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Original article:

Detection of malignancy of oral cavity leukoplakia using toluidine blue stain

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Abstract:

Background: Oral cancers when caught an early stage is often curable, inexpensive to treat and affords better quality of life (QOL) for affected person over time. In order to assess the malignancy, potential various adjuncts have been tested, out of that toluidine blue staining is used in this study.

Aim: To assess the malignancy potential of oral cavity leukoplakia (OL), using toluidine blue stain.

Settings and Design: Hospital based study (prospective)

Material and Methods: Total of 100 subjects were selected for study out of which, 79 were males and 21 were females of age group ranging from 21 years to 70 years. The entire 100 patients have one or more addiction of tobacco and its' products.

Results: 36% patient failed to retain the stain they all were benign, 64% patients retained the stain, staining pattern observed was of uniform light blue with diffuse boundaries. Out of 64 dye positive patient, 56 were dysplastic including malignant and 8 were benign. Sensitivity of toluidine blue in determining the dysplastic changes was 100% and specificity was 81.81%. In the present study the specificity of Toluidine blue in leukoplakia lesions was 81.81%.

Conclusion: Toluidine blue staining is highly reliable source for the detection of dysplasia and carcinoma. Staining with these stains is an adjunct to clinical judgment and not a substitute.

Key Words: malignant tumors, oral cavity, precancerous lesions, toluidine blue.

INTRODUCTION:

Although the oral cancer accounts for 3.6% of all malignancy there are approximately 27,000 new cases and 9,000 death each year which would lead to more than 1,00,000 individuals suffering from disease in population in any given year. Less than 40% of oral cancers are diagnosed at early stage as it is difficult to detect either to patients or inexperienced doctor, thus making high mortality rates unfortunately, oral cancer is usually detected when it becomes symptomatic and, at this stage, at least two thirds of the patients present an advanced disease. This requires treatment which gives rise

to a high rate of morbidity and mortality and, furthermore, early detection of oral pre-malignant lesions is important to improve the survival rate and quality of life [1].

In many cases, clinicians have difficulty in recognizing patients at high risk of developing oral cancer. The major problem is when and where the biopsy should be taken from suspected lesions and this depends on the clinical ability to differentiate pre-malignant lesions from reactive and inflammatory diseases [2].

Furthermore, in many cases, this may be very difficult because often, when a white patch or plaque, like a clinically defined leukoplakia, is observed, it is difficult to define it as another disorder (inflammatory or reactive). In a lesion that appears as a leukoplakia, in 16% of the cases, the lesion is already malignant [3]. Various techniques have been developed to support clinical examinations, with the aim of improving early oral cancer diagnosis.

Oral cancers when caught an early stage is often curable, inexpensive to treat and affords better quality of life (QOL) for affected person over time. With this aim number of techniques have been developed to supplement clinical examination and thus improve diagnosis of premalignant and early malignant lesion [4].

In the present study, the use of toluidine blue staining was taken into consideration to identify clinically doubtful oral lesions and to compare the clinical evaluation with toluidine blue stain and with the histological evaluation.

MATERIALS AND METHODS:

The study was carried out during May 2009 to May 2011 period. A total of 100 patients were selected, of which, 79 were males and 21 were females. The study was approved by institutional ethics committee. All patients of leukoplakia visiting OPD, admitted in wards and diagnostic camp cases were included in the study during a period. There are no specific criteria for age, sex etc.

The study subjects were comfortably seated in the dental chair and examined under artificial illumination. Examination was carried out following the methods described by Kerr DA, Ash MM, Millard [5] and the relevant data were entered into proforma. After obtaining Informed consent from every subject to perform a biopsy prior to application of toluidine blue stain.

PREPARATION OF 1% TOLUIDINE BLUE DYE STAIN---• Toluidine blue 1gm

• Acetic acid 10 ml • Absolute alcohol 5 ml • Distilled water 86 ml • pH adjusted to 4.5

STAINING PROCEDURE [6]

1. After recording the clinical features and photograph of clinically suspicious lesions, the lesion areas were applied prior with 1% acetic acid with cotton bud for 20 seconds. The procedure was repeated one more time and further rinsed with water.
2. Toluidine blue was applied with cotton bud for 10 to 20 seconds.
3. The lesion was decolorized with 2% acetic acid using cotton bud for 20-30 seconds and photograph was taken

Interpretation of the Toluidine blue stain

Dark blue was considered as positive for lesions suspicious of malignancy, light blue retention was considered as positive for premalignant lesions unless proved otherwise by biopsy ,and lesion without retention was considered negative [7].

BIOPSY--Incisional biopsy obtained from the lesion following the methodology of Reichtar PA [8] for the all study subjects and preserved in 10% formalin for histopathological diagnosis.

Histopathological grading for Leukoplakia, Speckled Leukoplakia and Erosive as suggested by **Axell T, Pindborg , van der Waal I [9]** which were grouped on the basis of degree of dysplasia into those with no dysplasia, mild

dysplasia, moderate dysplasia and severe dysplasia.

STATISTICAL ANALYSIS: Data was analyzed into percentage, Chi-square test and was used to compare the groups and assess the association. Diagnostic validity tests listed below were performed to determine the utility of test results for predicting the various condition of the disease. **Sensitivity** [10] and **Specificity [8]** of test were calculated. Also positive predictive value (PPV) and negative predictive value (NPV) calculated. **Diagnostic validity / Diagnostic accuracy [11]:** Expresses the ability of a test to separate or distinguish those who have the disease from whom, do not.

RESULTS:

The present study was done to assess the malignant potential of OLs using toluidine blue staining adjuncts. All 100 study subjects who were undergone all procedure analyzed. The data obtained from the study were entered into master chart. The data from master chart further statically analyzed.

TABLE 1 Male and Female distribution of study subjects:

Sex	No. of Cases	Percentage
Male	79	79%
Female	21	21%

TABLE 2 Age wise distribution of study subjects:

Age in years	No. of cases	Percentage
< 20 year	0	0%
21 to 30	6	6%
31 to 40	21	21%
41 to 50	20	20%
51 to 60	18	18%
61 to 70	25	25%
> 70	10	10%
Total	100	100%

In 100 study subjects of leukoplakia 79(79%) were male patient and 21(21%) were female patient. Out of 100 study subjects no one was below 20 year age .6% patient from 21to 30 year, 21% were from 31to 40 years of age, 20% patients from 41 to 50 years of age, 18% patients from 51 to 60 years of age ,25% of patients from 61 to70 years of age, 10% of patients were more than 70 years of age.

TABLE 3 Site wise distributions:

Site of lesion	No. of Cases	Percentage
Buccal Mucosa	58	58%
Tongue	17	17%
Gums	10	10%
Floor of Mouth	6	6%
Lip	9	9%

Out of 100 study subjects most common site was buccal mucosa (58%), 17% patient having lesion on tongue, 10% patient having lesion on gums, 6% patient having lesion on floor of mouth, 9% patient having lesion on lip.

TABLE 4 Type of addiction

Sr. No.	Type of addiction	No of Cases	Percentage
1	Tobacco of lime	30	30%
2	Alcohol	15	15%
3	Pan	07	7%
4	Smoking	02	2%
5	Gutkha	11	11%
6	Tobacco lime and Alcohol	03	3%
7	Tobacco lime and Pan	13	13%
8	Tobacco lime and Smoking	03	3%
9	Tobacco lime and Gutkha	05	5%
10	Alcohol and Pan	05	5%
11	Smoking and Gutkha	06	6%
	TOTAL	100	100%

All the 100 study subjects have one or more addiction. Tobacco and its products are major addiction. Out of 100 patients 30% patient have addiction of tobacco of lime,15% have alcohol as addiction, 7% have pan as addiction , 2% have smoking as addiction and 11% have gutkha as addiction. 3% have both tobacco lime and alcohol as addiction, 13% have tobacco lime and pan as addiction, 3% tobacco lime and smoking as addiction, 5% have tobacco lime and gutkha

as addiction, 5% have alcohol and pan as addiction,6% have smoking and gutkha as addiction.

TABLE 5 Distribution according to Duration of Symptoms

Duration of Symptoms	Number	Percentage
Less than 6 months	13	13%
6months-1year	19	19%
1-2years	16	16%
2-5years	16	16%
5-10years	18	18%
More than 10years	18	18%

Out of 100 study subjects studied 13% patients showed symptoms less than 6 month, 19% patient showed symptoms between 6 month to 1 year,16% patient showed symptoms between 1 to 2 year,16% patient showed between 2 to 5 year,18% patient showed symptoms between 5 to 10 year and more than 10 year.

TABLE 6 Diagnostic accuracy of toluidine blue

Dye Retention	Histological Diagnosis		Total
	Dysplastic+Malignant	Benign	
+	56	8	64
-	0	36	36
TOTAL	56	44	100

Of 100 study subjects 36% benign patients were failed to retain the stain, whereas 64% patients retained the stain. Staining pattern observed was of uniform light blue with diffuse boundaries.

Out of 64 dye positive patient, 56 were dysplastic including malignant and 8 were benign.

Sensitivity of toluidine blue in determining the dysplastic changes was 100% and specificity was 81.81%. The positive predictive value was 87.5% and negative predictive value was 100%. Diagnostic accuracy of toluidine blue staining in distinguishing early leukoplakia was 92%

TABLE 7 Distribution according to HPR report

Type of Lesion	Number	Percentage
Benign Lesion	44	44%
Mild Dysplasia	22	22%
Moderate Dysplasia	18	18%
Severe Dysplasia	9	9%
Positive for Malignancy	7	7%

Out of 100 study subjects of leukoplakia HPR report 44% benign lesion, 22% have mild dysplasia, 18% have moderate dysplasia, 9% have severe dysplasia and 7% have positive for malignancy.

TABLE 8 Association of duration of symptoms and HPR report

Duration	Benign (%)	Mild (%)	Moderate (%)	Severe (%)	Positive for HPR (%)	Total (%)	χ^2 (p value)
Less than 6 months	12 (27.3)	0 (0)	1 (5.6)	0 (0)	0 (0)	13	76.054 (0.000)
6months-1 year	15 (34.1)	3 (13.6)	1 (5.6)	0 (0)	0 (0)	19	
1year-2years	8 (18.2)	6 (27.3)	1 (5.6)	1 (11.1)	0 (0)	16	
2years-5years	5 (11.4)	7 (31.8)	3 (16.7)	1 (11.1)		16	
5years-10year	3 (6.8)	3 (13.6)	9 (50)	1 (11.1)	2 (28.6)	18	
More than 10 years	1 (2.3)	3 (13.6)	3 (16.7)	6 (66.7)	5 (71.4)	18	
Total	44 (100)	22 (100)	18 (100)	9 (100)	7 (100)	100	

Out of 100 cases of leukoplakia more than 50% of benign HPR report has symptom duration up to 1 year. More than 50% of mild dysplasia patient has symptom duration 1 to 5 year. In case of moderate dysplasia 50% of patient has symptom duration 5 to 10 year. In case of severe dysplasia 66.7% has symptom duration more than 10 year.In case of positive for malignancy patient, 28.6% patient has 5 to 10 year symptom duration and 71.4% patient has symptom duration more year. And P value is significant (0.00).Association of symptoms duration and HPR report s/o as duration of

symptoms increases degree of HPR reports also increases.

DISCUSSION:

The concept of two-step process of cancer development in the oral mucosa, i.e., the initial presence of a precursor (premalignant or pre-cancerous) lesion subsequently developing into cancer is well established [12] and the early detection of oral mucosal epithelial dysplasia could potentially halt the progression of these lesions into malignant transformation. Thus, establishment of useful and objective techniques adjunctive to clinical judgments and microscopic diagnosis have contributed to the control of oral cancers [13].

In vivo staining reveals cytological details that might otherwise not be apparent, however staining can also reveal where certain chemicals or specific chemical reactions are taking place within cells or tissues [14] and thus aid in accelerating biopsies, diagnosis and treatment. Toluidine blue, an acidophilic metachromatic dye of thiazine group selectively stains acidic tissue components (sulfates, carboxylates and phosphate radicals) thus staining DNA and RNA. It is used as an in vivo stain based on the fact that dysplastic and anaplastic cells may contain quantitatively more nucleic acids than normal tissues. Also malignant epithelium may contain intracellular canals that are wider than normal epithelium, which may facilitate penetration of the dye [5].

The present study of TB staining in premalignant lesions was seen to be highly efficient in the detection of dysplasia, with a sensitivity of 100%. This result was in accordance to the findings of Silverman Jr S, Migliorati C, Barbosa J [15] who reported a sensitivity of 100% with Toluidine blue stain in Precancerous lesion. Another study by Epstein JB, Scully C and Spinelli JJ [5] reported a value of 93% sensitivity in detecting premalignant lesions, which was in correlation to our study. However our results of sensitivity differed from the findings of Mashberg A [16] and Warnakulasuriya Kaas,Johnson NW [17] who reported a sensitivity of 25% and 79.5% respectively.

Though the staining techniques used by Mashberg A [16] was similar to our study the difference in the values could be attributed to interindividual differences in considering the staining pattern, as light blue staining was considered negative by the author. The difference in sensitivity between our study and as reported by Warnakulasuriya Kaas, Johnson NW [17] can be attributed to the difference in the methodology of the staining techniques as the authors study was carried on with Toluidine blue mouth rinse while our study was carried out with application of Stain to suspicious areas. Inability of the rinse to disclose lesions in the posterior areas might have minimized the sensitivity of TB rinse as compared to our study.

In the present study the specificity of Toluidine blue in leukoplakia lesions was 81.81%. Our result was differed to the findings of Mashberg A [16] Warnakulasuriya Kaas who reported the specificity value of 92% in premalignant lesion. Our results of specificity also differed from the findings of Onofre MA, Sposto MR, Novarro CM Scully C [18], Johnson NW [17] who reported values of 44% and 62% respectively. The difference in specificity between our study and as reported by Mashberg [16] can be attributed to the difference in methodology carried out by Mashberg as he insisted a 10 to 14 day waiting period for all the lesions so that inflammatory lesions resolved before application of the stain.

In our present study PPV, NPV and diagnostic accuracy of toluidine blue stain was 87.5%, 100% and 92% respectively which were in accordance to the findings reported by Silverman JR S, Migliorati C and Barbosa J [15] who reported values of 90% of PPV, 92% of NPV and 90% of Diagnostic Accuracy. Another

study conducted by Epstein JB, Scully C, Spinelli JJ [5] reported a PPV and DA of 84% and 83% respectively which were in correlation to our study while the NPV showed a significant difference of about 20%. This can be attributed to the false negative results of the stain i.e. failure of dye to retain in dysplastic / malignant lesions and these results have significantly reduced the NPV in the author's study. Bhattacharya I, COHEN DM, Silverman JR S [18] reported that: Leukoplakia frequently affected the men with predominant sites being buccal mucosa, vermilion border of lip and gingival Mashburg A, Samit AM [7] and Silverman S JR [5] reported that leukoplakia is a disease of increasing age and approximate 90% of cases occur in people older than an 40 years with an average age at diagnosis of approximately 60 years. Neville BW, Damm DD, Allen CM, Bouquot JE [19] suggested that the most common intraoral site for cancer was tongue (>50%) usually the posteriolateral and ventral surface, followed were floor of the mouth (35%), lip vermilion, soft palate, gingival and buccal mucosa. They further stated that males were three times more affected than females. IN our study also male are more (79%) affected than females (21%). IN our study also leukoplakia more common in age more than 40 year, approximate 73% of patients are more than 40 year.IN our study most common site of leukoplakia is buccal mucosa(58%) followed by tongue (18%).

In another study by Jerry e. bouquet and Robert J Gorlin [20] in American white shows that leukoplakia was the most common of all lesions diagnosed and Americans over 35 years of age and was twice as high for males as for females. In another study by Roed Peterson, P.C. Gupta, J.J Pindborg [21] showed that males are most commonly affected in leukoplakia. Incidence of leukoplakia increases with age. Buccal mucosa and labial commissure is common site for leukoplakia followed by lip.

In our study all the 100 patients have one or more addiction. Tobacco and its products are the major addiction. IN another study by Roed Peterson, P.C. Gupta, J.J pindborg [21] showed that tobacco is major etiological factor and people having smoking habits have labial commissure as commonest site, people having chewing habits Buccal mucosa is commonest site (71.7%) of leukoplakia.

CONCLUSION:

It was also found that Toluidine Blue staining is highly reliable source for the detection of dysplasia and carcinoma. Staining with these stains is an adjunct to clinical judgment and not a substitute. Incidence of leukoplakia increases with age .It is commonly seen in 40 to 70 years of age. It is four times common in male .As duration of leukoplakia increases its degree of dysplasia or malignant potential increases, so that early detection and diagnosis of leukoplakia is important.

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