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RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL DETECTION OF EBV IN ORAL LEUKOPLAKIA.

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Abstract

Background & Objectives: Oral Leukoplakia (OL) has multifactorial etiology with tobacco being the major etiological factor; however other factors can also influence its prognosis. The association of Epstein Barr virus (EBV) in etiology of OL is being investigated as it can cause proliferation of oral squamous epithelium after reactivation by tobacco use. Hence, the study was aimed to detect Epstein Barr virus antigen expressivity in oral leukoplakia by immunohistochemistry.

Methods: 60 cases of oral leukoplakia were selected according to selection criteria and were subjected to incisional biopsy, followed by histopathological grading, OLEP staging and immunohistochemical staining for detection of EBV expressivity. The obtained values of these parameters were tabulated, statistically analyzed and observations were drawn.

Results: Non-homogenous leukoplakia was significantly associated with strong EBV expressivity. Also, moderate epithelial dysplasia was significantly associated with moderate as well as strong EBV expression (p value < .05).

Conclusion: The present study demonstrated the presence of EBV expressivity in various clinical forms of oral leukoplakia and with higher grades of epithelial dysplasia.

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Introduction:-

The etiology of OL is multifactorial, tobacco in different forms has been established as the main etiological factor as 80% of OL are present in tobacco users. Individuals smoking tobacco for more than 10 years have nearly 11 times greater risk of developing OL than non-smokers.¹ About 35-40 % of tobacco consumption in India is in the form of smokeless tobacco and it causes OL in 18-64 % of its users at the site where it is held.^{2,3} Besides this, infections by *Candida*, human papillomavirus (HPV), and more recently Epstein Barr virus (EBV) have been identified as cofactors that may affect the prognosis of established OL.⁴ Association of EBV in subgroups of leukoplakia namely oral hairy leukoplakia and proliferative verrucous leukoplakia has been observed and is related with their clinical appearance and subsequent clinical course.⁵

EBV is a double stranded DNA virus, member of Herpes viridae family with a serologic prevalence of about 95% in adults worldwide.⁶ Following primary infection, it enters latent phase in oropharyngeal, salivary gland epithelial

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cells and in a small proportion of B-lymphocytes. But these can go into reactivation cycle leading irreversibly to death of infected cells with release of mature virions, which are then shed in saliva. These virions can then enter oral epithelial surface via intercellular micro-defects and invades the terminally differentiating keratinocytes, inducing their proliferation.⁷

Studies have demonstrated that purified products of smokeless tobacco can cause recurrent EBV reactivation in a dose dependent manner and deficiency in local immune defense with decrease in number of Langerhans cells and natural killer cells.^{8,9,10} The end products of smoking tobacco increases levels of cytokines and growth factors, which contributes to local immune suppression.¹¹ In congruence, this might result in shedding of EBV from natural reservoirs and its replication.¹²

Literature review also suggests that association of EBV is more in tobacco users and EBV may have a synergistic effect with tobacco in etiology of OL and also in its malignant transformation. Hence, identifying the association of EBV in different clinical forms of OL with tobacco habit may alter their subsequent clinical course, prognosis and management. However there is scarcity of studies demonstrating the expression of EBV in OL amongst tobacco users in India. Realizing the paucity this study was designed to detect EBV in OL with different habits of tobacco.

Aims:-

1. To detect EBV antigen expressivity in oral leukoplakia associated with smoke and smokeless tobacco habits.
2. To associate different clinical forms, histological grades of oral leukoplakia with EBV antigen expressivity

Material & Method:-

This study was conducted on 60 subjects of OL and 10 healthy age and sex matched controls who were selected based on the selection criteria during the period of December 2014 to June 2016 visiting Department of Oral Medicine and Radiology, Government Dental College and Research Institute, Bangalore. The study was conducted in full accordance with ethical principles and was reviewed and approved by an ethical board of the institution. All the selected subjects were informed about the details of the study in their known local language and a written informed consent was obtained. A detailed case history, thorough clinical and oral examination was then carried out and documented on a specially designed case history proforma.

Inclusion Criteria For Study (Cases) Subjects:-

1. Patients with OL associated with smoke and smokeless tobacco habits in the age group of 18-60 years.
2. Patients with clinically diagnosed and histologically proven case of OL.
3. Patients not on any medication for OL for the past 2 weeks.

Inclusion Criteria For Controls:-

Patients visiting the Department of Oral Medicine and Radiology, Government Dental College and Research Institute for extraction of teeth and fulfilling the following criteria:

1. Age and Sex matched subjects with that of the study group.
2. Subjects without habit of tobacco.
3. Subjects without any mucosal lesion.

Exclusion Criteria For Controls And Study Subjects:-

1. Patients with OL associated with factors other than tobacco habits.
2. Patients with history of already established diagnosis of EBV associated malignancies like Burkitts lymphoma, Nasopharyngeal Carcinoma, Infection Mononucleosis, T cell lymphoma, Post Transplant Lymphoproliferative Disease.
3. Patients with other oral lesions such as lichen Planus, Oral Submucous Fibrosis, Leukoedema, White Sponge Nevus, and Cheek bite.
4. Pregnant and Lactating women.
5. Patients with autoimmune diseases.
6. Patients on systemic steroids, immunosuppressive drugs, anticoagulant drugs.
7. Patients with any other systemic diseases

Clinical Categorization, histologic Grading and OLEP staging of Oral Leukoplakia:-

All the selected cases were subjected to detailed examination of the lesion and were grouped into two main types: Homogeneous and Non-homogeneous leukoplakia according to Pindborg et al 1997 (WHO International Histological Classification of Tumors) following which incisional biopsy was done from the most representative area.¹³ A systematic histopathologic assessment and grading of the H & E stained sections was done by an oral pathologist according to WHO classification of oral epithelial dysplasia (2005) followed by staging based on Modified classification and staging system for oral leukoplakia (OLEP).^{14,15}

Immunohistological Study & evaluation of stained sections:-

Sections of 5-micron thickness from paraffin blocks of 60 study subjects and 10 controls were immunohistochemically stained using My Biosource & Impress Detection Kit and were viewed by a pathologist for the presence of EBVNA1 positivity that was clearly identified by its brown color nuclear staining under a magnification of 40x (Figure 1 A & B). All sections that showed nuclear staining were considered positive and were graded qualitatively according to the following scale.

Grading System For Staining¹⁴⁸: -

STAINING	GRADE
Negative	0
Weakly	1
Moderately	2
Strongly	3

Results:-

The study population comprised of 60 cases and 10 controls. All the 60 cases were in the age range of 26 to 60 years with mean age of 40 ± 9.24 years. Among 60 cases, 49 were males and 11 were females. The tobacco habit varied from chewing tobacco, smoking, both variants and some patients had the habit of alcohol consumption along with the tobacco (Graph 1). Graph 2 shows distribution of EBV expressivity with different tobacco habits. Overall there was statistically significant association of tobacco & alcohol group with EBV expression (p value < .05).

The maximum number of lesions was noticed on buccal mucosa alone (46) followed by commissures only (7) & combined buccal mucosa and commissure had 7 lesions. The association of EBV expressivity with these sites varied as depicted in Graph 3.

Among 60 subjects, homogenous form was noticed in 38 (63.3%) subjects, followed by non-homogenous form in 22 (36.6%) subjects. When compared to other subtypes, nodular non-homogenous type was associated with higher degree of EBV expressivity and overall the association between different clinical type and EBV expressivity was statistically significant with a p value of <0.001 (Graph 4).

The histological examination of the biopsy specimens revealed maximum number of cases with mild dysplasia (35) followed by moderate dysplasia (22) and hyperplasia (2). Overall, the association between grading of epithelial dysplasia and EBV expressivity was found to be statistically significant (P value < .05) (Table 1)

On OLEP staging, none of the case could be categorized into Stage I, 33 cases were in Stage II, 18 cases were in Stage III followed by 7 cases in stage IV. The EBV expressivity with different OLEP staging varied as shown in Table 1.

Graph 5 shows EBV expressivity in cases & controls.

Discussion:-

Oral leukoplakia is the most common potentially malignant disorder of the oral mucosa. The risk of malignant transformation of the OL is difficult to assess, the clinical risk factors for the OL transformation vary among different study populations. The presence of epithelial dysplasia is a marker of the malignant potential of oral leukoplakia, and the risk of an individual leukoplakic lesion to progress to carcinoma increases with the increase of the grade of the epithelial dysplasia.

In the present study mean age of subjects with oral leukoplakia was 44.6 ± 7.3 years ranging from 26 to 60 years with male predominance in the ratio of 4:1. The reason for male predominance could be attributed to peer pressure, tension reduction/relaxation, addictive smoking and habit/automatism. The majority of subjects were with the combined habit of smoke and smokeless tobacco. These observations with habit, gender and age are in concurrence with the study conducted by **Birur et al 2014**,¹⁶ and **Bisht et al 2016**.¹⁷ The predominant form noticed was Homogenous OL (63.3%) with buccal mucosa most common site for its occurrence (92.1%). These observations were in accordance with the studies done by **Sharma et al 2011**¹⁸; **Ramesh et al 2013**¹⁹; **Varshney et al 2015**²⁰; **Birur et al 2014**¹⁶

The association of EBV can be best studied by demonstration of its expressivity at the lesion site, hence in the present study was an attempt to demonstrate the expressivity of EBV in the biopsy specimen of oral leukoplakia by immunohistochemistry which is one of the most specific method for its detection in early stages and can also distinguish latent from replicative infection based on the expression profiles.

On assessing EBV expressivity in study group, we found that 72.7 % of oral leukoplakia cases were positive for EBV. This was not in accordance with the findings of the study by **Horiuchi et al 1995**²¹ who observed 5.3% cases of oral leukoplakia positive for EBV while **Salehi MR et al 2011**²² observed EBV positivity in 44% of oral leukoplakia and in 83% of oral hairy leukoplakia. The discrepancies in prevalence rate may be attributed to the method of sample collection and technique of detection, as oral smears, scrapings, and throat washings seem to give a higher EBV prevalence.¹⁷

The present study evaluated EBV expressivity at different sites in oral cavity and it was found that 67.2% of the leukoplakic lesions involving buccal mucosa showed EBV expression. This observation is in concurrence with study conducted by **Cruz et al 1997**.²³ This is based on the assumption that EBV replicates in epithelial cells of buccal mucosa, thereby accounting for its increased expression. Also, virions that shed into the oral fluid from EBV replication in the oropharyngeal tissue can penetrate micro-defects in the oral epithelial surface where the virus can invade terminally differentiated keratinocytes and induce their proliferation.¹²

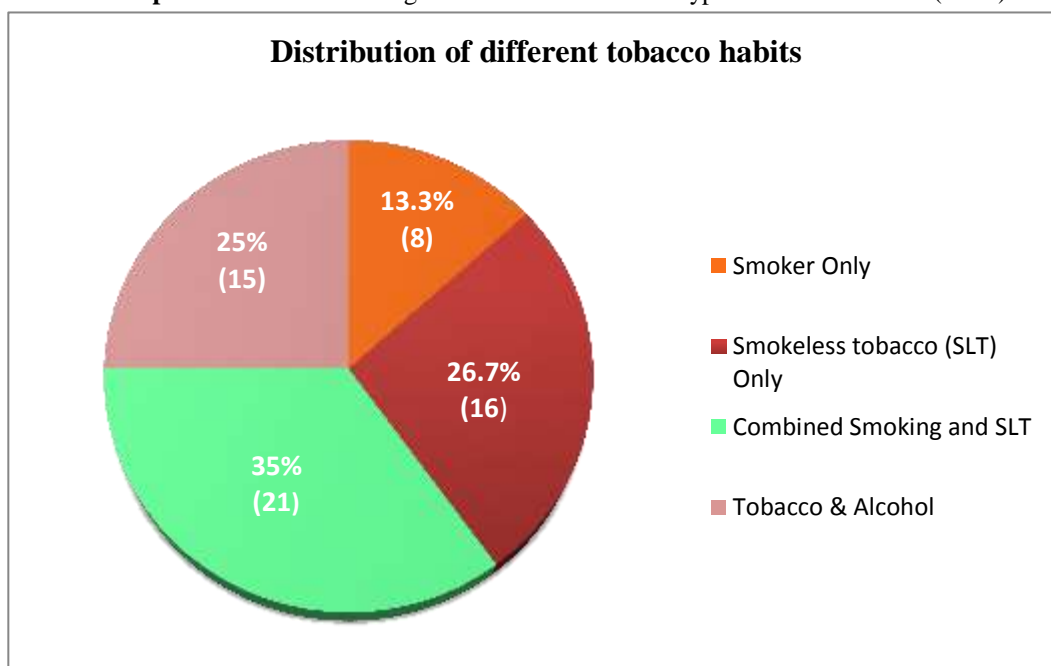
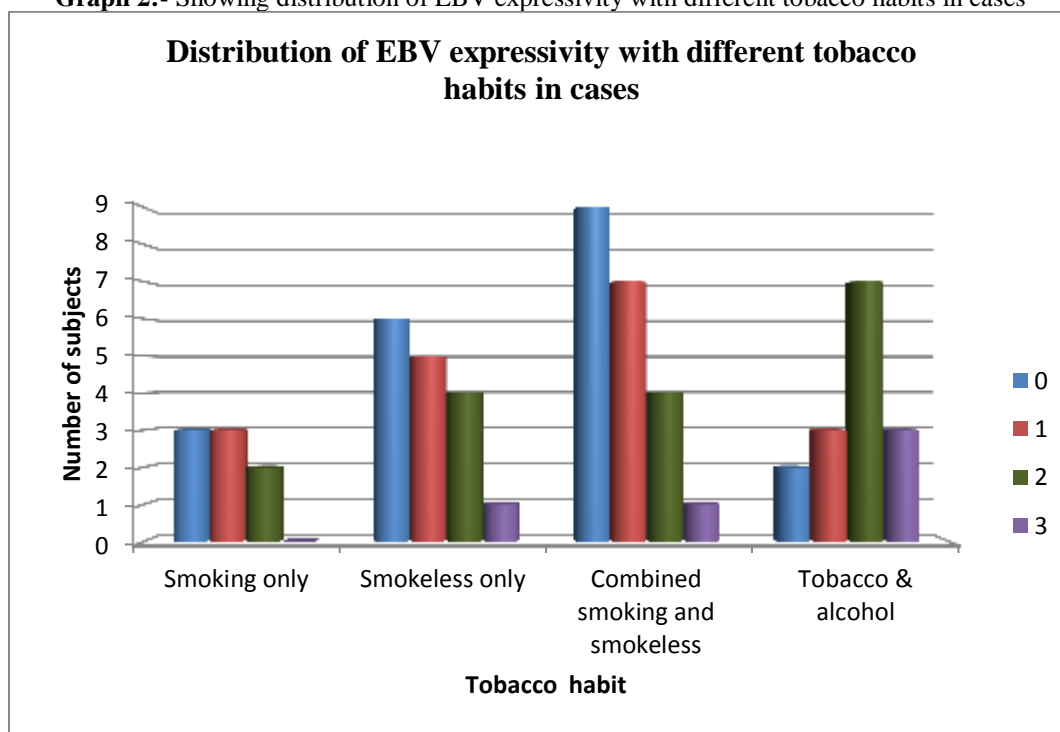
On association of EBV expressivity with different clinical forms of leukoplakia, it was observed that homogenous leukoplakia is more associated with mild EBV expressivity (23.3%) whereas non-homogenous type was more commonly associated with higher grades of EBV expression i.e. moderate (45.5%) and strong (22.7%).

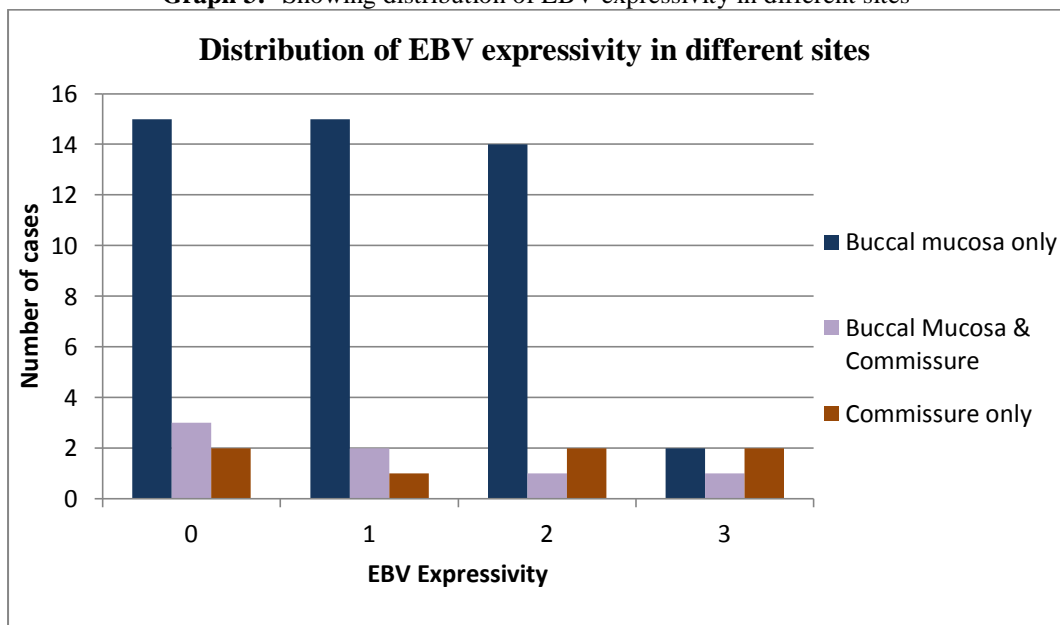
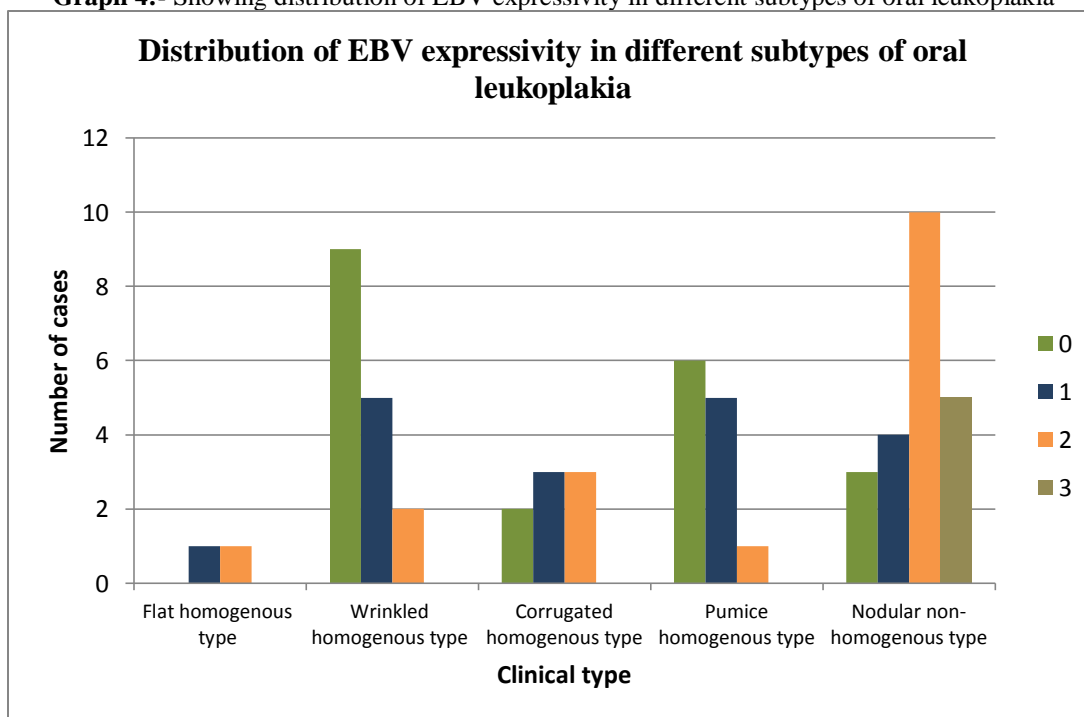
On association of EBV expressivity with tobacco habit, it was found that, 62.5% subjects in smoking group were positive for EBV. This finding is based on the fact that smoking increases levels of cytokines and growth factors i.e. IL-6, IL-10, TGF, which contributes to immunosuppression, thereby leading to increased EBV replication.¹⁶

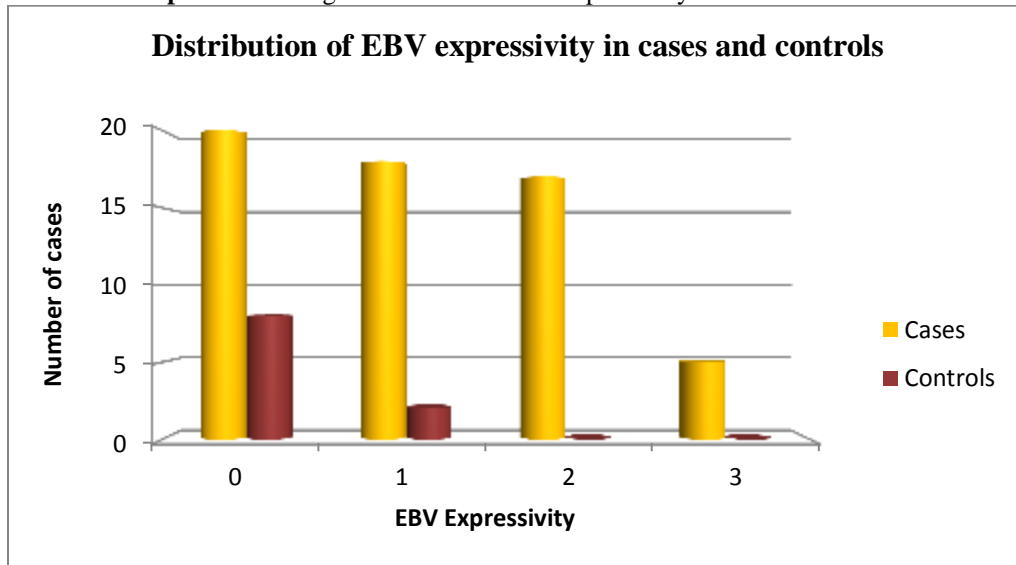
Similar findings were noted in patients with habit of smokeless tobacco wherein 62.5% were positive for EBV but with higher grades of expressivity. This is based on the assumption that smokeless tobacco products can cause recurrent EBV activation in a dose dependent manner leading to accumulation of genomic instability of host cells, which is one of the hallmarks of carcinogenesis.^{13,14} Also, it causes deficiency in local immune defenses thereby leading to increased shedding of EBV from natural reservoirs.¹⁷

EBV expression was also related to advanced stages of epithelial dysplasia as is evident from the observation that EBV is expressed more in subjects with moderate dysplasia (86.9%) as compared to mild dysplasia (45.7%). The findings were in accordance with study by **Rensberg et al 1995**²⁴ and **Cruz et al 1997**²³ who reported up regulation of EBV expression in dysplasia and carcinoma. Also, higher grades of EBV expressivity were associated with stages III & IV of OLEP.

There were certain limitations to this study, as a small sample size of 60 cases and 10 controls was included. Also leukoplakia involving tongue was excluded as tongue is the most common site for oral hairy leukoplakia, which is more often associated with immunocompromised individuals who were excluded from our study so as to assess the role of EBV and tobacco in oral leukoplakia. The habit of alcohol was present in some subjects, which in combination with tobacco may lead to higher grades of dysplasia. The site of biopsy and contamination of specimen with saliva could have resulted in higher detection of EBV in our study.

Graph 1:- Pie chart showing distribution of different types of tobacco habits (cases).**Graph 2:-** Showing distribution of EBV expressivity with different tobacco habits in cases

Graph 3:- Showing distribution of EBV expressivity in different sites**Graph 4:-** Showing distribution of EBV expressivity in different subtypes of oral leukoplakia

Graph 5:- Showing distribution of EBV expressivity in cases and controls**Table 1:-** Showing association between different grades of EBV expressivity with grading and staging of oral leukoplakia

Parameter	EBV expressivity				χ^2 value	'p' value
	0	1	2	3		
Hyperplasia (2)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	4.14	.2467
Mild dysplasia (35)	15 (42.9%)	16 (45.7%)	4 (11.4%)	0 (0.0%)	24.67	<.000**
Moderate dysplasia (23)	3 (13.04%)	2 (8.70%)	13 (56.5%)	5 (21%)	28.75	<.000**
Severe dysplasia (0)	0 (0%)	2 (8.70%)	0 (0%)	0 (0%)	0	0
CIS (0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
Total	20	18	17	5		
Stage I (0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0	0
Stage II (33)	16 (48.4%)	13 (39.3%)	4 (12.1%)	0 (0%)	20.12	<.000**
Stage III (18)	2 (11.11%)	4 (22.2%)	11 (61.1%)	1 (5.56%)	14.32	<.002**
Stage IV (9)	2 (22%)	1 (11.1%)	2 (22.22%)	4 (44.44%)	18.36	<.000**
Total	20	18	17	5		

CIS- Carcinoma In Situ

**denotes highly significant 'p' value

Figure 1:- Stained Section Of Leukoplakia Showing Weakly Stained (A) & Strongly Stained (B) Brown Nuclei Of Epithelial Cells Suggestive Of EBNA1 Positivity

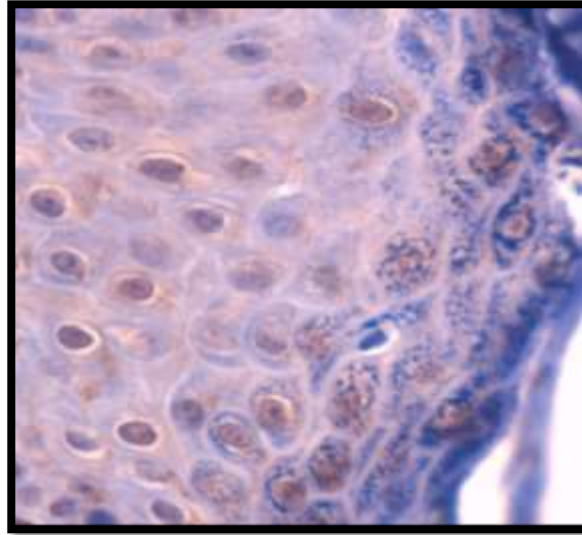


Figure 1(A)

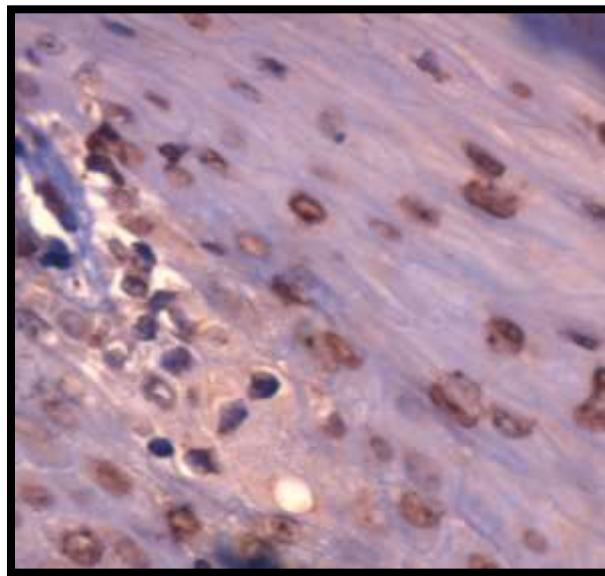


Figure 1(B)

Conclusion:-

This study demonstrated the presence of EBV expressivity in various clinical forms of oral leukoplakia and with higher grades of dysplasia. We are reporting this study on small number of cases, but our positive results encourage further investigations with larger sample size to ascertain the role of EBV in oral leukoplakia.

Bibliography:-

1. Boker M, Vuckovic N. Cigarette Smoking As a Risk Factor With Oral leukoplakia. *Archive of Oncology* 2002;10:67-70.
2. Gupta PC, Ray CS. Smokeless Tobacco And Health In India And South Asia. *Respirology* 2003;8:419-431.
3. Connolly GN, Winn DM, Hecht SS, Henningfield JE, Walker B, Hoffmann D. The Reemergence of Smokeless Tobacco. *N Engl J Med* 1986;314:1020-1027.

4. Calatayad AM, Estrada RB, Sebastian JV, Jimenez OS, Barona CG. Oral Leukoplakia: Clinical, Histopathologic, and Molecular Features and Therapeutic Approach. *Actas Dermosifiliogr.* 2009;100:669-684.
5. Bagan JV, Jimenez Y, Murillo J et al. Epstein Barr virus in oral proliferative verrucous leukoplakia and squamous cell carcinoma : A Preliminary study. *Med Oral Pathol Oral Cir Buccal.* 2008;13:110-113.
6. Baumforth KRN, Young LS, Flavell KJ, Constandinou C, Murray PG. The Epstein-Barr virus and its association with human cancers. *J Clin Pathol: Mol Pathol* 1999;52:307–322.
7. Khammissa RA, Fourie J, Chandran R, Lemmer J, Feller L. Epstein-Barr Virus and Its Association with Oral Hairy Leukoplakia: A Short Review. *International Journal of Dentistry* 2016;2016:4941783.
8. Huang SY, Fang CY, Tsai CH, et al. N-methyl-N'-nitro-N-nitroso guanidine induces and cooperates with 12-O-tetradecanoylphorbol-1, 3-acetate/sodium butyrate to enhance Epstein-Barr virus reactivation and genome instability in nasopharyngeal carcinoma cells.. *Chem Biol Interact.* 2010; 188:623-634.
9. Daniels T, Hansen L, Greenspan J, et al. Smokeless tobacco-associated epithelial and Langerhans cell changes. Smokeless tobacco or health, Monograph 2. NIH, Bethesda, Maryland, USA 1992: 66-73.
10. Johansson S, Hirsch JM, Johnson D et al. Effect of repeated oral administration of tobacco snuff on natural killer- cell activity in the rat. *Arch Oral Biol* 1991;36: 473- 476.
11. Sopor LM, Kozak W. Immunomodulatory effects of cigarette smoke. *J Neuroimmunol* 1998;83:148–156.
12. Sand L, Wallstroem M, Jalouli J, Larsson P-A, Hirsch J-M. Epstein-Barr virus and human papillomavirus in snuff-induced lesions of the oral mucosa. *Acta Otolaryngol.* 2000;120:880-884.
13. Pindborg JJ. Precancerous Lesions. In: Pindborg JJ, Reichart PA, Smith CJ, Vander wall I, editors. Histological typing of oral cancer and precancer of oral mucosa. 2nd edition, Berlin Heidelberg New York, Springer 1997; p. 43-47.
14. Branes L, Eveson JW, Reichart P, World DS. Tumours of the oral cavity and oropharynx. *Pathol Genet* 2005;67:177-179.
15. Van der Waal L, Schepman KP, Van der Meij EH et al. A modified classification and staging system for oral leukoplakia. *Oral Oncol* 2000;36:264-266
16. Birur P, Shubhasini AR, Bhanushree R, Mendonca P. Correlation of oral homogenous leukoplakia with grades of oral epithelial dysplasia. *IOSR J Dent Med Sci.* 2014;13:98-103.
17. Bisht RS, Singh AK, Sikarwar V, Darbari A. Study over the clinical picture and histopathology of leukoplakia and to establish the correlation between causative factors in the patients of Garhwal hill region. *Natl J Maxillofac Surg* 2013;4:177-180
18. Sharma N, Mubeen K. Non -invasive diagnostic tools in early detection of oral epithelial dysplasia. *J Clin Exp Dent.* 2011;3:184-188.
19. Ramesh S, Jhincy T, Rani PM, Daniel VA, Sunila T, Vivek V. Frequency of leukoplakia in patients visiting a dental college located in a rural area of south kerala:A Pilot Study. *J Indian Aca Oral Med Radio* 2013;25:109-111.
20. Varshney S, Sandhir S, Mishra S. A study of oral pre-malignant lesions and related risk factors. *Indian J Comm Health.* 2015;27:130-134
21. Horiuchi K, Mishima K, Ichijima K. Epstein Barr virus in the proliferative diseases of squamous epithelium in the oral cavity. *Oral Surg Oral Med Oral Pathol Oral Radio L Endod* 1995;79:57-63.
22. Salehi MR, Khozeimeh F, Noshadian H. Comparative evaluation of the presence of Epstein-Barr virus in common leukoplakia, oral hairy leukoplakia and healthy mucosa by polymerase chain reaction (PCR). *J Isfahan Dental School* 2011;7:402-408.
23. Cruz I, Van den Brule JC., Steenberg DM. Prevalence of Epstein-Barr Virus in Oral Squamous Cell Carcinomas, Premalignant Lesions and Normal Mucosa- a Study Using the Polymerase Chain Reaction. *Oral Oncology* 1997;33:182-188
24. Van Rensburg EJ, Engelbrecht S, Van Heerden W, Raubenheimer E, Schoub BD: Detection of EBV DNA in oral squamous cell carcinoma in a black African population sample. *In Vivo* 1995;9:199-202.