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Original Article

Dentistry Section

Assessment of DNA Ploidy in Oral Potentially Malignant Disorders using a VELscope

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ABSTRACT

Introduction: The device VELscope (Visually enhanced lesion scope) is intended as an aid to the clinician to complement a conventional white light exam (whether it be a general oral cavity examination or examination of a particular lesion) to detect abnormal tissue that might have otherwise been overlooked. In this study, it was used concomitantly with ploidy status to evaluate the malignancy potential of Oral Potentially Malignant Disorders (OPMD).

Aim: To evaluate potential of Deoxyribonucleic Acid (DNA) ploidy as a preliminary adjunct to oral biopsy to identify patients with aneuploidy for the necessity of biopsy, and to assess the sensitivity of VELscope in the determination of accurate clinical parameters of the lesions in the oral mucosa.

Materials and Methods: This in-vivo cross-sectional study was carried out in Department of Oral and Maxillofacial Pathology and Oral Microbiology, Bharati Vidyapeeth Deemed to be University, Pune from February 2020 to February 2021. Clinically diagnosed OPMD were included in the study, after verification of clinical extension boundaries by VELscope. DNA ploidy status was evaluated with DNA image cytometry and exclusive individuals with aneuploidy were biopsied followed by histopathological evaluation and surgical removal of the lesion. One way Analysis of Variance (ANOVA) test was used to compare the mean in 4 study groups [Oral leukoplakia, Oral submucous fibrosis, Oral lichen planus and Tobacco pouch keratosis] vs control. Sensitivity, specificity, positive predictive value and negative positive value was calculated by using the ROC (Receiver Operating Characteristic Curve) analysis. A p-value <0.05 was considered as significant for evaluating sensitivity of VELscope and evaluation of ploidy status.

Results: The study included patients whose mean age was 45.8 years. The groups had tobacco habits in one of the various forms [smoke or smokeless] both in experimental and control group. VELscope could identify the clinical borders of the OPMDs considered via the loss of autofluorescence. Lesion borders were identified precisely with the loss of autofluorescence. It was quite helpful especially in aneuploid cases where the whole margin could be removed leaving no genetically aberrant cells behind. Oral submucous fibrosis was concluded to have maximal sensitivity of 86.7%, 100% in specificity and positive predictive and 88.2% negative predictive value.

Conclusion: The DNA ploidy could decipher the malignancy potential and could identify the individuals who needed biopsy. VELscope was able to mark the clinical diameters which were not visible to naked eyes clinically. Oral submucous fibrosis was found to be grouped with the maximum potential of malignancy followed by Oral Lichen Planus, Oral Leukoplakia and Tobacco Pouch Keratosis.

Keywords: Deoxyribonucleic acid, Loss of autofluorescence, Malignancy potential, Ploidy status, Visually enhanced lesion scope

INTRODUCTION

The early detection and management of epithelial dysplasia in OPMD is an important preventative step against malignant transformation. The current guideline recommends Conventional Oral Examination (COE), which involves visual examination and tactile palpation under white light with biopsy being gold standard for confirmation [1]. Any approach that simplifies the visualisation of a dubious lesion could help an oral clinician to detect malignancy transformation in its early stages. Hence, the evolution of several light-induced fluorescence visualisation appliances like the VELscope. Due to the increased demand for non invasive diagnostic evaluation which might promulgate as the regular white light oral examination for the diagnosis of OPMDs. It presents with a sensitivity of 98% and specificity of 96%-100% [2]. Shah S et al., had successfully investigated the efficacy of autofluorescence in diagnosing OPMDs [2]. Xiaobo Lu et al., a systemic review, on the use of autofluorescence to diagnose OPMD, has implied that it was more suitable for specialist clinics than for primary care [1,3].

Aneuploidy is defined as an abnormal balance of either intact chromosomes or segments of chromosomes or both. Aneuploidy stands out as the most consistent marker of malignancy. A simple aneuploidy is the earliest and most distinctive preneoplastic genotype [4]. Currently, the relationship between DNA aneuploidy and the clinicopathological risk factors of OPMDs, including smoking, patient age, lesion site, and dysplasia, are unclear [5]. Ploidy analysis utilising DNA image cytometry is known to predict malignant transformation [6].

Oral biopsy is an invasive procedure, associated with bleeding, has risk of infection, delays the diagnosis, and requires expertise of the performer [7]. This cross-sectional study was done to observe how ploidy status can be used as an adjunct to oral biopsy and how every individual with OPMD need not get exposed to biopsy noted in daily practice. The study aimed to know if DNA ploidy could be applied as a primary assessment concurrently using VELscope to detect involved areas clinically.

MATERIALS AND METHODS

This in-vivo cross-sectional study was carried out in Department of Oral and Maxillofacial Pathology and Oral Microbiology, Bharati Vidyapeeth Deemed to be University, Pune, Maharashtra, India, from February 2020 to February 2021. It was approved by the Institutional Ethics Committee bearing number (EC/NEW/INST/ 2019/329).

Inclusion criteria: Patients with clinically visible OPMD like oral submucous fibrosis, oral lichen planus, oral leukoplakia, tobacco pouch keratosis were included [8]. Each disorder included 30 cases along with 30 controls were considered with age ranging from 20-60 years. This age range was selected since most cases of OPMD

were observed in this range with the initiation of deleterious habits that start around early 20s.

Exclusion criteria: Cases with frank oral squamous cell carcinoma were excluded.

Study Procedure

A convenient sampling method was used without performing any power analysis. The lesions were scanned with VELscope [VELscope Vx PN LD 300-0001.manufactured by LED Dental Inc, 580 Hornby St Suite 810, Vancouver BC, V6C 386 Canada]. Brush biopsy was done under VELscope scan. The smear was collected in a vial consisting of ethanol (22.5% by weight) as a preservative and was sent to the lab for Deoxyribonucleic acid (DNA) image cytometry (ICM) evaluation. Slide-Slick (Emulsion) with 220 mL of distilled water was added to 2 mL of slide-slick prepared for 10 minutes and dried till no fluid residue. One conical-bottomed tube labelled with specimen number and one round-bottomed test tube per sample was required. Using pipette 100 µL of glucyte cell was added to round tube and specimen was twirled in the vial for 10 seconds. 6 mL of the specimen was placed in the centrifuge tube, and then it was centrifuged at 1500 rpm for 5 minutes. The cell pellet-containing fluid was removed from the centrifuge tube. Until there was no fluid, the tube was placed upside down on a cloth that absorbed all the fluid. The glucyte manual method was used, to measure the size of the pellet, the appropriate amount of distilled water was added, and then the mixture was vortexed until the water and cell pellet were well-combined. Two drops of the cell mixture was transferred from the centrifuge into a round-bottomed tube filled with glucyte. The centrifuge tube had 50-100 L of glucyte which was added to it directly and vortexed for 5 seconds. Two droplets to the middle of each slide was transferred with a label, then it was air-dried for an hour and it was sent for dehydration after being stained with feulgen-eosin [9,10].

DNA-ICM assessment: The preparation of the slides till assessment of the DNA indices were in accordance with the principles mentioned by Aubele M et al., [6] and Biesterfeld S et al.,[9]. The usual precision of recent DNA ICM allowed the DNA stem lines to be identified as abnormal (or aneuploid), if they deviate more than 10% from the diploid (2c) or tetraploid region (4c), i.e., if they are outside 2c +/- 0.2c or 4c +/- 0.4c. A DNA-stem line ideally be identified as polyploid within the duplication position of a G0/1- phase-fraction 0.2c +/- (at 4c), and +/- 0.4c (at 8c), respectively, with an error probability p-value <0.05 if, the coefficient of variation (CV) of the ratios between modal Integrated Optical Density (IOD)-values of non pathologic G0/1-and G2/M-phase-fractions in a series of measurements is <2.5%.

DNA-ICM ploidy measurement: Slides were stained with Feulgen Eosin and scanned with an automated DNA image cytometer (Motic Easy Scan Pro: Motic Inc., Xiamen, China) and analysed using Motic imaging software (Moti Classify Version: 1.0.0.1004). The DNA content of each cell was measured by the integrated optical density normalised to a measure known as DNA index. Sample reporting was done in accordance with the European Society for Analytical Cellular Pathology (ESACP) guidelines [10]. According to Motic guidelines, a DNA-ICM diagnosis is considered reliable when the sample contains more than 3000 cells and has an integrated optical density of >90. Cells were automatically divided into three groups based on their DNA Index [DI] in accordance with Motic guidelines- diploid (0.9-1.2), cycling or proliferating (1.2-2.45) and aneuploid (DI≥ 2.5) [9,10].

Interpretation of ploidy report:

- Green-Normal cells
- Orange-Neoplastic changes
- Red-Malignant changes

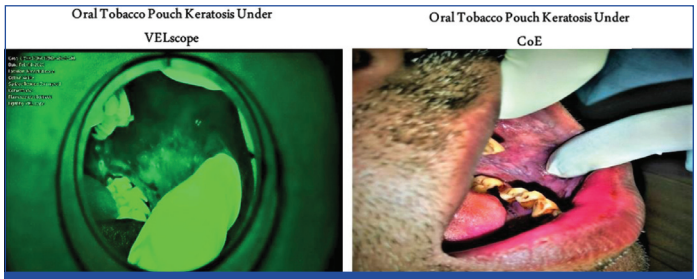
STATISTICAL ANALYSIS

A ROC curve (Receiver Operating Characteristic Curve) analysis was done to analyse the specificity, sensitivity, negative and positive predictive value [NPV, PPV]. The predetermined cut-off was 2.5 [10], and we obtained a second cut-off number using the ROC curve analysis. There are thus two cut-off values. Frequencies of test outcome were considered for n1 patients with diploid lesions and n2 patients with aneuploid lesions. In diagnostic test with dichotomous outcome (positive/negative test results), the sensitivity and specificity were used as measures of accuracy of test in comparison with gold standard status. When the test results were recorded in ordinal scale [diploid/ aneuploid] or when the test results were reported on continuous scale, the sensitivity and specificity was computed across all the possible threshold values [11]. According to the Area under the ROC Curve (AUC), the reference point was 1.94 for oral leukoplakia, 1.88 for tobacco pouch keratosis, and 1.94 for oral submucous fibrosis and oral lichen planus [11].

RESULTS

The mean age of the population was 45.8 yrs. The groups had tobacco habits in one of the various forms [smoke or smokeless] both in experimental and control group.

Tobacco pouch keratosis: Amongst 30 subjects 4 were females rest 26 were males; 26 cases were found to be diploid and sent for periodic screening without the need for oral biopsy. It accounted to 86.66% who did not require an invasive detection methodology. The average range of aneuploid DNA indices ranged between 2.61-3. ROC curve analysis was conducted with the mean DNA Index value of 1.88 as the point of validation. Although the sensitivity was 50%, the specificity was 93%. While the positive predictive value was 88.2% and the negative predictive value was 65.1%. The subjects were mostly counselled to quit the habit followed by periodic follow-up to observe for resolution of keratosis. VELscope could successfully detect 15 cases of which 4 were aneuploid lesions with a sensitivity [Table/Fig-1,2,3] summing upto 50%.



[Table/Fig-1]: Case of tobacco pouch keratosis.

Diseases	Sensitivity values	True positive values	True negative values	False positive values	False negative values
Control			30		
Oral leukoplakia	0.766	23	0	0	7
Oral lichen planus	0.633	19	0	0	11
Oral submucous fibrosis	0.866	26	0	0	4
Tobacco pouch keratosis	0.5	15	0	0	15
Grand total	2.766	83	30	0	37

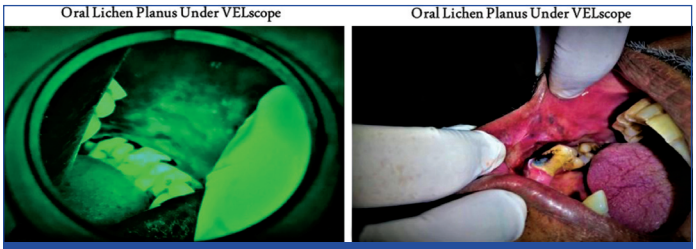
[Table/Fig-2]: Representation of the sensitivity of VELscope scan.

Oral lichen planus: Amongst 30 subjects 9 were females rest 21 were males; 12 cases were found to be diploid and sent for periodic screening without the need for oral biopsy. It accounted to 40% who did not require an invasive detection methodology. The average range of aneuploid DNA indices ranged between 2.50-3. ROC curve analysis was conducted with the mean DNA Index value of 1.94 [AUC Segment value] as the point of validation. The sensitivity was 63.3%, the specificity was 100%. While the positive

Diagnosis	DNA Index	Cases	Controls	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Oral leukoplakia	>1.94	23	0	23	76.7	100.0	100.0	81.1
	≤1.94	7	30	37				
	Total	30	30	60				
Oral submucous fibrosis	>1.94	26	0	26	86.7	100.0	100.0	88.2
	≤1.94	4	30	34				
	Total	30	30	60				
Oral lichen planus	>1.94	19	0	19	63.3	100.0	100.0	73.2
	≤1.94	11	30	41				
	Total	30	30	60				
Tobacco pouch keratosis	>1.88	15	2	17	50.0	93.3	88.2	65.1
	≤1.88	15	28	43				
	Total	30	30	60				

[Table/Fig-3]: Representation of the sensitivity, specificity, negative predictive value, positive predictive value of the four study groups [based on ROC curve analysis].

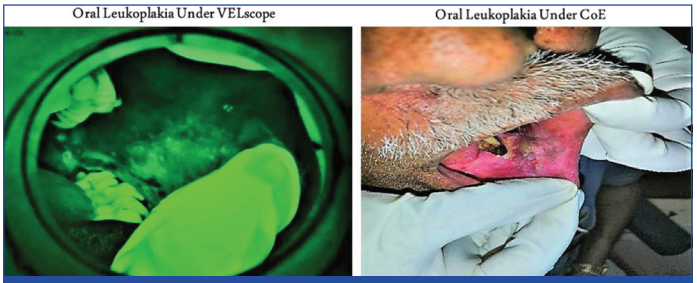
predictive value was 100% and the negative predictive value was 73.2%. The 18 aneuploid lesions [60%] were advised for biopsy and the level of dysplasia was evaluated. Some were detected as lichenoid dysplasia for which corticosteroids were advised followed with surgical removal. The rest 12 with diploidy were exclusively treated with corticosteroids without surgical intervention. VELscope could successfully detect 19 cases of which 12 were the aneuploid lesions with the sensitivity [Table/Fig-2,3,4] summing upto 63%.



[Table/Fig-4]: Case of oral lichen planus.

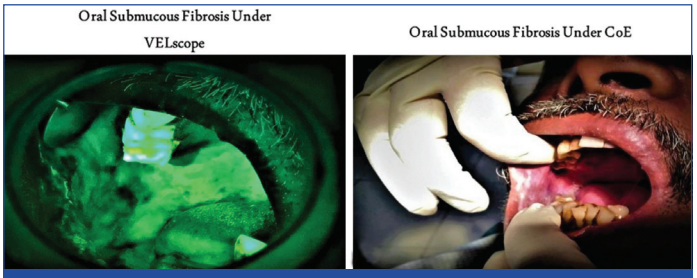
Oral leukoplakia: Amongst 30 subjects 11 were females rest 19 were males; 12 cases were found to be diploid and sent for periodic screening without the need for oral biopsy. It accounted to 40% who did not require an invasive detection methodology. The average range of aneuploid DNA indices ranged between 2.56-3. ROC curve analysis was conducted with the mean DNA Index value of 1.94 [AUC segment value] as the point of validation. Although the sensitivity was 76.7%, the specificity was 100%. While the positive predictive value was 100% and the negative predictive value was 81.1%. The 18 subjects who had aneuploidy were recommended for biopsy to check the gradation of dysplasia. Post to which surgical removal was carried out. The ones who did not show aneuploidy were kept on antioxidants, isoretinol and multivitamins as well as counselled for quitting of habit that led to regression of the lesions. VELscope could successfully detect 23 cases of which 18 were aneuploid lesions and 3 diploid with the sensitivity [Table/Fig-2,3,5] summing upto 77%.

Oral submucous fibrosis: Amongst 30 subjects 4 were females rest 26 were males; 11 (36.66%) cases were found to be diploid and sent for periodic screening without the need for oral biopsy. The average range of aneuploid DNA indices ranged between 2.50-3. ROC curve analysis was conducted with the mean DNA Index value of 1.94 [AUC segment value] as the point of validation. Although the sensitivity was 86.7%, the specificity was 100%. While the positive predictive value was 100% and the negative predictive value was 88.2%. This group exhibited maximal subjects showing 19 aneuploid lesions which mandated histopathological analysis. The ones with dysplasia needed surgical flap intervention, while the remaining 11 cases without aneuploidy were counselled for habit and intralesional therapy was administered comprising of dexamethasone, hyaluronic acid and placentrax. VELscope could successfully detect 26 cases of which 19 were aneuploid lesions



[Table/Fig-5]: Case of oral leukoplakia.

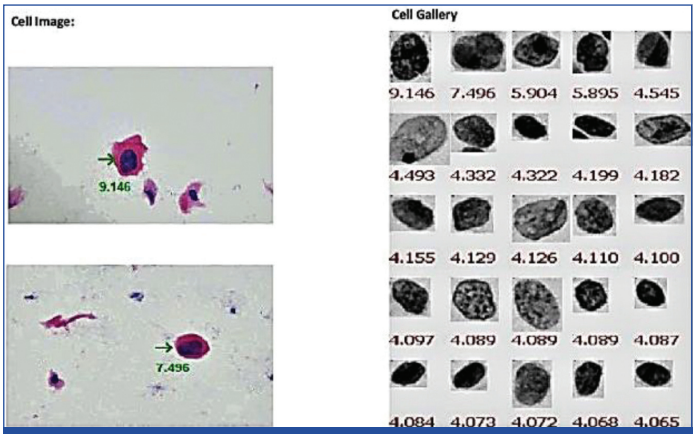
and 7 diploid with the sensitivity of detection [Table/Fig-2,3,6] summing upto 87%. The DNA indices and the pictorial presentation of DNA diploidy and aneuploidy are shown in [Table/Fig-7-9].



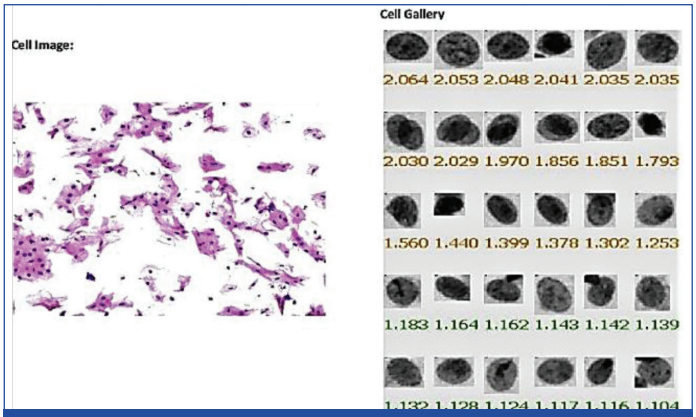
[Table/Fig-6]: Case of oral submucous fibrosis.

Study groups	DNA ploidy	Cell count
Oral submucous fibrosis	2.45	1750.9
Oral lichen planus	2.39	1689.73
Oral leukoplakia	2.31	1617.36
Tobacco pouch keratosis	1.92	1223.46
Control	1.56	412

[Table/Fig-7]: The cell count and DNA Indices of the four study groups vs control.



[Table/Fig-8]: Representation of DNA aneuploidy indicated by red marked indices [biopsy recommended].



[Table/Fig-9]: Representation of DNA diploidy indicated by green marked indices while neoplastic changes by orange marked indices [follow-up].

DISCUSSION

The need for the hour is to cut down on needless repeated biopsies for every OPMD encountered in clinical practice. The invasive procedure is quite intimidating at the level of primary care. This cross-sectional research was undertaken after significant observations by the investigators that patients with OPMDs were lost to follow-up whenever they were advised oral biopsy at chairside detection. The VELscope was utilised since loss of autofluorescence in dysplastic and cancerous tissue is believed to reflect a complex alteration due to the breakdown of the collagen matrix and elastin composition with decrease in flavin adenine dinucleotide concentration and increase in the reduction form of nicotinamide adenine dinucleotide associated with progression of the disease [12]. VELscope is useful in confirming the boundaries of the OPMDs, that went undetected with naked eyes. When compared to the traditional oral examination, Shah S et al., highly recommend the use of autofluorescence, which is consistent with the current findings [2]. Exclusive use of VELscope cannot segregate high and low risk lesions [12]. This is where DNA ploidy analysis comes as DNA ICM can predict malignant transformation in OPMD [13-15]. Zaini ZM et al., determined the number and tissue organisation of aneuploid cells in oral dysplasia [15]. It was mentioned that DNA ICM ploidy analysis is a gross DNA measurement technique that works well to predict development of malignancy in clinical practice. ROC analysis revealed a good response with ICM in DNA ploidy in terms of good sensitivity and specificity. ROC curve analysis was applied in the present study as well, to estimate the sensitivity, specificity, NPV and PPV. Values indicated significant high NPV and PPV as well as sensitivity and specificity. Despite the fact that the category for tobacco pouch keratosis only gave a 50% chance that it was a reactive lesion. While the other 3 study group [oral leukoplakia, oral submucous fibrosis and oral lichen planus] indicated quite significantly high sensitivity and specificity [16,17].

The DNA ploidy was measured using Flow Cytometry (FCM) by T Vijayavel and N Aswath in 2013 to correlate histopathological grading and ploidy status in OPMDs [18]. After conducting the analysis of sensitivity, specificity, PPV and NPV it was found that oral submucous fibrosis had the maximum potential to undergo malignancy transformation with 100% value in specificity and PPV and with maximum value of NPV 88.2% and sensitivity of 86.7%

Sperandio M et al., determined that the overall PPV for malignant transformation caused by DNA aneuploidy was 38.5% and that it was 39.5% for severe dysplasia grade [14]. While the four study groups in this present research by the authors specificity ranged from 93 to 100%, their sensitivity ranged from 50-86.7%. Oral leukoplakia and oral submucous fibrosis had the highest values. Combining DNA ploidy analysis with dysplasia grading gives a higher predictive value than either of the technique alone. The lesions could be better analysed in terms of its malignancy potential, subjects could be convinced better and had less apprehension to biopsy and were not lost to follow-up; thus were more conforming to the protocol. Last but not the least 100% clinical detection was assured aided by loss of tissue autofluorescence through VELscope scan that reduced potential of malignant transformation.

The research was successful since it gave validity to the hypothesis put forth that utilisation of ploidy analysis along with precisive scan by VELscope, to determine the boundaries beyond the affected whitish area by the loss of autofluorescence could be more scientific and a personalised mode of diagnostic protocol could be devised before suggesting an invasive assessment methodology of oral biopsy.

Limitation(s)

The methodology of DNA ploidy evaluation aided with VELscope is a technique sensitive procedure as collection of cytology smear seems to be a challenge. Lesions that are exposed to bleeding during digital manipulation may prevent a sufficient number of cells from being counted, which could lead to inaccuracies in the evaluation of DNA indices since the calculation of indices depends on the number of cells collected. The ability to visualise through VELscope requires adequate technical training.

CONCLUSION(S)

Patients with OPMDs are more wary of the recommended course of treatment when they are given general advice to get a biopsy. Thus, ploidy status would provide a better approach to determine which patient specifically needs the invasive procedure of biopsy. VELscope showed an elaborate and enhanced diagnostic approach with sensitivity as high as 86.7% and specificity of 100% as a chair-side clinical assessment tool for OPMDs.

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