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RESEARCH ARTICLE

EFFICACY OF IN VIVO STAINGING IN THE EARLY DETECTION OF ORAL POTENTIALLY MALIGNANT AND MALIGNANT DISORDERS IN COMPARISON WITH EXPRESSION OF Ki-67 PROLIFERATIVE MARKER

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ABSTRACT

Introduction: Oral squamous cell carcinoma has a potential fatal outcome that accounts for 90% of oral cancers diagnosed worldwide. A variety of new emerging diagnostic aids and adjunctive techniques are currently being used in the screening of oral potentially malignant disorders (OPMDs). In vivo Staining methods namely, toluidine blue(TB), and methylene blue (MB), are advocated as simple, inexpensive and fairly sensitive, chair side investigative method for OPMDs detections.

Aims: To determine and compare the diagnostic efficacy and reliability of TB and MB in early detection of OPMDs and Malignant disorders (MDs), with expression of Ki-67 antigen proliferative marker.

Materials and Methods: The study group diagnosed as OPMDs and MDs included 20 patients and were equally divided into two groups of 10 randomly selected subjects who were stained with either TB or MB. The biopsy site was chosen where stain retention was positive. Histopathological evaluation for grades of dysplasia and carcinoma were determined and further subjected for Ki-67 proliferative marker evaluation and were graded accordingly as mild, moderate and severe proliferation index. The data collected was subjected to statistical analysis.

Results: The overall diagnostic accuracy in distinguishing OPMDs and MDs with expression of Ki-67 proliferative marker showed statistically significant (p=0.003) results with TB stain, while MB stain showed insignificant results (p=0.414).

Conclusion: The diagnostic efficiency of TB with expression of Ki-67 proliferative marker was found to be better than MB and thus, can be recommended as an adjunct diagnostic tool for diagnosis of OPMDs and MDs.

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INTRODUCTION

Cancer is Latinized from Greek word 'Karkinos', meaning crab, denoting how carcinoma extends its claws like a crab into the adjacent tissues. Oral cancer is one of the most common neoplasms and is ranked sixth in the cancer incidence worldwide. It is estimated that more than one million new cases are being detected annually in the Indian subcontinent and the prevalence is highest in men (George *et al.,* 2011). Many malignancies are not diagnosed until the late stage of disease. Early detection of cancer is of critical importance because survival rates markedly improve when identified at an early stage (Joel *et al.,* 1992) but early detection of OPMDs still pose a diagnostic challenge for most of the clinicians (Nagaraju *et al.,* 2010). In vivo stains are prompt resources, which have emerged, in the recent years, to aid in detecting early premalignant and malignant lesions. This technique has been applied in the oral setting since 1963 by means of dyes like Toluidine blue and Lugol's iodine (Chaudhari *et al.,* 2013). Markers of epithelial proliferation such as Ki-67indicate the progression of malignancy.

A significant increase in its number can be seen in case of dysplasia and carcinoma of the oral cavity. Ki-67 may thus serve as a standard in early diagnosis of malignant transformation and determine the prognosis of OPMDs. (Maheshwari *et al*., 2013) With this objective, the present study was intended to assess the efficacy of In Vivo stains, Toluidine blue and Methylene blue in early detection of OPMDs and malignant disorders, with expression of Ki-67 proliferative marker which serves as a standard.

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Aims and Objectives

- To determine and compare the diagnostic efficacy of Toluidine blue (TB) and Methylene blue (MB) in the early detection of OPMDs and Malignant disorders, with expression of Ki-67 antigen proliferative marker as standard for the study.
- To utilize In vivo staining as a safe, non-invasive adjunct for mass screening of OPMDs and occult malignant disorders so that appropriate treatment strategies can be implemented for management of OPMDs.

MATERIALS AND METHODS

The present study was conducted from May 2014 to July 2015 in the Department of Oral Medicine and Radiology, JSS Dental College and Hospital, Mysuru. Ethical approval was obtained from Institutional Review Board in accordance with the Helsinki declaration following which 20 subjects presenting with OPMDs and Malignant Disorders were selected and equally divided into 2 groups. The subjects were randomly assigned to either Toluidine Blue or Methylene Blue group for in vivo staining.

Inclusion Criteria

- Patients with clinically diagnosed OPMDs and any oral lesion suspicious of malignancy
- Those willing to be a part of the study, and who gave a signed the informed consent were included.

Exclusion Criteria

- Benign oral lesions easy to differentiate from suspicious malignant lesion by visual examination and clinical findings.
- Any individuals known to be hypersensitive to Toluidine blue and Methylene blue stains.
- Pregnant and lactating females.

Methodology

A detailed extra and intra oral clinical examination were carried out. The patients were informed about the study and signed informed consent was taken. The study group was categorized into two groups based on the in vivo staining procedure employed.

❖Group A: comprising of 10 subjects in whom Toluidine blue in vivo staining was done.

❖Group B: comprising of 10 subjects in whom Methylene blue in vivo staining was done.

The procedure employed for staining was in accordance with the previous investigations Perfomed by Nagaraju *et al*., 2010 and Riaz *et al*., in 2013. The area of dye retention and clinical appearance of the lesion was than biopsied. The sites where dye retention was not evident, clinical judgment alone would guide the site of biopsy. The specimen was preserved in 10% formalin for histopathological diagnosis and then subjected to Ki-67 proliferation marker estimation adopted from Shrishail and Verheuen *et al.* in 2014. The sections were interpreted for any dysplastic or carcinomatous changes and graded by pathologist (blinded for the study). The section stained for Ki-67 proliferation were evaluated using scores from 1 to 3 (Robbins *et al.,* 1984).

- Score 1: + -- Low proliferation- 10 to 30% positive cells.
- Score 2:++ - Moderate proliferation- 30 to 50% positive cells.
- Score 3: +++ - High proliferation->50% positive cells.

Statistical analysis

The data was tabulated and subjected to statistical analysis. Descriptive statistical procedures including means, SD, frequency and Percentages were used to summarize all variables. Chi-Square test was done for all the tests to measure of the association between two nominal variables. A p-value of 0.05 or less was considered as statistically significant. Efficiency and efficacy of invivo stains were assessed using the sensitivity, specificity, positive predictive value and negative predictive value. The results obtained were analysed using SPSS software for windows (version 22).

RESULTS

Twenty subjects of OPMDs and Malignant disorders were selected using purposive sampling and divided into Toluidine Blue (TB) group and Methylene Blue (MB) group. Table 1, and Graph 1 shows the demographic data of the study subjects. Of 20 subjects, 15 cases were clinically diagnosed as OPMDs, of which 9 were leukoplakia, 1 erythroplakia, 1 oral submucous fibrosis (OSMF), 4 oral lichen planus (OLP) and 5 cases were diagnosed as malignant disorders. Of 20 subjects, 10 subjects in TB group, 8 cases were diagnosed as OPMDs, out of which 5 were leukoplakia and 3 OLP. There were 2 case diagnosed as malignant disorders. Of 10 subjects in MB group, 7 cases were diagnosed as OPMDs, out of which 4 cases were diagnosed as leukoplakia, 1 erythroplakia, 1 OSMF, and 1 OLP.

There were 3 cases diagnosed as malignant disorders. Of 20 subjects, 17 cases were found in buccal mucosa, 1 in corner of mouth, 1 in soft palate and 1in lingual alveolar mucosa, and all 20 subjects showed dye retention in both the group when applied in the area suspected for clinical lesions. Of 10 subjects in TB group, 8 cases were diagnosed clinically as OPMDs, out of which 5 cases were diagnosed with severe dysplasia histopathologically, in which 4 cases showed high proliferation and 1 case showed moderate proliferation of Ki-67 marker. There were 3 cases of moderate dysplasia and all the 3 showed moderate proliferation of Ki-67 marker. There were 2 cases clinically diagnosed as malignant disorder, and histopathologically diagnosed as moderately differentiated carcinoma, and both cases showed low proliferation of Ki-67 marker. The results obtained were statistically significant.(p=0.03) (Table 2 and Graph 2), (Figure: 1).

Table 1. Demographic data of the study subjects

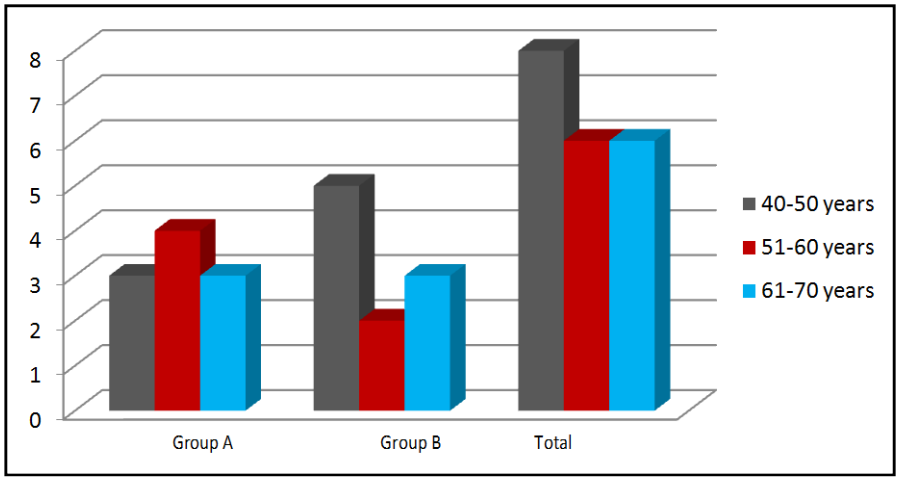
Crosstabulation								
			Stain				Total	P value
			Group A	N	Group B	N		
Ages	40-50	Count	3		5		8	
		% within stain	30.0%	47	50.0%	47	40.0%	
	51-60	Count	4		2		6	
		% within stain	40.0%	58	20.0%	59	30.0%	0.558
	61-70	Count	3		3		6	
		% within stain	30.0%	63	30.0%	63	30.0%	
Sex	M	Count	9		8	54	17	
		% within stain	90.0%	57.3	80.0%		85.0%	
	F	Count	1		2	53	3	
		% within stain	10.0%	48	20.0%		15.0%	

GROUP A-Toluidine Blue Stain, GROUP B-Methylene Blue Stain, N-Mean Age
M- Male, F-Female

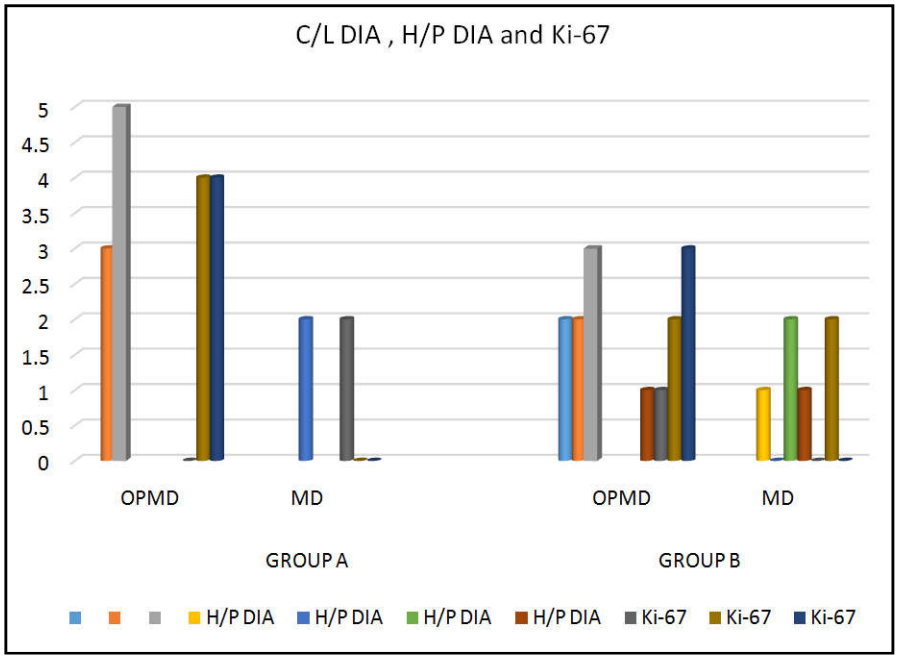
Table 2. Association b/w clinical and histopathological and KI-67 proliferation index

C/L DIA and H/P DIA andKi-67														
STAIN				H/P						Ki-67				HP	
				MIL DYS	MOD DYS	SEV DYS	WELL DIFF	MOD DIFF	POOR DIFF		NEG	LP	MP		P value
		OPMD	Count		3	5	0.0%	0.0%	0.0%	Count	0	4	4		
			% within H/P		37.5%	62.5%	0.0%	0.0%	0.0%	% within KI67		0.0%	50.0%	50.0%	
			% within C/L		100.0%	100.0%	0.0%	0.0%	0.0%	% within C/L		0.0%	100.0%	100.0%	
		MD	Count			0	0.0%	2	0.0%	Count		2	0	0	
			% within H/P			0.0%	0.0%	100.0%	0.0%	% within KI67		100.0%	0.0%	0.0%	
GROUP	C/L DIA		% within C/L			0.0%	0.0%	100.0%	0.0%	% within C/L		100.0%	0.0%	0.0%	
A	Total		Count		3	5	0.0%	2	0.0%		Count	2	4	4	0.003
			% within H/P		30.0%	50.0%	0.0%	20.0%	0.0%		% within H/P	20.0%	40.0%	40.0%	
			% within C/L		100.0%	100.0%	0.0%	100.0%	0.0%		% within C/L	100.0%	100.0%	100.0%	
		OPMD	Count	2	2	3	0.0%	0.0%	0.0%	Count	1	1	2	3	
			% within H/P	28.6%	28.6%	42.9%	0.0%	0.0%	0.0%	% within KI67	14.3%	14.3%	28.6%	42.9%	
			% within C/L	100.0%	100.0%	100..0%	0.0%	0.0%	0.0%	% within C/L	50.0%	100.0%	50.0%	100.0%	
		MD	Count				1	0	2	Count	1	0	2	0	
	C/L DIA		% within H/P				33.3%	0.0%	66.7%	% within C/L	33.3%	0.0%	66.7%	0.0%	
			% within C/L				33.3%	0.0%	66.7%	% within Ki-67	50.0%	0.0%	50.0%	0.0%	0.414
	Total		Count	2	2	3	1	0.0%	2		2	1	4	3	
GROUP			% within H/P	20.0%	20.0%	30.0%	10.0%	0.0%	20.0%		20.0%	10.0%	40.0%	30.0%	
B			% within Ki-67	100.0%	100.0%	100.0%	100.0%	0.0%	100.0%		100.0%	100.0%	100.0%	100.0%	

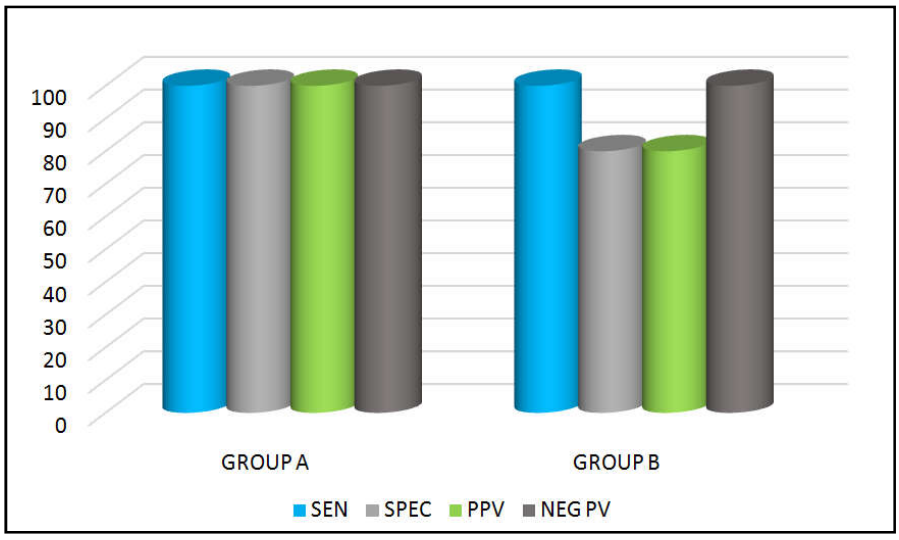
GROUP A-Toluidine Blue, GROUP B-Methylene Blue, MIL DYS-Mild Dysplasia, MOD DYS-Moderate Dysplasia, SEV DYS-Severe Dysplasia, WEL DIFF-Well Differentiated Tumor, MOD DIFF-Moderately Differentiated Tumor, POOR DIFF-Poorly Differentiated Tumor, NEG-Negative, LP-Low Proliferation, MP-Moderate Proliferation, HP-High Proliferation.



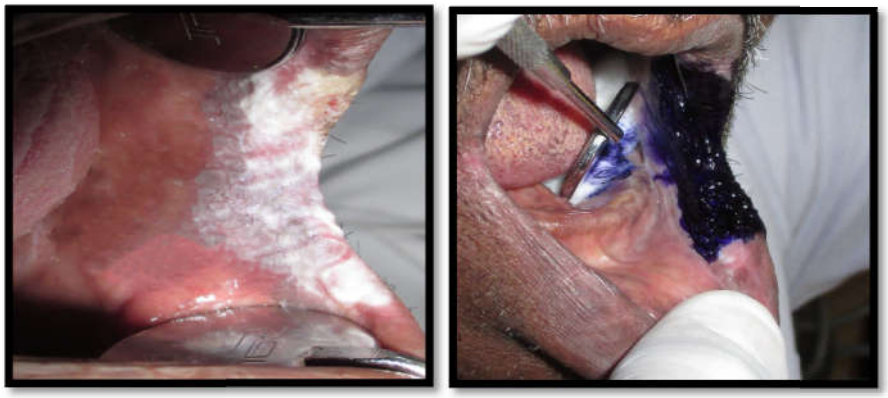
Graph 1. Age distribution



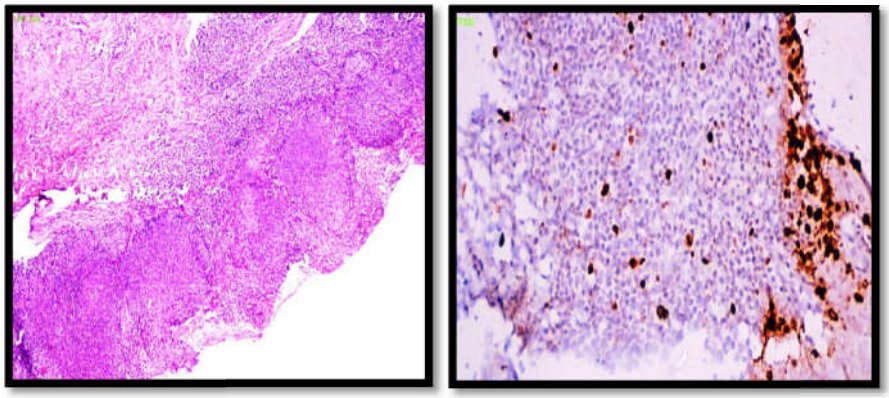
Graph 2. Clinical, Histopathological Diagnosis and Ki-67



Graph 3. Sensitivity, Specificity, PPV and NPV



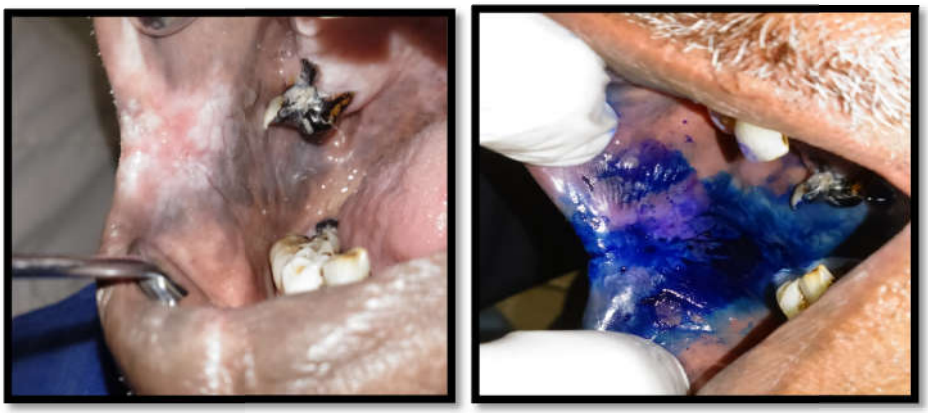
Pre staining Post staining



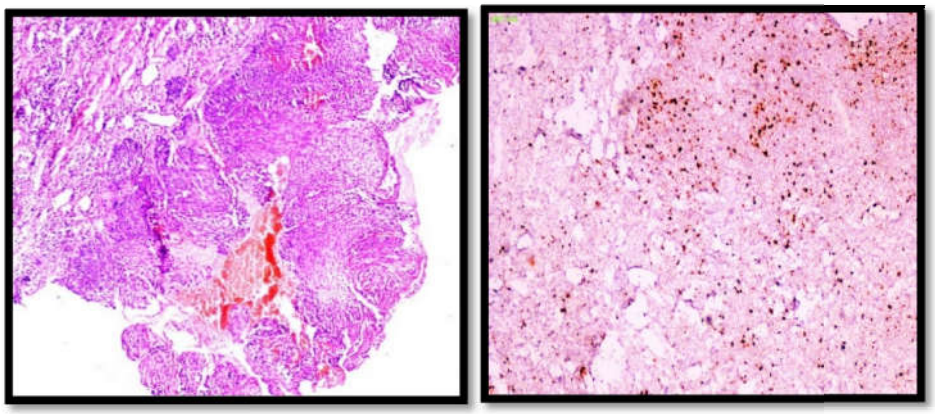
H/P Diagnosis
Severe Dysplasia

Ki-67 Marker
High Proliferation

Figure 1a. Oral Leukoplakia



Pre staining Post staining



H/P Diagnosis
Moderate Dysplasia

Ki-67 Marker
Moderate Proliferation

Figure 1b. Oral Leukoplakia

Figure 1. Toluidine Blue stain

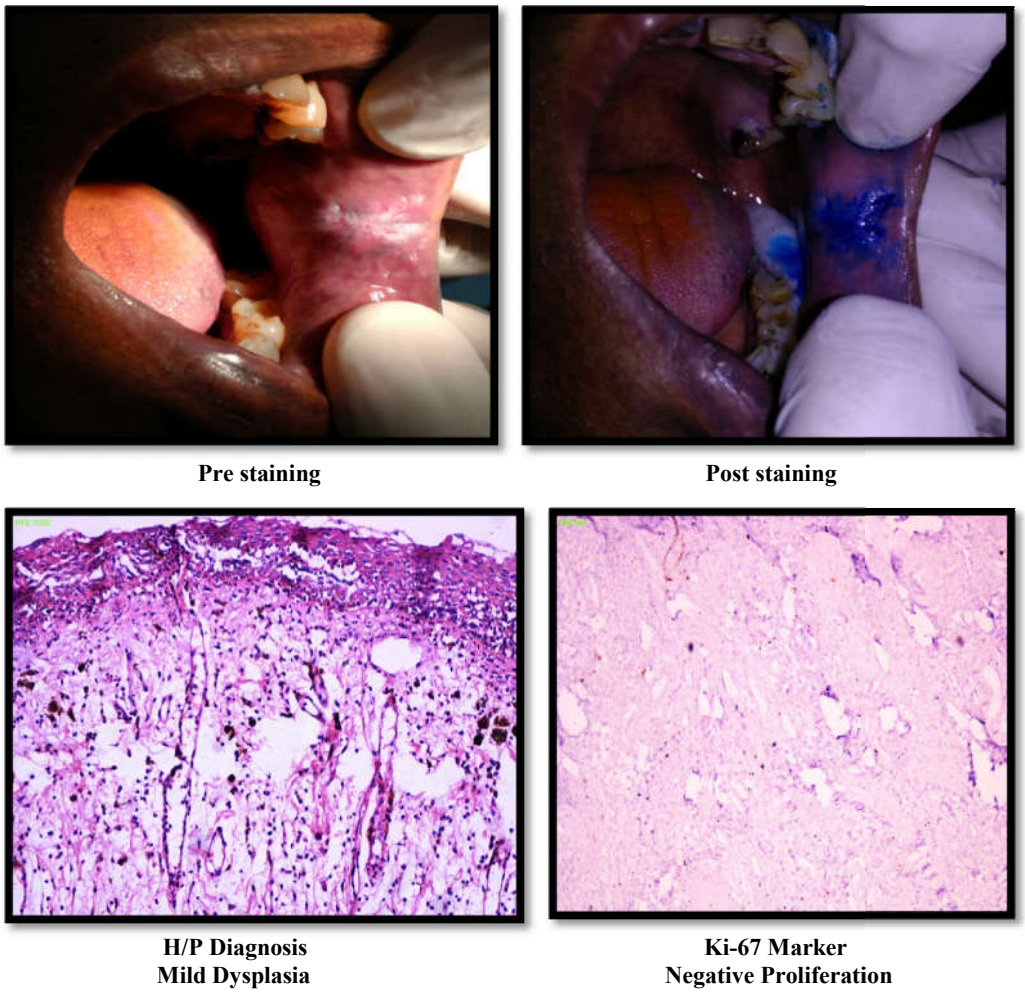


Figure 2. Methylene blue stain

Of 10 subjects in MB group, 7 cases were diagnosed clinically as OPMDs, of which 3 cases were diagnosed with severe dysplasia and all the 3 showed high proliferation of Ki-67 marker. There were 2 cases diagnosed as moderate dysplasia and both the cases showed moderate proliferation of Ki-67. There were 2 cases diagnosed as mild dysplasia, of which 1 case showed low Ki-67 proliferation index, and other was negative for Ki-67 proliferation index. There were 3 cases clinically diagnosed as malignant disorder, of which 2 were poorly differentiated carcinoma which showed moderate proliferation of Ki-67 index and 1 case was diagnosed as well differentiated carcinoma which was negative for Ki-67 proliferation marker index. The results obtained were statistically insignificant. (p=0.414) (Table 2 and Graph 2), (Figure: 2). The sensitivity and specificity of TB group was 100% in staining OPMDs and Malignant disorders whereas MB group showed 100% sensitivity and 80% specificity in staining OPMDs and Malignant disorders (Graph: 3). The PPV and NPV of TB stain was 100% in comparison with MB stain, PPV of which was 80% and NPV was 100%. The overall diagnostic validity of in vivo stain when retained was found to be 100% in TB group, in comparison to MB group which exhibited 80% diagnostic validity in diagnosing OPMDs and Malignant disorders. The results obtained were statistically significant for TB group in comparison to MB group. (Graph: 3).

DISCUSSION

Oral cancer is one of the most common neoplasms occurring worldwide. Of all malignant neoplasms, approximately 3% occur in the oral cavity, thereby posing a Significant universal health problem (George *et al*., 2011). Squamous cell carcinoma, which arises from the oral mucosal lining, accounts for over 90 percent of these tumors. Early detection and prompt treatment of oral cancer offers the best chance for cure (Mahima, 1995). OPMDs are morphologically altered tissues which have greater than normal risk of transforming into a malignancy. Some may even contain microscopic foci of cancer at the time of diagnosis. Several white and red oral lesions have been recognized by the World Health Organisation as OPMDs (Warnakulasuriya *et al.,* 2007; Lingen *et al.,* 2008; Warnakulasuriya *et al.,* 2008). A variety of new and emerging diagnostic aids and adjunctive techniques are currently proposed and are available to potentially assist in the screening of healthy asymptomatic patients for the detection of OPMD's or otherwise occult oral cancerous lesions. These include in vivo staining with toluidine blue and methylene blue, auto fluorescence, chemiluminescence, brush biopsy, salivary diagnostics etc (Warnakulasuriya *et al.,* 2007; Lingen *et al.,* 2008; Warnakulasuriya *et al.,* 2008). In vivo Staining is advocated as a simple, inexpensive and fairly sensitive, chair side investigative method for OPMDs detection

(Warnakulasuriya *et al.,* 2008) and has been applied in oral setting since 1963 (Nagaraju *et al.,* 2012). The molecular biological markers have been also suggested to be of some value in the diagnosis and prognostic evaluation of precancerous lesions. Markers of proliferation, epithelial differentiation and genomic markers, eg: p53 and Ki-67 could potentially be good indicators for improving the prognostic evaluation of precursors of oral cancer (Rastogi *et al.,* 2013). An uncontrolled proliferation of Ki-67 is a hallmark of cancer with poor prognosis of disease (Maheshwari *et al.,* 2013). With this objective, this study was undertaken to assess the efficacy of In Vivo staining procedures using two dyes, Toluidine blue and Methylene blue in the early detection of Oral Potentially Malignant Disorders, considering expression of Ki-67 epithelial proliferative marker as a standard. Villa *et al*. in 2014, Mortazavi *et al*, in 2013, Diajil *et al.* in 2013, reported that more than two thirds of patients with OPMD's and malignant disorders were aged above 40 years. A clinical and epidemiological data collected from 3,142 OPMDs patients found that most of the patients were in the age range of 42 to 59 years, with mean age of around 54.3 years (George *et al.,* 2011). In the present study, a total number of 20 subjects were enrolled between the age group 40-70 years with a mean age of 55.10 year which was in accordance with the above mentioned studies. George *et al.* in 2011, Villa *et al* and Camile *et al*, in 2014 reported a male preponderance in their study. Neville *et al.* in 2002 found that 80% of males were affected and male to female ratio was 2:1. In the present study, there were 17 males and 3 female patients with the male: female ratio being 5.6:1.Chang *et al.* in 2012 stated that the prevalence of leukoplakia was 7.44% and that of erythroplakia was 0.02% to 0.83%. Pindborg *et al* reported 0.02-0.4% prevalence of OLP. Shafer *et al* in 2002 stated 0.2 to 0.5% prevalence of OSMF.

In the present study there were a total number of 20 OPMDs and Malignant disorder patients. Fifteen (75%) cases presented with OPMDs, of which 9 (50%) cases were clinically diagnosed as leukoplakia, one (5%) case as erythroplakia, one (5%) as OSMF and 4 (15%) as oral lichen planus. Five (25%) of the 20 cases were clinically diagnosed as malignant ulcero-proliferative growth. Neville *et al* in 2002, Warnakulasuriya *et al* in 2007, George *et al* in 2011and Mortazavi *et al* in 2013 stated that usually OPMDs were found in buccal mucosa, followed by gingivae, tongue and floor of the mouth. In the present study, of 20 cases 17 (85%) cases were found in buccal mucosa, one (5%) corner of the mouth, one (5%) in soft palate and one (5%) case in lingual alveolar mucosa. Nagaraju *et al* in 2010, Upadhyay *et al* in 2011, in their study stained 60 and 47 subjects respectively with toluidine blue stain and Riaz *et al* in 2013 stained 120 subjects with methylene blue stain to check for dye retention in OPMDs and Malignant disorders and stated that all cases were positively stained for clinically suspicious lesions. In the present study all 20 subjects in both groups showed dye retention which indicates both the dyes could positively stain OPMDs and malignant disorders. The obtained results are in accordance with the above mentioned studies. Neville *et al* in 2002, Warnakulasuriya *et al* in 2007, Nagaraju *et al* in 2010, stated that staining procedure may be a valuable chair side investigation tool in identifying the biopsy site to exactly co relate the clinical and histopathological grades of dysplasia. In the present study, of 20 subjects, 10 subjects in TB group, all (100%) were histologically diagnosed for potentially malignant disorders and graded for dysplasia and carcinoma. Five (62.5%) of the 8 (80%) OPMDs were histopathologically graded as severe dysplasia, and 3 (37.5%) as moderate dysplasia. There were 2 (20%) cases of malignant disorders, and both were diagnosed as moderately differentiated carcinoma. In MB Group, Of the 10 subjects, all (100%) were histologically diagnosed as premalignant and malignant disorders and graded for dysplasia.

Two (28.6%) cases of 7 (70%) OPMDs were graded histopathologically as mild dysplasia, 2 (28.6%) as moderate dysplasia, and 3 (42.9%) as severe dysplasia. There were 3 (30%) cases diagnosed as malignant disorders, of which 2 (66.7%) cases were diagnosed as poorly differentiated carcinoma, and 1 (33.3%) as well differentiated carcinoma. Saxena *et al* in 2010 stated that the expression of Ki-67 proliferative marker was high in severe dysplasia where the active multiplication of cells are taking place, moderate proliferation in moderate dysplasia and low proliferation in mild dysplasia. Similarly, well differentiated carcinoma showed low or absence of proliferation, moderately differentiated carcinoma showed moderate proliferation, and high proliferation was seen in poorly differentiated carcinoma. In the present study of total 20 subjects, 8 (80%) of the 10 cases in TB group were diagnosed clinically as potentially malignant disorders, of which 5 (71.4%) cases were diagnosed severe dysplasia histopathologically, in which 4 (57.1%) cases showed high proliferation, and 1 (14.3%) case showed moderate proliferation of Ki-67. There were 3 (37.5%) cases of moderate dysplasia, and all the 3 (100%) cases showed moderate proliferation of Ki-67. In MB group, of 10 cases, 7 (70%) cases were diagnosed clinically as potentially malignant disorders, of which 3 (42.9%) cases were diagnosed as severe dysplasia and all the 3 cases showed high proliferation of Ki-67 marker Index. There were 2 (28.6%) cases diagnosed as moderate dysplasia and both the cases showed moderate proliferation of Ki-67. There were 2 (28.6%) cases diagnosed as mild dysplasia, of which 1 case showed low Ki-67 proliferation index and 1 case was negative for Ki-67 proliferation marker index. There were 3 (30%) cases clinically diagnosed as malignant disorder, of which 2 cases were diagnosed as poorly differentiated carcinoma which showed moderate proliferation of Ki-67, and 1 case was diagnosed as well differentiated carcinoma which was negative for Ki-67 proliferation marker index.

Ling *et al* in 2008 and Nagaraju *et al* in 2010 in a review, mentioned the sensitivity and specificity of TB range from 78-100% and 31-100% in detection of OPMDs. In the present study, sensitivity, specificity, PPV, and NPV of TB stain in staining OPMDs and malignant disorders was found to be 100% which was in accordance with previous studies. This states that TB stain is highly effective in identifying the oral potentially malignant and malignant disorders. Akhtar *et al* in 2013 stated the sensitivity and specificity of MB stain was 91.4% and 76.6% respectively. The PPV was 97.7% and NPV was 33.3% in detection of OPMDs. In the present study, the sensitivity of MB stain in staining oral potentially malignant and malignant disorders was found to be 100% but specificity was found to be 80%. The PPV of methylene blue stain in

staining OPMDs and malignant disorders was 80% and NPV was 100% which was in accordance with the above studies. This states that MB stain could stain healthy mucosal tissue and thereby imparting a false positive impression of dysplasia Not many studies have been done to find an association between in vivo staining of toluidine blue and methylene blue with expression of Ki-67 proliferative marker index. In the present study we assumed that a link is certain with positive toluidine blue staining and Ki-67 proliferation marker index, when compared with that of methylene blue. The results obtained in this study co relate well with this assumption. Hence, one must consider invivo staining with toluidine blue in early detection of oral potentially malignant and malignant disorder patients.

Conclusion

The following inferences can be drawn from this study.

- In vivo staining with Toluidine blue and Methylene blue was found to be effective in early detection of oral potentially malignant disorders histopathologically.
- The diagnostic efficiency of Toluidine blue in early detection of oral potentially malignant disorders with Ki-67 proliferative marker showed statistically significant results.
- Invivo staining with Toluidine blue and Methylene blue can be used as an adjunct in early diagnosis and in case of mass screening of OPMDs and malignant disorders.

To conclude, favourable results were obtained with the Toluidine blue stain when compared to Methylene blue with expression of Ki-67 proliferative marker in detection of oral potentially malignant and malignant disorders. Nevertheless, further studies with larger sample size should be undertaken in future to elucidate the effectiveness with newer stains such as Rose Bengal stain in diagnosing Oral Potentially Malignant and Malignant disorders.

Conflict of interest: None

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Full name of review committee: Institutional ethical review committee JSS Dental College.

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