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Diagnostic Aids in Screening of Oral Cancer – An Update.

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Abstract

The diagnosis and treatment of oral premalignant lesions and squamous cell carcinoma are currently based on histopathologic features, site of involvement and stage of disease. Recent advances in techniques for detecting lesions and predicting their progression or recurrence are reviewed here. Adjuncts for detection of lesions and selection of biopsy sites include vital tissue staining (with toluidine blue) and exfoliative cytology. Advances in diagnosis and staging at the molecular level are expected to affect choice of treatment and patient outcomes. Oral health care providers should be aware of these advances in the evaluation and diagnosis of oral premalignant lesions and squamous cell carcinoma.
Keywords: Oral cancer, Screening, Premalignancy, Diagnosis.

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INTRODUCTION

Oral cancer occurs as a multistep process, progressing from a precancerous stage to the stage of cancer. This offers the advantage of diagnosing it in an early stage before it progresses into a cancer. In spite of occurring in stages, most often it is diagnosed in its advanced stages. The prognosis still remains poor with the 5-year survival rate approximately 50% for the last 50 years.

There are numerous diagnostic adjuncts available for its early diagnosis. Cytological methods, tissue staining techniques, and molecular methods have been used and tried. Supravital staining has long been used as an adjunct in the early diagnosis of malignant lesions. In 1928, Schiller reported the use of Lugol's iodine solution in carcinoma of the uterine cervix. *In vivo* staining has been extensively used in gynecological practice for the detection of malignant change of the cervix during colposcopy. The technique has been applied in the oral setting for over 30 years by means of the dye toluidine blue (TB). Apart from TB, other stains such as methylene blue (MB), Lugol's iodine, and acetic acid have also been tried in the diagnosis of cancerous lesions.

Early detection and prompt treatment offer the best chance for cure. Early diagnosis of oral cancer can speed proceeding to treatment and can improve the prognosis. This requires patients to seek an oral and dental examination at an early stage. Studies have demonstrated that the survival and cure rate dramatically increase when oral cancer is detected in its precancerous stage or at an early asymptomatic stage [1].

This section provides a brief insight into the available diagnostic aids in detection of oral cancer.

Conventional oral examination (COE) is the standard method of revealing Premalignant lesions and Oral malignancy confirming the clinical suspicion by biopsy and histopathological examination.

Clinical diagnosis of Oral cancer [2]

Since there may be widespread dysplastic mucosa ("field change") or a second primary neoplasm, the whole oral mucosa should be examined often, along with examination of the rest of the upper aerodigestive tract. The cervical lymph nodes must always be carefully examined by palpation.

The most frequent complaint is sore or irritation in the mouth. Early carcinomas of the oral cavity may be painless associated with only a mild irritation. Pain usually occurs when it becomes ulcerated. Rarely patient seeks consultation because of lump in the neck that represents metastasis from oral lesion.

Classic features of oral malignancy include ulceration, nodularity, induration and fixation and cancer must be suspected especially when there is a

single oral lesion persisting for more than 3 weeks (Figure-1).

OSCC may present variously as an indurated lump/ulcer i.e., firm infiltration beneath the mucosa:

- Granular ulcer with fissuring or raised exophytic margins
- White or mixed white and red lesion
- A non-healing extraction socket
- A lesion fixed to deeper tissues or to overlying skin or mucosa. Fixation of nodes to adjacent tissue due to invasion of cells through the capsule is a late occurrence and evidence of aggressive disease.
- Other features include cervical lymph node enlargement, especially if there is hardness in a lymph node or fixation. Enlarged nodes in a patient with oral carcinoma may be caused by infection, reactive hyperplasia secondary to the tumor, or metastatic disease.
- Dysphagia, odynophagia, otalgia, limited movement, oral bleeding, neck masses and weight loss may occur with advanced disease.

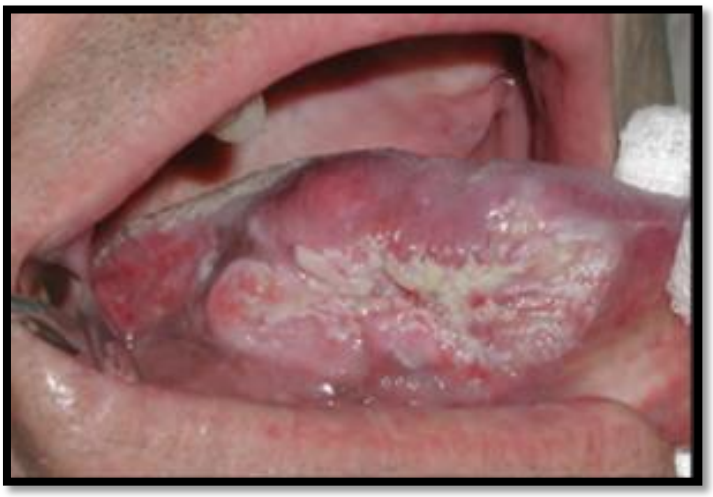

Fig-1: An ulceration on the lateral surface of the tongue with suspected malignant features.

Oral carcinomas even if clinically visible can resemble oral Premalignant lesions and some common benign oral lesions. Thus, the reliable differentiation of malignant lesions from benign lesions by clinical inspection alone is unreliable. Malignant transformation of potentially malignant lesions cannot be accurately predicted based solely upon clinical characteristics.

The only method currently available to reliably determine the diagnosis and give an indication of prognosis is the laboratory histopathological examination of a tissue sample. Histopathology for many years has been the *gold standard* in the diagnosis of OSCC. When histopathological examination shows there is dysplasia extending through the full thickness of the epithelium, the diagnosis is *carcinoma in situ*. When basement membrane is violated, *carcinoma* is diagnosed.

Progression of a Premalignant (potentially malignant) lesion to OSCC is as high as 36% when moderate or severe epithelial dysplasia is present and occurs in up to 50% in lesions with severe dysplasia.

The following are the various diagnostic modalities employed for the detection of Oral cancer.

EXFOLIATIVE CYTOLOGY

It can be an effective and non-invasive means of detecting dysplasia and early carcinoma in those patients who are either asymptomatic or in those with minor symptoms who do not warrant immediate biopsy [3]. It could serve as a useful adjunct to biopsy. Use of oral cytology to test potentially precancerous epithelial lesions has lost popularity for several decades after studies from the late 1960s through early 1970s had false-negative rates high as 31%.

BRUSH CYTOLOGY (BRUSH BIOPSY; ORALCDX)

It was introduced in 1999 as an alternative to conventional exfoliative cytology for investigating persistent oral epithelial lesions.

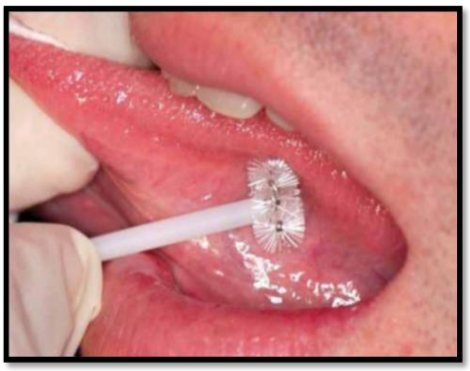

Fig-2: The Oral CDx brush biopsy

The *brush biopsy* or *Oral CDx* test has overcome the shortcoming by screwing a bristle covered wire through the thick surface keratin to the basal layer of the epithelium (Figure-2). This relatively painless procedure captures the deeper epithelial cells on the bristles and the entire brush is sent to a lab, where the cells are removed and plated on a microscopic slide. A computer-associated optical scanner compares the size of each individual cell with the size of its nucleus. Large, dark nuclei are found in dysplastic or immature cells [4, 5]. The brush biopsy can be used as an adjunct for oral dysplasia, the clinician should properly identify the most "severe" area to brush (Figure-2).

According to Joel B. Epstein *et al.*, collection of exfoliated epithelial cells by cytobrush may yield more complete sampling of the epithelium, but the data obtained will still be less than that available through biopsy, as the relationship between epithelial cells and the connective tissue cannot be assessed from exfoliated cells [3].

TOLUIDINE BLUE – IN VIVO STAINING OF DNA

Toluidine blue (TB) staining is a simple and inexpensive diagnostic tool that uses a blue dye to highlight abnormal areas of mucosa. TB is a basic *metachromatic nuclear stain* which stains nuclear material of malignant lesions and premalignant lesions [6, 7]. TB is chemically referred to as tolonium chloride. Its molecular formula is $C_{15} H_{16} N_3 S^+$ and it has a molecular weight of 270.374 g/mol. It is soluble in water (up to 3.5%) and in alcohol (up to 0.5%).

It is an acidophilic dye of the thiazine group that selectively stains acidic tissue components (carboxylates, sulfates, and phosphate radicals) such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It has the staining property of *metachromasia*, which is due to the presence of repetitive phosphate groups in the nucleic acids and is dependent on temperature and the pH. The recommended pH is 6.0-7.0.

The temperature should not exceed 30°C above which the metachromatic property diminishes in strength. TB is a cationic dye and it binds with the nucleo histones in the DNA by two ways. One method is by intercalation and other by aggregation or stacking. The dye attaches to phosphate bonds and the extent of binding depends on the amount of DNA, which is related to number and size of nuclei present in the superficial layers. Its use in *in vivo* is based on the fact that dysplastic and anaplastic cells contain quantitatively more nucleic acids than normal tissues, shows loss of cell cohesion and increased mitosis. In addition, malignant epithelium may contain wider intracellular canals; a factor that enhances penetration of the dye. It stains to the depth of 2 to 10 cell layers, and hence just reflects only the epithelial changes, the invasion into the underlying connective tissue, or the changes in the sub mucosa cannot be appreciated.

The TB solution can be prepared in the laboratory or it is also available commercially as ready to use kit, which consists of three component systems. One component is 1% TB solution and the other two are the pre-and post-rinse solutions consisting of 1% acetic acid.

Technique of Staining

TB can be used in two forms. It is either applied to the site of the lesion with a cotton applicator (Figure-3) or it is used as mouth rinse [7].

The procedure of staining is as follows

- Oral examination
- Rinsing the mouth twice with water for 20 s to remove the debris
- Application of 1% acetic acid for 20 s to remove any ropey saliva
- Application of 1% TB solution for 20 s either with cotton swab when a mucosal lesion is seen or given as a rinse when no obvious lesion is detected
- Application of 1% acetic acid to reduce the extent of mechanically retained stain
- Rinsing oral cavity with water
- Oral examination and recording of the stained areas.

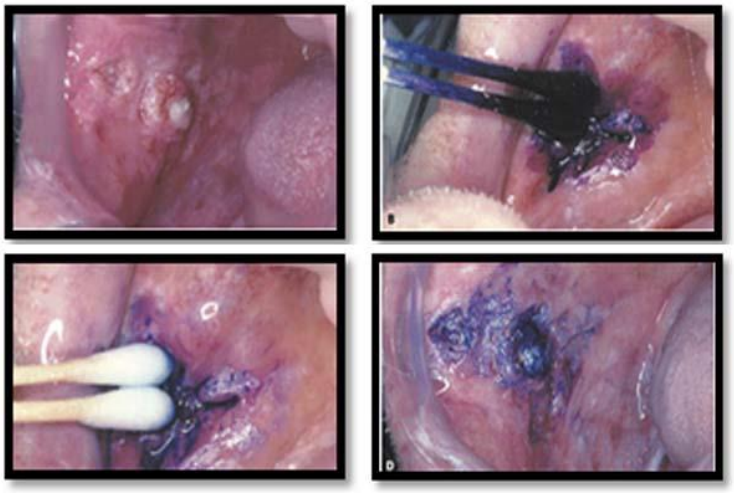

Fig-3:

Interpretation

A dark blue (royal or navy) stain of either the entire lesion or a portion of it is considered as positive stain, lack of color absorption by the lesion as negative stain, and light or pale blue staining as doubtful. These cases are usually due to mechanical surface retention or inadequate removal of the stain.

Mashberg suggests some areas not to be considered positive if it retains stain. These areas include the nucleated scales covering the papillae on the dorsum of the tongue, pores of seromucinous glands in the hard palate, dental plaques, gingival margins around each tooth, diffuse stain of soft palate transferred from the retained stain on dorsum of tongue, and ulceration lesions. Confusion prevails over the interpretation of pale colored staining. Some recommend rewiping of the lesion with cotton swab dipped in 1% acetic acid. If the staining disappears it indicates a negative result and if it persists it indicates biopsy.

Advantages

- TB staining is a simple, quick, non-invasive, and highly cost effective procedure.
- It is used as an adjunctive aid in the detection of premalignant and malignant lesions, in selecting biopsy site
- In the screening of second primaries of the oral cavity
- For the detection of multicentric tumors
- In obtaining the marginal control of carcinoma
- During the follow-up of treated lesions.

Epstein JB *et al.,* evaluated the topical application of toluidine blue and revealed that it assists in identifying sites of malignant change and possible high-grade dysplasia and concluded that toluidine blue has high sensitivity, no false-negative results and good positive predictive values [8].

LUGOLS IODINE

Lugol's iodine, also known as Lugol's solution, first made in 1829 is a solution of elemental iodine. Naturally occurring iodine is a single isotope with 74 neutrons and potassium iodide in water, named after the French physician J. G. A. Lugol. Lugol's iodine solution is often used as an antiseptic, for emergency disinfection of drinking water, and as a reagent for starch detection in routine laboratory and medical tests.

Earlier, Lugol's iodine has been used for studying cervical and esophageal epithelium. During colposcopic examination of uterine cervix, Lugol's iodine is applied to identify dysplastic epithelium and this test is called as Schiller's test [9].

Lugol's solution consists of iodine and potassium iodide. It has been used in varying concentrations: 1, 1.25, 1.5, 2, 3, and 10%. Staining with 3% Lugol's solution, followed by 5% has been found to be more effective.

The basic principle with iodine staining is its affinity for carbohydrates and starch in the tissues. As the malignancy is associated with reduction in the glycogen content of the tissues, the malignant tissue remains unstained and on the contrary the normal epithelium gets stained brown or black [10]. This selective staining delineates the inflammatory and carcinomatous epithelium from the normal epithelium.

Iodine infiltrates and reacts with the glycogen mainly in the upper superficial layer of the nonkeratinized epithelium. Iodine solution can penetrate normal epithelium to a maximum depth, but iodine-stained areas are completely consistent with glycogen distribution only in the upper superficial layer. Glycogen content is inversely related to the degree of keratosis, suggesting a role of glycogen in keratinization. Throughout the oral mucosa, the content of glycogen varies with the type of keratinization of the

mucosa. This may limit the use of Lugol's iodine in keratinized lesions and in such case its uptake should be assessed carefully. This technique also cannot be used for the detection of subepithelial infiltrating tumors.

Procedure

- Photograph of untreated lesion
- Application of 1% acetic acid for 20 seconds
- Rinse with water
- Apply 1% toluidine blue with cotton tip applicator for 10-20 sec
- Decolorize with 1% acetic acid solution for 20-30 sec. Take a photograph.
- Apply Lugols iodine for 10-20 sec
- Take a photograph.

Epstein JB, Scully *et al.,* evaluated the use of toluidine blue and Lugol's iodine to assess the value of using both stains in assessment of oral lesions and concluded that either of the stains were positive and sensitivity is greatest but that specificity was reduced [8].

Methylene blue staining (MB Staining)

Another dye that has been studied recently is MB. The technique of MB staining was originally described by Japanese investigators for improving the diagnosis of early gastric cancer. Its application has been reported recently in detecting some gastrointestinal abnormalities such as Barrett's esophagus, gastric cancer, prostate cancer, and also bladder cancer [11, 12]. However, its application in detecting oral lesions by far is very limited.

The physicochemical properties and chemical structure of MB are similar to TB except that, it is less toxic to the human body. The uptake of MB dye in epithelial cells is still not very clear. It is acidophilic in nature and may penetrate into cells with an abnormal increase in nucleic acids, thus resulting in different uptake between normal and highly dysplastic and malignant cells. MB dye system includes two bottles of solution. Bottle A, the dye rinse solution containing MB and bottle B containing pre- and post-rinse solution.

The application of MB involves:

- Rinsing with bottle B for 20 s to remove food debris and excess saliva
- Gently draining the target area with gauze and power air spray to ensure that the lesion is not contaminated with saliva
- Rinsing with 1% MB dye; bottle A for 20 s
- Rinsing again with bottle B for 20 s to wash out the excess dye.

The pattern of dye retention is assessed by the intensity of stain retention on the lesion. Local, stippled, patchy, and deep blue stains are marked as positive reaction. Wide, shallow, or faint blue stains are marked as negative reaction. If the blue stain is washed out, negative reaction is recorded.

APPLICATIONS

- MB is indicated for early detection of oral cancer and precancerous lesions.
- It has recently been used for intraoperative detection of canal isthmuses in molars during endoscopic periradicular surgery.
- To identify the areas of incomplete excision during peripheral osteotomy of aggressive lesions like odontogenic keratocyst (OKC) and ameloblastoma.
- This technique has been claimed to ensure complete removal of the lesion and hence decrease in the recurrence.

ACETOWHITE STAINING

Acetic acid staining has been used as a part of colposcopic examination since 1938. It is also used as a component in other staining techniques such as TB and chemiluminescence for cancer screening, where it is used in the concentration of 1% acetic acid both pre- and post-applications of TB stain or the light stick. With these techniques, it functions to remove the ropey saliva and to reduce the extent of mechanically retained stain. Since it is relatively inexpensive and easy to use, interest has emerged in using acetic acid alone in the assessment of premalignant and malignant lesions.

PROCEDURE

Acetic acid is used in the concentration of 3-5%. A piece of gauze soaked with 5% of acetic acid is applied on to a cleaned and dried lesion for 60 s. A positive finding is designated as a lesion that changes color to opaque white, while a negative finding is a lesion that shows no change or changes to transparent white. It acts by causing dehydration of the cells, thereby producing a white appearance. The acetic acid removes the mucus by coagulating it and thus allows the visualization of abnormal areas [13].

It also cause swelling of the epithelium and reduces its transparency by producing a transient coagulation of nuclear proteins. Thus, the higher nuclear content in premalignant and malignant lesions reacts with the acetic acid producing a aceto white appearance. Study by Bhalang *et al.,* recorded very high sensitivity of 83.35%, specificity of 84.21%, and accuracy of 83.64% in detecting oropharyngeal squamous cell carcinomas. The results also correlated with the expression of p53 in the cellular level [14].

Diagnostic ability of acetic acid staining in oral HPV infections was studied by Kellokosi *et al.,* They recorded a specificity of 50%. The staining was significantly associated with smoking and age, but was not related to alcohol consumption, histological and cytological findings. The ageing was related to the degenerative changes which reduced the reactivity of epithelium with acetic acid.

COLPOSCOPY

Colposcopy is the gold standard tool in gynaecology for diagnosis of cervical abnormalities. It provides an enlarged view of the area, to visually distinguish the abnormal appearing tissues from the normal to get the best representing biopsy sites [15].

Colposcopic examination takes very less time and requires no anaesthesia. It is safe and painless procedure. The normal squamous epithelium of oral mucosa is pink and smooth which defines fine, regular vessels. This normal vascularity increases in various inflammatory, benign and malignant lesions and conditions.

The surface pattern, clarity of demarcation, color tone and opacity can be more easily identified by direct oral microscopy than by routine clinical examination. This will aid in early detection of premalignant lesions and conditions. Colposcopic directed biopsies avoid false negative results,

OPTICAL SYSTEMS

Interaction of light with tissues may highlight changes in tissue structure and metabolism. Optical spectroscopy systems to detect changes rely on the fact that the optical spectrum derived from a tissue will contain information about the histological and biochemical characteristics of that tissue. Such optical adjuncts may assist in identification of mucosal lesions, premalignant lesions and oral cancer and assist in biopsy site selection and enhance visibility of surface texture and margins of lesions and may also assist in identification of cellular and molecular abnormalities not visible to the naked eye on routine examination.

THE VIZILITE – HIGHLIGHTING THE KERATIN

Early detection of mucosal lesions can be enhanced by the use of a dilute acetic acid rinse and observation under a chemi- luminescent light [1].

Acetic acid produces swelling of both squamous and columnar epithelium and reduces its transparency by producing a transient coagulation of nuclear proteins. The areas that stain white after acetic acid wash are called aceto white lesions**.** Areas of the tissue which turn white after the application of acetic acid often considered for biopsy (Figure 4a and b).

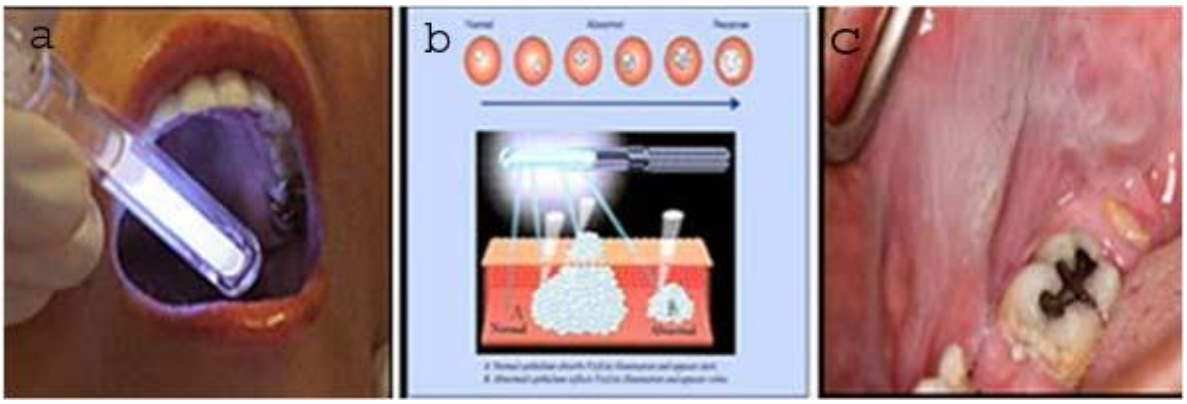

Fig-4a: Application of Vizilite; b: Principle of action of Vizilite, c:Detection of keratinized area of premalignant lesion using Vizilite

Early detection of mucosal lesions can be enhanced by the use of fluorescence. All tissues have a tendency to glow (fluoresce) in the dark, either spontaneously (auto-fluorescence) or if an external sensitizer is applied to the tissues.

PHOTOSENSITIZERS

Topical or systemic application of chemical agents called photosensitizers can render pathologic tissues fluorescent when exposed to specific wavelengths of light.

ORAL AUTOFLUORESCENCE – WHEN THE MUCOSA DOESN'T GLOW

Endogenous Fluorescence

Another approach to fluorescence-based oral diagnosis uses endogenous emissions, or autofluorescence. The use of excitation and emission peaks at one specific set of wavelengths targeting specific fluorophores within the tissues. The wavelengths which excite the greatest fluorescence in oral mucosa range from 400 to 460 nm, i.e. violet and blue light [16].

The VEL Scope (R) uses a blue light with peak intensity at approximately 436 nm; this wavelength especially stimulates a green fluorescence (Figure 5a and b).

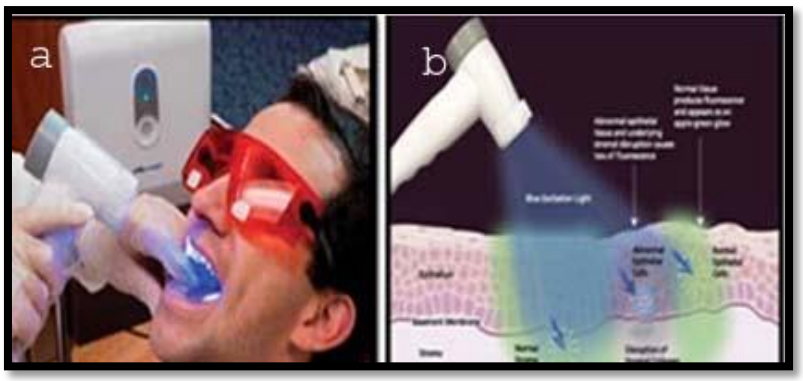

Fig-5a: Application of VELScope; b: Principle of action of VELScope

The Identafi (R) 3000 Ultra shines a violet light of approximately 405 nm, which especially stimulates a blue/violet fluorescence. This device also provides two other types of light: a white light suitable for a conventional visual examination, and a green-amber light that highlights keratinized mucosa and submucosal blood vessels [16].

Scheer *et al*., studied autofluorescence imaging with VEL scope and suggested that the method can assist in the identification of malignant and potentially malignant oral lesions from normal mucosa in high risk patient [17].

PHOTOACOUSTIC IMAGING

It relies on the measurement of light-induced acoustic emission. When a laser pulse passes through a tissue, some of the energy is absorbed and generates a sound wave. The image contrast is provided by native light absorbing chromophores but it can also be used to image microvascular networks that may be important in early malignancy.

SPECTROSCOPY [1, 10]

LIGHT-INDUCED FLUORESCENCE SPECTROSCOPY

An alternative approach is to stimulate synthesis of photosensitizing agents in situ with a photo inactive precursor. The photosensitizer, protoporphyrin IX (PpIX) is an immediate precursor of heme in the biosynthetic pathway, for heme is determined by the rate of synthesis of 5-aminolevulinic acid ALA. The presence of exogenous ALA inducing the intracellular accumulation of photosensitizing concentrations of PpIX. A selective accumulation of PpIX occurs in areas of increased metabolism such as tumor cells. Interrogation with blue light results in a fluorescence signal which is then captured using a CCD camera which allows specific measurement of red and green fluorescence.

ELASTIC SCATTERING SPECTROSCOPY

It requires light to be fired into tissue and the resulting signal is detected by fibers and fed into a spectrometer interfaced with a computer. When light enters the tissue it may be elastically scattered, or absorbed. The amount the light scatters depends on nuclear size, shape and orientation. In addition, light will be scattered by intracellular organelles and there will also be other changes depending on tissue thickness. Elastic scattering spectroscopy recordings from normal and OSCC tissue helps in differentiating cancer and dysplasia from benign lesion.

TRIMODAL SPECTROSCOPY

Trimodal Spectroscopy Uses three independent optical diagnostic techniques- fluorescent spectroscopy, diffuse scattering spectroscopy and elastic scattering spectroscopy to achieve better results, sensitivity and specificity of 96% in differentiating between normal oral mucosa and dysplasia, and oral cancer.

ORTHOGONAL POLARIZATION SPECTRAL

Orthogonal Polarization Spectral (OPS) imaging for in vivo visualization of the human microcirculation facilitates high resolution images of the oral mucosa. Oral squamous cell carcinomas are characterized by chaotic and dilated vessels accompanied by numerous areas of hemorrhage and this may be detectable by OPS.

MULTIPHOTON EXCITED FLUORESCENCE

MPF is a nonlinear, high-resolution optical method used in a variety of biological imaging applications. The image forming signal in MPF arises from the simultaneous interaction of two or more photons with the sample. Two-photon interactions in MPF result in second harmonic generation and two-photon excited fluorescence(TPF).TPF can be observed, depending on energy dissipation pathways of chromophores present in tissue, non invasive diagnostic modality for oral premalignancy and malignancy.

OPTICAL COHERENCE TOMOGRAPHY

OCT is a new high-resolution optical technique that enables minimally invasive imaging of near-surface abnormalities in complex tissues. Both ultrasound and OCT provide real-time structural imaging, but unlike ultrasound, OCT is based on low-coherence interferometry, using broadband light to

provide cross-sectional, high-resolution subsurface tissue images.

Optical Coherence Tomography

The imaging was carried out along the long axis at the centre of each lesion using either a fiber optic high-resolution 3D OCT probe with a scan length of up to 10 mm. Contra lateral healthy tissues were scanned in a similar fashion. The acquisition required approximately 5-180 seconds per 3D scanning and 15 seconds for 2D scanning, totally less than 15 minutes for each patient (Figure-6).

Fig-6: Optical Coherence tomography

The image is produced by analyzing interference of the recombined light waves. Cross-sectional images of tissues are constructed in real time, at near histologic resolution. It has the potential to give great resolution in a non-invasive way, yields information about the early changes associated with invasive cancer [4, 18]

CONFOCAL REFLECTANCE MICROSCOPY

Confocal imaging with reflected light allows for detailed images of cell morphology and tissue architecture using back scattering by various tissue components to provide contrast with high-resolution confocal images of tissue *in vivo* in near real time resembling histological tissue evaluation, except that three-dimensional subcellular resolution is achieved non-invasively (Figure-7) [18].

Fig-7: Confocal reflectance microscopy

It provides the potential to image oral epithelial tissues with subcellular resolution in a clinical setting. It play a significant role in the clinical evaluation of oral lesions, real time identification of tumor margins, and monitoring of response to therapeutic treatment. This type of imaging precisely identify cancerous cells and could aid in determining the margins of lesions during surgery.

NANO TECHNOLOGY

Nano technology is being explored to improve the above mentioned imaging techniques. Nano particles may be injected into tissue, applied topically onto tissue, injected intravenously into blood stream. Electrons on the surface of nanoparticles interact with photons to produce unique effects. *Gold and silver nano particles* can be designed as spheres, rods, cubes, each scatter and absorb light at different wavelengths. Micro injection of Gold nanoparticles deliver this particles under the surface of the oral tissue. The contrast of OCT images taken after injection was significant.

When conjugated to antibodies that bind specifically to proteins present on the surface of cancer cells but not on normal cells, thousands or millions of nano particles will attach to a single cancerous cell, and this high density causes the cell membrane to appear

many times brighter than normal cells when viewed through confocal microscopy.

CONCLUSION

Oral cancer is nowadays an increasing extended disaster around the world. Early diagnosis of oral cancer and its pre-malignant lesions, and especially preclinical screening for high risk population may have a major impact on survival and quality of life.

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