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Original Research

Efficacy of Toluidine Blue and Brush Biopsy in Oral Lesions

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Abstract

Background: Oral cancer is a global health problem and its early detection is of prime importance. In vivo stains and brush biopsy have emerged in recent years to aid as clinical diagnostic tools. **Aims and objectives**: In our study we assessed the efficacy and accuracy of Toluidine blue and brush biopsy in comparison to wedge biopsy in patients with oral premalignancies and malignant lesions. **Materials and methods**: The study group comprised of 172 subjects with clinically suspicious premalignant and malignant oral lesions. All the lesions were stained with toluidine blue and dye retention were recorded with photographs. Brush biopsy was done for early assessment of cancer. Wedge biopsy was done for confirmation of diagnosis. The data thus compiled were statistically analyzed using statistical software SPSS version 16. **Results:** In the present study 127 cases were females and 45 were males; among them 84.3% had the habit of areca nut/tobacco. Maximum cases (62%) were seen in buccal mucosa. Histopathologically 73% carcinomas, 27% premalignant and benign lesions were diagnosed. Toluidine blue showed a few false positive results in inflammatory lesions. Sensitivity and Specificity of combined evaluation of toluidine blue and brush biopsy in malignant lesions were 93% and 95% respectively and in premalignant lesions were 88% and 90% respectively. Positive predictive value was 96%. **Conclusion:** Combined evaluation of toluidine blue and brush biopsy technique is non invasive, painless outpatient procedure which has high sensitivity and specificity in detecting premalignant and malignant lesions. This is an ideal screening tool which can minimize false negatives.

Key words: Brush Biopsy; Oral Cancers; Toluidine Blue; Tolonium Chloride; Biopsy; Tobacco Habit; Areca Nut; Sensitivity and Specificity.

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Introduction

Early detection of cancer is of prime Importance. It helps us to reduce morbidity and mortality. Around 300,000 patients are annually estimated to have oral cancer worldwide and in India it constitutes 30-40% of cancer load. The rising trend of usage of pan masala, gutka owes to the cancer.[1,2] Despite the easy accessibility of the oral cavity during physical examination, many malignancies are not diagnosed until late stages of disease. Multiple screening, detection techniques and different modalities of treatment to prevent progression of premalignant to malignant lesions have been tried. Clinical diagnosis of oral squamous cell carcinomas (OSCC) is not difficult when the lesion is obviously invasive or functional limitation is present. Conversely, it is more difficult to diagnose dysplasia and potentially malignant epithelial lesions.[3]

Supravital stain Toluidine Blue (TB) has been used to mark the area for biopsy and to mark the full extent of premalignant lesion. It has been reported that toluidine blue stains premalignant and malignant lesions, but, not the benign lesions and normal mucosa.[2,4] Toluidine blue is a member of thiazine group of metachromatic dyes.[4] In vivo staining may identify early lesions which could be missed on clinical examination.[5] Moreover it can outline the full extent of the dysplastic epithelium or carcinoma when excisions are planned.[6] It helps in selecting the biopsy sample site in premalignant lesions. Also, it can help the follow up of patients with oral cancers.[7] Oral cytological examination has been tried for non scalpel intervention in patients with suspicious lesions. Histopathological diagnosis by scalpel biopsy is the gold standard for accurate diagnosis.[3,5] Patients often fear the routine biopsy as it is an invasive Out Patient (OPD) procedure. Brush Biopsy (BB) which involves scraping of surface epithelium and is less traumatic, can be used as a routine procedure. In our study we attempted to assess the efficacy and accuracy of TB and brush biopsy in

comparison to wedge biopsy in the diagnosis of premalignant lesions.

Materials and Methods

A total of 172 patients who presented with either a plaque/ulcer/tumor in oral cavity, to the outpatient clinics of Otorhinolaryngology and Dental surgery, from time interval of September 2009 to March 2011 were screened with in-vivo toluidine blue staining, oral brush biopsy and wedge biopsy. The ethical clearance was obtained from the institutional Ethics Committee. **Inclusion Criteria**: All patients with clinically suspicious oral precancerous and cancerous lesions. **Exclusion Criteria**: Patients with any dental conditions such as Orthodontic or other fixed prostheses which may interfere with the examination and previously treated for cancerous condition by surgery or irradiation were excluded for the present study.

Patients with suspicious premalignant or malignant lesions of the oral cavity, irrespective of site, stage and sex were selected. Relevant history, epidemiologic data, with special emphasis on age, duration of disease and exposure to possible carcinogenic substances were recorded in a detailed structured questionnaire. On local examination, the intra oral location, lesion size, extent of local infiltration, oral hygiene and cervical lymph node enlargement were assessed. Written consent was obtained from all patients after explaining the various steps and hazards. All the patients were screened with in-vivo toluidine blue, brush biopsy and wedge biopsy.

In vivo toluidine blue staining: 1% aqueous TB was applied to the lesion for 30 seconds, Followed by tap water or normal saline rinse. Then rinsed by 1% acetic acid for 30 seconds to reduce the background staining.[2]

Oral brush biopsy: Using a small hard tooth brush (commercially available), a trans-epithelial brush biopsy was taken. Removed cells were transferred to a glass slide by distributing the obtained material evenly over the glass surface and smears were fixed with Biofix spray. Smears were stained with Papanicolaou stain, hematoxylin and eosin stain followed by light microscopic examination. In case of interpretation of brush biopsy, the smears were analyzed for enlarged nuclei, variation in nuclear/cytoplasmic ratio, number of nuclei, hyperchromatism, and discrepancy in maturation. Smears were categorized as benign, suspicious for malignancy or malignant lesions.

Wedge biopsy: The biopsy was taken from the site where maximum staining of TB was seen. In sites where no retention of dye occurred, clinical judgment directed biopsy. The biopsy obtained was fixed in 10% formalin saline, processed and thin sections (3-4µm in thickness) were made. They were stained with hematoxylin and eosin. The sections were examined under light microscope for confirmation of diagnosis which was considered gold standard for the diagnosis. The brush biopsy cytological smears and corresponding histopathology slides were manually examined by two pathologists in a double blind fashion. If there were any discrepancies, a third opinion was obtained to reach a final diagnosis.

Statistical analysis was carried out using statistical software SPSS version 16. Statistical significance was determined using Chi square test and a p value of <0.05 was taken as statistically significant. To test the diagnostic accuracy of the screening tests, sensitivity, specificity and predictive value were calculated.

Results

The age of the patients varied from 18 years to 90 years. Mean age was 55 years. The maximum numbers of cases (69.7%) were seen in the age group over 46 years. An overall female preponderance was noted, with a female to male ratio of 2.8:1. Eighty four percent had a habit of areca nut / tobacco chewing. Majority of patients (57%) developed cancer between 11-30 years of exposure. Only 11 cases had an exposure of below 10 years. The average exposure time was less than ten years for the premalignant lesions. Maximum number of cases were seen in buccal mucosa (62.8%), next highest was tongue (12.8%) and lowest incidence were in lip (1.2%) (Table1). All the patients showed poor oral hygiene. Among malignant lesions most of the cases 33% were less than two cm and in premalignant lesions maximum cases 15% were of less than two cm. Most of the lesions 57.6% were ulcero-proliferative.

In vivo toluidine blue staining evaluation

Of the 172 cases 124 (73%) were positive for TB (Fig 1a) and negative were 48 (27%) (Fig 1b) as depicted in table 2. All patients

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were followed by wedge biopsy. Sensitivity of TB screening for malignant lesions was 92%, Specificity 82%, Positive predictive value 93%, Negative predictive value 79%, False negatives 7%, False positives 17%. Sensitivity of TB screening for premalignant lesions was 61%, Specificity 80%, Positive predictive value 91%, Negative predictive value 36%, False negatives 38%, False positives 20%.

Site	Number of cases	%
Buccal mucosa	108	62.8
Floor of mouth	5	2.9
Hard palate	10	5.8
Lip	2	1.2
Tongue anterior 2/3rd	22	12.8
Upper alveolus	9	5.2
Lower alveolus	8	4.6
Retro molar trigone	8	4.7
Total	172	100

Table 1: Percentage distribution of various oral lesions according to the site

TB stain	Malignant Lesions	Benign Lesions	Total
Positive	116 (67%)	8 (5%)	124 (73%)
Negative	10 (6%)	38 (22%)	48 (27%)
Total	126	46	172

Table 2: Comparison of toluidine blue staining with histopathological diagnosis

Oral brush biopsy evaluation

Of the 172 cases, 119 (69%) cases were reported as positive for malignancy on cytology (Fig 1c) and 53 (31%) cases were reported as negative for malignancy on cytology as shown in table 3.of 119 cases,115 cases truly matched with histopathology as malignancy. The other 4 cases were benign on histopathology and hence were false positives. Of 53 cases, 42 cases truly matched with histopathology as benign lesions. The other 11 cases turned out to be malignant on histopathology and hence were false negatives.

Brush biopsy screening	Malignant lesions	Benign lesions	Total
Positive	115 (67%)	4(2%)	119 (69%)
Negative	11 (6%)	42(24%)	53 (31%)
Total	126	46	172

Table 3: Comparison of brush biopsy with histopathological diagnosis

All patients were followed by histopathology for confirmation. Sensitivity of brush biopsy screening for malignant lesions was 91%, Specificity 91%, Positive predictive value 96%, Negative predictive value 79%, False negatives 8.7%, False positives 8.6%. Sensitivity of brush biopsy screening for premalignant lesions 83%, Specificity 90%, Positive predictive value 96.7%, Negative predictive value 60%, False negatives 16.66%, False positives 10%.

Among malignant cases where biopsy was performed, 41% were of well differentiated oral squamous cell carcinoma followed by moderately differentiated OSCC (23%) and severe dysplasia (5%). Least cases were of poorly differentiated OSCC (1%) and microinvasive carcinoma (0.5%).There were about 17% of dyskeratosis cases without dysplasia, 3% of dyskeratosis cases with mild dysplasia and least being 0.5% each of Kimura's disease, Candidiasis, Submucous fibrosis and Lobular angioma (Table 4).

Combined evaluation of in-vivo toluidine blue staining and brush biopsy

Sensitivity of combined TB and BB screening for malignant lesions was 93%, Specificity 95%, Positive predictive value 99%, Negative predictive value 84%, False negatives 6%, False positives 4%. Sensitivity of combined toluidine blue and brush biopsy screening for premalignant lesions 88%, Specificity 90%, Positive predictive value 96%, Negative predictive value 69% False negatives 11%, False positives 10% (Table 5).

Discussion

The present study showed that about 20% of all cancer seen in otorhinolaryngology and oncology department was oral malignancies. Individuals having habit of smoking, alcohol consumption, areca nut chewing were reported to have 123 times the risk of developing cancer than people without such habits.[8] The difference in 5 year survival rate between early and late detection of oral squamous cell carcinoma is 80.55% vs 18.3%.[9]

Epidemiological and clinical aspects

Our study showed that oral cancers were more common in female patients. Chewing tobacco/pan (quid) is fairly common practice among women of this region. Among carcinogens, tobacco (84%) was the most common substance of abuse in one form or the other.[10] The buccal mucosal and anterior

two third of tongue were commonly involved. This could be attributed to individuals as they chewed tobacco and kept in mouth for long time. Mehrotra et al.,[11] detected 63 new cases of oral cancer per annum. Our centre detects 81 new cases of oral cancer per annum. Properly structured site specific data like this can augment National Cancer Registry Programme (NCRP) and is essential indicator of the magnitude and pattern of cancer problem in India.[11]

Varshney et al.,[12] showed that maximum cases (52%) with 15-20 years exposure to carcinogens had developed cancer. Our study showed maximum patients (68%) who were exposed for a period of 21-30 years developed cancer. In our study, clinical diagnosis showed false negative rate of 6.34% and false positive rate of 21.73%, similar to an earlier study that had false negativity of 4.8% and false positivity of 28.5%.[9]

Malignant cases		Benign cases	
Well differentiated squamous cell carcinoma	71 (41%)	Dyskeratosis without dysplasia	30 (17%)
Moderately differentiated squamous cell carcinoma	40 (23%)	Dyskeratosis with mild dysplasia	6 (3%)
Poorly differentiated squamous cell carcinoma	2 (1%)	chronic ulcer	2 (1%)
Verrucous type squamous cell carcinoma	3 (1%)	Pseudo-epitheliomatous hyperplasia	2 (1%)
Mucoepidermoid carcinoma	4 (2%)	Squamous papilloma	2 (1%)
Microinvasive carcinoma	1 (0.5%)	Kimura's disease	1 (0.5%)
Severe dysplasia	5 (3%)	Candidiasis	1 (0.5%)
		Submucous fibrosis	1 (0.5%)
		Lobular angioma	1 (0.5%)
Total n=172	126(73%)		46 (26%)

Table 4: Distribution of various histopathologically diagnosed cases after wedge biopsy

Statistical analysis	Premalignant/Benign lesions			Malignant lesions		
	TB	BB	TB+BB	TB	BB	TB+BB
Sensitivity (%)	61	83	88	92	91	93
Specificity (%)	80	90	90	82	91	95
Positive predictive value (%)	91	96	96	93	96	99
Negative predictive value (%)	36	60	69	79	79	84

Table 5: Statistical analysis of in-vivo Toluidine blue staining, Brush Biopsy and combined TB and BB of oral lesions.

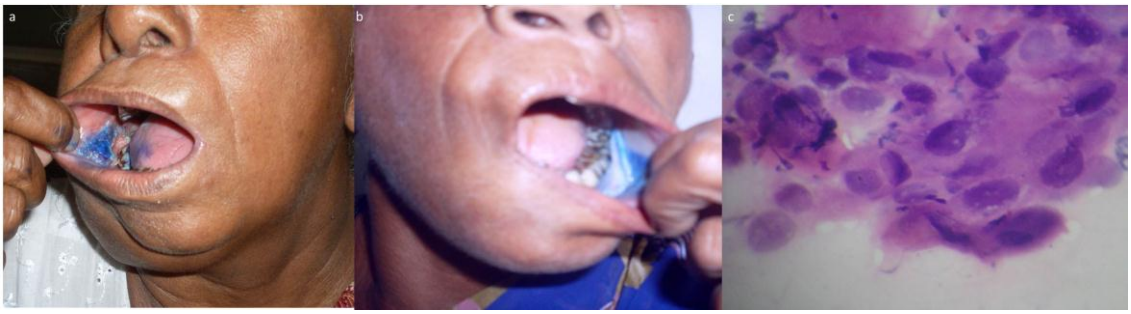

Figure 1: The clinical picture of oral squamous cell carcinoma patient showing positivity for toluidine blue (a) and oral leukoplakia showing negative staining for toluidine blue (b). The photomicrograph of oral brush biopsy from squamous cell carcinoma patient from the buccal mucosa showing high nuclear/cytoplasmic ratio, intracellular keratinization in a necrotic background.

Supravital toluidine blue staining:

An easy economic technique is required for the purpose of mass screening. Supravital staining method has been adapted to aid in early diagnosis since many years. TB (supravital dye) was first used by Richart in 1963 in medicine for detecting cervical dysplasia and carcinoma insitu.[13] TB is a

cationic metachromatic vital dye that binds to sulphates, phosphates and carboxylates. Cells which are dysplastic or cancerous in nature have leaky cell membrane, allowing the dye to enter and bind to phosphate group of nucleic acids. Also malignant epithelial cells may contain intracellular canals that are wider than normal epithelium, which may facilitate penetration of the dye.[2,14]

Sensitivity in the published data for TB ranged from 93.5% to 97.8%. In our study, in-vivo TB staining was highly sensitive and efficient in detecting malignant disease (92%). Warnakulasuriya et al.,[15] found TB staining to be less useful in detecting premalignant lesions. They found a false negative rate for oral epithelial dysplasia of 22%. Martin[16] and colleagues found false negative staining rates for carcinoma in situ of 42% and 58%, respectively for moderate and severe dysplasia. We had 10 cases of false negatives of which were cases of mucoepidermoid carcinoma (4), microinvasive carcinoma (1), dysplasia (4), carcinoma in situ (1).

In aspect of specificity of TB staining, we obtained a value of 82.60% with a resulting false positive rate of 17.39%. The false positives were three cases of dyskeratosis without dysplasia, three cases of dyskeratosis with mild dysplasia and two cases of chronic ulcers. These false positives could be related to the retention of stain in inflamed and trauma areas. Other causative factors may include the irregular, papillary or digital surfaces of the lesions, which may cause the mechanical retention of dye, contamination of saliva and plaque, retention of dye material in papilla of the tongue or minor salivary gland ducts over mucosa.[13]

For TB, false positive results are quite common in ulcerated, inflammatory, or traumatic lesions as reported by previous workers.[2,3] TB appears to stain only 3-4 cell layers deep. Therefore, early cancer that might be surfaced by intact epithelium, which are not exposed to environment, do not stain.[13] In our study, two patients with mucoepidermoid cancers presented as plaques, did not take the stain. It is important to understand that only those lesions which have some ulceration of mucosa will take the stain and those which are completely mucosal covered will not.

Zhang et al.,[17] found that more than 6 fold elevation in cancer risk was observed for TB positive lesions with positive retention of dye present in 12 of 15 lesions, that later progressed to cancer (p=0.0008). Martin et al[16] demonstrated TB to be highly efficient in detecting invasive malignant disease with a sensitivity of 100%. A recent prospective longitudinal study showed TB staining and allelic loss is predictive of malignant transformation, even in benign lesion or those with low grade dysplasia and is important for further study and use of TB.[17] A two week waiting period is required before staining, assuming that patients will return for follow up, will reduce false positive findings, secondary to inflammatory condition in a general population.[9]

Exfoliative cytology

Brush cytology has the potential to assist the diagnostic portion of the “screening gap” which currently challenges the early detection of many epithelial cancers. It is a noninvasive technique, based upon the fact that dysplasia and cancer cells tend to “slough off” or exfoliate preferentially and can easily be collected from surface of the lesion.[4] The oral Brush Biopsy was introduced to the dental profession in 1999 which uses special brush to scrape all 3 layers of lesion, basal, intermediate and superficial layers. It does not require topical anesthesia and causes minimal bleeding and pain. Full thickness sampling indicated by pinpoint bleeding during procedure, is essential for evaluation of collected cells to yield representative findings. Many dysplastic cells first develop in basal layer may be lost as the cells mature and keratin is produced.[18]

In our study, of 119 cases reported as malignant on cytology, 115 cases truly matched with histopathological results (i.e true positives). The other 4 cases, i.e dyskeratosis with mild dysplasia (2) and chronic ulcer (2) were false positives. Among 53 cases which were reported as negative for malignancy on cytology, 42 cases were true negatives and 11 cases were false negatives i.e mucoepidermoid carcinoma (4), verrucous carcinoma (1), dysplasia (4), microinvasive carcinoma (1) and carcinoma in situ (1). Our false positive rate for brush biopsy was 8.6% and false negative rate for BB was 8.7%.

The two cases of mucoepidermoid carcinoma on cytology showed only mucous

and few squamous cells. One case which was diagnosed clinically as leukoplakia, showed only benign squamous cells with yeast forms of candida. The true BB negatives were truly benign on histopathology suggesting that BB is specific for benign lesions. Potter et al.,[19] found four BB false negatives of a total 115 cases analyzed. Although the number of false positive cases is small in his study it is important to emphasize that mean delay time in diagnosing a cancer in these cases was 117.25 days.

Mehrotra et al.,[20] studied 79 patients with adequate transepithelial BB. Their sensitivity was 76.8% ($p<0.5$) and specificity was 93.3%. They had four false negatives which turned out to be dysplasia/malignancy on histopathology. The biggest pitfall with BB is risk of false negative results if sample is too superficial. Dysplastic lesions might show normal cytology results in the upper layers of oral mucosa, therefore a sufficient number of cells must be removed to reach the deeper cell layer of the lesion. It has been suggested that a sufficient sample should cause pinpoint bleeding at the site of lesion. False negative test results using BB have been reported. So a negative cytology result must be interpreted in the light of clinical pretest probability of cancer. Highly suspicious lesions with negative BB results should be repeated or the patient should be advised for an excisional biopsy.[21]

Svirsky et al.,[22] studied 243 patients with abnormal BB and showed 38% positive predictive value, suggesting that these patients have strong positive predictive value for dysplasia or cancer. Similar to study by Gupta et al.,[2] combined evaluation of toluidine blue staining and oral brush biopsy showed an increase in sensitivity in premalignant cases, but in malignant cases, no additional advantage was obtained. Rammerbach et al.,[23] studied 1328 exfoliative smears of which 332 lesions were compared with histology. Additionally, nuclear DNA content was measured after Feulgen restaining using a TV image analysis system. The sensitivity of cytologic diagnosis in addition to DNA image cytometry on oral smears for detection of cancer cells was 98%, specificity 100%, positive predictive value 100% and negative predictive value 98%. However, in our study DNA image cytometry was not done. Highly sophisticated diagnostic techniques like cytomorphometry, DNA cytometry and molecular analysis have become the recent trend.

Though molecular markers was not used in our study, further study including epigenetic alterations (hypermethylation of promoter regions) and genomic instability such as loss of heterozygosity and micro satellite instability can help in knowing the pattern of carcinogenesis.

Conclusion

Early detection of oral cancer is possible even at precancerous stage by using noninvasive, painless outpatient procedure of combined in vivo supravital toluidine blue staining and brush biopsy. This technique increases the sensitivity and specificity in detecting premalignant lesions and also to minimize false negatives. Toluidine blue showed a few false positive results, especially in inflammatory lesions. This can be used as an ideal screening tool even in rural areas where sophisticated diagnostic detection is not possible.

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