Original Article

# Toluidine Blue Staining in Identification of a Biopsy Site in Potentially Malignant Lesions: A Case–control Study

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## ABSTRACT

**Objective:** Oral cancer is a significant threat to public health all over the world, especially in Southeast Asia. At the present time, screening of oral cancer, its premalignant stages as well as its early detection, is still largely based on visual examination of the mouth. Visual examination is highly subjective and hence lacks the specificity and sensitivity. The objective of this study was to determine the usefulness of toluidine blue in marking a biopsy site in potentially malignant disorders. **Methods:** In this study, a total of 500 patients were screened. The study was a case–control study which included 17 lesion cases and 23 normal controls. Toluidine blue staining was taken into consideration to identify clinically doubtful oral potentially malignant lesions and to compare the clinical evaluation with toluidine blue stain followed by a punch biopsy and histological evaluation. SPSS Statistics version 16.0 and Chi-square test were used for statistical analyses. **Results:** The most common site for potentially malignant lesions was found to be the buccal mucosa. The sensitivity of toluidine blue was found to be 88.89%, while specificity was found to be 74.19%. The positive predictive and negative predictive values were 50% and 97.83%, respectively. *P* = 0.000672 was considered statistically significant. **Conclusions:** The results seem to be promising, but many such studies have to be done at larger scales to exactly help us in identifying the capability of toluidine blue in the long run.

**Key words:** Oral cancer, potentially malignant lesions, punch biopsy, screening, toluidine blue

## Introduction

Oral dysplastic lesions arise due to a wide number of reasons such as tobacco chewing, smoking, betel nut chewing, and reverse smoking. Oral cancer is a significant threat to public health all over the world, especially in Southeast

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Asia. More than one million new cases of oral cancers are being detected annually in the Indian subcontinent. Nearly 92%–95% of all oral malignancies are found to be oral squamous cell carcinomas. Around 300,000 patients are annually estimated to have oral cancer worldwide, and in India, it forms for about 30%–40% of cancer load.[1]

Early detection of oral cavity carcinoma is, however, far from being straightforward. Precancerous lesions are not easy to detect due to a high likelihood of false positivity.[2] The major problem is when and where the biopsy should be taken from suspected lesions and this depends on the clinician's ability to differentiate premalignant lesions from reactive and inflammatory diseases.[3] Histopathology continues to be used as the reference standard test. Unfortunately, oral cancer is usually detected when it becomes symptomatic, and at this stage, at least two-third of the patients present an advanced disease.[4]

In spite of so much advancements in technology, the 5-year survival rate is about 40%–50%.[4] Despite the general accessibility of oral cavity during routine examination, most of the lesions are diagnosed at their last stages. Early detection of cancer is extremely important as it remarkably increases the 5-year survival rate in patients.[1]

Oral cancer when caught at an early stage is often curable, inexpensive to treat, and affords a better quality of life. Detection of potentially malignant lesions at an early stage, especially in high-risk groups, is of utmost importance to prevent further morbidity, as they have shown a rate of progression and cancer transformation of up to 17% within a mean of 7 years after diagnosis.[5]

It is established by researchers that almost all oral cancers are preceded by visible clinical changes in the oral mucosa, usually in the form of white or red patch. Prevention and early detection of such potentially malignant disorders (PMDs) help in decreasing the incidence and improving the survival of those who develop oral cancer. Lack of public awareness about the signs, symptoms along with the lack of knowledge for early detection by health-care providers is believed to be primarily responsible for the delay in identifying the PMDs.[3]

Preliminarily assessed data suggest that toluidine blue stain may be preferentially retained by PMDs with high-risk molecular clones.[6] No specific method has yet been introduced to exactly mark a biopsy site noninvasively and at a low cost, hence this research can lay a good foundation for such site identification. This technique also aids in improving the ability of oral health-care professionals to detect relevant potentially malignant lesions or cancerous lesions at their earliest or most incipient stage.[3]

Literature on toluidine blue shows that it is a practical, rapid, inexpensive, and an effective adjunct diagnostic tool in identification of various potentially malignant lesions.[7] It is thought that the increased amount of DNA and RNA in neoplastic cells and the wider intercellular canals compared to normal epithelial cells are mainly responsible for staining of malignant and dysplastic cells.[8] Its clinical application in staining malignant cells was first described by Richart in 1963 to stain cervical carcinoma *in situ*.[9]

### *Aim of study*

This study aimed to determine the usefulness of toluidine blue in marking a biopsy site in PMDs.

### *Objective*

The main objectives of this study are as follows:

- To determine the usefulness of toluidine blue in marking a biopsy site in PMDs
- To compare the effectiveness of toluidine blue in detecting dysplasia in potentially malignant lesions with clinical examination
- To determine whether toluidine blue can be used as an effective screening tool for potentially malignant lesions.

In this study, the use of toluidine blue staining was taken into consideration to name clinically doubtful oral potentially malignant lesions and to compare the clinical evaluation, with toluidine blue stain and histological evaluation. Toluidine blue staining is both an affordable and a convenient staining technique for potentially malignant lesions.[7] This study was done particularly because no specific universal tool is standardized to detect dysplasia in potentially malignant lesions till date.[10]

## Methods

### *Sample selection and description of participants*

This study was conducted at Chettinad Dental College and Research Institute in Kanchipuram district, India. The study was conducted from January 2015 to December 2015 (1 year). In this study, a total of 500 patients were screened at the Department of Oral Medicine and Radiology at our institute using simple random sampling method. Of the 500 patients, 185 patients had a history of tobacco smoking, chewing tobacco, and/or alcohol consumption and only 40 patients were compliant with our inclusion criteria (tobacco smoking or chewing for at least 2 years, in the age range between 18 and 65 years, and patients not undergoing treatment for potentially malignant lesions) and exclusion criteria (habit history of <2 years, age below 18 and above 65 years, patients who had presence of frank malignancy or those patients who had any systemic disease that interferes with or are contraindications to biopsy procedure or undergoing treatment for tobacco-related disorders were excluded from

the study). The patients were asked to give a written consent for the study following which the staining and biopsy were performed. The patients were segregated into a study group and a control group. The study group comprised 17 patients who had clinically suspicious oral potentially malignant lesion while the control group had patients with all factors similar to study group but did not have an oral potentially malignant lesion. Following initial screening, history charting was done and information about the patients' habits of tobacco and alcohol use, its type, duration, and frequency was recorded.

### *Technical information*

Patients were asked to rinse their oral cavity with water for 20 s to remove debris prior to rinsing with 1% acetic acid for 20 s. Toluidine blue (1% W/W) was given as an oral rinse for 20 s and then 1% acetic acid was used for 20 s to eliminate mechanically retained stain. Lesions that showed dark blue staining [Figure 1] were considered to be positive for potentially malignant tissue, while those that stained light or did not take up the stain were considered negative for potentially malignant lesion. Staining was performed for both the study and the control groups. For the control group, a biopsy was performed from the buccal mucosa and sent for histological evaluation. The biopsy was performed under local anesthesia using 2% lignocaine Hcl.

A sterile disposable punch was used to take biopsy of the site that showed greatest staining and sent for histopathologic examination. For lesions with large toluidine blue staining, the biopsy was done from anterior aspect of the staining lesion. This was done to aid in ease of biopsy and suturing. For the statistical analysis, we used histopathological examination as the gold standard to which clinical examination and toluidine blue stain retention were compared. The data were then analyzed and the results were interpreted. This study was mainly focused on identification of biopsy site in potentially malignant lesions and not on its detection *per se*.

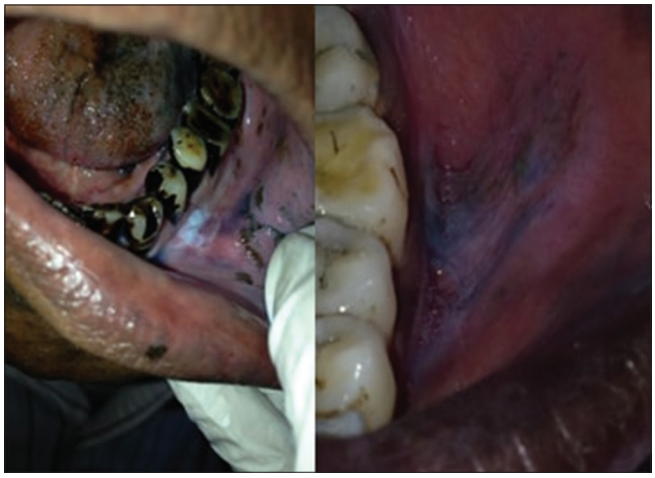

**Figure 1:** Areas staining darkly with toluidine blue indicating dysplastic changes at the site should be used as the site for biopsy

### *Ethics*

Ethical clearance was obtained from our institute's Ethical Committee. The ethical clearance number assigned was IHEC/02/2014 Desp No: 371.

### *Statistical analysis*

SPSS Statistics version 16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Student's *t*-test and Chi-square test were used to compare the study and control groups.

## Results

Out of forty patients examined in the study, all patients were male. Of the forty patients, 13 patients consumed alcohol. Of these 13 patients, 11 consumed 80 ml alcohol daily while the rest 2 consumed alcohol occasionally. Among the forty patients, thirty patients consumed smokeless tobacco more than 5 times a day. Smoking was noted among ten patients who smoked 4–5 cigarettes a day. Pan chewing was noted in nine of the forty patients. It was observed that the most common habit of patients was consumption of smokeless tobacco and alcohol in 13 patients (32.5%) followed by only smokeless tobacco in 11 patients (27.5%), smokeless tobacco chewing along with smoking in seven patients (17.5%), pan chewing and smokeless tobacco consumption in six patients (15%), and pan chewing and smoking in three patients (7.5%) [Table 1].

The most common sites for potentially malignant lesions included buccal mucosa in 12 lesions (70.58%) followed by labial mucosa and buccal vestibule in 2 lesions (11.76%) each, and followed by labial vestibule in 1 lesion (5.8%) [Figure 2]. The result of toluidine blue staining showed that 16 (94.12%) out of 17 potentially malignant lesions stained positive while one result was negative (5.88%) to toluidine blue staining. Histopathological examination of toluidine

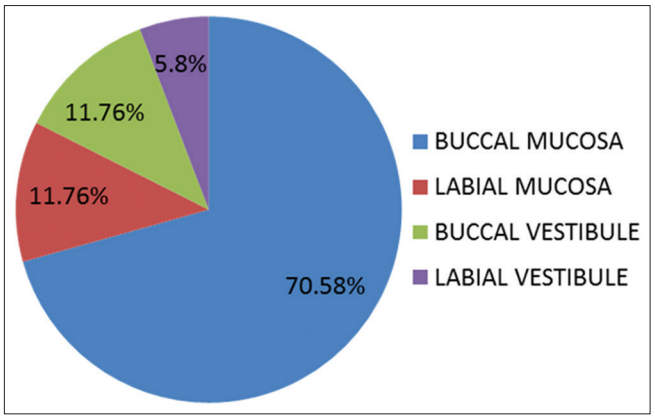


**Figure 2:** Location of oral potentially malignant lesions

blue-positive cases showed eight lesions (47.05%) to be benign (hyperkeratosis, hyperparakeratosis, hyperplasia), six lesions (35.29%) were diagnosed as oral submucous fibrosis, one lesion (5.88%) showed moderate dysplasia, and the rest of two lesions (11.77%) showed mild dysplasia [Figure 3].

Histopathological examination of toluidine blue-negative cases showed 23 lesions (95.83%) to be benign and 1 lesion (4.17%) was diagnosed as oral submucous fibrosis. The results of the toluidine blue test and histology were compared to calculate sensitivity, i.e., true positives and specificity (true negatives). The sensitivity of toluidine blue was 88.89% while specificity was 74.19%. The positive predictive and negative predictive values were 50% and 97.83%, respectively. $P$ =0.000672 (<0.05) was considered statistically significant.

# Discussion

## *Key findings*

Toluidine blue staining has high sensitivity and a very high negative predictive value and hence can be an effective screening tool for treating oral potentially malignant lesions.

## *Strengths and significance of the study*

This study showed that, if a lesion stained negative, it is almost always a mild variation from normal rather than a potentially malignant lesion and hence unnecessary biopsies can be avoided. Another important strength of the study was that biopsies were performed on patients without the lesion but with habits of tobacco smoking and/or chewing, hence validating the results better.

**Table 1: Habit pattern noted among patients**

| Habit | *n* (%) |
|---|---|
| Smokeless tobacco and alcohol | 13 (32.3) |
| Only smokeless tobacco | 11 (27.5) |
| Tobacco chewing along with smoking | 7 (17.5) |
| Pan chewing and smokeless tobacco consumption | 6 (15.0) |
| Pan chewing and smoking | 3 (7.5) |

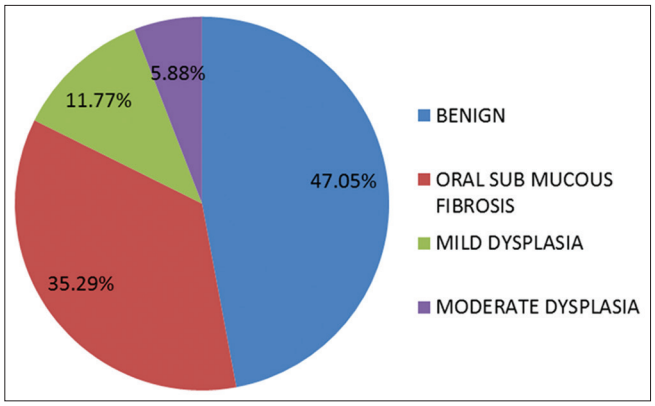


**Figure 3:** Histopathology of biopsies of study group

## *Limitations*

The main limitation of this study was the sample size of potentially malignant lesions.

## *Confounding factor*

Alcohol consumption is an important confounding factor in occurrence of potentially malignant lesion, as its usage along with tobacco increases the penetrance of tobacco alkaloids into the mucosa, thus enhancing the adverse effects of tobacco. However, alcohol consumption in itself is not directly linked to the formation of potentially malignant lesions.

## *Interpretation and implications*

Results of our study showed good sensitivity and specificity at 88.89% and 74.19%, respectively. In a study conducted by Allegra *et al*.,[4] the comparison was made between clinical and histological results of toluidine blue staining and they found the sensitivity and specificity to be 96.2% and 77.7%, respectively, which is at par with our study. Pallagatti *et al*.[5] in their study included patients only with suspected lesions without a control group and found the sensitivity and specificity to be 95% and 71.45%, respectively, which are similar to our study. Kumbhare and Taralekar[11] in their study compared Vizi Lite and toluidine blue staining for potentially malignant lesions and found sensitivity and specificity of toluidine blue staining to be 87% and 81%, respectively, and their results were similar to our study. Rahman *et al*.[12] in their study compared exfoliative cytology and toluidine blue staining and found the sensitivity and specificity to be 81.35% and 66.67%, respectively, which is similar to our study. Singh and Shukla[13] in their studies showed that sensitivity and specificity were a staggering 97.8% and 100%, respectively, higher than any previous study conducted and they were significantly higher than our study. Their results could possibly be due to the fact that the lesions in their study were at a more advanced stage and were present for longer durations than our study.

Cancela-Rodríguez *et al*.[14] in their study included both precancer and cancer patients and found the sensitivity and specificity of toluidine blue staining to be 65.5% and 73.3%, respectively. The sensitivity of toluidine blue staining in the study by Cancela-Rodríguez *et al*.[14] was much less than that of our study, probably as they used both cancer and precancer cases in their study and their initial diagnosis of carcinoma was also made clinically. Ramanathan *et al*.[15] in their study compared Vizi Lite with toluidine blue staining and found the sensitivity to be 55.5% which is significantly

less than that of our study, while the specificity was 91.6% which is significantly more than that of our study. This is probably as the toluidine blue staining was done for longer duration than our study. Awan *et al.*[16] in their study had significantly lower sensitivity and specificity of toluidine blue staining than our study. The reason for the lower sensitivity and specificity is because their study also included cases of frictional keratosis which are not part of potentially malignant lesions, thus affecting the results of their study. Cancela-Rodríguez[14] in their studies showed that toluidine blue had a high negative predictive value, which is similar to results obtained in our study. Therefore, it was observed that toluidine blue can help clinicians in reducing the number of biopsies to approximately half while identifying potentially malignant lesions. Therefore, toluidine blue-negative lesions need not to be subjected to further histopathological examination, thus saving time and resources.

Furthermore, it was observed that, while comparing the dysplastic changes occurring at various sites with the histopathology, no statistically significant differences were observed, which depicts that it could not be used as a tool for site-specific dysplastic changes but as an adjunctive tool in identification of potentially malignant lesions in oral cavity as a whole; it proved to be a good tool and was specific and sensitive in identification of these potentially malignant lesions. Toluidine blue is useful in raising or confirming clinical suspicion of malignancy or premalignancy and has the capability to reduce the number of biopsies being done. It has been proved in our study that, when the lesion stained faintly, it came out to be histopathologically negative in most of the cases.

### *Future research directions*

Our study showed that toluidine blue could be a useful tool for identifying biopsy site in potentially malignant lesions. It proved to be an excellent tool to rule out potentially malignant lesions. Further studies with larger sample sizes have to be done to make the use of toluidine blue more widespread.

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### *Conflicts of interest*

There are no conflicts of interest.

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