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ADVANCED DIAGNOSTIC MODALITIES IN DETECTION OF ORAL POTENTIALLY MALIGNANT DISORDERS

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Abstract

The screening of patients for signs of oral cancer and potentially malignant disorders has always relied upon the conventional oral examination. Several commercially available conventional diagnostic aids and adjunctive advanced techniques potentially assist in the screening of occult cancerous change or to assess the biologic potential of clinically abnormal mucosal lesions. This manuscript systematically and critically reviews the literature associated with recent technological advances in oral cancer and potentially malignant disorder screening for a more reliable and accurate diagnosis.

Key words – Chemiluminescence, Pre-malignant, Oral cancer, Velscope

Introduction

Oral cancer and potentially malignant disorders are the most common conditions affecting oral cavity and are most prevalent in Indian Scenario in past few decades. Increasing cases are contributed due to lack of awareness among individuals and lack of proper early diagnostic facilities in the majority of set ups. One approach to this problem would be to improve the ability of oral health care professionals to detect relevant potentially malignant lesions or cancerous lesions at their earliest or most incipient stage and by generating awareness among public regarding adverse consequences of delaying the screening of such lesions and their timely management to improve overall quality of life of patients. Although conventional and radiographic diagnostic aids have proved efficient in screening such disorders but for more precise diagnosis advancements have come about both through a greater degree of research activity in this field, with these recent technical innovations, the art of diagnosis has become much more of a science and the attitude of clinician has changed from clinic centric to technocentric. Advances in diagnostic oral medicine are aimed at reducing the morbidity and mortality associated with oral diseases.

Light based techniques

Chemi-luminescence light:

The chemi-luminescence technique **(Vizilite, Zila pharmaceuticals, Phoenix, Arizona)** is a technique that was approved in **2002** in the USA. It serves the purpose of improving the identification, visualization, and monitoring of potentially malignant disorders.(1)

Chemi-luminescence involves emission of light from a chemical reaction between hydrogen peroxide and acetylsalicylic acid inside a capsule light stick. This reaction emits blue/ white light (430-580 nm) whose principle is based on the reflective properties of tissues that present cellular alterations such as a higher nuclear/cytoplasmic rate. The aceto-white lesion is more defined, whereas the normal tissue is dark. Chemi-luminescence was first applied for the detection of dysplasia in the cervix. The test has been adapted and proposed for oral mucosal examination based on the hypothesis that oral mucosal tissues may exhibit features like the cervical epithelium when subjected to chemi-luminescence. One of the components of chemiluminescent examination is acetic acid pre-rinse. It is mainly done to remove the debris and glycoprotein layer for enhanced penetration and reflection of light. But acetic acid is also known to cause cellular dehydration and protein coagulation that reduces the transparency of the epithelium. This could be one of the reasons for the aceto-white appearance of the white lesions. (1,2)

Vizilite: This technique is based on the ability of acetic acid to highlight areas of thickened keratin surface. It is stored between 150 and 300^0 Celsius using a technology which is effective in detecting soft tissue abnormalities. (3)

A Vizilite examination is essential for those more susceptible to oral cancer. It aids in early detection of asymptomatic oral cancer patients. Vizilite, in combination with a regular visual examination, provides a comprehensive oral screening procedure for the patients who are at a high risk for oral cancers. It is a painless, effective, rapid and lifesaving procedure.

The Vizilite kit comprises of 1% acetic acid solution, a capsule (which emits light), retractor and the manufacturer's instructions for patients at high risk for oral cancer.
The capsule is formed by an outer shell of flexible plastic and an inner vial of fragile
glass to activate it, the capsule is bent for breaking the glass vial, so that the
Chemical products react with them and produce a bluish-white light with a wavelength of 430-580nm that lasts for around 10 min. sensitivity and specificity are about 77.3% and 27.8% respectively. (3,4)

Fig1: Vizilite kit

Procedure:

- The patient is asked to do a 1-minute mouth wash with the acetic-acid solution for drying the oral mucosa and for removing the glycol-protein barrier.
- The intensity of the focused light is dimmed, and a diffuse bluish-white chemiluminescent light is applied.
- The normal cells that absorb the light are depicted in a bluish color.
- The abnormal cells reflect the light back with a high nucleus-cytoplasm ratio, and the epithelium with excess keratinization, hyper para-keratinization and a pre-dominant inflammatory infiltrate, appears ***aceto-white***, with more brightness and distinguished borders.

Fig 2: Chemi-luminescent illumination positivity

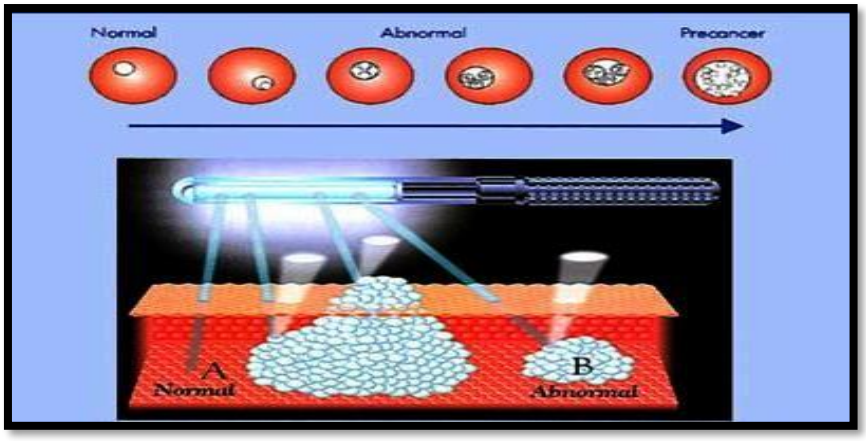


Fig 3: Normal mucosa shows reflection of light whereas the pre-malignant site shows absorption of light.

Vizilite, as has been described above, may be used with or without the Vizilite plus accessory eyewear, depending on the operatory environment. The Vizilite plus accessory eyewear consists of lenses that filter the ambient light outside the wavelength transmission range of the chemiluminescent light.

Vizilite plus with toluidine blue in oral lesion is used for identification and marking system with conventional head and neck examination to identify, evaluate, monitor and mark abnormal oral lesions suspicious for pathology including precancerous cells and cancer that may be difficult to see during a regular visual examination. Any lesion highlighted with Vizilite may show the pathological site and help in clinical diagnosis and management of potential malignant disorders. [3]

Fig 4: Vizilite plus kit

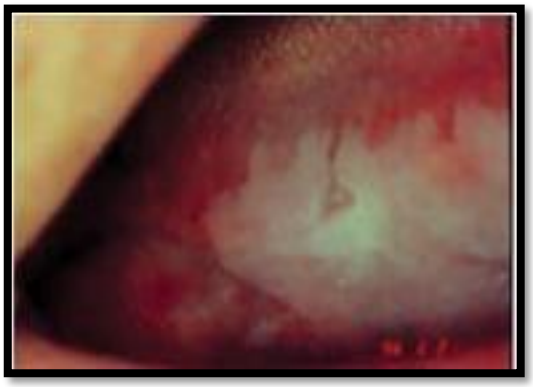


Fig 5a: Premalignant site

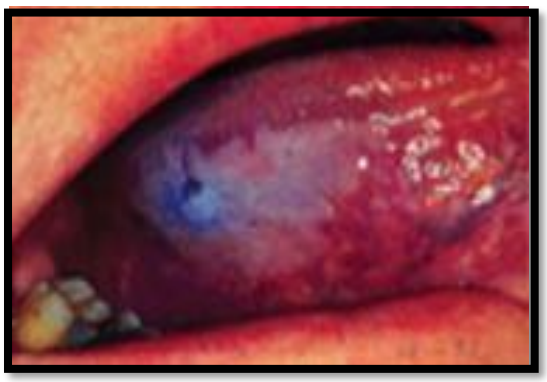


Fig 5b: Appearance under Vizilite

Some investigators have found that Vizilite enhances visual lesions to about 60%. Several studies were conducted to evaluate the efficacy of Vizilite and concluded that chemi-luminescence increases the brightness and margins of oral mucosal white lesions and thus assist in identification of mucosal lesions not detected under conventional visual examination.

Ram *et al.* (2004) concluded that Vizilite was 100% sensitive with low specificity of 12.5 % for detection of oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC) required for biopsy and histological verification. 31 lesions disclosed 14 SCC (45.2%), 10 epithelial dysplasia (32.3%), 5 lichen planus (16.1%) and 2 benign lesions (6.4%]. [(5)]

Rajmohan *et al. (2001)* assessed oral mucosa in normal, pre-cancer and cancer patients using Vizilite and found 77.8 % sensitivity for detecting dysplasia and 90 % sensitivity for detecting OSCC. [(2)]

Awan *et al. (2007)* concluded that the majority of mucosal disorders were positive (acetowhite) for chemi-luminescence (75.4%). [(6)] Vizilite was useful in enhancing the visibility and sharpness of majority of the oral leukoplakia, making the clinically evident lesions more prominent and distinct from surrounding oral mucosa. 50% of erythroplakia lesions were Vizilite positive.

The limitations associated with Vizilite includes requirement of a dark environment, high cost, no permanent record unless photographed, low specificity for dysplasia, contributing to high referral rate and over-treatment, unable to detect some red lesions.

Mucosal surface reflectance, inability to objectively measure the visualization results. This visualization adjunct gives information only about the horizontal extent of the lesion (one dimension). The depth of the lesion which is more important in predicting the malignant behavior cannot be assessed through this modality. [(7)]

Vizilite when compared with toluidine blue, showed better diagnostic values. the re ported sensitivity of Vizilite ranged from 0[8] to 84%, [9,10] and the specificity ranged from 15 [10]to 91%.

Light emission technique

The light emission technique (microlux dl) operates on a principle of light emission similar to that of chem-iluminescence light and helps to sharpen the lesion edges as well as to improve visualization. (10)

This method involves rinsing the patient's mouth with 1% acetic acid, and then a battery powered light source is used. An advantage of this system is that it is reusable and has an autoclavable light guide.

Another similar system uses LED (orascopticdk (orascoptic, akerrcompany, middle ton, wis.) with a re-chargeable battery to screen the oral mucosa and claims to improve visualization. (11)

Mcintosh l (2009) assessed the efficacy of acetic acid mouthwash and diffused light illumination (microlux /dl), as a diagnostic tool in the visualization of oral mucosal potentially malignant lesions and it showed a sensitivity of 77.8% and a specificity of 70.7%. (10)

Fig 6: microlux dl

The auto-fluorescence of tissue and its potential use in cancer detection were first described in 1924. It is a phenomenon that utilizes an extrinsic light source to excite endogenous fluorophores such as certain amino-acids, metabolic products, and structural proteins. Within the oral mucosa, the most relevant fluorophores are nicotine amide adenine dinucleotide (NADH) and flavin adenine dinucleotide (fad) in the epithelium and collagen crosslinks in the stroma. The fluorophores absorb photons from the exogenous light source and emit lower energy photons which are present clinically as fluorescence. Each fluorophore is associated with specific excitation and emission wavelengths. When irradiated with wavelengths between 375 and 440 nm, the fluorochromes show fluorescence in the green spectral range and normal, unaltered mucosa emits apple-green auto-fluorescence when viewed through a selective, narrow band filter. A proper filtration is crucial, due to the intense light used for excitation of the fluorochromes. Without proper filtration, it would be impossible to visualize the pale and narrow auto-fluorescence signal. However, dysplastic tissues lose Fluorescence emission power due to a disruption in the distribution of the fluorochromes and appear darker in color in comparison to the surrounding healthy tissue.

A number of methods based on the principle of soft tissue fluorescence have been described for use in the oral cavity, including exogenous fluorescence, auto-fluorescent spectroscopy and auto-fluorescent imaging. Both exogenous fluorescence and auto fluorescent spectroscopy due to practical purposes are unlikely to be applied as screening aids. In exogenous fluorescence, there is a delay before the fluorophore reaches an adequate concentration and the fluorophore also causes temporary photosensitization to the subject, which may be deemed unacceptable to the individual.

In auto fluorescence spectroscopy, small optical fibers are used to expose the oral mucosa to different wavelengths and is impossible to screen the entire oral cavity, therefore limiting its application. (11)

Velscope: (visually enhanced lesion scope)

It is an example of narrow-emission tissue fluorescence technique involving tissue exposure to different wavelengths (400 to 460 nm) in order to observe differences between normal and abnormal mucosa. (12)

Fig 7: Velscope

This system involves the auto fluorescence due to cellular fluorophore after excitation. The abnormal tissue has a different fluorophore concentration that results in changes in color. This method uses a small optic fiber and consequently does not cover the entire mouth, so it is employed only for isolated lesions, lesion edge, and determination of cancerization field.

While the normal mucosa glows and emits color (pale green), the abnormal mucosa shows decreased levels of fluorescence and acquires a dark magenta, brown, or black color, as it absorbs fluorescence.

Velscope utilizes blue light excitation between 400 and 460 nm wavelength to enhance oral mucosal abnormalities by direct tissue auto fluorescence. At these excitation wavelengths, normal oral mucosa displays pale-green fluorescence when viewed through a filter, whereas abnormal tissue is associated with a loss of auto fluorescence and appears dark. Neoplastic tissues are expected to cause fluorescent visualization loss (FVL) and thus appear as a dark area. (12)

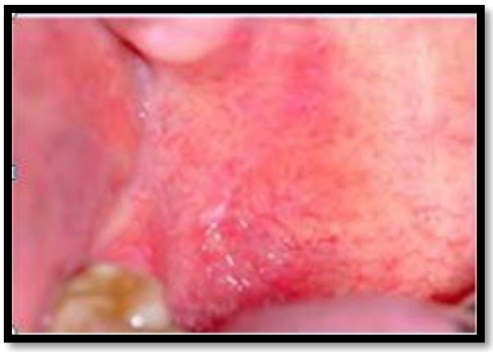


Fig 8a: Normal appearance

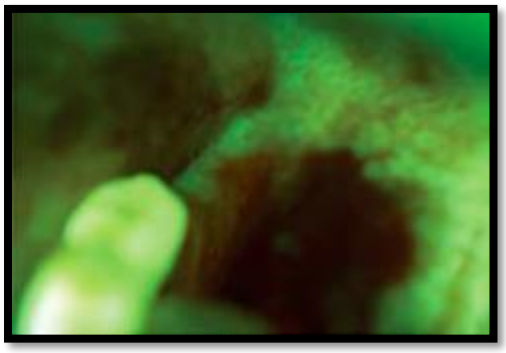


Fig 8b: dark area seen under Velscope

This technique is likely to be helpful in lesion detection, but its use in the differentiation of malignant from benign lesions is relatively less. Despite its applicability, the system is expensive, and color interpretation is difficult, which could lead to an erroneous diagnosis. Some studies in the literature referred to this technique as having sensitivity values from 97%to 98%and specificity from 94% to 100%.

Several studies have investigated the effectiveness of the Velscope system as an adjunct to visual examination for purpose of improving the distinction between normal and abnormal tissues (both benign and malignant changes) and to be able to differentiate between benign and dysplastic/malignant changes and identify dysplastic/malignant lesions that are visible to naked eye under white light. Whether it can distinguish between dysplasia and benign inflammatory lesions is questionable. Benign inflammatory conditions can result in an increased blood supply to a lesion. The increase in hemoglobin content (chromophores) may absorb light and cause FVL mimicking neoplasia.

Hanken h et al. (2013) examined 120 patients with suspicious oral lesions and postulated that Velscope has a higher sensitivity (22.0%), and a lower specificity (8.4%). Also, it is more promising than clinical visual examination in detecting potentially malignant disorders. (12)

Koch et al (2011) showed a higher sensitivity (97%) and specificity of (95.8%) of Velscope to diagnose OSCC. (13) The positive predictive value (PPV) was calculated at 41% and negative predictive value (NPV) was 75-80%.

Rana *et al (2012)*. observed that Velscope leads to higher sensitivity (100% vs.17%), but a lower specificity (74% vs. 97%) as compared to clinical visual examination. The major lack of the study was the large number of false-positive test results. (14)

Mcnamara *et al. (2012)* concluded that clinical oral examination is more valid than auto fluorescence examination with Velscope in routine screening for OPMD. (15)

Multi-spectral fluorescence and reflectance (*identafi 3000* (trimira, Houston, texas)

It is a new technique based on the tissue fluorescence principle that uses three types of lights: white, violet (405 nm), and amber (560 nm). It is cordless, handheld, and resembles a dental mirror, with a band that can be rotated to toggle among the three colors of light. It comes with tinted glasses for both the doctor and the patient.

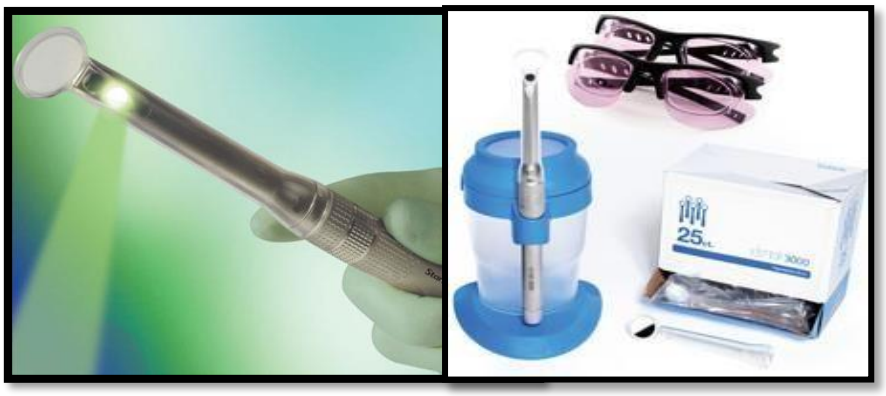


Fig 9: Identify 3000 kit.

According to the manufacturer**, white and violet lights** use the same principle as tissue reflectance and fluorescence, while **amber light** improves vascular architecture visualization in normal and abnormal tissue. Normal tissue appears defined, while abnormal tissue has a diffuse vasculature.

The identafi® uses white, violet and amber wavelengths of light to excite oral tissues indistinctly and in unique ways. As a result, biochemical changes can be monitored with fluorescence, while morphological changes can be monitored with reflectance. The combined system of fluorescence and reflectance uses the body's natural tissue properties as an adjunctive tool for oral mucosal examination. Conventional examination of tissue is performed using a highly concentrated **white** light. Wearing reusable identafi® filtered eyewear enhances visual effects and allows transmission of reflected light, thus the health professional then switches to **violet** for a second observation.

The clinician's photosensitive glasses block the violet excitation light and allow the observance of the tissue's natural fluorescence. **Violet** light enhances normal tissue's natural fluorescence; however, suspicious tissue appears dark because of its loss of fluorescence.

When suspected abnormalities are present this selector is switched to **amber** light, which enhances normal tissue's reflectance properties so the clinician may directly observe the difference between normal and abnormal tissue's vasculature. (16)

Messadi et al and **younnai et al** explained that the abnormal tissue has a diffuse vasculature, where in normal tissue vascularity is clearly defined. The combination of all three multi-spectral wavelengths provides the clinician with more visual information, improved results over direct visual exam alone and increased confidence for recommending biopsies. (17)

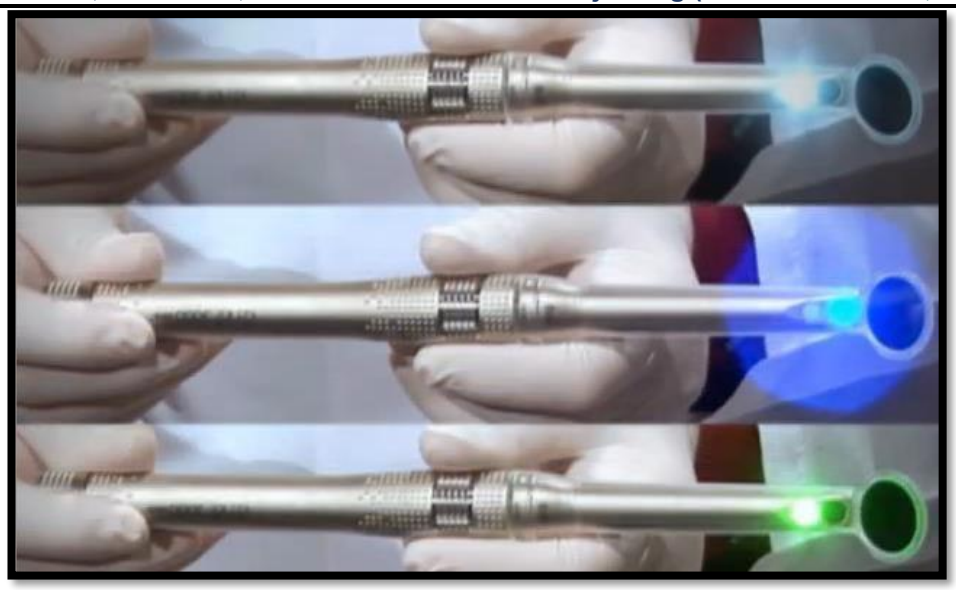
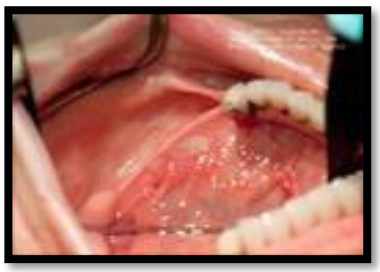
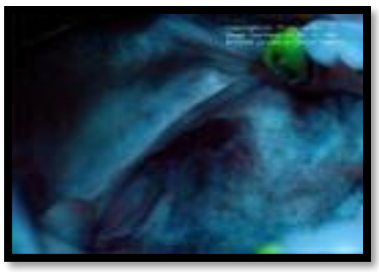


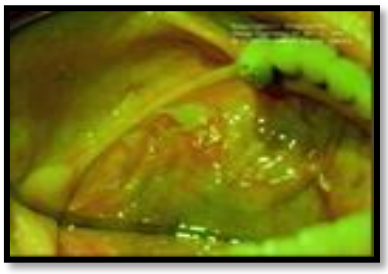
Fig 10: Light of three different wavelengths i.e., white, violet and amber



White light



violet light



amber light

Advantages of the device include detection of abnormal tissue with accuracy, assessment of depth of tissue involvement, ability to distinguish between benign and malignant lesions and providing less false positive diagnosis.

Photo-diagnosis

Light-induced fluorescence (LIF) spectroscopy is a technique with a potential to improve the diagnostic accuracy and efficacy for early cancer detection in various organ sites including the oral cavity.

The principle of LIF technique is based on the detection of the endogenous tissue auto fluorescence or the exogenous fluorescence of photosensitizers selectively accumulated in tumor tissue.

Light- induced fluorescence after ALA exposure can differentiate between the different stages of premalignancy and malignancy. Its ability to differentiate between healthy tissue and early pathology is particularly interesting.

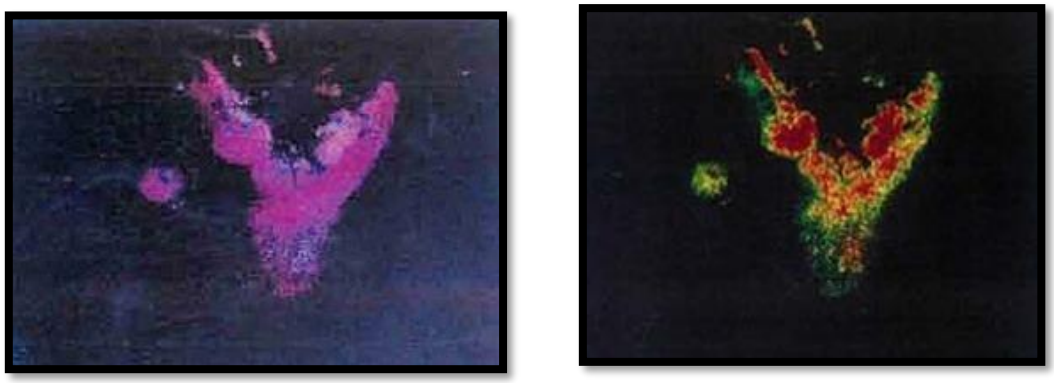
LIF has a 2-way approach in diagnosing malignancy; firstly, it involves systemic administration of a drug like hematoporphyrin derivative (HPD) which is selectively retained by the tumor. When photo excited with light of appropriate wavelength the drug localized in the tumor fluoresces. This fluorescence is used for detection and imaging of the tumor. Photo excitation also leads to populating the triplet state via intersystem crossing. The molecule in excited triplet state can directly react with biomolecules or lead to generation of singlet oxygen which is toxic to the host tissue. The resulting destruction of the host tissue is exploited for photodynamic therapy of tumor. From a diagnostic point of view, this approach has two drawbacks, a possible dark toxicity of the drug and the possibility of drug induced photosensitization.

Secondly, the other approach is of no usage of exogenous tumor markers. Instead, it exploits for diagnosis the subtle changes in the spectrum as well as the decay time of fluorescence from native tissues as it transforms from normal to malignant state. The studies carried out over the last few years have shown considerable promise of this approach for diagnosis of the cancer of various organs like uterine cervix, esophagus, lung, breast and oral cavity. [18]

5-aminolevulinicacid (5-ALA):

It is one of the most promising photosensitizers today which do not fluoresce by itself but induces protoporphyrin ix (PPIX) fluoresces. Topical or systemic administration of 5-ala causes a selective accumulation of PPIX in neoplastic tissues, resulting in a high contrast as compared to the surrounding normal tissue.

5-ALA-PPIX fluorescence endoscopic images (ALA mode) recorded showing dysplastic regions in the palate (FIG. 11)



Topical or systemic administration of 5-ALA can lead to a selective accumulation of PPIX in neoplastic tissues, thereby resulting in a high contrast as compared to the surrounding normal tissue. ALA has a further advantage of being excreted from the human body relative to other photosensitizers such as hematoporphyrin derivatives (HPD) or Photofrin.

Therefore, more recently there has been an increasing interest in applying the 5-ALA fluorescence based technique into clinical oncology. **Allison et al (2010)** have reported promising results on ALA-induced fluorescence endoscopic imaging for the detection of premalignant and malignant oral mucosa with a high sensitivity of 95–100 %. [19]

However, benign oral lesions are often misdiagnosed as positive, resulting in a lower diagnostic specificity during ALA fluorescence endoscopy. In addition, most of the available ALA-mediated fluorescence imaging systems cannot be used for quantifying the fluorescence images, hampering the ability to explore the relationship between the fluorescence intensity and the histopathology of diseased tissues.

The system mainly consists of an illumination console, a fluorescence detection unit, a video display, a recording unit and a computing system for image acquisition, display and processing. A 300w short arc xenon lamp (d-light AF system, Karlstorz, Tuttlingen, Germany) is used for both the white light illumination (WL mode) and the ALA fluorescence excitation (ALA mode) filtered by a band pass filter (380–450nm). The irradiance of the violet-blue light at the endoscope tip is approximately 50mw/cm^2 used for both illumination and observation of tissues of area of interest via modified endoscope integrated with a long pass (LP) filter (cut-off wavelength at 470nm). This observation LP filter in the eyepiece of the endoscope only transmits 8% of the diffuse back-scattering excitation light with a peak at 450 nm (blue light), while has a transmission of over 98% in the 470–800 nm range, thus the back-scattered blue light, the green tissue auto fluorescence and the red PPIX fluorescence can pass through the endoscope efficiently. The fluorescence and white light images were recorded by a 3-chipcharge-coupled device (CCD) video camera (Tricamsl-PDD, Karlstorz, Tuttlingen, Germany) connected to the modified endoscope. The white light acquisition mode (WL) can be switched to the ala fluorescence acquisition mode rapidly if required, and vice versa. The RGB video outputs of the CCD camera can be grabbed simultaneously by a frame grabber (Matrox genesis-lc), and the digitized image can be stored for image processing, and the processed image can be displayed on the computer screen in near real-time for tissue diagnosis. Meanwhile, the RGB video signals can also be displayed on a video monitor in real-time and recorded by a video recorder enabling documentation of the entire examination process. The white light examination should be performed before the topical administration of 5-ALA or after the fluorescence endoscopic imaging to avoid photo bleaching of PPIX fluorescence and ensure the fluorescence measurement reflecting the initial fluorescence intensity.

PPIX-fluorescence imaging on the computer generates sequences that represent porphyrin fluorescence alone, both for tumor and normal tissues as red fluorescence. The intensity of fluorescence is stronger in neoplastic tissue compared with normal tissue. Tumor borders are less well defined than under CFD, as transition from tumor to normal tissue usually shows only a gradual decrease in fluorescence intensities. The dorsum of tongue and gingival plaques generally show nonspecific red fluorescence after application of 5-ALA. Furthermore, chronically irritated, or inflamed tissue also often show false-positive red fluorescence. (20)

Allisson et al (2010) conducted a study on 31 patients to assess the native cellular fluorescence of neoplastic upper aero digestive mucosa and found that neoplastic mucosa of the upper aero digestive tract within an individual patient expresses native cellular fluorescence properties in vivo different from those found in normal upper aero digestive epithelium. (19)

Weizheng et al (2002) demonstrated that quantifying ALA-PPIX fluorescence endoscopic images associated with the red-to-blue intensity ratio as a diagnostic algorithm can provide good differentiation between the different stages of oral pre malignancy and malignancy. [18]

Applying the red-to-blue intensity ratio (i_r/i_b) as a diagnostic algorithm yielded a sensitivity of 92% and 98%, and specificity of 96% and 96%, for differentiating benign tissue from dysplasia, and cancer tissue, respectively, and a sensitivity and specificity of 98% and 92% respectively.

Comparing the main sites of malignancies with the various diagnostic methods are subjective to tumor localization and demarcation, thus these properties lead to that PPIX fluorescence as prime mode for diagnosis of tumors at the rim of the tongue, whereas auto fluorescence is a better mode for diagnosis of tumors of floor of the mouth and the oropharynx. [19,20]

Advantage:

It gets excreted from the human body in less than 24 hours, thereby avoiding skin photosensitization. If a problem is encountered other photosensitizers, such as HPD or Photofrin can be used. Therefore, there has been an increasing interest in applying the 5-ALA fluorescence-based technique into clinical oncology. Several groups have reported promising results on ALA induced fluorescence endoscopic imaging for the detection of potentially malignant and malignant oral lesions with a high sensitivity of 95-100 %.

Disadvantage:

- Benign oral lesions during ALA fluorescence endoscopy are often mis-identified as positive, leading to a lower diagnostic specificity.
- Most of the ALA-mediated fluorescence imaging systems are unable to quantify the fluorescence images as they are available only for visual inspection, hindering the ability to explore the relationship between the fluorescence intensity and the histopathology of diseased tissue. [19,20]

Tissue fluorescence-spectroscopy

Auto-fluorescence spectroscopy:

The common procedure for detecting potentially malignant disorders consists of visual inspection, followed by biopsy. However, benign lesions which are very common and diverse may present very similar to early malignant or potentially malignant lesions, which makes it difficult to distinguish them even for experienced clinicians. Therefore, a technique that can distinguish between different lesion types in a reliable and non-invasive way would be very useful. Such a device would be particularly useful for finding the most optimal dysplastic biopsy site, so that the risk of under diagnosis and need for repeated biopsies is avoided. Another important clinical improvement would be to detect malignant changes at an earlier stage, preferably before visual detection is possible. It has been claimed that auto fluorescence spectroscopy and imaging can assist in oral oncology for the detection and classification of lesions.

In case of malignancy there are changes in the physical and chemical characteristics of the tissues due to the subcellular architectural changes in cancer, such as nuclear grade and nuclear to cytoplasm ratio, mitochondrial size and density, amount of keratin, and elastin to collagen ratio, and it is well known that all tissues fluoresce and malignant tissues fluoresce relative to normal tissues, have different spectral characteristics. Studies of these methods in normal oral mucosa have shown increased green fluorescence in comparison to neoplastic lesions upon ultra-violet (UV) or near UV-light source.

Auto fluorescence is a method based on illumination due to absorption of tissue fluorophore molecules (NADH and FAD in the epithelial layer and collagen and elastin in the stroma) in ultraviolet visible spectrum leading to the emission of lower energy photon that can be detected as fluorescence from the oral surface mucosa.

Optical fibers may be introduced into the tissues through a hollow needle; the tissue signals are interpreted by spectrometers. The reported sensitivity in fluorescence spectroscopy technologies was up to 81% and specificity was 100%.

A short history of fluorescence spectroscopy and imaging-auto fluorescence for the detection of malignant lesions emanated from photodynamic therapy, is a technique for cancer treatment. In this therapy, the light-sensitive drug ("photosensitizer") is localized in a tumor, either through systemic or topical application or by administration of a precursor, such as PPIX. The photosensitizer produces single oxygen upon excitation with light of a certain wavelength, which damages vital cell organelles inducing death of cells in the direct environment. Since some of the sensitizers were believed to accumulate in malignant tissues, they could possibly serve for diagnostics as well. The use of exogenous fluorescence for tumor detection has been investigated for various organs. For the oral cavity, some promising results have been obtained. However, the use of exogenous fluorophores has some major drawbacks. A certain waiting time after application is necessary for the fluorophore to reach its optimal fluorescence intensity. Furthermore, the application of photosensitizers leaves the patient temporarily sensitive to light, which negatively affects his daily life. This makes the technique impractical, especially for use in regular screenings of high-risk patient groups. Finally, the specificity of the photosensitizers appeared to be less than expected. In the late 1970s, it was discovered that auto fluorescence (also called natural or endogenous fluorescence), which had until then been regarded only as a disturbing background signal in exogenous fluorescence detection, could be used for cancer detection as well. [(20)]

Development of auto fluorescence imaging techniques are capable of sampling several square centimeters at a time. Tissue is illuminated with a light source, mostly in the near- Ultraviolet to green range of the spectrum, and images of the fluorescence produced in the tissue and altered by absorption and scattering events are recorded using a camera. Imaging has the advantage of providing 2D information, which allows spotting of lesion-specific features such as in homogeneities, while the recording of large areas makes the technique potentially useful for localizing new lesions.

Studies on auto fluorescence imaging in oral oncology-

Harris and Werkhaven (1987) noticed endogenous auto fluorescence around 630 nm in 188 tumors of the oral mucosa, but also in healthy oral mucosa. This fluorescence is associated with porphyrins. The occurrence of red auto fluorescence could explain false positives when applying photosensitizers for tumor detection. They therefore thought of auto fluorescence as disturbing rather than as a contributing factor. [21]

Onizawa et al (1999) [22] applied an instant photography camera with ultra-violet flash lamp and a 480 nm long pass filter and observed an orange fluorescence in 14 of 16 malignant tumors and in only one of 16 benign lesions. This porphyrin-like fluorescence was probably produced by micro-organisms living on ulcerating or necrotic surfaces, which is consistent with the observation that the fluorescent materials could be wiped off. In a study with a larger patient population, auto fluorescence photographs were acquired with a similar set-up in 130 patients (79 malignant tumors and 51 benign lesions). They found 91.1% sensitivity and 84.3%specificity for distinguishing malignant from benign lesions. When dysplastic lesions were regarded as potentially malignant, they increased to 93.8% and 95.5%. Thus, in accordance with the results, it can be concluded that the appearance of orange auto fluorescence accompanies the transformation from benign into dysplastic lesions, rather than from dysplastic into malignant lesions.

Yang et al (2004) proposed to detect tumors by using 630 and 690 nm peaks that are associated with porphyrins, that are believed to be localized and retained in malignant tumors.

Both auto fluorescence imaging and spectroscopy give good results for the distinction of lesions from normal mucosa. In the case of imaging, this is very useful because it gives the clinician a tool to scan the oral cavity for new lesions, and possibly to assess invisible extensions of visually detected lesions. Auto fluorescence imaging can be indicated to detect lesions that are not easily noticed by visual inspection. [15]

Raman spectroscopy It is a complementary technique in the discovery of the Raman Effect in 1928. Similar to IR spectroscopy, Raman entails the coupling of incident radiation with molecular vibrations and the resultant spectrum is similar characteristic of the material. IR spectroscopy involves the absorption of radiation, whereas 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 IR spectra, whereas Raman is particularly suitable as a probe of symmetric, non-polar groups. Notably, OH vibrations of water are very strong in IR spectra, but are extremely weak in Raman spectra, rendering Raman a potentially more suitable technique for in vivo applications.

It is a laser-based technique that enables chemical characterization and structure of molecules in the sample. It helps to obtain a vibrational spectroscopic picture of the tissue content, thus providing immediate real time histology.

This technique is issued to observe vibrational, rotational, and other low-frequency modes in a system. The Raman Effect occurs when light impinges upon a molecule and interacts with the electron cloud and the bonds of that molecule.

For the spontaneous Raman Effect, which is a form of light scattering, a photon excites the molecule from the ground state to a virtual energy state. When the molecule relaxes it emits a photon and it returns to a different rotational or vibrational state. The difference in energy between the original state and this new state leads to a shift in the emitted photon's frequency away from the excitation wavelength. The reported sensitivity of this technique was 80.5% and specificity of 86.2%.

Schut et al. (2000). [23] analyzed applications of Raman spectroscopy in oral cancer diagnosis of normal and dysplastic tissues in a rat model by inducing dysplasia in the palate by topical application of the carcinogen 4-nitroquinoline1-oxide and observed sensitivity and specificity of 100%.

Venkata Krishna et al (2003) [24] recorded spectra of 49 human oral cancer biopsies and obtained an average classification efficiency of 88%.

Molecular methods

Saliva has been found to contain constituents that reflect the diseased or physiological state of the human body, and hence could be utilized for diagnostic purposes. The search for reliable salivary biomarkers for early detection of OSCC has developed rapidly, spurred on by the fact that collecting saliva is relatively easy and non-invasive, compared to the drawing of blood. From the late 1990s until the present, more than 40 research studies have been published and more than 100 different salivary constituents have been suggested as potential OSCC salivary biomarkers. [25,26]

Polymerase chain reaction(PCR) has emerged as one of the most universally used technique for the amplification of genes and the IR RNA transcripts. It is used as a screening technique for detection of malignant cells in human secretions in urine, sputum and saliva by studying the low numbers of unique DNA fragments.

Saliva as a diagnostic tool:

The whole saliva is unique and complex, both in its sources and composition. It consists not only of secretions from the three major salivary glands and the minor glands, but also gingival crevicular fluid, oral mucosa transudate, secretions from nasal and pharyngeal mucosa, non-adherent bacteria, desquamated oral epithelial cells, keratin debris, blood cells and perhaps food or medication residuals. The various chemical components of saliva include water, inorganic compounds (ions), organic compounds (non-proteins and lipids), protein/polypeptides, and hormones.

More than 2300 proteins and peptides have been found in human saliva. The most abundant proteins are α-amylase, albumin, cystatins, histatins, secretory-IgA, lactoferrin, mucins, lysozymes, proline rich proteins, statherin and transferrin–which together account for more than 98% of the total salivary proteins. Most of the potential OSCC salivary biomarkers are also salivary proteins. However, except for three, α-amylase, statherin, and transferrin, those proteins, as well as the non-protein OSCC salivary biomarker candidates, are present in a very low concentration in saliva and require methods/ instruments with high sensitivity for detection. [27]

Saliva from patients has been used in a novel way to provide molecular biomarkers for oral cancer detection. Discovery of analytes in saliva of normal and diseased subjects suggests a very promising role of saliva and its use as a diagnostic fluid meets the demands for an ***inexpensive, non-invasive and accessible diagnostic tool.*** Till date saliva has been used to detect caries risk, periodontitis, oral cancer, breast cancer, salivary gland diseases and systemic disorders such as HIV, HCV, SARS CoV-2 etc.

However, due to lack of knowledge of disease markers and an overall low concentration of these markers in saliva when compared to serum, the diagnostic value of saliva has not been fully realized.

Nowadays, highly sensitive and high-through put assays such as DNA microarray, mass spectrometry and nanoscale sensors can measure protein and RNA markers at low concentrations in saliva, thus expanding the utility of saliva as a diagnostic tool.

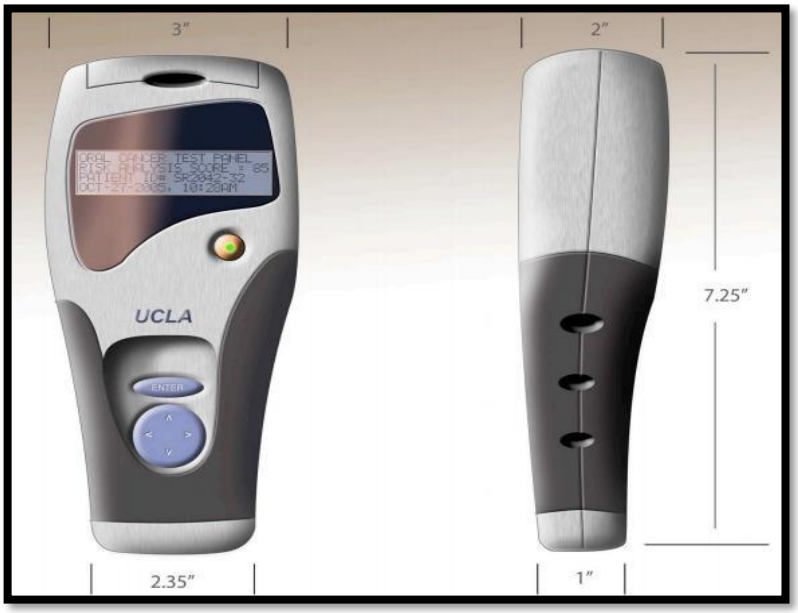


Fig 12: Handheld oral nano sensor for saliva testing

Biomarkers:

Since the introduction of molecular techniques such as examination for abnormal protein expression, including tumor suppressor genes (TSGS) and other genetic changes, molecular markers have revealed neoplastic changes in PMNLs (and furthermore may predict involvement of tumor resection margins and lymph nodes, and prognosis).

The most predictive of the molecular markers thus far available and assessed in OSCC development include the TSG p53 protein expression, chromosomal polysomy (DNA ploidy), and changes (termed loss of heterozygosity; Loh) in chromosomes 3p or 9p (probably due to changes in the TSG p16).

The use of such biomarkers as adjuncts to routine histopathological examination may help prognostication and effective management of PMLs but their routine use is still hampered by the cost and complexity of the tests, the lack of facilities in some laboratories, and limited outcome studies to date.

More readily available markers, such as those of cell proliferation **(ki-67antigen)** and apoptosis **(bax, bcl-2),** may also play a diagnostic role: apoptotic bcl-2 expression decreases significantly in dysplastic and early invasive lesions and then increases almost to normal tissue level in consequent stages while ki-67 expression increase sharply in initial stages of OSCC, but significantly decreases in later stages.

A more aggressive tumor behavior and worse prognosis may also be signified by changes in a range of biomarkers, such as reduced e-cadherin expression, laminin (ln) chain expression, and decreased tumor cell transmembrane proteo-glycansyndecan-1. [24]

Reported potential salivary biomarkers for oral cancer detection.

1. Non-organic compound biomarkers.

2. Peptide or protein biomarkers.

3. DNA, mRNA or microRNA biomarkers.

4. Metabolomic biomarkers.

5. Miscellaneous biomarkers (chemical and enzyme activity).[25,26]

Clinical implications of salivary biomarkers of oral cancer

Numerous salivary tumor markers are found to be significantly increased in the saliva of oral cancer patients.

Several reports on these salivary biomarkers in oral cancer have shown significant clinical usefulness for oral cancer. **Shiptzert et al (2009)** found that various biomarkers namely cycd1, ki67, ldh, mmp-9, ogg1, maspin were significantly altered in oral cancer and found to be useful as a supportive tool for diagnosis, prognosis and post-operative monitoring. [26]

Sudbo et al (2001) and Femiano et al (2010) have found that potentially malignant lesions with an euploidy convert into cancer more frequently than lesions with normal DNA content irrespective of the histopatho-logical grade of dysplasia. DNA aneuploidy appears to be associated with advanced stage carcinomas and lymph node metastasis. [28,29]Hence, DNA content of a tumor may help in predicting the aggressiveness of the cancer.

Zhangetal (2012) found 75% positive expression of telomerase in saliva of oral cancer patients suggesting its utility as a supportive marker to diagnose oral cancer and also suggested that human telomerase reverse transcriptase (HTERT) analysis may be a potential biomarker for the diagnosis of oral cancer. [30]

Jou YJ et al (2010) concluded that actin and myosin are promising salivary biomarkers for distinguishing potentially malignant and malignant oral lesions. Salivary transferrin was also validated as a biomarker for detection of early-stage oral cancer. (31)

Studies by S**chwartz et al (2004)** have also been carried out on carboxylation (indicative of oxidative damage to proteins) because of its irreversible and irreparable nature, which becomes cytotoxic and is associated with cancer. (32)

The levels of salivary soluble cd44 were shown increased in the majority of OSCC and could distinguish cancer from benign disease with high specificity.

3 tumor markers, cytokeratin 19 fragment cyfra21-1, tissue polypeptide antigen, and cancer antigen 125, were found significantly raised in the saliva of OSCC patients, and combined use of these markers resulted in similar diagnostic value as sera of OSCC patients.

The level of p53 autoantibody in saliva was also found correlated with its serum levels in OSCC and examination of p53 autoantibody in saliva may present a specific method for recognition of a subset of OSCC with p53 aberrations.

7 m-RNA molecules; il-8, and il1β which play a part in signal transduction, proliferation, inflammation and apoptosis, s100p (s100 calcium binding protein p)with a role in protein binding and calcium ion binding, dusp1 (dual specificity phosphatase1) which takes part in signal transduction, protein modification and oxidative stress,oaz1 (ornithinedecarboxylaseantizyme1) helps in polyamine biosynthesis, h3f3a (h3 histone, family 3a) possessing a DNA binding activity and sat (spermidine/spermine n1-acetyltransferase) which is included in enzyme and transferase activity, to be significantly increased in OSCC patients.(33)

Colposcopy

Colposcopy (direct oral microscopy) of oral mucosal lesions helps in selecting more representative sites for biopsy than routine clinical examination alone. Because of its precision, versatility, ease of use, and being a non-invasive technique, colposcopy might prove to be a useful step toward continuing to learn and improve the care for the patients. (34)

Since ages colposcope has been routinely practiced in gynecology. It was invented by P**rof. Hans hinselmann**(35). It was only in the year **2000, Goran gynther** used colposcope for oral lesions. (36)

A colposcope is typically defined as a stereoscopic binocular field microscope with a long focal length and powerful light source. The parts of a colposcope include a colposcope head, a height adjustment knob to adjust the height of the instrument, head inclination knob to adjust the angulation. Illumination is provided by a halogen lamp via a fiber optic cable connected to a system of lenses. It can magnify the tissue from 4

to 40 folds. The colposcope head consists of a pair of diopter knobs, 45° angled lens, objective lens, three step magnification knob for low, medium and high power Various light filters are available to highlight different aspects of the surface of the tissue. Colposcope uses a green or blue filter to facilitate the examination of vascular changes and color tone because the unfiltered white or yellow light reduces the contrast between the terminal vessels and the surrounding tissue. The green filter removes red light thereby enhancing the vascular details by making blood vessels appear dark. The focal length of the microscope is 200 mm, providing an optimal working distance. It provides a 3-dimensional image of tissue surfaces examined. [36]

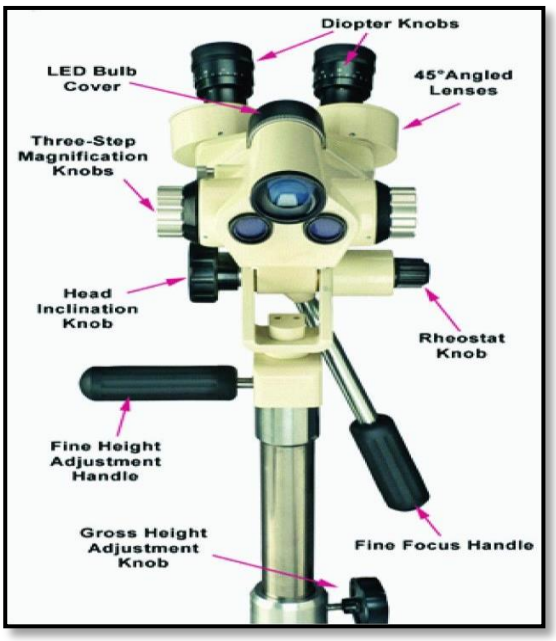


Fig 13: the colposcope

In a few studies conducted by **dhakal et al (2013) and pazouki et al (1997)** it was concluded that there was a close relationship between vascularity and tumor progression in oral mucosa. The vascularity changes described in colposcopy can be used for selecting biopsy sites in oral cavity. One of the most frequently used indices is the reids index. [37,38]

Colposcopic Sign	Zero Point	One Point	Two Point
Margin	Condylomas Micropapillary areas Pale acetowhitening satellite lesion and acetowhitening extending beyond the transformation zone	Regular lesions with smooth outlines	Rolled peeling edge. Internal demarcation between areas of different appearances
Color	Shiny snow white Pale acetowhitening	Shiny grey	Dull oyster white
Vessels	Fine caliber vessels	Absent vessel	Define punctuation and mosaicism
Iodine Staining	Positive staining or minor iodine negativity	Partial iodine uptake	Negative staining

Reids colposcope index-

The normal squamous epithelium of the oral mucosa is pink and smooth, and it demonstrates as fine, regular vessels. This normal vascularity can be altered in various inflammatory, benign, and malignant lesions and conditions.

Colposcopic findings suggesting invasion are vascular pattern, inter-capillary distance, surface pattern, color tone and opacity as well as the clarity of demarcation of the mucosal lesions. Because of the increase in vascularity, necrosis of the surface epithelium occurs and in some cases production of keratin leads to color change.

With the application of 3-5% acetic acid, the 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.

A lesion with an internal border that is a lesion within a lesion is typically high grade. These results are based on the vascular and tissue changes. The capillary changes preceding tumor growth with the pattern of tumor angiogenesis are different from the usual neo-vascularization taking place during repair and regeneration processes.

At a cellular level, various molecules such as vascular endothelial growth factor, b basic fibroblast growth factor, and transforming growth factor alpha are implicated. Direct optical visualization of these patterns would be helpful in the early determination of the underlying pathology and also aid in marking out the site of biopsy. [(39)]

Blood vascular pattern: the criteria for vascular changes described in colposcopic literature for the selection of biopsy site has been described as follows.

Normal: in the normal mucosa, two basic types of capillary networks can be seen with the colposcopy procedure -network capillaries and hairpin capillaries.

Abnormal: in the abnormal epithelium, three different types of capillary networks are included.

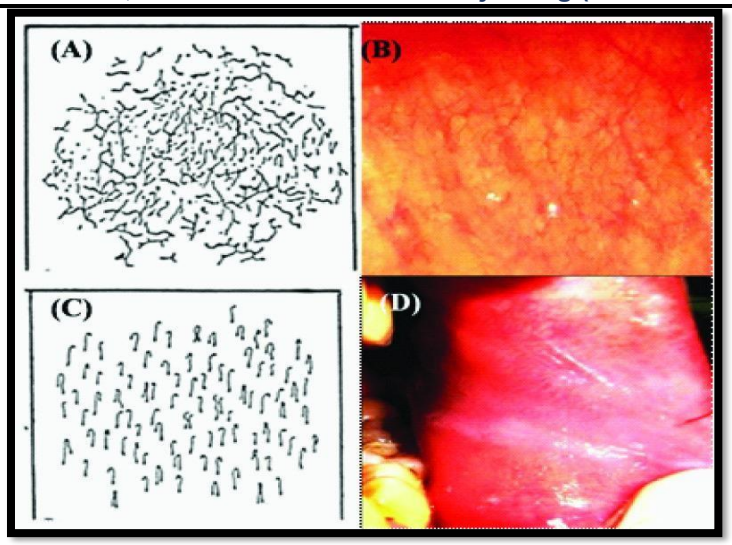


Fig 14:(a) illustration of network capillaries in colposcopic view. (b) network capillaries in normal buccal mucosa (c) illustration of hairpin capillaries in colposcopic view. d) Hair pin pattern in normal buccal mucosa

In areas of dysplasia and carcinoma in situ of the uterine cervix or oral mucosa, a specific vascular pattern, punctuation, is common, characterized by dilated, often twisted, irregular, hairpin-type vessels. These dilated capillaries terminating on the surface appear from the ends as a collection of dots and are thus referred to as punctuation. Another pattern of the vessels in dysplasia is called mosaic. Terminal capillaries surrounding roughly circular or polygonal blocks of aceto-white epithelium crowded together are called mosaic because their appearance is similar to a mosaic tile. These vessels form a basket around the blocks of abnormal epithelium. When it is difficult to describe the pattern of the vessels, the term a typical vessel is used. Atypical Vessels are terminal vessels that are irregular in size and shape and coarse and such arrangement indicates neoplasia.

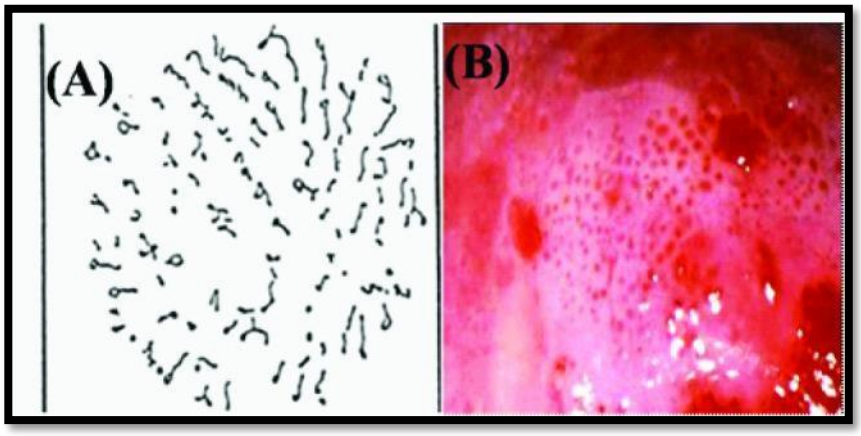


Fig 15: illustration of punctate capillaries in colposcopic view (b) punctate vessels in leukoplakia

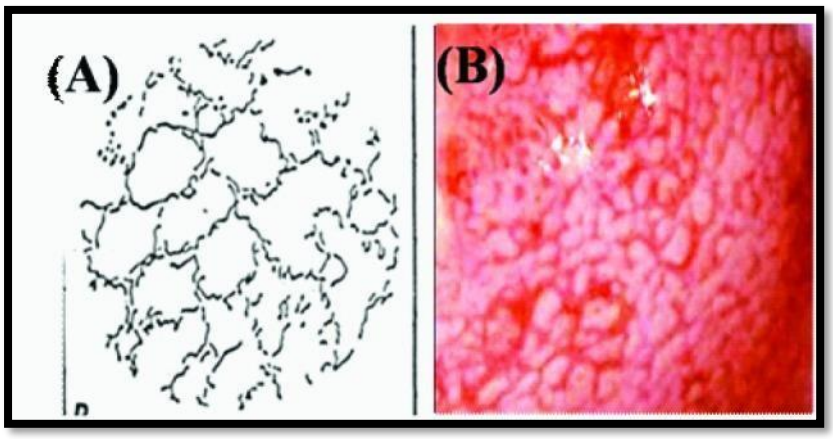


Fig 16:(a) illustration of mosaic capillaries in colposcopic view(b) mosaic pattern in leukoplakia pointing towards high grade of dysplasia.

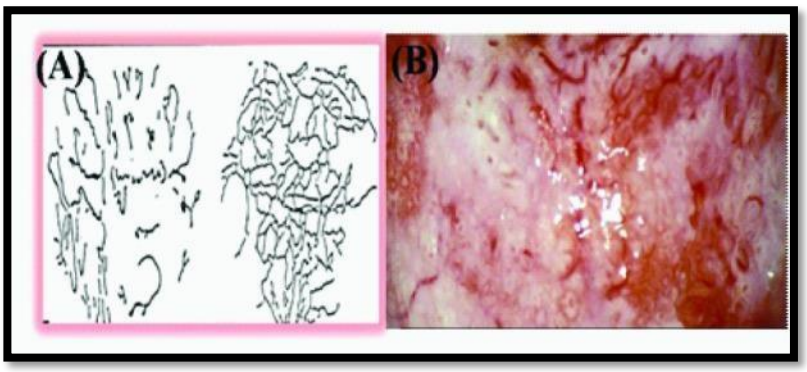


Fig 17: (a) illustration of atypical capillaries in colposcopic view. (b) atypical l pattern in leukoplakia suggesting of epithelial dysplasia and ongoing malignant transformation

Fine punctuation and mosaicism which are created by narrow vessels and uniform inter-capillary distances typify low-grade lesions. A coarse pattern resulting from a wider and more variable vessel diameter and spacing indicates higher grade abnormalities. The mosaic tiles with central punctuation indicate carcinoma in situ. Fracturing of previously intact mosaic and punctuate patterns with the production of predominantly waste thread like vessels is a nearly colposcopic warning sign of squamous micro invasion or cancer. Thus, dilation and proliferation of the resulting punctuate, and mosaic patters increase with the degree of neoplastic change.

As the neoplastic growth process proceeds and the need for oxygen and nutrition increases, angiogenesis occurs as a result of tumor and local tissue production of vegf, pdgf, egf, and other cytokines, resulting in the proliferation of blood vessels and neovascularization. Atypical vessels may be looped, branched, or reticular. Sharp turns, dilations, and luminal narrowing also characterize these vessels. The surface epithelium may be lost in these areas leading to irregular surface contour and friability. Common to all these vascular patterns is irregular vessel dilatation and inter-capillary distances greater than the normal distance of 50-200 μm with the increasing degree of dysplasia, the distance increases so that the maximum distances may exceed

700 µm [37]

In Sweden, research conducted by **Gorongnyther (2000)**, among 35 patients, 29 patients (83%) showed changes in the vascular picture on microscopy, according to the colposcopy criteria. The study concluded that direct oral microscopy of mucosal lesions seems to offer advantages in selecting more representative sites for biopsy than routine clinical examination alone. [36]

Ellenhhopman (2010), in his study stated colposcopy as an effective tool for diagnosing cervical intra-epithelial neoplasia. It was suggested that micro-invasive carcinoma was suspected when mosaic, punctuation and aceto-white epithelium was seen with a thick white epithelium that had a clear and elevated margin with an irregular surface contour and raised capillaries. [39]

Compared with other methods, the advantages of colposcopy include high resolution, good magnification, good illumination, good storage capacity, detects lesions at an early stage, painless, non-invasive, with an accuracy of 80-90%. The chief disadvantages are the complexity and cost, and these should be evaluated by further comparative studies. [40]

Conclusion

Increased diversity and sophistication are developing in the areas of molecular biology, basic science, and medical sciences. These will transform our traditional approaches to oral and dental disease management. Combined with an increased public awareness of oral cancer in general, robust advanced diagnostic aids allow clinicians to detect lesions unseen by conventional examination techniques and should help more affected patients become long-term survivors of this challenging disease.

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