**ADVANCED DIAGNOSTIC AIDS IN ORAL CANCER**

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**INRODUCTION**

Oral cancer is the 6th most common cancer worldwide with 5 year survival rate with high mortality rates. Most of the oral cancers were Oral squamous cell carcinoma (OSCC). Most common site was Tongue –postero lateral and ventral (40%) followed by Floor of mouth , gingival mucosa, buccal mucosa, labial mucosa, and hard palate . High mortality rate was due to difficulty in diagnosis by routine clinical examination. Early diagnosis of OSCC will decrease both the morbidity and mortality.

**CONVENTIONAL ORAL EXAMINATION**

Alterations in the surface texture , integrity, color, size or contour, and mobility of structures indicates a suspicion for oral leukoplakia or squamous cell carcinoma. Red or white lesions or a long standing ulceration is the characteristic clinical appearances of malignancies. Small fraction of these lesions undergo malignant change

**VITAL STAINING**

Vital staining is a procedure where living cells take up certain dyes, which selectively stains some elements in the cells like mitochondria, lipid vesicles, lysosome, etc. They are

1. Rose Bengal stain
2. Lugols iodine
3. Toluidine blue

**Toluidine blue**

Toluidine blue has been used for more than 40 years as an aid to detection of mucosal abnormalities of the cervix and the oral cavity. Toluidine blue is a metachromatic acidophilic dye that binds preferentially to tissues undergoing rapid cell division (inflammatory, regenerative, and neoplastic tissue), resulting in preferential staining of abnormal tissue. It has affinity to selectively stain the acidic tissue components, sulfate, carboxylate and phosphate radicals such as DNA and RNA, but not normal mucosa.

**Stain preparation** : 1% toluidine blue

* toluidine blue – 1 gm
* 1% acetic acid - 10 ml
* absolute alcohol - 4.19 ml
* Distilled water – 86 ml

Mechanism of action

Nuclei of malignant cells have an increased uptake of the dye, and this manifests as increased DNA synthesis. Rapid dye penetration will occur through randomly arranged tumor cells

Procedure

* Identify the suspected lesion
* Rinse with toluidine blue/ apply
* Neutralize with 1% acetic acid/ apply
* Observe for stained areas

Inference

Malignant lesions will appear dark blue. Dysplasia will present with different shades of blue and non-malignant areas of inflammation may not stain. Re-stain all positive lesions within 14 days- to minimize false positive result

**False positive** results are seen with following lesions:

Epithelial hyperplasia, hyperkeratotic lesions, inflammatory and traumatic lesions, hyperplastic candidiasis can retain 60% of stain. The decision making can also be attributedto the experience of the clinician. Repeat the test after 10-14 days to allow the inflammatory lesions to resolve. This reduces the false positive by 8.5%.

**False negative** results are recognized in:Low grade dysplasia, lichenoid dysplasia

Disadvantage

* Low positive predictive value of 43.5% for potentially malignant lesions
* A false negative rate as high as 20.5% for pre-malignant lesions also have been reported.

**LUGOL’S IODINE**

Lugol’s iodine was first made in 1829, which is named after the French physician Lugol (1786–1851).

Composition

* + Iodine: 2gm
	+ Potassium iodide: 4 gm
	+ Distilled water: 100 cc

Mechanism of action**:** The principle is based on glycogen content of the cytoplasm and the reaction is known as the iodine–starch reaction, visualized by a colour change. As there is enhanced glycolysis in cancer cells, do not promote the iodine–starch reaction. The vital dye with Lugol’s solution is also called Schiller’s test

Procedure

* 1% acetic acid is applied to the lesional tissue for 20 Sec and rinse with water
* Apply Lugol’s iodine at the lesion with a cotton bud for 10-20s
* If the lesions shows brown stain it is considered as normal mucosa.

Advantages

Lugol iodine is cheap, widely available, easy to use and only about 5 min are necessary to perform the staining procedure.

**DOUBLE STAINING TECHNIQUE**

Staining Toludine Blue along with Lugol’s iodine is known as double staining technique. It is used for clinical determination of the degrees of differentiation of malignant lesions because poorly differentiated malignant lesions without glycogen content do not show Lugol’s iodine retention. This technique is indicated in high risk patients and selecting biopsy sites for patients with wide field cancers.

**ROSE BENGAL STAINING**

It is 4, 5, 6, 7 tetrachloro-2, 4, 5, 7 tetraiodo derivate of fluorescein, can stain the desquamated ocular epithelial cells. RB staining is used to delineate the extent of the corneal and conjunctival neoplasms and even oral epithelial dysplasia and OSCC

**CYTOLOGICAL TECHNIQUES**

Oral Exfoliative cytology is a cost effective and perhaps the best procedure for the initial evaluation and diagnosis of oral lesions. Their types vary based on the method of cytological slide preparations. They are

* Oral Brush biopsy (Oral CDX)
* Liquid Based Cytology
* Centrifuged Liquid Based Cytology

**ORAL BRUSH BIOPSY (OralCDx)**

Computer-assisted cytology is based on the use of dedicated instruments that allow the identification of both preneoplastic and neoplastic lesions with a sensitivity equal to, or higher than, that of manual screening, without loss of specificity. The Oral CDx Brush Test System (CDx Laboratories, Suffren, NY) takes advantage of a method of collecting a trans-epithelial sample of cells that are automatically examined by a computer-assisted system

Contraindications

* Highly keratinized leukoplakia as it does not allow enough basal cells to be gathered.
* Inflammatory conditions

Advantages

* Simple procedure that does not cause bleeding or require anesthesia
* Oral brush biopsy provides cytological evaluation of cellular dysplastic changes
* Sensitivity - 71.4% to 100% , Specificity- 32% to 100%
* With molecular techniques –
	+ - Increases its specificity
		- Identification of genetic anomalies, such as mutations of the tumor suppressing gene p53
		- Genomic instability

Disadvantage

* Expensive automated devices and materials
* Trained users for interpretations
* Increased processing costs
* Loss of specificity

**Centrifuged LBC (CLBC)**

It is a modification of Liquid based Cytology. Smear is taken and flushed in a solution 🡪 centrifuged 🡪 the obtained cell pellet is resuspended in 95% alcohol 🡪 left for 2 hours 🡪 stained with PAP stain.

The advantages are clearer background, reduced number of unsatisfactory slides, and reduced false-negative results.

**HISTOPATHOLOGY**

* Gold standard for diagnosis and staging of many diseases. Grading systems have been developed to predict tumor aggressiveness. Reliable and inexpensive method for detection of precancer and cancer. Less sensitive and prone to a lot of errors, time-consuming, and there is an increase in inter-observer variability

**IMMUNOHISTOCHEMISTRY (IHC)**

IHC is a technique for identifying cellular or tissue constituents (antigens) using antigen-antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method. It has an apparent advantage over traditionally used special and enzyme staining techniques that identify only a limited number of proteins, enzymes, and tissue structures. The advantages of IHC are that it is compatible with standard fixation and embedding procedures, it can be performed retrospectively in archival material, it is sensitive and specific and is applicable to almost any immunogenic molecule.

**MOLECULAR LEVEL TECHNIQUES**



**Polymerase chain reaction**

The polymerase chain reaction (PCR) is a scientific technique in molecular biology which can be used in the diagnosis and study of infectious diseases and malignancies associated with micro organisms. PCR helps in the study of cancer and provide clearer understanding of the pathogenesis of neoplasia. PCR can be used to detect mutations in cancer-associated oncogenes (e.g., K-ras, Nras), tumor suppressor genes (e.g., p53, p16) etc. and aids as an important detection tool

Steps in PCR

There are three major steps involved in the PCR technique: denaturation, annealing, and extension. In step one; the DNA is denatured at high temperatures (from 90 - 97 degrees Celsius). In step two, primers anneal to the DNA template strands to prime extension. In step three, extension occurs at the end of the annealed primers to create a complementary copy strand of DNA. This effectively doubles the DNA quantity through the third steps in the PCR cycle

**Microarray technology**

Microarray technology helps in the quantitative study of mRNA. The expression levels of thousands of genes are assessed at the same time. Provides a unique profile of panel of genes, increased or decreased in a given disease

Application of micro array

* To detect precancer and cancer
* To perform guided biopsies
* To estimate the drug dosage during chemotherapy
* To Assess the surgical margins
* Role in sentinel node biopsy

**Nanodiagnostics**

Nanodiagnostics is the term used for the application of nanobiotechnology in molecular diagnosis which is based on pharmacogenetics, pharmacogenomics, and pharmacoproteomics information. It involves the application of nanoparticles, the use of manufactured nanorobots to make repairs at the cellular level. It is used in the discovery of biomarkers and the management of cancer through personalized medicine. Use of nanotechnology for clinical diagnostic purposes developed to meet the demands for increased sensitivity and earlier detection of disease.

It has the capability to detect even a single cancerous cell in vivo and deliver the highly toxic drugs directly to the cancerous cells. Types of nanodiagnostic aid in oral cancer are

* Nanoscale cantilevers: Elastic beams used to attach with cancer‑linked molecules
* Cantilever array sensors: Ultrasensitive mass detection technology
* Nanopores: Small holes that enable DNA passage one strand at a time, thus making DNA sequencing highly efficient
* Nanotubes: Carbon rods that can detect affected genes and also localize their location
* Quantum dots: These glow very brightly in ultraviolet light. They attach to proteins associated with cancer cells, thus localizing tumors
* Nanoelectromechanical Systems: Convert biochemical to electric signal
* Multiplexing modality: Sensing large numbers of different biomolecules simultaneously.
* Gold NPs can provide an optical contrast to discriminate between cancerous and normal cells and their conjugation with antibodies also allows them to map the expression of relevant biomarkers for molecular imaging.

**Insitu hybridization**

* ISH is the study of the macroscopic distribution and cellular localization of DNA and RNA sequences in a heterogeneous cell population. DNA or RNA in a cell is identified *in situ* using a complementary probe of RNA or DNA*.* The labeled probe is detected using an antibody directed against the label. The complex is then visualized using a fluorochrome or by peroxidase reaction of a substrate, similar to IHC

STEPS



Applications

* ISH helps in detection of various infective agents
* The study of cell development
* Human gene mapping
* Cytogenetics

Disadvantage:Itcannot provide information on translational and post-translational modifications.

**OPTICAL TECHNIQUES**

* + Photodiagnosis
	+ Flow cytometry
	+ Laser induced fluorescence
	+ Ratio imaging
	+ Elastic scattering spectroscopy
	+ Optical coherence tomography
	+ Raman spectroscopy
	+ Multiphoton excited fluorescence
	+ Nuclear magnetic resonance spectroscopy

**Photodiagnosis**

Non-invasive procedure which provides tissue diagnosis in real time through optical spectroscopy.Used in performing guided biopsies, tissue perforation in free flap surgeries, detection of dysplasia, assessment of surgical margins, and in sentinel node biopsy.

**Chemiluminescence (Vizilite)**

Chemiluminescence by definition is the emission of light from a chemical reaction. Vizilite, a diagnostic tool for the early detection of oral cancer is based on the principle of chemiluminescence. The kit contains 1% acetic acid solution, a capsule with an outer shell of flexible plastic and an inner vial of fragile glass, and a retractor. Activation requires breakage of the glass vial by bending the capsule. This permits the chemical products to react and produce a bluish-white light with a wave length of 430-580 nm that lasts for around 10 min. Under diffuse bluish-white chemiluminescent light, normal mucosa absorbs the light and appears blue, whereas the light is reflected by abnormal cells with a higher nucleus: cytoplasm ratio and by epithelium with excessive keratinization, hyperparakeratinization, and / or significant inflammatory infiltrate, which appear acetowhite with brighter, more marked, and more distinguishable border.

**Tissue fluorescence imaging (Velscope system)**

Use of tissue autofluorescence has been used for the screening and diagnosis of pre-cancerous and early cancers of the lung, uterine cervix, and skin. The changes in the structure and metabolism of the epithelium and sub-epithelial stroma alter their interaction with intense blue light (400 to 600 nm). As the optical fiber can sample only a small mucosal area, this technique is not suitable to detect new lesions or to demarcate large lesions,thus limiting the use of spectroscopy for evaluating well-defined small mucosal lesions that has been already diagnosed through clinical inspection, with the attempt to clarify its benign or (pre) malignant nature

Velscope system is a tissue fluorescence imaging system

**Identafi 3000**

The Identafi 3000 ultra shines a violet light of approximately 405 nm, which especially stimulates a blue/violet fluorescence. This technology combines three concepts Fluorescence, Fiber optics and Confocal microscopy. It also examines tissue reflectance based on changes in angiogenesis with green-amber light

**Flow cytometry (FCM)**

Flow cytometry has the ability to measure the optical and fluorescence characteristics of a single cell or any other particle such as microorganisms, nuclei and chromosome preparations in a fluid stream when they pass through a light source. Size, granularity and fluorescent features of the cells, derived from either antibodies or dyes, are also examples of parameters used to analyze and differentiate the cells

**Components of flow cytometers**

* Fluidics - directing liquid containing particles to the focused light source.
* Optics (excitation and collection) - excitation optics, focuses the light source on the cells/particles while collection optics transmits the light scatter or fluorescent light of the particle to an electronic network.
* An electronic network (detectors) - detects the signal and converts the signals to a digital data that is proportional to light intensity
* Computer –analyze data

Procedure

The cells or biological particles are led in a stream past an illumination and light detection system. As the cells traverse the illumination spot one by one, a micro objective collects the scattered and fluorescent light from the cells and directs it to a set of photomultipliers. Temporal, spatial, and chromatic filters eliminate background light and separate the signals from different fluorophores. The digital acquisition electronic equipment measures the intensity of the light pulses from each of the photomultipliers. A cell sorter adds a means for separating the cells of interest from a heterogenous mixture after they have been measured and classified based on the electrostatic charge of the particles.

Applications

* Detects DNA- aneuploidy 🡪 information about the occurrence and number of abnormal stem lines
* Cell cycle fractions
* Occurrence of rare aneuploid cells with an abnormally high DNA content
* Detection of loss of heterozygosity and thus aids in detection of oral precancer and cancer.

Demerits

Expensive and requires high current levels. The operator should be aware of the optimal excitation and emission wavelengths of the dyes used. Complex biosafety systems must be employed to reduce the potential of infection for the operator

**Laser-induced fluorescence (LIF)**

LIF is a non-invasive, easy tool used for the detection of structural and chemical alterations of the cells. Atom or molecule is excited to a higher energy level by the absorption of laser light and then followed by spontaneous emission of light. Absorption bands in the 300-500 nm range, they give rise to fluorescence in the 350-700 nm range. The excited species de-excite and emit light at a wavelength longer than the excitation wavelength usually in the order of few nanoseconds to microseconds. This fluorescent light is typically recorded with filtered photodiodes or photomultiplier tube. Autofluorescence is due to the presence of fluorophores in tissue matrix and intracellular molecules such as elastin, collagen, and nicotinamide adenine dinucleotide hydrogen (NADH).

Applications

* Study of the structure of molecules
* Detection of selective species
* Flow visualization

**Ratio imaging**

 It is a method to analyze ion concentration change in living cells such as calcium ion and pH. It requires cameras with high stability and high quantitative precision for capturing multiple spectrum images. Ratio imaging compares a photochemical or metabolic end-product of the intracellular compound, one which is increased in disease state, and another that is decreased in the same diseased state. The use of ratiometric fluorescent probes allows measurement of intracellular pH and calcium concentration at the single cell level, thus helping in the study of a multitude of cellular processes. Advantages include non-invasiveness and semiquantitative assessment.

**Elastic scattering spectroscopy**

Generates a spectrum dependent on wavelength, which reflects changes in structure and morphology of tissues at scattering centers such as the chromatin, nucleus, sub-cellular organelles, structural proteins, lipids, and erythrocytes. The cellular and sub-cellular changes are identified using the refractive indices of the cellular components. The light emitted by cellular and sub-cellular organelles ranges from 330 to 850 nm, which is within the near ultraviolet and visible part of the spectrum.

**Optical coherence tomography (OCT)**

Optical coherence tomography (OCT) was first reported by Fujimoto et al. in 1991 .OCT is an imaging technique that uses light to capture micrometer-resolution, three-dimensional (3D) images from within optical scattering media like tissue specimen. It is based on low-coherence interferometry, employing near-infrared light. It provides cross-sectional, high-resolution subsurface tissue images. This technique detects areas of inflammation, dysplasia, and cancer by recording subsurface reflections to build a cross-sectional architectural image of the tissue. Contrast enhancement of the images may be done with the use of surface plasmon resonant gold nanoparticles. In an abnormal oral epithelium containing dysplastic cells, the cell size, shape, nucleus size, and arrangement become more randomly distributed, when compared with healthy oral epithelium. Spatially average scattering intensity in a dysplastic oral epithelium is generally higher than a normal oral epithelium. Imaging range for the oral mucosa is with a tissue penetration depth of 1 mm to 2 mm

Advantage

* Cross sectional images of normal or abnormal tissues can be obtained without biopsy and no preparation of the sample or patient is required.
* As it is an optical technique and uses the light for imaging the tissues there is no exposure of the patient to ionizing radiation.

**Fluorodeoxyglucose-positron emission tomography(FDG-PET)**

FDG-PET examination demonstrates precise and prognostic significance while defining lymphatic condition. This aids in timely assessment and diagnosis of oral malignancy in affected patients. This can identify and distinguish surgical and radiation-induced variations from residual or recurrent neoplasias because cancerous cells uphold greater FDG for lengthier intervals of time as compared to infectious and inflammatory structures

**Raman spectroscopy**

Raman spectroscopy is a complementary technique to Fourier-transform infrared spectroscopy. Raman spectroscopy is a scattering technique, whereby the incident radiation couples with the vibrating polarization of the molecule and thus generates or annihilates a vibration.Vibrations of asymmetric polar bonds thus tend to be strong in infrared spectra, whereas Raman is particularly suitable as a probe of symmetric, nonpolar groups.

***Bakker Schut et al****, 2000-* analysed raman spectroscopy in normal and dysplastic tissue in a rat model. ***Malini et al in 2006***discriminated normal, cancerous, precancerous, and inflammatory conditions and found that lipid rich features in normal conditions and prominent protein features in tumors and other pathological conditions. ***Shyam Sunder et al****, 2011*  thatoral carcinomas of different pathological grades can also be differentiated on the basis of the relative intensities of bands associated with lipids and proteins.

**Multiphoton excited fluorescence [MEF]**

Multiphoton Microscopy (MPM) has emerged as a powerful tool to explore the structure and function of biological samples, and especially of tissues. This is mainly because MPM techniques can non-invasively acquire optical sections (virtual biopsies) in unlabeled tissues, containing information that is very relevant for diagnostic purposes. These non-linear techniques are based on the theory of quantum transition through photons proposed by Nobel Laureate Maria Göppert-Mayer. During the non-linear processes that take place, the sample absorbs two or three infrared photons and emits a unique photon of shorter wavelength. This can occur via different physical processes, that may take place quasi-simultaneously, e.g., fluorescence or harmonic generation.

Principle : the energy (i.e., light) released by fluorophores during MEF allows the visualization of different biological components, such as elastin, keratin, melanin, nicotinamide adenine dinucleotide (NAD+/NADH) or flavin adenine dinucleotide (FAD). Selective probing of these autofluorescent tissue components by MEF followed by arithmetic operations for distinct signals enable the non-invasive assessment of important information such as cell morphology, size variation of cell nuclei, blood vessel hyperplasia, or inflammatory reaction related aspects

3D images of endogenous tissue fluorescence can effectively distinguish between normal, precancerous, and cancerous epithelial tissues with high‐resolution capability

**Nuclear magnetic resonance spectroscopy**

An NMR spectrometer instrument can help researchers to categorize metabolites, the intermediates and products of metabolic processes in a biological system based on the magnetic properties of their nuclei. Biomarkers of metabolism can be the metabolites detected in urine or blood cells. Nuclear magnetic resonance provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. . All living matter contain cells which have atoms. Each atom has a nucleus that contain subatomic particles called electrons (negatively charged particles), protons (positively charged particles) and neutrons (neutral particles). The charge of an atom depends on how many of each of the different types of subatomic particles it has.

An NMR spectrometer investigates atoms by using its magnet to create a magnetic field that impacts the nuclei of atoms in different ways due to their individual charges. The results are plotted on a diagram showing NMR spectra – the peaks created for separate constituents of the nuclei. This helps scientists to derive the chemical structures.  NMR spectroscopy is powerful method for identification of small molecules in biological fluids such as in saliva. Identification of new salivary biomarkers would help us to diagnose HNSCC in its early stages, which is highly advantageous and can help in selecting the most appropriate treatment modalities.

**Other techniques**



**Salivary biomarkers**

Saliva is known to be capable of mirroring the status of both oral and systemic health. It contains locally expressed proteins and end-products of different metabolic pathways (i.e., metabolites) that are known to alter greatly in their concentrations in various diseases. Therefore, these substances, called as salivary biomarkers, are good indicators of an individual's health status.

Measurement of specific salivary macromolecules and examination of proteomic or genomic targets such as enzymes, cytokines, growth factors, metalloproteinase, endothelin, telomerase, cytokeratins, mRNAs, and DNA transcripts can be done by the saliva. carcino-embryonic antigen (CEA), SCC, CA125, and CA19-9 are the most studied epithelial serum circulatory tumor markers in the saliva of carcinoma patients.

**LAB ON A CHIP**

Microfuidics technology , micro-total-analysis systems are the other names for lab on chip system. It is the adaptation, miniaturization, integration and automation of analytical laboratory procedures into a single device or “chip”. Microfluidics are suited for handling living cells in a three-dimensional, biologically relevant environment.

Principle:

 The LOC comprises a microfluidic system and a detection system. There are eight inlets for the reactants and is installed with a unique inlet for the sample to be tested. The sample is mixed with the respective reactants.

Many candidate genes associated with OSCC tumor progression such as p53, cyclin D1, and epidermal growth factor receptor gene have been identified. Microarray analysis of several tumor types has demonstrated that global expression profiling that distinguishes tumor cells from normal cells

Advantages

1. Small sample size with minimal invasive technique

2. Short processing times

3. Reduced reagent consumption, reproducibility, consistency

4. Reduced exposure to hazardous materials or infectious agents

5. Minimal risk of sample contamination

The disadvantagesis the electrokinetic methods in microchannels suffer from limitations of buffer incompatibility, solvent evaporation and electrophoretic demixing.

**COLPOSCOPY**

The colposcope was invented in 1925 by Professor Hinselmann of Hamburg, Germany, specifically for the purpose of detecting early cervical cancer. It functions as a lighted binocular microscope to magnify the view of the cervix, vagina, and vulvar surface and other similar tissues. Illumination is provided by a halogen lamp via a fibreoptic cable connected to a system of lenses.

The colposcope is fitted with a green or blue filter to facilitate the examination of vascular changes and color tone as unfiltered white or yellow light reduces the contrast between the terminal vessels and the surrounding tissue. The focal length of the microscope is 200mm, providing an optimal working distance. Accurateness of colposcopy was 70%-98%

In the normal mucosa, two basic types of capillary networks can be seen with the colposcopy procedure: Network capillaries and hairpin capillaries. The vascular patterns associated with abnormal epithelium include punctuation, mosaicism, and atypical vessels. High-grade lesions demonstrate a more persistent duller shade of white, whereas low-grade lesions are translucent or bright white and fade quickly. Low-grade lesions have feathery margins and irregular borders whereas high-grade lesions have straighter, sharper outlines and well-defined borders

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