Toluidine Staining as a Screening Tool for Soft Tissue Margins of Cancerous Oral Lesions

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Abstract

Background:

As the world is becoming advanced, the number of diseases also seems to be increasing. Mouth cancer and oral lesions are known as dangerous and fatal diseases. The experimentation is in the process. The problem is not new; it has been the cause of deaths of many patients since many decades. The increase in cancer patients has been recorded up to 43% in the last 8.1 years and it has been observed that most of the cancerous cells are malignant at the time of diagnosis and treatment.

It is mandatory for the physician or the dental experts to diagnose oral cancer in the early stage and remove the tumor along with safe margins to decrease the chances of reoccurrence of the disease.

The objective of the study is to detect the usefulness of the toluidine blue dye as a cost effective, less time consuming and a user-friendly screening tool for the tumor margins intra-operatively.

Methods:

Fifty patients with biopsy proven oral squamous cell carcinoma who were advised surgical resection as a treatment plan, independent of the grade and stage of the tumor, by the department of Oral and Maxillofacial surgery of Dow University of Health Sciences were considered to undergo intra-operative toluidine blue dye staining and the results were compared and confirmed by the frozen biopsy section of the same margin.

Results:

Total fifty patients were participated in this study involving multiple margins. 86% agreement was present between the dye and the biopsy and the sensitivity of the dye appears to be 74% as 20 cases out of 27 showed true positive results yet specificity of the dye remains 100% as all 23 cases displayed true negative results.

Conclusion:

Toluidine blue dye has demonstrated to be a safe, convenient and time saving method of screening of the margins as it takes less than 5 minutes in its application and result starts to appear in within 3 to 5 minutes. Yet bigger lesions are yet to be focused as they have chances of giving false results. Moreover there is room for research in this aspect as the screening character of this dye is yet to be proved.

Background

Every year 350,000 to 400,000 new identified cases of oral cancer are seen worldwide, making it the major public health problem. Different incidences and cases are seen that are different in countries and geographical areas but mostly it is observed commonly in developing countries.² Oral and pharyngeal

cancer constitutes 2% of the malignant tumors or malignancy. The diagnosis and treatment of oral cancer can decrease the death rate associated with the treatment and ensures long-term survival.³ Delay in the diagnosis of oropharyngeal cancer may be linked with poor prognosis.⁴ When lesions start to show symptoms they often complain of pain, bleeding, a mass, dysphagia or odynophagia. However, before the patients become symptomatic the lesions keep growing or metastasizing.⁵ The Late arrival of the patient is important as advanced disease requires more radical treatment and association with poor prognosis.⁶

The eighth most common cancers worldwide out of ten is the squamous cell carcinomas of the oral cavity (OSCCs) accounting for 3-5% of all the malignancies. Oral cancer is associated with high death and morbidity rate mostly due to late diagnosis with less than 50% patients cured as in early stages it is asymptomatic and in advance stages, it shows many symptoms at the time of diagnosis. Early detection and identification of oral cancer are critical and crucial to increasing the survival rate. Identification and detection of oral potentially malignant disorders allow delivery of interventions to reduce the evolution of these disorders to malignancy. A variety of new and emerging diagnostic aids and adjunctive techniques are currently available. Delay in diagnosis includes two stages. The first period is the time from the onset of symptoms to the initial visit to a dental or medical professional is called the patient delay. Second is the professional delay which is the time during which the patient is under the care of the doctor and final diagnosis has been made that is the tumor is confirmed histologically to be malignant.⁷

The diagnosis is followed by prompt surgical removal of the lesion with safer margins as to decrease the chances of re-occurrence to zero which practically seems to be impossible. There is a need to identify active soft tissue margins of the tumor intra-operatively so that they can be eradicated. Frozen section biopsy is the most common method used at present with a sensitivity as high as 88.8% and sensitivity 98.9%.^{8,9}

Frozen sections biopsy may not be used frequently by the surgeons as it costs approximately RS 5000-7000 for margins. Prior to the surgery patient is informed and asked to pay the expense of biopsy which they might not afford.¹⁰ Secondly, frozen sections should be delivered to the pathologists within 10 minutes therefore department of pathology should be present in the same institute or near by the operation theatre vicinity or else it cannot be performed accurately. It has been observed that the techniques have been improving with the intervals of time but the actual problem arises when the doctors use different techniques without having proper knowledge of their effectiveness and use. The recent techniques include the use of toluidine blue, ViziLite Plus with Blue, Vizilite and Orascopic DA yet biopsy remains the gold standard.¹¹

The above-mentioned information reveals that the cases of oral cancer patients are increasing day by day and the techniques for the treatment are not showing effective results yet. The risk factor of the disease is more in male than female and is more common in the people above forty years of age. This research will help in determining whether toluidine will prove an effective screening technique for oral cancer or not. Toluidine blue is used in staining tissues rich in DNA and RNA because it contains high affinity for acidic tissue components and it is basic thiazine

Metachromatic dye. It has many applications including staining living tissue and a special stain due to its metachromatic property. This dye is widely incorporated in vivid to identify dysplasia and carcinoma of the oral cavity. It is mainly used to highlight components such as mast cells granules, mucin, and cartilages. It is also called toluidine blue which is known because of its selectively staining acid tissue components such as sulfates, carboxylates and phosphate radicals.¹²

Toluidine blue dye has been proved to be an expensive and simple diagnostic tool for the oral lesions.¹³

Methods

It is an analytical cross sectional study conducted in the Dental OPD in the Department of Oral Surgery at Dow University of health sciences. All the diagnosed cases of oral squamous cell carcinoma which were undergoing surgery were considered as sample while patients allergic to dye, previous oral cavity surgery, ongoing radiation therapy were excluded from the sample

A sample size of 40 patients has been calculated. Using PASS version 11 test for one sample sensitivity and specificity with 95% confidence interval and 80% power of the test, dye prevalence of 19.6%¹⁴ with 100% sensitivity and 84.9%¹⁵ specificity including 5% margin of error, an estimated population size of 45 patients during 5-6 months, the calculated sample size is 40.¹⁶ Purposive sampling technique has been used for sampling.

The procedure was explained to the patient and an informed consent was signed by both the patient and the guardian undertaking that they allow us to perform the concerned procedure. The collected data was only utilized in this study and will be kept private and confidential. There was no differentiation on the basis of gender, ethnicity and religion.

After the approval from the internal project committee IRC the data was collected. Patients appearing in the dental OPD of Dow University of Health Sciences with suspicious lesion on oral mucosa were the subject for study. Patients were selected regardless of their age, race and gender. Patients who appear with the intention of treatment and follow up were entertained. Undertaking was signed by the patient prior to application of the toluidine blue dye.

Perform the following test:

After the resection of the major bulk of the tumor procedure for the dye application involved irrigation of the margins with normal saline and 1% acetic acid was applied to it for 20 seconds. Dried softly with the available helping material and then 1% TB was applied for 30 seconds. The cotton swab was used for the purpose. Next, the saline solution was used again gradually and 1% acetic acid was applied to slow the extent of retained stain. Then perform biopsy of the same lesion

Frozen biopsy was performed from the dye retained regions of the margin and the specimens was sent to histopathology labs within 10 minutes.

As the time of transfer of the specimen to the lab was minimum therefore there weren't any significant effect on the cell growth.

The cost of the frozen section was bared by the researcher. A record of the biopsy report was maintained to compare the results.

Data were analyzed using SPSS24. The tables and graphs were formatted by using excel 2013. The demographic data includes age and gender. The gender is represented by the frequency count and percentage whereas the age has been represented by the mean and standard deviation. In addition to it the study setting is also represented by frequency count and percentage. Moreover the distribution of the sites is analyzed by chi-square goodness of fit test and the distribution of sites in relation to lymph nodes is analyzed using chi-square test for association. The demographic component like age in relation to lymph nodes is also analyzed by chi square test for association whereas age and site in comparison to tumor staging is graphically representation.

The sensitivity and specificity of the dye was also calculated using cross tables comparing the result of both the dye and the biopsy and the agreement between the dye and the biopsy was calculated by AC1 GWETZ statistics.

Results

The study comprises of fifty patients with their approved consent, out of which 6 (44%) patients were female and 44 (88%) were male with an average age 39.56 ± 7.514 . The sites which were considered for sample collection included buccal mucosa, tongue, retro-molar trigon, both the upper and lower alveolus and floor of the mouth. The most common site involved was buccal mucosa as out of 50 cases 24 (48%) involved buccal mucosa whereas second most common involvement was of retro-molar trigon with 12 (26%) patients followed by lower alveolus with 8 (16%) patients and floor of the mouth with 5 (9%) patients (Table- 01).

Table 1
DISTRIBUTION OF SITES

Site	N (%)	P-Value
Buccal mucosa	24 (48)	0.001**
Retro-molar trigon	12(26)	
Lower alveolus	8(16)	
Floor of the mouth	5(9)	
Upper alveolus	0	
Tongue	0	
**Significant at 1%		

Thirty six (72%) samples were collected from the operation theatre of Dow University of Health Sciences OJHA Campus and 14 (28%) from the operation theatre of Civil Hospital Karachi respectively (Table-02).

Table 2
DEMOGRAPHIC
REPRESENTATION

N=50	
Age (years)	
Mean $\pm$ SD	39.56 $\pm$ 7.514
Min - Max	21 - 55
Hospitals	
Ojha Campus	36 (72%)
Civil Hospital	14 (28%)
Gender	
Male	44 (88%)
Female	06 (12%)

From these patients there were 32 (64%) of T3, 10 (20%) of T3 and 8 (16%) patients of T4 (Table-03).

Table 3
DISTRIBUTION OF
SATGE

Stage	N (%)
T2	32(64%)
T3	10(20%)
T4	8(16%)

In order to observe the association between the age and site with respect to lymph nodes, association test were applied but the result remained insignificant illuminating that there is no major association between them as displayed in tables: 4 and 5.

Table 4
DISTRIBUTION BETWEEN SITES AND LYMPHNODES

SITE	LYMPHNODE INVOLVEMENT			P-VALUE
	LEVEL 1	LEVEL 2	LEVEL 3	
Buccal Mucosa	4.2	29.2	66.7	0.815~
Retro-molar Trigon	0	23.1	76.9	
Lower Alveolus	0	50	50	
Floor of the Mouth	0	40	3.3	
~Cells proportion>20%				

Table 5
DISTRIBUTION BETWEEN AGE AND LYMPHNODES

LYMPHNODES	AGE GROUP		P-VALUE
	<40	≥40	
No Lymph node Involvement	0	1	0.381**
Level 1	7	9	
Level 2	19	15	
**Significant at 1%			

Age and site of the tumor with respect to the stage of the tumor is graphically represented to give an idea of their frequency of occurrence. (Figure 1 and 2)

50 patients participated in the study from which 27 patients were those who stained positive for toluidine blue dye. Out of these 27, 20(40%) patients were those whose frozen biopsy report confirmed that dysplastic cells are present. While 7(14%) patients result were not supported by biopsy result.

While in all the cases where toluidine blue stain produced negative result the biopsy result supported it as all 23 cases which produced negative dye result were supported and confirmed negative by the biopsy.

Therefore the sensitivity of the dye appears to be 74% as 20 cases out of 27 showed true positive results yet specificity of the dye remains 100% as all 23 cases displayed true negative results. (Table no 6)

Table 6
CROSS TABLE FOR SENSITIVITY AND
SPECIFICITY

	DYE (+)	DYE (-)	TOTAL
BIOPSY (+)	20	0	20
BIOPSY (-)	7	23	30
TOTAL	27	23	50
SENSITIVITY: $20/27 \times 100 = 74\%$			
SPECIFICITY: $23/23 \times 100 = 100\%$			

The observed agreement between the toluidine blue dye and frozen biopsy, which was manually calculated by true positive and true negative results, was 86%. Subsequently the value of statistical analysis by AC1 GWETZ statistics was estimated 0.72 (0.098) which describes that there is a strong agreement between the dye and biopsy, therefore this can be deduced that toluidine blue dye be able to be used as a substitute of frozen biopsy section intra-operatively.

Discussion

Surgical resection of the oral squamous cell carcinoma is considered to be the primary treatment of choice while chemotherapy and radiotherapy may also aid in fighting against the disease. Yet remains biopsy is the gold standard diagnostic tool.¹⁷ While going through a metastatic review about toluidine blue dye Patton deduced that it is suitable to use toluidine blue dye as a diagnostic agent but it is not appropriate to say that it is a reliable screening agent due to lack of research based evidence.¹⁸ However prior studies of some very old times states that toluidine blue dye can be used as a screening adjunct in population where oral squamous cell carcinoma is a high risk disease.¹⁹ A study held by E. Allegra and fellows check the efficiency of diagnosis based on toluidine blue dye and recommended that 77.7% results which were negative in the study were those lesions which were histologically benign and had no dysplastic cells present.²⁰

Keeping in mind the spread of the disease through lymphatic system and recurrence rate, complete resection of the involved soft and hard tissue is very important. In order to assure that there are no dysplastic margins left behind which may be the cause of recurrence, surgeons emphasis on leaving behind margins free of dysplastic cells. For this purpose intra-operative frozen biopsy is carried out which demands quick transfer of the sample to laboratory along with high cost.²¹ This study was carried out in a tertiary care center where cost for each frozen section per margin costs 6000 to 7000 Pkr. As being a developing country the low socioeconomic status should be taken into consideration because most of the patients cannot bear the cost of frozen biopsy therefore, there is a need to find one fine, user friendly and cost effective replacement which does not require the involvement of laboratory skill. Moreover the

laboratory transfer of the frozen section extracted from the tumor margin takes around 10–15 minutes. This contributes to increase in the time required to complete the surgical procedure.²²

In view of current situation this study was carried out to find, if not better, than at least equivalent substitute of frozen biopsy. This study outshines the fact that toluidine blue dye can not only be used as a diagnostic tool but also for screening purposes for tumor margins intra-operatively. The results reveal adequate agreement and association between the two; frozen biopsy and toluidine dye however there are also cases of where results were false positive. Yet false positive results may also be due to traumatic resection of margins.²³ Nonetheless true positive and true negative cases outshines the result.

A similar study was carried in in Agha Khan University in the year 2013 in which the use of toluidine blue dye as screening tool was highlighted. The study was carried out on 50 patients and results revealed that in smaller lesions where tumor size is T1 and T2 the results were reliable and true positive while false positive results were in T3 lesions where there were more chances traumatic handling margins as the size of the mass to be excised was bigger. Same as this study the site predominantly involved was buccal mucosa yet no attention was paid towards the histopathological grading, nodal involvement, age and the data was collected from only one tertiary care center. Yet both the studies have male predominance in gender.²⁴

Oral cancer is such a vast topic which regardless of all the research and evidence of surgery still needs to be explored for innovation with respect to the diagnosis and its treatment. A lot of research has been done on toluidine blue dye to prove its diagnostic ability yet a lot research and a large cohort study is still required to verify its screening ability as there is not enough evidence to support this cause.

Conclusion

Use of toluidine blue dye intra-operatively is easy, quick and a user friendly method of screening the margins of oral squamous cell carcinoma. It is a very cost effective method of screening in comparison to frozen section biopsy. Moreover it is effective method for use in underdeveloped and developed countries where the facility of frozen biopsy the not readily available or is not present in the vicinity. Taking in to consideration the time and cost of frozen section biopsy, toluidine blue dye should be considered as a strong candidate to substitute it yet one should also keep in mind the probability of false positive in large lesions. However, more research on a large scale involving more tertiary care centers and a larger sample should be carried out to resolve the controversies on this topic.

Declarations

Ethics approval and consent to participate

This STUDY was approved by the Institutional Internal Project Committee of Dow University of Health Sciences (Ref: DIKIOHS/PROJ-APPROVED/19/03-01). For all subjects consents were obtained.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AA: Conceived the study, principal investigator and a major contributor in data collection, manuscript writing and literature search.

AHS: Co-investigator assisted in the selection of title, analysis and interpretation of data

WAF: Statistical analysis

SF: Contribution in data collection

All the authors have read and approved the manuscript

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Figures

Figure 1

Distribution of age with respect to tumor stage

Figure 2

Distribution of site with respect to tumor stage