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**EVALUATION OF IMMUNE EXPRESSION OF STAT3 AS EARLY EVENT FOR
CELLULAR ALTERATION IN TOBACCO STRESSED ORAL MUCOSA**

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ABSTRACT

Background: Tobacco contains various known chemical component which act as direct or indirect carcinogenic agent in development oral cancer. Toxins presence in tobacco has a immunomodulatory effect. Signal transducer and activator of transcription-3(STAT3) regulates inflammation through transmitting signals from cytokines and growth factor receptor to target the genes. It has been proposed that due to tobacco carcinogen there is dysregulation and constant activation of STAT3 signaling pathway concerned in malignant transformation. In the present study, we observed expression of STAT3 in tobacco stressed mucosa and in oral Leukoplakia. To the author's knowledge, the STAT3 expression was first time analyzed in oral mucosa stressed under various forms of tobacco and oral leukoplakia.

Methods: Tissue sample of normal oral mucosa without tobacco habit (n=50) and with tobacco habit (n=50) were selected for the study to analyze STAT3 expression. Paraffin embedded tissue sections were

processed for immunohistochemistry. Hematoxylin and eosin stained slides and the presence of STAT3 in nucleus as well in cytoplasm was analyzed by two independent observers.

Results: Among the 50 samples of tobacco stressed mucosa all slides shows variation in immunoreactivity. On the basis of immunoreactivity score, 70% cases shows no staining i.e. score 0-2 and 30% cases shows weak expression i.e. 3-4 immunoreactive score. In comparison in different tobacco form smoking form observed to be high expression score of mean than mixed followed by smokeless which was 2.8, 2, and 0.002 respectively. No immunoexpression were observed in sample without tobacco habit. Statistical analysis were calculated by using one way ANOVA with post hoc and unpaired 't' test.

Conclusion: The STAT3 expression in tobacco stressed mucosa suggests its participation in modulation of molecular mechanism is the early event before obvious cellular structural changes occurs, which may further transform in to malignancy.

Keywords: Signal transducer and activator of transcription-3, STAT3, tobacco habits, oral carcinogenesis, leukoplakia

INTRODUCTION:

Oral Mucosa undergoes various symptomatic and asymptomatic tribulations due to constant exposure to numerous toxic and non toxic agents. Constant irritation affects the structural and molecular mechanism of mucosal lining leading to serious oral health problem like precancerous and cancerous lesions. The occurrence of oral lesions is usually related to various factors like stress, tobacco related habits, inflammation and genetic. Globally, the use of tobacco is among largest factor responsible for cancer where tobacco products are consumed worldwide and also remain as addiction throughout life.¹ Worldwide estimated prevalence of oral lesions are ranging from 15% in the Gizan, Saudi Arabia² and 5 to 51.1% in Spain.³ In India prevalence of regular use of smoking tobacco is 16.2% and

chewing tobacco is 14%.⁴ The overall prevalence of smoking, drinking alcoholic beverages and chewing were 15.02%, 8.78% and 6.99% respectively.⁵ These habits have been associated with oral lesions like oral submucous fibrosis (OSF), leukoplakia and oral lichen planus, which has great potential for malignant transformation.⁶ Tobacco and its related products are known to be carcinogenic due to its chemical composition. This tobacco related disorders have received great research attention due to transformation in to oral cancer and it is known that oral squamous cell carcinomas generally occur with a history of smokeless tobacco, smoking and/or alcohol abuse. The young patients with these histories have a greater potential to develop these disease

despite for shorter duration of exposure to carcinogenic substances.

The harmful ingredient of tobacco and cigarette smoke is the major factor when exposed constantly to mucosa induces inflammatory and apoptotic responses and even cellular hypoxia in the vicinity. It has been observed that the inflammatory component like cytokines and various growth factors plays a crucial role in the cell regulation and get affected with cellular dysfunctions through activation of STAT proteins.

The STAT proteins (signal transducers and activators of transcription) were first identified in the last decade as transcription factors that was critical in mediating virtually all cytokine driven signaling.⁷ In addition to normal signaling, various oncoproteins activates STATs and its continuous activation participates in oncogenesis.⁸ STATs oncogenic activity and its constant activation in absence of cytokine signaling is the result of genetic mutation or polymorphisms.⁹ Activated STAT3 binds enhancer element of genes and induces pro-inflammatory genes, indeed loss of STAT3 expression lowers cytokines expression claims their role in cellular function through activated JAKs-STAT pathway.¹⁰ Even the recent study on lung carcinoma demonstrates

the relation of cigarette smoke carcinogen with activated STAT3 which induces IKBKE oncogen.¹¹ It has been observed by many researchers that aberrant activation of STAT3 contributes in tumorigenesis and its progression towards cancer.¹²

The exploration of these aspects, etiology, history, risk factors and molecular mechanism related to oral mucosal pathologies is crucial for early prevention, diagnosis and timely treatment of these fetal diseases. In the current study, we have explored the expression of activated Stat-3 in oral epithelium exposed to various tobacco related products, which will further helpful for researcher in early diagnosis and their potential towards malignant transformation as well in understanding the pathway of early event in carcinogenesis.

MATERIAL AND METHODS:

Tissue Specimen

Total 100 participant included in the study who reported to MGM Dental College and Hospital, Kamothe, Navi Mumbai for surgical removal of third molar tooth as well individuals undergoing surgery for non-inflammatory and nononcogenic tissue like crown lengthening, non-inflammatory gingivectomy surgical procedure. We grouped total participants as in Group I (50 without tobacco or related habit), and Group

II (50 with tobacco or related habit without any significant oral lesion). The detailed case history and medical reports were evaluated carefully. Gingival tissue was resected when tooth extraction and while surgical procedure were planned for Group I and II. The written informed consent from the patient was obtained at the time of surgery. The ethical approval for the study was obtained from the ethical committee board of MGM institute of Health Sciences. All biopsy were processed for hisopathological staining (H&E) and observed for cellular alteration and underlying inflammation.

Immunohistochemistry:

All formalin fixed tissue processed routinely and embedded in paraffin wax block. Thin tissue sections were maintained up to five micrometer and mounted on silanized glass slides and kept at 58°C in an oven for 1 hour. After dewaxing the sections were rehydrate and incubated for 30 min in H₂O₂ (3%) for quenching endogenous peroxidase. The sections were again heated for 20 min in 0.1M citrate buffer at pH 6 and then washed in phosphate buffer saline. The primary Mouse monoclonal antibody Stat-3 (F-2), dilution 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunostaining and incubated for overnight at 4°C. For chromatin substrate

peroxidase reaction were carried out using 3-3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and sections were counterstained with hematoxylin. Positive control and negative control sections were also prepared for authenticity.

Slide analysis:

Hematoxyllinan and eosin stained slides were observed for cellular alteration and levels of inflammatory infiltrate. In immunohistochemistry, the expression of STAT3 was determined by semiquantitative assessment method. The percentage of marked positive cells and staining intensity were assessed. The percentage was rated as 1 from 1 to 10%, 11 to 50% as 2, 51 to 80% as 3 and more than 80% as 4. The staining intensity was scored as 0 is no color reaction, 1 is mild reaction, 2 as a moderate reaction, 3 as an intense reaction. By calculating these two immunoreactive score were calculated as 0 to 1 is negative (Score 0), 2 to 3 is mild (score 1), 4 to 8 is moderate (score 2), and 9 to 12 is strong positive (score 3).¹³ All slides were analyzed under 40X magnification by two independent observer and average score were used.

RESULTS:

Stat-3 Expression:

The primary objective was to observe the immunoreactivity of localized STAT3 in 100

tissue sample of patients of having no tobacco habit and having tobacco habit. In Group II of 50 tissue sample 18 % (09 of 50) showed mild STAT3 expression, 52% (26) showed moderate and 30% (15) showed no expression of STAT3. The immunostaining was observed in both cytoplasmic and nuclear localization. Statistical significant correlation was observed between STAT3 and various parameters like tobacco habit form. The Group I participants were in range of 0 to 1 i.e. negative for expression of

STAT3 in both nuclear and cytoplasmic localization. The secondary objective was to correlate expression with clinical parameter and within different forms of tobacco habits. The results were assessed according to clinical parameter like age, gender and tobacco habits. **(Table 1)** Statistical correlation was observed between STAT3 expression in clinical parameter like age, gender, tobacco habit and within tobacco habit.

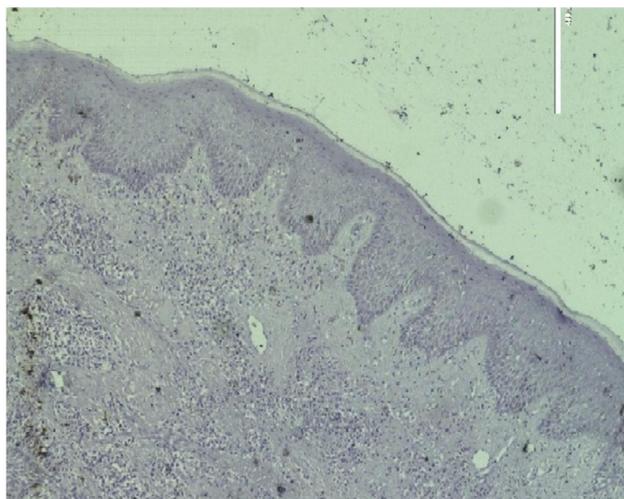


Figure 1: Tissue section of control group without tobacco habit shows no expression of STAT3. (10x)

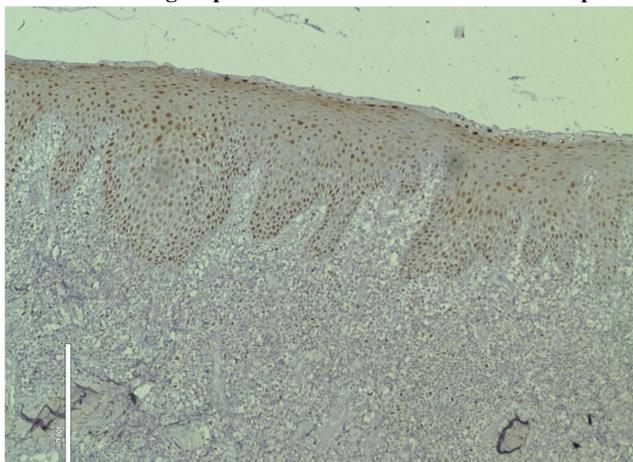


Figure 2: Tissue section of oral mucosa with tobacco habit shows expression of STAT3 from basal cell layer to superficial layer. (10x)

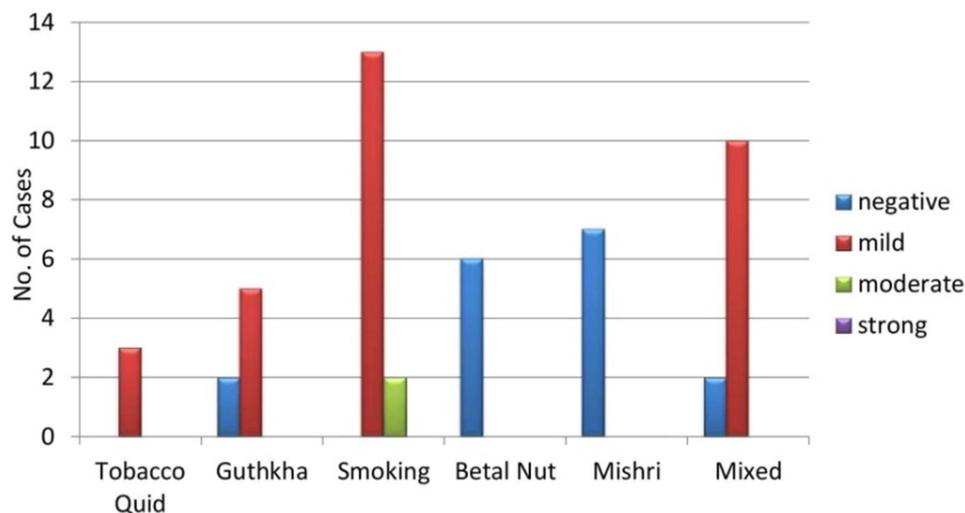


Figure 3: Graph showing Immunoreactive score in comparison with different forms of tobacco.

Table1: Demographic distribution and comparison of expression of STAT3 with clinical parameter in GroupII. († - One way ANOVA with post hoc, ‡ – unpaired ‘t’ test.)

Factors	parameter	No of cases	Epithelial Atrophy	Basal Hyperplasia	Acanthosis	Expression Mean ± SE	P value	Inference
Habit (n=50)	Smoking (A)	15	2 (4%)	12 (24%)	6 (12%)	2.80 ± 0.1746	A Vs B- 0.001	** p<0.01(†)
	Smokeless (B)	23	8 (16%)	8 (16%)	6 (12%)	1.08 ± 0.2259	A Vs C - 0.451	Insignificant (†)
	Mixed (C)	12	00 (0%)	9 (18%)	4 (8%)	2.00 ± 0.1741	B Vs C - 0.014	* p<0.05(†)
Age (n=50)	Below 40	28	6 (12%)	19 (38%)	12 (24%)	2.18 ± 0.303	0.0059	* p<0.05(‡)
	Above 40	22	4 (8%)	10 (20%)	4(8%)	1.30 ± 0.303		
Gender (n=50)	Male	41	7 (14%)	28 (56%)	14 (28%)	2.15 ± 0.332	0.0001	*** p<0.01(‡)
	Female	9	3 (6%)	01 (2%)	2 (4%)	0.33 ± 0.332		
Study group (n=50)	With Tobacco	50	0.12±0.167				0.0001	*** p<0.01(‡)
Control group (n=50)	Without tobacco	50	1.82±0.167					

Statistical Analysis:

The statistical analysis for determining the significance of STAT-3 staining with normal vs with tobacco habits was performed. STAT3 expression score compared with inflammatory grades and with various clinical parameters like gender, age and habits. Statistical analysis with in different tobacco habits with immune expression scores were performed using one way

ANOVA test. The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that the one or more habits are significantly different. The Tukey HSD test, post-hoc tests used to identify which of the pairs of habits are significantly different from each other.

DISCUSSION:

Despite of having advanced diagnostic and therapeutic approach oral squamous cell

carcinoma had a leading cause of death due to late diagnosis, metastasis, and recurrence. The abundant studies throw light on tobacco as main etiological factor for oral cancer. The association between tobacco and oral cancer is unambiguous and conformed by International Agency for Research on Cancer with nitrosamine is a main carcinogenic constituent.¹⁴ The chronic exposure of tobacco component to cells of oral cavity modulates signaling pathways and favors carcinogenesis. In tobacco, carcinogen such as polyaromatic hydrocarbons causes DNA adducts and gene mutation¹¹ and become responsible for deviation from normal.

Other than tobacco carcinogen, inflammatory components (or in combination) are also accountable for carcinogenesis and been reported in various cancer. In human lung cell cigarette smoke extract exposure shows increased STAT3 phosphorylation (Tyr 705) and DNA binding activity and also stimulates IL-6 resulting in expression of DNA damage, suggesting STAT3 mediates response to cigarette smoke extract.¹⁵ Current evidence on extensive work on primary tumor and cell lines wires the crucial role and inappropriate high frequency activation of STAT3 in variety of cancer. Grandies *et al* ., reported constant activation of STAT3 is linked with production of variety of growth factors

(transforming growth factor, Epidermal growth factor receptor)¹⁶ in addition to that nicotine shown to induce certain growth factor and cytokines which ultimately alter systemic physiology.¹⁷

The pro and anti-inflammatory effect of STAT3 may be due to disease and cell specific mechanism that determines the STAT3 role. There are several studies on STAT3 discusses limitation and merits. Smithgall *et al.*, 2000 utilized STAT3^{-/-} mice to suggest cell maturation and differentiation and lack of STAT3 activation altered the immune system and mice are more responsive to exposed to cigarette smoke.¹⁸

In present study, we focus primarily on type of tobacco form and grades of inflammation with expression of STAT3. We observed cytoplasmic as well nuclear accumulation of STAT3, suggesting participation of STAT3 in signal transduction and activation of transcription of various genes. It was observed in normal epithelium stressed under tobacco as well in without tobacco habit mucosa in no to mild form. In the recent study, Jatin K Nagpal *et al* in 2002,¹⁹ had not detected any accumulation of STAT3 in normal epithelium in correlation with premalignant lesion to developed cancer in tobacco habitual patients. In present study, STAT3 expression was detected in normal

epithelium, very few cases were expressed STAT3 (07 cases) in mild form were histologically cellular hyperplasia (11 cases) were observed with mild inflammatory cell infiltrate (18 cases). Accumulation of STAT3 in normal epithelium may validate association with the cellular hyperplasia and inflammatory component justifying link between inflammation and cellular STAT3 accumulation.

Participants were broadly categorized in to smoking, smokeless and mixed (smoking + smokeless+ alcohol) habits. To be for more precise, smokeless includes tobacco quid, gutkha, mishri, betel nut habits. Participant with variety of tobacco habit shows significant increase in expression of STAT3 levels. Smokeless tobacco shows mean (0.0021) expression which is statistically

significant with the mean of smoking and mixed habits (2.8 and 2 respectively). The result shows smoking habit as well mixed habit patients had high accumulation of STAT3 in comparison with smokeless tobacco (**Table 1**) suggesting definite alteration at molecular level. When tobacco habits subdivided in to definite tobacco form like tobacco quid, gutkha, smoking, betel nut, mishri and mixed. STAT3 expression was significantly high in smoking habit followed by mixed habit. The expression of STAT3 in smoking habit is statistically significant with betel nut, mishri and mixed. Tobacco quid and gutkha expression was statistically significant with betel nut and mishri. In comparison of smoking and gutkha chewing were found to be statistically insignificant (**Table 2**).

Table 2: Comparison of STAT3 expression among different tobacco habits (one way ANOVA with post hoc) (A-Tobacco Quid, B- Gutkha, C- Smoking, D- Betal Nut, E- Mishri, F- Mixed)

Tobacco habits	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inference
A vs B	1.0856	0.8999947	insignificant
A vs C	1.6582	0.8289826	insignificant
A vs D	5.8267	0.0021454	** p<0.01
A vs E	7.1338	0.0010053	** p<0.01
A vs F	1.1605	0.8999947	insignificant
B vs C	3.9277	0.0805618	insignificant
B vs D	6.0592	0.0013001	** p<0.01
B vs E	7.8082	0.0010053	** p<0.01
B vs F	0.0000	0.8999947	insignificant
C vs D	10.7006	0.0010053	** p<0.01
C vs E	13.0457	0.0010053	** p<0.01
C vs F	4.6421	0.0232054	* p<0.05
D vs E	1.4427	0.8999947	insignificant
D vs F	6.7420	0.0010053	** p<0.01
E vs F	8.7756	0.0010053	** p<0.01

When STAT3 expression was compared with the duration of contact of tobacco with mucosa were found to be extremely significant. It signifies that higher the contact time of carcinogen to the oral epithelium more the expression of STAT3. In comparison with basilar hyperplasia STAT3 expression was observed to be 58% (29) cases positive and 42% (21) negative cases.

J Guo in 2013, showed in non-small cell lung carcinoma that cells on exposure to component of cigarette smoke (nicotine and nicotine derived nitrosamine ketone) induces oncogen called Serine/threonine kinase IKBKE through constitutively active STAT3. While IKBKE abrogated on inhibition of STAT3.¹¹ In the study conducted by Arredondo J, *et al* 2006 on oral keratinocytes, the cells were stimulated by nicotine or side stream cigarette smoke showed increased in the STAT3 mRNA and protein levels.²⁰ In comparison with those study results, our study also suggests that ingredients of different tobacco form contribute in activation of the STAT3 and can mediate carcinogenesis.

It has been widely accepted that inflammation plays major role in pathogenesis of variety of malignancy. The translocation of activated STAT3 to nucleus begins once it form homo- or heterodimers

due to interaction of cytokines and JAK family members.²¹ To assure said process, we observed expression of STAT3 score percentage in relation with different grades of inflammation in tobacco stressed mucosa. STAT3 immunohistochemistry score as negative, weak and mild were found to be maximum percentage was in grade 2 inflammation as 47%, 48% and 50% respectively. In present study 62% cases were fall in weak expression score of IHC had showed significant up relation with grade 2 inflammation. Comparisons within inflammatory grades, severe dysplasia observed to be statistically significant with no, mild and moderate grade of inflammation suggesting tobacco associated inflammation gives raise to expression of STAT3. (Table 3) In nutshell, IHC expression score ladder was in raised with increased grades of inflammation. The persistent inflammation may give rise to stable expression of STAT3 which may further land up in to initiation of irreversible changes at cellular level and can develop epithelial dysplasia. Further extended study on pSTAT or mRNA is needed in premalignant and malignant lesion to suggest the role and pathway of STAT3 in initial stages of carcinogenesis.

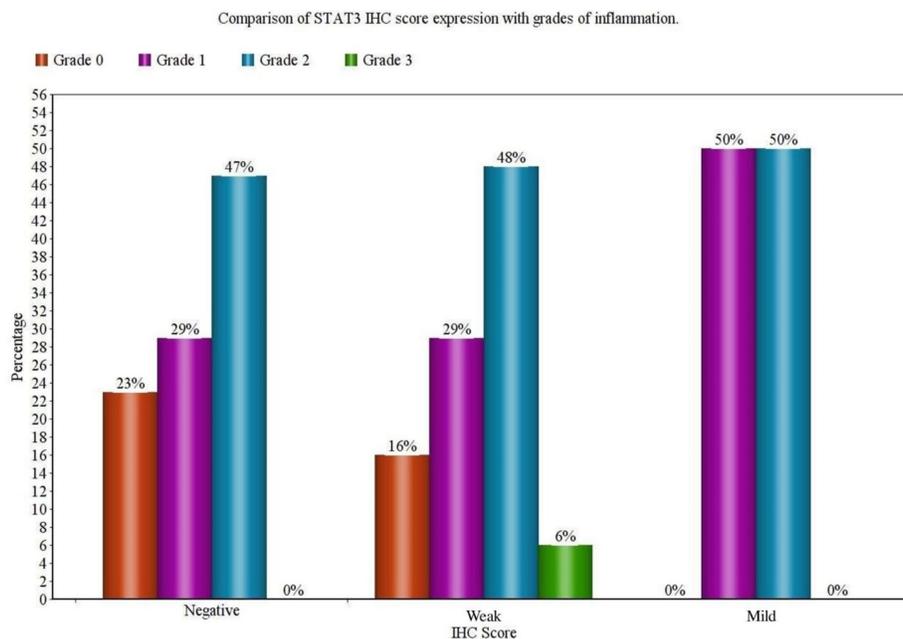


Figure 4: Graph represents STAT3 expression percentage in different grades of inflammation

Table 3: of STAT3 IHC score expression within different grades of inflammation (No Inflammation- A, Mild Inflammation- B, Moderate Inflammation- C, Severe Inflammation- D) (one way ANOVA with post hoc)

Marker	Inflammation	Within group	Significance	95% Confidence Interval	
				Lower Bound	Upper Bound
STAT 3	A Mean±SD 11.48±9.10	B	1.000	-12.848	12.199
		C	1.000	-12.058	11.161
		D	.041	-47.180	-.742
	B Mean±SD 11.81±9.93	A	1.000	-12.199	12.848
		C	1.000	-9.900	9.652
		D	.035	-45.995	-1.278
	C Mean±SD 11.93±11.85	A	1.000	-11.161	12.058
		B	1.000	-9.652	9.900
		D	.031	-45.372	-1.653
	D Mean±SD 35.45±20.85	A	.041	.742	47.180
		B	.035	1.278	45.995
		C	.031	1.653	45.372

CONCLUSION:

In current study on in depth observations of STAT3 expression with prime agents participates in oral carcinogenesis suggest that STAT3 activation is a critical mediator of tobacco and tobacco related carcinogen and may provide environment for initial steps for carcinogenesis.

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