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The utility of toluidine blue staining and brush cytology as adjuncts in clinical examination of suspicious oral mucosal lesions

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Abstract. The objective of this study was to investigate the utility of toluidine blue and brush cytology in patients with clinically detected oral mucosal lesions. Clinical examination of 35 patients was completed before toluidine blue application, oral brush cytology and scalpel biopsy. Lesions were photographed before and after stain application; followed by brush cytology. All findings were compared with histopathologic results. Severe dysplasia and carcinoma-in-situ were determined as 'positive'; no dysplasia and mild to moderate dysplasia were defined as 'negative'. The sensitivity, specificity, positive and negative predictive values of clinical examination and toluidine blue were the same: 0.923, 0.433, 0.414, and 0.929, respectively. Those of brush cytology were 0.923, 0.517, 0.462, and 0.938. The concordance of all methods was 30% for benign and 61% for malignant lesions. Adjuncts identified 92% of carcinoma-in-situ and squamous cell carcinoma as confirmed by histopathology, in contrast to clinical findings alone in which 62% of these lesions were identified ($p = 0.046$). In conclusion, adjunct diagnostic methods decreased the level of uncertainty for the diagnosis of oral malignancies and lichenoid dysplasias when applied as adjuncts to clinical examination.

Keywords: oral cancer; squamous cell carcinoma; toluidine blue; brush biopsy.

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It is anticipated that diagnosis of high-risk oral premalignant lesions (OPLs) and early stage cancer decreases the morbidity of treatment and mortality due to oral squamous cell carcinoma (OSCC)[14,29]. OPLs and oral premalignant disorders may present clinically as leukoplakia, erythroplakia[8,32], ulceration[16], oral submucous fibrosis[23], and oral lichen planus[1,6].

The malignant risk cannot be determined using standard clinical examination. Adjunct methods and devices have been introduced to improve detection, thereby promoting diagnosis[8,9,16,19,28,29,36]. The most evaluated adjunct for lesion detection is toluidine blue (Tblue). Tblue is a metachromatic dye that binds to deoxyribonucleic acid and retention has been associated

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with loss of tumor suppressor gene (TSG) loci on specific chromosomes. TSG loss has been shown to predict progression of OPLs to cancer. The utility of Tblue in the identification of OPLs and early diagnosis of OSCC[13,15,21,22,28,46], to assess margins of OPLs and SCC of the lesions before biopsy, to assist in biopsy site selection[4], and to accelerate the decision to biopsy has been examined. Retention of Tblue is also seen in ulcerated and potentially inflammatory lesions although the pattern of staining may be different, thus, retention may result in a false-positive outcome. In order to assist clinicians, a 2 week review of lesions not felt to be at high risk of cancer at first evaluation is suggested[22].

Oral exfoliative cytology has been evaluated since the 1950s as a method to collect epithelial cells in order to examine cell morphology under a light microscope. Exfoliative cell collection using a bristle-brush (brush cytology) has been reported to obtain a full thickness collection of epithelial cells including basal epithelial cells[18]. It is promoted as a fast, inexpensive and well-tolerated method that may reduce or increase the need for biopsies in clinically benign lesions[18,23,35,36]. While a number of reports have shown the efficiency of brush cytology for early detection of OSCCs[18,19,36], others reported large numbers of false-positive and potentially false-negative results[8,15,26,31] ranging between 30–84%[33] and 63% for dysplastic lesions[27].

The goal of the present study was to examine the utility of Tblue and brush cytology in patients with clinically detected oral mucosal lesions by comparing the results of Tblue application and the characteristics of brush cytology with the findings of scalpel biopsy.

Materials and methods

Thirty-five patients with oral mucosal lesions identified by the Orofacial Lesions Council of Ege University, İzmir, Turkey, were seen for further evaluation. Informed consent was obtained from the patients and thorough clinical head, neck and intraoral examinations were completed before Tblue application, oral brush cytology and scalpel biopsy. The clinical appearance, location and size of each lesion were recorded on a standard form; thus, all evaluations were performed on the same area of each lesion. All clinically identified lesions underwent biopsies irrespective of the findings with Tblue staining and the results of brush cytology. Scalpel biopsies were performed under local anesthesia following Tblue staining and brush cytology, without any significant delay (not more than 2 weeks) between the 3 methods of investigation. For 12 lesions, repeat evaluation was conducted after a 2 week period, while the remaining patients were evaluated in one visit. All clinical examinations were performed by the same examiner (P.G.) who is experienced in evaluating oral mucosal lesions and therefore the clinically suspicious nature of the lesions was affirmed in advance. Surgical biopsies were performed by an experienced oral and maxillofacial surgeon.

Clinical examination

A photograph of each lesion was obtained before and after the procedure using a digital camera (Olympus Camedia C-2500 L, Melville, NY, USA). An example of the lesions that were evaluated in this study is presented in Fig. 1. Lesions selected for further examination with Tblue staining and brush cytology were homogenous and nonhomogenous leukoplakia[2,26], reticular[1,2,38] or erosive/ulcerated lichenoid lesions[37], and superficial ulcerations suspicious of malignancy.

Toluidine blue staining

To decrease false-positive rates, a waiting period of 10–14 days after the initial clinical examination was conducted for lesions not highly suspicious of cancer. Potential causative agents (factors related to traumatic or inflammatory changes, including ill fitting dentures, non-hygienic/defective restorations, orthodontic brackets, cheek biting) were treated to prevent false-positive results with staining at follow-up. At recall, examination and tissue testing were conducted, including Tblue staining and brush cytology and tissue biopsy. Tblue was prepared as an oral rinse[22], since there is no pharmaceutical grade Tblue available in Turkey. Toluidine blue rinse (1%) was compounded at Faculty of Pharmacy, Ege University, as follows[21,26,31]: 1 g tolonium chloride powder (Merck KGaA, Darmstadt, Germany); 10 ml acetic acid (Merck KGaA, Darmstadt, Germany); 4.19 ml absolute alcohol (Merck KGaA, Darmstadt, Germany); and 86 ml of distilled water[31] without flavoring. The pH value of the solution was 4.5. One hundred milliliters of 1% acetic acid rinse was prepared by adding 1 ml glacial acetic acid to 99 ml distilled water[31]. The oral rinsing protocol was: 20 s pre-rinse with 30 ml of 1% acetic acid; 20 s water rinse; 20 s rinse/gargle with 10 ml of the 1% tolonium chloride solution; 20 s post-rinse with 30 ml of 1% acetic acid (twice); a final water rinse.

Each lesion was photographed before and after application of the Tblue and findings were recorded on the standard form. The pattern of dye retention and the intensity of stain retention were recorded (2, dark blue staining; 1, minimal blue staining; 0, no blue staining). Occasionally, normal mucosa also appeared light blue, but this staining was not interpreted as positive.

Brush cytology

Brush cytology was performed using a Cytobrush Plus GT (Medscand Medical AB, Malmo, Sweden) which was rotated on the lesion site with pressure, until pinpoint bleeding was observed. The harvested cells were transferred to a slide (SuperFrost Plus; Menzel, Braunschweig, Germany) by a 360° turning and rolling motion with the brush and the slides were

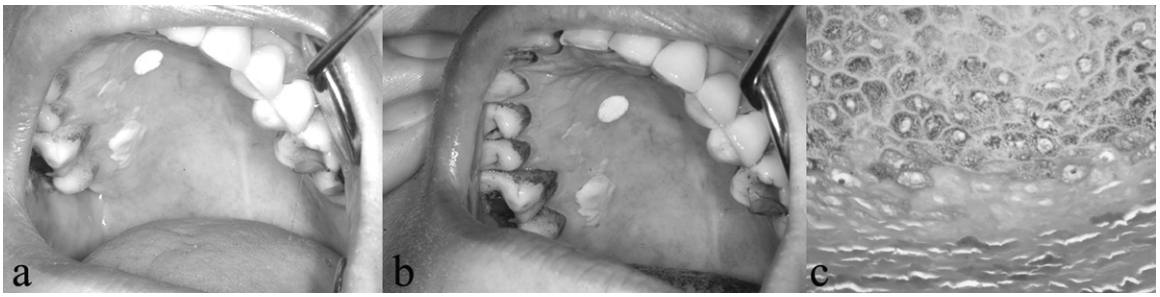

Fig. 1. A white hyperkeratotic lesion that was evaluated using clinical examination, Tblue staining, brush cytology and scalpel biopsy.

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washed rapidly with ethyl alcohol for fixation.

Cytology specimens were stained with hematoxylin–eosin, and examined by an oral pathologist (A.C.K.) who was experienced in oral cytology and was blinded to clinical findings. The brush cytology results were classified as malignant, atypical (suspicious), benign tissues or inadequate sample[35].

Scalpel biopsy

All lesions were subject to scalpel biopsy with selection based on the clinical appearance of the lesion. All areas retaining Tblue were biopsied; in sites with no retention of staining, clinical judgment guided the biopsy procedure. Multiple biopsies were performed in large lesions to represent the entire lesion based on clinical findings.

The scalpel biopsy specimens were submitted in formalin for hematoxylin–eosin staining. After embedding, 5 μm thick sections were prepared. Pathologic interpretation was based on established criteria[45] and classified as squamous cell carcinoma, epithelial dysplasia, hyperkeratosis, lichen planus, and other benign lesions.

Data analysis and statistics

Histological results were accepted as the gold standard and therefore, the interpretation of Tblue retention and the findings of brush cytology were compared with the histopathologic results. Lesions with severe dysplasia, carcinoma-in-situ and SCC were classified as 'serious pathology', lesions with no dysplasia were considered 'benign'. Mild and moderate dysplasias were referred 'non-serious pathology'[12]. In the present study, the summary statistics (accuracy, sensitivity, specificity, prevalence, true-positive/negative results, false-positive/negative results) were calculated to aid in the analysis of the diagnostic tests[8]. In these analyses, biopsies confirmed as severe dysplasia, carcinoma-in-situ or SCC were considered 'positive'; no dysplasia, mild and moderate dysplasia were 'negative'[12]. The significance of the distribution of malignant and benign lesions among tobacco users and non-users was evaluated using Fisher's exact test; the z test was used to compare proportions and p was set as 0.05.

Table 1. The diagnostic outcomes of the lesions provided by all 4 methods.

Lesions	Clinical assessment	Toluidine blue	Brush cytology	Histology	Histology cut-off
1	Suspicious	2	Serious pathology	Pleomorphic adenoma	Benign
2	Benign	0	Suspicious	Squamous hyperplasia	Benign
3	Benign	0	Suspicious	Squamous hyperplasia	Benign
4	Suspicious	2	Benign	Lichen planus	Benign
5	Suspicious	2	Suspicious	Lichen planus	Benign
6	Benign	0	Benign	Squamous hyperplasia	Benign
7	Benign	0	Suspicious	Squamous hyperplasia	Benign
8	Benign	1	Suspicious	Lichen planus	Benign
9	Benign	0	Benign	Inflammation	Benign
10	Benign	0	Suspicious	Squamous hyperplasia	Benign
11	Suspicious	1	Suspicious	Lichen planus	Benign
12	Suspicious	2	Benign	Nonspecific ulcer	Benign
13	Suspicious	2	Inadequate	Nonspecific ulcer	Benign
14	Suspicious	0	Suspicious	Nonspecific ulcer	Benign
15	Serious pathology	2	Benign	Pyogenic granuloma	Benign
16	Serious pathology	2	Benign	Reperative granuloma	Benign
17	Benign	0	Suspicious	Squamous hyperplasia	Benign
18	Suspicious	2	Benign	Pemphigus	Benign
19	Suspicious	0	Suspicious	Lichen planus	Benign
20	Suspicious	2	Benign	Lichen planus	Benign
21	Suspicious	2	Benign	Lichen planus	Benign
22	Serious pathology	2	Benign	Squamous hyperplasia	Benign
23	Benign	0	Benign	Squamous hyperplasia	Benign
24	Suspicious	1	Benign	Squamous hyperplasia	Benign
25	Benign	0	Benign	Squamous hyperplasia	Benign
26	Benign	2	Suspicious	Squamous hyperplasia	Benign
27	Benign	0	Benign	Squamous hyperplasia	Benign
28	Benign	0	Benign	Squamous hyperplasia	Benign
29	Suspicious	2	Serious pathology	Lichenoid dysplasia (mild dysplasia)	Non-serious pathology
30	Suspicious	2	Serious pathology	Lichenoid dysplasia (mild dysplasia)	Non-serious pathology
31	Serious pathology	2	Serious pathology	Verrucous carcinoma	Serious pathology
32	Suspicious	2	Serious pathology	Adenocarcinoma	Serious pathology
33	Suspicious	2	Serious pathology	SCC	Serious pathology
34	Serious pathology	1	Serious pathology	SCC	Serious pathology
35	Serious pathology	2	Serious pathology	SCC	Serious pathology
36	Serious pathology	2	Serious pathology	SCC	Serious pathology
37	Suspicious	2	Serious pathology	SCC	Serious pathology
38	Serious pathology	2	Serious pathology	SCC	Serious pathology
39	Serious pathology	2	Serious pathology	SCC	Serious pathology
40	Serious pathology	2	Serious pathology	SCC	Serious pathology
41	Benign	0	Benign	SCC	Serious pathology
42	Serious pathology	2	Serious pathology	SCC	Serious pathology
43	Suspicious	2	Serious pathology	SCC	Serious pathology

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Table 2. The assessment scores of 3 diagnostic methods with respect to histology results.

Histology		Clinical assessment			Tblue staining			Brush cytology*		
		Serious pathology	Suspicious	Benign	Serious pathology (Tb+)	Suspicious (Tb±)	Benign (Tb−)	Serious pathology	Suspicious	Benign
Benign	Pleomorphic adenoma	0	1	0	1	0	0	1	0	0
	Squamous hyperplasia	1	1	11	2	1	10	0	6	7
	Lichen planus	0	6	1	4	2	1	0	4	3
	Inflammation	0	0	1	0	0	1	0	0	1
	Nonspecific ulcer	0	3	0	2	0	1	0	1	1
	Pyogenic granuloma	1	0	0	1	0	0	0	0	1
	Reperative granuloma	1	0	0	1	0	0	0	0	1
	Pemphigus	0	1	0	1	0	0	0	0	1
Non-serious pathology	Lichenoid dysplasia	0	2	0	2	0	0	2	0	0
Serious pathology	Verrucous carcinoma	1	0	0	1	0	0	1	0	0
	Adenocarcinoma	0	1	0	1	0	0	1	0	0
	Squamous carcinoma	7	3	1	9	1	1	10	0	1

* One specimen was discarded from brush cytological examination because of inadequate sampling.

Results

Thirty-five patients (13 men; 22 women, mean age 56.2 years) with 43 lesions were enrolled. Most of the lesions were observed on the buccal mucosa (56%), the tongue (19%), and the hard palate (14%). Thirteen of 35 patients (37%) had malignant lesions and 61% of these patients were using tobacco. Twenty-two patients (63%) had benign lesions of which 38% were tobacco users ($p = 0.18$). Of 13 malignant lesions, 12 were SCC (92%) and 1 was adenocarcinoma (8%) (Table 1). The adenocarcinoma was located on the soft palate, adjacent to the maxillary tuberosity, presenting as a mucosal ulcer. Most of the 30 benign lesions were squamous hyperplasia (46%). One patient had bilateral buccal mucosal lesions, both with a suspicious clinical appearance, that were diagnosed histologically as lichenoid dysplasia having mild dysplastic features and were stained dark blue with Tblue stain, no other lesions were reported as dysplastic on histopathology (Table 1).

Clinical assessment

Of 43 samples, clinical examination suggested 14 benign (33%), 11 serious pathology (25%) and 18 suspicious (42%) lesions (Table 2). Eight of 13 malignant diagnoses (62%) and 13 of 28 histologically benign lesions (46%) were clinically diagnosed accurately. The two lichenoid lesions were considered 'suspicious' with clinical examination. The summary statistics of clinical examination were established as sensitivity 0.923; specificity 0.433; positive predictive value (PPV) 0.414; negative predictive value (NPV) 0.929 (Table 3).

Tblue staining

In 43 samples, Tblue stain was negative in 14 (33%), dark stained in 25 (58%) and pale stained in 4 (9%) lesions. Of 13 malignant lesions, 10 SCCs and 1 adenocarcinoma were dark stained (84%), 1 SCC was pale stained (8%). The total stain of 'serious pathology' was 92%, and 1 SCC did not retain stain (8%). Of 30 benign lesions, 14 were dark stained (46%), 3 were pale stained (10%) and 13 were not stained (44%) (Table 2). Both lichenoid dysplasias were dark stained (100%). The summary statistics of Tblue staining were determined as sensitivity 0.923; specificity 0.433; PPV 0.414; NPV 0.929 (Table 3).

Brush cytology

One sample (2%) contained inadequate number of cells for interpretation; the histological examination of that specimen yielded a benign diagnosis. Considering that an adjunct was anticipated to assist the clinician to determine the need for biopsy, this shortcoming was accepted as a false-positive for this specimen and all analyses were performed over 43 lesions thereafter. Among 42 lesions, brush cytology results indicated 16 benign (38%), 15 serious pathology (malignant) (36%) and 11 suspicious (26%) lesions.

Twelve of 13 histologically confirmed 'serious pathology' (92%) and 15 of 30 benign lesions (50%) were successfully classified using brush cytology, whereas 11 lesions including 2 lichenoid dysplasias were established as 'suspicious' (37%) (Table 2). The summary statistics of brush cytology were calculated as sensitivity 0.923; specificity 0.517; PPV 0.462; NPV 0.938 (Table 3).

Concordance of the methods

In this study, all methods yielded similar results in 9 histologically benign lesions (30%) and 8 histologically confirmed malignancies (62%). Clinical assessment

Table 3. Dichotomous analyses of diagnostic methods in order to provide sensitivity, specificity, positive and negative predictive values of the methods.

Histology	Clinical assessment		Tblue staining		Brush cytology*	
	Serious pathology + suspicious	Benign	Serious pathology + suspicious	Benign	Serious pathology + suspicious	Benign
Benign + non-serious pathology	17	13	17	13	15*	15
Serious pathology	12	1	12	1	12	1

* One specimen was omitted due to the lack of material to be examined and this benign lesion was considered as ''false positive'' with brush cytology.

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agreed with Tblue staining in 40% of benign lesions and in 62% of histologically confirmed cancers (Table 2). Its concordance with brush cytology was 30% for benign lesions and 62% for serious pathologies. Tblue staining and brush cytology methods predicted 43% of benign and 92% of the cancers (Table 3). Considering the adjunct tools in addition to clinical assessment, the 5 suspicious cases (3 OSCCs and 2 lichenoid dysplasias) decreased to 1 with Tblue application and to 0 with brush cytology. The adjuncts utilized (Tblue and brush cytology) identified 92% of the serious pathology accurately in contrast to 62% based on clinical examination ($p = 0.046$).

Discussion

None of the lesions included in this study was clinically undetectable and pathology identified malignant lesions, benign lesions and mild dysplasia without cases of moderate or severe dysplasia. Clinical estimations of the malignant potential of these lesions and determination of the biopsy sites/surgical margins were difficult. Adjunct methods may be used to assist in treatment planning for clinically identified lesions. The high number of OSCC in this sample confirms the high risk nature of the patients referred and enrolled in this study.

Following histopathological examination, 2 lesions were diagnosed as 'lichenoid dysplasia' since they presented characteristics of mild dysplasia as defined by WHO and had a bandlike lymphocytic infiltrate underneath dysplastic epithelium. Even though the term 'lichenoid dysplasia' is controversial[40], it has been used to describe lichen planus-like histopathological aspects in dysplastic lesions[40,41]. Considering the level of dysplasia[11], the rate for malignant transformation of lichenoid lesions[41], and the recent US Food and Drug Administration (FDA) guidelines on terminology and classification of potentially malignant lesions[43], these lesions were considered benign and further analyses were performed accordingly.

The classification of grade of dysplasia is variable[11,43,44], and may be more difficult when a dichotomous outcome is desired. Mild dysplasia (low-grade squamous epithelial lesion) is accepted as 'non-serious pathology' because of the potentially reversible nature of the lesion[12,43,44]. Establishing the malignant transformation potential of mild to moderate dysplasia is not possible based on histopathology alone[43,44]. The reliability of histopathology as a guide to estimate the malignant nature of a lesion continues to be debated. When all suspicious lesions were accepted as 'serious pathology'[30,34] and histologically 'non-serious pathologies' were included in the benign lesions category, 62% of serious pathologies and 43% of benign lesions were identified successfully with clinical examination. Ninety-two percent of serious pathologies and 43% of benign lesions were identified with Tblue. With dichotomous analysis, 92% of serious pathologies and 50% of benign lesions were identified with brush cytology. Using this classification of histologic diagnosis both adjuncts provided additional information and identified dysplasia and OSCC in 92%, compared with 62% based on clinical examination alone, even in clinically detected lesions referred to a speciality clinic.

Both of the lichenoid dysplasias with mild dysplastic features were identified with Tblue stain and brush cytology. The dark blue staining of the lichenoid lesions in these patients raised clinical concerns, of serious pathology; the molecular patterns of the lesions were not assessed, rather, the patient was followed up clinically. Several studies suggest that Tblue stained lesions present loss of heterozygosity (LOH) (allelic loss)[13,17,46]. Guo et al. reported that in addition to all SCC cases, 82% of carcinoma-in-situ or dysplasia and 59% of cases without dysplasia showed LOH in at least one marker[17]. Three-quarters of the lesions identified by Tblue were clonal and had the potential to progress to malignancy[17]. Zhang et al. showed that progression of OPLs to SCC was significantly higher in stain positive areas, with a 4-fold higher risk of progression to SCC even in lesions with benign histopathology or mild dysplasia[46]. After 44 months, 33% of the Tblue positive OPLs with or without dysplasia progressed to SCC, but this was observed in only 5% of the Tblue negative mucosal lesions ($p = 0.0002$)[46]. These findings suggest that Tblue positivity represents risk, even in lesions with benign histopathology, and suggest that false-positive Tblue results based on histomorphology alone, may represent molecularly true positive lesions with risk of progression to OSCC[46].

In studies investigating the efficacy of Tblue staining in oral mucosal malignancy diagnosis, some authors considered any uptake of blue dye as positive while others classified partial staining as positive or negative, or assigned to another category[28]. The sensitivity/specificity and PPV/NPV values vary between the reports; the ranges were sensitivity 38–98% (median 85%), specificity 9–93% (median 67%), PPV 33–93% (median 85%) and NPV 22–92% (median 83%)[7,8,10,13,21,22,26,28,31,38,42,46]. Gandolfo et al. reported 100% sensitivity, 0% specificity, with 50% PPV and 100% NPV[15]. The present results (sensitivity 92%, specificity 43%, PPV 41% and NPV 93%) fell within this wide range. It should be noted that Tblue staining was implemented only on clinically suspicious lesions rather than innocuous appearing ones and this study design is expected to influence the sensitivity and PPV of Tblue findings.

Brush cytology may be used in patients previously treated for OSCC or those who have widespread instability of oral mucosa[25,34]; but sampling errors (between 71 and 100)[24,25] affect the value of exfoliative cytology[19]. The sensitivity of Oral CDx ranged from 71 to 100% and the specificity was 25–94%[30,34,35,39]. The PPV of Oral CDx ranged from 33 to 86%[3,30,34,39]. The absence of scalpel biopsy results[28,35], the characteristics of the sample population[5,30], necrotic and/or super-infected OSCC lesions and the delay between brush cytology and scalpel biopsy may affect the results in Oral CDx studies[30,33,34,39]. In the present study, conventional exfoliative cytological examination was performed. The sensitivity, specificity and PPV values observed in this study (92%, 52% and 46%, respectively) were similar to previous Oral CDx studies. While it has been reported that false-negative rates of oral exfoliative cytology for oral cancer exceed 30%[35], the low false-negative rates (2%) reported here may be due to the irregular clinical features of oral lesions in this trial. Limitations in evaluating morphological features of disaggregated cells[4], and novel adjunct evaluation techniques, such as molecular assessment of cytologic samples should be considered[4,25,34].

In the current study, concordance between examination methods was greater for malignant diagnoses, although the adjuncts used identified carcinoma-in-situ and squamous cell carcinoma in 92%, in contrast to clinical appraisal which predicted 62% of these diagnoses. There was less concordance between examination methods in benign lesions. The sensitivity values of clinical examination, Tblue staining and brush cytology were comparable, although analysis that included dysplastic lesions and OSCC showed utility of the adjuncts studied in detecting histopathologic abnormality. The accuracy of benign lesion diagnosis was identical for

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clinical examination and Tblue (43%), and was slightly higher for brush cytology (51%). The first two methods may be more prone to subjectivity compared with brush cytology, because clinical examination and Tblue staining are qualitative methods that rely on visual perception, so the observers' shortcomings may affect the final clinical decision when these methods are utilized. The similar clinical appearance of many benign conditions may affect the performance of the subjective methods. Even though the diagnostic accuracy was higher for malignant lesions, standard clinical examination performed less well (62%) than Tblue (92%) and brush cytology (92%). The expertise of the clinician performing the clinical diagnoses might influence the accuracy of clinical examination[20]. This case series, including clinically suspicious lesions, as suggested by the high number of malignant diagnoses, had an impact on the outcomes. Application of adjunct diagnostic methods to clinical inspection decreased the level of uncertainty for diagnosis of OSCCs and lichenoid dysplasias and similar results were reported by Epstein et al.[12]. In contrast to brush cytology, which requires specimen collection and a laboratory procedure, Tblue is a noninvasive method that provides real-time clinical information that may assist in completing a biopsy, biopsy site selection and/or referral.

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Competing interests

The authors have no competing interests to declare.

Ethical approval

Ethical Approval was granted by Ege University Faculty of Medicine Clinical Research Ethical Committee; the verdict no. was 10-4.1/26.

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