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Research Protocol

Dentistry Section

CTLA4 Methylation and its Expression as a Prognostic Biomarker in Tobacco Users with and without Oral Squamous Cell Carcinoma- A Protocol for Cross-sectional Study

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ABSTRACT

Introduction: Oral Squamous Cell Carcinoma (OSCC) is characterised by immunosuppression mediated by evasion of 'immune checkpoints' by tumour cells. Cytotoxic T Lymphocyte Associated Protein 4 (CTLA4) is one of the immune checkpoint molecule whose role in oral carcinogenesis remains to be elucidated. Deoxyribonucleic Acid (DNA) methylation of CTLA4 promoter region holds potential as a biomarker for diagnosis and assessment of individuals at risk of developing OSCC.

Need of the study: Immunotherapy is an emerging treatment modality in some cancers. Thus, better understanding of CTLA4 methylation role in OSCC paves a way for newer strategies in immunotherapeutic.

Aim: To assess the DNA methylation pattern of promoter region of CTLA4 gene and evaluate its expression in tobacco users with and without OSCC so that it can contribute as biomarker for diagnosis and prognosis of OSCC.

Materials and Methods: The proposed longitudinal cross-sectional study will be conducted at Sharad Pawar Dental College and Hospital, Maharashtra, India. It will evaluate promoter methylation and protein expression of CTLA4 gene in Smokeless Tobacco (SLT) users. There will be three groups: group A OSCC patients; group B normal individuals with SLT habit without any oral lesion; and group C normal individuals without SLT habit. Formalin Fixed Paraffin Embedded (FFPE) tissue blocks will be prepared from the biopsy obtained from total thirty nine participants. DNA methylation of CTLA4 promoter will be assessed using Methylation-Specific PCR (MS-PCR). In addition, the quantitative expression of CTLA4 will also be assessed using Immunohistochemistry (IHC).

Keywords: Carcinogenesis, Cytotoxic T lymphocyte associated protein 4, Epigenetics, Hypomethylation, Immune checkpoints

INTRODUCTION

There has been a surge in morbidity and mortality due to OSCC worldwide since last few decades. Majority of these cases are from South-east Asian continent [1]. Though, there is an easy access to oral cavity still most of the cases remain undiagnosed and later present with an advanced lesion. This can be attributed due to lack of awareness and easy accessibility to tobacco and associated products. Hence, there is an urgent and unmet need for early diagnostic, as well as, prognostic biomarkers. Initially, environmental factors and genetic factors were thought to play independent role in carcinogenesis. But recent evidences states that both these factors are linked through epigenetics [2]. Epigenetics is an upcoming research field as the genes have the advantage to reverse back to normal state. Moresoever, unlike genetic changes it does not cause change in the DNA sequence.

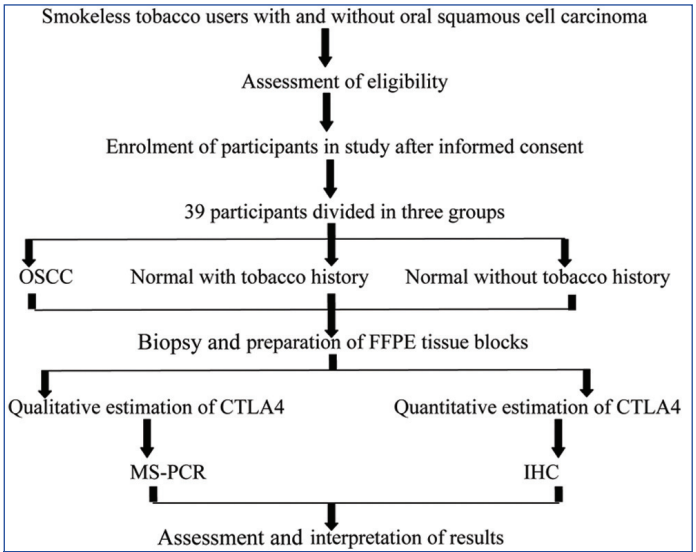
The OSCC formation and progression is interplay between stromal cells, cancer cells and host defense mechanisms [3]. The crosstalk between stromal cells and cancer cells causing inhibition of various tumour suppressor genes and activation of oncogenes has been well-documented. However, the immune defense mechanism elicited by host and tumour cells is now classified as hallmark of cancers [3]. OSCC is characterised by an immunosuppressive state, with scarce Natural Killer (NK) cells, lymphocytes and specific antigens as compared to normal tissues. The immune system maintains equilibrium between immune recognition and tumour development with a dual capacity to either promote or suppress tumour growth [4]. The basic mechanism in patients with OSCC is the escape of cancer cells through immune checkpoints [5]. Immune checkpoint molecules include: PD-1(Programmed Death 1), PD-L1 (Programmed Death Ligand 1), PD-L2 (Programmed Death Ligand 2), CTLA-4 (Cytotoxic T-lymphocyte-associated Protein 4), TIM-3 (T-cell Immunoglobulin and Mucin domain containing-3), LAG-3 (Lymphocyte Activation Gene 3), IDO (Indoleamine 2, 3-Dioxygenase), VISTA (V-domain Ig Suppressor of T-cell Activation), TIGIT (T-cell Immunoreceptor with Ig and ITIM domains), B7-H4 {V-set domain containing T-cell activation inhibitor 1 (VTCN1/B7x/B7S1/B7 homologue 4)} and more [6-8]. These immune checkpoint molecules have been used as prognostic biomarker and predictive biomarker for therapeutic response in various cancers including OSCC [7]. Immune Checkpoint Blockade (ICB) therapy has gained tremendous importance recently as a major therapeutic modality for oral cancer cure. It blocks immune checkpoint pathways, thus, preventing tumour cells from evading immune control [9].

The CTLA4 provides a negative feedback signal to T-cells and thus plays a crucial role in immune regulation. CTLA-4+ cells contribute to Treg-mediated immunosuppression. It is transiently expressed on activated T-cells [10,11]. Recently, anti-tumour immune responses have been enhanced by using specific antibodies that block CTLA-4+ cells [12,13]. The current knowledge of fundamental biological role of CTLA4 promoter methylation in OSCC is limited. CTLA4 hypomethylation in OSCC might be a surrogate biomarker for the state of T-cells exhaustion. The role of tobacco use on methylation of CTLA4 gene holds potential to enhance our understanding of early oral carcinogenesis. Therefore, the present study would give insights into the actual role of immune checkpoint molecule CTLA4 promoter methylation in tobacco users with and without OSCC and can lead to emergence of early diagnostic and prognostic biomarker in OSCC. The objectives will be to assess the methylation

pattern in promoter region of CTLA4 gene using MS-PCR and to quantitatively assess the presence of CTLA4 cells in tissue sections using Immunohistochemistry (IHC).

MATERIALS AND METHODS

A longitudinal cross-sectional study design will be adopted for conducting the present study. The present study is a genetic study with primary focus on the promoter region of CTLA4 gene. The methodology will be carried out by using two techniques i.e., MS-PCR and IHC. The study design is presented in a flow diagram [Table/Fig-1]. The present study protocol has been reviewed by the Institutional Ethical Committee of Sharad Pawar Dental College and Hospital, DMIMS (DU), Sawangi, Maharashtra, India. An ethical approval was obtained (Ref. No. DMIMS(DU)/IEC/2017-18/6263). The present study protocol is registered in Research Registry with registration number 7545. Informed consent will be obtained from each participant.



[Table/Fig-1]: Flow diagram of study design.

The study will be conducted at the Sharad Pawar Dental College and Hospital, DMIMS (DU), Sawangi, Wardha, India. It is a tertiary centre offering various speciality services to patients. The present study will be being conducted by Department of Oral and Maxillofacial Pathology and Microbiology in collaboration with Department of Oral and Maxillofacial Surgery. The biopsy samples would be recruited from outpatients attending the Department of Oral and Maxillofacial Surgery. The samples will then be processed to Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks in the Department of and Maxillofacial Pathology and Microbiology for Immunohistochemical (IHC) analysis. Then, the FFPE tissue blocks will be sent to PAR Life Sciences and Research Pvt. Ltd., Tiruchirapalli, Tamil Nadu, India for methylation analysis.

The study group will include patients recently diagnosed clinically and histopathologically with OSCC and with the history of use of SLT. The control group will be further divided into two groups- one group will include participants who do not have any oral precancer or cancer but have the habit of SLT use (i.e., continuous use for atleast six months before being enrolled in the study) and other group of normal healthy individuals (without any habit of tobacco use in any form). All the patients consenting to participate will be include in the study, if they satisfy the inclusion/exclusion criteria as follows:

Inclusion criteria: Those with habit of using SLT in any form but without any oral precancer or cancer; those with habit of using SLT in any form with OSCC; those who are histopathologically diagnosed cases of OSCC; normal healthy individuals without any habit and without oral precancer or cancer. Both the genders will be included.

Exclusion criteria: Those known to have or had any other cancers; those who have completed treatment or partially treated for OSCC; those oral cancer patients with recurrence; those with any other inflammatory condition like sharp tooth, gingivitis, periodontitis, etc., in SLT users without disease and in normal healthy individuals; those who did not give consent.

The study will have three groups: Group A will consist of patients diagnosed histopathologically with OSCC, group B will consist of individuals who have the history of habit of SLT use but without any oral precancer or cancer and group C will include individuals without habit of SLT use and without oral precancer and cancer. The sample size is calculated using two proportion- hypothesis testing-large proportion-equal allocation method where the estimated risk difference is 0.5, power (1-β) of the study is 80% and α error is 5%. Hence, the total sample size is 39. Each group will have 13 participants. The participants will be recruited using a purposive sampling method.

Study Procedure

Tissue samples/biopsies will be obtained from all the participants. The tissue samples of control group will be obtained from those individuals who will approach the OPD for minor surgical procedures other than precancerous or cancerous lesions like disimpaction, crown lengthening procedures, etc. A 10% neutral buffered FFPE tissue blocks will be prepared. Two sections will be taken on two different slides. One 4 µm section will be cut serially and taken on 3-amino propyl tri-ethoxy silane coated slides for IHC to evaluate expression of CTLA4 antigens and another section on plain glass slide for routine Haematoxylin and Eosin (H&E) staining. The FFPE tissue block will then be sent to PAR Life Sciences and Research Pvt. Ltd., Tiruchirapalli laboratory for methylation analysis. Hence, the present study will have two modes of intervention and assessment-methylation analysis and IHC analysis.

Methylation protocol:

- **DNA extraction and modification:** Genomic DNA will be extracted from FFPE tissue blocks using QIAamp® DNA FFPE Tissue Kit (Qiagen, USA) as per the manufacturer protocol. Quality and quantity of DNA will be estimated on standard agarose gel method with ethidium bromide [14].
- **Methylation Specific PCR (MSP):** DNA methylation assay will be performed using methylated and unmethylated primers of CTLA4 promoter sequence as reported by Mousavia M and Tajoddinib S in their study [15]. Primers sequences and annealing temperatures are listed in [Table/Fig-2]. Each 25 µL Polymerase Chain Reaction (PCR) reaction will contain: double distilled water- 15 µL, buffer- 2.5 µL, Hot Start Taq- 0.5 µL (GCC Biotech, India) cat#G7120A, dNTP mix- 1 µL, of MgCl2- 2.5 µL, DNA- 1.5 µL (80 ng), and each primer- 1 µL. The MS-PCR amplification program will be set as follows: 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 seconds, the variable step of cycle will be 52°C for methylated primer and 50°C for unmethylated primer annealing for 30 seconds and extension step at 72°C for 30 seconds. The last extension will be completed by incubation at 72°C for 10 minutes. Finally, 10 µL of each PCR product will be loaded in 2% agarose gel with ethidium bromide, and visualised under ultraviolet illumination [15].

Product	Sequences (5'→3')	Annealing temperature (°C)	Amplicon size (bp)
CTLA4-U	F:GAGATTAGTTTGGTTAATATGGTGA R:CCCAAATTAAAATACAATAACACAAT	50	183
CTLA4-M	F:GAGATTAGTTTGGTTAATATGGCGA R:CCAAATTAAAATACAATAACGCGAT	52	182

[Table/Fig-2]: CTLA4 promoter primer sequences and annealing temperatures.

Immunohistochemical (IHC) protocol:

- **Immunohistochemical (IHC) staining:** The universal immunoenzyme polymer method will be used [16]. The tissue sections will be deparaffinised with xylene and rehydrated through descending grades of ethanol prior to IHC. For antigen retreival the slides will be heated in microwave oven for 12 minutes in 0.01 M sodium citrate buffer (pH 6.0) and bench cooled for 20 min. Endogenous peroxidise activity will be blocked by incubating the section with 3% H_2O_2 in methanol for 30 minutes [16]. Then the sections will be washed three times by gentle shaking in Tris-Buffered Saline (TBS) for five minutes. The tissue section will be incubated with prediluted CTLA4 antibody {CTLA4 mouse monoclonal antibody (Clone ID:UMAB249), OriGene Technologies, US} at room temperature in humidifying chamber for 60 min and then at 4°C overnight [16] followed by incubating with secondary antibody {PolyExcel HRP/DAB detection system, PathnSitu (Cat#PEH002), India} at room temperature in humidifying chamber for 30 minutes. Tonsillar tissue will be used as positive control. For negative control, the primary antibody will be omitted from one section in every batch and incubated with TBS/serum. A freshly prepared chromogen solution of 3'3- Diaminobenzidine (DAB) will give a coloured precipitate at tissue antigen site and the sections will be counterstained in Harris's haematoxylin to visualise the antigen-antibody reaction. The sections will then be dehydrated, cleared and mounted. Finally, the sections will be examined by conventional light microscope (Leica DMLB2) at 400x magnification.
- **Immunohistochemical (IHC) assessment:** The immunohistochemically stained sections will be assessed quantitatively for the expression of CTLA4 protein. All the IHC-stained slides will be independently evaluated by two investigators in a blinded manner. The results will be recorded based on the intensity of the staining reaction on the cytoplasm/cell membrane, as well as, the percentage of positive tumour cells [16].

A study specific variables and data sources along with their assessment methods are as mentioned below:

- Independent variable: SLT use- A detailed case history will be taken with emphasis on types (ghutka, betel quid with tobacco, khaini, pan masala with tobacco, oral application), total duration, amount, frequency/day, placement-quadrant/overall, duration of chewing, etc.
- Dependent variable: CTLA4 methylation- The DNA methylation of promoter region of CTLA4 gene will be assessed by MSP. The CTLA4 methylation pattern shall be correlated with the expression of CTLA4 protein in the tissue section demonstrated by IHC.
- Outcome variable: There is an association between exposure of interest and the disease outcome. Hence, the outcome variable for the present study is OSCC. This outcome is due to exposure to SLT.
- Socio-demographic factors: Age, sex, religion, marital status, socioeconomic status, education, occupation, etc., will be documented in a detailed case history proforma.
- Confounding factors: These could be any other habit like alcohol, betel nut chewing, etc.; individual genetic susceptibility, any undiagnosed systemic/local condition and unknown. Adjustment for possible confounders will be done.

Expected Outcome

The results expected from methylation analysis are hypomethylation of CTLA4 promoter gene in almost all cases of group A (OSCC cases) and group B and none of group C. The results expected from IHC analysis are increased expression of CTLA4 positive cells in descending order in group A, group B and group C.

STATISTICAL ANALYSIS

It will be performed using the predictive analytics software Statistical Package for the Social Sciences (SPSS), version 16.0. Descriptive statistical methods shall be used depending on categorical or continuous variables (percentages, frequencies, means and standard deviations). Repeated measures one-way Analysis of Variance (ANOVA) (with Post-hoc as Bonferroni correction) shall be used to compare the continuous variables among the groups. Chi-square test or Fisher's-Exact test, as appropriate shall be used to analyse categorical variables. Pearson's/Kendall's Tau correlation coefficients and relevant partial correlation coefficients shall be calculated for assessing correlation. A p-value of <0.05 will be considered statistically significant. There is a possibility of gender bias and age bias that shall be removed by regression analysis.

REVIEW OF LITERATURE

The global tobacco epidemic has been the leading cause of oral cancer. The oral cavity is a persistent site for microbial, immunologic, carcinogenic, and clinical effects of tobacco use. In South-east Asian countries, there is more frequent use of SLT than smoked tobacco. In India, 21.4% is used in the form of SLT out of estimated 28.6% tobacco use [17]. SLT use is associated with impaired immunity. Moreover, oral cancer especially OSCC is characterised by defective immunological state with tumour cells escaping the inhibitory T-cells "immune checkpoints" mediated by CTLA4 and others. These immune checkpoints are epigenetically controlled. DNA methylation of immune checkpoints is linked to certain immunological processes, such as T-cell differentiation and T-cell exhaustion [18]. Furthermore; prior study investigating HNSCC has suggested immune checkpoint methylation as a prognostic or predictive biomarker [19]. DNA methylation is a stable molecular alteration of DNA, that occurs early and most commonly in cancer, its easy to detect in small amounts and is known to occur in response to environmental factors such as use of tobacco, etc. DNA methylation is also known to regulate messenger Ribonucleic Acid (mRNA) expression of several genes. Promoter methylation and gene body methylation are often associated with a decrease and increase in mRNA expression, respectively [20]. However, certain studies assessing the prognostic value of the inhibitory CTLA-4 showed inverse relation. Jones PA and Basu B et al., in their studies found better overall survival of patients with high CTLA-4 expression which they attributed to higher immune infiltration of tumours and it being a measure for immune infiltration [19,21].

CONCLUSION(S)

Although, the function of CTLA-4 is well studied, limited knowledge exists regarding its epigenetic regulation in association with tobacco use. There also exists inadequate data related to the genotoxic effects of SLT use in individuals without oral cancer or precancer. These genotoxic agents might act synergistically with epigenetic machinery towards oral cancer pathogenesis. Thus, the present study can pave a way in the our understanding of immunological effects in oral carcinogenesis. Also, CTLA4 promoter methylation patterns in tobacco users can serve as a biomarker for state of T-cells exhaustion and thus be helpful as an early diagnostic marker to identify individuals at risk of developing oral cancer. Moreover, the blockade of CTLA4 pathway can be considered for clinical trials in OSCC patients wherein, it can predict the response to anti-CTLA4-immunotherapy and/or prognosis. The findings of CTLA4 promoter hypomethylation in tobacco users with and without OSCC thus, hold a therapeutic potential.

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