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Expression of proinflammatory cytokine INTERLEUKIN 6 in oral submucous fibrosis and oral squamous cell carcinoma: Immunohistochemical study

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ABSTRACT:

Introduction: Interleukin 6 is a proinflammatory cytokine indicated in many types of cancer. Increased cytokine levels are found in oral submucous fibrosis which is a premalignant condition. Immunohistochemical evaluation of interleukin 6 will help in better understanding of progression of OSF and OSCC

Materials and methods: Formalin fixed, paraffin embedded oral submucous fibrosis tissue blocks were obtained from departmental archives. 40 samples each of early, moderately advanced and severe OSF were randomly selected. 20 samples of normal mucosa and 20 samples of OSCC were selected. Immunohistochemistry was performed, quantitatively and qualitatively assessed for expression of IL 6.

Results: The test results demonstrated a significant difference in the percentage of expression in the IL-6 expression between 3 groups (P<0.001).. Higher intensity and percentage of expression was found in OSCC and the expression was cytoplasmic. Despite having an increasing trend in the expression of IL-6 based on tumour grade, there was no statistically significant difference noted. OSF group showed higher mean Q scores as compared to control group but the difference was not statistically significant. Despite having an increasing trend in the expression of IL-6 based on tumour grade, there was no statistically significant difference noted.

Conclusion: Interleukin 6 is consistently expressed in oral squamous cell carcinoma but not in OSF tissues. Immunohistochemical marking of interleukin-6 in surgically excised oral squamous cell carcinoma specimen may help in understanding prognosis.

Key words: Interleukin 6, oral squamous cell carcinoma, oral submucous fibrosis, immunohistochemistry

INTRODUCTION:

Interleukins are a group of cytokines that were first seen to be first expressed by white blood cells. The name interleukin was chosen in 1979 during the second international lymphokine workshop in Switzerland. The term interleukin derives from inter- "means of communication between" and leukin – from leukocytes and act on leukocytes. This term was coined by Dr Vern Paetkau.[1,2]

Interleukin-6 (IL-6) is a well-known inflammatory mediator and fibrogenic cytokine. IL-6 plays a major role in the response to injury or infection and is involved in the immune response, inflammation, and haematopoiesis. Its deregulation impacts numerous disease states, including many types of cancer. IL-6 was cloned first by Hirano et al., in 1986. Interleukin 6 was originally identified as T cell derived lymphokine that induces maturation of B Cells into anti body producing cells. Il 6 is produced by various types of cells, including lymphocytes, monocytes, and fibroblasts but majorly by Macrophages. IL-6 is secreted by many different cells, including T cells, B cells, monocytes, endothelial cells, fibroblasts, and some tumor cells.[3]

IL-6 level has been correlated with tumor progression in multiple cancer types. Serum level IL-6 has been identified as prognostic marker in many types of cancer, including ovarian cancer [4], prostate cancer [5], breast cancer [6], colon cancer [7], melanoma [8] and HNSCC [9].

Oral submucous fibrosis (OSF) is a chronic fibrotic disease of the oral cavity and oropharynx characterized by fibroelastic change in the mucosa which leads to progressive inability to open the mouth. In India overall incidence is about 0.2 – 0.5%. Malignant transformation in OSMF ranges 3 to 7.6%. After comparing the risk ratio, it has been estimated that people with OSF are 19.1 times more likely to develop oral cancer than those without it. [10]

Cytokines and growth factors produced by inflammatory cells within the lesion may promote fibrosis by inducing proliferation of fibroblasts, upregulating collagen synthesis and downregulating collagenase production. Haque et al [11] has lately found that oral submucous fibrosis tissues express higher levels of IL-6 than comparable healthy tissues. Gallo et al[12] found high serum IL-6 levels in HNSCC patients. Therapeutic targeting of IL-6 and its receptor in cancer has strong biologic rationale. In the present study we have studied expression of IL 6 in different grades of OSF, oral squamous cell carcinoma [OSCC] samples compared to normal mucosal tissue.

MATERIAL AND METHODS:

Formalin fixed, paraffin embedded oral submucous fibrosis tissue blocks were obtained from departmental archives. 5 µm sections of samples were stained with routine haematoxylin and eosin and analysed under light microscopy. 40 samples each of early, moderately advanced and severe OSF were randomly selected. 20 samples of normal mucosa and 20 samples of OSCC were selected.

IHC procedure: Immunohistochemistry was performed with avidin biotin technique and 5µm sections were placed on positive charged slides. Sections were deparaffinised, rehydrated and quenched. IHC staining was done with IL 6 Monoclonal antibody (ab6672) (ABCAM clone rabbit anti human). Antigen Retrieval was done using EDTA Solution with pH 8 Sections were covered with MACH1 HRP Polymer incubated with secondary antibody. Antigen antibody binding was

detected with Betazoid DAB Chromogen and sections were counter stained with Mayers Haematoxylin Counterstain. Expression of the marker was evaluated using scoring methods Quantitative scoring methods [13,14,15]

Scoring of % of expression

Score	0	1+	2+	3+	4+
Positive Cells	<10%	10-25%	25-50%	50-75%	>75%

Scoring of intensity of expression

Score	1	2	3
Intensity of Staining	weak staining	moderate staining	strong staining

Quick score (Q):
Results are scored by multiplying the percentage of positive cells (P) by the intensity (I).
Formula: Q = P x I; Maximum = 300

RESULTS

In the present study immunohistochemical expression of interleukin 6 was evaluated based on quantitative methods in 80 samples of normal mucosal tissue, OSF and OSCC tissues. Chi Square test, Kruskal Wallis Test and multiple comparisons of mean difference b/w diff groups using Mann Whitney Post hoc Test was used for statistical analysis.

The test results demonstrated a significant difference in the percentage of expression in the IL-6 expression between 3 groups at P<0.001. OSCC group showed a significant proportion of IL-6 expression varying from 25-50 to >75% in 25 to 30% of cases as compared to 0 to 5 % in control and 0 to 15% in OSF cases. And contrastingly, predominant expressions were noted with <10% [65% in control & 40% in OSF] & 10-25% [32.5% in OSF & 30% in Control] as compared to OSCC group [<10% in 0% cases & 10-25% in 15% cases]. These differences in the percentage of IL6 expression between OSCC and OSF & control group were statistically significant at $P \leq 0.001$ respectively. However, no significant differences were noted between OSF & Control groups. [Table 1, 2]

Similar findings were noted for the intensity of staining of IL-6 between 3 groups, which was statistically significant at P<0.001. A significant proportion of OSCC cases showed moderate to Strong intensity of staining [15 – 40%] as compared to Control and OSF groups which showed predominantly with no expression [65% in Control & 40% in OSF group] and weak expression [42.5% in OSF group and 35% in Control group]. These differences in the intensity of staining of IL-6 between different groups was statistically significant between OSCC and OSF & Control group at P<0.001. However, a borderline significance was noted between OSF and Control group with P=0.07. [GRAPH 1]

The mean Q scores for OSF group was 34.38 ± 41.09, for OSCC group was 111.25 ± 74.11 and in control group was 10.00 ± 14.96. This difference in the mean Q Scores between 3 study groups was statistically significant at P<0.001. [graph 2]

Multiple comparison of mean difference between different study groups showed that OSCC group showed significantly highest mean Q Scores [graph 3]as compared to OSF and control groups at P<0.001. This was followed by OSF group showing a significantly higher mean Q scores as compared to control group at P=0.03. This infers that OSCC group showed significantly highest mean Q scores, followed by OSF group and least value in Control group. [Table 3, 4]

The test results indicated that 40% of T_1 tumour showed both 10-25 and 25-50% with very few cases of >75%, whereas 42.9% T_2 tumour showed >75% expression, followed by 28.6% of 25-50%, with very few cases with 10-25% & 50-75% expressions (14.3%) and majority of the T3 tumour cases showed 50-75% expression (50%) and remaining 50% cases showed with 25-50% &>75% expression (25% each). Despite having an increasing trend in the expression of IL-6 based on tumour grade, there was no statistically significant difference noted.

In terms of intensity of IL-6 staining, majority of the T1 showed weak expression (60%), as compared to T2 with 28.6% cases with strong expression and 50% of the cases in T3 with moderate intensity of IL-6 staining. However, no significant differences were noted between different grades of tumour. [Graph 4]

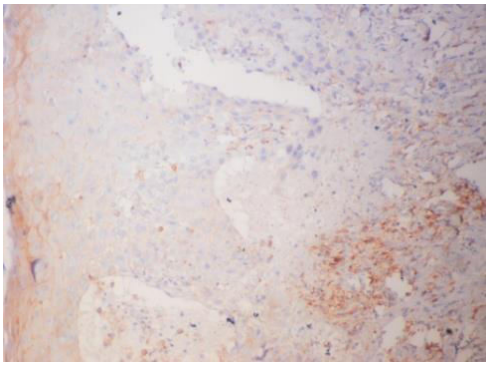


Figure 1: uptake in OSCC

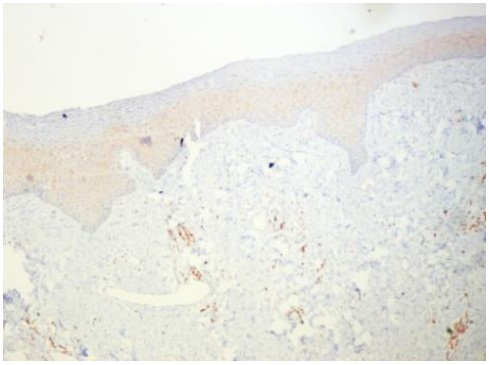


Figure 2: more uptake is observed in basal layers

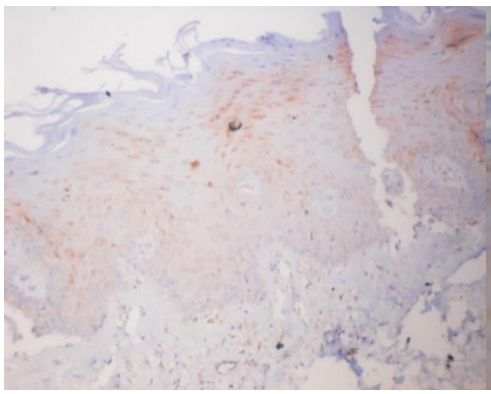


Figure 3: Uptake in OSF tissue

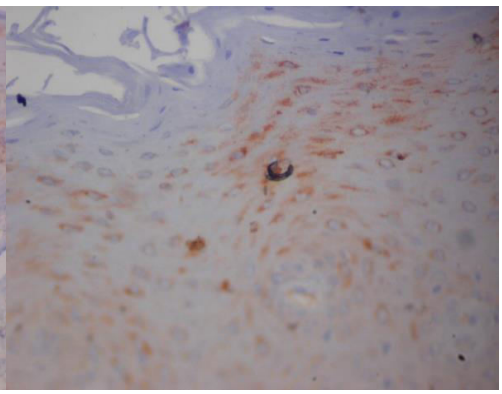


Figure 4: cytoplasmic uptake evident

Comparison of Percentage and Intensity of Expression of IL-6 between different groups using Chi Square Test									
Variable	Category	OSF		OSCC		Control		χ^2 Value	P-Value
		n	%	n	%	n	%		
Percentage o	< 10%	16	40.0%	0	0.0%	13	65.0%	40.434	<0.001*

Expression	10-25%	13	32.5%	3	15.0%	6	30.0%		
	25-50%	6	15.0%	6	30.0%	1	5.0%		
	50-75%	5	12.5%	5	25.0%	0	0.0%		
	> 75%	0	0.0%	6	30.0%	0	0.0%		
Intensity of Expression	No Expression	16	40.0%	0	0.0%	13	65.0%	29.838	<0.001*
	Weak	17	42.5%	9	45.0%	7	35.0%		
	Moderate	7	17.5%	8	40.0%	0	0.0%		
	Strong	0	0.0%	3	15.0%	0	0.0%		

TABLE 1 :**Comparison of Percentage and Intensity of Expression of IL-6 between different groups**

Multiple comparison of Percentage and Intensity of Expression of IL-6 between different groups			
Groups	OSF vs OSCC	OSF vs Control	OSCC vs Control
Percentage of Expression	<0.001*	0.15	<0.001*
Intensity of Expression	0.001*	0.07	<0.001*

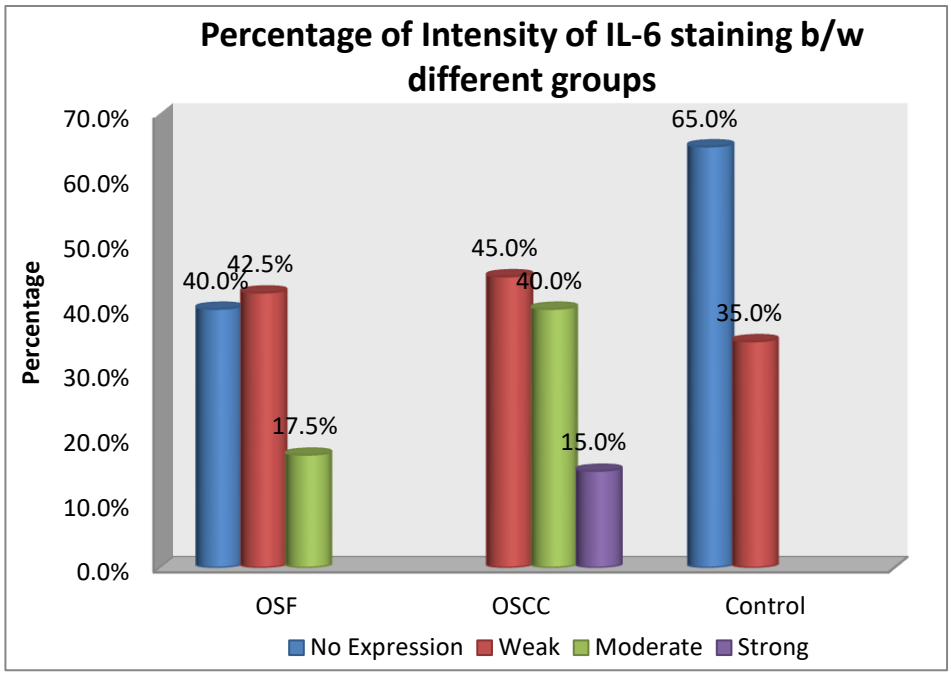
TABLE 2: **Multiple comparison of Percentage and Intensity of Expression of IL-6 between different groups**

Comparison of mean Q Score between different study groups using Kruskal Wallis Test							
Groups	N	Mean	SD	Median	Min	Max	P-Value
OSF	40	34.38	41.09	25	0	150	<0.001*
OSCC	20	111.25	74.11	100	25	300	
Control	20	10.00	14.96	0	0	50	

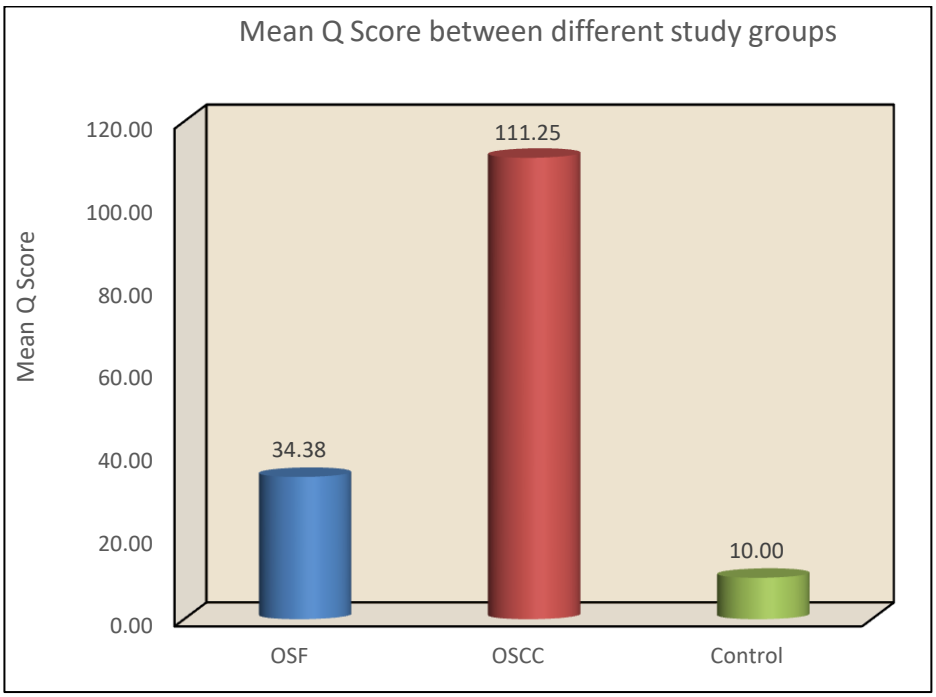
TABLE 3 **Comparison of mean Q Score between different study groups using Kruskal Wallis Test**

Multiple comparison of mean difference in Q Scores b/w diff groups using Mann Whitney Post hoc Test					
			95% CI for the Diff.		
(I) case	(J) case	Mean Diff. (I-J)	Lower	Upper	P-Value
OSF	OSCC	-76.88	-108.03	-45.72	<0.001*
	Control	24.38	-6.78	55.53	0.03*
OSCC	Control	101.25	65.28	137.22	<0.001*

TABLE 4:**Multiple comparison of mean difference in Q Scores b/w diff groups using Mann Whitney Post hoc Test**



GRAPH 1



GRAPH 2

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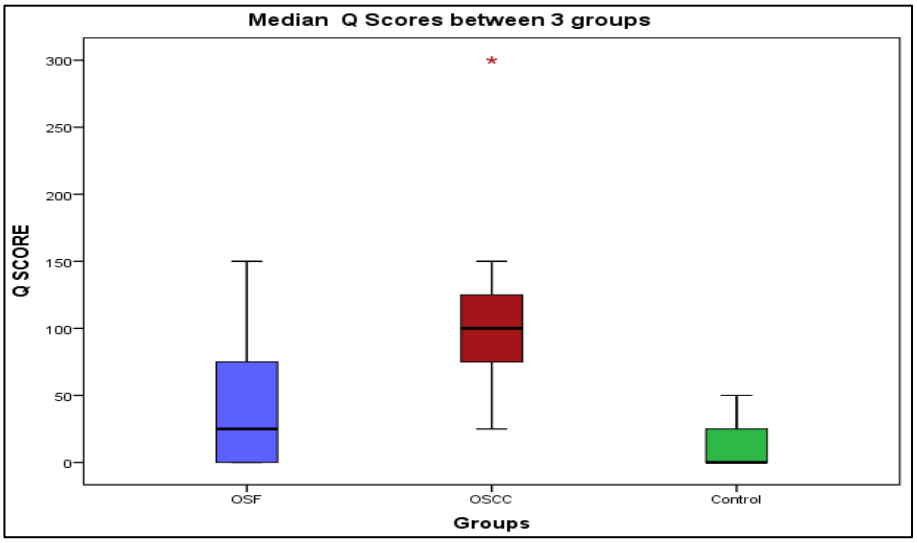
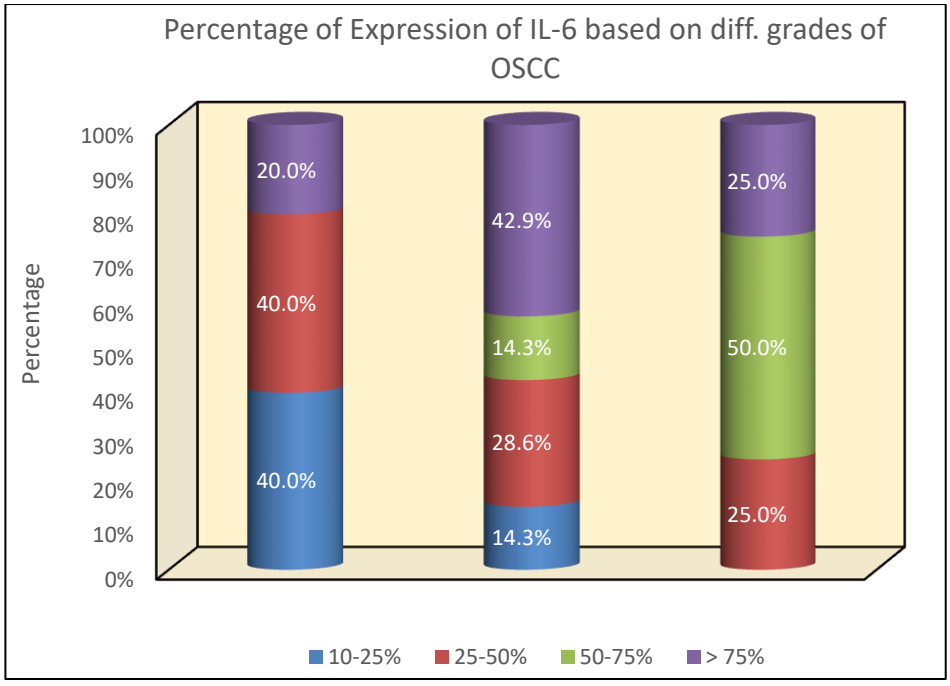


GRAPH 3



GRAPH 4

DISCUSSION

Cancer cells have ability to self renew and differentiate leading to uncoordinated growth.

Cancer stem cell theory describes the importance of molecular mechanism and inability to target these cells the reason for failure in cancer treatment. [16]. Kim et al hypothesized that endothelial cell-secreted interleukin-6 (IL-6) contributes to tumor progression by enhancing the migratory phenotype and survival of cancer stem cells. They also showed that blockade of the IL-6 pathway with a humanized anti-IL-6R antibody (tocilizumab) inhibited endothelial cell-induced motility in vitro and decreased the fraction of cancer stem cells in vivo.[17]

Hence IL 6 can act as a diagnostic marker and be therapeutic target.

Patients with head and neck squamous cell carcinoma were found to have increased serum level of interleukin 6 in various studies [9, 18-20] salivary interleukin 6 in OSCC has been evaluated in

few studies [20-22] which show increased IL 6 levels similar to our study. These studies are based on ELISA, we have done immunohistochemical evaluation.

We found increased immunopositivity for IL 6 in OSCC compared to OSF and normal tissue. Interleukin 6 was found more in cytoplasm, expressed more towards the epithelial basement membrane and perivascular tissue. Adjacent connective tissue showed positive uptake occasionally. Very faint expression or negative expression was found in OSF tissue. Shinagawa et al [23] has assessed IL 6 by IHC and have found immunopositivity at 78.4% in OSCC samples which can be correlated to our study.

Haque 24 et al have shown increased expression of IL 6 in OSF tissues. In our study faint expression of IL 6 was found in epithelium of OSF tissue. The frequency and intensity of expression was low in OSF tissue. The increase in expression when compared to normal tissue was not statistically significant. This may suggest that interleukin 6 is more sensitive indicator of dysplasia than fibrosis. Salivary and serum cytokine concentrations were measured using enzyme-linked immunoassay kits by Kaur et al [25].And found that levels of serum and salivary IL 6 were increased in oral submucous fibrosis in contrast to normal healthy subjects.

Immunofluorescence staining showed that IL-6R was strongly expressed by most cells at the invasive fronts of HNSCC We found that high IL- 6R expression in the invasive front of the tumor correlated with poor overall survival [17]

Intensity of IL 6 expression was more in T3 tumours compared to T2 and T1 tumour samples in the present study. Despite having an increasing trend in the expression of IL-6 based on tumour grade, there was no statistically significant difference noted. The limitation may be due to difference in sample size and small number of samples. Serum level of IL 6 was increased in patients with advanced stage tumours in study by Riedel et al [18]. Nakano et al [26] found that larger-sized tumors (T3, 4) contained significantly greater levels of IL-6 proteins than small-sized tumors (T1, 2).

Chang et al [27] found that serum interleukin-6 levels were associated with increased tumor burden and aggressiveness of oral cavity squamous cell carcinomas and may be useful as a prognostic indicator after treatment. Many studies showed IL-6 is observed at higher levels in the elderly patients [28, 29]. We did not correlate the age in the present study as the specimens were obtained by archives. Age specific and progression associated study might add more value to the study

CONCLUSION

Interleukin 6 is consistently expressed in oral squamous cell carcinoma but not in OSF tissues. IL-6 has been proposed as a malignancy predictor, with sensitivity and specificity of about 60–70% and 58–90%, respectively [30]. However, there are limited studies available that might be used to define cut-off values for IL-6 as a diagnostic tool. Therefore immunohistochemical marking of interleukin-6 in surgically excised oral squamous cell carcinoma specimen may help in understanding prognosis. Increased interleukin 6 levels if found in OSF tissue can be correlated to dysplastic changes and progression into carcinoma.

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