphoma.

► Centroblasts are large centrofollicular cells with pale round nuclei and two or more marginal nucleoli. These latter are almost attached to the nuclear membrane on histological sections and often just marginal on the smears. Centroblasts can be significantly present in follicular hyperplasia and high-grade follicular lymphoma

► Immunoblasts of either B or T phenotype are the largest lymphoid cells with pale nuclei and one or two centrally located very large nucleoli. A well-defined deeply basophilic rim of cytoplasm may be typical of these large “blue cells”. Immunoblasts may be numerous in viral, postvaccinial and mononucleosis lymphadenitis and immunoblstic NHL.

► Macrophages are large cells with wide cytoplasm, often engulfed with phagocyted cellular debris, and one or two eccentrically located nuclei. These latter may be round or oval pale with “dusty” chromatin and small or inconspicuous nucleoli. Macrophages are predominant in sinus histiocytosis or intermingled among lymphoid cells in reactive hyperplasia, “starry sky” pattern in lymphoblastic lymphoma.

► Lymphoblasts are undifferentiated medium-size lymphoid cells with “dusty” dispersed chromatin and one or two small nucleoli. Nuclear fragility, high mitotic rate and nuclear crashes are typical of this cytotype. Monotonous pattern in lymphoblastic lymphoma.

REACTIVE LYMPHADENOPATHY

- Non-specific hyperplasia
- Viral and post-vaccinial
- HIV infection
- Mononucleosis
- Sinus histiocytosis
- Kikuchi’s disease
- Suppurative lymphadenitis
- Granulomatous lymphadenitis
- Dermatopatic lymphadenitis

- **Non-specific hyperplasia**

FNC of most lymph nodes enlargements shows a variable mixture of different normal cell type constituents. Vascular structures and phagocyting histiocytes may be present conferring a polymorphous appearance to the smear. When lymphadenopathy is maintained by follicular centre expansions smears show numerous centrocytes and centroblasts intermingled with small mature lymphocytes, plasma cells and immunoblasts. In cases of interfollicular expansion the smear shows a prevalence of mature lymphocytes, plasma cells and immunoblasts. In both the cases, in less polymorphous smears differential diagnosis with NHL is pointed out.

Differential diagnosis: follicular lymphoma (see NHL).

- **Viral and post-vaccinial**

FNC shows small lymhocytes and numerous centrocytes and centroblasts intermingled with small mature lymphocytes, plasma cells and immunoblasts. Phagocyting histiocytes and eosinophils may be also present.

Differential diagnosis: follicular lymphoma (see NHL).

- **HIV infection**

The cytological pattern may be variable ranging from suppurative to granulomatous or non-specific. In case of florid follicular hyperplasia smear show numerous centrocytes and centroblasts intermingled with small mature lymphocytes and plasma cells.

Differential diagnosis: follicular lymphoma (see NHL).

- **Mononucleosis**

FNC shows normal cell types constituents including centrocytes and centroblasts. Numerous immunoblasts with large nucleoli and a rim of blue cytoplasm are present.

Differential diagnosis: Hodgkin lymphoma (see HL).

- **Histiocytosis**

Is mainly observed in lymph nodes draining inflamed tissues or organs or cancers. Corresponding smears may show histiocytes, small lymphocytes and macrophages with engulfed cytoplasm.

Differential diagnosis: Sinus histiocytosis with massive lymphadenopathy.

- **Kikuchi's disease**

Is a necrotizing histiocytes lymphadenitis that affect young women with fever and cervical lymph nodes enlargement. The aetiology is unknown. Smears show macrophages engulfed with nuclear debris in a necrotic background and small lymphocytes in the background.

- **Suppurative lymphadenitis**

May be observed in lymph nodes draining bacterial infections. Smears show lymphoid cells and a variable amount of granulocytes in a necrotic background.

- **Granulomatous lymphadenitis**

Granulomatous lymphadenitis may be determined by several infective agents or determined in different pathological processes being sarcoidosis and tuberculosis the most frequent. Smears show epithelioid cells isolated or in a granulomatous arrangement in a lymphoid background.

HODGKIN LYMPHOMA (HL)

- General features
- Classification
- FNC evaluation
- Cytological findings

- **General features**

HL represents approximately 30% of all lymphomas and has a bimodal age curve with a peak at 15-35 years and a second peak >40. Usually arises in cervical lymph nodes and involves adjacent lymph nodes. Mediastinum is involved mainly in nodular sclerosis and the spleen in mixed cellularity. HL is nowadays curable in high percentage of cases; patients may be asymptomatic or may show systemic symptoms, designed as B in the staging, consisting of fever, itching, night sweats and body weight loss. The lymph node architecture is effaced by variable number of scattered large mononucleated cells: Hodgkin cells (HC), “popcorn cells” (L&H) and multinucleated cells: Reed-Sternberg cells (HRS) in a background composed by an heterogeneous admixture of fibrous stroma and non-neoplastic lymphoid cells, neutrophils and eosinophils.

- **Classification**

Nodular lymphocyte-predominant Hodgkin lymphoma

Classical Hodgkin lymphoma:

Nodular sclerosis classical Hodgkin lymphoma

Mixed cellularity classical Hodgkin lymphoma

Lymphocyte-rich classical Hodgkin lymphoma

Lymphocyte-depleted classical Hodgkin lymphoma

- **FNC evaluation**

Smears may be very or scanty cellular; the patterns are variable depending on the corresponding histotypes, the amount of fibrosis and the different cell composition in the different areas of the lymph node. General appearance of the smears is polymorphous with mature lymphocytes and follicular centre cells and a variable amount of neutrophils and eosinophils. Epithelioid cells may also be present; nuclear crushes and lymphoid tangles are expression of concomitant fibrosis. Diagnosis is performed in presence of HC and HRS cells that may be present in variable amount and are generally related to the corresponding histotypes: few in lymphocyte-rich and nodular sclerosis, more in mixed cellularity and

lymphocyte depletion. In nodular lymphocyte-predominant Hodgkin lymphoma the diagnosis is related to the identification of L&C in a lymphoid back ground. Diagnostic cells are identified by ICC demonstration of CD15 and CD30 positivity. ICC demonstration of CD20+ of L&H cell may be equivocal because immunoblasts may be CD20+ as well.

- **Cytological findings**

Nodular lymphocyte-predominant Hodgkin lymphoma: is characterized by a diffuse lymphoid background in which there are large neoplastic monucleated cells known as lymphocytic and/or histiocytic Reed-Sternberg cell variants (L&H). The nuclei are often folded or multilobated, the chromatin is mostly vesicular, the nucleoli are usually multiple and smaller than those seen in classical HRS cells. Histiocytes and some polyclonal plasma cells can be found at the margin of the nodules; the diffuse areas are mainly composed of small lymphocytes with admixed histiocytes.

ICC: L&H cells: CD15-, CD30-, CD20+, CD79a+, BCL6+ and CD45+; immunoglobulin light chains frequently positive.

FC: CD4/CD8 co-expression (Rahemtullah A)

Classical Hodgkin lymphoma: HRS cells are large and show abundant slightly basophilic cytoplasm and two nuclear lobes or nuclei. Diagnostic RS cells have at least two nucleoli in two separate nuclear lobes. Mononuclear variants are termed Hodgkin cells. Hodgkin cells represent only a minority of the cellular infiltrate; the composition of the reactive cellular infiltrate varies according to the histological subtype.

HRS cells are positive for CD30 in nearly all cases, and for CD15 in the majority of cases; are usually negative for CD45. Most HRS cells express the proliferation-associated nuclear antigen Ki-67.

Classical Hodgkin lymphoma is classified as:

Nodular sclerosis, Lymphocyte-rich, Mixed cellularity and Lymphocyte-depleted

- *Nodular sclerosis* contains a variable number of HRS cells, small lymphocytes and other non-neoplastic inflammatory cells. The HRS cells of this subtype tend to have more lobated nuclei with smaller lobes, and less prominent nucleoli. Eosinophils may be numerous.
- *Mixed cellularity* HRS cells are typical; the background cells consist of a mixture of eosinophils, neutrophils, histiocytes and plasma cells. One of these cell types may predominate. The histiocytes may show pronounced epithelioid differentiation and may form granuloma-like clusters.
- *Lymphocyte-rich*: scattered HRS cells in a background characterized by an abundance of small lymphocytes. Eosinophils and neutrophils may be present in small numbers. The small lymphocytes may be admixed with a large number of histiocytes with or without epithelioid features.
- *Lymphocyte-depleted*: is rich in HRS cells and/or depleted in non-neoplastic lymphocytes. In some cases pleomorphic HRS cells may predominate, producing a sarcomatous appearance.

ICC: HC and HRS cells: CD15+, CD30+, CD20-, CD45-.

NON HODGKIN LYMPHOMA (NHL)

- General features
- Classification and frequency
- Role of cytology
- Diagnostic criteria for NHL

- **General features**

NHL is an heterogeneous group of neoplasm that represents 2-3% of all the tumours in the Western World. They encompass the slowest and the fastest growing tumours, ranging from chronic lymphatic leukemia/small lymphocytic lymphoma which in early stages may be untreated (watch and wait) to lymphoblastic lymphoma which require timely and intense therapy. Moreover some entities are clinically well identified by clinical, phenotype, genetic and molecular profiles - as generally happens for small cell lymphomas. Others such as diffuse, large, B-cell lymphoma (DLBCL) seem to be a container for molecular and clinically different entities. Clinical signs show the same variability: weight loss, itch, fever, weakness may be variably complained of. Sometimes symptoms and signs related only to the lymph nodal growth are present. Even the involved lymph nodes are quite variable whereas anterior cervical are the most frequently involved followed by supra-clavicular and axillary. Histological classification has been reformulated several times reflecting the huge amount of knowledge acquired in the field of immunology and genetic of lymphomas. The most accepted classification is the revised European American Lymphoma (REAL) classification.

- **Role of cytology**

Cytological diagnosis of NHL is hampered by objective difficulties. Many NHLs lack significant nuclear atypia, as happens in other neoplasms, because they are the clonal expansion of lymphocytes in a specific stage of maturation. Moreover these cells may be intermingled with different amounts of other lymphoid cells and reactive cells.

In practice the cytological diagnosis of NHL is determined by a combination of microscopic features and ancillary techniques. Differentiated B-cell lymphomas generally show a more monomorphous cell pattern but a definitive diagnosis may be assessed only by light chains clonality (κ/λ >4:1 or <1:1) or IGH rearrangement. Attention should be paid to quantitatively small imbalance, mainly if detected by FC (less than 20% of the gated cells). In fact small imbalance, in absence of lymphoma may occur in reactive lymph nodes mainly in cases of autoimmune or immunodeficiences syndromes. There are other phenotypic patterns, mainly assessed by FC, which define a lymphoid cell population as B-lymphoma and are frequently sub-type specific: CD5/CD19 co-expression (CLL/SLL or MCL); CD10/Bcl-2 co-expression (FL), CD10+ in >50% of the gated cells, combined with a specific cytological pattern (BL).

T-cell lymphomas are more complex; peripheral T-cell generally express CD2/3/7 and alternatively CD4 or CD8. Loss of one antigen (often CD7) may suggest a clonal proliferation. CD3/CD56 over expression is indicative of NK lymphoma.

Large cell B-cell lymphoma (DLBCL), generally shows more evident nuclear atypia in all its microscopic and phenotypic variants (centroblastic, immunoblasic, anaplastic). In these

cases cytological features and phenotypic assessment can be diagnostic (CD19+, aberrant phenotypes by FC, ALK+,CD30-, EMA+ in DLBCL ALK+).

Cytological features combined with ancillary techniques allow a correct diagnosis and classification in most of the cases. However, cytopathologists should be aware that objective limitations still exist in the diagnosis as well as in classification of lymphoprolipherative processes. Therefore, rather than repetition, surgical excision and histological examination should always considered in primary processes as well as in follow-up of known cases.

- **Diagnostic criteria**

Chronic lymphatic leukemia/small lymphocytic lymphoma (CLL/SLL)

Cytology: smears show monotonous, dispersed cell population composed by small lymphocytes with clumped chromatin and occasional, small, nucleoli; mitoses are rare if any. Accelerate phases of CLL/SLL show larger, nucleolated, cells (paraimmunoblasts) and mitoses and should not confused with a Richter's syndrome which is a large-cell "histiocytic" lymphoma arising on a CLL/SLL background.

ICC: κ or λ light chains restriction, CD5+, CD20+, CD23+, Ki67+ in less than 10% of the cells.

FC: CD5/CD19+, CD19/CD23+, CD19+/CD10-, κ or λ light chains restriction.

FISH: trisomy 12 (Liu K), 11q22 (ATM), 12, 13q14.3, 13q34.3 (LAMP1), and 17p13.1 (p53) as prognostic markers (Karaway N)

Differential diagnosis: Non-specific hyperplasia

Lymphoplasmacytoid Lymphoma, Immunocytoma (LPCL)

Cytology: dispersed cell population composed by small lymphocytes with plasmacytoid differentiation; mature plasma cells are also present.

ICC: CD20+, CD5-,CD10-, CD23-, Ki67+ in a variable percentage (10-20%) of the cells

FC: CD5-/CD19+, CD19+/CD23+, CD19+/CD10-, CD19+/CD38+, κ or λ light chains restriction.

Differential diagnosis: Non-specific hyperplasia

Mantle cell lymphoma (MCL)

Cytology: dispersed cell population composed by medium-size lymphocytes with irregular nuclei and a thin rim of pale cytoplasm. Nuclei may be slightly irregular in shape or cleaved and have clumped chromatin and small nucleoli. Mitoses are scanty whereas may be numerous in "blastoid" variant

ICC: CD20+, CD5+,CD10-, CD23-, Ki67+ in a variable percentage (10-20%) of the cells

FC: CD5+/CD19+, CD19/FMC7+, CD19+/CD23-, CD19+/CD10-, κ or λ light chains restriction.

FISH: t(11;14)(q13;q32) (Caraway N)

Differential diagnosis: Non-specific hyperplasia, SLL/CLL,FL.

Follicle centre lymphoma (FL)

Cytology: dispersed cell population composed by a spectrum of medium-size to large lymphocytes with irregular nuclei and a thin rim of pale cytoplasm. Nuclei range from slightly irregular in shape or cleaved with clumped chromatin and inconspicuous nucleoli in grade I - to large nuclei with dispersed chromatin and two or more marginal nucleoli in grades II and III.

A variable amount of mature B-lymphocytes may be present among follicular cells.

ICC: CD20+, CD5-,CD10+, CD23-, Ki67+ in a variable percentage (10-20%) of the cells

FC: CD5-/CD19+, CD19+/FMC7+, CD19+/CD23-, CD19+/CD10+, CD10/bcl-2+, κ or λ light chains restriction.

FISH: t(14;18)(q32;q21) (Kishimoto Richmond)

Differential diagnosis: Non-specific hyperplasia, SLL/CLL,MCL.

Marginal zone B-cell lymphoma (MZL)

Cytology: dispersed cell population composed by small lymphocytes with irregular nuclei and a wider rim of pale cytoplasm. Nuclei may be slightly irregular in shape or cleaved and have dense chromatin and indistinct nucleoli. Monocytoid cells and plasma cells may be intermingled. Mitoses are scanty.

ICC: CD20+, CD5-,CD10-, CD23-, Ki67+ in a variable percentage (10-20%) of the cells

FC: CD5-/CD19+, CD19+/FMC7-, CD19+/CD23-, CD19+/CD10-, κ or λ light chains restriction.

FISH:

Differential diagnosis: Non-specific hyperplasia, SLL/CLL,MCL, FL.

Diffuse large B-cell lymphoma (DLBCL):

Cytology: dispersed cell population composed by large lymphoid cells with irregular nuclei, one or more large nucleoli and a wider rime of pale cytoplasm. Morphological variants are centroblastic, immunoblastic T-cell/histiocytes rich and anaplastic. Nuclei may be slightly irregular in shape or cleaved and have dense chromatin and indistinct nucleoli. Monocyoid cells and plasma cells may be intermingled, Mitoses are scanty.

ICC: CD19+, CD20+, 22+, CD79a+, Ki67+ in a variable percentage (10-20%) of the cells

FC: CD5-/CD19+, CD19+/FMC7-, CD19+/CD23-, CD10-, κ or λ light chains restriction.

Lymphoblastic Lymphoma (Burkitt-like lymphoma) (BL)

Cytology: dispersed cell population composed by undifferentiated medium sized lymphocytes with dispersed chromatin and one or more small basophilic nucleoli; cytoplasm are basophilic and sometimes vacuolated. Immature nuclei are fragile resulting in frequent nuclear crushes in the smear; a high mitotic index is observed. Scattered macrophages with engulfed cytoplasm may give a starry sky pattern to the smear.

ICC: CD20+, CD5-, CD10+, Bcl-6+, CD23-, Ki67+ in a high percentage (10-20%) of the cells

FC: CD5-/CD19+, CD19+/FMC7-, CD19+/CD23-, CD19+/CD10+, light chain often unexpressed.

FISH: t(8;14)(8q24;q32) (troxell)

Peripheral T-cell lymphoma (PTL) (represents 7.6% of all the NHL)

Cytology: dispersed and polymorphous medium-sized or large cells with irregular shape vesicular nuclei and evident nucleoli. Nuclei have irregular shape, coarse chromatin and one or more large nucleoli. Binucleated cells are frequently observed. The background is generally polymorphous with mature lymphocytes granulocytes and eosinophils.

ICC: CD20-, CD3+, CD4+ CD10-, CD23-, Ki67+ in a variable percentage of the cells

FC: CD5+, CD19-, CD10-, CD2+ CD3+ CD7-.

PCR: TC RECEPTOR REARRANGED IN MOST OF THE CASES

T-cell lymphoblastic lymphoma (TLL)

Cytology: dispersed and monomorphous medium-sized undifferentiated cells. Nuclei are round or convoluted with dispersed chromathin and small nucleoli. High mitotic index and nuclear fragility.

ICC: CD20-, CD3+, CD10-, TDT+, Ki67+ in a high percentage of the cells

Anaplastic large cell lymphoma (k1-l)

Cytology: dispersed and polymorphous large-sized or large cells with very irregular shape. Multilobated, horseshoe bi and multinuclecleate cells are present. Nuclei have coarse chromatin and larger nucleoli. The background is generally polymorphous with mature lymphocytes granulocytes and eosinophils. Atypical mitoses and necrosis may be seen.

ICC: CD20-, CD45+ CD15-, CD30+, Ki67+ in a variable percentage of the cells

FC: CD5+/-, CD19+/-, aberrant phenotype may be observed.

CYTOLOGICAL DIAGNOSTICS OF BREAST FNAC

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Introduction

Breast fine needle aspiration cytology (FNAC) represents first choice diagnostic tool to determine mammographic lesions and clinically palpable breast nodules. This method is considered to bring more advantages, as far as invasiveness and as costs if compared with other techniques like core biopsy or surgical biopsy, especially when it is performed under ultrasound guidance, which guarantees perfect targeting of lesions.

Most literature's critics towards FNAC are due to the different realities about where the clinical examination is performed and about the variety of breast lesions examined. The most reliable results with FNAC are obtained in those clinical groups where pathologist, who is also a cytopathology expert, actively joins the aspiration sessions taking care of collecting patients' clinical data, radiological and ecographical features of lesions to be examined. This way, the pathologist can evaluate macroscopic characteristics of aspirated material (fluid, dense, whitish, jelly like, smelly etc..) that will be spread on smears and evaluated right away through a fast stain. Pathologist will be able to evaluate specimen's adequacy and to get an idea of clinical issues the case may arise.

FNAC is affected anyway by a certain degree of subjectiveness, that is more evident with the so-called "grey zone " cases (the ones not clearly benign or not clearly malignant) and when there are some doubts about material adequacy. These situations may create some misunderstandings between clinicians and pathologists with possible over- or under-treatment for patients. The need for clear communication is extremely important, but it becomes a priority in senology (study of the breast) because of the multidisciplinary aspects of the field. For this reasons a breast cytology reporting system has been proposed since 1993 within a U.K. mammographic breast screening programme. The reporting system's aim is to communicate to clinicians in an extremely precise way pathologist's evaluation about cytological cases:

Diagnostic categories

C1 = INADEQUATE; includes all those cases which do not provide the possibility to solve a specific diagnostic problem (poor cellularity, bad technical preparation, excessive inflammatory or blood elements , etc.);

C2 = benign; there's no evidence of malignancy. It includes all cases characterized by absence of nuclear or morphological alterations.

C3 = probably benign; includes all cases in which the smear's cells are not certainly interpretable as benign. Management of such cases requires correlation of cytology with clinical and / or radiological aspects.

C4 = suspicious for malignancy ; the cellular appearance, although highly suggestive for malignancy, is not conclusive. This category includes the cases with few highly atypical cells and some very well-differentiated tumors. These lesions must undergo biopsy to obtain a conclusive diagnosis or, in cases with low cellularity, FNAC can be repeated.

C5 = MALIGNANT; cytological features are diagnostic for malignancy. Sometimes, through FNAC the histotype of malignancy can be determinated.

The five diagnostic categories proposed in the U.K in 1993 were later adopted at a European level; using this system does not limit pathologists' possibility to give a description of morphological aspects observed on smears, but it requires a conclusive short diagnostic assessment that all other breast screening unit members can undoubtedly understand to allow precise and exact indications regarding further exams or treatment to be given. Diagnostic categories have also been revealed as being an extremely useful tool for all the audit activities each anatomic pathology laboratory has to respect within an organized screening programme, providing periodically data that show quality control's respect. As a matter of fact, all the activity's reports and correlations regarding cytology's diagnostic efficacy are based on working out data obtained by diagnostic categories and by comparison of cytologic diagnosis with following histology or clinical follow up. Making use of the five diagnostic categories also permits easy information exchange and comparison among labs in different regions or states and any observed abnormality can be easily discussed and corrected.

Diagnostic categories for multidisciplinary management and audit

We introduced the five diagnostic categories system to report breast cytology in our lab since 1995 in an experimental way; and they have become an essential part of cytological reports since 2000. Mammographic breast screening programme, which has started in our region, Friuli Venezia Giulia, since 2006, has made them mandatory for all cytological reports of pathologists involved in the screening programme. This allows the Public Health Regional Agency (encharged to control and to coordinate the programme) to monitor cytology's reports diagnostic quality.

Each diagnostic category is strictly associated with a diagnostic pathway, which needs well-founded reasons to be modified. C1, for example requires repetition of the examination or histology, which becomes mandatory for those cases with suspicious radiology; in the case of doubtful radiology the patient can also be recalled early for instrumental follow up. Thanks to the pathologist's presence during FNAC sessions, our inadequate rate is very low; and besides the screening detected lesions, cytology has represented the first morphological examination for about 90% of women with breast abnormalities.

In the first table we have represented the number of FNACs in the period 2004-2010 (Table 1). This distribution is representative of breast cytology's performance in the Trieste's area and we can observe the activity's continuous increase.

For each nodule the correlation with histological examination was performed or, for benign lesions not undergoing surgery, correlation with instrumental follow-up was done. No case was lost at follow-up and the correlation was possible even for those lesions with histology performed at other regional facilities due to the possibility offered by the Health Information System of Regione Friuli Venezia Giulia (INSIEL) which allows to share on the network all histological reports made by the Anatomical Pathology labs operating on the regional territory. The outcome of cyto histological correlation is summarized in Table 2

Thanks to the cyto-histological correlation, it was possible to calculate the quality indicators (sensitivity, specificity, predictive values, false positives and inadequate and

compare them with the corresponding reference standards proposed in the guidelines for screening mammography summarized in Table 3.

Meeting the imposed reference standards has been made possible by the direct and continuous participation of the pathologist in the cytologic sample's collection. This has helped to keep the inadequate number very low and to minimize false positives and negatives. Using the rapid cytology to immediately discuss with the radiologist about patient management in the same session provides great results for the patients.

Conclusion

From our experience we think only a cytological exam with well demonstrated and well- documented quality which satisfies those criteria suggested by guidelines will be able to play a key role in patients' clinical management . It will allow to reduce as much as possible diagnostic biopsies on benign lesions and frozen sections to confirm malignant lesions. We believe that a good quality cytology can provide an important and relevant contribution to define breast lesions' nature without using other invasive procedures.

Bibliography

(1) Guidelines for Cytology Procedures and Reporting in Breast Cancer Screening - Cytology Sub-Group of the National Coordinating Commitee for Breast Screening Pathology NHS-BSP No. 22 Sept 1993.

(2) European guidelines for quality assurance in breast cancer screening and diagnosis - fourth edition, 2006.

TABLE 1

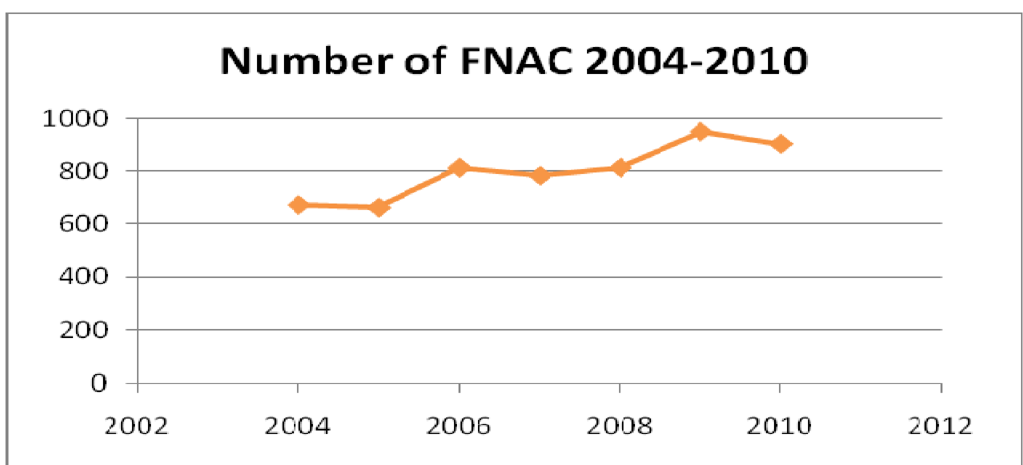

TABLE 2

DATA RECORDS 2004-2010

2004-2010 Data	C5	C4	C3	C2	C1	Total
Malignant histology	1935	217	48	5	53	2258
Benign histology	2	58	228	128	62	478
Malignant at follow-up	93	15				108
Benign at follow-up			235	2353	164	2752
Total FNA	**2030**	**290**	**511**	**2486**	**279**	**5596**

TABLE 3

Indicators	**Data 2004-2010**	**Standard**
Absolute sensitivity	**86,3%**	>60%
Complete sensitivity	**97,5%**	>80%
Specificity (biopsy cases only)	**26,8%**	
Specificity (full)	**76,8%**	>60%
VPP C5	**99,9%**	>95%
VPP C4	**78,9%**	70-80%
VPP C3	**9,4%**	<20%
False-negative rate	**0,2%**	<5%
False-positive rate	**0,1%**	<1%
Inadequate rate	**5,0%**	<25%
Inadequate rate from cancers	**2,2%**	<10%
Suspicious rate	**14,3%**	<20%
Total FNA 2004-2010	**5596**	

Thyroid cytopathology - classical patterns, pitfalls and challenges

Professor Aleš Ryška

Thyroid FNAC has dramatically changed current clinical approach to patients with thyroid lesions. It has led to significant reduction in number of thyroidectomies performed; still the number of diagnosed and treated malignancies has increased. In experienced hands it is a valuable method with high sensitivity and specificity. However, it has - as any other method - its limitations.

Typically, the thyroid lesions are classified in following categories:

1. Hyperplastic goiter (including macrofollicular nodule, microfollicular nodule, regressive and posthaemorrhagic changes, post-therapeutic changes)
2. Chronic inflammation (including Hashimoto thyroiditis, focal lymphocytic thyroiditis, postpartum thyroiditis)
3. Granulomatous thyroiditis (including de Quervain subacute thyroiditis, tuberculosis, sarcoidosis)
4. Hyperfunctional changes (including Graves-Basedow disease, toxic hyperplastic nodule, toxic adenoma)
5. Follicular neoplasia (including follicular adenoma, minimally invasive follicular carcinoma, widely invasive follicular carcinoma)
6. Papillary carcinoma (including classical and various variants)
7. Medullary carcinoma
8. Poorly differentiated carcinoma
9. Undifferentiated (anaplastic) carcinoma
10. Malignant lymphoma
11. Metastasis from other primary tumor

Most important issues in thyroid FNAC

Staining

No definite recommendation - 3 options

1. Papanicolaou – excellent nuclear features - wet fixation needed
2. May-Grünwald-Giemsa – drying out at room temperature, less nuclear details, better cytoplasmic features and other structures
3. Haematoxylin-eosin

Evaluation

- all smears, whole area (periphery!)
- morphologic features in context – synthesis of information
- CAVE! - under- or overdiagnosis based on a single morphologic detail
- close clinico-pathologic cooperation is a must!
- limitations of the method - FNAC is not supposed to replace histology
- second opinion, ancilliary techniques

Criteria of adequacy

- variable at different institutions
- 5-6 cell groups, 10-15 follicular cells each
- 10 cell groups, 20 follicular cells each
- counting of cells = limited evaluation
- "benign structures only, amount of material is limited" - repeat the aspiration

Non-diagnostic smears - most frequent causes

- missing small nodule
- abnormal structure of the lesion (cystic, highly vascularized or regressively changed nodules)
- non-diagnostic aspirations 1.6 - 21% cases - when more than 10% - discuss the aspiration with your clinician

Individual diagnostic entities

Hyperplastic goiter

- any enlargement without defined reason
- diffuse goiter (homogenous, no nodular rearrangement)
- children – virtually always diffuse goiter
- variable proliferative activity - selection of populations with different features, somatic mutations
- hyperplastic nodules – nodular goiter

  Cytologic features
  - variable cellularity of smears
  - abundant colloid, mosaic pattern
  - hyperfunction - anisokaryosis, vacuolization of cytoplasm, "lace-like margin"
  - macrofollicular – large flat fragments and sheets
  - non-transparent tissue-fragments
  - regressive change – nuclear shrinkage, vacuoles, granules of hemosiderin
  - siderophages, foamy histiocytes, cholesterol crystals

Inflammations

Acute suppurative thyroiditis

- rare, relatively more frequent in children under 10 Y (piriform sinus fistule); immuno-compromized patients
- FNAC - pus, cultivation!

  Cytologic features
  - neutrophillic granulocytes, histiocytes, macrophages
  - degenerative changes of follicular cells

Subacute granulomatous thyroiditis (de Quervain)

- viral etiology, heredity (antigen HLA-B35), often follows acute respiratory infection
- may be unilateral (single lobe) - presents as a solitary nodule!

  Cytologic features

- moderate cellularity
- cellular debris, small amounts of colloid, regressive changes of follicular cells
- lymphocytes, neutrophils and macrophages
- epithelioid cells - „wavy" nuclei - granulomas
- multinucleated giant cells (reaction to colloid) - not specific for subacute thyroiditis !!!

Palpation thyroiditis

- granulomatous inflammation, no clinical importance
- granulomas with multinucleated giant cells
- reaction to ruptured follicles

Autoimmune thyroiditis

Thyroiditis	Clin.course	Characteristic features
with goiter (Hashimoto)	chronic	goiter, lymphoid infiltrate, oncocytic transformation of follicular cells, fibrosis
atrophic (primary hypothyroidism)	chronic	atrophy, fibrosis
juvenile	chronic	lymphocytic infiltrate
focal lymphocytic	self-limited	present in 20% of goiters at autopsy
post-partum	transient	small goiter, lymphocytic infiltrate
silent	transient	small goiter, lymphocytic infiltrate

Hashimoto thyroiditis

- goiter, variable size and character, small changes of thyroid size (fast progression - suspicious of malignancy)
- clinical symptoms of hypothyroidism
- ultrasound - hypoechoic, inhomogenous structure

Focal lymphocytic thyroiditis

- up to 20% of goiters, no clinical symptoms
- cytologically - differentiation from HT may be difficult - small or moderate amount of lymphoid elements, predominance of mature lymphocytes

Diffuse toxic goiter (Graves-Basedow disease)

- most frequent cause of thyroid hyperfunction, organ specific autoimmune disorder
- autoantibodies against TSH receptor
- thyroid growth + increased production of T3, T4
- indication for FNAC = nodule (present in 40% of GB goiters, carcinoma in 3,5% of GB goiters)

- probability of malignancy of the nodule = 11%

  Cytologic features
  - high cellularity
  - minimal amount of colloid
  - follicular cells in small and middle-sized groups, moderate cohesivity
  - marginal vacuoles at periphery
  - background with small amount of mature lymphocytes

Tumors

Papillary carcinoma

- most frequent thyroid malignancy (65-80%) derived from follicular cells
- tumor papillae and/or specific nuclear features
- F:M = 2-3:1, any age, microCa more frequrent in males !
- more frequent in the bckground of lymphocytic thyroiditis (RET/PTC rearrangement in up to 95% HT)
- RET/PTC rearrangement, BRAF mutation
- typical morphologic nuclear features (deformation of nuclear membrane chromatin clearing) – ground glass nuclei - not in all neoplastic cells!
- early metastasizing to cervical LN

  Architectural patterns
  - classical PC
  - follicular variant of PC (including macrofollicular, encapsulated, multifocal)
  - tall cell PC
  - columnar cell PC
  - diffuse sclerosing PC
  - cribriform-morular PC
  - oncocytic + Warthin-like PC
  - PC with nodular fasciitis-like stroma
  - papillary microcarcinoma

  More aggressive behavior
  - tall cell PC
  - columnar cell PC
  - diffuse sclerosing PC

  Less aggressive
  - encapsulated PC
  - papillary microcarcinoma

  Cytological features

- high cellularity
- increased N/C ratio
- intranuclear pseudoinclusions – higher frequency
- multinucleated giant cells wihout presence of lymphoid elements
- thick colloid (chewing-gum like)
- intranuclear grooves
- papillary fragments
- psammoma bodies

Follicular neoplasms
Hyperplastic nodule
Follicular adenoma
Atypical follicular adenoma (UMP)
Follicular carcinoma
- minimally invasive – without vascular invasion
- minimally invasive – with vascular invasion
- widely invasive

Follicular variant of PC
Mixed tumors

There are no reliable cytologic criteria, distinguishing benign and malignant follicular tumors

Follicular carcinoma
- malignant conterpart of follicular adenoma, more frequent in elderly and in regions with iodine defficiency
- Evrope 27%, USA 10%
- transcapsular invasion and/or vascular invasion
- biologic behavior different from PC (hematogenous spread, metastases into bones, brain and lungs)
- ras mutations

  Cytologic features of follicular neoplasia
  - high cellularity
  - low cohesivness of cells
  - microfollicular formations (rosette-like)
  - dense colloid in microfollicles
  - absence of colloid in the background
  - nuclear anisomorphy (no prognostic significance)

Oncocytic (Hürtle cell) tumors (in last WHO classification not a separate category)
- >75% of oncocytes (Askanazy, oxyphillic or Hürthle cells), abundant mitochondriae
- oncocytic adenoma
- oncocytic carcinoma

- follicular (subcategory of follicular carcinoma)
- papillary (oncocytic variant of papillary carcinoma)

Anaplastic (undifferentiated) carcinoma

- rare - USA 1,7%, Germany 3,6%
- M:F = 1,5:1; max. 7. decade
- rapidly growing mass, extrathyroidal spread
- necrosis, calcifications, bone formation
- large cells, variation of size and shape, bizzare nuclei, multinucleated cells, mitotic and apoptotic activity, vascular invasion
- histologic types: squamous
- giant-cell
- spindle-cell (sarcomatoid)
- frequently mixed features

Cytological features

- high cellularity
- absence of colloid
- necrotic debris and neutrophills
- poorly cohesive cell groups
- large polygonal cells
- spindle cells
- bi- or multinucleated cells

Medullary thyroid carcinoma (MTC)

- relatively rare - sporadic or as a part of hereditary syndromes (25%) - MEN 2A, MEN 2B, FMC
- typical growth pattern x atypical manifestations
- unusual patterns small cell
  giant cell
  squamous cell
  amphicrine
  mucinous
  pigmented
  follicular
  oncocytic

Cytological features

- moderately to highly cellular smears
- poorly cohesive cells, absence of colloid
- fragments of amyloid (1/4 cases)
- round, oval, triangular, spindle cells

Thyroid lymphomas

- relatively rare - 2% of extranodal lymphomas, 5% of all thyroid malignancies

- max. in 7. decade, M:F = 3:1
- thyroid enlargement, growth of nodule
- virtually always in the background of HT
- non-Hodgkin ML, 98% B cells
- high-grade transformation from MALT-lymphoma

Secondary neoplasms

- rare (autoptic series 24%, bioptical series – significantly less)
- direct growth (larynx, hypopharynx, esophagus)
- hematogenous – tumor generalization
- solitary metastasis, long time span
- serious diagnostic problem
  renal, lung, breast, GIT ca, melanoma

Pitfalls and limitations

In cases, where classical morfological features are present, main limitations of thyroid cytology are in following areas:

1) FNAC is not able to distinguish follicular adenoma from follicular carcinoma (the same is valid for oncotytic (Hürtle cell) tumors)

2) To distinguish florid chronic inflammation (e.g. Hashimoto thyroiditis) from low-grade malignant lymphoma (MALT ML) is extremely difficult and often even impossible on cytological level.

3) Differentiation of follicular tumor from parathyroid lesion - it is recommended to add examination of PTH level in aspirated fluid (which is extremely high in case of parathoroid lesion and low in case of follicular neoplasia)

Regarding the follicular neoplasia dilemma, no one technique tested so far has brought any improvement - many work on a statistical basis, but in an individual case, they are useless. One must admit this limitation, therefore a common category of follicular lesion (or follicular neoplasia, or follicular proliferation) is used to diagnose these lesions. This diagnostic category is a signal for a surgeon that the tumor should be removed, as there exists 10-20% risk of malignancy.

A lot of debate has been taken about the borderline category - classified in the Bethesda classification as "follicular lesion of uncertain significance". This category, however, is so far not precisely defined and thus a threshold between "follicular neoplasia" and "follicular lesion of uncertain significance" is probably set differently in different labs.

Other pitfalls are hidden in cases, where the cytological features are obscured or blurred - typically due to concurrent regressive changes. Thus, cystically degenerated papillary carcinoma can sometimes be misinterpreted as regressive changes in macrofollicular hyperplastic goiter - and vice versa. The nuclear changes can easy mimic malignancy in severe regressive changes, such as haemorrhage or pseudocystic degeneration of hyperplastic nodule.

Thyroid FNAC is a reliable method in experienced hands. As in many other areas, with increasing experience, the diagnostic accuracy is growing. Thus, thyroid specimens should be examined by cytopathologists with sufficient number of cases and in an ideal setting also with simultaneous histopathological experience, which provides an essential feedback

CYTOLOGY OF CEREBROSPINAL FLUIDS

Professor Torill Sauer

CSF cytology deals with disease processes involving the ventricular system and leptomeninges. These include infections, inflammatory/reactive conditions and neoplastic lesions, both primary and metastatic.

CSF is secreted from the choroid plexus and produced at a rate of 500mL/day (or 350µL/min). It is circulated through the ventricles and subarachnoid space. The ventricular system is lined by ependymal cells, which form a leaky barrier to the cerebral interstitium. The fluid is absorbed into the venous system. The total volume of intracranial CSF is about 100mL

Sampling and preparation for cytological examination

Usually about 10-15mL is sampled, but for cytology we usually only get 2-3mL.

Ask for more if malignancy is suspected. Fluid should be transported to the cytology lab within 2 hrs (ideally 30min) and stored at 4 ^{0}C. The fluid is centrifuged and we should try to estimate the need for ICC or other special stains. Membrane filter methods are also used. Both Papanicolaou and Giemsa stains are appropriate as routine stains, preferable both.

CSF cytology is likely to be diagnostic when there is clinical or radiological suspicion of involvement of the subarachnoid space or ventricular system by a cellular infiltrative process, a raised CSF cell count or protein or a previous history of CNS or other tumour.

Normal cell types **are lymphocytes (mainly T-cells), monocytes with 0-4 cells/mm^3 or µL and a ratio of lympho-/monocytes 2-3:1 between the two.**

Chondrocytes, fibroblasts, cells from choroid plexus, ependymal cells, meningeal or leptomeningeal cells, squamous cells, corpora amylacea are contaminants**.**

Artifacts **may occur due to poor cell preservation and viability and when processing is delayed, resulting in sample deterioration.**

In infectious diseases **CSF may confirm an inflammatory CNS disorder. It might help in diagnosing an underlying tumour. Differential cell count help distinguish acute from chronic inflammation. In a Giemsa stain we can easily identify eosinophilic granulocytes. PAS and Grocotte stains may help identify fungi. Acute, bacterial infections will reveal a high polymorph count. Gram stain may identify organism (in addition low glucose; high lactate). Macrophages are often present.**

In tuberculous meningitis **we find lymphocytosis (small and reactive cells), increased neutrophils in the early stages and plasma cells. Epithelioid cells are rarely found.**

Causes of chronic lymphocytic non-viral meningitis**: tbc, syphilis, Cryptococcus, Listeria, Brucella, Mycoplasma, neurocysticercosis and toxoplasmosis**

Further causes of chronic lymphocytic meningitis:

Viral infection (Enterovirus, arbovirus, HIV, HSV)

Inflammatory conditions (SLE, vasculitis, sarcoidosis, MS)

Neoplasms (Carcinoma,lymphoma)

In viral, (aseptic) lymphocytic meningitis neutrophils may be found during first 6 hrs. The common finding is lymphocytosis with reactive lymphocytes, and there is a spectrum of size and activation of lymphocytes. Occasional plasma cells may be seen.

CSF cytology for tumour diagnosis

Most tumour diagnosed in CSF are metastases from solid tumours, Lymphomas and leukemias. Malignant meningitis is a process of multifocal seeding of the leptomeninges by malignant cells. Neoplastic meningitis is found in 3-8 % of cancer patients. Tumour cells reach the meninges through venous plexus or arteries, direct extension from a tumour mass or along perineural routes. CSF in primary CNS tumours make out about 10-20 % of CSF positive specimens. It is estimated that 15-20 % of glial tumours have positive CSF. PNET, pineal tumours and germ cells tumours have a predilection to seed through the CSF.

Cytological diagnosis of metastases to the CSF

5-20 % of cancer patients with neurological symptoms have evidence of meningeal involvement (postmortem studies). Neurological symptoms may occasionally be the presenting symptom of cancer. Carcinoma cells probably enter the CSF through blood vessels; less often from direct invasion.

Metastases from breast and lung carcinoma as well as malignant melanoma are the most common.

Cytological findings in metastatic solid tumours

Single or clusters of large, pleomorphic cells. In adenocarcinoma there is often cytoplasmic vacuolisation as well as frilly or ruffled cell borders. There are usually FEW cells.

ICC panel in suspected metastases: cytokeratins, EMA, PanMel, Melan A, S-100, HMB45, synaptophysin, chromogranin

Malignant lymphomas and leukaemias make up 10-40 % of malignant CSF's.

Most of them are secondary in patients with known ML/leukaemia. Lymphomatous meningitis is found in 65 % of primary CNS lymphoma (PCNSL).

CLL rarely involves CSF, whereas ALL is common. AML rarely involves CSF;

CML occur occasionally, mainly during blast crisis. The most common malignant lymphomas are diffuse, large B-cell lymphoma, Burkitt's lymphoma, lymphoblastic lymphoma and mantle cell lymphoma.

Cytological findings**: increased number of lymphoid cells, often consisting of large, blasts with prominent nucleoli, abnormal chromatin and irregular nuclear contours. A restricted ICC panel may be used (CD3, CD10, CD20) in suspected ML/lekaemias.**

Conclusions 1

- When finding carcinoma cells in CSF without any known primary: lung is the most probable origin
- Breast carcinomas are usually known in advance

Conclusions 2

- When finding malignant cells in CSF, the probability of tumour type is (in decreasing order):
  - Malignant lymphoma/Leukaemia
  - Metastasis from a carcinoma
  - Metastasis from malignant melanoma
  - Primary CNS tumour

Serous effusion cytopathology

Professor Torill Sauer

Anatomy, histology and cytology

Serous effusions obtained from

- Pleural cavities
- Pericardial cavity
- Peritoneal cavitiy
- Cavities lined by a flat monolayer of mesothelial cells (parietal and visceral)
- Various stimuli make the mesothelial cells undergo reactive changes making them more cuboidal in appearance
- Mesothelial cells are derived from the mesoderm, but have many of the morphological and biological features of epithelial cells

Mesothelial cells (1)

- 15-30µm (1.5 – 2 times the size of neutrophils)
- Solitary or small cohesive clusters
- Monolayer sheets in peritoneal washings
- Cytoplasm with narrow, pale outer zone associated with microvilli and a dense perinuclear zone containing a higher density of intermediar filaments

Mesothelial cells (2)

- Round cell borders with smooth contours and blebs along a ruffed surface
- Mesothelial "window" due to microvilli
- Nuclei usually central or slightly off centre; when eccentric the nuclear membrane does not touch the cell border (due to microvilli)
- Nuclei round or oval with smooth contours

Mesothelial cells (3)

- Cytoplasmic vacuolization indicates how long the cells have been in the fluid
- Size of nucleoli and mitotic figures indicate reactive cells
- Produce hyaluronic acid (PAS and Alc positive: hyaluronidase)

Reactive mesothelial cells (1)

- Size up to 50 µm
- Variable amount of cytoplasm
- Enlarged nucleus with some variation in size and shape
- Some cells may have scant cytoplasm, high n/c-ratios and prominent nucleoli

- Conspicous nucleoli
- Binucleation and multinucleation frequent
- Cohesive clusters including micropapillary configurations

Reactive mesothelial cells (2)

- Gigantic nuclei due to fusion of degenerate cells
- Phagocytic activity leads to transformation into foamy macrophages
- Distinuishing histiocytic macrophage from reactive, foamy mesothelial cells not important
- Dd of cytoplasmic vacuolization

Cell-in-cell configuration

- One cell may wrap around another
- Commonly associated with mesothelial cells, but may also be seen in malignant cells in serous fluids

Cohesive clusters

Micropapillary structures

Pitfalls reactive mesothelial cells

- Hepatomegaly associated with congestive heart failure
- Hepatocellular carcinoma
- Ischaemic processes
- Trauma
- Large retroperitoneal masses
- Pelvic inflammatory diseases
- Postoperative

Macrophages

- Histiocytic or mesothelial origin
- May be the dominant cell population
- Non-cohesive
- Scattered, with well to ill-defined cell borders
- May also occur in small ill-defined, loose groups
- Kidney-shaped nuclei, usually eccentric with the nuclear margin closely approximated to the cell membrane
- Indistinct nucleoli
- Cytoplasmic vacuolization

Blood-derived cells

- Red blood cells,
- Neutrophils
- Eosinophils
- basophils

Other components

- Psammoma bodies in benign conditions
  - Ovarian cystadenoma
  - Cytadenofibroma
  - Endosalpingiosis
  - Endometriosis
  - Papillary mesothelial hyperplasia
- Megakaryocytes
- Collagen balls
- Detached ciliary tufts

Collagen balls

- Fragments of collagen covered by mesothelial cells
- Found in 4-29 % of peritoneal washings
- Originate from the surface of the ovaries?
- Most common in pelvic washings
- Usually in women
- Have been reported in asitic fluid from men with encapsulated peritonitis

Types of effusions

- Reactive
- Malignant (recurrent, hemorrhagic)
  - lung, GI-tract and pancreas in men
  - breast, lung and ovaries in women
  - in ascites: GI-tract, ovaries and pancreas
- Transudates (hypocellular, mostly mesothelial cells)
- Exudates (hypercellular with inflammatory cells and reactive mesothelial cells; with or without malignant cells)
- Chylous (many lymphocytes and some lipid-laden macrophages)

Paedriatic effusions

- Most are benign
- Secondary to small round blue cell tumours, mostly lymphoma and leukaemia
- Main dd neoplasms of small cell type versus mononuclear inflammatory cells

Pleural, pericardial and peritoneal fluid cytology

Aspirated fluid should be placed in a plain bottle and sent for processing the same day as collection; ideally, 25-50ml in volume (22). Natural fibrin clots should be processed as histological sections and reported alongside the cytology slides.

A sample of fluid should be spun down and processed as a cell block for immunocytochemistry if required. One air-dried and one alcohol-fixed cytospins are prepared for MGG and Papanicolaou (or H&E) stains. Bloodstained samples should be but in Cytolyt (Hologic) or the equivalent before cytospins are prepared.

There are no normal cells in serous fluids, since an effusion is a pathological process. The following cell types are seen in reactive effusions, and may help identify the cause of the effusion.

- *Neutrophil polymorphs*: **t**hese denote inflammation in the underlying lung, but not empyema, which is characterised by purulent fluid, containing degenerate polymorphs and histiocytes and fibrin.

- *Lymphocytes*: these denote chronic inflammation or obstruction to outflow of serous fluid though the hilar lymphatics. Usually monomorphic, with few if any plasma cells or blast cells. More than 80% of the cells are T-cells. Common causes are malignancy (lung or breast cancer, lymphoma) and tuberculosis (primary or TB pleurisy).

- *Eosinophils*: these usually denote previous aspiration, or any other cause of air being introduced into the serous cavity (pneumothorax). Eosinophilic effusions may be seen in the recovery phase of pneumonia.

- *Mesothelial cells*: these may be exfoliated in any form of effusion, even transudates. Any insult to the pleura (introduction of air, blood, saline, transudate, exudate) may result in mesothelial cell losing their cellular attachments and populating the serous fluid. Mitotic figures and double nuclei are common in these cells, as they regenerate to replace the damaged membrane.
  - Reactive mesothelial cells are usually two-dimensional and do not form true papillary clusters but show a wide variety of appearances, which may result from non-neoplastic processes above or below the diaphragm.
  - Reactive mesothelial cells are positive for Cam 5.2, MNF116, HBME-1, Ca 125 and calretinin.
  - They are negative for EMA (usually), CEA, BerEP4 and other "carcinoma-specific" antibodies, including TTF1.

Cytology of effusions in malignancy

Squamous cell carcinoma

Squamous cell carcinoma is rarely seen in pleural fluid cytology in which metastatic lung cancer is usually from adenocarcinoma, small cell carcinoma or poorly differentiated non-small cell carcinoma.

On the rare occasions when metastatic squamous cells are seen in pleural fluid, metastasis from elsewhere should be considered, especially head & neck cancer now that cervical carcinoma is so rare.

Immunoprofile of pulmonary squamous cell carcinoma

This should be used to support a morphological diagnosis, which is not primarily made by immunocytochemistry.

- SqCC should be positive for CK5, CK5/6 and p63 (23,24). Normal basal cells also stain with these antibodies.
- Well differentiated SqCC is usually CK7 and CK20 negative but poorly differentiated SqCC may be CK7 positive (23).
- TTF1 is usually negative.

Non-small cell carcinoma, NOS

This diagnosis may be given when there are no clear features in morphology to suggest adenocarcinoma, squamous cell carcinoma or neuroendocrine differentiation – or when the immunoprofile is inconclusive (for example CK5 and CK7 both positive or both negative, TTF1 negative or focally positive).

Small cell carcinoma (oat cell carcinoma)

Different sub-types of small cell carcinoma cannot be distinguished, except occasionally combined types. Cytological features of small cell carcinoma are:-

- Coarse "salt and pepper" chromatin pattern
- Nuclear moulding
- Sparse cytoplasm
- Cell streaking and crush artefact
- Individual cell necrosis

Immunoprofile of small cell carcinoma

- Positive staining with CD56
- Synaptophysin may be positive, chromogranin less often positive
- Granular (dot-positive) staining for cytokeratin
- CK5, 5/6 and p63 negative
- CK7 positive, CK20 negative

The distinction between small cell carcinoma and poorly differentiated squamous cell carcinoma may be difficult – the immunoprofile can help as small cell carcinoma is usually TTF1 and CK7 positive; CK5 and p63 negative.

While the phenotype of small cell carcinoma is similar to adenocarcinoma (CK7+, CK5/6-, TTF-1+), neuroendocrine markers are positive in the former and the morphology usually quite different.

The typical morphological features or small cell carcinoma are most classically seen in sputum and other exfoliative specimens. Cells may appear larger in air-dried preparations, and may be in cohesive groups in pleural fluid.

Non-small cell (large-cell) neuroendocrine carcinoma

This diagnosis may be suspected when some features of small-cell carcinoma are seen (moulding, granular chromatin), but the cells are more cohesive and structured and there is less cell death, streaking etc. The immunocytochemistry is similar, in that CD56 and synaptophysin (sometimes also chromogranin) are positive. Cytokeratin staining may not be granular and tends to define islands of cells.

Adenocarcinoma

Pulmonary adenocarcinoma is common, and is now more frequency seen than squamous cell carcinoma in resected specimens. Adenocarcinomas may be central, peripheral or diffuse. Many are poorly differentiated and difficult to distinguish cytologically from anaplastic large cell carcinoma.

The characteristic features of adenocarcinoma are:

- Vesicular nuclei with prominent nucleoli
- Eccentric nuclei and vacuolated cytoplasm
- Three-dimensional papillary clusters

Other features in pulmonary adenocarcinoma include

- Intra-nuclear holes and nuclear grooves
- Plump columnar cells resembling bronchial cells
- Clean background, no diathesis

Immunoprofile of pulmonary adenocarcinoma

- Most are CK7 and TTF1 positive; CK20, CK5/6 negative (23)
- Some may be focally CK 5/6 (and occasionally CK20) positive
- P63 is variable and may be negative or heterogeneously positive (26).
- TTF-1 may rarely be positive in adenocarcinomas from other sites and has been described in breast, ovarian clear cell and endometrial carcinomas.

Well-differentiated adenocarcinoma may be difficult to diagnose cytologically, especially when the cells are admixed with exfoliated ciliated cells. It may arise as a localised mass, diffuse bronchiolar-alveolar cell carcinoma or as metastasis. These may be hard to distinguish without clinical correlation.

Poorly differentiated adenocarcinoma is usually impossible to recognise cytologically, in which case it is best described as carcinoma, not small cell unless the immunoprofile indicates the diagnosis.

Now that *targeted chemotherapy* is available, material from all adenocarcinomas ideally should be analysed for EGFR and k-RAS (27), which may be carried out on cytological or histological material (including cell block sections).

METASTATIC TUMOURS

The best examples are seen in pleural fluid cytology, where characteristic features of common primary sites may be compared.

The commonest metastases to be seen in the pleura are from ovary, breast and lung.

Many types of metastasis are seen in the lung. Breast, colon and kidney are probably the commonest, but sarcomas and melanomas should also be watched out for.

Although lung metastases may be clinically obvious clinically when multiple, they may also present as solitary metastases mimicking a primary lung tumour (especially renal, colon, breast, melanoma).

Metastatic breast carcinoma

There are three main patterns of metastasis listed below, demonstrated in:-

- Uniform "cell balls" with smooth borders
- Cells-within-cells (owl's eye)
- Free cell pattern, with mucin secretion
- Adenocarcinoma, NOS
- Cells are usually positive for EMA, may be positive for ER or c-erb B2 and may be negative for CEA.
- The cytokeratin pattern (CK7+, CK20-) is usually the same as for lung cancer.

Breast cancer may be recognised in bronchial washings, sputum, BAL, EBUS and EUS FNAC when cancer cells are well differentiated, mucin-secreting or in tight clusters. Clinical correlation and/or immunocytochemistry are usually needed to make this diagnosis.

Metastatic lung carcinoma

Lung cancer may be diagnosed and staged by FNA of palpable lymph nodes, usually in the neck or axillae. CEA is usually positive in lung cancer, as are other carcinoma-specific antibodies and EMA. TTF-1 is often positive.

Lung carcinoma in the pleura is usually adenocarcinoma, small cell or undifferentiated carcinoma and rarely squamous cell. There are no specific morphological features to suggest a lung primary.

Metastatic lung carcinoma may be diagnosed in mediastinal lymph node biopsies in the absence of a known primary.

Metastatic ovarian carcinoma

This is common in pleural fluid and typically presents as abundant vacuolated adenocarcinoma cells, positive for Ca 125. CEA may be focally positive but is often negative which helps distinguish ovarian from G-I tract carcinomas. Ovarian carcinoma may be positive for oestrogen receptor. The cytokeratin pattern (CK7 positive, CK20 negative) is usually the same as for lung cancer.

Metastatic G-I tract carcinoma

Gastric carcinoma may be suggested by a free cell pattern in pleural fluid with signet ring cells and mucin secretion. The differential diagnosis is breast carcinoma.

Colon carcinoma may be recognised in lung cytology specimens when malignant cells are palisaded with oval nuclei.

Colon carcinoma is usually positive for CK20 and negative for CK7, whereas lung carcinoma is usually the other way round.

Immunocytochemistry for metastatic carcinoma

- Cytokeratin profile may help (CK20+, CK7- colorectal; CK7+, CK20+ urothelial carcinoma; CK7-, CK20- renal cell carcinoma) but breast and stomach may be CK7+, CK20- like lung cancer. Consider pancreas if CK20 is positive but this is variable.
- TTF1 is usually negative in carcinomas other than lung and thyroid but positive staining has been reported at other sites (endometrium, ovary) and may be seen in neuroendocrine carcinomas from any site.
- ER and PR suggest breast cancer but may also be found in gynaecological cancers.
- Specific antibodies are used as indicated by clinical findings (PSA, melan-A, S100).
- Cytokeratin in spindle celled tumours to help exclude melanoma and sarcoma.
- CD45, Leu M1 (CD15), CD30, Alk-1 to help exclude or suggest Hodgkin or non-Hodgkin lymphomas, which may mimic carcinoma.

Malignant mesothelioma

Malignant cells are usually exfoliated in pleural fluid in epithelioid-type mesotheliomas, but rarely in mesenchymal mesothelioma. Patterns in pleural fluid cytology are as follows:

- No malignant cells, lymphocyte-rich or occasionally polymorph-rich effusion.
- Background of hyaluronic acid, with or without malignant cells.

- Morulae of mesothelial cells with rather uniform nuclei; morulae have scalloped edges (looking like raspberries) and may have collagen cores and are hollow in sections.
- Free cell pattern of mesothelial cells with pleomorphic nuclei and prominent nucleoli. Nuclei are larger than in reactive mesothelial cells (and larger than in morulae).
- Mixed free cell and morulae

Immunoprofile of malignant mesothelioma

- Malignant mesothelioma cells will all show the same immunoprofile (no dual cell population)
- Calretinin, EMA positive, CEA; BerEP4, TTF1 negative.
- CK5/6 is more likely to be positive in mesothelioma than carcinoma.
- Otherwise the cytokeratin pattern (CK7+, CK20-) is the same as lung cancer.

Malignant lymphoma

Non-Hodgkin's malignant lymphoma

Involvement of the pleural space by NHL is relatively uncommon, but is associated with a monomorphic population of lymphoma cells. Recognisable NHL is usually high-grade. Similar features are seen at any site and characteristics of high-grade NHL are:

- Large cells with prominent nuclei and thin rim of deep blue cytoplasm
- Karyorrhexic (individual cell death)
- Lymphoblasts are usually smaller, but have similar cytoplasm
- Myeloma may be recognisable by monotonous plasma cells and plasmacytoid cells with double nuclei
- Vacuolated cytoplasm may be seen in Burkitt's type NHL

Low-grade non-Hodgkin malignant lymphoma

These may be difficult or even impossible to diagnose cytologically without clinical correlation or immunocytochemistry.

Reactive effusions are predominantly T-cell and most low-grade lymphocytic lymphomas and leukaemias are B-cell. Similarly reactive lymph nodes are usually predominantly T-cell (80:20 T-cell:B-cell).

Low-grade T-cell lymphomas need molecular analysis to demonstrate monoclonality.

Hodgkin lymphoma

Direct involvement of the pleural fluid by Hodgkin lymphoma is extremely rare and is only seen in advanced stage IV disease. Flow cytometry may be unhelpful and cell blocks for immunocytochemistry will be more useful.