DETERMINATION OF KI-67 EXPRESSION IN ORAL LEUKOPLAKIA IN SNUFF USERS AND NON-USERS IN KHYBER PAKHTUNKHWAI PROVINCE OF PAKISTAN.

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ABSTRACT... Objectives: The aim of the article was to evaluate Ki-67 expression in oral leukoplakia in snuff users and non-users. Study Design: Descriptive Cross sectional study. Setting: At different hospitals of Khyber Pakhtunkhwa province. Period: From August 2016 – March 2017. Material & Methods: Clinically diagnosed and histopathologically confirmed cases of oral leukoplakia in snuff users and non-users 30 cases each were immunohistochemically examined and percentage expression of nuclear Ki-67 was evaluated by a semi quantitative method in basal, parabasal and suprabasal layers of the lining epithelium. Results: The relationship of expression of Ki-67 in parabasal and suprabasal layers of affected epithelium in snuff users was found to be statistically significant with a p value of 0.007 and 0.002 respectively. Conclusion: The present study concludes that the proliferative index is high in snuff users as compared to non-users which can be a factor for malignant transformation.

Key words: Dysplasia, Ki-67 Antigen, Leukoplakia Oral, Tobacco Smokeless.

INTRODUCTION

Oral leukoplakia is defined according to WHO as a “white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”.¹ The worldwide prevalence of oral leukoplakia is approximately 0.2 - 11.7%.² There is marked geographic variation in prevalence even in the different regions of the same country.³ In a Karachi based Pakistani study the estimated prevalence of oral leukoplakia in Pakistan was found to be 5% and another study conducted in Islamabad reported 7% cases of oral leukoplakia.⁴⁵

It is a potentially malignant disorder of oral cavity which carries a high risk of conversion into frank malignancy.⁶⁷ The global malignant transformation rate of oral leukoplakia ranges from 0.13% to 34% depending on the site.⁸

Although etiology of oral leukoplakia is unknown however most common associated factors are tobacco use⁹⁻¹¹, mechanical trauma, electro galvanic stimulation produced by different restorative metals, Ultraviolet radiations¹², and Human Papilloma Virus 16 and 18.¹⁰¹³

There is strong evidence of carcinogenic effect of tobacco and its smokeless forms on oral mucosa.¹⁰ Of smokeless tobacco users 40% develop oral leukoplakia as compared to 1.5% of non-users.¹⁴ Tobacco use is very common in Pakistan. Smokeless form called Paan is common in Karachi and certain areas of Punjab whereas oral snuff or naswar is widely used in Khyber Pakhtunkhwa and Baluchistan.¹⁵¹⁶

Ki-67 is a human nuclear protein. Its expression is strictly associated with proliferation of the
cell. Therefore it can be reliably used as a marker of proliferative activity.\textsuperscript{17,18,19}

Usually the epithelial dysplasia evaluated by microscopy determines the potential for malignant transformation.\textsuperscript{20} However, there is probability of false negativity in this method. Ki-67 being a marker of proliferative activity can specifically predict the conversion of oral leukoplakia into malignancy.\textsuperscript{21}

The aim of this article is to evaluate and compare Ki-67 expression and proliferative activity in basal, parabasal and suprabasal layers of epithelium in oral leukoplakia of both snuff users and non-users in Khyber Pakhtunkhwa province of Pakistan.

**MATERIALS AND METHODS**

It was a multicenter study which was conducted at different hospitals of Khyber Pakhtunkhwa province from August 2016 –March 2017.

The study comprised of 60 clinically diagnosed cases of oral leukoplakia which were divided into two groups. Group A consisted of 30 cases with a history of snuff use and Group B consisted of 30 cases without any history of snuff use. Cases were independently confirmed by two histopathologists. The relevant data of all the cases was entered in a predesigned proforma.

Immunohistochemistry for Ki-67 was carried out with Dako Envision™ FLEX detection system (Monoclonal Ki-67 Antibody). Tonsillar tissue was taken as positive control for Ki-67. Epithelial cells stained with clear brown color irrespective of staining intensity were taken as positive.

Ki-67 positivity was assessed using a scoring system proposed by Kannan.\textsuperscript{22,23,24} The details of scoring interpretation are as follows:

Score 0 = 0 - 5%
Score 2 = 6- 25%
Score 4 = 26 - 60%
Score 6 = 61 - 99%\textsuperscript{22}

Statistical analysis was carried out using Statistical Package for Social Sciences version 19. Frequency and percentages were calculated for categorical variables, i.e., level of expression of Ki-67 in basal, parabasal and suprabasal layers of epithelium. Mann Whitney test was used for comparing percentages of Ki 67 expression in snuff users and non-users. Probability value of less than or equal to 0.05 (P value ≤ 0.05) was considered statistically significant.

**RESULTS**

Among these 60 cases of oral leukoplakia 43 (71.7 %) were males and 17 (28.3%) were females. Male to female ratio was 2.5:1.

All 60 cases of oral leukoplakia were evaluated for expression of Ki-67 marker in epithelial layers with IHC. Nuclear expression in oral leukoplakia in snuff users and non-users is shown in Tables-III and IV respectively.

| Males | Females | Total |
|-------|---------|-------|
| Snuff Users | Non-Users | Snuff Users | Non-Users | Snuff Users | Non-Users |
| 30 (100%) | 13 (43.3%) | 0% | 17 (56.7%) | 60 |

*Table-I. Gender distribution in oral leukoplakia in snuff users and non users*

| S. No | Histopathological Features | Snuff Users | Non-users |
|-------|--------------------------|------------|----------|
|       | No of Cases (%)          |            | No of Cases (%) |
|       |                          |            |            |
| Dysplasia | 6(20)                   | 6(20)       |
| Hyperplasia | 3(10)                   | 11(36.7)    |
| Hyperkeratosis | 5(16.7)             | 7(23.3)     |

*Table-II. Distribution of most common histopathological features in oral leukoplakia in snuff users and non-users*
Relationship of expression of Ki-67 in snuff users and non-users in parabasal and suprabasal was significant with a p value 0.007 and 0.002 respectively.

**DISCUSSION**

In our study oral leukoplakia was more common in males (71.7%) than in females (28.3%). This finding is consistent with the international study conducted by Nair et al. In the present study 20% cases showed histopathological features of dysplasia in oral leukoplakia which is in contrast with an Indian study by Kumar et al with 52% cases of dysplasia. However, work by Marne et al., showed no dysplasia in snuff induced oral leukoplakia. The marked difference in the results of these studies from different parts of the world may be due to the composition of snuff which varies from country to country.

Clinical identification of oral leukoplakia with risk of malignant transformation is a challenge for clinicians. On histological examination malignant transformation begins as a mildly dysplastic lesion which may or may not progress to full thickness dysplasia (carcinoma in situ). So early detection of abnormal changes is of utmost importance and helps to reduce morbidity and mortality by adopting the right approach to management of the patient. For this purpose different immunological markers are used for assessment of cellular proliferation which includes PCNA and Ki-67. Among these markers Ki-67 is more commonly used because its nuclear expression in specific period of cell cycle (G1 and M phases) makes it more reliable as an indicator of the level of mitotic activity. Moreover it has a short half-life so it produces less residual staining in cells which have passed through proliferative stage.
Most of the proliferation takes place in basal layer of epithelium while in rest of the layers maturation of cells takes place without proliferation. Therefore any proliferative activity above the basal layer carries an alarming sign.\textsuperscript{30} In snuff users the mean of percentages of Ki-67 expression in basal layer was 60.93±30.6 and in non-users it was found to be 57.10±35.7. Relationship of Ki-67 expression in basal layer in snuff users and non-users was statistically not significant (p value 0.79). It means that it was expressed in almost the same percentage in snuff users and non-users in basal layer with a cut off value ranging from 25-30%.

The mean of percentages of Ki 67 expression in parabasal layer in snuff users was 64.9±19.7 and in non-users it was 51.03±22.0. Relationship of expression of Ki-67 in snuff users and non-snuff users was significant (p value .007) which revealed that it is highly expressed in parabasal layer in snuff users than non-users.

The mean of percentages of Ki-67 expression in suprabasal layer in snuff users was 20.4±21.0 and in non-users it was 6.23±10.7. Relationship of Ki-67 expression in snuff users and non-users was statistically significant (p value 0.002), showing that there is a marked difference of its expression in suprabasal layer in snuff users.

In our study hyperkeratosis was found more in non-snuff users (23.3%) than snuff users (16.7%). Several authors tried to correlate hyperkeratosis with OL but they did not associate it with the use of snuff.\textsuperscript{24,26,30,31}

Similarly, international literature regarding immunohistochemical expression of Ki-67 in snuff users in basal, parabasal and suprabasal layers for comparison with this study could not be found on extensive search. Though snuff is used worldwide but the methods of manufacturing and application of snuff in oral cavity is diverse in different parts of the world and even in various regions in the same country.\textsuperscript{32}

**CONCLUSION**

Ki-67 expression was specifically found in parabasal and suprabasal layers in snuff users indicating an increased proliferative activity, so chances of malignant transformation are more in snuff users than in non-users.

**REFERENCES**

1. Villa A and Woo SB. Leukoplakia—a diagnostic and management algorithm. Journal of oral and maxillofacial surgery. 2017; 75: 723-34.

2. Starzyńska A, Pawlowska A, Renkielska D, Michajłowski I, Sobjanek M and Błażewicz I. Oral premalignant lesions: epidemiological and clinical analysis in the northern Polish population. Advances in Dermatology and Allergology/Post-py Dermatologii i Alergologii. 2014; 31: 341.

3. Bokor-Bratić M. The prevalence of precancerous oral lesions: Oral leukoplakia. Archive of Oncology. 2000; 8: 169-70.

4. Rana Z, Khoso N, Bajaj D and Arshad O. Risk factors for precancerous lesions of oral mucosa. Ann Pak Inst Med Sci. 2009; 5: 220-3.

5. Memon IM, Iqbal SM, Hussain SI and Baig MN. Pattern of oral malignancies at tertiary care hospitals. Pak J Surg. 2014; 30: 268-71.

6. Parlatescu I, Gheorghe C, Coculescu E and Tovaru S. Oral leukoplakia—An update. Maedica. 2014; 9: 88.

7. Warnakulasuriya S, Johnson NW and Van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. Journal of oral pathology & medicine. 2007; 36: 575-80.

8. Anderson A and Ishak N. Marked variation in malignant transformation rates of oral leukoplakia. Evidence-based dentistry. 2015; 16: 102.

9. Kharadri U, Onkar S, Birangane R, Chaudhari S, Kulkarni A and Chaudhari R. Treatment of oral leukoplakia with diode laser: a pilot study on Indian subjects. Asian Pac J Cancer Prev. 2015; 16: 8383-6.

10. Kumar M, Nanavati R, Modi TG and Dobariya C. Oral cancer: Etiology and risk factors: A review. Journal of cancer research and therapeutics. 2016; 12: 458.

11. Amagasa T, Yamashiro M and Uzawa N. Oral premalignant lesions: From a clinical perspective. International journal of clinical oncology. 2011; 16: 5-14.
12. Kharadi U, Onkar S, Birangane R, Chaudhari S, Kulkarni A and Chaudhari R. Treatment of oral leukoplakia with diode laser: A pilot study on Indian subjects. Asian Pacific journal of cancer prevention: APJCP. 2014; 16: 8383-6.

13. Sathyanarayanan R, Karthigeyan R and Dinesh D. Awareness about oral cancer among Non medical university students of Puducherry. JIDENT. 2012; 1: 1-5.

14. Rödström P-O, Jontell M, Mattsson U and Holmberg E. Cancer and oral lichen planus in a Swedish population. Oral oncology. 2004; 40: 131-8.

15. Begum N, Naheed G, Nasreen S and Khan A. Oral cavity cancers in north west Pakistan: A hospital based study. Journal of Postgraduate Medical Institute (Peshawar-Pakistan). 2011; 23.

16. Shah SH and Shah SN. Prevalence and pattern of tobacco use in rural area of Peshawar. Journal of Ayub Medical College Abbottabad. 1993; 6: 5-8.

17. Birajdar SS, Radhika M, Paremala K, Sudhakara M, Soumya M and Gadivan M. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. Journal of oral and maxillofacial pathology: JOMFP. 2014; 18: 169.

18. Sobecki M, Mrouj K, Camasses A, et al. The cell proliferation antigen Ki-67 organises heterochromatin. Elife. 2016; 5: e13722.

19. Maheshwari V, Sharma S, Narula V, Verma S, Jain A and Alam K. Prognostic and predictive impact of Ki67 in premalignant and malignant squamous cell lesion of oral cavity. Int J Head Neck Surg. 2013; 4: 61-5.

20. Smith J, Rattay T, McConkey C, Helliwell T and Mehanna H. Biomarkers in dysplasia of the oral cavity: A systematic review. Oral oncology. 2009; 45: 647-53.

21. Yagyuu T, Obayashi C, Ueyama Y, et al. Multivariate analyses of Ki-67, cytokeratin 13 and cytokeratin 17 in diagnosis and prognosis of oral precancerous lesions. Journal of oral pathology & medicine. 2015; 44: 523-31.

22. Kannan S, Chandran GJ, Pillai KR, et al. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: Correlation with expression of Ki67. Clinical molecular pathology. 1996; 49: M170.

23. Humayun S and Prasad VR. Expression of p53 protein and ki-67 antigen in oral premalignant lesions and oral squamous cell carcinomas: An immunohistochemical study. National journal of maxillofacial surgery. 2011; 2: 38.

24. Mondal K, Mandal R and Sarkar BC. A study of Ki-67 expression and its clinicopathological determinants in nondysplastic oral leukoplakia. Contemporary clinical dentistry. 2016; 7: 493.

25. Nair DR, Pruthy R, Pawar U and Chaturvedi P. Oral cancer: Premalignant conditions and screening-an update. Journal of cancer research and therapeutics. 2012; 8: 57.

26. Kumar P, Kane S and Rathod GP. Coexpression of p53 and Ki 67 and lack of c-erbB2 expression in oral leukoplakias in India. Brazilian oral research. 2012; 26: 228-34.

27. Merne M, Heinaro I, Lähteenoja H and Syrjänen S. Proliferation and differentiation markers in snuff induced oral mucosal lesions. Journal of oral pathology & medicine. 2002; 31: 259-66.

28. Kumar V, Abbas AK, Fausto N and Aster JC. Robbins and Cotran pathologic basis of disease. Philadelphia: Elsevier Saunders, 2005.

29. Nagler R, Bahar G, Shpitzer T and Feinmesser R. Concomitant analysis of salivary tumor markers—a new diagnostic tool for oral cancer. Clinical Cancer Research. 2006; 12: 3979-84.

30. Dwivedi N, Chandra S, Kashyap B, Raj V and Agarwal A. Suprabasal expression of Ki-67 as a marker for the severity of oral epithelial dysplasia and oral squamous cell carcinoma. Contemporary clinical dentistry. 2013; 4: 7.

31. Sinanoglu A, Soluk-Tekkesin M and Olgac V. Cyclooxygenase-2 and Ki67 Expression in Oral Leukoplakia: A Clinicopathological Study. Journal of oral & maxillofacial research. 2015; 6: e3.

32. Bakaris S, Okur E, Yildirim I and Kilinc M. Ki-67 protein expression in smokeless tobacco (Maras powder)-induced oral mucosal lesions. Toxicology mechanisms and methods. 2007; 17: 567-74.
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| 1     | Tehmina Naushin           | Acquisition of data.                                                |                    |
| 2     | M. Muntaz Khan            | Final approval of the version to be published.                      |                    |
| 3     | Sajjad Ahmed              | Conception and design.                                              |                    |
| 4     | Mahmood-ul-Hassan         | Drafting the work.                                                  |                    |
| 5     | Fatima Iqbal              | Analysis and interpretation of the data.                            |                    |
| 6     | Nasih Bashir              | Analysis and interpretation of the data.                            |                    |
| 7     | Abbas Saleem Khan         | Critical review of the work.                                        |                    |