Original Research Article

Evaluation of extracellular signal regulated kinase 1 expression in premalignant lesions of oral cavity

Manisha Yadav[1], Vipin Arora[1]*, Showket Hussain[2], Sandeep Sisodiya[2], Preeti Diwaker[3], Ashish Goyal[4], Harish C. Taneja[5]

[1]Department of ENT and Head Neck Surgery, [3]Department of Pathology, [4]Department of Medicine, University College of Medical Sciences, Delhi, India
[2]Division of Molecular Oncology, ICMR, NICPR, Noida, Uttar Pradesh, India
[5]Department of ENT and Head Neck Surgery, Maulana Azad Medical College, New Delhi, India

*Correspondence:
Dr. Vipin Arora,
E-mail: vipinar@gmail.com

ABSTRACT

Background: Most oral squamous cell carcinomas are preceded by premalignant lesions like leukoplakia, erythroplakia etc. Identification of these lesions by molecular markers and intervention at an early stage can reduce mortality, morbidity and cost of treatment associated with oral cancer. Extracellular signal regulated kinase 1 (ERK 1) is one of the various markers involved in carcinogenesis.
Methods: We studied the expression of ERK 1 in twenty-five oral premalignant lesion biopsies using immunohistochemistry (IHC) and western blot analysis.
Results: Ninety-two percent of premalignant lesions showed positive expression of ERK 1 on IHC and the same were confirmed by western blot analysis. ERK 1 expression was increased in premalignant lesions as compared to peripheral normal tissue but the difference between them was not statistically significant. On comparing the level of expression of peripheral normal and premalignant tissue on IHC, moderate and high level of expressions were significantly higher in premalignant lesions (24%) than peripheral normal tissue (12%).
Conclusions: There was an increased expression of ERK 1 in oral premalignant lesions as compared to peripheral normal tissue, although, this difference was not statistically significant.

Keywords: ERK 1, Oral premalignant disorders, Mitogen-activated protein kinases, Immunohistochemistry, Western blotting

INTRODUCTION

Oral cancer is one of the most common type of cancer in world and there are around 6,57,000 new cases of oral cancer per annum accounting for 3,30,000 deaths overall (WHO statistics 2019). Oral and oropharyngeal cancers have an overall 5year survival rate of 65% but it increases to 84% if diagnosed at an early stage. Oral premalignant disorders (OPMD) refer to all epithelial lesions with an elevated risk of malignant transformation with a worldwide prevalence ranging from 1-5%.[1-3] They are predominantly associated with the use of tobacco, heavy alcohol, smoking and areca nut chewing. However, approximately 10% patients do not have any known causative factor and are placed under idiopathic etiology.[4] OPMD have a malignant transformation rate mainly to oral squamous cell carcinoma (OSCC) of 0.13-36.4% at an annual rate of 1.6% (95% CI- 0.69-2.03%).[4]

OPMDs include leukoplakia, erythroplakia, oral submucosal fibrosis (OSMF), oral lichen planus, proliferative verrucous leukoplakia and squamous

papilloma. Oral Leukoplakia (OL), the most common OPMD, is a white plaque of questionable risk having excluded other non- disease or disease that do not carry high risk for cancer.[5] Oral erythroplakia (OE) comprises any bright red velvety plaque in oral mucosa which cannot be placed under any recognizable disease clinically or pathologically. The prevalence ranges from 0.02%-0.83 % and predominantly affects the middle age and elderly.[6-8] Oral submucous fibrosis (OSMF) is a chronic insidious inflammatory disease originating from areca nut chewing and smoking which is distinguished by loss in elasticity of the oral mucosa and submucosa. Oral lichen planus (OLP) is a chronic inflammatory disease characterized by a T-lymphocyte mediated immune response against epithelial basal cells, causing basal cell degeneration, which may result in mucosal erosion and ulceration and commensurate oral soreness.[9] Oral lichenoid lesion (OLL) is clinically similar to oral leukoplakia which is usually unilateral as compared to OLL which involve oral mucosa bilaterally. Smoker's palate, the most common clinical consequences of smoking on oral mucosa, is white leathery lesions of the palate and cheeks. Oral leukoplakia may have a multifocal presentation, known as proliferative verrucous leukoplakia which can have homogenous and non-homogenous features. It has a weak association with tobacco because it may occur in both smokers and nonsmokers.[10]

OPMDs carry a high risk of malignant transformation, hence, their identification and management at an early stage has potential to control secondary development to OSCC. This holds a great promise in reducing the overall morbidity and mortality associated with oral cancer but the management of OPMD remains a major challenge due to the lack of reliable clinical or histopathological markers to predict the conversion of OPMD into malignancy. Hence, there is a need of understanding the molecular mechanisms associated with the conversion of OPMD to malignancy and aiding the discovery of new predictive markers for diagnosis and prognosis. Our study aims at identification of level of expression of such molecular markers like ERK 1 (a member of MAPK family) and facilitates the identification of new targeted therapies in preventing development of OSCC.

Several genetic alterations are associated with OSCC which are frequently associated with the mutation of onco-suppressor p53 and p16, ras oncoprotein and EGF receptor one of the major signaling routes downstream of EGFR is the mitogen-activated protein kinase (MAPK) pathway.[11,12] MAPKs are a family of protein kinases by which cells transduce a vast array of extracellular signals into intracellular responses controlling proliferation, differentiation, cell motility and apoptosis. To date, three important MAPK members have been reported in detail i.e. ERK1 and 2 (extracellular signal regulated kinase 1 and 2), p38-MAPK and JNK/SAPK. ERK1 and 2, also called p44ERK1 and p42ERK2, were first identified as growth factor activated protein kinases, and the

constitutive activation of this pathway has been mediated by mutations of ras or raf oncogenes in different types of cancer. The activation of ERK leads to the phosphorylation and activation of multiple cytoplasmic substrates, such as cytoskeletal proteins, or downstream protein kinases. In addition, ERK1/2 can regulate the transcription of different genes as phosphorylated ERK1/2 can translocate to the nucleus and activate several transcription factors. Recent observations about ERK pathway namely NF-kB, AP-1, ETS-1,c-Myc, STATS, which are responsible for upregulation of cell proliferation, differentiation, survival, immortalization and angiogenesis.[13-15] ERK 1/2 has been contemplated as an important regulator of various cellular activities such as cell proliferation, motility and survival, being suggested in the malignant transformation.

In present study, we specifically evaluated the total ERK1 in OPMD.

METHODS

This was an observational, cross-sectional study which was conducted in the Department of Otorhinolaryngology and Department of Pathology at University College of Medical Sciences & GTB Hospital, Delhi & ICMR-National institute of cancer prevention and research (NICPR), Noida from November 2018 to April 2020. Since this is a pilot study so a convenient sample size of 25 patients was chosen. All consenting histologically confirmed patients with oral premalignant lesions were included in the study. Patients who have received any form of previous treatment in the form of surgery, radiation and chemotherapy, poor general condition and unfit for surgery, refused consent were excluded from the study. Institutional ethical committee approved for this research work.

A detailed clinical history and Head and neck examination was taken. Biopsy from primary lesion including advancing radial margin which included at least 1mm of the apparently normal tissue was taken. In total 25 oral premalignant lesions were biopsied and level of expression of ERK 1 was studied by conducting immunohistochemistry (IHC) in both premalignant lesions and peripheral normal tissue and western blotting in premalignant lesions only to validate the findings of IHC.

Western blotting

Whole cell protein was extracted from biopsies following the method of Kültz and Somero16 with modifications by Hussain et al.[17] Frozen oral tissue biopsies were homogenized in 1×cold phosphate-buffered saline (PBS) and centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant was discarded, and remnant was washed with 1×PBS in 0.3-0.5 mL of radioimmunoprecipitation assay lysis for 45 minutes while tapping after every 10 minutes. Extraction mixture was centrifuged at 14,000 rpm for 30

minutes at 4°C. The whole cell lysate (WCL) was taken and 50 μg of WCL was loaded per lane in 12% SDS polyacrylamide gel and were electro transferred in semi dry trans blotter to immobilon-PSQ polyvinylidene difluoride membranes which was kept in 5% BSA for 2 hours at 4°C. Rabbit monoclonal antibody against ERK1 (ab32537; Abcam, UK) was then used and incubated overnight in 1×PBS supplemented with 2% BSA and 0.05% tween 20 (Sigma-Aldrich, Chemie GmbH, Germany). Bands were visualized using goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotech, CA) at a dilution of 1:5000 by chemiluminescence Amersham ECL™ detection kit (GE Healthcare, Chicago, IL) using X-ray and exposing at different time intervals. After the exposure, blots were developed in the developer. Protein estimation was done by Qubit® 3.0 fluorometer. The expression was graded based on an arbitrary scale as nil (<5000), mild (5000-10000), moderate (10000-20000) and high (>20000).

Immunohistochemistry

Four-micron thick sections were cut and dewaxed in xylene and rehydrated through graded concentrations of alcohol. Antigen retrieval was done by boiling in tris buffer (Dako-EnVision, Denmark). Endogenous peroxidase enzymatic activity blocked by hydrogen peroxide by keeping in the dark for 10 minutes and then HPR conjugation done for 45 mins followed by buffer washing and incubated with polyclonal rabbit primary antibody against ERK1 (ab32537; Abcam, UK) with the standardized dilution (1:50) overnight at 4°C. Then slides were washed and incubated for 45 minutes with the horseradish peroxidase conjugated secondary antibody followed by staining with chromogen, diaminobenzidine for 2 to 5 minutes in the dark and then counterstained with Mayer's hematoxylin and finally mounted in DPX. Semi quantified expression of the protein was taken with regard to the intensity of stained cell which was graded as 0 to 3 (0 for negative staining; 1 for low positive; 2 for positive and 3 for high positive).

Statistical analysis

Data was entered in MS excel and analyzed using SPSS 20.0 software. Comparison of expression of ERK 1 proteins between patients with oral premalignant disease and normal controls was done using Chi-square test. P value of less than 0.05 was considered statistically significant.
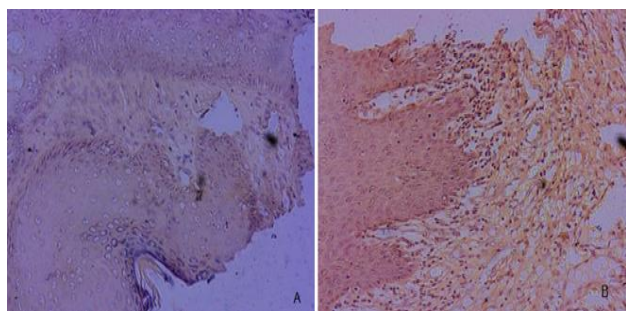
RESULTS

Buccal mucosa has been the most common site of lesion in our series, followed by tongue and lip. Patient characteristics are given in (Table 1).
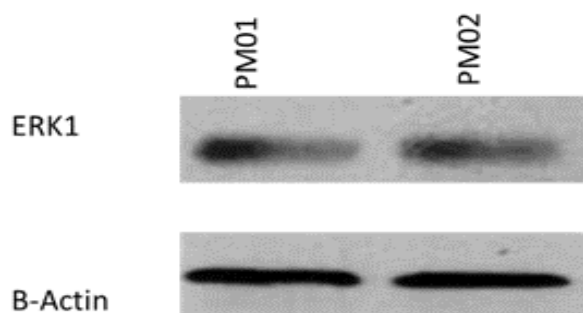
Table 1: Patient characteristics (n=25).

Characteristics		
Age (years)	Mean= 36.3 yrs (Range- 21-76)	
Male:female ratio	5.25:1	
Site of lesion	No. of cases	Percentage (%)
Tongue	3	12
Buccal mucosa	17	68
Lip	3	12
GB sulcus	2	4
Histological distribution		
Dysplasia	8	36
Leukoplakia	2	8
Oral submucous fibrosis	13	52
Lichen planus	1	4
Squamous papilloma	1	4

In our study, we observed that 92% of premalignant lesions showed positive expression of ERK 1 as compared to 84% in peripheral normal tissue on IHC. However, this difference was not statistically significant (p=0.66). Similar findings were validated by Western Blotting which also showed positive expression of ERK 1 in 92% of premalignant lesions.

Table 2: Expression of ERK 1 in premalignant lesions by immunohistochemistry and western blotting.

ERK-1 expression	Level of expression*	CASE (lesion)	Control (peripheral normal tissue)	P value
		N (%)	N (%)	
Immunohistochemistry	0 - no expression	2 (8)	4 (16)	0.66
	1 - mild positive	16 (64)	18 (72)	
	2 - moderate positive	6 (24)	3 (12)	
	3 – high positive	1 (4)	0	
Western blotting	<5,000- no expression	2 (8)	-	-
	5-10,000- mild positive	7 (28)	-	
	10-20,000 - moderate positive	16 (64)	-	
	>20,000 – high positive	0	-	

*Based on scale – IHC (based on pixel count of staining intensity of slide images by IHC profiler), WB (based on chemiluminescence of the protein bands)

Figure 1: ERK 1 IHC (20X) showing mild positivity in premalignant oral cavity lesions.

Figure 2: Immunoblot experiment showing expression pattern of ERK 1 in oral premalignant lesion biopsies.

Also, on comparing the level of expression of normal and premalignant lesions on IHC, we found that moderate and high level of expression was significantly higher in premalignant lesions as compared to peripheral normal tissue (24% vs 12% respectively). Buccal mucosa was the most common site of lesion which most commonly expressed moderate level of ERK 1 expression on both western blotting (36%) and IHC (48%). OSMF was the most common premalignant lesion and showed mild level of positivity on both IHC and western blotting (Table 2, Figure 2).

In our study, on IHC, maximum number of cells which were positive for ERK 1 expression showed cytoplasmic staining for anti-ERK 1 antibody but in some lesions like moderate to severe dysplasia, few of the cells on the edges of the lesion showed high level of nuclear staining for anti-ERK 1 antibody (Figure 1).

DISCUSSION

The development of oral cancer from premalignant lesions is a multifactorial and multistep process. Among the various molecular pathways involved in the tumorigenesis of OSCC, MAPK cascade is a distinguished pathway that transmits various signals from extracellular membrane to the nucleus. EGFR activation activates the MAPK. MAPK, which has various subfamilies of proteins like ERK1/2, further, activates the downstream proteins which finally reach the nucleus of the cell and act on various transcription factors. This ultimately controls cell proliferation and apoptosis. In this study we evaluated the expression of ERK 1, a MAPK subfamily protein as it plays an important role in tumorigenesis. Identification of such precise pathways will further lead to development of more precise targeted therapies.

In our study we observed that patients with premalignant lesions presented at an early age. Most patients were in the 4th decade of their life. 36% patients were in the age group of 30-39 years and 32% were in the 40-49 years age group. The youngest was 21 years and the oldest was 76 years of age. As compared to the western population, where the average age of presentation is 5th and 6th decade, 18 Indian population with premalignant lesions usually belong to the 2nd and 3rd decade.[19] This can be attributed to the early age of use of causative factors like smoking (most common), tobacco chewing and alcohol consumption.

Among the premalignant lesions on IHC, 2 (8%) had no expression, 16 (64%) had low positive expression, 6 (24%) had moderately positive expression and 1(4%) had high positive expression. On comparing the expression of ERK 1 in premalignant and peripheral normal tissue, we found no significant difference between the two groups (p=0.66) on IHC.

In our study, we observed that the stain was highly expressed in the cytoplasm of maximum cells but there was a differential pattern of staining in cytoplasm and nucleus in some of the lesions like moderate and severe dysplasia. This signifies that nuclear staining is a characteristic feature of actively proliferating variants which have greater propensity of transforming into tumor cells.

Yan Dong et al conducted a similar study on 21 oral squamous cell carcinoma (OSCC) and 25 oral leukoplakia (OLP) specimens.[20] He also reported similar findings that is, total ERK 1 & 2 was positively expressed in the cytoplasm of epithelial cells. Eighty one percent of OSCC tumor cells and 68% of OLP cells were stained positive. However, on comparing the two groups, there was no statistically significant difference between them. He also studied phosphorylated ERK 1 & 2 in both OSCC and OLP in which 81% of OSCC cells were stained positive while 76% of OLP cells were stained negative. In his study, he concluded that phosphorylation of ERK is associated with malignant transformation of OLP.

On conducting western blotting to validate the findings of IHC in our study, we found that similar to IHC, 92% of biopsied specimens showed positive expression of ERK 1. But, overall maximum premalignant lesions 16 (64%) showed moderate level of ERK 1 expression. Among the histological variants, OSMF was the most common

variant in which 7 (54%) patients showed mild levels of expression and 4 (30%) patients showed moderate levels of expression while all degrees of dysplasia combined as well as lichen planus and squamous papilloma showed moderate levels of expression. Although, in literature, there are few studies on ERK 1 expression in premalignant lesions, using western blotting techniques.

Gholizadeh et al in 2019 did a case control study in 26 OSCC, 20 OLP and 20 healthy control tissue specimens to assess the level of expression of ERK 1/2 gene by quantitative reverse transcriptase polymerase chain reaction (T-PCR). He observed a significant increase in the ERK 1/2 gene in OLP and OSCC specimens as compared with healthy controls (p<0.001).[21]

Limitations

As the sample size of the study is small so further studies are required on a larger number of patients with longer patient follow up duration to validate the findings in our series.

CONCLUSION

This study evaluated the expression of ERK 1 in oral premalignant lesions. There was an increased expression of ERK 1 in oral premalignant lesions as compared to peripheral normal tissue although; this difference was not statistically significant. Further studies are required to validate the increased expression of ERK 1 in higher number of patients and a variety of different techniques and markers to increase the understanding of tumorigenesis in premalignant lesions.

REFERENCES

1. Warnakulasuriya S, Johnson NW, Van Der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007;36:575-80.
2. Petti S. Pooled estimate of world leukoplakia prevalence: A systematic review. Oral Oncol. 2003;39(8):770-80.
3. Smith LW, Bhargava K, Mani NJ, Malaowalla AM, Silverman Jr S. Oral cancer and precancerous lesions in 57,518 industrial workers of Gujarat, India. Ind J Canc. 1975;12(2):118.
4. Napier SS, Speight PM. Natural history of potentially malignant oral lesions and conditions: an overview of the literature. J Ora Pathol Medic. 2008;37(1):1-0.
5. Van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Or Oncol. 2009;45(4-5):317-23.
6. Reichart PA, Philipsen HP. Oral erythroplakia - A review. Oral Oncol. 2005;41:551-61.
7. Shafer WG, Waldron CA. Erythroplakia of the oral cavity. Canc. 1975;36(3):1021-8.
8. Hashibe M, Mathew B, Kuruvilla B, Thomas G, Sankaranarayanan R, Parkin DM, et al. Chewing Tobacco, Alcohol, and the Risk of Erythroplakia 1. 2000.
9. Roopashree MR, Gondhalekar RV, Shashikanth MC, George J, Thippeswamy SH, Shukla A. Pathogenesis of oral lichen planus–a review. J Oral Pathol Medic. 2010;39(10):729-34.
10. Arduino PG, Bagan J, El-Naggar AK, Carrozzo M. Urban legends series: oral leukoplakia. Oral Disea. 2013;19(7):642-59.
11. Tokman B, Gultekin SE, Sezer C, Alpar R. The expression of p53, p16 proteins and prevalence of apoptosis in oral squamous cell carcinoma. Correlation with mode of invasion grading system. Sau Medic J. 2004;25(12):1922-30.
12. Diniz-Freitas M, García-Caballero T, Antúnez-López J, Gándara-Rey JM, García-García A. Pharmacodiagnostic evaluation of EGFR expression in oral squamous cell carcinoma. Oral Disea. 2007;13(3):285-90.
13. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncoge. 2007;26(22):3203-13.
14. Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncoge. 2007;26(22):3100-12.
15. Katz M, Amit I, Yarden Y. Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochim Biophysi Acta (BBA)-Molecul Cell Resea. 2007;1773(8):1161-76.
16. Kültz D, Somero GN. Differences in protein patterns of gill epithelial cells of the fish Gillichthys mirabilis after osmotic and thermal acclimation. J Comp Physiol - B Biochem Syst Environ Physiol. 1996;166(2):88-100.
17. Hussain S, Bharti AC, Salam I, Bhat MA, Mir MM, Hedau S, et al. Transcription factor AP-1 in esophageal squamous cell carcinoma: Alterations in activity and expression during human papillomavirus infection. Brit Med Cent Cancer. 2009;9(1):329.
18. Mortazavi H, Baharvand M, Mehdipour M. Oral potentially malignant disorders: an overview of more than 20 entities. J Dent Res Dent Clin Dent Prospects. 2014;8(1):6-14.
19. Srivastava R, Devina S. Prevalence of oral submucous fibrosis in patients visiting dental OPD of a dental college in Kanpur: A demographic study. J Fam Med Primary Care. 2019;8.
20. Dong Y, Li Z, Gao M, Liu C, Liu H, Chen Z, et al. Immunohistochemical detection of aurora a and ERK pathway in oral leukoplakia and oral

squamous cell carcinoma. J Hard Tissue Biol. 2014;23(1):71-6.

21. Gholizadeh N, Emami Razavi A, Mohammadpour H, Tavakol F, Sheykhbahaei N. Association of MAPK and its regulatory miRNAs (603, 4301, 8485, and 4731) with the malignant transformation of oral lichen planus. Mol Biol Rep. 2020;47(2):1223-32.