

ADVANCED DIAGNOSTIC AIDS IN ORAL CANCER

Abstract

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer globally with high mortality rates. Early diagnosis of OSCC helps in reducing the mortality rates. Various diagnostic methods have been used globally in diagnosing of oral carcinoma. In this chapter we will be discussing some basic and conventional diagnosing aids as well as advanced diagnostic aids with their merits and demerits in OSCC diagnosis.

Keywords: Diagnostic Aids, Oral Cancer, OSCC, Vital staining.

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I. INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer globally with 5 year survival rate with high mortality rates. The common site of occurrence was Tongue (postero lateral and ventral surface) followed by Floor of the mouth, gingival mucosa, buccal and 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.

II. CONVENTIONAL ORAL EXAMINATION

Oral leukoplakia, a premalignant condition or squamous cell carcinoma is indicated by changes in the surface grain, color, integrity, size and mobility of lesion structure. Red or white lesions of oral mucosa or a non healing ulceration is the distinguishing clinical feature of malignancies. Small portion of these lesions may turn into malignant lesion.

III. VITAL STAINING

Vital staining technique selectively stains viable cytoskeletal structures like mitochondria, lipid vesicles, lysosome. There are many vital stains available most commonly used stains are

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

1. Toluidine Blue (TB Stain): TB stain has being used to detect any mucosal abnormalities of the cervix and the oral cavity. It is a metachromatic acidophilic dye that binds preferably to the tissues which are under rapid cell division like regenerative, inflammatory, and neoplastic tissue. This stain has attraction to preferentially stain the acidic constituents of tissue such as carboxylate, sulfate and phosphate radicals of DNA and RNA only and doesn't stain 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:** The malignant cell nucleus has an improved uptake of the dye, and this evident as augmented DNA synthesis. Quick dye penetration will occur in haphazardly arranged tumour cells.
- **Procedure**
 - Identify the suspected lesion
 - Rinse with toluidine blue/ apply
 - Neutralize with 1% acetic acid/ apply

➤ Observe for stained areas

- **Inference:** Dysplastic lesions will appear dark blue. Dysplasia will present with different shades of blue and non-malignant inflammatory cell may not stain. To avoid false positive results re-staining should be done within 14 days
- **False Positive Lesions:** Epithelial hyperplasia, inflammatory, hyperkeratotic, traumatic lesions will show false positive results. Chronic hyperplastic candidiasis can hold 60% of stain. The final diagnosis is attributed to the clinician's experience.
- **False Negative Lesion:** Low grade dysplasia and lichenoid dysplasia
- **Disadvantage:**
 - For potentially malignant lesions, Low positive prophetic value - 43.5%
 - For pre-malignant lesions (leukoplakia), A false negative rate as 20.5%

2. **Lugol's Iodine (LI Stain):** Lugol's iodine stain was first prepared by French physician Lugol in 1829

- **Composition**

Iodine	2gm
Potassium iodide	4 gm
Distilled water	100 cc

- **Mechanism of Action:** Iodine–starch reaction occur in lugol's iodine staining mechanism and it rely on cytoplasmic glycogen contents and it is visualized by a colour change. Due to improved glycolysis in cancer cells, there will be no iodine–starch reaction taking place. The vital dye preparation along with LI solution is called as Schiller's test
- **Procedure:**
 - 1% acetic acid is spread to the suspected lesion tissue for 20 seconds and rinse with water
 - Apply the stain at the lesional site with a cotton applicator for 10-20seconds
 - If the lesional site shows brown stain it is considered as normal mucosa.
- **Advantages:** Lugol's iodine is inexpensive, generally available, easy to use and not time consuming it only takes about 5 min to perform the staining procedure.

3. **Double Staining Technique:** Staining Toluidine Blue down with LI stain is known as double staining technique. It is used for clinical identification of the degrees of differentiation of malignant cell since poorly differentiated cell without glycogen content

do not show LI retention in the cells. This technique is indicated in patients with high risk of malignant changes and to choose biopsy sites in wide field cancers patients.

- 4. Rose Bengal Staining:** It is a vital staining technique and it constitute of 4, 5, 6, 7 tetrachloro-2, 4, 5, 7 tetraiodo derivate of fluorescein. This stain had an affinity to stain desquamated ocular epithelial cells. RB staining is used to demarcate the degree of the corneal and conjunctival neoplasms, oral epithelial dysplasia and Oral squamous cell carcinoma

IV. CYTOLOGICAL TECHNIQUES

Exfoliative cytology in oral cavity is the best procedure for the preliminary investigation of oral lesions. Their types vary based on the method of cytological slide preparations. They are

- 1 Oral Brush biopsy (Oral CDX)
- 2 Liquid Based Cytology
- 3 Centrifuged Liquid Based Cytology

- 1. Oral Brush Biopsy (OralCDx):** Computer-assisted cytology use specialized mechanics that allow the detection of premalignant and malignant lesions with sensitivity greater than, that of physical screening methods, without losing the specificity. The Oral CDx system takes improvement in the method to accumulate epithelial cell sample that are automatically inspected through a computer-aided system

- **Contraindications:**

- Highly keratinized leukoplakia has increased keratinization and it will be difficult to collect the basal cells
- Inflammatory conditions

- **Advantages**

- Simple technique that does not induce bleeding or require anesthesia for the procedure.
- Sensitivity will be 71.4% to 100% and Specificity will be 32% to 100%
- When used along with some molecular techniques –
- There will be increases its specificity
- Identification of genetic abnormalities, like mutation in tumor suppressing gene p53
- Genomic instability can be identified

- **Disadvantage**

- Need trained technician for interpretations
- Automated devices and materials were little expensive
- Loss of specificity
- Increased processing charge

- 2. Centrifuged LBC (CLBC):** It is a modified technique of Liquid based Cytology. Cytological smears scrappings were taken and mixed in a solution then it is centrifuged and the attained cell pellet is suspended in 95% alcohol and in alcohol it is left for 2

hours then the slides were stained with PAP stain. Their advantages are clearer background, reduced false-negative results and lesser number of unsatisfactory slides.

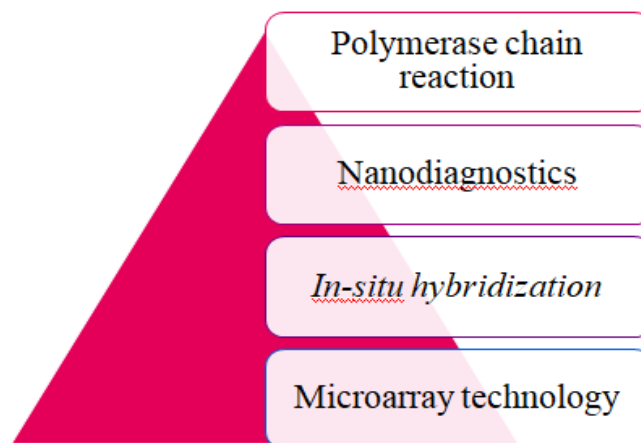
V. HISTOPATHOLOGY

Biopsy and histopathological technique is considered as a gold standard for identification of many lesions and conditions. Grading systems of carcinomas have been build up to represent tumor aggressiveness. It is a cost effective and reliable for detection of precancer and cancerous lesion. The main disadvantages are it is not as much of sensitive and subjected to more errors and the procedure takes lot of time also there is greater inter-observer variability

VI. IMMUNOHISTOCHEMISTRY (IHC)

Immunohisto chemistry is a histo chemical method of demonstrating antigens of cells or tissue of preparations using antigen-antibody reaction, the spot of which the antibody binds will be identified either by secondary labeling method or direct labeling of the antibody. It has an obvious benefit over conventionally used enzyme and special staining techniques that recognize only a restricted number of enzymes, proteins and tissue structures. The benefit of IHC are that it is well-suited with regular fixation and embedding technique, also it can be done retrospectively in archival or stored tissue blocks, and is highly sensitive and specific and it can be applicable to several immunogenic tissue.

VII. MOLECULAR LEVEL TECHNIQUES



VIII. POLYMERASE CHAIN REACTION (PCR)

PCR is a molecular level procedure used in molecular biology, used to understand infectious diseases and tumour associated with microorganisms. PCR aids in the diagnosis of carcinomas and to understand the pathogenic mechanism of tumor. It helps to find out mutations in tumor suppressor genes, cancer-associated oncogenes like K-ras, Nras genes

Steps in PCR

1	Denaturation	DNA is denatured at high temperatures (90 - 97 degrees Celsius).
2	Annealing	Primers anneal to the DNA template strands to prime extension
3	Extension	Extension occurs at the end of the annealed primers to create a complimentary copy strand of DNA

This efficiently doubles the DNA quantity in the PCR cycle.

1. Microarray Technique: Microarray technique aids in the quantitative reading of mRNA. The expression stages of many of genes are evaluated at one time. It provides a sole summary of panel of genes, greater or lesser in a provided malignancy. The commonly used microarray techniques are *oligonucleotide* microarray and *spotted* microarray.

- **Principle:** Microarray process can be classified into two steps:
 - Probe production
 - Target (cDNA) production.

Definite sequence of genome are immobilized to a plane and acted with labelled cDNA targets. A sign resultant from hybridization of the labelled target with the specific immobilized probe determines which RNAs are present in the unknown target sample.

Applications of microarray are, they detect precancer and cancerous lesion and to perform guided biopsies. Used to rule out metastatic potential of cancer cell. They help in estimation of amount of drug delivered during chemotherapy, helpful in assessing surgical margin and supportive in sentinel node biopsy

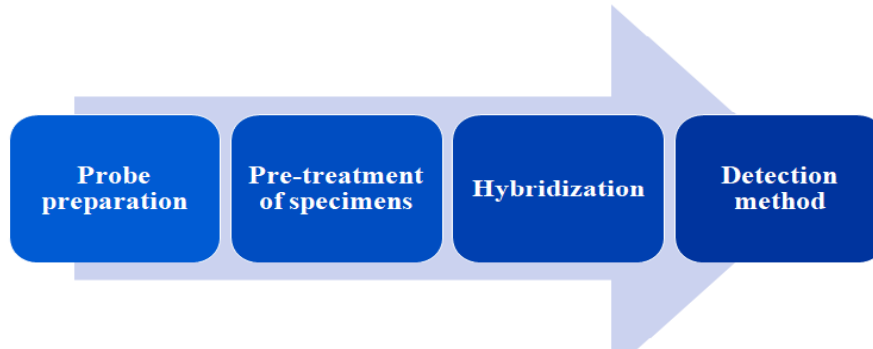
2. Nanodiagnostic Technology: Nanodiagnosis is a terminology in nanobiotechnology used for molecular diagnosis in pharmacogenomics, pharmacogenetics and pharmacoproteomics information. With the help of nanorobots genetic level changes are possible with nanoparticles. To identify cancer biomarkers and personalized medicines of management of carcinomas and early diagnosis of cancers are being done with nanodiagnosis. These nanoparticle has ability to detect even a single neoplastic cells *in vivo* and delivers drugs directly to the single neoplastic cell. The types of nanodiagnostic aid in oral cancer are

1. Nanoscale cantilevers
2. Cantilevers array detectors
3. Nanopores
4. Nanopores
5. Quantum dots
6. Nanoelectromechanical Systems
7. Multiplexing modality
8. Gold Nanoparticles

3. Insitu Hybridization: ISH is a technique used for of the cellular localization and cellular distribution of DNA and RNA sequences in a preserved tissue section, whole tissue or

heterogeneous cell population. Hybridization of complementary strand made of nucleotide probe was made to make a sequence of particular nucleotide. These hybrid nucleotide can be identified by various detection methods like enzymatic detection, autoradiography or indirect methods (haptent- Biotin, Digoxigenin, or Fluorescein)

- **Steps**



- **Applications**

- Determination of various infective agents
- Revelation of mechanism of Virus Dissemination and Transmission
- Localization of active infection
- Human gene mapping
- Cytogenetics

- **Disadvantage:** It fails to provide explanation on post-translational and translational modification of nucleotide sequence

IX. OPTICAL TECHNIQUES

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

1. **Photodiagnosis:** This is a procedure which aids in diagnosis of tissue in true time through optical spectroscopy. Mainly useful to perform guided biopsies of malignant lesion as well as tumors, early diagnosis of dysplasia, surgical margin assessment, and in biopsy of sentinel nodes.

- **Chemiluminescence:** The principle of chemiluminescence is when there is tissue level chemical reaction that causes emission of light from the tissue. Vizilite is a tool that follows the chemiluminescence principle. The vizilite kit consist of acetic acid 1% solution, a capsule which is made of elastic cover externally and a vial internally and a retractor. The vizilite is activated by folding the capsule that make the glass vial to

rupture and chemical reaction to take place and a 430 -580 wavelength blue light is produced that light will last only for 10 min. This emitted light got absorbed by normal mucosa and appears blue, but the dysplastic epithelium will appear white with more prominent border.

- **Tissue Fluorescence Imaging (Velscope System):** This technique aids in assessing pre-malignant lesions and carcinoma in situ of the uterine cervix, lung and skin. Because of the metabolic and structural changes in epithelium and connective tissue of neoplastic epithelium, the blue light (400-600nm) interactivity will be changed. The limitation is that this optical fiber can evaluate only very few mucosal area, hence it is not fit to identify any new lesions or to delineate substantial lesion. This system was very helpful to identify prominent tiny lesion that has been previously evaluated as malignant lesion in clinical inspection, and to confirm its malignancy status. Velscope is one of a tissue fluorescence imaging apparatus commercially available. The Identafi 3000 is also a tool in photodiagnosis which uses violet light (wavelength 405 nm). It inspects tissue reflectance depending on modification in vasculogenesis with ambergreen light

2. **Flow Cytometry (FCM):** Flow cytometer is a device used in flow cytometric technique. This technique uses fluidic mechanism to quantify the optical and fluorescence feature of any cell or particle like chromosome, microorganism etc. The size of the particle, its granularity and its fluorescent nature are the parameters used to discriminate and study the cells.

The underlying principle of flow cytometry is related to the property of light scattering as well as fluorescence emission which happens when light (laser) from excitation source hits the moving particles.

- **Components of Flow Cytometers:**
 - Fluidics – It directs the liquid that contains cells to the determined light source.
 - Optics – This light system, excitation optics centres the laser light on the particles then the collection optics will transfer the scattered light or fluorescent particle light to a computerized system.
 - Electronic detector – the signals are absorbed and they are converted to a digital record that data is relative to the intensity of light.
 - A monitor to analyze the data
- **Applications:** Encounters aneuploidy in DNA and analyzes data regarding the event & amount of atypical cell lines. Cell cycle fragments can be examined. Study of aneuploidy cell with an unusual DNA content. Aids in identification of heterozygosity loss in oral precancer and cancer cells.
- **Demerits:** Procedure is costly and needs high electrical consumption. The machinist must be attentive of finest emission and excitation wavelengths of the indicator dye used. Intricate biological safety protocols should be employed to decrease the possibility for operator acquiring infection

- **Laser-Induced Fluorescence (LIF):** Laser-induced fluorescence uses the principle that the atoms are excited to an energy level which is higher by light absorption and then reemission of the light. The excited atom or molecule got de-excited and release light of lengthier wavelength for few seconds(microsec, nanosec). The fluorescent emissions will be classically documented by photodiodes filtered. It demonstrates the presence of fluorphores in collagen fibres, eulanin, elastin, niacinamide and adenine dinucleotide hydrogen of connective tissue matrix
 - **Applications:** To understand the structure of molecules, cells and tissues. Helps in identification of particular species of microorganism and to detect flow visualization
- 3. Ratio Imaging:** Ratio imaging technique, examine the ion concentration (calcium ion and pH) alteration in viable cells. It needs cameras with increased permanence and quantitative precision for reproducing various spectrum images. Ratio imaging contrasts a metabolic productivity of the intracellular substances, one of which is improved in infection state and a different that will be reduced in a similar infected state.

This ratioimaging probes helps in quantification of calcium level and pH intracellularly at the individual cell stage thus aiding in understanding of large amount of cellular progression. Advantages include non incursive and semi quantitative assessment.

- 4. Elastic Scattering Spectroscopy:** The alteration in morphology and structure of cancerous cell at scattering regions like nuclear chromatin, nuclei, mitochondria, lipids, proteins and blood cells were measured by the spectrum which is rely on wavelength. This sub cellular and cellular level variations were recognized by means of cellular components refractive index. . 330 to 850 nm wavelength of light is discharged by these organelles, which is in range near UV light and visible light spectrum part.
- 5. Optical Coherence Tomography (OCT):** OCT technique was initially described by Fujimoto and his colleagues in 1991. OCT technique uses light ray to record 3D image, micron resolution images from optical scattering medium like tissue or surface specimens. This technique relays on low-coherence interferometry involving light which is nearer to infrared spectrum. It provides high-quality, cross-sectional subsurface images. This procedure identifies regions of dysplasia, malignancy, inflammation by the structures reflection and make a cross-sectional, high quality image of subsurface region. Atypical oral epithelium containing malignant cells, the cellular atypia and architectural derangement become more haphazardly scattered, when compared with normal oral epithelium. Standard scattering concentration in a dysplastic oral epithelium was commonly greater than a usual oral epithelium.

- **Advantage**
 - Cross sectional depiction of tissues can be acqiured with no need for biopsy and tissue sections of specimen
 - Optical technique utilizes the low-coherence light that views the tissues and exposure to harmful radiation is minimized.

6. **Fluorodeoxy Glucose-Positron Emission Tomography(FDG-PET):** FDG- PET assessments exhibit accurate and prognostic importance while explaining lymphatic condition. This technique helps in appropriate evaluation and analysis of oral malignancy and premalignancies. This can recognize and discriminate radiation and surgical induced changes from recurring neoplasias since cancer cell sustain larger FDG for longer period as contrasted to inflamed tissue.
7. **Raman Spectroscopy:** This technique corresponds to Fourier-transform infrared spectroscopy. It is a scattering method, according to it the vibrations of the polarizing molecule combine with the incident radiation to produce vibrations.

Bakker Schut et al, 2000- studied raman spectroscopy method in normal and neoplastic epithelium in a animal study. *Malini et al, 2006* discriminated normal, cancerous, precancerous, and inflammatory lesions and concluded that high lipid character in standard state and major protein character in neoplasm and other diseased conditions. Another study by *Shyam Sunder and his colleagues in 2011* evaluated oral malignancy of various grades can be discriminated based on the comparative bands intensity of proteins and lipids.

8. **Multiphoton Excited Fluorescence [MEF]:** Multiphoton microscopy has comeout as a potential tool to study the structure of viable cells and tissues and their functions are explored. The MPM procedure is non incursive and the virtual biopsies are taken by optical sectioning of affected tissue samples. This technique was developed by Maria Göppert-Mayer and the technique employs quantum transition theory through photon. In this process the tissue samples absorb 2 or 3 photons and emit a specialized photon of wavelength shorter. 3Dimensional image of this technology can efficiently differentiate among normal mucosa, premalignant and malignant epithelium with resolution ability high.
9. **Nuclear Magnetic Resonance Spectroscopy:** This apparatus can aid in classifying metabolites in metabolic process that occur in cells and tissue. From urine or blood, the salivary biomarkers are identified. This technology gives details about the information of how the metabolite reacts, its structure, its dynamics, biochemical reactivity of particular molecule.

All viable particle contain cells which have atoms. Each atom consists of a nucleus that enclose subatomic particles called electrons, protons, and neutrons. The charge of an atom depends on how many of every unique type of subatomic particles it has. An NMR spectrometer examines atoms by means of its magnet to produce a magnetic field that contact the nuclei of atoms in various ways due to their individual charges. The results are plotted on a picture showing NMR spectra – the peaks produced for divided constituents of the nuclei. This aided in researchers to obtain the chemical structures. NMR spectroscopy is influential technique for detection of little molecules in biological fluids such as in saliva. Recognition of new salivary biomarkers would help us to detect HNSCC in its early stages, which is highly beneficial and can aid in choosing the most suitable treatment methods.

- **Other Techniques:**



10. Salivary Biomarkers: Saliva is known to be proficient of mirroring the grade of both oral and systemic health. It encloses locally conveyed proteins and final-products of different metabolic that are known to modify vastly in their concentration in numerous diseases. Therefore, these substances, called as salivary biomarkers which are excellent indicators of an individual's health grades.

Saliva contains numerous enzymes, proteins and other macromolecules , their concentration can be assessed and their concentration variation can be helpful in understanding pathological conditions. Salivary biomarkers such as CA125, SCC, carcino-embryonic antigen (CEA), and CA19-9 are the mainly considered epithelial tumor marker in carcinoma patients

X. LAB ON A CHIP

Microfluidics technology , micro-total-analysis systems are the alternate names for lab on chip system. It is a integration, revised, tininess, and computerization of logical lab measures into a single device or “chip”. Microfluidics is suited for handling viable cells in a three-dimensional, biologically relevant environment.

1. Principle: This system consist of microfluidic and detection system. There are 8 inlet vent for the reactive agent and a special inlet for testing the sample. The testing sample is assorted with particular reactants. In this technology, the progression of carcinomas can be related by the recognition of cyclin D1, p53 and epidermal growth factor receptor genes and helps in differentiating normal cell and neoplastic cell.

2. Advantages:

- Requires little sample size with limited invasive procedure
- Processing time for procedure is less
- Decreased reagent usage, reproducibility, consistency

The disadvantage is the electrokinetic techniques in microchannels undergo restriction of incompatibility of buffer, evaporation of solvent.

XI. COLPOSCOPY

The colposcope was invented in 1925 by Professor Hinselmann of Hamburg, specially for the purpose of identifying 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. Light source is given via a fiberoptic cable joining to a system of lenses by a halogen lamp.

This apparatus is fixed with a green or blue filter to aid in evaluation of angiogenic changes and color tone as unfiltered white or yellow light decreases the difference between the end vessels and the adjacent tissue. Accurateness of colposcopy was 70%-98%

In non neoplastic epithelium, capillary network can be visualized by two variants they are hairpin capillaries and Network capillaries. The vascular patterns of neoplastic epithelium shows mosaicism, punctuation and unusual vessels

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