**ADVANCED DIAGNOSTIC AIDS IN ORAL CANCER**

**AUTHORS:** PORKODI SUDHA J, SWATHIRAMAN J

**REFERED BY:** Dr. J DINESH SHANKAR **. EDITOR ID:** IIPER1675422015

**CONTENT**

* Introduction
* Conventional Methods
  + Vital staining
  + Liquid-based cytology (LBC)
  + Centrifuged LBC (CLBC)
  + Histopathology
  + Immunohistochemistry (IHC)
* Molecular level techniques
  + Polymerase chain reaction
  + Nanodiagnostics
  + *In‑situ* hybridization
  + Microarray technology
* 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
* Other Newer techniques
* References

**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, gingiva, 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.

**CONVENTIONAL ORAL EXAMINATION**

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

**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. Toludine blue
2. Lugols iodine
3. Rose Bengal stain

**Toluidine blue**

Toluidine blue 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 affinity to preferentially stain the acidic components of tissue such as sulfate, carboxylate and phosphate radicals of 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

The malignant cell nuclei have an improved uptake of the dye, and this manifests as augmented DNA synthesis. Quick dye penetration will occur through haphazardly arranged tumor 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 and traumatic lesions. Chronic hyperplastic candidiasis can hold 60% of stain. The final diagnosis is attributed to the clinician’s experiance.

False negative lesion

Low grade dysplasia and lichenoid dysplasia

Disadvantage

* Low positive prophetic value of 43.5% for potentially malignant lesions
* A false negative rate as 20.5% for pre-malignant lesions like leukoplakia.

**LUGOL’S IODINE**

Lugol’s iodine 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 is based on cytoplasmic glycogen content 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 along with Lugol’s iodine solution is called as Schiller’s test

Procedure

* 1% acetic acid is applied to the suspected lesion tissue for 20 seconds and rinse with water
* Apply Lugol’s iodine at the lesional site with a cotton bud 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.

**DOUBLE STAINING TECHNIQUE**

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

**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 has an affinity to stain desquamated ocular epithelial cells. RB staining is used to demarcate the extent of the corneal and conjunctival neoplasms and also oral epithelial dysplasia and Oral squamous cell carcinoma

**CYTOLOGICAL TECHNIQUES**

Oral Exfoliative cytology is a cost effective and possibly the best procedure for the preliminary 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 use specialized instruments that allow the detection of both preneoplastic and neoplastic lesions with a sensitivity higher than, that of manual screening method, without loss of specificity. The Oral CDx Brush Test System (CDx Laboratories, Suffren, NY) takes improvement in the method of collecting a trans-epithelialcell sample that are automatically examined through a computer-assisted 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

**Centrifuged LBC (CLBC)**

It is a modification of Liquid based Cytology technique. Smears were taken and mixed 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 false-negative results and lesser number of unsatisfactory slides.

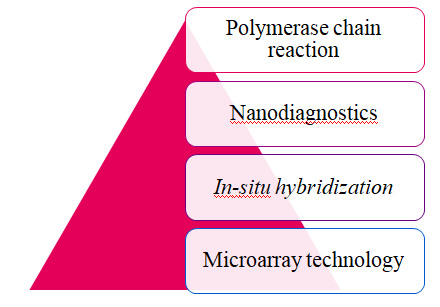
**HISTOPATHOLOGY**

Biopsy and histopathology is considered as a gold standard for diagnosis of many lesions and conditions. Grading systems of carcinoma’s have been developed to represent tumor aggressiveness. It is a cost effective and reliable for detection of precancer and cancerors lesion. The main disadvantages are it is less sensitive and subjected to a lot of errors and the procedure is time-consuming also there is an increase in inter-observer variability while histopathological diagnosis.

**IMMUNOHISTOCHEMISTRY (IHC)**

IHC is a histochemical method of identifying cellular or tissue constituents (antigens) of preparations using antigen-antibody interactions, the site of which the antibody binds will be identified either by direct labeling of the antibody, or a secondary labeling method. It has an obvious advantage over conventionally used special and enzyme staining techniques that recognize only a restricted number of proteins, enzymes, and tissue structures. The advantages of IHC are that it is well-suited with standard fixation and embedding procedures, it can be done retrospectively in archival tissue blocks, and it is highly sensitive and specific and it is applicable to any immunogenic tissue molecule.

**MOLECULAR LEVEL TECHNIQUES**



**Polymerase chain reaction**

The polymerase chain reaction (PCR) is a molecular level technique used in molecular biology which can be used in study of infectious diseases and malignancies associated with micro organisms. PCR helps in the diagnosis of cancer and aid in understanding of the pathogenesis of neoplasia. It can be used to find mutations in cancer-associated oncogenes (e.g., K-ras, Nras), tumor suppressor genes ( p53, p16) etc.

Steps in PCR

The steps involved in the PCR technique:

1. Denaturation
2. Annealing
3. Extension

In denaturation, the DNA is denatured at high temperatures (90 - 97 degrees Celsius).

In annealing, primers anneal to the DNA template strands to prime extension. In third step, 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.

**Microarray technology**

Microarray technique aids in the quantitative study of mRNA. The expression levels of many of genes are assessed at the same time. It provide a sole profile of panel of genes, increased or decreased in a given malignancy. The commonly used microarray techniques are *oligonucleotide* microarray and *spotted* microarray.

Principle:

Microarray process can be divided into two main steps:

1. probe production
2. Target (cDNA) production.

Specific sequence of genome are immobilized to a surface and reacted with labelled cDNA targets. A signal 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 drug dosage during chemotherapy, helpful in assessing surgical margins and supportive in sentinel node biopsy

**Nanodiagnostics**

Nanodiagnostics is the expression used for the operation of nanobiotechnology in molecular opinion which is based on pharmacogenetics, pharmacogenomics, and pharmacoproteomics information. It involves the operation of nanoparticles, the use of manufactured nanorobots to make repairs at the cellular position. It's used in the discovery of biomarkers and the operation of cancer through substantiated drug. Use of nanotechnology for clinical individual purposes developed to meet the demands for increased perceptivity and earlier discovery of complaint.  
It has the capability to descry indeed a single cancerous cell invivo and deliver the largely toxic drug directly to the cancerous cells.

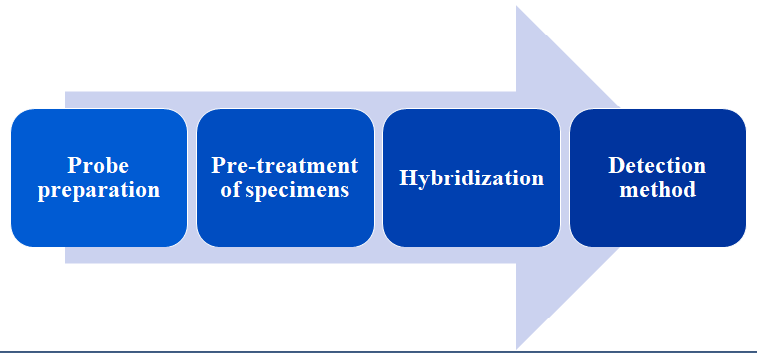
The types are nanodiagnostic aids are  
Nanoscalecantilevers: Elastic shafts used to attach with cancer linked molecules

Cantilevers array detectors:  Ultrasensitive and helps in mass screening technology  
Nanopores:  Small holes that enable DNA passage one beachfront at a time, therefore making DNAsequencing largely effective  
Nanotubes: Carbon rods that can descry affected genes and also localize their position  
Quantum dots:  These glow veritably brightly in ultraviolet light. They attach to proteins associated with cancer cells, therefore localizing excrescences  
Nanoelectromechanical Systems:  Convert biochemical to electric signal  
Multiplexing modality:  seeing large figures of different biomolecules contemporaneously.  
Gold NPs can give an optic discrepancy to distinguish between cancerous and normal cells and their conjugation with antibodies also allows them to collude the expression of applicable biomarkers for molecular imaging.

**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. By hybridizing the complementary strand of a nucleotide probe to a particular sequence. These hybrids can be visualized by various detection methods like enzymatic detection, autoradiography or indirect methods (hapten- 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:Itfails to provide explanation on translational and post-translational modifications.

**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

**Photodiagnosis**

This is a procedure which aids in tissue diagnosis in real time through optical spectroscopy. Mainly used to perform guided biopsies of malignant lesion as well as tumors, to avoid tissue perforation in free flap surgeries, early diagnosis of dysplasia, surgical margins assessment, and in sentinel node biopsy.

**Chemiluminescence (Vizilite)**

Chemiluminescence is the emission of light from a chemical reaction occurring at tissue level. Vizilite, a indicative tool for the early detection of oral cancer and it follows the principle of chemiluminescence.

The vizilite kit contains 1% acetic acid solution, a capsule with an external shell of elastic cover and an internal vial of delicate glass, and a retractor. Activation requires rupture of the glass vial by folding the capsule. This result in chemical products of vial to react and produce a bluish-white light with a wave length of 430-580 nm that will last only for 10 min. Under diffuse bluish-white chemiluminescent light, the light is absorbed by normal mucosa and appears blue, whereas the light is reflected by dysplastic cells with a higher nucleus: cytoplasm ratio and by epithelium with extreme keratinization (hyperparakeratinization, and / or significant inflammatory infiltrate), and appear as white with intense, more noticable, and more prominent border.

**Tissue fluorescence imaging (Velscope system)**

Application of tissue autofluorescence has been aid in screening and diagnosis of pre-cancerous and carcinoma insitu of the lung, uterine cervix, and skin. The commute in the structure and metabolism of the epithelium and sub-epithelial connective tissue change their interactivity with intense blue light of wavelength 400 to 600 nm. The limitation is that the optical fiber can evaluate only few mucosal area, hence it is not suitable to identify new lesions or to delineate substantial lesions. To assess well-defined tiny mucosal lesions that has been diagnosed before through clinical inspection, with the venture to elucidate its benign or (pre) malignant nature

Velscope system is one of a tissue fluorescence imaging apparatus used commercially available

**Identafi 3000**

The Identafi 3000 ultra emits a violet light of wavelength 405 nm, which particularly invigorate a blue/violet fluorescence. This equipment combines three theories 1. Fluorescence 2.Fiber optics 3.Confocal microscopy. It also inspect tissue reflectance depend on modification in angiogenesis with green-amber light

**Flow cytometry (FCM)**

Flow cytometry has the capacity to measure the optical and fluorescence features of a single cell or any particle like microorganisms cell nuclei and genomic chromosome preparations in a fluid stream when they through a light source. Particle size, granularity and fluorescent nature of the cells, derived from any antibodies or fluorescent dyes, are also parameters that help to study and discriminate the cells

**Components of flow cytometers**

* Fluidics – It directs the liquid that contain cell or particles to the focused light source.
* Optics – this light system, excitation optics centres the light source on the cells/particles then the collection optics will transfer the light scatter or particle fluorescent light to an electronic system.
* An electronic detectors – they absorb the signal and changes the signals to a digital data that is relative to light intensity
* An monitor to analyze the data

Applications

Encounters DNA- aneuploidy and analyze data about the event and amount of atypical stem lines. Cell cycle fragments can be examined. Study of rare aneuploid cells with an atypical high DNA content. Identification of loss of heterozygosity in oral precancer and cancer cells.

Demerits

Procedure is costly and needs high electrical consumption. The machinist should be attentive of the finest excitation and emission wavelengths of the indicator dyes. Intricate biosafety methods must be employed to decrease the possible of infection for the operator

**Laser-induced fluorescence (LIF)**

LIF is a non-incursive, simple diagnostic aid to identify structural and chemical changes of the cells. Atom or molecule is excited to a higher energy level by the assimilation of laser light and then go after by unprompted emission of light. 300-500 nm range bands will give rise to fluorescence in the 350-700 nm range. The excited species de-excite and emit light at a wavelength lengthier than the excitation wavelength frequently in the direct of few nanoseconds to microseconds. This fluorescent light emission is classically documented with filtered photodiodes or photomultiplier tube. Autofluorescence is owed to the occurrence of fluorophores in cell/tissue matrix and intracellular molecules such as collagen fibres, eulanin, elastin, and niacinamide, adenine dinucleotide hydrogen (NADH).

Applications: To understand the structure of molecules, cells and tissues. Helps in identification of particular species of microorganism and to detect flow visualization

**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 photochemical or metabolic output of the intracellular compound, one which is improved in disease state, and another that is reduced in the similar diseased state. The application of ratiometric fluorescent probes permits quantification of intracellular pH and calcium concentration at the individual cell level, thus serving in the identification of a large amount of cellular processes. Advantages include non incursive and semi quantitative assessment.

**Elastic scattering spectroscopy**

This technique produces a field relying on wavelength, which reflects alterations in structure and morphology of cancerous tissues at scattering regions such as the nuclear chromatin, nuclei, sub-cellular organelles like mitochondria etc, structural proteins, lipids, and blood cells. The cellular and sub-cellular alterations are recognized by means of the refractive indices of the cellular components. 330 to 850 nm wavelength of light is discharged by cellular and sub-cellular organelles, which is in range near ultraviolet and visible part of the spectrum.

**Optical coherence tomography (OCT)**

Optical coherence tomography (OCT) was initially described by Fujimoto et al. in 1991. OCT is an optical imaging technnology that utilizes light to record micrometer-resolution, three-dimensional (3D) images from or inside optical scattering medium like tissue specimens. It is relying on low-coherence interferometry, engaging near-infrared light. It issues cross-sectional, high-resolution subsurface images. This procedure identifies regions of inflammation, dysplasia, and malignancy by documenting subsurface reflections to make a cross-sectional architectural reflection of the tissue. Comparative improvement of the images can be done with the application of surface plasmon resonant gold nanoparticles. In an atypical oral epithelium containing dysplastic cells, the cellular atypia and architectural derangement become more haphazardly scattered, when compared with normal oral epithelium. Standard scattering intensity in a dysplastic oral epithelium is commonly greater than a normal oral epithelium. Imaging array for the oral epithelium is with a tissue infiltration depth of 1 mm to 2 mm

Advantage

* Cross sectional depiction of normal or abnormal tissues can be acqiured without biopsy and no need for preparation of the tissue or specimen is required.
* Optical technique utilizes the light for viewing the tissues and there is no exposure to harmful ionizing radiation.

**Fluorodeoxyglucose-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 surgical and radiation-induced changes from residual or recurrent neoplasias since cancer cells sustain greater FDG for longer period of time as contrasted to infectious and inflammatory tissue

**Raman spectroscopy**

Raman spectroscopy is a corresponding technique to Fourier-transform infrared spectroscopy. It is a scattering method, according to the incident radiation combine with the vibrating polarization of the molecule and thus produces a vibration. Vibrations of unsymmetric polar bonds thus prefer to be strong in infrared spectra, whereas Raman is meticulously appropriate as a probe of symmetric, nonpolar groups.

***Bakker Schut et al****, 2000-* studied raman spectroscopy method in normal and dysplastic epithelium in a rat model. ***Malini et al in 2006***discriminated normal, cancerous, precancerous, and inflammatory lesions and concluded that lipid rich character in normal conditions and major protein character in tumors and other pathological conditions. ***Shyam Sunder et al****, 2011* said thatoral malignancy of various pathological grades can also be discriminated on the basis of the comparative intensities of bands allied with lipids and proteins.

**Multiphoton excited fluorescence [MEF]**

Multiphoton Microscopy (MPM) has come out as a influential tool to discover the structure and function of biological tissues and cells. This is mostly because MPM procedure can be non-incursively obtain optical sections (virtual biopsies) in unlabeled tissues, holding details that are extremely related for diagnostic point. This non-linear technology rely on the theory of quantum transition all the way through photons put forward by Nobel Laureate Maria Göppert-Mayer. Through the non-linear procedure that take place, the sample engross two or three infrared photons and discharge a exclusive photon of smaller wavelength. This can happen via diverse physical processes, that may take place quasi-simultaneously, like fluorescence or harmonic generation.

Principle : the energy (light source) liberated by fluorophores through MEF permit the apparition of various biological components, such as collagen, eulanin, elastin fibres, keratin filament, melanin pigment, (NAD+/NADH) or flavin adenine dinucleotide (FAD). discerning probing of these autofluorescent tissue mechanism by MEF proceeded by arithmetic operations for discrete signals allow the non-invasive evaluation of important information such as cellular morphology, abnormality in cell size, cell nuclei, blood vessel. hyperplasia or inflammatory reaction related abnormalities

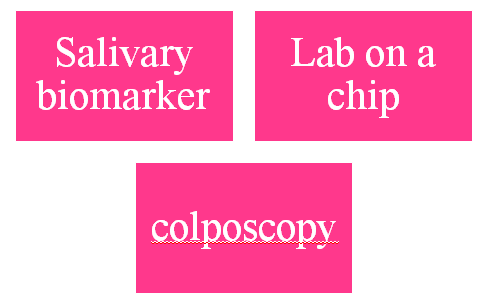
3D images of endogenous tissue fluorescence can efficiently differentiate among normal, precancerous, and cancerous epithelial tissues with elevated resolution ability

**Nuclear magnetic resonance spectroscopy**

An NMR spectrometer instrument can aid investigator to classify metabolites, intermediates and the products of metabolic processes in a biological organization established on the magnetic properties of their nuclei. Biomarkers of metabolism can be the metabolites identified in urine or blood cells. Nuclear magnetic resonance gives characteristic details about the structure, dynamics, reaction state, and chemical environment of molecules. . 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 examine 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**



**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.

Concentration of particular salivary macromolecules and assessment of proteomic or genomic target such as enzymes, cytokines, growth factors, metalloproteinase, endothelin, telomerase, cytokeratins, mRNAs, and DNA transcripts can be completed by the saliva. carcino-embryonic antigen (CEA), SCC, CA125, and CA19-9 are the mainly considered epithelial serum circulatory tumor markers in the saliva of carcinoma patients.

**LAB ON A CHIP**

Microfuidics technology , micro-total-analysis systems are the alternate 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 viable 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 assorted with the particular reactants.

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

Advantages

1. Requires little sample size with limited invasive procedure

2. processing time for procedure is less

3. Decreased reagent usage, reproducibility, consistency

4. limited exposure to hazardous solution, reagent or infectious agents

5. decreased risk of sample contamination by microorganism

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

**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. Illumination is given by a halogen lamp via a fibreoptic cable joining to a system of lenses.

The colposcope is fixed with a green or blue filter to aid in evaluation of vascular changes and color tone as unfiltered white or yellow light decreases 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 variant of capillary networks can be visualized 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 show a more constant 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

**REFERENCES**

* Park PK. Textbook of Preventive and Social Medicine. 18th ed. Jabalpur: M/S Banarsidas Bhanot; 2005. p. 302-5.
* Sharma G. Diagnostic aids in detection of oral cancer: An update. World Journal of Stomatology 2015;4(3):115.
* Saeed S, Hasan S, Kuldeep K, Singh Parmar S. Conventional and Recent Diagnostic Aids in Oral Candidal Infections: A Brief Overview. Biomedical and Pharmacology Journal 2017;10(1):419-426.
* Shamimul H, elongovan S. Conventional and advanced diagnostic aids in oral cancer screening – the journey so far. International Journal of Pharmacy and Pharmaceutical Sciences 2015;7(1)
* Nigam P. Advanced Diagnostic Aids in Early Detection of Oral Cancer. Indian Journal of Dental Advancements 2014;06(03).
* Masthan K, Babu N, Dash K, Elumalai M. Advanced Diagnostic Aids in Oral Cancer. Asian Pacific Journal of Cancer Prevention 2012;13(8):3573-3576.
* Nambiar K, Haragannavar V, Augustine D, Sowmya S, Rao R. Diagnostic aids in detection of oral precancer and cancer: Past to present. International Dental & Medical Journal of Advanced Research - VOLUME 2015. 2016;2(1):1-7.
* Yadav Karthik D*.* “Diagnostic Aids for Oral Cancer: Detect Early to Treat Early’”. Acta Scientific Cancer Biology 3.4 (2019): 18-22.
* Chaudhary R, Shah A, Shah DM, Singh S, Thakkar P, Goyal S. Advanced Diagnostic Aids in Detection of Oral Cancer. Int J Dent Med Res 2014;1(3):139-143.
* Singh S, Ibrahim O, Byrne H, Mikkonen J, Koistinen A, Kullaa A et al. Recent advances in optical diagnosis of oral cancers: Review and future perspectives. Head & Neck. 2015;38(S1):E2403-E2411.
* Ehtisham M, Wani F, Wani I, Kaur P, Nissar S. Fundamentals of In situ Hybridization: A Review. Int Res J Cli Med 2016;1(4):23-29.
* Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Critical Reviews in Biotechnology 2016;37(2):163-176
* Suvarna S, Layton C, Bancroft J. Bancroft's theory and practice of histological techniques.
* C.F.A Culling. Handbook of Histopathological and Histochemical Techniques: Including Museum techniques). 3rd ed: Butterworth & Co; 1983.
* Bernard PS, Wittwer CT. Real-time PCR technology for cancer diagnostics. Clin Chem 2002;48:1178-85.
* Poonia M, Ramalingam K, Goyal S, Sidhu SK. Nanotechnology in oral cancer: A comprehensive review. J Oral Maxillofac Pathol 2017;21:407‑14.
* Kah JC, Kho KW, Lee CG, James C, Sheppard R, Shen ZX, *et al. Early* diagnosis of oral cancer based on the surface plasmon resonance of gold nanoparticles. Int J Nanomedicine 2007;2:785‑98.
* Carlson R, Lewis SW, Lim KF. Seeing the light: using chemiluminescence to demonstrate chemical fundamentals. Aust J Chem Ed 2000;14:51-3.
* Martínez-Ojeda RM, Pérez-Cárceles MD, Ardelean LC, Stanciu SG and Bueno JM Multiphoton Microscopy of Oral Tissues: Review. Front. Phys. 2020; 8:128.
* Divya P, Anil KN, Sreedevi R*.* Lab-on-a-Chip – Oral Cancer Diagnosis at Your Door Step.Journal of International Oral Health2015; 7(11):122-128
* Pallagatti S, Sheikh S, Puri N, Gupta D, Singh B. Colposcopy: A new ray in the diagnosis of oral lesions. Indian J Dent Res 2011;22:810-5.