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Dentistry Section

Expression of Salivary Secretory Leukocyte Protease Inhibitor in Smokers, Leukoplakia and OSCC

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ABSTRACT

Introduction: The unsatisfactory prognosis and morbidity by contemporary treatment modality of carcinoma and late diagnosis due to lack of simple non-invasive early marker has driven researchers to explore and identify new molecules which can be used as markers or for targeted therapy for cancer. Secretory Leucocyte Protease Inhibitor (SLPI) is one such molecule of interest, whose association with smoking and cancer is controversial and less researched.

Aim: To estimate and compare the salivary levels of SLPI in healthy control, smokers with and without leukoplakia and oral squamous cell carcinoma.

Materials and Methods: A prospective, case-control study was conducted in which whole unstimulated saliva samples were collected from total of 80 participants which included 20 controls, 20 smokers without leukoplakia, 20 smokers with leukoplakia and 20 cases of Oral Squamous Cell Carcinoma (OSCC). Salivary SLPI levels were measured using enzyme-linked immunosorbent assay and comparison of SLPI levels between groups was done using Mann Whitney Test.

Results: The study results showed a higher concentration of SLPI in controls than leukoplakia and OSCC. The median SLPI of controls was 144 ng/mL, while concentration of SLPI in smokers without leukoplakia, with leukoplakia and squamous cell carcinoma were 101 ng/mL, 112 ng/mL and 113 ng/mL, respectively.

Conclusion: Results from this study suggest that smoking, premalignancy and OSCC are associated with lower concentrations of salivary SLPI compared to controls. Hence, salivary SLPI could be a potent biomarker in cancer diagnosis and can have a great value in immune therapy of OSCC.

Keywords: Enzyme-linked immunosorbent assay, Saliva, Squamous cell carcinoma, Targeted therapy

INTRODUCTION

OSCC is a multifactorial disease, where numerous intrinsic and extrinsic factors play a role in its causation. The increasing prevalence of cancer has created a major impact in living standards of people across the world in all stratas of the society. Globally, over 1 million new cases are reported every year among a population of 1.2 billion. Though the overall incidence rate of cancer is lower in India when compared to western countries, the incidence of oral cancer is higher, representing up to 50% of all cancers and OSCCs representing 95% of them [1].

Data based on six cancer registries across India revealed an increase in lung and oral cancer in Chennai. This clearly suggests that tobacco plays a major role and its prevention and control should remain the prime concern to decrease the burden of oral cancer. The International Agency for Research on Cancer predicts that the incidence of Cancer in India to be more than 1.7 million in 2035 and the death rates to increase from 6,80,000 to 1-2 million during the same period [1].

Most of the oral carcinomas inspite of its easy access for examination is diagnosed later which makes its prognosis poor. Although, early-stage cancers have a favourable prognosis, there are no reliable methods for the early detection of OSCCs. Over half of all OSCCs present with advanced disease, highlights the need for further studies to investigate molecular biomarkers indicative of OSCC risk, early diagnosis, targeted therapy and to assess prognosis [2].

Oncological research have identified molecular pathways connecting inflammation and cancer. This has suggested that cancer-related inflammation represents one of the hallmarks of cancer. This link was found as early as 1863 by Virchow, who hypothesised that origin of cancers, was at sites of chronic inflammation [3,4].

SLPI is an innate immunity-associated protein functioning mainly to protect local tissues against the detrimental consequences of inflammation. The anti-inflammatory action of SLPI is mainly through its inhibition of Nuclear factor kappa B, one of the prime movers of molecular pathway involved in cancer-related inflammation. In recent years, Protease inhibitors are of huge interest among researchers as they are over-expressed in many types of cancers including lung cancers and nasopharyngeal carcinoma. With regards to its role as a biomarker in cancers, the expression of SLPI seems to be a subject of controversy where SLPI is highly upregulated in pancreatic, papillary thyroid, uterine cervix, endometrial, ovarian cancer and colorectal cancer. However, by contrast, SLPI is downregulated in OSCC, Nasopharyngeal carcinoma, bladder tumours, and some breast carcinomas [5,6].

It is of particular interest to know that proteomic analysis of oral premalignant and OSCC tissue showed that SLPI protein stood out based on its decreased expression in both potentially malignant oral disorders and OSCC lesion tissues compared to healthy normal tissues [7].

Interestingly, the expression of SLPI to tobacco also has yielded varied results. The initial study in the literature regarding the role of tobacco smoke on oral SLPI was by Chan KH et al., who showed that total neutrophil elastase concentration and activity as well as SLPI activity was increased following exposure to cigarette smoke in a rat model [8].

Following this a preliminary study by Hoffmann M et al., investigated the role of SLPI in healthy mucosa of non-head and neck squamous cell carcinoma patients and correlated SLPI expression with smoking habits. In accordance with the data obtained in the rat model, they showed that moderate/strong SLPI expression was mostly found in patients with smoking habits [9].

In contrast, studies on the expression of SLPI in the lung mucosa of smokers showed decreased production of SLPI suggesting that cigarette smoking jeopardises the protective screen provided by functional SLPI [10].

Considering these controversial results and minimal literature regarding the effects of tobacco on SLPI, this study aimed to quantify the levels of SLPI in patients with smoking and oral squamous cell carcinoma.

MATERIALS AND METHODS

A prospective case-control study was conducted during the period from January 2016 to February 2017. A written and informed consent was obtained from all the study participants before enrolling in the study. The study was approved by the Scientific and Institutional Ethical Committee of SRM University, Chennai (SRMDC/IRB/2015/MDS/No.603). The recruitment of study subjects was done from the outpatient department of SRM Dental College, referrals from SRM General Hospital and private dental clinics. Study subjects were recruited by professionally qualified, well-trained and experienced Oral Pathologists. The study group included a total of 80 participants comprising of 20 healthy controls (Group I), 20 smokers without leukoplakia (Group IIa), 20 smokers with leukoplakia (Group IIb) and 20 OSCC subjects (Group III). Demographic data, habit history including type of smoking, frequency, and years of smoking were recorded. Clinical and histological characters of leukoplakia and OSCC in terms of size of lesion, type of leukoplakia and grading of OSCC was done [Table/Fig-1]. As one of the objectives was to assess the levels of SLPI in smoking, only male subjects were included in this study.

Exclusion and Inclusion Criteria for Study Subjects

Healthy Control Group (Group I)

The control subjects were recruited from volunteers of SRM Dental College and Hospital. Subjects (n=20) were recruited with the following eligibility criteria: Age matched male subjects, with no current or previous use of tobacco, no chronic/acute illnesses, no oral lesions, no acute or chronic, no pathological dry mouth syndrome, or inability to collect sufficient saliva samples on a reliable basis.

Smokers without Leukoplakia (Group IIa)

Subjects (n=20) with the following eligibility were recruited: Only male subjects with age range 30-60 years were considered. Those with history of only smoking and without any clinical evidence of tobacco associated lesions, who were not undergoing or having undergone any form of treatment for leukoplakia were also considered. Subjects with underlying systemic illnesses and presence of oral inflammatory conditions such as gingivitis, periodontitis, and oral ulcers were excluded.

Smokers with Leukoplakia (Group IIb)

Subjects (n=20) with the following eligibility were recruited: age range 30-60 years, only male, comprised of patients who had oral potentially malignant lesions (leukoplakia) which were clinically diagnosed and later histopathologically confirmed and who were not undergoing or having undergone any form of treatment for these lesions. Patients with underlying systemic illnesses and presence of oral inflammatory conditions such as gingivitis, periodontitis, and oral ulcers were excluded. Only histopathologically confirmed cases were included in the study. Due to rarity of erythroplakia occurrence in South India region, only leukoplakia and was included and OSMF being a disorder with different aetiology was excluded from the study.

OSCC group (Group III)

Subjects (n=20) were recruited with the following eligibility criteria: age range 39-70 years, only male, comprised of patients with clinically and histologically confirmed cases of OSCC with only smoking habit, who were not currently undergoing or having undergone any form of definitive therapy for OSCC in the form of radiation, chemotherapy or any other adjunctive treatments. All oral lesions which were included in the study were subjected to routine exfoliative cytology studies with Papanicoloau stain and potassium hydroxide stain to rule out presence of candida hyphae.

Category		Control (Group I)	Smoking		OSCC (Group III)
			Without leukoplakia (Group IIa)	With leukoplakia (Group IIb)	
Subjects (n)		20	20	20	20
Age range (years)		30-74	30-60	30-60	39-70
Sex	Male	20	20	20	20
	Female	-	-	-	-
Smoking status					
Type of smoking	Cigarette	-	12	11	8
	Beedi	-	8	6	7
	Both	-	-	3	5
Years of smoking	<15 yr	-	9	10	5
	>15 yr	-	11	10	15
Frequency	<10 /day	-	10	12	6
	>10 /day	-	10	8	14
Clinical characters of leukoplakia					
Clinical type	Homogenous	-	-	14	-
	Non-Homogenous	-	-	6	-
Size	<2 cm	-	-	7	-
	2-4 cm	-	-	10	-
	>4 cm	-	-	3	-
Site	Buccal mucosa	-	-	8	-
	Vestibule	-	-	3	-
	Buccal mucosa and vestibule	-	-	2	-
	Tongue	-	-	4	-
	Commissure of lip	-	-	3	-
Clinicopathological data of OSCC					
Site	Buccal mucosa	-	-	-	12
	Tongue	-	-	-	6
	Lip	-	-	-	2
Histological grading	Well-differentiated	-	-	-	13
	Moderately-differentiated	-	-	-	5
	Poorly-differentiated	-	-	-	2

[Table/Fig-1]: Demographics and subclassification of study groups.

Collection of Saliva and Storage

The subjects were instructed to rinse the mouth with water and whole unstimulated saliva was collected. Following collection, saliva was immediately centrifuged in a cooling centrifuge at 2500 rpm for 15 minutes at 4°C to remove squamous cells and cell debris. The resulting supernatant was separated into 1 mL aliquots and stored at -80°C freezer. Not more than one freeze thaw cycle was allowed for each sample.

Estimation of SLPI Concentration

Quantification of salivary Secretory leukocyte protease inhibitor (SLPI) was done by each individual expectorated 5 mL of saliva under non-stimulatory conditions in a quiet room into a sterile centrifuge

tube. Commercially, available ELISA kit (Bioassay Technology) which is based on Biotin double antibody sandwich technology. Briefly, standards and samples were added to the precoated SLPI antibody microplate and incubated and then washed with buffer solution. Horseradish Peroxidase (HRP) conjugate bound with detection antibody was added to the washed plate. A chromogen substrate was added to the wells which resulted in the development of blue coloured complex. The colour development was then stopped by addition of stop solution making the resultant final product yellow. The intensity of the colour development is proportional to the concentration of the SLPI present in the sample and measured in a microplate reader which is capable of reading at a wavelength of 450 nm. The optical density thus obtained was used for calculation of SLPI in the sample.

STATISTICAL ANALYSIS

Statistical analysis of the data obtained was done using SPSS software version 16. As SLPI concentrations were skewed, values are expressed as median and Inter-quartile range and non-parametric Mann-Whitney U-tests were performed to find the significance of the observed differences between groups statistical methods were used. Median SLPI concentrations and Inter-quartile range were calculated and compared between the groups.

RESULTS

Demographic, habit history and clinical characters of subjects are outlined in [Table/Fig-1]. A total of 80 samples comprising of 20 control subjects (Group I), 20 smokers without leukoplakia (Group IIa), 20 smokers with leukoplakia (Group IIb) and 20 OSSC subjects (Group III) were included. The premalignant lesions were from buccal mucosa (n=8), vestibule (n=3), together in buccal mucosa and vestibule (n=2), tongue (n=4), commissure of lip (n=3). Similarly, the OSCC lesion were from buccal mucosa (n=12), tongue (n=6), lip (n=2).

The median and interquartile range for SLPI concentration in control subject (Group I) is 144 (66.25-176), smokers without leukoplakia (Group IIa) is 101 (57-142), smokers with leukoplakia (Group IIb) is 112 (103-125) and OSCC (Group III) is 113 (82-127.5) ng/mL [Table/Fig-2]. Among groups, smokers without lesion showed the lowest SLPI concentration and healthy controls with no habits showed the highest concentration. The statistical analysis between different groups showed lower concentrations of SLPI in smokers with and without leukoplakia and OSCC compared to controls [Table/Fig-3].

Group	Sample size (n)	Age group (Years)	SLPI levels (ng/mL)		
			Mean	Median	Interquartile range
Control (Group I)	20	30-74	128.20	144	66.25-176
Smoker (Group IIa)	20	30-60	106.75	101	57-142.5
Leukoplakia (Group IIb)	20	30-60	102.65	112	103-125
OSCC (Group III)	20	39-70	113.90	113	82-127.5

[Table/Fig-2]: Descriptive Statistics for concentration of SLPI in control and study groups.
The levels of salivary SLPI were assessed by ELISA in all the study subjects. The concentrations are expressed in nanogram per milliliter, and the values represent mean, median and interquartile range

Group	Sample size (n)	Mean SLPI (ng/mL)	Mean rank	p-value
Control (Group I)	20	128.20	23.03	0.172
Smoker (Group IIa)	20	106.75	17.98	
Control (Group I)	20	128.20	22.75	0.223
Smokers with leukoplakia (Group IIb)	20	102.65	18.25	
Control (Group I)	20	128.20	16.63	0.322
OSCC (Group III)	20	113.90	13.25	
Smoker (Group IIa)	20	106.75	20.55	0.978
Smokers with leukoplakia (Group IIb)	20	102.65	20.45	
Smoker (Group IIa)	20	106.75	14.30	0.291
OSCC (Group III)	20	113.90	17.90	
Smokers with leukoplakia (Group IIb)	20	102.65	15.10	0.725
OSCC (Group III)	20	113.90	16.30	

[Table/Fig-3]: Comparison of salivary SLPI levels between groups.

DISCUSSION

The increasing prevalence of cancer has created a major impact in living standards of people across the world in all stratas of the society. Globally, over 1 million new cases are reported every year among a population of 1.2 billion [11]. Though the overall incidence rate of cancer is lower in India when compared to western countries, the incidence of oral cancer is higher, representing up to 50% of all cancers and OSCCs representing 95% of them [12].

Identification of many inflammatory pathways involved in tumourigenesis such as cyclooxygenase and Nuclear Factor kB, Epidermal Growth Factor Receptor (EGFR) and Signal Transducer and Activator of Transcription (STAT) protein have driven the research towards identifying key molecular players in these pathways which can be used in immune targeted therapy and as early markers of cancer [3].

In cancer research, SLPI is gaining popularity for its significant role in modulation of inflammation by its antiproteolytic properties and regulation of cell proliferation, differentiation and apoptosis. SLPI plays a complex role in tumourigenesis, its role seems to differ depending on the anatomical site, species, endocrine effect and infective agent. SLPI levels are found to be upregulated in ovarian, pancreatic, papillary thyroid, uterine cervix, endometrium and colorectal cancer, whereas its levels are downregulated in OSCC, nasopharyngeal carcinoma, bladder tumour and some breast carcinomas [6].

The present study finding of lower level of SLPI in premalignancy and squamous cell carcinoma in comparison with controls was in accordance with previous studies. Wen J et al., studied the immunohistochemical expression of SLPI in OSCC and showed significant decrease in SLPI in OSCC compared to normal oral epithelium. Furthermore, an inverse correlation between SLPI and histological parameters associated with tumour progression was evident [13]. On the contrary, raised plasmin and elastase were positively correlated with histological parameters of tumour invasion. Plasmin is highly expressed in tumour tissues and protected from inhibition by α2-antiplasmin. However, SLPI orchestrates the blocking and prevents the conversion of plasminogen to its active form plasmin, by binding with receptor Annexin A2. As the migration and invasion of tumour cells is associated with protease plasmin, decreased levels of SLPI accelerated the invasive nature of the tumour and substantiated the SLPI's antitumourigenic effect in OSCC [13].

Furthermore, Cordes C et al., showed a strong statistically significant association between lower SLPI expression and increased risk of lymph node metastasis suggesting a protective effect of SLPI on metastasis [14].

Yang Y et al., analysed SLPI levels in soluble proteins isolated from whole cell lysates from oral brush biopsy samples of potentially malignant lesions and squamous cell carcinoma, and found SLPI concentration was twenty five-fold lower in OSCC tissue when compared with epithelium of healthy controls and inferred the potential for SLPI as a non-invasive biomarker of oral cancer progression with potential in preventive treatment [7].

Interestingly, the expression of SLPI to smoking also has yielded varying results. It is generally considered that smoking has a direct effect on oral immune status and SLPI levels are increased. Smoking has been shown to enhance the expression of signal transducer

and activator of transcription (STAT1 protein), which in turn leads to increased SLPI expression [8,16]. However, most of the available data in the literature regarding effects of smoking and SLPI are on the respiratory mucosa or the secretions of the respiratory tract and have shown significantly increased levels of SLPI on exposure to smoke.

Chan KH et al., studied the expression of SLPI in a rat model by exposing it to cigarette smoke and found that cigarette smoke caused an increase in SLPI levels. They found cigarette smoke caused oxidative stress and imbalance in protease/anti-protease. They also found that cigarette smoke increased level of neutrophil elastase and consecutively the level of anti-trypsin alpha and SLPI in bronchial lavage [8].

Following this, Quabius ES et al., investigated the role of SLPI in healthy mucosa of Non-HNSCC patients and correlated SLPI expression with smoking habits. In accordance with the data obtained in the rat model, they showed moderate/strong SLPI expression in patients with smoking habits [15].

Contrarily, studies on the expression of SLPI in lung mucosa of smokers showed decreased production of SLPI [10]. Hence, to address these controversial results on SLPI and smoking, the relationship of SLPI between healthy individuals and saliva of smokers was done. The decreasing levels of SLPI concentration in smokers (median-101 ng/mL) in comparison with healthy individuals (median-144 ng/mL) were found in the present study. This suggests that that cigarette smoking jeopardises the protective screen provided by functional SLPI.

However, contrary results were shown by Pierce Campbell CM et al., who did a follow-up study on smokers who developed OSCC and showed increasing levels of SLPI in OSCC than controls. In this nested case-control study, although a statistically significant SLPI median value was observed in smokers in comparison with non-smokers, the values were positively skewed showing a wide variation. Similarly, among 50 smokers who had OSCC 34 subjects showed an increased concentration of SLPI and 16 subjects showed lower SLPI concentrations. With these results they speculated that there can be varied SLPI levels in smokers, but concluded smokers with increased SLPI concentrations levels are at increased risk of developing HNSCC [16,17].

Wang X et al., investigated the correlation between the abundance of SLPI protein and the different histological grades of leukoplakia by immunohistochemistry and the results specified that the level of SLPI was negatively correlated with the histological grades of the oral premalignant lesions, signifying that it may be a potential prognostic tool for the malignant transformation [18].

Amiano N et al., demonstrated that the inoculation of mammary tumour cells genetically modified to express high levels of SLPI does not develop tumours in BALB/c mice. They also showed that genetically modified SLPI over-expressing non-irradiated tumour cells which do not develop tumour in immunocompetent mice, act as a vaccine that partially restrain the tumour growth, probably by apoptosis that boosts an adaptive immune response [19].

The concentration of SLPI is found to be affected by many factors such as country, age, oral hygiene habits, diet, sexual habits, gingival inflammation, alcohol use etc. Hence these aforementioned factors may have contributed to the contrasting results of present study as far as smokers are concerned. Furthermore, the previous studies concerning smoking and SLPI are carried out primarily on respiratory epithelium and their secretions such as nasal fluid and bronchial lavage.

In addition, the limited data in the literature, in relation to oral cavity have concentrated on the cellular expression in which intracellular form of SLPI contributed to major part rather than salivary SLPI. Few other studies done on saliva used oral gargle samples in which its alcohol content, vigorousness and duration of gargle might have imparted the difference. Alcohol, a factor that has known to affect the SLPI concentration could have also affected present study results owing to prevalent co-habit in smokers.

LIMITATION

Due to the regional lifestyle the habit of smoking is far less in females and so were excluded from the study. Furthermore, since the present study was primarily focused on effect of smoking on SLPI, we selected OSCC patients only with smoking habits and this probably could have influenced the statistical significance, because HPV associated OSSCs have significantly decreased SLPI. Hence to ascertain the potential diagnostic value of SLPI, it would be ideal when the clinical endpoint of development of OSCC is observed.

CONCLUSION

Collectively, the observed findings suggest that SLPI, like nearly all previous reported data has shown a definitive decrease in concentration in premalignancy and OSCC though not statistically significant. Hence, the use of salivary SLPI as a potent biomarker in cancer diagnosis can be speculated and have a great value in immune therapy of OSCC. However, in view of the contrasting results with regards to smoking from previous literature, additional research is required to evaluate the various characters that influence the concentration of SLPI and larger samples including these various influencing factors might give a clear representation of SLPI levels.

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