Application of *In Vivo* Stain of Methylene Blue as a Diagnostic Aid in the Early Detection and Screening of Oral Squamous Cell Carcinoma and Precancer Lesions

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Background: Early detection of oral malignant or precancerous lesion by screening individuals with high-risk factors may identify candidates who should receive treatment to prevent cancer progression and reduce patient mortality. Among the diagnostic tools, *in vivo* staining is advocated as a simple, inexpensive, and fairly sensitive method.

Methods: The present study involved the examination of fifty-eight patients suspected of having oral malignant or precancerous lesions by methylene blue staining. The results of methylene blue uptake were compared with a simultaneous biopsy of these lesions. The pathologically confirmed precancers and cancers were the positive targets of this screening, while benign epithelial lesions were sorted as negative subjects of screening.

Results: The results revealed sensitivity of 90%, specificity of 69%, positive predictive value of 74%, and negative predictive value of 87%.

Conclusion: We consider that methylene blue staining is a useful diagnostic adjunct in a large, community-based oral cancer screening program for high-risk individuals. [*J Chin Med Assoc* 2007;70(11):497–503]

Key Words: methylene blue, oral cancer, oral precancer

Introduction

Oral cancer is a significant threat to public health all over the world, especially in South and Southeast Asia, where betel use is very popular. In Taiwan, it accounts for an average of 1,436 deaths annually, being the 5th highest cause of death owing to its malignancy.[1] Despite advances in the detection and treatment of many other malignancies, the overall survival rate of oral cancer has remained disappointingly low for the last 10 years and was reported as less than 50% in the literature.[2] Not surprisingly, this reflects the fact that the treatment outcome depends not mainly on the improvement of surgical techniques but on the characteristics of the cancer itself. According to recent statistical data reported in Taipei Veterans General Hospital in Taiwan, the 5-year survival rate of stage I oral cancer patients was 66.2%, while for stage IV, the survival rate declined to only 22.2%.[3] The paper suggested that the early detection of oral cancers and treating patients in their early stages were the most important factors in improving the survival rate.

Periodic clinical examination of the oral cavity is the mainstay for early detection of oral cancers. It was shown to reduce mortality from oral cancer by 32% in high-risk individuals.[4] Additionally, using adjunctive aids such as toluidine blue (also referred to as tolonium chloride) has been widely accepted to improve the effectiveness in large-scale screening for oral cancer diagnosis.[5–8] However, it is hazardous if swallowed, and was shown to have toxicity to fibroblasts. Another kind of dye material, methylene blue, has a similar chemical structure and exhibits similar physicochemical properties to toluidine blue. It is less toxic to the human

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body and has recently been proposed for screening some gastrointestinal or prostate tumors. The application of this material in detecting oral lesions has so far not been addressed. The objective of this study was to evaluate the sensitivity and reliability of *in vivo* staining with methylene blue as a diagnostic adjunct in screening for oral malignant or precancerous lesions.

Methods

Subjects

This project was approved by the institutional review board of Taipei Veterans General Hospital (VGH), with informed consent signed by patients. Fifty-eight patients at the Oral and Maxillofacial Surgery, Department of Dentistry, Taipei-VGH between October 2002 and August 2003 with the presence of abnormal oral manifestations were included in this study. All the patients had no history of oral cancer or previous oral surgery. The patients' clinical profiles such as gender, age, and habits of betel use were collected. All the patients were subjected to a systematic oral examination with clinical diagnosis as: (1) homogeneous leukoplakia: white, uniform, flat lesion with a smooth, wrinkled, or corrugated surface, not able to be scraped; (2) nonhomogeneous leukoplakia: white lesion with irregular and exophytic surface; (3) erythroplakia: red lesion with ill-defined margin; (4) ulceration: localized and superficial lesion that does not heal after local treatment. The control group comprised 20 dental students from National Yang-Ming University who volunteered and who were randomly enrolled, with a mean age of 22 years. All volunteer students had no habits of betel quid chewing, smoking, or alcohol drinking.

Gargling solution

A set of methylene blue dye system includes 2 bottles of solution. The dye rinse solution (Bottle A) contains active ingredient methylene blue 1%, with the addition of 1% malachite, 0.5% eosin, glycerol, and dimethylsulfoxide. Pre- and post-rinse solution (Bottle B) contains 1% lactic acid, raspberry flavor, and purified water.

Staining procedure

The application of methylene blue was as follows. A 5-minute teeth brushing procedure was required before testing. All patients rinsed their mouths with Bottle B for 20 seconds to remove food debris and excess saliva and to provide a consistent oral environment. The mucosa of the target area was gently dried with gauze and power air spray to ensure that the lesion was not contaminated with saliva. Patients gargled and rinsed with 1% methylene blue dye (Bottle A) for 20 seconds, then expectorated. Patients then rinsed again with Bottle B for 20 seconds to wash out the excess dye. The pattern of dye retention was assessed by the intensity of stain retention on the lesion. Local, stippled, patchy and deep blue stains were marked as positive (+) reaction. Wide, shallow or faint blue stains were marked as negative (–) reaction. For equivocal staining, Bottle B solution was applied with cotton rolls to wipe out the staining surface. If the blue stain was washed out, negative reaction was recorded and vice versa. If the patient had a highly suspicious lesion that was not all stained by the solution, the patient was instructed to revisit within 14 days to repeat the test in order to reduce the false-negative rate. The results of methylene blue dye staining were recorded with photographs, and biopsy was performed simultaneously in the suspected lesions to compare the accuracy of the diagnostic capability of methylene blue.

Biopsy

Incision biopsy was performed in the most obvious staining area of the suspicious lesion of patients under local anesthesia. If there was no dye uptake in the lesions, the biopsy specimen was taken from the area judged by a specialist's experience. The specimens were then fixed in 10% neutral buffered formalin and processed in the pathology laboratory for initial routine pathologic diagnosis.

Histologic examination

All the specimens were microscopically evaluated by pathologists who were blind to the results of methylene blue stain. The pathology reports of the lesions were classified as: (1) benign lesions including epithelial hyperplasia, lichen planus, hyperkeratosis; (2) precancerous lesions including verrucous hyperplasia, dysplasia; and (3) malignant lesions including verrucous carcinoma and squamous cell carcinoma.

Data analysis

The pathologically proven cancers and precancerous lesions were the targets of screening. The results of positive/negative uptake of methylene blue in each lesion were correlated with the histopathologic diagnosis. Statistical analysis was performed, including sensitivity, specificity, positive and negative predictive values. The association of methylene blue uptake and pathologic diagnosis among the precancer/cancer group, benign group, and normal group were analyzed using Fisher's exact test. A p value of less than 0.05 was considered significant.

Results

Subject characteristics

Seventy-eight people (58 patients, 20 students who volunteered) were enrolled in this study. The patients' ages (patient group) ranged from 31 to 82 years (41 ± 15 years), with the ratio of male to female being 51:7. The students' ages (control group) ranged from 20 to 24 years. Two-thirds of patients ($n=38$) had a history of betel quid chewing, and 52 patients had a history of cigarette smoking. The suspected lesions were distributed over the buccal mucosa ($n=25$), tongue ($n=16$), gingivae ($n=9$), lip ($n=3$), floor of the mouth ($n=2$), palate ($n=2$), and retromolar trigone ($n=1$).

In the control group, as methylene blue dye was not used to examine the oral cavity, it was necessary to verify that the dye would not be retained on normal mucosa. The results demonstrated that there was no retained dye in the control group.

Methylene blue staining related to grade of pathology

The clinical and histopathologic diagnosis of oral lesions and the results of staining are shown in Table 1. The pathologic grade was classified as benign lesions, precancer lesions and cancer lesions as previously described in the methods section. The following statistical terms were used to describe and analyze the relationship between the grade of pathology and the uptake of methylene blue staining.

Sensitivity represents the proportion of histologically proved cancer/precancerous lesions which are detected by positive methylene blue staining. In the current study, 26 of 29 pathologically proved cancer/precancerous lesions were positive with deep and focal methylene blue staining (Figure 1B). The overall sensitivity was 90%. Among the 3 false negative cases, 2 were clinically presented as chronic ulcers with induration over the tongue and were later proved as squamous cell carcinoma after biopsy, and 1 was a homogeneous leukoplakia on the buccal mucosa with a pathologic diagnosis of epithelial dysplasia. They were stained with a faint blue color (Figure 2B).

Specificity suggests the proportion of pathologic benign lesions, neither precancerous lesions nor cancers, which are correctly identified as negative staining of methylene blue (Figure 3B). In our study, 20 of 29 benign lesions showed negative staining; thus, the specificity was 69%.

The results of staining with methylene blue for all lesions correlated well with the pathologic diagnosis and are summarized in Table 2. Fisher's exact test showed significant differences among cancer/precancerous lesions, benign lesions, and normal control groups ($p<0.05$). Overall, the positive predictive value was 74% (26/35), and the false predictive value was 87% (20/23).

Discussion

Oral cancer is very common in Southeast Asia, including Taiwan. Areca nut chewing is the main etiologic factor inducing carcinogenesis in oral mucosa. Individuals with all the habits of smoking, drinking and areca nut chewing were reported to have 123 times the risk of

Table 1. Clinical and histologic diagnosis of oral lesions and results of staining

	Clinical diagnosis							
	Homogeneous leukoplakia ($n=36$)		Nonhomogeneous leukoplakia ($n=11$)		Erythroplakia ($n=5$)		Ulcerations ($n=6$)	
Histologic diagnosis	+	–	+	–	+	–	+	–
Malignancy ($n=16$)								
Squamous cell carcinoma	10	–	–	–	3	–	1	2
Precancerous lesion ($n=13$)								
Dysplasia	4	–	3	–	1	–	–	–
Epithelial hyperplasia	–	1	4	–	–	–	–	–
Benign lesion ($n=29$)								
Epithelial hyperplasia	–	9	1	–	–	–	–	–
Hyperkeratosis	–	2	3	–	–	–	–	–
Lichen planus	3	7	–	–	1	–	1	2
Total ($n=58$)	17	19	11	–	5	–	2	4

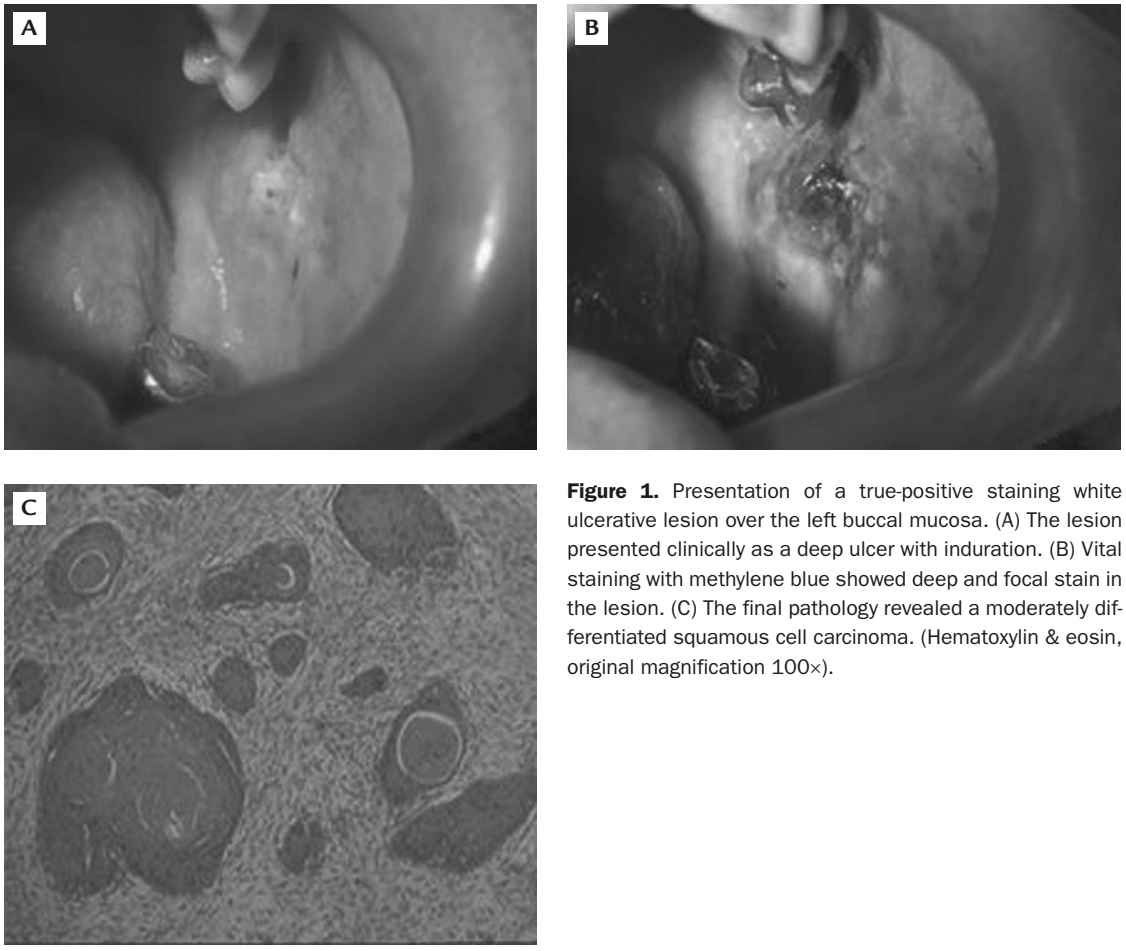

Figure 1. Presentation of a true-positive staining white ulcerative lesion over the left buccal mucosa. (A) The lesion presented clinically as a deep ulcer with induration. (B) Vital staining with methylene blue showed deep and focal stain in the lesion. (C) The final pathology revealed a moderately differentiated squamous cell carcinoma. (Hematoxylin & eosin, original magnification 100×).

people without such habits.[9] Thus, oral cancer screening of high-risk individuals is very important in these countries.

For a large-scale community screen, some dye materials help to identify abnormal mucosa tissue which raise oral examiners' attention and refer the patients with suspicious lesions to oral surgeons for further examinations. This vital staining method was used at first in medicine for detecting cervical dysplasia and carcinoma *in situ* in the 1960s.[10] Niebel and Chomet were the pioneers who used dye material to detect oral cancer in 1964.[11] Toluidine blue dye is known as 1 of the diagnostic adjuncts to detect oral cancer/precancerous lesions. The efficacy of this technique has been evaluated in many reports with diverse results. It has yielded sensitivities between 72% and 100%,[5–8] and specificities between 45% and 67%, in detecting suspicious malignancies. However, the Material Data Safety Sheet indicates that toluidine blue is probably toxic by ingestion, and it is seldom used for detecting cancers in other parts of the human body.

Methylene blue is another recently proposed dye for *in vivo* staining used in endoscopic examination. Its application has been reported recently in detecting some gastrointestinal abnormalities such as Barrett's esophagus,[12–14] gastric cancer,[15] prostate cancers,[16,17] and also bladder cancer.[18] The exact mechanism for the uptake of methylene blue dye in epithelial cells is still not very clear, but it resembles toluidine blue dye in its acidophilic characteristic and may penetrate into cells with an abnormal increase in nucleic acid, thus resulting in different uptake between normal and highly dysplastic/malignant cells.

Usage of the methylene blue technique in detecting oral cancer or precancerous lesions has not been reported thus far. Among all the statistical values, sensitivity rate and false-negatives are the most important in evaluating the efficacy of certain diagnostic tools for detecting abnormal lesions. In the current study, 26 of 29 pathology-proven precancer/cancer lesions showed positive staining with deep and focal methylene blue dye. Overall, 90% sensitivity (88% for malignancy and 92% for precancerous lesions) was reported, with a false-negative rate of 10%. Compared to the 72–100% sensitivity reported in the previous studies,[5–8] these values indicated that using methylene blue dye in the

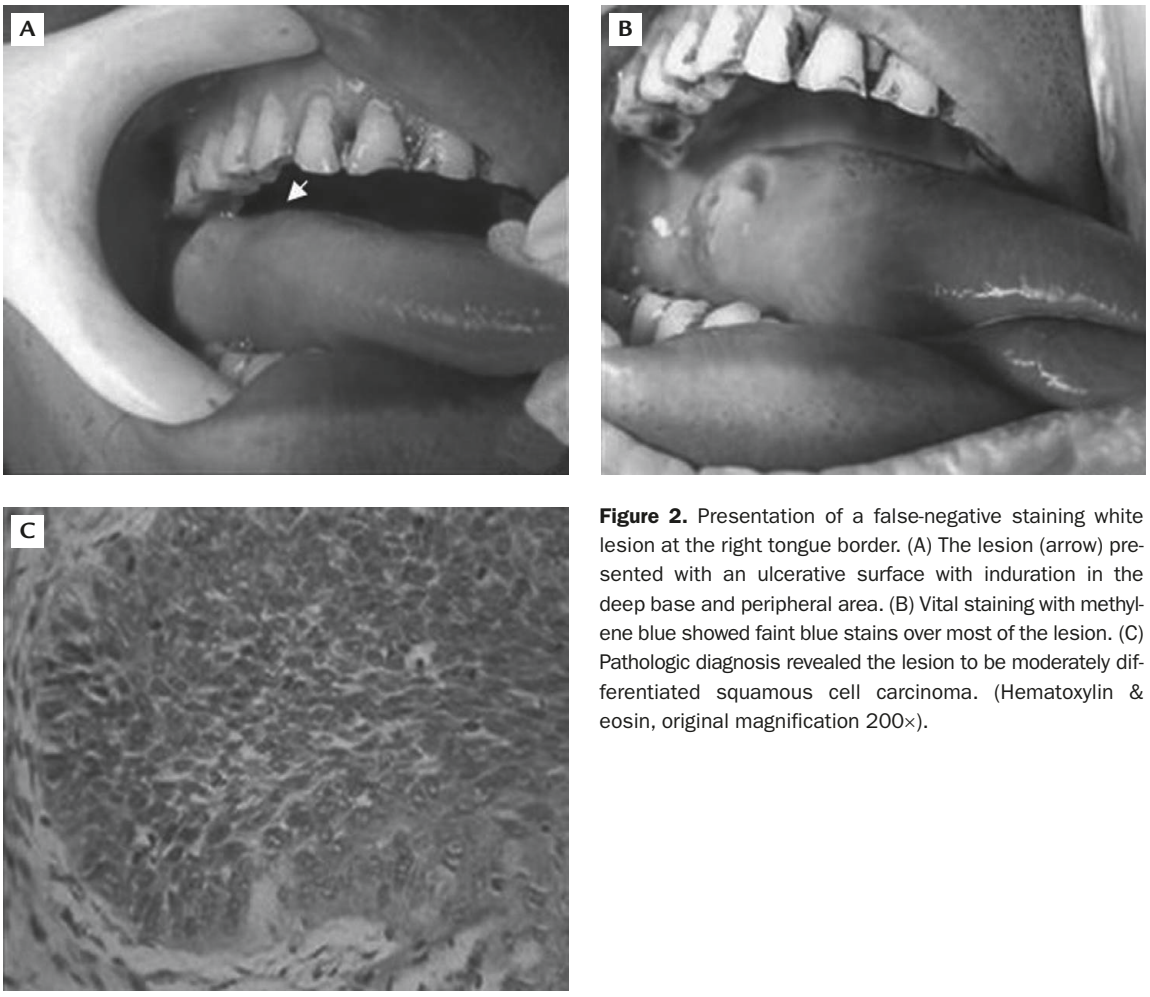

Figure 2. Presentation of a false-negative staining white lesion at the right tongue border. (A) The lesion (arrow) presented with an ulcerative surface with induration in the deep base and peripheral area. (B) Vital staining with methylene blue showed faint blue stains over most of the lesion. (C) Pathologic diagnosis revealed the lesion to be moderately differentiated squamous cell carcinoma. (Hematoxylin & eosin, original magnification 200×).

detection of cancer or precancerous lesions is acceptable. As for false-negatives, we consider that the ambiguous light blue stains which may be misinterpreted as negative but clinically suspicious of malignancy still need further biopsy to prove the diagnosis pathologically.

In the aspect of specificity, we obtained a value of 69% (20/29) with a resulting false-positive rate of 31%. The 9 false-positives were homogeneous leukoplakia ($n=3$), nonhomogeneous leukoplakia ($n=4$), erythroplakia ($n=1$), and an ulceration ($n=1$). The high false-positive rate was discussed to be related to the retention of stain in inflamed and trauma areas.[19] Other causative factors may include the irregular, papillary or digital surfaces of the lesions, which may cause the mechanical retention of dye, contamination of saliva and plaque, retention of dye material in papilla of the tongue or minor salivary gland ducts over the mucosa. The high number of false-positives in this study means that more patients received biopsies. Nevertheless, rational management for patients with suspected oral lesions who have either a positive or negative methylene blue stain remains biopsy of the lesion.

Applying this method to screen high-risk patients with the habits of betel quid chewing or smoking, a large group of individuals may include those with obvious oral lesions and those with normal oral mucosa. To study these people and to re-evaluate the efficacy of methylene blue in detecting oral cancers/precancerous lesions, a large proportion of the people with normal oral mucosa will lower the rate of false-positives and result in higher specificity. Although we had a control group with fully normal oral mucosa, there was a flaw in the experimental design that these students had no habits of betel quid chewing and histories of smoking. However, individuals who had these habits without lesions were also not very suitable to be our control group because performance of biopsy in normal mucosa would not be ethical.

In conclusion, this study shows that methylene blue staining has nearly 90% sensitivity in detecting oral cancers or precancerous lesions. Considering its low toxicity and the fact that it is cheaper than toluidine blue, it may be convenient to substitute it for toluidine blue in large-scale oral screening of high-risk

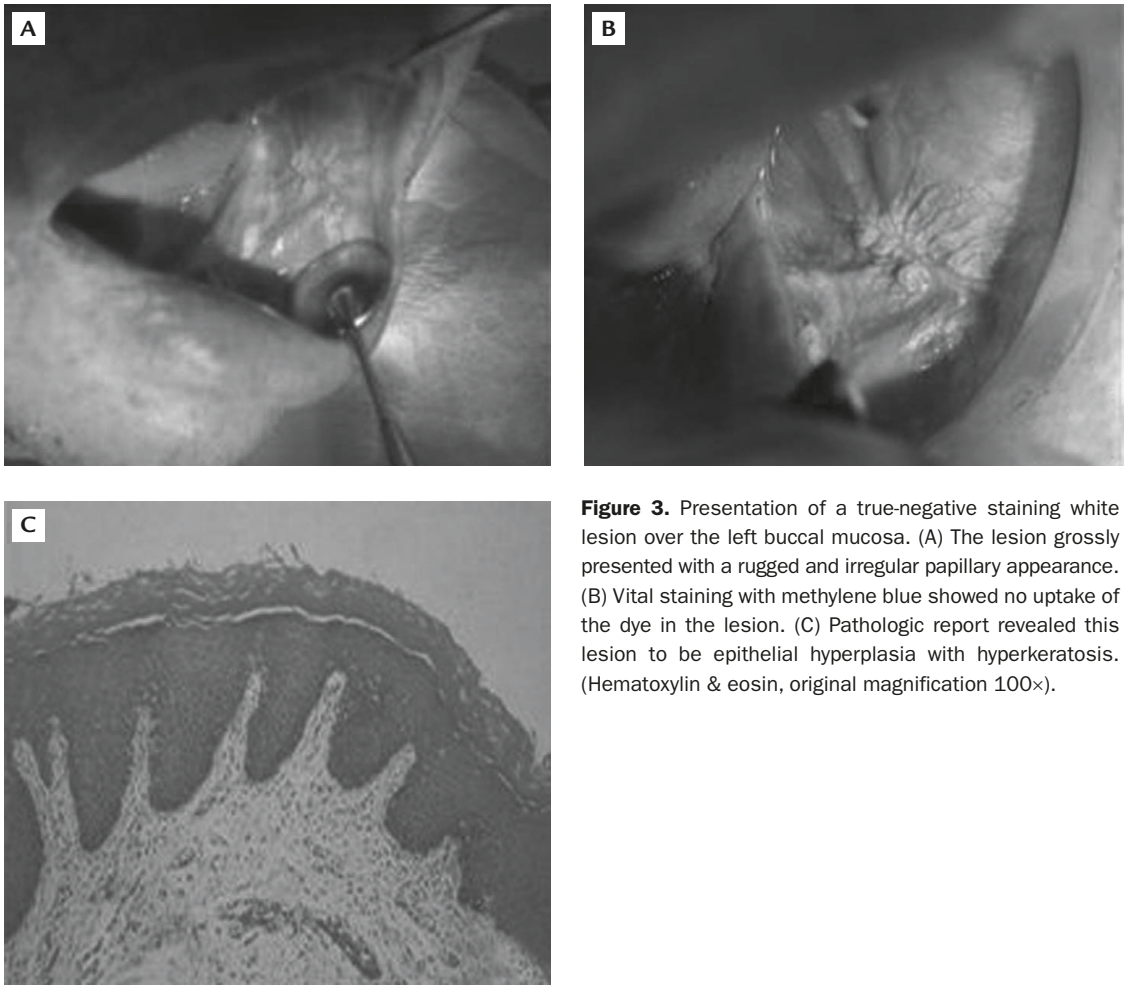

Figure 3. Presentation of a true-negative staining white lesion over the left buccal mucosa. (A) The lesion grossly presented with a rugged and irregular papillary appearance. (B) Vital staining with methylene blue showed no uptake of the dye in the lesion. (C) Pathologic report revealed this lesion to be epithelial hyperplasia with hyperkeratosis. (Hematoxylin & eosin, original magnification 100×).

Table 2. Efficacy of methylene blue application in pathologically proved cancer/precancerous lesion

Type of tissue	Positive (%)	Negative (%)	*p*	
Cancer/Precancer ($n=29$)	26 (90)*	3 (10)	<0.001	<0.001
Benign ($n=29$)	9 (31)	20 (69)†		
Normal ($n=20$)	0 (0)	20 (100)	0.0067	
Positive predictive value	26/35 (74)			
Negative predictive value		20/23 (87)		

**Sensitivity; †specificity.*

patients. Nevertheless, the pathology report from biopsy is still the gold standard to accurately diagnose the lesion before a treatment modality is determined.

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