Review Article

Vital tissue staining in the diagnosis of oral precancer and cancer: Stains, technique, utility, and reliability

Udyavara Sridhara Sudheendra, Huchanahalli Sheshanna Sreeshyla[1], Raju Shashidara

Departments of Oral Pathology and Microbiology, Coorg Institute of Dental Sciences, Virajpet, [1]Oral Pathology and Microbiology, JSS Dental College and Hospital, Mysore, Karnataka, India

ABSTRACT

Early diagnosis is the single most factors that improve the prognosis and survival rate of cancer patients. Numerous adjuncts are available to aid in its diagnosis. Vital tissue staining is one such adjunct used in the diagnosis of cancer. Though not a new technique, its application to cancer diagnosis especially in its premalignant stage is still uncertain. While the efficiency of toluidine blue (TB) is established to certain extent, the role of other vital stains needs to be researched. This article reviews the various vital tissue staining techniques available in the diagnosis of oral precancer and cancer.

Key words: Diagnosis, oral cancer, toluidine blue, vital stain

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.[1] 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.[2,3] The technique has been applied in the oral setting for over 30 years by means of the dye toluidine blue (TB).[2,4] 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.

Access this article online	
Quick Response Code:	Website: www.ccij-online.org
	DOI: 10.4103/2278-0513.130156

TB

TB was first used by Richart in 1963 to stain uterine cervical carcinoma *in situ* and dysplasia.[5] He compared the results of TB and Schiller's iodine staining in 200 patients with dysplastic tissue changes and concluded TB to be more reliable than Schiller test and it delineated neoplastic epithelium in 95% of cases. Later studies including the one by Richart did not mention about the false negatives or if mentioned, they remained unexplained.[6] However, presently numerous studies have been done to evaluate the effectiveness of TB in diagnosis of premalignant and malignant lesions.

Properties and principle

TB chemically is referred to as tolonium chloride. Its molecular formula is $C_{15}H_{16}N_3S^+$ 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%).[7] 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

Address for correspondence: Dr. Huchanahalli Sheshanna Sreeshylai, Hampi Circle, Vijaynagara 2nd Stage, Mysore - 570 017, Karnataka, India. E-mail: sreeshylahs@gmail.com

acid (RNA).[3,4] 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 nucleohistones 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.[6] Its use *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.[3,4,7] It stains to the depth of two to 10 cell layers, and hence just reflects only the epithelial changes, the invasion into the underlying connective tissue, or the changes in the submucosa cannot be appreciated.[2,8]

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.[5,9]

Technique of staining

TB can be used in two forms. It is either applied to the site of the lesion with a cotton applicator or it is used as mouth rinse. The procedure of staining is as follows[10,11]

- 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.

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.[7,8,10] 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.[5,8,10] 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.[10]

TB staining is a simple, quick, noninvasive, and highly cost effective procedure.[4,10,12] 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, and during the follow-up of treated lesions.[3,4,7,12]

Studies using TB

Several studies have been carried out to establish the diagnostic role of TB in oral precancer and cancer diagnosis. They have recorded a varying sensitivity and specificity of 93.5-97.8% and 73.3-92.9%, respectively.[7,13-15] The recorded sensitivity for oral dysplasia alone is comparatively less ranging from 42 to 87%.[14] Thus, most studies have shown high sensitivity but a low specificity. To decrease the number of false-positive results, Mashberg recommends that all irritating, inflammatory, and traumatic lesion should be allowed to heal and if not responding they should be reevaluated and stained after 10-14 days, and a positive stain should be considered as suspicious of carcinoma.[5,10] Mashberg in his study, upon following this protocol showed reduction of false positives by 8.5%.[10] Discrepancies in the results may be due to the nature of the sample analyzed, study pattern of inflammatory/traumatic lesions, staining method employed, variation in interpretation of color of the stain, and lack of histological correlation. TB staining is highly sensitive and efficient in detecting *in situ* carcinoma and invasive carcinomas.[3,4,7,8,12,14,15] The carcinoma could be detected with a sensitivity of 100% even with the use of TB oral rinse.[9] However, few studies have shown high false negative values even with *in situ* carcinoma and invasive carcinomas.[4] The staining capability of premalignant lesions is variable and limited.[4,7-9] Myers has demonstrated positive staining in malignant tumors other than epidermoid carcinoma such as melanoma, fibrosarcoma, and lymphosarcoma. However, all these tumors in the study had ulceration at one or the other part of the lesion. The author suggested that only lesions with ulceration take up the stain and not the once which are completely mucosal covered.[16] The stain can be taken up by some benign lesions as well and is mostly related to ulceration or erythema.[7,16] When used along with chemiluminescent technique, it is

reported to reduce the number of false positive biopsies without increasing the false negatives.[15]

Molecular studies on TB stained lesions have reported a link between carcinoma and loss of heterozygosity (LOH) at 3p, 17p, and multiple loci; while dysplasia resulted related to LOH at 9p, even if the only LOH at 3p and 17p were linked to TB uptake.[7,14] The study by Zhang, *et al*., showed that the TB stains lesions with clinical features associated with risk, premalignant lesions with increased frequency of histologic progression and lesions with high-risk molecular patterns. The authors suggested that the TB staining predicts risk and outcome for oral premalignant lesions with minimal or no dysplasia. The study points to the need to reaccess TB stain not just with its association with histology, but also with molecular risk predictors and with outcome.[17] Invasive margins at the tumor front have been studied with TB, which was found to be effective; but it could not delineate positive resection margins due to carcinoma *in situ* or severe dysplasia.[2]

There is lack of agreement on the color perception of the stain. Few authors suggest to consider dark color alone as positive, while others consider pale blue stain as well.[4,7,10] Study by Epstein showed presence of molecular changes even in sites of pale blue staining.[14] With the existing data it can be said that the staining with TB is an adjunct but not a substitute for either judgment or biopsy.[8,11,16] It is very useful in the developing countries like India because of the cost effectiveness and easy technique.

MB

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. However, its application in detecting oral lesions by far is very limited.[18-20]

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.[18] 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:[18]

- 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.

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 and 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.[18,20]

Study by Chen, *et al*., showed a sensitivity of 90%, specificity of 69%, positive predictive value of 74%, and negative predictive value of 87% in suspected oral lesions like leukoplakias, erythroplakia, and ulcerations. The authors suggested that being economical, MB can be used as an useful diagnostic adjunct in a large, community-based oral cancer screening program for high risk individuals.[18]

Lugol's iodine

Lugol's iodine, also known as Lugol's solution, first made in 1829, is a solution of elemental iodine.

Iodine, is a chemical element that has the symbol I and atomic number 53. 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.[21,22] 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.[3,23]

Lugol's solution consists of iodine and potassium iodide.[3,22,24] It has been used in varying concentrations: 1, 1.25, 1.5, 2, 3, and 10%.[22,23] Staining with 3% Lugol's solution, followed by 5% has been found to be more effective.[23] 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.[3,22,24,25] This selective staining delineates the inflammatory and carcinomatous epithelium from the normal epithelium.[24] 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 neighboring the parabasal layer, but iodine-stained areas are completely consistent with glycogen distribution only in the upper superficial layer.[26] 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.[3,22.25] This may limit the use of Lugol's iodine in keratinized lesions and in such case its uptake should be assessed carefully.[3,22] This technique also cannot be used for the detection of subepithelial infiltrating tumors.[22]

Study by Epstein, *et al*., and Nagaraju, *et al*., recorded a sensitivity of 87.5 and 92.7% and specificity of 84.2 and 60%, respectively.[3,24] Molecular analysis of stain subjected areas with PCNA, p53, Ki67, GLUT 1, telomerase, and cytokeratins have shown promising results for Lugol's staining.[22,25,26] It is found to be useful in determining the adequacy of surgical margins for local resection and hence thereby useful in reducing the locoregional recurrence.[27-29] Colorimetric analysis of iodine stained areas showed a possibility of histological diagnosis based on the colors measured from the lightly stained or unstained areas.[23] Being simple, inexpensive, noninvasive, and easy to use; further studies are essential to assess the effectiveness of Lugol's iodine.[22,28]

Acetowhite staining

Acetic acid staining has been used as a part of colposcopic examination since 1938.[30] 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.[15] 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.

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.[30-32] The acetic acid removes the mucus by coagulating it and thus allows the visualization of abnormal areas. It also cause swelling of the epithelium and reduces its transparency by producing a transient coagulation of nuclear proteins.[32] Thus, the higher nuclear content in premalignant and malignant lesions reacts with the acetic acid producing a acetowhite 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.[31] 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.[30]

Double staining

Studies have been done wherein few authors have used combination of two dyes to aid in the assessment of oral malignant diseases. TB and Lugol's iodine combination has been used by Epstien, *et al*., and Nagaraju, *et al*., for the assessment of oral malignant diseases.[3,24] TB will stain the abnormal epithelium, whereas Lugol's solution binds to glycogen present in the normal epithelium. The use of Lugol's iodine may be limited on lesions arising from keratinizing mucosa. Thus, the use of both tissue stains can overcome this potential limitation of Lugol's iodine. On contrary, the consecutive application of Lugol's iodine increases the specificity of the staining technique.[3] Zhu, *et al*., have studied the combination of MB and Lugol's iodine double staining in the detection of esophageal carcinoma. The basis of the mucosal double staining technique was that MB stains lesion blue and Lugol's iodine reversibly stains glycogen brown. Normal squamous epithelium appears unstained because it does not absorb MB, but in abnormal mucosa the superficial epithelium is often stained blue because it absorbs MB. Therefore, the area stained blue indicates the existence of carcinoma, the area stained brown belongs to normal squamous mucosa and the area between both the colors clarifies the invasive lesion of carcinoma.[19]

CONCLUSION

Being simple, economical, widely available, noninvasive, and easy to use; vital staining can be used in all the clinical settings especially in developing countries where the advanced diagnostic modalities are unavailable. Though numerous studies have been carried out using TB, the same are lacking for other vital stains. Studies with well-designed methodologies with due consideration to the nature of

samples are essential to evaluate the efficacy of MB, Lugol's iodine, and acetic acid; so that their role in the diagnosis of precancer and cancer can be accurately determined.

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Cite this article as: Sudheendra US, Sreeshyla HS, Shashidara R. Vital tissue staining in the diagnosis of oral precancer and cancer: Stains, technique, utility, and reliability. Clin Cancer Investig J 2014;3:141-5.

Source of Support: Nil, **Conflict of Interest:** None declared.