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Original research

The influence of toluidine blue staining on decision-making for the selection of biopsy sites in oral disorders

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Purpose
The aim of this study is to evaluate the influence of toluidine blue (TB) staining on decision-making processes for the identification of biopsy sites in oral lesions.

Materials and Methods
Original and TB-stained images of oral mucosal lesions that required histopathological examination were accessed through archives. Twenty dental specialists were asked to select and mark their choice of biopsy sites on both original and TB-stained images using a standard circle selection tool of software. The X and Y coordinates of the center points of selections were calculated by a custom-made image processing algorithm in Matlab. In order to assess the dispersity of the selected centers from the mean center, the distances of the selected points from this average point were calculated using Euclidean distance. Data was analysed using Wilcoxon test.

Results
The study involved original and TB-stained images of 5 suspicious lesions. The histological diagnoses of oral lesions were reported as bening for 3 cases and malignant for 2 cases. 20 dental specialists marked their choice of biopsy site on a total of 200 images. The results revealed that the biopsy selections varied significantly between original and TB-stained images in certain cases. In 60% of cases, specialists showed statistically significant agreement in biopsy site selection on TB-stained images compared to original images ($p < 0.05$).

Conclusion
While the study did not evaluate the accuracy of site selection against a gold standard, the observed consensus among specialists suggests the potential utility of TB staining in enhancing the consistency and objectivity of biopsy site selection for suspicious oral lesions.

Keywords: *Oral mucosal lesion, tissue biopsy, biopsy site selection, vital staining, toluidine blue*

Introduction

Oral carcinogenesis is a multi-step process where normal tissue progresses through a series of oral potentially malignant disorders (OPMDs) including varying degrees of dysplasia, carcinoma in-situ and finally oral cancer. OSCC lesions are predominantly preceded by precursor lesions that can be white (leukoplakia) or red (erythroplakia). Additionally, other inflammatory disorders of the oral mucosa such as lichen planus and submucous fibrosis have been associated with an increased risk of OSCC development (1). Some potentially malignant lesions are relatively common (1-5%) (2,3), may appear innocuous, and be easily overlooked during intraoral screening and examination (1). Currently the clinical standard for monitoring oral abnormalities is visual inspection followed by histopathological examination in suspected cases. Visual examination is subjective, and it is challenging for general dentists to discriminate between

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benign and malignant lesions, as well as determining the lesion borders (4). This is underlined by the fact that most of the patients diagnosed with OSCC had received visual examination for oral cancer screening and been pronounced healthy within last years prior to being diagnosed (5).

A variety of non-invasive commercial diagnostic aids and adjunctive techniques for screening/detection of oral premalignant lesions have been developed in the past decades, claiming to enhance oral mucosal examinations and facilitate the detection of early cancerous mucosal changes that can be occult to visual inspection (6). However, incisional or excisional scalpel biopsy and histopathological examination are still considered as the gold standard for diagnosis of OPMD and OSCC (7-9). One of the most important drawbacks of tissue biopsy is the fact that oral cancer is subjected to the "field cancerisation phenomenon", having the highest risk of development of second primary tumors of any cancer (10). When dentists perform biopsy, they visually select the biopsy site or sites and there is considerable risk that they either miss additional sites of cancerization that are not visible to the naked eye and/or biopsy a site that is not representative of the most severe pathology that is present (11). In particular, large or multiple lesions are challenging for clinicians during selection of biopsy site, which is influenced by subjective criteria such as knowledge and professional experience (11). To obtain a tissue sample that will reflect the histologically altered oral epithelium is of vital importance in order to avoid false negative results (12). The presence of nodular, verrucous or indurated areas within the lesion may facilitate biopsy site selection compared to early-stage lesions which may be overlooked by clinicians due to lack of clinical suspicion. These early lesions may require utilization of adjunctive methods before tissue biopsy for more precise results (13). TB staining, a chair-side, non-invasive and inexpensive method has been evaluated as an adjunct before selection of biopsy site in a number of studies (14-16). Due to its affinity against nucleic acids, TB binds to mucosal areas with high nucleic and mitotic activity (13,17). The wide intracellular canals in dysplastic and malignant tissues increase the penetration of the dye, and suspicious lesions appear as dark blue, an indication of increase in nucleic material (17).

The sensitivity and specificity of TB staining in detecting the premalignant/malignant oral lesions by using histopathologic assessment as the gold standard has been evaluated in a number of studies, and the results suggest that TB staining as an adjunctive method may be utilized as a useful diagnostic tool in detecting early OPMLs and malignant lesions (17-23). On the other hand, despite the available literature on the diagnostic efficacy of TB staining, its impact on consensus in the determination of biopsy site selection for oral suspicious lesions has not been previously explored. Therefore, the aim of this study is to examine the influence of TB staining on the decision-making process of observers concerning the selection of biopsy sites for oral suspicious lesions, employing an objective analytical methodology.

Materials and Methods

Ethical approval

The study protocol was approved by the Ethics Committee of the Medical Faculty of Ege University (Protocol No: 16-2/47) and was performed in accordance with the Declaration of Helsinki.

Data collection and vital staining protocol

Intraoral images from patients who applied to outpatient clinic of Ege University, School of Dentistry, Department of Oral and Maxillofacial Radiology with a suspicious oral mucosal lesion that requires histopathological examination were enrolled. After through intra and extra oral examination, as a part of routine clinical protocol, intraoral images are obtained under standart conditions from all patients that apply to the outpatient clinic with an oral mucosal lesion. In order to facilitate biopsy site selection, the lesions are stained with TB and imaged again before referral to surgical departments for tissue biopsy. Vital staining was performed using acetic acid and TB solutions as previously described (8). After rinsing the mouth with water to remove debris, 1% acetic acid solution (30 ml), water and 1% TB solution (10 ml) were applied for 20 seconds, respectively. The regions with dark blue were accepted as "positive staining" The patient data, intraoral images and histopathological diagnosis are recorded in the patient file in the archive. The archive was screened retrospectively to identify TB-stained and unstained images of oral mucosal lesions. The inclusion criteria were high quality images and the presence of 2 intraoral images from the same lesion before and after vital staining with TB. The selected images were accessed using a software (Adobe Photoshop CS6) and a total of 20 specialists with a mean experience of 15 years from Oral and Maxillofacial Radiology and Oral Surgery Departments were asked to select and mark their choice of biopsy site on both stained and unstained images using a standart circle selection tool (5 mm). The assessments were performed in an under dimmed light using an 18.5-inch LED monitor (Asus VS197DE; ASUSTek Computer Inc.Taipei, Taiwan) with a resolution of 1366 x768 pixels and 0.3 mm pixel pitch.

Analysis of observers' biopsy site selection

For the purpose of comparison and analysis, distinct colors were assigned to the marked biopsy sites, each corresponding to a specific specialist. Subsequently, these marked sites were overlaid onto a single image, facilitating visual representation of the concurrent biopsy site selections made by different specialists (Figure 1).

This methodology facilitated the assessment of observers' decisions and the identification of any disparities or consen-

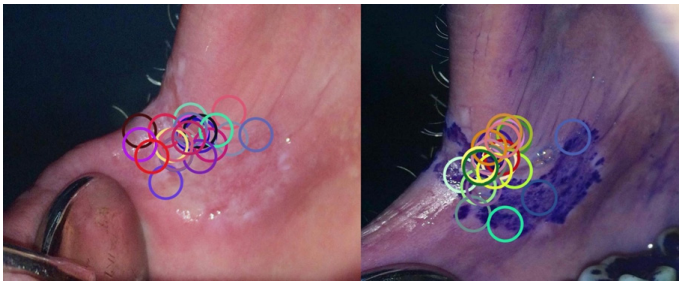

Figure 1. *Twenty specialists preferred biopsy site selections on the original and TB-stained images of the same lesion (Case 5).*

sus among the specialists' choices. Threafter, centers of the circular marks were identified, and the X (longitude) and Y (latitude) coordinates were calculated by a custom-made image processing algorithm in Matlab (The MathWorks, Inc., USA) (Figure 2).

Mean and standard deviation of X and Y coordinates were calculated for TB-stained and original images, separately. Standard deviations were normalized due to differences in the calculated means. Subsequently, in order to assess the dispersity of the individual centers from the mean center, the distances of the selected points from this average point were calculated using Euclidean distance, and statistical properties of these distances for each lesion were determined (Figure 3).

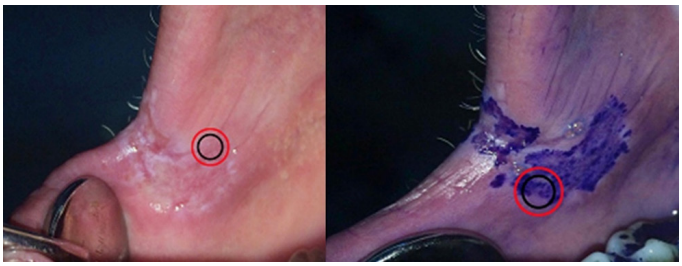

Figure 2. *The markings provided by the code on the original and TB-stained image, displaying the assessment of the observer's selection for the biopsy (Case 5).*

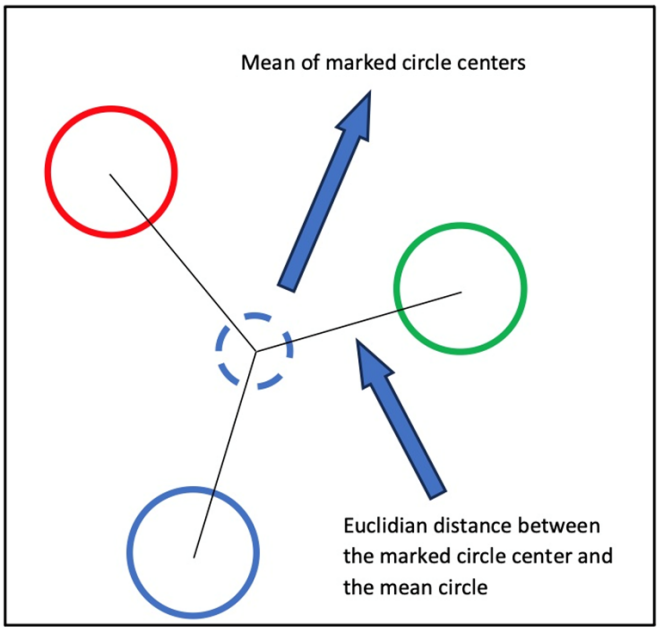

Figure 3. *Euclidian distance between the mean center and individual centers.*

Statistical analysis

Statistical analysis of the data was performed using SPSS Statistics, Version 25 (IBM Corp., Armonk, NY, USA). The Wilcoxon ranked test was then applied to assess whether there was a statistically significant difference between the mean values of these two distance data sets for both conditions. P-value was set as 0.05.

Results

A total of 10 original and TB-stained images from 5 lesions were evaluated by 20 specialists, leading to 200 images for further data investigation (Figure 4).

The histological diagnoses of oral mucosal lesions were reported as bening disorders for 3 cases (Cases 1, 3, and 5) and malignant for 2 cases (Cases 2 and 4). The differences in biopsy site selections on the original and TB-stained images of the lesions varied in each case. For Case 1, the cumulation of the selected biopsy sites on the original and TB-stained images differed significantly (p=0.01414). This was also observed for Cases 3 and 4 as well (p=0.03658, and p=0.006099, respectively). On the other hand, the biopsy site selections did not vary significantly on the original and TB-stained images for Cases 2 and 5 (p=0.3408, and p=0.08853, respectively) (Table 1). When all cases were considered, TB staining of oral mucosal lesions provided higher consensus on the biopsy site selection for 3 cases (60%, p<0.05). For the remaining 2 cases, staining the lesions with TB did not affect the biopsy site selection of 20 observers (30%, p>0.05).

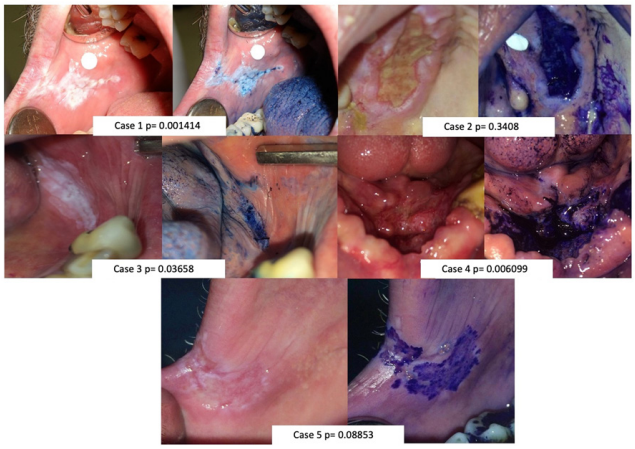

Figure 4. *Original and TB-stained images of all oral lesions with the significance of the differences between their site selections for the biopsy (white round paper was used as a calibration material to provide geometric and illumination standardization of the images).*

Table 1. *The distribution of selected biopsy sites with respect to the mean of center point coordinates, provided with administration of Euclidean distance analysis. (* p<0.05).*

	Histopathological diagnosis	Original images	TB stained images	p
Case 1	Bening disorder	111.983±17.388	178.400±17.910	0.01414*
Case 2	Malignant	174.001±30.033	143.229±24.105	0.3408
Case 3	Bening disorder	315.661±20.828	86.805±12.037	0.03658*
Case 4	Malignant	203.139±28.658	96.990±16.077	0.006099*
Case 5	Bening disorder	81.446±13.602	107.394±13.457	0.08853

Discussion

The exploration of field cancerization has become a critical concept in recent molecular studies, as it reveals the significance of oral cancers' distinct and intricate nature, characterized by a range of genetic and molecular changes (10). Field cancerization, also referred to as field effect or field defect, describes the presence of abnormal tissue in the tumor region, which can lead to local recurrence or the development of secondary tumors, even after the complete removal of the initial tumor and its surrounding region. It was reported that the molecular abnormalities observed in the mucosal field neighboring the tumor have a direct impact on the survival rate of oral cancer patients (10,24). In a case study by Tsui *et al*. (15), development of two genetically unrelated OSCCs within a 10 mm area of lateral tongue was demonstrated, indicating the extremely dynamic field effect of oral cancer. Additionally, the authors reported that a nodular area, 25 mm adjacent to the primary lesion which appeared as normal during dental examination using incandescent light and conventional dental instruments, exhibited dark staining when examined using a light-based detection system (Velscope-dark brown) and vital staining (TB-dark blue) (15). These findings underscore the high likelihood of abnormal tissue or potential cancerous alterations occurring in the surrounding oral mucosa of the primary lesion. They emphasize the importance of field cancerization, highlighting the need for supplementary diagnostic techniques when determining biopsy locations, ensuring sufficient surgical margins, and monitoring suspicious lesions during follow-up (15). Others underlined that the patients diagnosed with OSCC had received visual examination for oral cancer screening and been pronounced "healthy" within the last years prior to being diagnosed with OSCC (5). Indeed, these findings highlight the necessity of employing adjunct diagnostic techniques to accurately identify appropriate biopsy locations, ensure an adequate surgical margin, and effectively monitor the suspicious mucosal lesion area during the follow-up period. By utilizing additional diagnostic tools, healthcare professionals can enhance their ability to detect any potential abnormal tissue or cancerous alterations in the surrounding oral mucosa, thus improving the precision and effectiveness of treatment strategies. This comprehensive approach that encompasses visual examination with adjunctive techniques helps to minimize the risk of incomplete excision and optimize patient outcomes (15).

Vital staining with TB has been utilized for the diagnosis of cervical dysplasia and carcinoma since 1960s, and there have been numerous studies in the literature evaluating its sensitivity, specificity, as well as positive and negative predictive values (8,18-23). The sensitivity of TB staining ranges from 51% to 92.6%, while the specificity is reported as 66-100% (8,18-23). It is important to mention that the majority of studies have shown a considerable variation in the values of sensitivity and specificity which can be attributed to specific factors, including the likelihood of positive TB staining in ulcerative and inflammatory lesions, thereby augmenting the count of false-positive cases. Conversely, in hyperkeratotic lesions, the thickness of the oral epithelium poses a challenge for TB to penetrate deeper layers effectively, resulting in an elevated number of false-negative cases. Nevertheless, recent meta-analyses have demonstrated that the diagnostic accuracy of TB for detecting OPMLs and oral cancer surpasses that of clinical examination alone (20,21). Therefore, it can be recommended as an adjunctive technique in combination with other methods. TB staining is also used for assessement of margins during surgical treatment for oral cancer to ensure a "safe" margin around the tumor (25-27). A number of studies compared the performances of TB staining and frozen biopsy sections during surgery and reported that TB is less specific but more sensitive than frozen sections for detecting positive mucosal margins of resected OSCC (26), and may increase the precision of the operating team in excising the dysplastic tissue surrounding the lesion (25). To the best of our knowledge, the evaluation of TB staining's influence on the selection of biopsy sites for suspicious oral lesions has not been previously assessed. Therefore, in this study, the aim was to provide preliminary information on this issue by examining both the original and TB stained intraoral images of suspicious oral mucosal lesions that had histopathological examination. A total of 20 specialists individually assessed both the original and TB stained images, and indicated their preferred biopsy sites utilizing a software. The dispersity/proximity of these sites in either one of the test conditions was evaluated by using the X (longitude) and Y (latitude) coordinates of each selected site. The dispersity observed in the TB-stained images indicates concordance among specialists in 60% of the cases, highlighting the effectiveness of TB as a tool to facilitate a more consistent and objective approach in selecting biopsy sites. In this investigation, the impact of TB staining on the observers' decisions regarding the biopsy site selection was evaluated with objective measures. However, it's essential to note that the efficacy of staining in terms of the accuracy of site selection against a gold standard was not aimed. The main limitation of the present study is the relatively small sample size, which may restrict the generalizability of the findings. However, despite this limitation, the preliminary results are promising and provide valuable insights. The observer consensus observed with the use of TB staining in the selection of biopsy sites for suspicious oral mucosal lesions in 60% of the cases indicates its potential as a valuable adjunctive technique, but further studies with larger sample sizes are required to validate these findings and to assess the reproducibility of the results. A significant strength of this study is its unique perspective, distinguishing it from previous research in the field. As the first of its kind in the literature, it provides valuable and objective findings that contribute to the existing knowledge base. The study also highlights the potential for further refinement in the study design, allowing for continuous improvement and future investigations, opening up opportunities for additional research to build upon these preliminary findings and explore the topic in greater depth.

Conclusion

This study aimed to investigate the impact of TB staining on the decision-making process for selecting biopsy sites in suspicious oral lesions. The findings revealed a significant difference in biopsy site selections between TB-stained and original images for certain cases, emphasizing the potential influence of TB staining. TB staining demonstrated a higher consensus among specialists in 60% of cases, suggesting its

utility in facilitating a more consistent and objective approach to biopsy site selection. However, it is important to note that the accuracy of site selection was not assessed against a gold standard in this study. Despite the relatively small sample size, the preliminary results are promising and provide valuable insights. This unique perspective contributes to the existing knowledge base, highlighting TB staining as a potential adjunctive technique. Further studies with larger sample sizes are warranted to validate these findings and assess reproducibility, paving the way for continuous improvement and deeper exploration of this topic in future research.

Türkçe Öz: *Ağız Hastalıklarında Toluidin Mavisi ile Boyamanın Biyopsi Yeri Seçimine Karar Verme Sürecindeki Etkisinin Değerlendirilmesi. Amaç: Bu çalışmanın amacı Toluidin mavisi (TM) ile boyamanın, oral lezyonlarda biyopsi yeri seçimine karar verme sürecindeki etkisini değerlendirmek. Gereç ve Yöntem: Çalışmaya histopatolojik inceleme gerektiren oral mukozal lezyonların orijinal ve TM ile boyalı arşiv görüntüleri dahil edildi. 20 adet uzman diş hekiminden biyopsi alanı olarak belirledikleri bölgeleri, yazılımın standart yuvarlak seçim aracını kullanarak hem orijinal hem de TM ile boyalı görüntüler üzerinde işaretlemeleri istendi. Uzmanlar tarafından belirlenen biyopsi alanlarının merkez noktalarına ait X ve Y koordinatları Matlab'da özel bir görüntü işleme algoritması ile hesaplandı. Seçilen merkezlerin ortalama merkez etrafındaki dağılımları ve ortalama merkeze olan uzaklıkları Öklid mesafesi kullanılarak belirlendi. Veriler Wilcoxon testi kullanılarak analiz edildi. Bulgular: Çalışmaya 5 adet şüpheli oral lezyona ait orjinal ve TM ile boyanmış görüntüler dahil edildi. Bu lezyonların 3'ü histopatolojik olarak benign, 2 olgu ise malign olarak rapor edildi. 20 adet uzman diş hekimi toplam 200 adet görüntü üzerinde biyopsi alanı olarak belirledikleri alanları işaratledi. Sonuçlar bazı olgularda orijinal ve TM boyalı görüntüler arasında biyopsi alanı seçimi önemli ölçüde farklılık olduğunu ortaya koydu. Olguların %60'ında, uzmanlar biyopsi yeri seçiminde TM ile boyalı görüntülerde orijinal görüntülere kıyasla istatistiksel olarak anlamlı şekilde fikir birliği sergilediler ($p<0.05$). Sonuç: Bu çalışmada biyopsi yeri seçiminin doğruluğu bir altın standartla karşılaştırılmamış olmakla birlikte, TM ile boyanan lezyonlarda uzmanlar arasında biyopsi alanı seçimine yönelik gözlenen konsensus, şüpheli oral lezyonlarda TM ile boyamanın biyopsi yeri seçiminde tutarlılığı ve objektifliği arttırmadaki potansiyel katkısını ortaya koymaktadır. Anahtar Kelimeler: oral mukozal lezyon, doku biyopsisi, biyopsi yeri seçimi, vital boyama, toluidin mavisi*

Ethics Committee Approval: The study protocol was approved by the Ethics Committee of the Medical Faculty of Ege University (Protocol No: 16-2/47) and was performed in accordance with the Declaration of Helsinki.

Informed Consent: Participants provided informed constent.

Peer-review: Externally peer-reviewed.

Author contributions CG, BI, BOG, PG participated in designing the study. CG, BI, PG participated in generating the data for the study. CG, BI, GB, PG participated in gathering the data for the study. BI, BOG, PG participated in the analysis of the data. CG, BI, PG wrote the majority of the original draft of the paper. CG, BI, PG participated in writing the paper. CG, BI, BOG, GB, PG has had access to all of the raw data of the study. CG, BI, BOG, PG has reviewed the pertinent raw data on which the results and conclusions of this study are based. CG, BI, BOG, GB, PG have approved the final version of this paper. CG, BI, BOG, GB, PG guarantees that all individuals who meet the Journal's authorship criteria are included as authors of this paper.

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