Diagnostic Cytopathology

Definition: Diagnostic or Clinical Cytology is the study of the normal and diseasealtered
cells obtained from various sites of the body (i.e., through the detection of abnormal morphologic characteristics of the examined dissociated human cells)

Cytopathology in Relation to Histopathology

Histopathology	cytopathology
• Deals with the form and the structure of the tissue. • Evaluation usually begins with a tissue biopsy. • More invasive traumatic procedure is needed; utilizing surgical instrumentation such as foreceps, scissors, etc..) • Needles if used should have a large gauge (i.e., Tru-cut needles measuring 14, 16) . • Diagnosis obtained after days. • Basic stain is H&E • Paraffin blocks are needed • Difficult to identify specific causative inflammatory pathogen	Deals with the structural changes within the nucleus and cytoplasm of individual cells • Evaluation requires cells only. • Inexpensive simple means of diagnosis which allows frequent repetition of cellular sampling (since it causes no tissue injury). • Fine needles with 22, 23 or 24 gauge are usually preferred. • Rapid diagnosis that could be obtained within minutes. • Basic stain is Pap stain (however H&E could be used as well) • Mainly slides are needed • Smears permit better evaluation of the nature of the inflammatory process. Fungi and parasites are usually easier to be diagnosed.

Limitations of Cytopathology:
As a rule, histopathology remains the gold standard technique in diagnostic pathology.
The whole presented tissue definitely provides a more accurate precise diagnosis of the disease in question. The interpretation of the morphological cellular changes is based mainly on individual observations and often cannot be forced into rigid criteria. Thus, in many cases the cytological diagnosis could not be final and needs confirmation by histopathology for the following reasons:

• Often the nature of the lesion is not so obvious as in a histological section.
• The location of the lesion is difficult to be pinpointed by cytology: for example a squamous cancer cells found in a cytology sputum sample may have originated from the buccal mucosa, pharynx, larynx or bronchi.
• The size of the lesion cannot be approximated by cytology.
• The type of the lesion –e.g., in situ carcinoma as compared with early invasion is more difficult to assess since the relationship of the cells to the surrounding stroma cannot be determined by cytology.

Sampling Techniques

In general, diagnostic cytology is based upon three basic sampling techniques :
(1) the collection of **exfoliated cells,**
(2) the collection of cells removed by brushing or similar **abrasive techniques,**
(3) the **aspiration biopsy** or removal of cells from non-surface –bearing tissue (or deep organs) by means of a needle, with or without a syringe

1- Exfoliative Cytology

Exfoliative cytology is based on spontaneous shedding of cells derived from the lining of an organ into a cavity, where they can be removed by nonabrasive means.
Typical examples are:

• The **vaginal smear** which is prepared from cells removed from the posterior fornix ofthe vagina. The cells that accumulate in the vaginal fornix are derived from several sources: from the squamous epithelium that lines the vagina and the vaginal portio of the uterine cervix, from the epithelial lining of the endocervical canal, and from other more distant sources such as the endometrium. These cells accumulate in the mucoid material and other secretion from the uterus and the vagina. In addition to the cells derived from the epithelial linings, the vaginal fornix often contains leukocytes and macrophages that may accumulate there in response to an inflammatory process, and a variety of microorganisms such as viruses, bacteria, fungi, and parasites that may inhibit the lower genital tract.

• Another example for the use of exfoliative cytology is the **sputum examination**. The sputum is a collection of mucoid material that contains cells derived from the buccal cavity, the pharynx, larynx, trachea, the bronchial tree and the pulmonary alveoli; as well as inflammatory cells, microorganisms, foreign material , etc…

The same principle applies to:
• **Voided urine**
• **Body fluids**
• **Nipple discharge**

Exfoliative cytology is the simplest of the three sampling techniques. To prevent the deterioration of cells by air-drying or by enzymatic or bacterial actions, the material should be processed immediately without delay. Alternatively, the use of fixatives, such as alcohol or other cell preservatives is indicated.

2- Abrasive Cytology

The purpose of this procedure is to enrich the sample with cells obtained directly from the surface of the target of interest. Hence, cell specimens are usually obtained through superficial scraping of the lesion (artificial mechanical desquamation) .
Examples include:

• Cervical scraping or the so called **Pap smear**: the cervical scraper introduced by Ayre in 1947 (i.e., Ayre's spatula) allows a direct sampling of cells from the squamous epithelium of the cervix and the adjacent endocervical canal

Buccal mucosal **smear**
• **Skin scraping** of various lesions
• **Brushing techniques** through using rigid endoscopic & fibroptic instruments to collect cell samples from the gastrointestinal tract, bronchial tree, etc…

The Pap Smear Test

Is used mainly to detect precancerous and cancerous lesions of the uterine cervix.It is based on the fact that cancer of the cervix is one of the most preventable cancers, because most of the cellular changes which may lead to ca rcinoma can be detected

and accordingly treated at an early stage before progression. In most cases, cervical carcinoma develops slowly (over a period of up to 10 years); whereby it passes into different pre-neoplastic conditions before it reaches the cancer stage; termed

dysplasia or **Ce rvical Intraepithelial Neoplasia** (CIN):
Mild Dysplasia --► Moderate Dysplasia --► Severe Dysplasia--►
CIN I   CIN II   CIN III
Carcinoma in Situ --► Carcinoma

Nomenclature of Dysplastic Lesions according to The Bethesda System
Squamous & Glandular abnormalities
The term squamous intraepithelial lesions (SIL) encompasses the spectrum of the precursors to invasive squamous cell carcinoma previously called dysplasia, carcinoma in situ, border line lesions and CIN .
Grading squamous intraepithelial lesions
The Bethesda System recommends a low-grade /high grade approach in classifying SIL. Most LSILs are secondary to transient infections that carry little risk of transition into carcinoma, whereas most HSILs are associated with viral persistence and a significant potential for progression to invasive cancer.
Low- grade squamous intraepithelial lesions
LSILs encompasses lesions that were previously described separately as "koilocytosis" (signifying Human Papilloma Virus infection) and mild dysplasia (CIN 1).

High- grade squamous intraepithelial lesions
HSILs encompasses lesions that were previously described as moderate dysplasia (CIN 2) and severe dysplasia (CIN 3)
Microscopical examination of the exfoliated cells from the uterine cervix through **Pap Smear test** should therefore lead to the detection of these pre-neoplastic lesions in their earliest stages (i.e., stage of mild dysplasia). Accordingly, giving the appropriate treatment would eventually prevent cervical cancer.
Pap Smear test is a simple procedure in which cells are collected from the cervix and examined cytologically to exclude abnormal changes. No anaesthesia is required, and the diagnosis could be obtained within few minutes. The procedure could be easily carried out in any clinic or specialized lab. It is recommended that all women between the ages of 18 and70 years, who have ever had sexual activity, to have the test every 2 years.
Pap smear could also allow the diagnosis of other microbiological agents such as Monilia, Trichomonas Vaginalis, Herpes Simplex or Human Papillomavirus "HPV". The latter might cause progression of dysplasias to carcinoma when untreated.

3- Fine Needle Aspiration Cytology (FNAC)
In general, the definitive diagnosis of any mass can be established by:
- Open biopsy
- Tissue core needle (Tru-cut) biopsy

- Fine needle aspiration biopsy.

Compared to FNA, Tru-cut biopsy is a more traumatic procedure which should be performed under local anaesthesia. It requires more time and special equipments that are more expensive. Pain, discomfort and bleeding are common complications. FNAC, on the other hand, provides many advantages to the surgeons being an easy, reliable, cost effective diagnostic technique which could give rapid results. The procedure could be performed in an office setting without anaesthesia. It is usually not more painful than a venipuncture and can be repeated immediately if the acquired material is inadequate.

The National Health Service Breast Screening Program (NHSBSP) displayed that many centers wished to include FNA as an additional test to provide preoperative diagnosis of breast cancer and to reduce the number of operations for benign breast diseases.

When reduced to its simplest terms, FNAC consists of:
• Using a needle and syringe to remove material from a mass.
• Smearing it on a glass slide.
• Applying a routine stain.
• Examining it under the microscope.

Technique of FNAC:

The skin over the palpable mass (within the breast, lymph node, or any other organ) is sterilized with an antiseptic. Utilizing the index and the middle finger or the thumb and the index finger, the mass is localized with the non-dominant hand. With firm downward pressure
on the skin over the mass, it should be compressed against a rib and stabilized.

Aspirates should be obtained using preferably a 23 gauge, 1 ½ inch disposable needle mounted on a 10 ml plastic syringe, held by the dominant hand. Larger needles (22 gauges)
are used in aspirating material from hard fibrous masses and in cases of suspected cyst or abscess.

Without using anesthesia, the needle should be gently introduced through the skin passing to
the level of the dominant mass. Having confirmed the position of the needle within the mass,
negative pressure should be created within the syringe by pulling back the plunger. The needle should move back and forth through the mass, in different rotational directions using
sewing-like-motion. Suction should be maintained throughout the process by outward pressure of the right thumb on the underside of the syringe. By this method the needle can cut
loose many small pieces of tissue that are then readily aspirated due to the suction applied by
syringe. These to and fro strokes should be repeated at least 6-8 times. In general most of the
aspirations are terminated when material begins to appear in the needle hub.

The following step should be designed to protect the material that has been obtained. All suction should be released before removing the needle from the mass. This is accomplished
by allowing the syringe plunger to return gently to its resting position. Then the needle should
be withdrawn gently from the mass. To limit haematoma formation from the site of the

puncture, firm pressure should be applied with a piece of cotton for two minutes.

Equipments needed for FNAC procedure:

Syringes

The key feature in a syringe is lightness in weight, adequate in volume, and one hand control. Modern sterile disposable plastic syringes are perfect for use in FNAC. Most practitioners use the 10 cc size, while some prefer the 20 cc size (specifically when a cyst is suspected).

Needles

Several variables should be considered in choosing the proper length and gauge. In general the length should be at least 1.5 inch (3-8 cm). This is desirable in the breast because many lesions are found deeper than expected. It has also been demonstrated that small gauges (22-23) are preferred since they are rigid enough and at the same time cause less sensation and haematoma. Needles with a plastic hub rather than a metal hub are preferred as well. This permits the aspirator to monitor the recovery of tissue, blood or other fluids as they appear in the hub.

Glass Slides

Common routine slides measure 75 mm X 25 mm X 1mm. An important procedure is slide labeling at the time of sampling. That is why slides which have one frosted end are preferred. Various substances are used to increase the adherence of specimens to the slide surface, the most commonly used being albumin.

Fixatives

These are applied to the smears as a spray or by immersion of the slide into a liquid. The most commonly used is 95 % Ethanol. This inexpensive readily available liquid provides excellent cytological details. Its only disadvantage is the need to store and transport slides in a container of liquid and to have them immersed in for at least 20 minutes.

Routine Stains

For cytological diagnosis, fixed material is usually stained by Papanicolaou Stain (which is preferred) or by Hematoxylin and Eosin (H&E) method. Both employ one of the Hematoxylins for staining of the nuclei. This dye colors the nucleic acids as a dark purple blue color. The counter staining with Eosin is designed to show cytoplasmic characteristics. With the Pap stain one hallmark is the use of Orange-G, which stains cytoplasmic keratin with a bright orange color.

Air-dried smears on the other hand are usually stained by any Romanowsky stains, which are various combinations of methylene blue and its breakdown products (Azure A, B, &C) with Eos in. Most rely on Methanol fixation.

Papanicolaou Stain

All slides should be wet fixed within 95% or absolute Ethanol before staining:

95% ethanol 10 dips
80% ethanol 10 dips
70% ethanol 10 dips
50% ethanol 10 dips
Distilled water 10 dips
Hematoxylin Stain 1-2 min.
Running Tap water 10 dips
50% ethanol 10 dips
70% ethanol 10 dips
80% ethanol 10 dips
95% ethanol 10 dips
Orange-G Stain 2 min.
95% ethanol 10 dips
Eosin Stain 2 min.
95% ethanol 10 dips
95% ethanol 10 dips
Absolute ethanol 10 dips
Absolute ethanol: Xylol (50:50 mixture) 5 min.
Xylol 20 min. Mount with Canada Balsam

Pitfalls to Reliable Results from FNAC
- No mass
- Vague mass
- Mass being too small (less than 1 cm)
- Mass being too deep
- Gross blood into the syringe (i.e., RBCs can mask the picture)
- Scanty cellularity in the aspirated mass
- Dense fibrosis
- Gross fluid

Indications for Cytopathology

1- Differentiation between benign and malignant lesions.
2- Diagnosis of malignancy and its type, as well as the identification of the neoplastic cells in primary, metastatic (secondary) or recurrent tumors.
3- Diagnosis of premalignant diseases: i.e., detection of precancerous or dysplastic cellular changes (figure 5). As explained earlier, dysplasia may be graded as mild, moderate and severe. In the uterine cervix for example, those lesions could be eliminated by treatment of the associated inflammation and causative pathogen or by excision of the lesion.
4- Detection of inflammation and certain types of pathogenic agents : i.e., the cytologist could prove that the cause of vaginal secretion is vaginitis due to Trichomonus vaginalis, Moniliasis or the cytology could show changes suggestive of Human Papilloma Virus infection (HPV – Figure 6). Similarly the cause of cystitis may be related to bilharziasis (through the detection of Bilharzial ova in smears of urine samples).
5- Study of the hormonal patterns and evaluation of the gonadal hormonal activity: through the examination of the squamous cells in vaginal smears; which are under the influence of ovarian hormones (figure 7).
6- Follow-up and monitoring of response to chemotherapy and irradiation; the latter producing certain cellular features which could be diagnostic on cytologic examination.
7- The identification of sex chromosome: if a newborn presents with ambiguous genitalia, one can not tell whether the sex is male or female. The presence of a dark dot attached to the nuclear membrane from inside (Barr body +ve) indicates that a sex chromosome is present, i.e., the genotype of the baby is XX (♀). Conversely the absence of this Barr body indicates that there is no X chromosome and accordingly the newborn is genotypically a male ((XY ♂).
8- Tumour markers study on cytological specimens.

Cytological Criteria of Malignancy

A) Nuclear Changes:
- Nuclear hypertrophy: nuclear enlargement that leads to increased N/C ratio.
- Nuclear size variation
- Nuclear shape variation
- Hyperchromatism and chromatin irregularity: refers to increased chromatin materials. In malignant cells the chromatin is not evenly distributed within the

nucleus; it is also distributed as coarse, clumps. This in contradistinction to normal cells, which have evenly distributed chromatin.

• Multinucleation: malignant cells may contain more than one nucleus. However, some normal cells such as hepatocytes and histiocytes may contain more than one nucleus. Multinucleated malignant cells differ from nonmalignant multinucleated cells by the fact that the nuclei of malignant cells are unequal in size (in contrast to that of normal cells).

• Irregularity of the nuclear membrane.

• Irregular and prominent nucleoli: giant nucleoli or multiple nucleoli may be present that differ in their sizes and shapes. It should be remembered, however, that normal columnar and goblet cells may contain 2 nucleoli.

B) Cytoplasmic Changes:

• Scantiness of cytoplasm: in consequence to the high N/C ratio.

• Cytoplasmic boundries: sharp and distinct in Squamous cell carcinomas and indistinct in undifferentiated carcinomas.

• Variation in size .

• Variation in Shape.

• Cytoplasmic staining: deep orange in keratinizing squamous carcinomas or basophoilic in immature poorly differentiated carcinomas.

• Cytoplasmic inclusions: e.g. melanin pigments in melanoma.

• Cytoplasmic and nuclear membrane relationship: cytoplasmic borders of malignant cells could be tightly molded against the nucleus, touching it in more than one place.

C) Changes in Malignant Cells as a Group:

• Cellular phagocytosis or Cannibalism: i.e., one cell appearing to be contained in a vacuole of the cytoplasm of another epithelial cell; indicating rapid growth of cells within a narrow cavity.

• Variation in the size and shape of the cell clusters or sheets.

• Lack of cellular adhesion: due to abnormalities in the desmosomes.

• Abnormal mitosis: malignant nuclei may show multiple poles e.g. three poles (tripolar mitosis; triploidy) or four poles (tetrapolar mitosis; tetraploidy). This is abnormal since normal mitosis has two poles (bipolar). Thus normal cells show euploid or diploid pattern on flow or image cytometry whereas malignant cells may show abnormal aneuploid patterns.

• Bloody background: fresh blood is meaningless, but malignancy is suspected when the blood is old, partially ingested by histeocytes or present within a specimen which is obtained without trauma.

• Foreign cellular structures: e.g. psammoma bodies in a routine vaginal smear.

• Degeneration and inflammation as a result of erosion or necrosis within the malignant tumour which could indicate invasion (i.e., tumour diathesis).

Perhaps the best demonstrable tool to observe the various types of malignant cells which could be encountered cytologically is through studying the different variants of **Bronchogenic Carcinoma.**

A routine recommendation for these patients is the examination of three successive early morning sputum samples.

Histologically, there are four major different types of bronchial cell carcinoma could be recognized on cytological examination:

1. Squamous Cell Carcinoma
Is the most common and easiest type to be diagnosed by cytologic examination of sputum because of its central position within the bronchial tree. Cytologically, most malignant cells exfoliate singly but some may be observed in clusters or sheets. Cells often assume variable sizes and shapes; could be amoeboid in shape, assuming pseudopod- like projections. Spindle or tadpole cells could be also detected. The background is usually obscured by necrotic debries, mucous and RBCs (tumour diathesis).

 The cytoplasm is abundant, dense and granular with a glassy orange appearance in well-differentiated types due to the presence of keratin (stained by Orane G). Anucleated deep orange squames may be evident in long standing cases. The nucleus may show nuclear size and shape variation with irregularity of the nuclear membrane. Marked hyperchromasia is often present; the nuclei appearing very dark or pyknotic. Cannibalism is common. Red prominent nucleoli could be present.

2. AdenoCarcinoma
Usually periphral in position. Cells exfoliate in clusters, sheets or acini; rarely singly. They are often round, oval and irregular in shape. Cytoplasmic vacuoles are observed in well-differentiated types due to the presence of mucous droplets. Large vacuoles may push or compress the nucleus to one side giving an irregular moonshaped
appearance: signet-ring pattern. Neutrophils maybe seen within these
vacuoles. Nuclei are often round or oval with moderate hyperchromatism and irregular nuclear membrane. Single or multiple diagnostic nucleoli are usually found. Multinucleatin may occur. Cells stain positive with PAS stain (due to the presence of mucin).

3. Undifferentiated Small Cell Carcinoma
The WHO classification recognizes three histological subtypes:
- Oat cell carcinoma.
- Small cell carcinoma – intermediate type.
- Combined Oat cell carcinoma (seen with foci of squamous cell
and/or adenocarcinoma).
The cellular morphology of small cell carcinoma varies with the method of specimen collection. In sputum, the exfoliated cells are often small in association with long strands of mucous. Dark lines of elongated compact masses of numerous small hyperchromatic neoplastic cells are seen with satellite single cells scattered around. The nucleus could be degenerated and pyknotic. In brushings and FNA samples, cells are usually better preserved, appearing larger and displaying more variations in nuclear chromasia and chromatin patterns.

4. Undifferentiated Large Cell Carcinoma
These are epithelial tumours comprised of malignant cells with large nuclei and prominent nucleoli that show no evidence of squamous, glandular or small cell differentiation. In the WHO classification, two variants are recognized:
- **Giant cell carcinoma** which contains a prominant component of highly pleomorphic multinucleated cells which maybe actively phagocytic. The hyperchromatic nuclei shows irregular chromatin clumping and large prominent macronucleoli.
- **Clear cell carcinoma** which is a rare variant composed of large cells with clear or foamy cytoplasm without mucin.

In general, during the evaluation of a smear, the cytopathologist should look for the following changes

• Modification of the normal nuclear and cytoplasmic cells.

• Maturation status.

• The quantity of the cells (hypo- or hyper-cellular)

• The mode of desquamation (clusters, sheets or scattered individually).

• The smear background (i.e., the presence of leukocytes, histiocytes, RBCs, necrotic cells and/or protein deposits).