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Diagnostic Reliability of Methylene Blue in Vivo Staining in Predicting Oral Premalignant and Malignant Lesions with Scrape Cytology and Histopathological Correlation

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ABSTRACT

Background and aim: To correlate the clinical diagnosis of the suspected lesions of oral cavity using methylene blue stain, histopathological and cytopathological methods.

Material and Methods: The study has been carried out in the Pathology department for a period of 1.5 years. The study includes in vivo application of methylene blue stain to clinically suspected premalignant and malignant lesions of oral cavity, followed by scrape cytology from the same site followed by its histopathological examination among 100 subjects which includes 60 cases and 40 controls. Scrape smear was collected from all the 100 subjects. Biopsy was done in 48 cases out of 60 cases.

Results: Out of 48 cases, maximum lesions were found to be malignant (71%), followed by premalignant (17%) oral lesions. Benign lesions were found in male patients only and malignant lesions (80%) and premalignant lesions (75%) were found in majority of males. Majority of patients (70%) had Well differentiated Squamous cell carcinoma, followed by 24% of patients having Moderately differentiated Squamous cell carcinoma.

Conclusions: The current study shows that majority of patients presented with carcinoma and very few at premalignant stage. There is prevalence of habits of tobacco chewing/smoking betel nut in India which are carcinogenic. So, the present study focused on highlighting the importance of screening of oral lesions to diagnose the oral lesions at early stage for its timely treatment and to improve the quality of life. In vivo methylene blue staining is simple, easy to perform, noninvasive, rapid, painless, inexpensive method.

1. Introduction

The oral cavity is a very valuable structure for humans. It participates in many important functions, including mastication, swallowing, taste, and phonation. Incapacitating these fundamental requirements has many consequences on both mental and physical health. Cancer, a modern epidemic among non-communicable diseases, is the second most common cause of mortality in developed countries and remains one of the ten most common causes of mortality in developing countries like India.[1] In economically developing countries, the burden of cancer increases due to the increased adoption of cancer-causing behaviors, particularly the use of tobacco products. Nearly 5,00,000 new oral and pharyngeal cancers are diagnosed in a year globally, out of which 65000 cases were reported from developing countries like India. The World Health Organization has estimated that in India, the death proportion due to tobacco-related disease will rise from 1.4% in 1990 to 13.3% of all deaths in 2020.[2] According to the World Health Organization's estimation, in 1991, in the next quarter of the century, the cancer cases will double, half of which will be in developing countries. In 2020, cancer of the lip and oral cavity was estimated to rank 16th in incidence and mortality worldwide and was a common cause of cancer death in men across much of South and Southeast Asia and the Western Pacific.[3] The knowledge of etiological factors for the development of oral cancers can make the disease preventable by avoidance of risk factors like tobacco consumption, betel-quid chewing, and alcohol abuse. Betel quid and areca nut chewing were significant risk factors evaluated by the International Agency for Research on Cancer (IARC) as carcinogenic to humans in the 2003 monograph evaluation. In Western countries, tobacco usually takes the form of cigarette, cigar, or pipe smoking. The etiologic role of oncogenic HPV infections in the development of oral cancer is being defined. Syphilis, nutritional deficiencies, sunlight (in cases of lip cancer), miscellaneous factors including heat (particularly heat from pipe steam in cases of lip cancer), trauma, sepsis, and irritation from sharp teeth and dentures also play a role in the etiology of oral cancers. Early oral cancers resemble transient,

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traumatic, and benign abnormalities. It is not surprising, therefore, that early oral cancers are rarely suspected, rarely biopsied, and rarely discovered. Most oral malignancies occur as Squamous cell carcinoma. Many Oral squamous cell carcinomas develop from premalignant lesions and conditions of the oral cavity. A wide array of conditions has been implicated in the development of oral cancer, including leukoplakia, erythroplakia, palatal lesion of reverse cigar smoking, oral submucous fibrosis, discoid lupus erythematosus, and hereditary disorders such as dyskeratosis congenita and epidermolysis bullosa. Multiple screening and detection techniques have been developed to address this problem and prevent the malignant transformation of these precursor lesions. Early detection of oral carcinoma is paramount as it can best be cured at its earliest stage. Among the diagnostic tools, in vivo staining is advocated as a simple, inexpensive, and fairly sensitive method.[4] Toluidine blue is a metachromatic acidophilic dye. Due to active cellular proliferation, dysplasia in premalignant lesions contains much more DNA and RNA than the normal epithelium. So, using toluidine blue in vivo staining is based on the fact that it selectively stains acidic tissue components such as DNA and RNA.[5] However, it is hazardous if swallowed and was shown to be toxic to fibroblasts. Another dye material, methylene blue, has a chemical structure similar to toluidine blue and exhibits physicochemical properties similar to toluidine blue. The methylene blue uptake mechanism in epithelial tissue may resemble toluidine blue in the acidophilic characteristics of cells with abnormal nucleic acid concentrations, resulting in differential uptake between normal/benign and highly dysplastic/malignant cells. To review the literature on diagnostic reliability of methylene blue in vivo staining in oral premalignant and malignant lesions. To correlate the clinical diagnosis of the suspected lesions of the oral cavity using methylene blue stain, histopathological, and cytopathological methods. To correlate predisposing adverse oral habits in the causation of various oral lesions.

2. Material and methods

This study was approved by the Ethical Committee of the Pathology department at Saurashtra University (IEC/Certi/23/2017). All the employees were informed about the study and were invited to complete the questionnaire. No one was forced to participate in the study. Moreover, all questionnaires were coded, and data were entered into the software according to their codes and documented on the questionnaires. All data are being kept confidential. The study was carried out in the Pathology department of a tertiary care hospital from May 2017 to October 2018. The study includes the in vivo application of methylene blue stain to clinically suspected premalignant and malignant lesions, followed by scrape cytology from the same site and its histopathological examination among 100 subjects. The subjects were divided into two groups: Cases and Controls.

Cases

Sixty patients with clinically suspected oral premalignant and malignant lesions were selected for the study, having habits of smoking tobacco, chewing tobacco, and alcohol consumption.

Controls

Forty controls without the habits of tobacco in any form and alcohol were included in this study. The clinical data regarding the patient's age, gender, and habits of tobacco use and alcohol consumption were collected. Subjects in the study and the control group were subjected to a methylene blue staining procedure. Formulation of tissue stain: Methylene blue dye system had two solution bottles. The dye rinse solution (Bottle A) had 1% methylene blue, 1% malachite green, 0.5% eosin, glycerol, and dimethyl sulfoxide. Pre- and post-rinse solution (Bottle B) had 1% lactic acid and purified water.

Statistical analysis

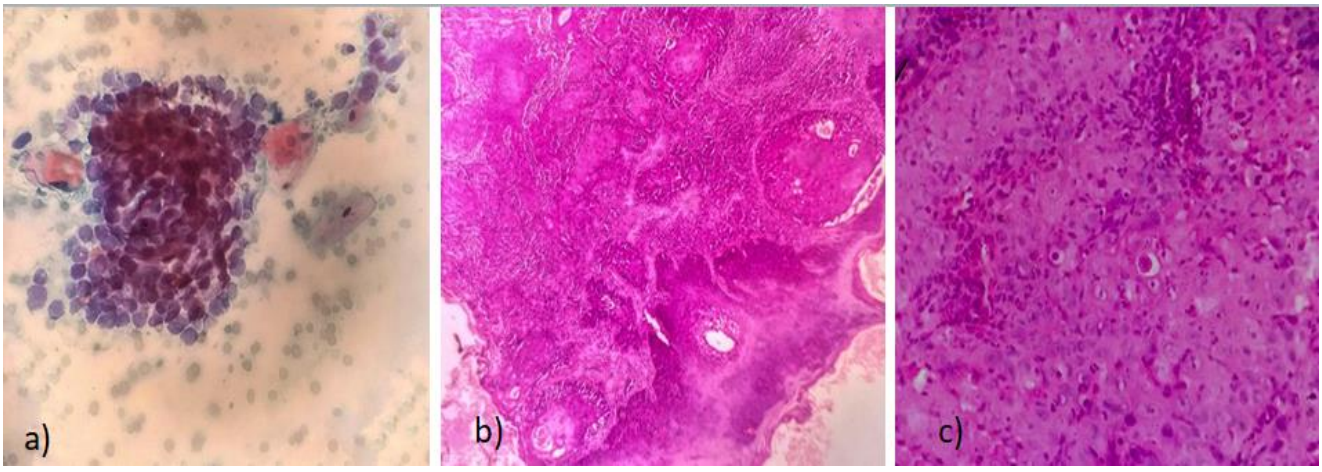
Statistical analysis included sensitivity, specificity, and positive and negative predictive values.

3. Results

Out of 100 subjects, there were 60 cases and 40 controls. The age range of cases was 20-90 years with a mean age of 49.8 years, and the age range of the control group was 20- 70 years with a mean age of 46. Out of 100 subjects, 86 were male and 14 were female. Out of 60 cases, 50 were males, and 10 were females, so the male-to-female ratio is 5:1, and out of 40 controls, 36 were males, and 4 were females. Out of 60 cases, 48(80%) patients had a biopsy done; in the rest, 12(20%) patients, methylene blue staining and scrape smear were done. (12 patients were not willing to biopsy). Out of 48 cases, 34 (71%) cases were malignant, 8 (17%) cases were premalignant, 2 (4%) were benign lesions, and in 4 (8%) cases, the material was inadequate to conclude. (One patient had severe pain, one patient had bleeding, one patient was not cooperative, and one patient was not able to open mouth due to submucous fibrosis). So, oral lesions were studied based on their clinical presentation, methylene blue stain results, scrape smear cytology, and histopathological study. Sixty cases had the habit of tobacco chewing, tobacco smoking, and alcohol consumption and had abnormal oral manifestations at advanced stages whenever they came for consultation. Forty controls had no habits, normal oral mucosa, or abnormal oral manifestations. Methylene blue staining and scrape smears were taken from 40 controls, and a biopsy was not done. Methylene blue stain was negative in the control group. So, out of 48 cases where all three diagnostic aids were done. methylene blue staining, scrape smear, and biopsy, maximum lesions were found to be malignant (71%), followed by premalignant (17%) oral lesions, which shows that people are still unaware about the timely oral check-up and consequences of the same and present at advanced stage. The majority of subjects belonged to the fifth decade (40-49 years) (36%), followed by the sixth decade (50-59 years) (22%) and then the fourth decade (30-39 years) (20%) The benign lesions were found in patients of the fifth decade (40-49 years). Most premalignant lesions were found in the patients of the sixth decade (50-59 years), followed by the fourth decade (30-39 years). Most malignant lesions were found in patients in the fifth decade (40-49 years), followed by the sixth decade (50-59 years). Out of 48 cases, 38 were males and 10 were females. Benign lesions were found in male patients only, and malignant lesions (80% of males) and premalignant lesions (75% of males) were found in most males. The majority of the patients (53%) presented with ulcerative lesions, followed by the presentation of ulcer proliferative lesions (25%). Benign lesions were equally found in buccal mucosa and lips. Most premalignant lesions were found at the buccal mucosa (75%). Malignant lesions were found maximally at the tongue (44%), followed by buccal mucosa (38%), and then the palate (12%). The majority of patients had the habit of smokeless tobacco (63%), followed by tobacco smoking (15%), followed by patients having the habit of both tobacco smoking and smokeless tobacco (10%). Most patients (40%) had the habit for 10- 19 years, followed by a duration of 1-9 years (33%). 1 out of 60 patients consumed 46-50 bidis/cigarettes per day, followed by one patient consuming 26-30 bidis/cigarettes per day. Twenty-seven patients consumed smokeless tobacco in the frequency of 1-5 per day, followed by 17 patients consuming the same in the frequency of 6-10 per day. Four patients consumed alcohol at

a frequency of 1-5 pegs per day. Most patients (70%) were diagnosed with well-differentiated squamous cell carcinoma, followed by 24% of patients having moderately differentiated squamous cell carcinoma. Of 2 benign lesions, 1(50%) lesion showed methylene blue positivity. Of 8 premalignant lesions, 5 (63% true positive) lesions showed methylene blue uptake, and 3 (37% false negative) lesions were negative with the stain. Of 34 malignant lesions, 33 (97% true positive) were positive for methylene blue, as shown in Figure 1, and 1 (3%) lesion was negative for methylene blue. 1 out of 2 histologically diagnosed benign lesions were reported as premalignant lesions. 3 out of 8 histologically proven premalignant conditions were cytopathologically concluded as malignant (Fig. 2a), and 3 out of 34 histologically proven malignant lesions (Figs. 2b and 2c) were cytopathologically diagnosed as premalignant lesions. The sensitivity of the present study is 90.47% (38/42), the specificity is 50% (01/02), the positive predictive value is 97.4%, and the negative predictive value is 20%. So, 6 out of 8 (75% sensitivity) cytologically diagnosed precancerous lesions stained positive with methylene blue, and 3 out of 4 (75% specificity) cytologically diagnosed benign lesions were stained negative with methylene blue (Table 1).

Figs. 1. (a) Growth on the hard palate, (b) Lesion stained with methylene blue, and (c) Lesion retained stain, it is positive for methylene blue.



Figs. 2(a). Squamous cell carcinoma, smear showing sheets of malignant squamous cells. (Papanicolaou stain x40). 2(b) and 2 (c).Well-differentiated squamous cell carcinoma, section showing sheets of malignant squamous cells and keratin pearl in the stroma. (Haematoxylin and eosin stain x40).

Table 1. Results of methylene blue staining compared with histopathological diagnosis (n=44).

Histopathological Diagnosis	Methylene Blue (+)	Methylene Blue (-)	Total
Benign lesions	01 (FP)	01(TN)	02
Premalignant lesions	05 (TP)	03 (FN)	08
Malignant lesions	33 (TP)	01 (FN)	34
Total	39	05	44

TP: True positive, FP: False positive, FN: False negative.

4. Discussion

In vivo, staining techniques, such as methylene blue, have been widely explored as diagnostic aids in diagnosing oral premalignant and malignant lesions. These techniques promise early detection of oral cancer, which would translate to a decrease in the mortality rate. Several studies have looked at the effectiveness of methylene blue staining, and these studies are useful in determining its sensitivity, specificity, and reliability. Riaz et al.[6] assessed methylene blue staining in a case series of oral lesions and found that the overall sensitivity was 91.4%. Of the 48 pathologically proven cancers, 44 showed deep and focal uptake of the stain, as did 42 46 precancerous lesions. Similarly, Chen et al.[7] reported 90% sensitivity in their study of 29 histopathology-confirmed precancerous and cancerous lesions, where methylene blue gave positive results in 26 lesions out of them. These investigations demonstrate the applicability of methylene blue in making a differential diagnosis between abnormal epithelial cells and normal tissue. Abraham et al.[8] published a report showing a sensitivity of 95%, with 55 of 58 pathologically proven malignant or potentially malignant lesions showing deep and focal staining. This result correlates with Gupta et al.[9] who found that a sensitivity of 89% is exhibited with methylene blue staining in 34 out of 38 pathologically confirmed cancerous or precancerous lesions. These studies highlight the promise of this agent, methylene blue, as an inexpensive, noninvasive-invasive screening tool for oral lesions. Another structurally similar dye, toluidine blue, has been studied widely for its role in detecting oral lesions. In a study by Cancela-Rodríguez et al.[10] and Pallagatti et al.[11] toluidine blue and methylene blue were compared. Both dyes have acidophilic properties and similar uptake mechanisms in dysplastic or malignant cells. However, the differences in study populations, staining protocols, and lesion characteristics have resulted in variability in diagnostic accuracy between studies. In the current study, methylene blue staining showed a sensitivity of 90.47%, a specificity of 50%, and a positive predictive value of 97.4% with a negative predictive value of 20%. [Table 2]These results are similar to all the previous studies, proving the reliability of methylene blue in diagnosis. The number of benign, premalignant, and malignant lesions in the present study is similar to the early studies. The demographic attributes of the target population surveyed, including age structure, sex, and habits like smoking and liquor consumption, followed the previously established studies [6, 7, 11]. The mechanism of methylene blue's uptake is not precise. However, the dye is believed to selectively stain cells with higher nucleic acid content, a characteristic feature of dysplasia and malignancy. This is similar to toluidine blue, which is extensively used for similar purposes. Considering the reliability of this stain in diagnosis, methylene blue staining, along with scrape cytology and histopathological correlation, is a promising approach for mass screening of oral lesions. Future studies should focus on standardizing staining protocols and exploring the underlying molecular mechanisms of methylene blue uptake in abnormal epithelial cells. Such investigations will enhance the diagnostic utility of this staining method and pave the way for its broader implementation in routine clinical practice.

Table 2. A statistical analysis of the present study with various previous studies.

	Present Study	Riaz et al.[6]	Chen et al.[7]	Abraham et al.[8]	Gupta M et al.[9]
Sensitivity	90.47	91.4	90	95	89
Specificity	50	66.6	69	70	91
Positive predictive value	97.4	97.7	74	91	97
Negative predictive value	20	33.3	87	80	73

5. Conclusion

In our current study, the majority of patients/subjects presented with advanced pathology, that is, cancer, and very few at the premalignant stage. It shows that people need to be sensitized about timely screening of oral lesions. There is a prevalence of habits of tobacco chewing/smoking betel nuts in India, which are carcinogenic. So, the present study focused on highlighting the importance of screening oral lesions to diagnose the oral lesions at an early stage for timely treatment and to improve the quality of life. In vivo, methylene blue staining is a simple, easy-to-perform, noninvasive-invasive, rapid, painless, and inexpensive method, and it can be used as a complementary screening method to cytological and histopathological examination. Scrape smear cytology is also a safe, rapid, reliable, inexpensive, noninvasive-invasive OPD procedure, so it can also be used as an adjunct to histopathology. So, with the help of in vivo methylene blue staining, the lives of many persons can be saved by timely diagnosis and treatment. Mass screening is needed for in vivo staining. Social health projects are needed in the present era. With the help of this awareness, youth who are at risk because of the increased trend of the habit of consuming tobacco in any form can be saved, and social health and welfare can be improved. Mass screening is possible with this in vivo staining, and even healthcare workers can perform this test after training. This in vivo stain should be kept at all Pan Galas. Only writing "injurious to health" in very small letters on tobacco packets will not help; it should be written in the local language so anyone can read it. Customers coming to buy any tobacco product should compulsorily go through this test by self-observation in the mirror.

Conflict of Interest

The authors declared that there is no conflict of interest.

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