COMPARATIVE ANALYSIS OF CD57 AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA - AN IMMUNOHISTOCHEMICAL STUDY

DISSERTATION

Submitted to The Tamil Nadu Dr. M.G.R Medical University in partial fulfillment of the requirement for the degree of

MASTER OF DENTAL SURGERY

BRANCH - VI

ORAL PATHOLOGY AND MICROBIOLOGY

2016 - 2019

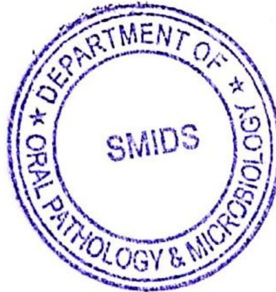
CERTIFICATE

Certified that the dissertation entitled: ***"Comparative analysis of CD57 and proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - An immunohistochemical study"*** is a bonafide record of the work done by **Dr. CS. Ani Simila** under our guidance during her post graduate study during the period of 2016-2019 under THE TAMIL NADU DR. M.G.R MEDICAL UNIVERSITY, CHENNAI, in partial fulfilment for the degree of MASTER OF DENTAL SURGERY IN ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH -VI. It has not been submitted (partial or full) for the award of any other degree or diploma.

Guide

Dr. T. ISAAC JOSEPH

Professor and Head



Co-Guide

Dr. GIRISH. KL

Professor

Department of Oral Pathology and Microbiology

Sree Mookambika Institute of Dental Science

Kulasekharam, Kanya Kumari District-629161

URKUND

Urkund Analysis Result

Analysed Document: Ani Plagiarism File - NEW.docx (D45701078)
Submitted: 12/13/2018 6:01:00 PM
Submitted By: anisimila.184@gmail.com
Significance: 3 %

Sources included in the report:

Dissertation-Dr tanvi Patel-Oral Pathology-2016-19.pdf (D44731025)
SWETHA THESIS.pdf (D45622452)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4525605/
http://repository-tnmgrmu.ac.in/9835/1/260426818sharifa.pdf

Instances where selected sources appear:

13

CERTIFICATE II

This is to certify that this dissertation work titled ***"Comparative analysis of CD57 and proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - An immunohistochemical study"*** of the candidate **Dr. CS. Ani Simila** with registration Number **241621301** for the award of **Master of Dental Surgery** in the branch of Oral Pathology and Microbiology [Branch –VI]. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **3%** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

Dr. T. Isaac Joseph M.D.S.
Professor & Head
Dept. of Oral Pathology & Microbiology
Sree Mookambika Institute of Dental Science
Kulasekharam - 629 161

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES, KULASEKHARAM

ENDORSEMENT BY THE PRINCIPAL / HEAD OF THE INSTITUTION

This is to certify that this dissertation titled ***"Comparative analysis of CD57 and proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - an immunohistochemical study"*** is a bonafide research work done by **Dr. CS. Ani Simila** under the guidance of **Dr. T. Isaac Joseph** M.D.S, Professor and Head, Department of Oral Pathology and Microbiology, Sree Mookambika Institute of Dental Sciences, Kulasekharam.

31/12/18

Dr. Elizabeth Koshi M.D.S.
Principal

Dr. Elizabeth Koshi MDS,
PRINCIPAL,
Sree Mookambika Institute of Dental Sciences.
V.P.M Hospital Complex,
Padanilam, Kulasekharam,
Kanyakumari District,
Tamil Nadu - 629 161

DECLARATION

I hereby declare that this dissertation titled **"*Comparative analysis of CD57 and proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - An immunohistochemical study*"** is a bonafide record of work undertaken by me and that this thesis or a part of it has not been presented earlier for the award of any degree, diploma, fellowship or similar title of recognition.

Dr. CS. Ani Simila
MDS Student,
Department of Oral Pathology and Microbiology,
Sree Mookambika Institute of Dental Sciences,
Kulasekharam, Kanyakumari District,
Tamilnadu.

ACKNOWLEDGEMENTS

"Good teachers are the reason why ordinary students dream to do extraordinary things.''

The success and final outcome of my dissertation required a lot of guidance and assistance from many people and I am extremely privileged to thank all my teachers at this moment who have been with me for the successful completion of my dissertation.

Thank you almighty for always guiding me in the right direction and for knowing what is best for me. Thank you god for all your blessings to me and my family, strength you give me each day and for all the people around me who make my life more meaningful.

I, respect and thank my Chairman **Dr. Velayuthan Nair** M.B.B.S., M.S. and my Director **Dr. Rema V. Nair** M.B.B.S.,M.D,D.G.O., for providing an opportunity to do my thesis work in our laboratory and for the access to the facilities available that made to complete my dissertation work on time.

I extend my thanks to my Principal, **Dr. Elizabeth Koshi** for her valuable comments, motivational words and encouragements.

I would like to express my sincere gratitude to my mentor and guide**, Dr. T. Isaac Joseph (Head of the Department)**, for his patience, motivation, cooperation, valuable suggestions and immense knowledge. With due respect, I thank him for giving me his guidance and support which made me to finish my thesis successfully.

I owe my special thanks to my Co-guide **Dr. Girish. KL (Professor)** for his valuable comments and encouragement during my thesis work, which motivated me to widen my research knowledge from various perspectives. His guidance and dedication have helped me in all the time of my research and during the writing of my thesis which is unforgettable.

I am thankful to my Professors **Dr. T. Prasanth** and **Dr. Geetha Varghese** and fortunate enough to get constant encouragement, support and guidance from them.

I would like to convey my gratefulness to the former Reader, **Dr. Pradeesh Sathyan**, who took keen interest on my dissertation work and for providing all the necessary information for developing a good dissertation.

I am thankful to my Senior lecturer **Dr. Angelin D,** for being with me in times of joy and despair. I cordially thank her for the love and support, she showered on me and always. I would not forget to remember the former senior lecturer **Dr. Deepa AG** for her kind support.

I would like to thank my senior lecturers **Dr. Jeslin Mary** and **Dr. Vidya S** for their valuable suggestions and encouragement.

I am thankful to my seniors **Dr. Akhil S, Dr. Aldrin Jerry** for their support and guidance. I thank my other colleagues **Dr. Ashitha. AS.** and my fellow postgraduate **Dr. Swetha D** for their suggestions and cooperation that pushed me to focus on my challenges. I also thank my juniors **Dr. Abilasha JV, Dr. Rajalekshmi MP, Dr. Jeya Priya S and Dr. Veena A** for their kind help and support.

I would like to thank **Mr. Sarath Babu K** for his contribution on the statistical analytical part of my study. I, personally thank my laboratory assistant **Mrs. Ringle Kripa** for assisting me in all my research work.

I heartily thank my Parents **Mr. Charles Stephen MC** and **Mrs. Michealammal C,** who have provided moral and emotional support in my life. I am also grateful to my other family members and friends who have supported me all along the way.

Thank you once again for your great support in the successful completion of my dissertation.

CONTENTS

S.NO	Index	Page No
1.	List of Abbreviations	i-ii
2.	List of Tables	iii
3.	List of Graphs	iv
4.	List of Colour Plates	v-vi
5.	List of Annexures	vii
6.	Abstract	viii-ix
7.	Introduction	1-4
8.	Aims and Objectives	5
9.	Review of Literature	6-43
10.	Materials and Methods	44-53
11.	Results and Observations	54-63
12.	Discussion	64-69
13	Summary and Conclusion	70-71
14.	Bibliography	x-xx
15.	Annexure	

LIST OF ABBREVATIONS

ADH	Alcohol Dehydrogenase
ALDH	Aldehyde Dehydrogenase
B3GAT1	Galactosylgalactosylxylosyl protein 3-beta-glucuronosyl Transferase 1
CD57	Cluster of Differentiation 57
CDK	Cyclin Dependant Kinase
CXCL	C-X-C motif ligand
CD8+ T CELLS	Killer or Cytotoxic T lymphocyte
DNA	Deoxy Ribonucleic Acid
DPX	Distrene Polystyrene Xylene
EGFR	Epidermal Growth Factor Receptor
EGF	Epidermal Growth Factor
FPG-PET	Fluorodeoxyglucose-Positron emission tomography
GDP	Guanosine Diphosphate
GTP	Guanosine Tri phosphate
GM-CSF	Granulocyte Macrophage –Colony Stimulating Factor.
HPV	Human Papilloma Virus
HNK-1	Human Natural Killer 1
H&E	Haematoxylin & Eosin
IARC	International Agency for Research on Cancer.
IDCL	Interdomain Connecting Loop
IHC	Immunohistochemistry
IL-12	Interleukin 12

IFN	Interferon gamma
LOH	Loss of Heterozygosity
MDSQCC	Moderately Differentiated Squamous Cell Carcinoma
MHC1	Major Histocompatibility Complex 1
MIP 1a	Macrophage Inflammation Protein1a
NK Cells	Natural Killer Cells
NKG2D	Natural Killer Group 2D
OSCC	Oral Squamous Cell Carcinoma
OCT	Optical Coherence Tomography
PCNA	Proliferating Cell Nuclear Antigen
PDSQCC	Poorly Differentiated Squamous Cell Carcinoma
PRb	Retinoblastoma Gene
ROS	Reactive Oxygen Species
RANTES	Regulated on Activation Normal T cells Expressed and Secreted
S phase	Synthetic Phase
TNF-α	Tumor Necrosis Factor –alpha
TNFR1	Tumour Necrosis Factor Receptor 1
TNFRSF	Tumour Necrosis Factor Receptor Super Family.
Th1 CD4+	Th1 subset of Helper T cells
WDSQCC	Well Differentiated Squamous Cell Carcinoma
WHO	World Health Organisation

LIST OF TABLES

TABLE NO	TITLE
Table 1	Mean labelling index of PCNA in different groups
Table 2	Comparison of mean labelling index of PCNA in normal mucosa with other groups
Table 3	Comparison of mean labelling index of PCNA in WDSQCC with other groups
Table 4	Comparison of mean labelling index of PCNA in MDSQCC with other groups
Table 5	Comparison of mean labelling index of PCNA in PDSQCC with other groups
Table 6	Multiple Comparison of mean labelling index of PCNA between the groups
Table 7	Mean labelling index of CD57 in different groups
Table 8	Comparison of mean labelling index of CD57 in normal mucosa with other groups
Table 9	Comparison of mean labelling index of CD57 in WDSQCC with other groups
Table 10	Comparison of mean labelling index of CD57 in MDSQCC with other groups
Table 11	Comparison of mean labelling index of CD57 in PDSQCC with other groups
Table 12	Multiple Comparison of mean labelling index of CD57 between the groups
Table 13	Correlation of PCNA labelling index with CD57 labelling index within the groups
Table 14	Correlation of PCNA labelling index with CD57 labelling index between the groups

LIST OF GRAPHS

GRAPH NO	TITLE
Graph 1	Mean age distribution in different grades of OSCC
Graph 2	Ratio of sex in different grades of OSCC
Graph 3	Comparison of mean labelling index of PCNA between the groups
Graph 4	Comparison of mean labelling index of CD57 between the groups
Graph 5	Comparative correlation of PCNA labelling index with CD57 labelling index within the groups
Graph 6	Correlation of PCNA labelling index with CD57 labelling index between the groups

LIST OF COLOUR PLATES

CP 1	Storage cabinet for archival blocks
CP 2	Semi-automatic microtome.
CP 3	Tissue floatation water bath.
CP 4	Slide warming table
CP 5	pH meter
CP 6	Digital weighing machine
CP 7	Reagents for preparation of buffer solutions
CP 8	Pressure cooker for antigen retrieval
CP 9	Primary antibodies - anti PCNA & anti CD 57
CP 10	Reagents used in immunohistochemical procedures
CP 11	Photomicrograph showing histology of normal mucosa (H&E staining; x100)
CP 12	Photomicrograph showing expression of PCNA in normal mucosa (IHC staining; x400)
CP 13	Photomicrograph showing expression of CD57 in normal mucosa (IHC staining; x400)
CP 14	Photomicrograph showing histopathology of WDSQCC (H&E staining; x100)
CP 15	Photomicrograph showing expression of PCNA in WDSQCC (IHC staining; x400)
CP 16	Photomicrograph showing expression of CD57 in WDSQCC (IHC staining; x400)
CP 17	Photomicrograph showing histopathology of MDSQCC (H&E staining; x100)

CP 18	Photomicrograph showing expression of PCNA in MDSQCC (IHC staining; x400)
CP 19	Photomicrograph showing expression of CD57 in MDSQCC (IHC staining; x400)
CP 20	Photomicrograph showing histopathology of PDSQCC (H&E staining; x100)
CP 21	Photomicrograph showing expression of PCNA in PDSQCC (IHC staining; x400)
CP 22	Photomicrograph showing expression of CD57 in PDSQCC (IHC staining; x400)

LIST OF ANNEXURES

Annexure No	Contents
I	Research Committee Certificate
II	Ethical Committee Certificate
III	Case record sheet
IV	Data entry sheet
V	Data analysis sheet

ABSTRACT

ABSTRACT

BACKGROUND:

Natural killer cells play an important role in the innate and adaptive immune system. The immune defense against tumour cells is mainly mediated by the natural killer cells. Cluster of differentiation 57 is a 110-kd glycoprotein, typically expressed by the natural killer cells, attack the cancer cells and inhibit the tumour development. Proliferating cell nuclear antigen (PCNA) is a 36 kd auxiliary protein for DNA polymerase delta, located on chromosome 20p12 correlates with the cell proliferation and DNA synthesis. This PCNA protein varies during cell cycle and accumulates in late G and S phase of the cell cycle. PCNA is essential component of the DNA replication, DNA recombination and repair.

AIMS AND OBJECTIVE:

To compare and correlate the expression of CD57 and PCNA in different grades of oral squamous cell carcinoma by immunohistochemistry.

MATERIALS AND METHODS:

Previously histopathologically confirmed 30 samples of different grades of oral squamous cell carcinomas and 10 samples of normal mucosa were included in this retrospective study. The histopathological sections were examined immunohistochemically for CD57 and PCNA expression. The statistical analysis was done by Anova (Post hoc) followed by Dunnet t-test and correlation between the markers was done by Pearson correlation test.

RESULTS

The CD57 expression in oral squamous cell carcinoma was found to be higher in well differentiated squamous cell carcinoma and lower in poorly differentiated squamous cell carcinoma whereas expression of PCNA was found to be lower in well differentiated squamous cell carcinoma and higher in poorly differentiated squamous cell carcinoma.

CONCLUSION:

CD57 expression was found to be decreasing from well differentiated squamous cell carcinoma to poorly differentiated squamous cell carcinoma. On the other hand, proliferative activity was found to be increasing from well differentiated squamous cell carcinoma to poorly differentiated squamous cell carcinoma. Therefore, the combination of CD57 and PCNA biomarkers appears to be good indicators of the immune status of the patient and the aggressiveness of the lesion.

Keywords: CD57, Proliferating cell nuclear antigen, Immunohistochemistry, Oral squamous cell carcinoma

INTRODUCTION

INTRODUCTION

Cancer is a multistep process with multifactorial etiology that involves initiation, promotion and progression of tumour[1]. At present, cancer is the second most common cause of morbidity and mortality in the world next to the cardiovascular diseases[2]. According to GLOBOCAN 2012, annually about 14.1 million new cancer cases and 8.2 million cancer-related deaths occurs worldwide[3]. In India, as per the study conducted by ICMR in 2016, 14.5 lakhs of new cases are added annually and about 7.3 6 lakh people die of these deadly diseases every year[4]. Oral cancer ranks as the sixth most common cancer in the world[2]. In India, oral cancer and breast cancer are the cancers that rank first in males and females respectively[4]. About 90% of the oral cancer reported is of oral squamous cell carcinoma.

Multicellular organisms can sustain their life only when all the cells function in accordance with the rules that regulate the cell growth and reproduction[5]. The cell numbers are maintained by controlling the rate of cell division as well as death of the cells. Thus, mitosis and apoptosis maintain the normal homeostasis of the body. Dysregulation of these mechanisms, may lead to either increase or decrease in the cell number. Hence, cancer develops as a disease of uncontrolled growth and proliferation whereby the cells escape the normal growth, reproductive control mechanisms and undergo limitless cell proliferation[6].

For a normal cell to acquire abnormal function, it requires genetic alterations over a longer period of time. These alterations may be due to inherited

mutations or by certain environmental factors such as UV rays, X rays, chemicals, tobacco products, virus[7]. The development of cancer is a multistep process that requires around four to seven events for the genetic changes to occur. These genetic alterations involve the classic hallmarks of malignancy and undergo immortalization[7]. Thus, cancer is referred as a disease of multistep process in which the cells undergo metabolic and behavioral changes, leading them to proliferate in an extensive and ultimate way[7].

Normal control systems prevent the tumour cells to undergo cellular proliferation and differentiation. When these control mechanisms gets altered, they undergo cellular proliferation that leads to uncontrolled cell growth even in the presence of the signals that normally inhibit cell growth and division[5]. These cancer cells acquire newer characteristics such as changes in cell structure, decreased cell adhesion, loss of normal architecture, breakdown of tissue boundaries, stromal changes, angiogenesis and invasion and metastasis[8]. Because of these newer characteristics, the cells exhibit uncontrolled proliferation and spreads rapidly resulting in invasion and metastasis. These abnormalities in the cancer cells are due to mutations in the proto oncogenes, tumour suppressor genes and DNA repair genes[5].

Regarding the incidence of the disease, there is a large amount of disparity exists between the developed and under developed countries. The incidence of the disease is higher in developed countries while the mortality rate is higher in under developed countries due to the lack of early diagnosis and the facilities of the treatment is poor in that countries[3]. In spite of the newer multi

therapeutic treatment modalities, the overall survival rate of oral squamous cell carcinoma is still questionable. Invariably, early detection of the disease provides better survival rate[6]. The development of molecular biological techniques may be helpful in detecting the early diagnosis of tumours and also to identify newer method to inhibit the growth of cancer that pave the way for more selective, less toxic, non-invasive forms of chemotherapy[9]. Biomarkers, that can act as indicators of the aggressiveness and recurrence of the lesion are useful in such case[5].

Natural killer cells (NK) play an important role in the innate and adaptive immune system. The immune defense against tumour cells is mainly mediated by the NK cells[2]. These cells detect and limit the development of the tumour directly without any priming or prior activation[9]. Natural killer cells identify the tumour cells by the lack of expression of major histocompatibility complex – I and secretes cytokines like interferon – gamma and tumour necrosis factor- α[2].

CD57 was identified on natural killer cells by using the mouse monoclonal antibodies Human Natural Killer-1 (HNK1). It was designated as cluster of differentiation, CD57 in 1989 by the fourth International Workshop of Human Leukocyte Antigens[10,11]. CD57 (cluster of differentiation 57) is a 110-kd glycoprotein which is typically expressed by the NK cells, attack the cancer cells and inhibit the tumour development[9].

Proliferating cell nuclear antigen (PCNA - Cyclin) is a 36 kd auxiliary protein for DNA polymerase delta, located on chromosome 20p12 correlates with the cell proliferation and DNA synthesis. This PCNA protein varies during cell cycle and accumulates in late G and S phase of the cell cycle[12]. This protein is

increased in the G1 and S phase and it is decreased in the G2 phase. Hence, this protein is a reliable indicator of cell proliferation[13]. PCNA is an essential component of the DNA replication, DNA recombination and repair.The malignant tissue is also characterized by an uncoordinated proliferation of this antigen namely, PCNA[14].

Since cellular proliferation and the presence of NK cells are important indicators for the aggressive nature and prognosis of the disease, these biomarkers are useful in predicting the aggressiveness of the disease as well as the immune status of the individual[12] and so this study aims to use CD57 and PCNA biomarkers to determine the immune status as well as aggressiveness or the biologic behavior of the disease thereby helping in planning of the treatment.

AIMS & OBJECTIVES

AIMS AND OBJECTIVES

AIM:

To compare and correlate the expression of CD57 and PCNA in different grades of oral squamous cell carcinoma by immunohistochemistry.

OBJECTIVES:

Primary Objective:

- Comparison and correlation of CD57 and PCNA expression in different grades of oral squamous cell carcinoma and also with that of control group.

Secondary Objective

- To evaluate the expression of CD57 and PCNA in different grades of oral squamous cell carcinoma

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Oral squamous cell carcinoma, also called as oral cancer is the utmost frequently occurring malignant tumour in the oral cavity.[15, 16]. Oral squamous cell carcinoma is defined as a malignant neoplasm of epithelial tissue with invasive behavior and variable degree of differentiation with or without keratinization[17]. Oral cancer is a multifactorial and a multistep process in which the cells undergo genetic mutation leading to proliferation of cells enormously and disregarding the rules of normal cell division[18]. Thus, oral cancer is the second most cause of mortality and morbidity in the world.

ANATOMY AND PHYSIOLOGY OF ORAL CAVITY:

The oral cavity arises from the lips externally and terminates to the junction of the hard and soft palate internally. It is surrounded anterolateraly by the gums, teeth and the alveolar arches of the jaws. The roof is formed by the hard palate and the soft palate while the floor of the oral cavity is formed by the tongue posteriorly and the sublingual region, anteriorly. Posteriorly, it communicates with the pharynx by the isthumus of fauces[19].

INCIDENCE AND PREVALENCE:

The incidence rate of oral cancer is highest in southern and Southeast Asian countries which constitutes a major health issues in these regions. According to WHO, oral cancer is considered to be the sixth most common cancer in males and in females, it is the tenth most common cancer in developing countries[20]. The international agency for research on cancer (IARC) in India estimated that the

incidence rate of oral cancer increases from 1 million in 2012 to more than 1 .7 million in 2035 and the death rate will also increases from 680000 in 2012 to 1-2 million in 2035[21].

According to the studies conducted by the urban and rural registries, oral cancer is more commonly seen in the northern part of India especially states like Uttar Pradesh because of the use of smokeless tobacco. Oral cancer is more common in western regions of the country among the males and east Khasi hills of Meghalaya records the highest number of oral cancer cases among females. Among the cancers about 30% accounts for the tobacco related cancers[21].

ETIOLOGY:

In India, the oral cancer is due to a number of etiological factors. Tobacco consumption either in the form of smoking and smokeless tobacco, alcohol consumptions, chewing betel quid with areca nut are the major risk factors in the pathogenesis of oral cancer in India. Viruses, immunosuppression, occupational risks, radiation, familial and genetic susceptibility are the other common causes of oral cancer.

Epigenetic factors

a. Tobacco

In India and other Asian countries, chewing tobacco with betel quid is the main causative agent in oral cancer. About 90 % of the oral cancers are caused by tobacco products. Various forms of tobacco are smokeless tobacco (Gutka, Paan, Zarda, Mawa, Kharra and Khaini), Betel quid, Cigarettes, Bidi & pipe smoking and Hookah) [22].

Tobacco smoke contains benzo-pyrene, tobacco- specific nitrosamines, arsenic and benzene. These products react with the DNA of keratinocytes and causes mutation in the DNA replication. In addition, tobacco pro-carcinogens are being metabolized by oxidizing enzymes like cytochrome P450 to promote the destruction and to counteract the protective effects of glutathione S- transferase, glutathione reductase and superoxide dismutase [23].

Marijuana, which is also called as bhang or ganja is used as smoking cigarettes. Tobacco which forms a part of the smoke contains potent carcinogen that predisposes to oral cancer formation [24].

Smokeless tobacco is used either as a dry snuff or wet snuff. Wet snuff is more common in north-western regions of India. Smokeless tobacco is kept inside the oral cavity where it contacts with the mucous membrane and the nicotine is absorbed by the mucosa which further leads to oral cancer [25].

b. Betel quid

In the Indian subcontinent, chewing betel quid is the most common and prevalent habit. Betel quid usually contains betel leaf, slaked lime, areca nut, and tobacco. Other ingredients such as cloves, cardamom or aniseed are also added to the quid in India and turmeric in Thailand[26]. Chewing betel nut alone is also said to be carcinogenic. The association of all these products develops precancerous lesions such as erythroplakia, leukoplakia, and oral submucous fibrosis. Betel quid chewing generates reactive oxygen species (ROS) and further induces the initiation of tumour [27].

c. Alcohol:

Alcohol are said to be carcinogenic in tumours of oral cavity, pharynx, larynx, oesophagus, and liver. Combination of alcohol and tobacco chewing possess an increased risk for oral cancer development than the usage of alcohol alone[26]. N-nitroso compounds, urethane, mycotoxins, inorganic arsenic are the carcinogens present in the alcohol. By the enzyme alcohol dehydrogenase (ADH), these substances are metabolized to acetaldehyde, which is then oxidized by means of aldehyde dehydrogenase (ALDH) to acetate. This acetaldehyde damages the DNA, interferes with the DNA synthesis and repair and thus initiates or promotes tumour formation. In oral mucosa, rapid oxidation of alcohol to acetaldehyde by ADH enzyme occurs and in contrast, acetaldehyde gets accumulated due to reduction in ALDH [aldehyde dehydrogenase] enzyme. Thus, genetic changes occur by these two enzymes that provides an increased risk of oral cancer [28].

Due to hepatic damage, the detoxification of carcinogenic substance like polycyclic aromatic hydrocarbons and N- nitrosamines are inhibited and thus leading to tumour formation. Chronic alcoholics with lack of nutrition are a contributory factor in the etiology of oral cancer[28].

d. Diet and nutrition:

According to IARC, reduced intake of fruits and vegetables may increases the risk for oral cancer. Increased intake of carrots, fresh tomatoes and green peppers reduces the risk of oral and pharyngeal cancer. The other food products like bread, legumes, cereals, vegetable oil, olive oil, fish, fresh meat, chicken, liver, shrimp and lobster have a protective effect against cancer[29]. Micro nutrients like vitamins A (retinol), vitamin E (alpha-tocopherol), vitamin C, carotenoids

(beta-carotene), selenium and potassium have antioxidant property. These anti-oxidants reduce the free radical reactions and minimize the genetic mutation[29]. Iron deficiency anemia and the Plummer-Vinson syndrome are associated with an increased risk of cancer on the upper respiratory and digestive tract[27].

e. Viral infections:

Highly oncogenic virus contains viral oncogenes in their genome. Viral oncogenes interact with the host genetic material and stimulate the proliferation of their host cells, immortalize the host cell facilitating oral carcinoma[26]. Human papilloma virus is the most common virus implicated in oral carcinogenesis. HPV are DNA viruses causing benign proliferative lesions such as papilloma, verruca vulgaris, condyloma accuminatum, and focal epithelial hyperplasia or Heck's disease. HPVs 16, 18, 31, 33, 35, and 39 are considered to be the highest risk types in oral cancer. Among them, HPV 16 has been identified in more than 90% of HPV positive oro-pharyngeal squamous cell carcinoma and oral cancer[30].

The primary mechanism involved in the pathogenesis of HPV related oral cancers are due to two major co-proteins such as E6 and E7. The E6 proteins binds to p53 and destroy p53 while E7 proteins binds to and destroy Retinoblastoma (pRb) gene respectively. This further disrupts the cell cycle with loss of control on DNA replication, repair, and apoptosis[31].

f. Fungal infections:

Studies showed that candidal species particularly candida albicans are known to cause oral premalignant lesions. Candida species are true causative agent or a secondary process in oral cancer is still in debate. In the immune compromised

individuals, candida infection coexist and provide synergistic action in the development of oral cancer[26].

g. Immunosuppression

Immunosuppressed individuals like HIV infected individuals; organ transplant patients are more prone to get oral squamous cell carcinoma[26].

h. Occupational risks:

Studies have showed that employees of certain occupations are more prone to develop oral and nasopharyngeal cancer. Evidence for increased risk for oral cancer are reported among metal workers, electrical workers, plumbers, machinists, painters and other individuals who are exposed to metal dusts[26].

i. Radiation:

Exposure to excessive UV radiation causes actinic chelitis which may transform into oral cancer. Exposure to X-ray radiation may cause chromosomal abnormalities. Low doses of ionizing radiation will not have any effects in our body. But each exposure to radiation builds up in our body and the risk of cancer increases [26].

j. Syphilis

Studies shown that syphilis have weak associations with the oral cancer. This can be due to the factor that the disease is detected and treated at an early stage[26].

k. Dental hygiene and other factors:

Poor oral hygiene, prolonged irritation of sharp teeth, dental sepsis are thought to be a causative factor in oral cancer. But some studies have shown that the oral cancer is due to the presence of coexisting risk factors like smoking and alcohol consumption. Several studies have shown that there is inverse

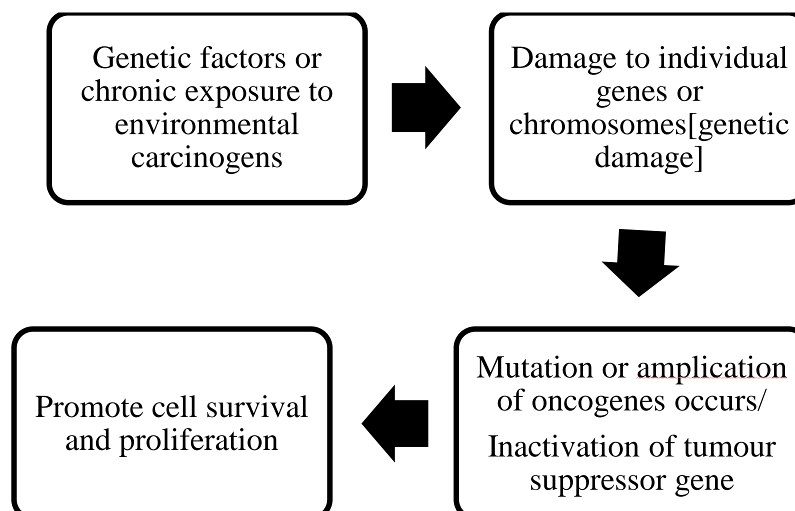
relationship between the oral hygiene and the occurrence of oral squamous cell carcinoma. Substantial evidence is needed to prove this hypothesis[26, 32].

l. Potentially malignant disorders:

Some precancerous lesions or precancerous condition can progress to oral squamous cell carcinoma. They are, [33]

- Leukoplakia
- Proliferative verrucous leukoplakia
- Erythroplakia
- Erythroleukoplakia (nodular or verrucous)
- Lichen planus (mainly the erosive and atrophic type)
- Submucous fibrosis
- Actinic cheilitis
- Sideropenic dysphagia (Plummer-Vinson syndrome)
- Discoid lupus erythematosis.
- Dyskeratosis congenital

PATHOPHYSIOLOGY IN ORAL SQUAMOUS CELL CARCINOMA



Molecular pathogenesis in oral squamous cell carcinoma:

Oral cancer develops through sequential events of genetic mutations followed by uncontrolled proliferation of cells. A normal cell require about 6-10 genetic events to become a cancerous cell. These alterations are due to inactivation of tumour suppressor genes and activation of oncogenes leading to uncontrolled proliferation of cells and cell death. Genetic alterations include point mutations, translocations, amplification and deletions. Point mutation is most commonly seen in p53 and K-ras. Gene amplifications and translocations are seen in malignant neoplasms[34, 35]. Recently, microsatellite analyses have shown that the allelic imbalance of chromosomal 9p is the most common mutation in head and neck squamous cell carcinoma [35].

The loss of heterozygosity (LOH) is a common genetic mechanism in cancer formation, whereby one allele is lost. This clearly indicates the absence of functional tumour suppressor gene in the lost region. The remaining recessive copy of the tumour suppressor gene can be inactivated by a point mutation, thereby leaving no tumor suppressor gene to protect the body. Loss of heterozygosity (LOH) was noted in the chromosome 9p21–p22 and in 72% of tumours. In precancerous lesions, allelic loss of 3p and 9p region that contain tumour suppressor genes are reported whereas LOH at chromosome 3p are seen more commonly in recurrent dysplastic lesions. Recent studies showed that about 77% of premalignant lesions having allelic loss defect, developed oral squamous cell carcinoma within five years[34].

Cyclin dependent kinase (CDK), cyclins and cyclin dependent kinase inhibitors are the regulators of cell cycle process. p16 is a protein that binds to

pRb [retinoblastoma protein] and inhibits the phosphorylation pRb with cyclin dependant kinase (CDK) inhibitors, CDK4 and CDK6. Mutation of the CDK inhibitors leads to dysregulation of p16 protein and this mutation is more commonly seen in oral premalignant lesions. Mutations of cyclin D, cyclin A, cyclin B lead to dysregulation of cell cycle process resulting in increased proliferation of cells[36].

a) Oncogenes:

Oncogenes are basically growth promoting genes and mutations in these genes either leads to increased function or overproduction of proteins. These oncogenes cause cellular alterations and are important initiators in the process of early changes of oral cancer. Several oncogenes are implicated in oral carcinogenesis[35]. They are listed as follows:

a. Abnormal expression of proto-oncogene epidermal growth factor receptor (EGFR)
b. Mutation of the c-myc oncogene
c. Mutation of the ras family
d. Bcl-1 gene
e. PRAD 1/cyclin D1 gene

In the early events of oral cancer, deregulation of growth factors and increased production of transforming growth factor-alpha (TGF-alpha) is seen. This TGF-alpha binds with the EGFR and stimulates cell proliferation. This TGF-alpha stimulates angiogenesis in later stage. Due to the increased production of TGF-alpha and epidermal growth factor (EGF), there will be

aberrant expression of EGFR. The interaction of TGF-alpha and EGF with the EGFR result in a cascade of events triggering the intrinsic pathway. Mutations of the EGFR send continuous growth stimulatory signals to the members of the ras oncogenes which is located inside the cell[37].

The ras onco-protein is present on the internal aspect of the cell membrane and transmits EGFR stimulatory signal to the nucleus. ras binds with guanosine diphosphate (GDP) and remains in the inactive state. When the cells are stimulated by the EGF and TGF-alpha, the ras oncogene gets activated. This activation is achieved by exchanging GDP for guanosine triphosphate (GTP). This activated ras protein will in-turn activates the raf protein, MEK, MAPK cytoplasmic kinases. Once the raf protein is activated by the enzyme guanosine triphosphatase (GTPase), the GTP gets hydrolyzed back to GDP. The active ras protein then return back to its inactive form. Thus the ras proteins remain active only for a limited period of time and regulate the cell proliferation. However, mutation of the ras protein, causes the ras gene to remain in its active form and sends continuous proliferative signals to the raf protein even without the binding of EGFR with the EGF[38].

c-myc gene induces both apoptosis and cell proliferation. c-myc induces apoptosis through tumour suppressor gene p53. Increased c-myc activates the transcription of cyclin D which further stimulates the cyclin-dependent kinase (CDK) enzyme. Active CDK inturn catalyzes the phosphorylation of the retinoblastoma tumour suppressor protein (pRb).Thus; on phosphorylation of retinoblastoma protein [pRb6], c-myc induces cell proliferation. Thus, c-myc

protein is overexpressed in all grades of OSCC due to gene amplification [34].The PRAD-1 gene is located on the chromosome 11q13 which encodes a protein called cyclin D. This cyclin D together with the Rb gene protein controls the G1 to S transition of the cell cycle. Amplification of PRAD-1 is associated with infiltrative growth pattern and metastases [39].

Normally bcl-2 is a protein that inhibits the apoptotic cell death. Tumour suppressor gene p53 blocks the activity of the bcl-2 gene by stimulating the transcription of Bax protein. Thus, the caspse-3 enzyme activity remains unchecked and apoptotic process occurs continuously. P53 suppress the bcl-2 gene transcription and further lead to apoptosis of cells associated with the DNA damage. In addition, hyperactive ras protein blocks the bcl -2 activity, thereby blocking the cell death and leading to uncontrolled proliferation [38].

b) Tumour suppressor genes:

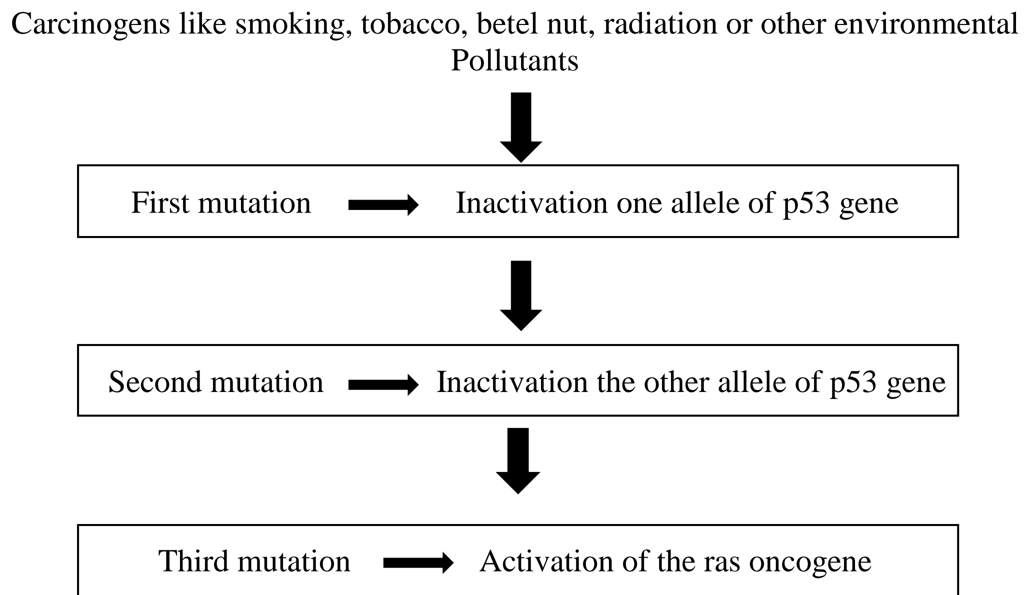
Tumour suppressor genes play a vital role in oral cancer. About 80% of the oral cancer is found to be associated with the mutation of tumour suppressor gene, p53[34, 38].

Normally, p53 protein detects the DNA damage and prevents the cells from entering into the cell cycle. Whenever, there is DNA damage there will be an increased expression of p53, in turn will stimulate the transcription of p21. This p21 gene inhibits the cyclin dependant kinase (CDK) enzyme and blocks the pRb phosphorylation which further prevents the release of E2F transcription factors and finally, DNA replication is blocked. Proliferating cell nuclear antigen (PCNA) is a protein which encircles and slides along the DNA. p21 protein binds

with the proliferating cell nuclear antigen (PCNA) and inhibit the replication of DNA Thus, p53 gene inhibits DNA replication via p21 and prevents DNA damaged cells from entering into the cell cycle [38].

Mutation of the p53 protein causes the DNA damaged cells to participate in the cell cycle and continue to divide and passing onto the next generation. Habits like smoking and tobacco chewing are associated with the p53 mutation in oral squamous cell carcinoma. The most common p53 gene mutation is the deletion of one allele accompanied by mutation of other allele [38].

As a summary, oral cancer results from the oral keratinocytes by three mutation of p53 gene which is depicted as a flow chart below:



c) Cell adhesion molecules:

E-cadherin is a cell adhesion molecule involved in invasion and metastasis. This protein is down-regulated in most of the oral cancers. Integrin is another cell adhesion molecule which mediates cell-cell adhesion and cell –

matrix interactions. Integrin plays a crucial role in the maintenance of tissue integrity, regulation of cell proliferation, differentiation and migration. Integrin expression is implicated in the tumour progression and metastasis[40].

In poorly differentiated squamous cell carcinoma, there is decreased expression of β1 integrin or α6β4 integrin suggesting that it is an early but nonspecific biomarker in oral malignancy. In metastatic oral carcinoma there is a strong expression of α2-6 integrins which significantly correlates with the mode of tumour invasion[41,42].

Metastasis is not an early event in carcinogenesis. However, because of the delay in diagnosis, invasion and metastasis are the major cause of morbidity. The alteration in the expression or in the function of the cell adhesion molecules indicates the process of tumour infiltration and metastasis[34].

TUMOUR IMMUNOLOGY:

Tumour immunology is defined as the branch of biology that describes the interaction of the host immune cells with the tumour cells and understanding the role of the immune system in the progression and the development of tumour[43]. The cells of the immune system identify and destroy the altered or the abnormal neoplastic cells thereby inhibiting the development of various tumours. Tumour immunology includes three main divisions

a. Immunosurveillance
b. Immunoediting
c. Immune evasion.

a) Tumour immunosurveillance:

The three primary roles of the immune system in the prevention of tumours are given below:

i. Elimination or inhibition of viral infection by host immune cells in viral induced tumours.
ii. Eradication of the pathogens and complete resolution of the inflammation will provide an unfavourable environment to carcinogenesis.
iii. Immune cells detect and destroy the tumour cells by the expression of tumour specific antigen. This process is known as immunosurveillance.

b) Tumour immunoediting:

It is a process by which the host immune cells reduce the immunogenicity of the primary tumour and control the tumour. Thus, cancer immunoediting process can promote complete eradication of tumours. This can be accomplished by 3E‘s of cancer immunoediting.[44] They are

i. Elimination
ii. Equilibrium
iii. Escape.

i. Elimination:

The growing tumour cells in the tissue cause local disruption of tissues which alerts the host immune cells. Because of this local disruption, remodelling of stromal cells occurs releasing the proinflammatory chemokines that attracts the immune cells to that particular site. The most important immune cells recruited are natural killer cells (NK) cells, T- lymphocyte and macrophages. Once these

cells are gathered towards the tumour site, natural killer innate immune cells identify the tumour cells through the lack of major histocompatibility complex-I (MHC-I) expression and release the cytotoxic granules like perforin or granzymes causing perforation of the target cells and subsequent cell death[45].

The tumour cells express tumour necrosis factor (TNF) receptor super family- like TRAIL receptors, Fas/CD95 and tumour necrosis factor receptor 1 (TNFRI). NK cells with their corresponding ligands bind with the tumour necrosis factor receptor super family (TNFRSF) receptors of cancer cells contributing to the cytotoxicity of the cells. In addition, NK cells secrete various cytokines and chemokines such as TNF, interferon-gamma (IFN-gamma), MIP-1a (Macrophage inflammatory protein-1a), Granulocyte-macrophage colony stimulating factor (GM-CSF), and RANTES (regulated upon activation, normal T cell expressed and secreted). Among the cytokines secreted, INF-gamma is considered to be crucial in priming the T-helper cells[46]. NK cells may also recognize developing tumours through the interaction T-cell receptor with either glycolipid-CD1 complexes or NKG2D ligands expressed on the tumour cells.[44].

Second step:

Interferon-gamma, released by the NK cells induces the production of CXCL (C-X-C motif ligand) -9,10 and 11 from the tumour cells as well as from the normal surrounding immune cells of the host, recruit more number of innate immune cells to the tumour site[47].

Interleukin -12 (IL-12) which is released by the macrophages, stimulate the NK cells to produce low amount of interferon-gamma. This interferon-gamma

further activates the macrophages to release IL-12 which in turn activates the NK cells to secrete interferon-gamma. Thus, this cyclic process gets repeated. NK cells receptor binds with the NKG2D ligands of the tumour cells and release more interferon-gamma. Interferon- gamma activates the process of killing the tumour cells by its anti-proliferative, proapoptotic and angiostatic property. Activated macrophages release oxygen and nitrogen metabolites that are cytotoxic to the cells [47].

Third step:

In the third step, the tumour antigens from the dead tumour cells activate the dendritic cells. These dendritic cells attracted to the tumour site and get activated by two mechanisms:

a. Innate immune cells secrete the cytokines and the cytokines attract the dendritic cell to that site.
b. By interaction with the NK cells

Activated dendritic cells act on the tumour cells either by direct or indirect mechanisms. Direct mechanisms involve ingestion of tumour cells directly. Indirect mechanisms involve the binding of the tumour antigen to the dendritic cell activates the tumour-specific Th1 CD4+ T-cells. These Th1 CD4+ T-cells in-turn activate the cytotoxic CD8+ lymphocytes [48].

Fourth step:

Activated Th1 CD4+ T cells and cytotoxic CD8+ lymphocytes involve in the process of killing the tumour cells. The Th1 CD4+ T cells produce interleukin-2 (IL-2) which along with IL-15 induces the action of cytotoxic CD8+

cells. Activated CD8+ lymphocyte kills the tumour cells either directly or it secretes interferon-gamma and promotes cell death.

Thus, this elimination process is a continuous process and it is repeated every time when the neoplastic cells arise. This is because of this reason tumour is more prevalent in old age where the immune status of the individual is low [47].

ii. Equilibrium:

The cells that have survived from the elimination phase enter into the equilibrium phase. This is the longest of all the three phases and occurs over many years. The original tumour cells are eliminated in the equilibrium phase but due to consequent mutations new tumour cells arise within the tumour site. This newly arisen tumour cells are highly resistant to the immune system of the host. Therefore, net result of the equilibrium phase is the newer population of tumour clones with reduced immunogenicity is seen[48].

iii. Escape:

Escape phase is the phase that represents the failure of the immune system to eliminate or control the transformed cells thereby allowing the tumour cells in an uncontrolled manner. Due to the genetic and epigenetic alterations, the tumour cells escape from both the innate and the adaptive immune system, thereby undergoing uncontrolled proliferation of cells [44].

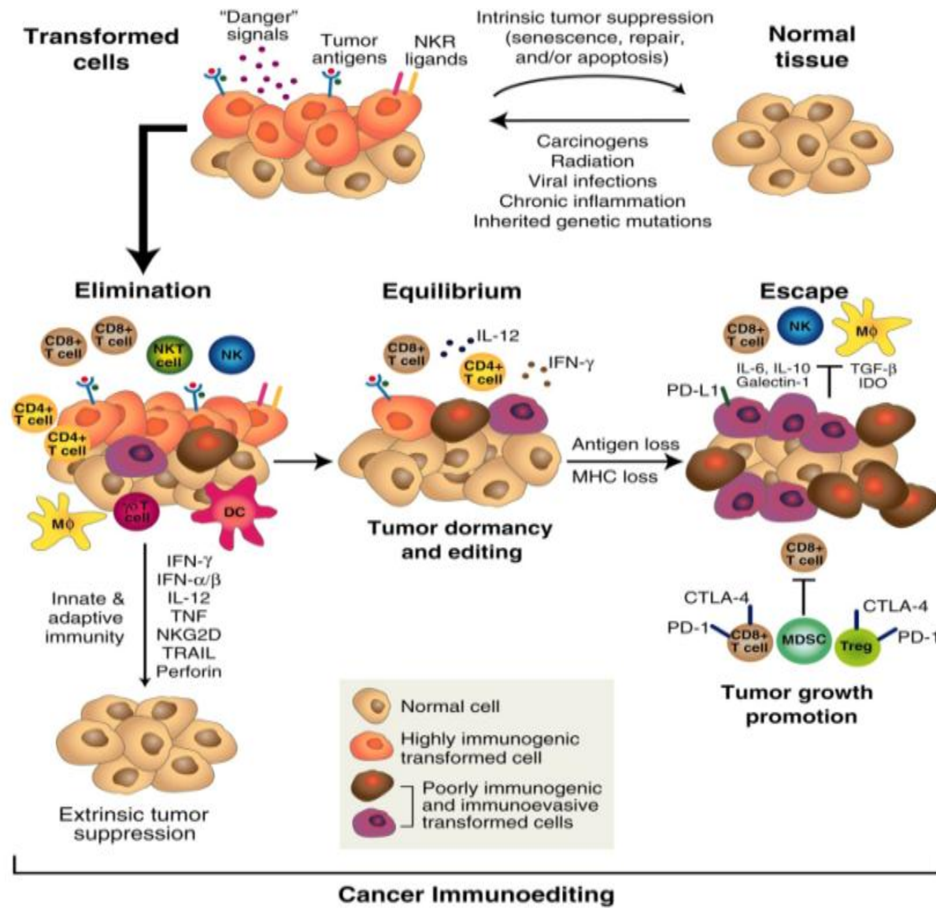


Figure 1: Three phases of cancer immunoediting

c) Immune evasion:

The ability of the tumour cells to evade the host immune response and is capable of uncontrollable growth thereby developing an antitumour response or inhibition of host immune functions. In case of an aggressive tumour, the aggressive tumour cells inhibits the functions of the immune cells such as T-lymphocytes, B-lymphocytes, macrophages, NK cells, Dendritic cells, granulocytes and mast cells. Thus the tumour cells hide from the immune system and avoid recognition of tumour cells [44].

CLINICAL FEATURES:

Oral squamous cell carcinoma apparently arising in normal mucosa are preceded by clinically visible potential malignant disorders like leukoplakia (white patch), erythroplakia (red patch), erythroleukoplakia (red and white patch), or verrucous leukoplakia. The appearances of these potential malignant lesions and the development of oral squamous cell carcinoma may ranges from 6 months to 39 years [49].

During the early growth of the lesion, there is minimal pain. Tongue and the floor of the mouth are the most common sites for the development of intraoral squamous cell carcinoma. Other sites of involvement are buccal mucosa, labial mucosa, gingiva, and hard palate. OSCC may manifest as the following:

- A white or mixed white and red lesion
- A red lesion (erythroplakia)
- An indurated lump/ulcer (ie, a firm infiltration beneath the mucosa)
- A granular ulcer with fissuring or raised exophytic margins
- A lesion fixed to deeper tissues or to overlying skin or mucosa
- A non-healing extraction socket
- Cervical lymph node enlargement, especially if hardness is present in a lymph node or fixation. Enlarged nodes in a patient with oral carcinoma may be caused by infection, reactive hyperplasia secondary to the tumor, or metastatic disease. Nodal enlargement is a feature particularly in oropharyngeal cancers[50].

HISTOLOGICAL GRADING OF ORAL SQUAMOUS CELL CARCINOMA:

Many years TNM clinical staging were used, but this system failed to clinically estimate the survival as well as the response to the therapy. It was Borders' in 1927 who introduced the quantitative grading of cancer based on the proportion of the neoplasm resembling the normal squamous epithelium. Many other researchers have also formulated the various grading systems to predict the biological behavior of oral squamous cell carcinoma. They are:

a. Border's system (1927)
b. Jakobbson et al (1973)
c. Fischer et al (1975)
d. Lund et al (1975)
e. Willen et al (1975)
f. Crissmann et al (1980)
g. Anneroth et al (1987)
h. Bryne'stumour invasive front grading system.(1989,1992)[51].

a) Broder's (1927) classification:

Accordingly, tumours were graded as follows:

Grade I : Well differentiated tumours - 75-100% of cells are differentiated

Grade II : Moderately differentiated tumours - 50-75% of cells are differentiated

Grade III : Poorly differentiated tumours - 25-50% of cells are differentiated

Grade IV : Anaplastic tumour - 0-25% of cells are differentiated[52].

DIAGNOSTIC TECHNIQUES OF ORAL SQUAMOUS CELL CARCINOMA:

The early identification of the diseases is of paramount important that reduce the mortality rate of the diseases. Diagnosis of oral squamous cell carcinoma is solely based on the expert clinical examination and histopathological examination of the affected area. However, tissue biopsy and histopathological examination remains a gold standard technique in the diagnosis of OSCC, the early identification of the disease is still remains a challenge. Early identification of the disease improves the survival rate as well as reduces the morbidity rate. In order to predict the early molecular changes, the introductions of protein biomarkers are of significant value [53].The most relevant techniques in the early diagnosis of the disease is listed below[54]:

VITALSTAINING	• 5% Acetic acid • Toluidine Blue • Methylene Blue • Lugol's Iodine • Rose Bengal • Iodine staining • Tolonium chloride
LIGHT BASED DETECTION SYSTEMS	• Tissue fluorescence imaging (Velscope, identafi 3000) • Chemiluminiscence (ViziLite plus, Microlux/DL) • Tissue fluorescence spectroscopy
HISTOLOGICAL TECHNIQUES	• Incisional biopsy • Excisional biopsy

CYTOLOGICALTECHNIQUES	• Oral Brush biopsy (Oral CDX) • Liquid Based Cytology • Laser Microdisection (LCMd)
MOLECULAR ANALYSES	• Gene alterations • Epigenetic alterations, loss of Heterozygosity and Microsatellite instability • Viral genome studies • Proliferation index and AgNOR Analysis • Immunohistochemical identification of tumour markers.
IMAGING TECHNIQUES	• FDG-PET [Fludeoxyglucose (FDG) molecule- • Optical Coherence Tomography (OCT)
OTHER TECHNIQUES	• Onco-chips

MOLECULAR ANALYSIS BY IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a technique for identifying the tissue or cellular antigens by means of antigen-antibody interactions. The introduction of prognostic and predictive biomarkers in immunohistochemistry has made a tremendous impact on early diagnosis as well as in the management of the lesion. This technique involves the utilization of specific tumour markers to detect whether a cancer is benign or malignant or to determine the stage and grade of a tumour or to identify the cell type and tumours of unknown histogenesis[55].

Role of tumour markers in oral squamous cell carcinoma:

When a cell becomes cancerous, newer antigens are expressed on the surface. These are called tumor antigens and the immune system of our body

identifies this antigen as foreign material and tries to destroy or eliminate these cancerous cells. Sensitive and reliable markers identify this antigen and provide information about the forthcoming behavior of an existing cancer. Henceforth, a tumour marker is defined as a substance present in or produced by a tumour or by the host immune response to the tumour that can be used to distinguish a tumour from normal tissue or to detect the presence of a tumour based on the measurement in the blood or secretions[56].

Criteria for an ideal tumour marker are[57]:

a. It should possess high sensitivity and specificity
b. It should possess high negative and positive predictive value
c. It should have the capability to distinguish the neoplastic and non-neoplastic disease
d. It should be able to predict prognosis and early recurrence of the lesion.
e. It should be able to identify the tumour at an early stage.
f. It should be easily assayable [57].

An ideal tumour marker should possess all these characteristics. The tumour markers are useful in various aspects that are listed below[56]:

a. Screening process for detecting early malignancy.
b. As a diagnostic aid for malignancy.
c. In determining the prognosis of the malignant lesion.
d. In the prediction for the efficacy of treatment.
e. In monitoring the therapy in advanced malignancy[56].

Recently, various tumour biomarkers are identified in the early detection and recurrence of oral squamous cell carcinoma. Some of those biomarkers are as follows[57]:

a. Albumin
b. Autoantibodies
c. Catalase
d. CD44
e. CD 59
f. Neural Wiskott-Aldrich syndrome protein (N-WASP)
g. Cofilin1[CFL1]
h. Cancer antigen 125[CA 125]
i. CYFRA21-1
j. Endothelins
k. Glutathione
l. Interleukin 1α,2β.IL-6,IL-8
m. Mac-2 binding protein
n. SCC antigen
o. S100 antigen
p. Tissue polypeptide antigen (TPA)
q. Tumour suppressor gene p53
r. Telomerase
s. Tumour necrosis factor-α (TNF-α)
t. Alpha amylase (α- amylase)
u. Cell proliferative markers (Cyclin A, B1& D, Proliferating cell nuclear antigen-PCNA, Ki-67)

The expressions of these biomarkers in OSCC are helpful in predicting the prognosis, patient survival and also with the grading and staging of tumour[57].

Role of CD 57 and PCNA in OSCC:

Cancer is a process of uncontrolled proliferation and the tumour growth is due to the reciprocal interactions of the tumour cells and the stroma in which they inhabitat. The infiltration of immune inflammatory cells into the tumour microenvironments either promote the progression of tumour or have an antitumor effect. Natural killer cells and cytotoxic T-lymphocytes (CD 8+) are two potent immune cells exhibiting the antitumor effect. Natural killer cell identify the tumour cells by the lack of expression of MHC-I (major histocompatibility complex) and kills the tumour cell directly without prior sensitization through the release of interferon-gamma or through perforin-granzyme enzymes. Hence forth, natural killer cells are important effector cells in the control of the disease[58].

CD57(Cluster of differentiation 57)

Gene profile:

Gene : *B3GAT1* gene [Galactosyl galactosylxylosyl protein 3- beta - Glucuronosyl transferase 1]

Location of the gene: 11q25 chromosomes

Function of the gene: A key enzyme in a glucuronyl transfer reaction during the biosynthesis of the carbohydrate epitope HNK-1 (human natural killer-1, also known as CD57 and LEU7)[59].

CD57 – designated as cluster of differentiation is defined as a terminally sulfated carbohydrate epitope. It was first described in 1981 on human natural killer cell (HNK) and it is also called as NK-1, LEU-7, or L2. This is a glycoepitope composed of GlcA attached in 1-3 linkage to a terminal galactose. This is synthesized by specific glucuronosyltransferases on terminal units of N-glycans. Glucuronylation is followed by 3-O-sulfation of the GlcA by one or more specific sulfotransferases. It is also described as O-glycans of glycoproteins, proteoglycans and glycolipids[60].

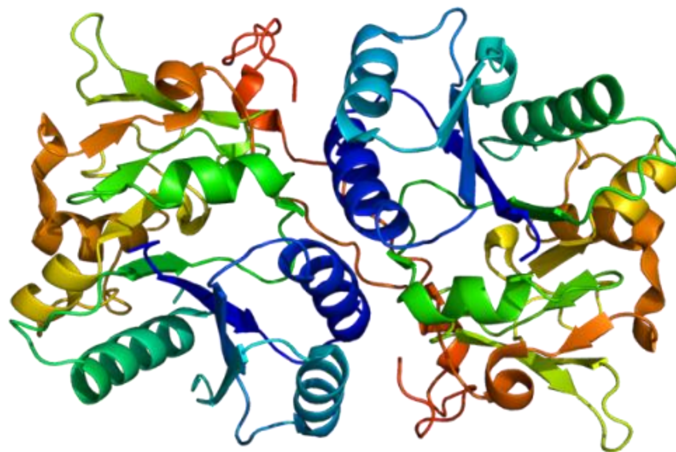


Figure 2: Structure of the B3GAT1 gene

Importance of CD 57:

CD57 is typically expressed by the natural killer cells and the cytotoxic CD8+ lymphocytes. In cytotoxic CD8+ lymphocytes, it is used as a marker of replicative senescence whereas in NK cells, CD57 expression is used to identify the peripheral NK cells maturation. NK cells expressing CD57 are highly cytotoxic and the presences of these cells are beneficial in certain non-communicable diseases and cancer. Therefore, it is suggested that CD57 can be used as a marker to indicate the immune senescence of the individual[61].

Since the presence of NK cell is an integral part of the tumour microenvironment and in the control process of the tumour, the expression of CD57 helps in evaluating the immune status of the patient[61].

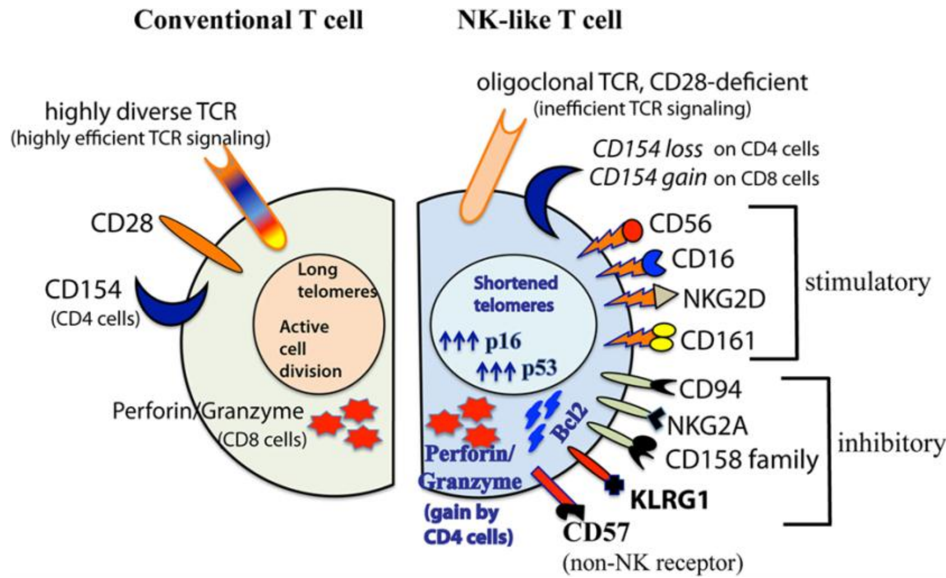


Figure 3: NK cells expressing CD57 antigen.

CD 57 expression and cancer:

CD57 and CD8+ lymphocytes are accumulated in individuals in various forms of cancer such as renal cell carcinoma, gastric carcinoma, melanoma, multiple myeloma, Hodgkin’s lymphoma, acute and chronic myeloid leukemia, and chronic lymphocytic leukemia. High expression of CD57 tumour cells are seen in cancer patients that has been attributed to less severity of the diseases and better outcomes [62].

This could be due to the immunosurveilance of the individual and the cytotoxic behavior of the NK cells. NK cells secrete the most important cytokines IFN-gamma after the stimulation of IL12/IL2, associated with the long term

survival of the patient. This suggests that heterogeneous group of NK cells consisting of CD57 subsets is useful in eliminating the neoplastic cells [62].

According to **Turkseven** and **Oygur (2010)**, in oral squamous cell carcinoma, low density of tumour infiltrating CD57 cells (NK Cells) and the higher expression of TNF- alpha are correlated with high aggressive behavior of the lesion and low survival rate[63].

According to **Sorskaar et al (1989)**, in acute lymphoblastic leukemia, increased numbers of CD57 and decreased NK cell activity and CD16 NK cells in bone marrow associated with complete remission[64].

Ortac et al. (2002) studies showed that the absence or low number of CD57 NK cells in the tumor tissue is associated with the relapse of Hodgkin's lymphoma and higher numbers of intratumoral CD57 NK cells are associated with no relapse and survival free in pediatric cases of non-hodgkin's lymphoma[65].

Vaquero et al. (2003) studies revealed that NK cells can infiltrate the melanoma, lung, breast, and renal carcinomas; but suggested that there is no correlation between numbers of infiltrating CD57 NK cells and apoptosis of malignant cells [66].

Lv et al. (2011) in his study showed that, tumour infiltrating CD57 NK cells in esophageal squamous cell carcinoma are positively associated with increased survival over 80 months [67].

Villegas et al. (2002) studies demonstrated that tumor infiltrating CD57 NK cells in squamous cell lung Carcinoma have positive correlation with the

survival rate. They showed that these patients have an increased survival rate of 2 years after surgery[68].

Thus, these studies proved that CD57 is a very valuable marker of NK cell maturation and identifying the cells with potent cytotoxic potential towards tumour cells.

Proliferating cell nuclear antigen (PCNA)

Gene profile:

Identity of the Gene : PCNA-proliferating cell nuclear antigen

Location of the gene : 20p12.3

Proliferating cell nuclear antigen (PCNA) is a well-established protein seen in all eukaryotic and archae species necessary for cell division and DNA replication. The other functions of PCNA are DNA repair, Chromatin remodeling, cohesion of sister-chromatid and control of cell cycle. Proliferating cell nuclear antigen (PCNA) is a highly characteristic marker for cell proliferation [69].

History:

PCNA protein is present on all eukaryotic species from the origin of species on earth. Despite of the evolutionary changes in the genetic material, this PCNA protein has not undergone any changes in its characteristic structure and functions over millions of years[69].

PCNA was discovered before 30 years ago in the patients of systemic lupus erythematosus as an antigen. Two years later, some other researchers found that 36 kd protein which was expressed during the cell cycle. They named it as

"cyclin". Later, the researchers found out that PCNA is associated with cell proliferation as well as in the neoplastic transformation. Finally, it was concluded that PCNA and the cyclin are the same protein[70].

PCNA structure:

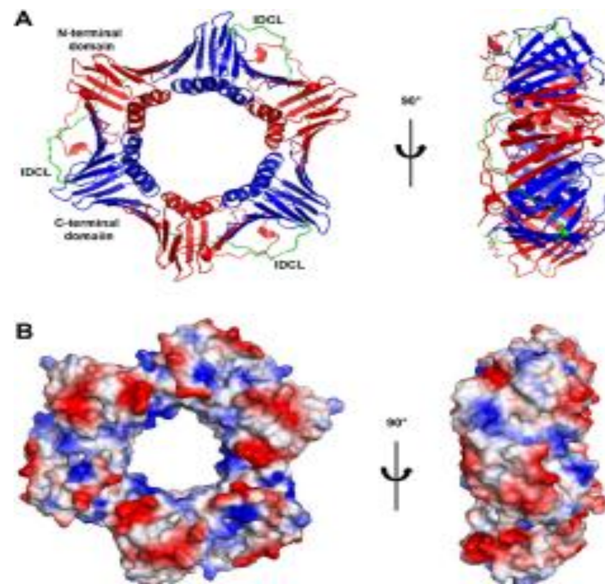


Figure 4: Three dimensional view of human PCNA – front view

PCNA belongs to the DNA sliding family DNA polymerase (pol) III b subunit and the T4 phage, gene45 protein. The DNA pol III b subunit is a homodimer with a pseudo-six-fold symmetry axis in which each monomer was composed of three repeated domains. The diameter of a central cavity ring in which the double helix of DNA is placed is 3.5 nm and allows for free and smooth sliding movements along the DNA molecule, thus preserving the physiological activity of this protein. In this DNA sliding family, PCNA acts as a binding site for DNA polymerases and also as a scaffold protein performing various functions like, DNA damage repair, chromatin remodeling, DNA replication and progression of cell cycle[69,71].

In contrast to the DNA pol III b ring with two subunits, the PCNA ring consists of three conjoined, identical monomers that are arranged in the form of head to tail. In addition, the PCNA possess a protruding C-terminal end which is designated as the ‘front’ end whereas the other side is designated as the ‘back’ end. The trimeric PCNA is ring shaped and encircles the DNA. The inner surfaces of the ring shaped PCNA is formed of 12 positively charged a-helices which interact with DNA, and the outer layer contains 54 ß-sheets and interdomain-connecting loops (IDCL) which makes significant contribution to the biological activity of PCNA. The IDCL not only serves to connect the N- and C-terminal domains of each monomer, but also an important docking site for different interacting proteins such as DNA pol d, DNA ligase 1 (DNA lig1), p21, DNA-(cytosine-5) methyltransferase and flap endonuclease (Fen1) [69,71].

Functions of PCNA:

a. DNA replication

The localization of the PCNA ring on DNA is ideal for allowing this protein to function in stabilization, recruitment and dynamic exchange of various replication proteins, thus making PCNA a key coordinator of the replication process.

b. Repair

c. Cell cycle control

PCNA interacts with cyclin A–Cdk2 complex and control the cell cycle. DNA damaged cells and aging cells leads to increased production of p21 protein. This p21 protein combines with cyclin dependant kinase (CDK) and

blocks the cell cycle from G1 phase to S Phase. This p21 is identified as a result of the complex formed by PCNA, Cyclin dependent kinases and cyclins[71].

Various studies conducted by using CD57 and PCNA in oral cancers:

Zain RV et al., in 1995 conducted a study to document the pattern of PCNA expression in oral squamous cell carcinoma. In this study, immunohistochemical staining of PCNA was done in 36 Oral cancer specimens and were investigated by statistical analysis. The results showed that PCNA can be used as a valuable marker in the differentiation of the hyperplastic and the dysplastic epithelium from the normal epithelium. This study further emphasizes that the PCNA expressions are present at the deep, infiltrating margins in conventional grading of different grades of OSCC[12].

Kurokawa H et al., in 1995 performed a study to evaluate the expression of PCNA in oral squamous cell carcinoma based on histological (mode of invasion, differentiation) and clinical findings (TNM clinical stage). This study was based upon the immunohistochemical expression of PCNA on various histopathological grades of OSCC. Analyzing the data obtained, low level of PCNA-positive cells are seen in well differentiated cases and cases with grade 4C and 4D revealed high level of PCNA-positive cells. They finally concluded that PCNA can demonstrate the proliferating activity of oral squamous cell carcinomas, especially in the degree of malignancy[72].

Myong H et al in 2006 had done a study to examine the relationship between proliferation markers and the rate of survival in oral squamous cell

carcinoma (OSCC) patients. This study was also conducted to determine the potentiality of proliferation markers in predicting lymph node metastasis. The study was based upon immunohistochemical expression of PCNA and Ki-67. Statistical analysis was done by univariate and multivariate analysis. From this result, it can be postulated that the cancer staging based on the TNM stage was a powerful prognostic variable and Ki-67 had a significant effect on the cumulative survival rate[73].

Turkseven MR et al in 2010 performed a study to find out the relationship between the biological behaviors of the tumor and the host local immune response by assessing the expressions of intratumoral natural killer (NK) cells and tumor necrosis factor-alpha (TNF-alpha) in oral squamous cell carcinomas. A total of 46 cases of oral squamous cell carcinomas were included in this study. The paraffin sections were immunohistochemical treated by CD57and TNF-alpha antibodies. The CD57 and TNF-alpha expression were analyzed according to the histopathologic grading and clinical staging groups. On analyzing the data obtained, low density of CD57+ cells (NK cells) and higher expression of TNF- alpha were seen in tumors graded as poor prognostic group compared to the cases in good prognostic group. These findings suggested that the increased secretion of TNF-alpha are associated with high invasive potential, facilitates the tumor invasion and are also responsible for the suppression of NK cells. This study finally concluded that because of the increased secretion of TNF-alpha, tumor cells are prevented from NK cell attacks and undergo invasion due to genetic alterations in the tumor microenvironment[63].

Watanabe S et al., in 2010 conducted an immunohistochemical study to investigate the expression of Ki-67, PCNA and cyclin B1 protein which relied on the pattern of cell invasion in OSCC.A total of 39 OSCC specimen and 13 normal samples were analyzed. Pearson's test, Mann Whitney test, Kaplan- Meier method & SPPS 11.0 were used as statistical methods. They concluded that Ki-67, PCNA, & cyclin B1 expression characterize the invasive tumour front that leads to better understanding of the behavior of OSCC[74].

Zancope E et al., in 2010 done a study to estimate the population of CD8 and NK (Natural killer) cells by immunohistochemistry on 70 OSCC and the results were analyzed using Cox regression analysis. The results showed that higher proportion of Cd8+ cells and NK cell were seen in OSCC indicating the lower neoplastic proliferation of the lesions. Therefore, the infiltration of NK cells and CD8+ cells reflect the distinctive tumor microenvironments that possess a favorable local cytotoxic response against neoplastic cells[75].

Kato K et al., in 2011 conducted a study to examine the expression of P53 and PCNA at the invasive front of OSCC by immunohistochemical staining and investigated their relationship with the prognosis of the patient. The study was conducted on 59 biopsy cases of OSCC and data were analyzed by using Mann-Whitney's U test and Kaplan - Meier method. On analysis, the study showed a high labeling index of p53 and PCNA in OSCC and it is associated with poor prognosis[76].

Fraga CA et al., in 2012 conducted a study to investigate the detection of CD57 inflammatory cells in HNSCC and their association with the overall

survival rate. A retrospective and analytical study was performed in 70 patients' specimen with anti CD57. Results were statistically analyzed by bivariate and multivariate analysis. The results indicated that predominant infiltration of CD57 inflammatory cells within the peritumoural stroma of HNSCC indicated a good prognosis and also added that high infiltration of inflammatory CD57 cells alone should not be considered as an independent prognostic marker in development of HNSCC.[9]

Iida M et al., in 2014 conducted a study to examine the significance of the presence of CD57 cells in peripheral blood from the patients of OSCC. The study was done in a total of 43 patients with OSCC by fluorescence-activated cell sorting analysis. They interpreted that CD57 cells are significantly increased with the clinical stage of the disease. Hence an increase in the CD57 cells is a potent valuable prognostic marker in the detection of the aggressiveness of OSCC[77].

Madan M et al., in 2015 performed a study to assess the proliferative index in potentially malignant lesions and malignant oral lesions using PCNA expression and AgNOR methods. A retrospective study was conducted on 30 cases of leukoplakia, 15 non-dysplastic, 15 dysplastic and 15 cases of OSCC for immunohistochemical detection of PCNA. The results were analyzed using ANOVA, Tukey's honest significant difference, Pearson correlation. Based on these findings it is concluded that AgNOR alone cannot be a valuable parameter and PCNA can be used as a useful biomarker in different grades of OSCC[14].

Poosarla CS et al., in 2015 conducted a study to evaluate the expression of PCNA in OSCC and to determine whether PCNA can be used an index for

clinical aggressiveness in oral premalignancy lesions and OSCC. This study was conducted on a total of 50 blocks that were previously histopathologically diagnosed as OSCC. The observed data were analyzed by one way ANOVA test, Fischer's test, and student's t test. The result showed a steady increase in the proliferative index from the normal to various grades of OSCC and therefore, PCNA index can be used to assess the cell proliferation and aggressiveness in dysplasia and different grades of OSCC[78].

Agarwal R et al., in 2016 performed a study to assess the expression of CD57 and to correlate the expression of CD57 with 3 years survival in patients with OSCC. A total of 100 patients of various grades of OSCC were included in the study. Further, these 100 patients were divided into two groups; group 1 with 50 dead patients and group II with 50 live patients. The results were obtained by using the spearman's correlation coefficient and student's unpaired t-test. They concluded that there is a significant correlation exists in between the CD57 and the prognosis of the patients[2].

Abdul Khadir SN et al., in 2016 conducted a study to evaluate PCNA and P53 expression in oral squamous cell carcinoma by immunohistochemistry. This was a retrospective study done on archival paraffin-embedded tissue blocks of 20 patients that were histopathologically diagnosed as oral squamous cell carcinoma. These archival blocks were analyzed by using the antibodies against P53 and PCNA. The results were analyzed by using Balanced ANOVA test and chi- square test. From the results, it was concluded that PCNA and P53 expression in OSCC has a prognostic significance and are associated with

biologically aggressive tumors. Hence PCNA can be used as a good indicator for detecting the aggressiveness of the lesion in all grades of OSCC[79].

In **2016 Taghavi N et al.,** conducted a study to evaluate the prognostic significance of CD 57, CD16 and TGF-β expression in OSCC. This is a retrospective study conducted on 57 patients that were primarily diagnosed as oral squamous cell carcinoma. Immunohistochemical examination of CD 57, CD 16 & TGF-β were done in 57 cases of histopathologically diagnosed OSCC. The relationship between marker's expression and clinicopathological data were analysed using bivariate and multivariate analysis. On analyzing the data obtained, CD57 expression and mode of invasion were concluded to be independent prognostic factors of survival in OSCC patients[80].

Fang J et al., in 2017 conducted a study to evaluate the importance of tumor infiltrating immune cell in patients with oral squamous cell carcinoma. This study was conducted in 78 OSCC patients with a follow up of 2 years. Immunohistochemical expression of T-bet, CD8, CD4, CD57and CD68 positive cells were assessed and the experimental data were analyzed by using Chi-square test, univariate and multivariate COX analysis. The predictive potential of immune cells for survival of OSCC patients was determined by using ROC (receiving operator characteristics) and AUC (area under curve) analysis. They concluded that the infiltration of CD57 and CD8 expression in the tumor stroma was associated with the status of lymphnode involvement and also predicts the survival of OSCC patients independently [81].

Ahmed S et al., in 2017 performed a study to compare the expression of AgNORs with PCNA in Oral squamous cell carcinoma (OSCC) and also to assess the reliability between PCNA and AgNOR. Previously confirmed 30 cases of oral squamous cell carcinomas were taken for the study and statistical analysis was done by ANOVA test. On analyzing the data obtained, PCNA expression and AgNOR count was found to be increased from well-differentiated OSCC followed by moderately differentiated and poorly differentiated OSCC. Therefore, they concluded that the proliferative activity is more in poorly differentiated OSCC. The combination of PCNA with AgNOR count appears to be an effective means of identifying the proliferating activity of squamous cell carcinoma[82].

Oliveira Maciel TA et al., in 2017 performed a study to evaluate the presence of NK cells and CD8+ T lymphocytes in lower lip Squamous Cell Carcinomas and its correlation with clinico-pathologic parameters. Thirty two cases of lower lip SCCs were included in the study for the immunohistochemical assessment of NK cells and CD8+ cells. Results were analyzed by Mann-Whitney test and spearman correlation coefficient. They found that CD57+ and CD8+ T cells were more abundant in clinical stages I/II, non-metastatic cases and low-grade malignancy tumors. They concluded that the high expression of CD57+ and CD8+ cells at the invasion front suggests that they are involved in anti-tumor defense[83].

MATERIALS & METHODS

MATERIALS AND METHODS

The current study was done in the department of oral pathology and microbiology at Sree Mookambika Institute of Dental Sciences in Kulasekharam.

a. **Study design**: Cross sectional study.

b. **Approximate total duration of the study:** 1 year.

c. **Number of groups to be studied**: Four Groups.

d. **Detailed description of the groups:**

Group1 : Tissue sections taken from the paraffin embedded blocks that are clinically diagnosed and microscopically confirmed cases of normal mucosa – (Control group)

Group 2 : Tissue sections taken from the paraffin embedded blocks that are clinically diagnosed and histopathologically confirmed cases of well differentiated squamous cell carcinoma (WDSQCC)

Group 3 : Tissue sections taken from the paraffin embedded blocks that are clinically diagnosed and microscopically confirmed cases of moderately differentiated squamous cell carcinoma (MDSQCC)

Group 4 : Tissue sections taken from the paraffin embedded blocks that are clinically diagnosed and microscopically confirmed cases of poorly differentiated squamous cell carcinoma (PDSQCC)

Hence forth, a total of 40 blocks were included in the study. Three sections were taken from each block and each section measured of 3-4 micron thickness. One section was stained with the haematoxylin and eosin stain to reconfirm the diagnosis while the other two sections were subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and CD 57 respectively.

e. Inclusion criteria:

- Paraffin embedded blocks of histopathologically diagnosed cases of well differentiated squamous cell carcinoma (WDSQCC).
- Paraffin embedded blocks of histopathologically diagnosed cases of moderately differentiated squamous cell carcinoma (MDSQCC).
- Paraffin embedded blocks of histopathologically diagnosed cases of poorly differentiated squamous cell carcinoma (PDSQCC).

f. Exclusion criteria:

Stored Archival blocks in which antigen retrieval could not be done.

SAMPLING:

Sample Size, $n = \frac{4pq}{d^2}$

n = Sample size

In reference to the study performed by Watanabe S et al., in 2010[74],

The obtained value of p = WHO - HMG = 79.4

q= 100 - p

d= 20% of p

n = Sample size

As per this calculation, a total sample of 40 patients was taken, and each group contained 10 patients respectively.

Group 1: Ten blocks of normal mucosa (n=10)

Group 2: Ten blocks of well differentiated squamous cell carcinoma (n=10)

Group 3: Ten blocks of moderately differentiated squamous cell carcinoma (n=10).

Group 4: Ten blocks of poorly differentiated squamous cell carcinoma (n=10)

g. **Sampling Technique**: Convenient sampling.

METHOD / TECHNIQUE / INSTRUMENTS / REAGENTS/ KIT

ARMAMENTARIUM

Apparatus and equipments:

- Semiautomatic microtome (Spencers, Model no: 1010-SMT-006, India)
- Microscopic Slide warming table (Science House Brand, India)
- Tissue flotation water bath (Science House Brand, India)
- Electronic weighing machine, (Infra Digital Limited, India)
- pH meter (Hannah instruments, India)
- Digital mint timer (Salter, United Kingdom)
- Pressure cooker (Pigeon, India)
- Poly-L lysine coated slides (Pathensitu technologies pvt ltd, India)
- Graduated measuring jars (Borosil Adelab scientific, Australia)

- Graduated measuring Beakers (Borosil Adelab Scientific, Australia)
- Coplin glass staining jars (Tru Laboratories, Philippines)
- Histology IHC Slide carrier (Shpc trading center, India)
- Volumetric micropipettes (Accupipetes, India)
- Stirring glass rods (Borosil Adelab scientific, Australia)
- Microscope Cover glass (Cover slips) (Blue star slides pvt ltd, India)
- Refrigerator (Electrolux smart series, India)
- Compound light microscope (labomed, India)
- Storage cabinet for archival blocks (India)
- Sterile gauze

Other reagents used in immunohistochemistry techniques are:

- Filtered deionized water (Roshan Industries, India)
- Ethanol 70%, 80%, 90%, 100% (Shree Krishna Chem enterprises, India)
- Hydrochloric acid-1Normal (RFCL limited- Rankem, India)
- Xylene (Nice chemicals, India)
- Mayer's Hematoxylin (Himedia labs, India)

Buffers

1. Antigen retrieval buffer

    Sodium citrate buffer (pH 6.0)

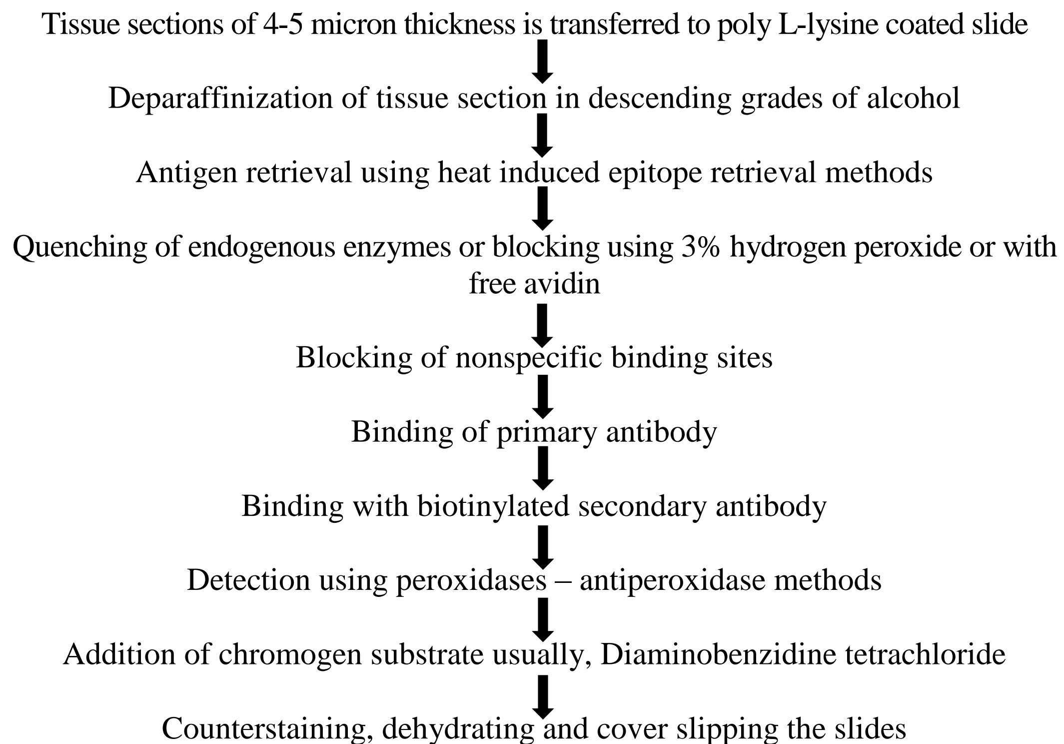
2. Wash buffer :

    TRIS- buffer saline (pH-7.6).

Antibodies employed in this present study are:

1. Primary antibody - (Pathensitu technologies pvt ltd, India)
    a. Rabbit Monoclonal PCNA antibody PC10 (cat# PR 065) for PCNA expression
    b. Mouse monoclonal - CD57 antibody (cat # PM 161) for CD57 expression
2. Secondary antibody - (Pathensitu technologies pvt ltd, India)
    a. Endogenous block (Hydrogen peroxide block)
    b. Protein block
    c. Horse radish peroxidase
    d. Chromgen DAB (Diamino benzidine tetra hydrochloride) detection system

Schematic representation of immunohistochemistry procedure:



Procedure:

Sectioning of tissues:

- Sectioning of tissue was carried out on a rotary semiautomatic microtome of 4-5 micrometer thickness. The serials of tissue sections were transferred to the tissue flotation bath and then onto the Poly L- lysine coated slides.

Preparation of buffer solutions:

a) Wash buffer

TRIS-Sodium chloride buffer

- TRIS buffer- 0.3g
- Sodium chloride- 4g
- Distilled water- 500ml
- 1 Normal hydrochloric acid- 2 ml

The ingredients were mixed and dissolved and the pH is adjusted to 7.6 with 1M Hydrochloric acid

b) Antigen retrieval buffer

Sodium Citrate buffer solution

- Tris Sodium citrate – 3.04 g
- Ethylene di amino tetra acetic acid- 0.37g
- Distilled water – 1000ml
- 1normal hydrochloric acid- 4ml

The ingredients were dissolved and standardized with pH meter and the pH is adjusted to 9.0

Deparaffinization /Dehydration:

The slides were dewaxed by heating at 60°c for 60 minutes followed by deparaffinization in xylene for half an hour. The slides were then kept in absolute alcohol for ten minutes followed by descending grades of alcohol [90%, 80%, and 70%] each for 10 minutes. The sections were then washed in distilled water for five minutes

Antigen retrieval method:

The slides with the tissue sections were put in sodium citrate buffer solution (pH 9.0) and kept in microwave oven for 10minutes at high power and allowed it to cool for few minutes. The same steps were repeated for another 8 minutes, 6 minutes and 5 minutes respectively. After these steps were done, the slides were cooled for 30 minutes.

Slides were washed in distilled water for 5 minutes and dried it completely. The slides are then immersed in wash buffer for five minutes.

Blocking of endogenous peroxidase enzyme:

The slides were treated with hydrogen peroxide for ten minutes to block the endogenous peroxidase enzyme activity and washed with wash buffer for five minutes.

Protein block:

The slides were then subjected to one drop of protein block and kept for ten minutes and the slides were blotted dry with the tissue paper. After this procedure, no wash buffer was used.

Incubation with primary antibody:

The tissue sections were then covered with primary antibody and incubated for one hour. For PCNA expression, rabbit monoclonal PCNA antibody PC10 and for CD57 expression, mouse monoclonal - CD57 antibody was used. The sections were then washed in two changes of wash buffer for five minutes each. The excess solution were wiped with the tissue paper.

Incubated with secondary antibody:

A drop of horseradish peroxide secondary antibody was added on the sections and incubated for thirty minutes. The sections were then washed in two changes of wash buffer for five minutes each. The slides were wiped off to remove the excess wash buffer.

Addition of chromogen substrate:

Freshly prepared chromogen substrate was added on to the tissue sections and kept for five minutes. Slides were washed in distilled water for five minutes.

Counter staining:

The slides were counterstained with Mayer's haematoxylin and rinsed in tap water for the process of bluing.

Mounting

The tissue sections were mounted with DPX. (Distrene polystyrene xylene)

Controls

Controls are necessary for validation of immunohistochemical staining results.

a. Positive tissue control:

A positive control includes tissue types that express the protein of interest. This indicates that the procedure is working and optimized.

b. Negative tissue control

Includes a tissue type in which the protein of interest is not expressed. This is to check for nonspecific signal and false positive results. In this study, tissues which were not stained properly with the omission of primary antibody were considered as a negative control.

Evaluation

- Cells are considered as positive for CD57 if there are intracytoplasmic DAB staining (chromogenic colour)
- Cells are considered as positive for PCNA staining when the nucleus of the highly mitotic cells stains up in light brown colour.

Parameters to be studied:

CD57 labelling index

CD57 was evaluated by counting the number of cells stained positively. A total of 1000 tumour cells were counted. CD57 labelling index was calculated according to Stelin S et al (2009)[84].

$$\text{CD57 labelling index} = \frac{\textit{Total number of positively stained cells}}{1000} \times 100$$

PCNA index

PCNA positive cells were obtained by counting from a minimum of 1000 tumor cells from 3 microscopic fields. The procedure will be repeated for

two times in order to eliminate the intraexaminer variability and the mean value was taken. PCNA index was calculated by the criteria given by Poosarla CS et al (2015)[78].

$$\text{PCNA labelling index} = \frac{\textit{Total number of positively stained cells}}{1000} \, x100$$

Statistical method of analysis:

The data obtained in this study were analyzed using the SPSS version 16.0 (Statistical package for social science) software. To compare and correlate the expression of CD57 and PCNA expression in oral squamous cell carcinoma, mean, standard deviation and Pearson's correlation coefficient test were used.

COLOR PLATES

COLOUR PLATES

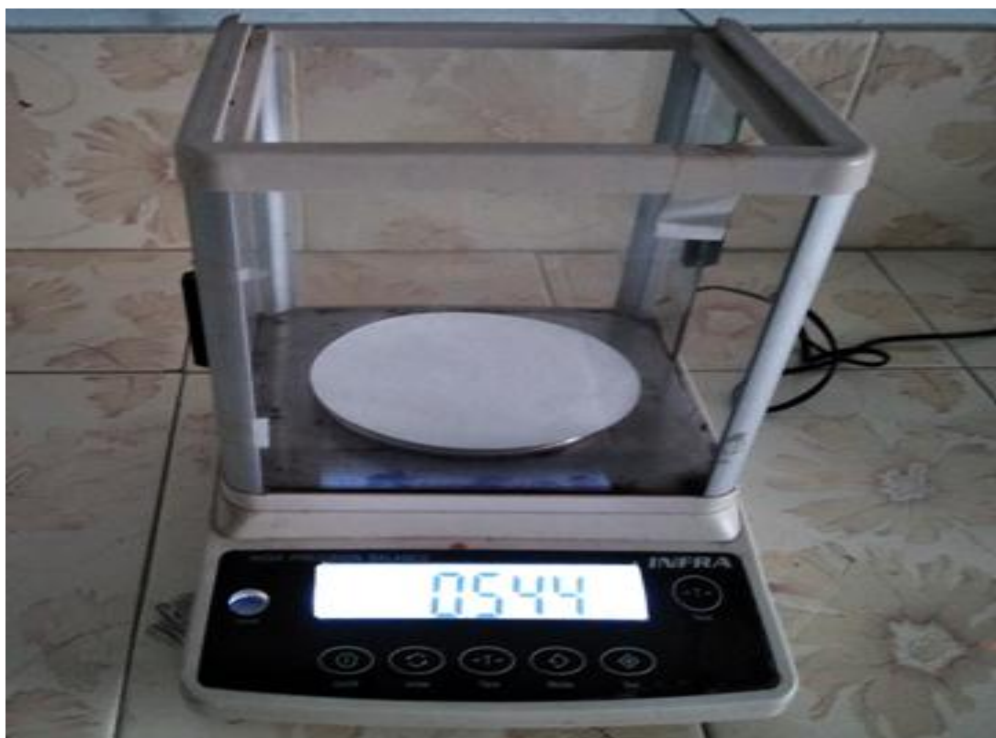
CP 1: Storage cabinet for archival blocks

CP 2: Semiautomatic microtome

CP 3: Tissue floatation water bath

CP 4: Slide warming table

CP 5: pH meter



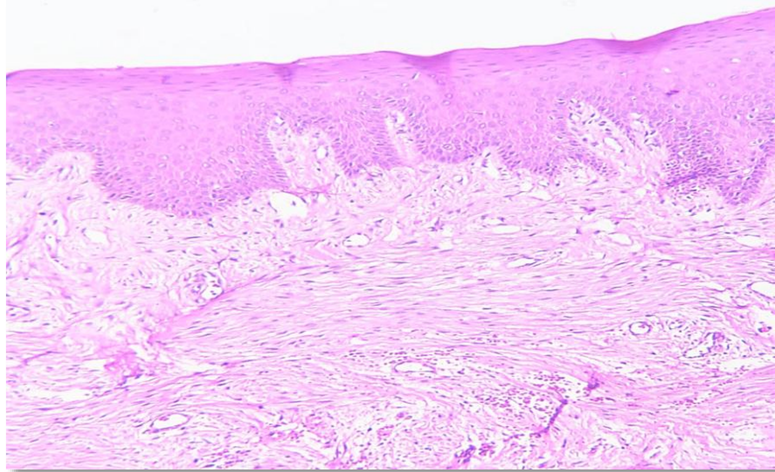
CP 6: Digital weighing machine

CP 7: Pressure cooker for antigen retrieval

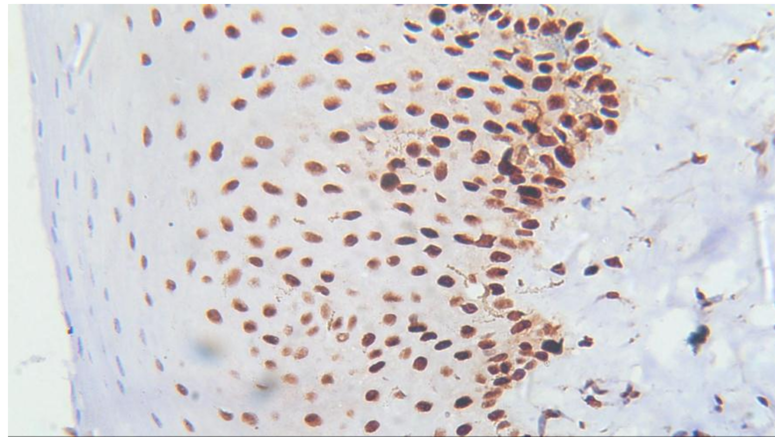
CP 8: Reagents used for buffer solutions

CP 9: Primary antibodies - anti PCNA & anti CD57

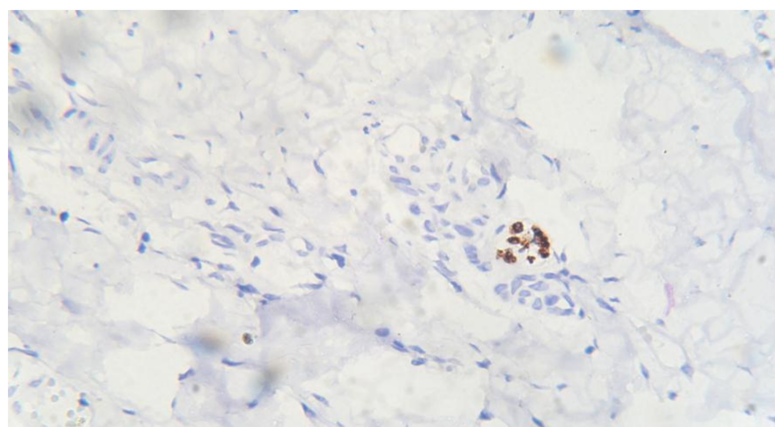
CP 10: Reagents used in IHC procedures



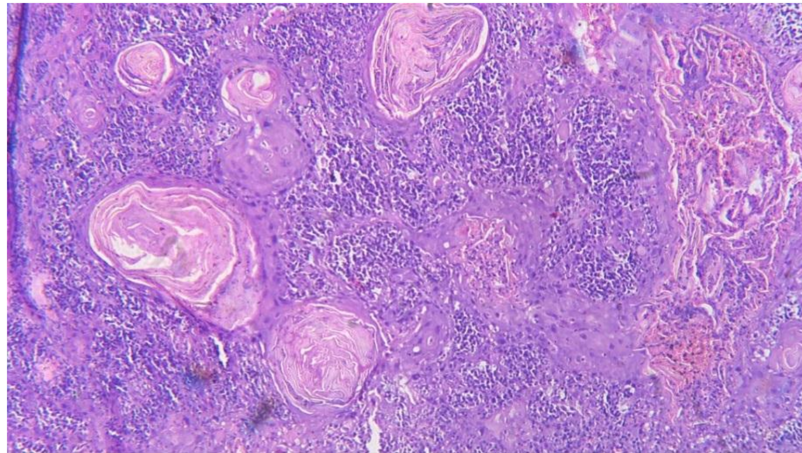
CP 11: Photomicrograph showing histology of normal mucosa (H&E staining; x100)



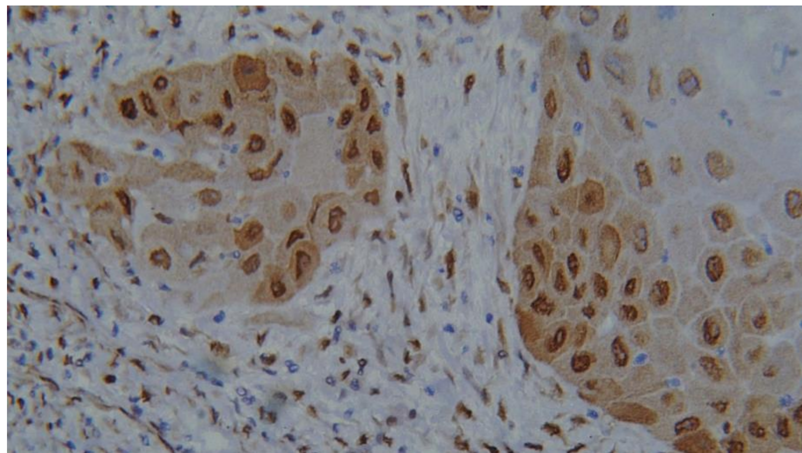
CP 12: Photomicrograph showing expression of PCNA in normal mucosa (IHC staining; x400)



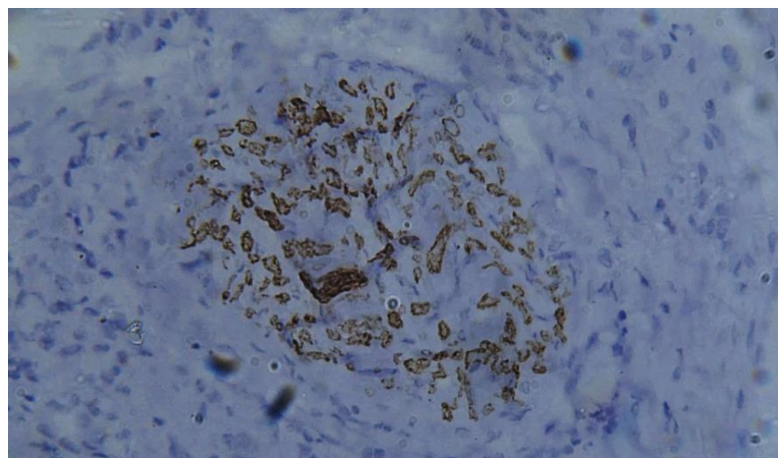
CP 13: Photomicrograph showing expression of CD57 in normal mucosa (IHC staining; x400)



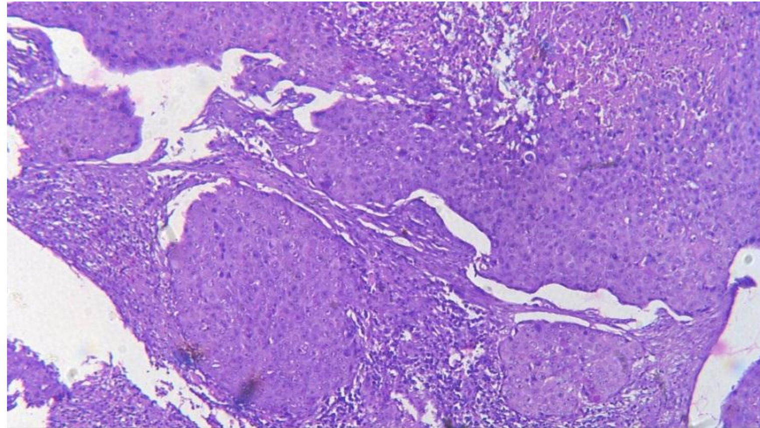
CP 14: Photomicrograph showing histopathology of WDSQCC (H&E staining; x100)



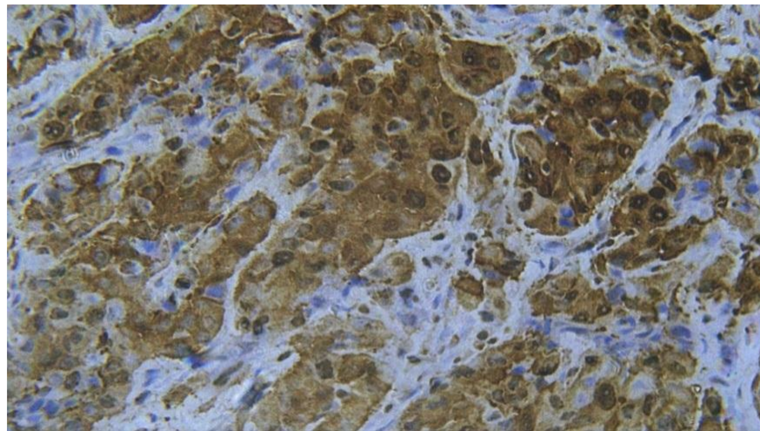
CP 15: Photomicrograph showing expression of PCNA in WDSQCC (IHC staining; x400)



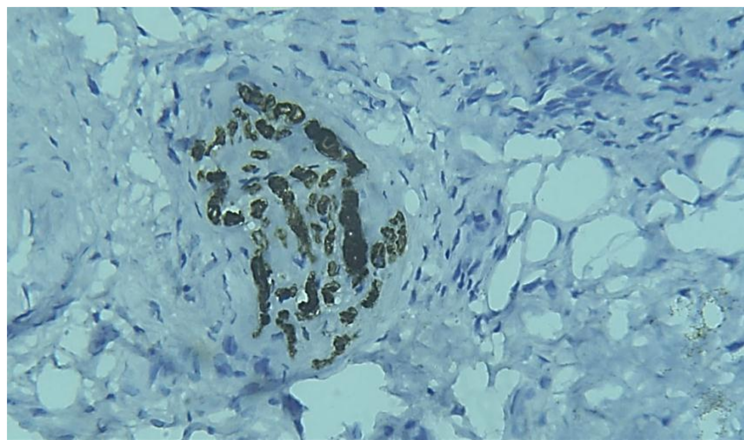
CP 16: Photomicrograph showing expression of CD57 in WDSQCC (IHC staining; x400)



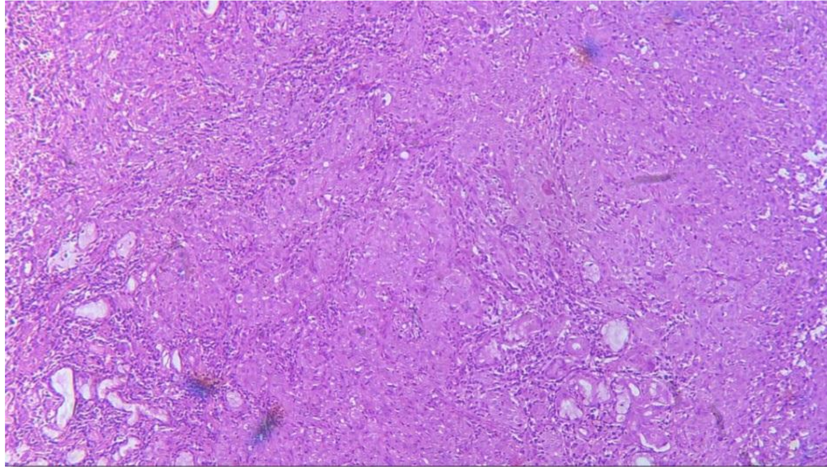
CP 17: Photomicrograph showing histopathology of MDSQCC (H&E staining; x100)



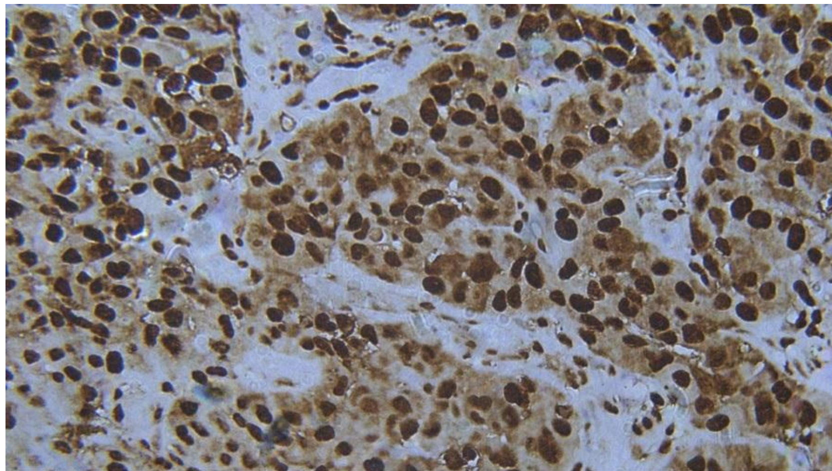
CP 18: Photomicrograph showing expression of PCNA in MDSQCC (IHC staining; x400)



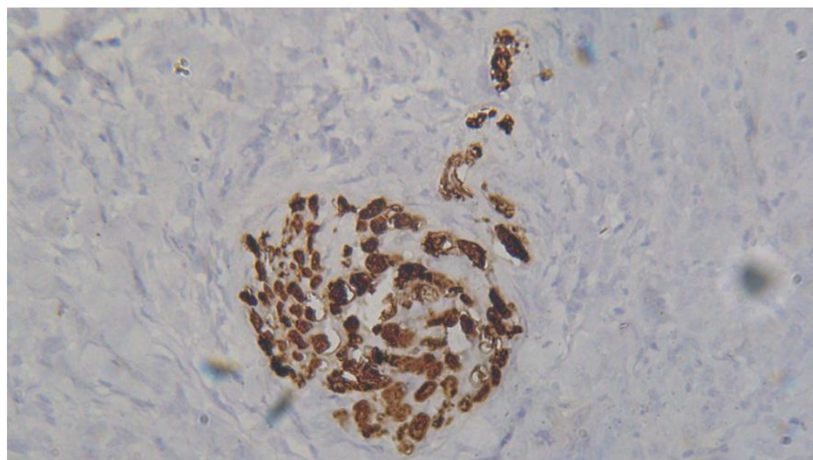
CP 19: Photomicrograph showing expression of CD57 in MDSQCC (IHC staining; x400)



CP 20: Photomicrograph showing histopathology of PDSQCC (H& E staining; x100)



CP 21: Photomicrograph showing expression of PCNA in PDSQCC (IHC staining; x400)



CP 22: Photomicrograph showing expression of CD57 in PDSQCC (IHC staining; x400)

RESULTS & OBSERVATIONS

RESULTS AND OBSERVATIONS

The current study was done in the department of oral pathology and microbiology at Sree Mookambika Institute of Dental Sciences in Kulasekharam. In the present study, the immunohistochemical expression of PCNA and CD57 was analyzed in different histopathological grades of oral squamous cell carcinoma. A total of forty samples were included in the study. The control group consisted of 10 samples of normal mucosa (Group 1) and the study group consisted of 10 samples each of well differentiated (Group 2; WDSQCC), moderately differentiated (Group 3; MDSQCC), and poorly differentiated (Group 4; PDSQCC), squamous cell carcinomas. PCNA and CD57 staining was done according to manufacturer’s protocol and the stained slides were observed under light microscope.

In this study, the age range for samples in the study groups were 39-72 years, 53-75 years and 35-70 years with a mean age of 56.79 years, 61.4 years and 55.7 years in well, moderate and poorly differentiated squamous cell carcinomas respectively. There was a male predilection in the ratio of 4:1 and 3:2 in well and moderately differentiated cases whereas equal sex predilection in the ratio of 1:1 was noticed in poorly differentiated squamous cell carcinoma. Documentation of the associated deleterious habits and common site of the lesion showed tobacco and betel chewing as the most prevalent cause and buccal mucosa as the most favorable site in all the three histopathological grades of squamous cell carcinoma.

Estimation of PCNA and CD57 were evaluated by the positive and negative staining of cells. Cells were considered as positive for CD57 if there was intra-cytoplasmic DAB staining (Chromogenic colour). Cells were considered positive for PCNA, when the nuclei of the highly mitotic cells stain as light brown in colour. The counts were performed by a single examiner in three different high power fields for both CD57 and PCNA. The counting was done twice in the same field to eliminate intra-observer variability. A total of 1000 cells from three different high power fields were counted and the number of positive cells was noted. The results were interpreted in terms of percentage positivity and expressed as labelling index (LI).

The results obtained were subjected to statistical analysis by using statistical package for social sciences (SPSS 16.0) software. Analysis of data between the groups was done by using ANOVA (Post hoc) followed by Dunnet t test. Correlation of PCNA and CD57 between the groups was done with Pearson correlation. P-value less than 0.05 ($p<0.05$) was considered statically significant.

The mean value of PCNA labelling index was 26.19±1.25, 45.88±2.20, 59.38±1.04, and 72.77±4.35 in normal mucosa, well differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC respectively. Multiple Comparison of expression of PCNA index between the study groups of WDSQCC, MDSQCC and PDSQCC was statistically highly significant with a p-value of 0.001. Comparison of expression of mean labelling index of PCNA between the study groups (WDSQCC, MDSQCC and PDSQCC) and the control group (normal mucosa) was also statistically highly significant with a p-value of

0.001. The PCNA labelling index was least in normal mucosa and increased in well differentiated OSCC followed by moderately differentiated OSCC and the highest was noted in poorly differentiated OSCC. These findings indicated that the proliferation of cells increased as the grades of OSCC increased.

Similarly the mean value of CD57 labelling index was 2.91±0.82, 16.63±2.33, 7.09±1.41 and 5.53±1.20 in normal mucosa, well differentiated OSCC, moderately differentiated OSCC and poorly differentiated OSCC respectively. Multiple comparison of expression of CD57 labelling index between the study groups of WDSQCC, MDSQCC and PDSQCC was statistically highly significant with a p-value of 0.001. Comparison of expression of mean labelling index of CD57 between the study groups WDSQCC, MDSQCC and PDSQCC with that of the control group (normal mucosa) was also statistically highly significant with a p-value of 0.001. The CD57 labelling index was least in normal mucosa and highest in well differentiated OSCC. The CD57 labelling index was decreased in moderately differentiated OSCC followed by poorly differentiated OSCC when compared with well differentiated OSCC but the mean values were higher than that of normal mucosa. These findings indicated that the natural killer cells or immune status of the individual decreases as the grades of OSCC increases.

In the present study on correlation of mean labelling index of PCNA and CD57, the Pearson correlation coefficient value within the groups were found not to be correlated which was statistically not significant ($p>0.05$) whereas the values were significant when compared between the groups ($p<0.05$).

TABLES

Table-1: Mean labelling index of PCNA in different groups

Groups	Description	PCNA index (MEAN±SD)
Group I	Normal Mucosa	26.19±1.25
Group II	Well differentiated squamous cell carcinoma (WDSQCC)	45.88±2.20
Group III	Moderately differentiated squamous cell carcinoma (MDSQCC)	59.38±1.04
Group IV	Poorly differentiated squamous cell carcinoma (PDSQCC)	72.77±4.35

Table-2: Comparison of mean labelling index of PCNA in normal mucosa with other groups

Groups	PCNA index (MEAN±SD)	p value
Normal mucosa	26.19±1.25	
WDSQCC	45.88±2.20*	0.001
MDSQCC	59.38±1.04*	0.001
PDSQCC	72.77±4.35*	0.001

(*p<0.05 significant when normal mucosa compared with other groups)

Table-3: Comparison of mean labelling index of PCNA in WDSQCC with other groups

Groups	PCNA index (MEAN±SD)	p value
Normal mucosa	26.19±1.25*	0.001
WDSQCC	45.88±2.20	
MDSQCC	59.38±1.04*	0.001
PDSQCC	72.77±4.35*	0.001

(*p<0.05 significant when WDSQCC compared with other groups)

Table-4: Comparison of mean labelling index of PCNA in MDSQCC with other groups

Groups	PCNA index (MEAN±SD)	p value
Normal mucosa	26.19±1.25*	0.001
WDSQCC	45.88±2.20*	0.001
MDSQCC	59.38±1.04	
PDSQCC	72.77±4.35*	0.001

(*p<0.05 significant when MDSQCC compared with other groups)

Table-5: Comparison of mean labelling index of PCNA in PDSQCC with other groups

Groups	PCNA index (MEAN±SD)	p value
Normal mucosa	26.19±1.25*	0.001
WDSQCC	45.88±2.20*	0.001
MDSQCC	59.38±1.04*	0.001
PDSQCC	72.77±4.35	

(*p<0.05 significant when PDSQCC compared with other groups)

Table-6: Multiple Comparison of mean labelling index of PCNA between the groups

Groups	PCNA index (MEAN±SD)
Normal mucosa	26.19±1.25
WDSQCC	45.88±2.20*
MDSQCC	$59.38 \pm 1.04^{*,\#}$
PDSQCC	$72.77 \pm 4.35^{*,\#,\$}$

(*p<0.05 significant when normal mucosa compared with other groups, $^{\#}$p<0.05 significant when WDSQCC compared with other groups, $^{\$}$p<0.05 significant when MDSQCC compared with other groups)

Table-7: Mean labelling index of CD 57 in different groups

Groups	Description	Labeling index (MEAN±SD)
Group I	Normal Mucosa	2.91±0.82
Group II	Well differentiated squamous cell carcinoma (WDSQCC)	16.63±2.33
Group III	Moderately differentiated squamous cell carcinoma (MDSQCC)	7.09±1.41
Group IV	Poorly differentiated squamous cell carcinoma (PDSQCC)	5.53±1.20

Table-8: Comparison of mean labelling index of CD 57 in normal mucosa with other groups

Groups	Labeling index (MEAN±SD)	p value
Normal mucosa	2.91±0.82	
WDSQCC	16.63±2.33*	0.001
MDSQCC	7.09±1.41*	0.001
PDSQCC	5.53±1.20*	0.001

(*p<0.05 significant when normal mucosa compared with other groups)

Table-9: Comparison of mean labelling index of CD 57 in WDSQCC with other groups

Groups	Labeling index (MEAN±SD)	p value
Normal mucosa	2.91±0.82*	0.001
WDSQCC	16.63±2.33	
MDSQCC	7.09±1.41*	0.001
PDSQCC	5.53±1.20*	0.001

(*p<0.05 significant when WDSQCC compared with other groups)

Table-10: Comparison of mean labelling index of CD 57 in MDSQCC with other groups

Groups	Labelling index (MEAN±SD)	p value
Normal mucosa	2.91±0.82*	0.001
WDSQCC	16.63±2.33*	0.001
MDSQCC	7.09±1.41	
PDSQCC	5.53±1.20*	0.001

(*p<0.05 significant when MDSQCC compared with other groups)

Table-11: Comparison of mean labelling index of CD 57 in PDSQCC with other groups

Groups	Labelling index (MEAN±SD)	p value
Normal mucosa	2.91±0.82*	0.001
WDSQCC	16.63±2.33*	0.001
MDSQCC	7.09±1.41*	0.001
PDSQCC	5.53±1.20	

(*p<0.05 significant when PDSQCC compared with other groups)

Table-12: Multiple Comparison of mean labelling index of CD 57 between the groups

Groups	CD 57 index (MEAN±SD)
Normal mucosa	2.91±0.82
WDSQCC	16.63±2.33*
MDSQCC	7.09±1.41$^{*,\#}$
PDSQCC	5.53±1.20$^{*,\#,\$}$

(*p<0.05 significant when compared normal mucosa with other groups, $^{\#}$p<0.05 significant when WDSQCC compared with other groups, $^{\$}$p<0.05 significant MDSQCC compared with other groups)

Table-13: Correlation of PCNA labelling index with CD 57 labelling index within the groups

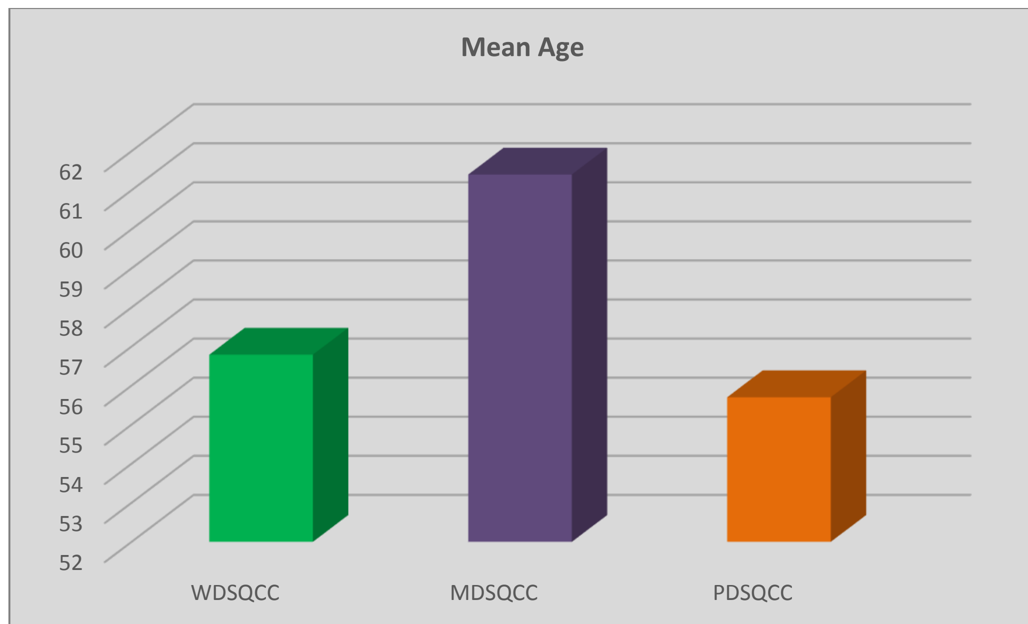
Groups		CD 57 labelling index (r value)	p value
Normal mucosa	Correlation PCNA	-0.43	0.20
WDSQCC		-0.43	0.21
MDSQCC		-0.58	0.07
PDSQCC		-0.59	0.06

Table-14: Correlation of PCNA labelling index with CD 57 labelling index between the groups

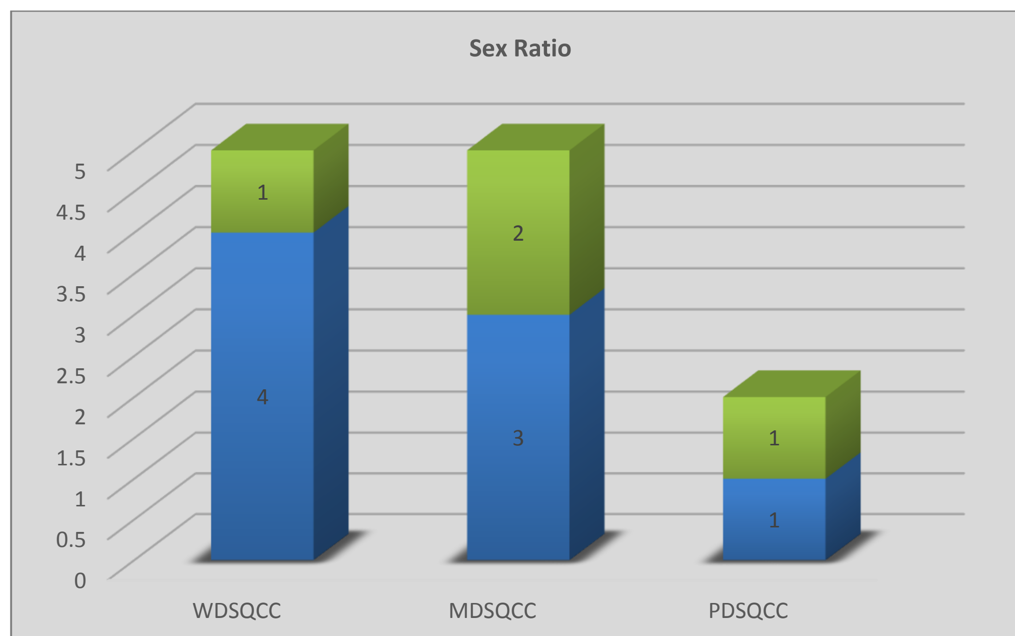
Correlation PCNA	CD 57 Labelling index (r value)				p value
	Normal mucosa	WDSQCC	MDSQCC	PDSQCC	
Normal mucosa	-0.43	-0.67*	-0.78*	-0.89*	0.04
WDSQCC	-0.64*	-0.43	-0.76*	-0.92*	0.03
MDSQCC	-0.68*	-0.71*	-0.58	-0.96*	0.03
PDSQCC	-0.75*	-0.74*	0.81*	-0.59	0.03

(*p<0.05 significant correlated between the groups)

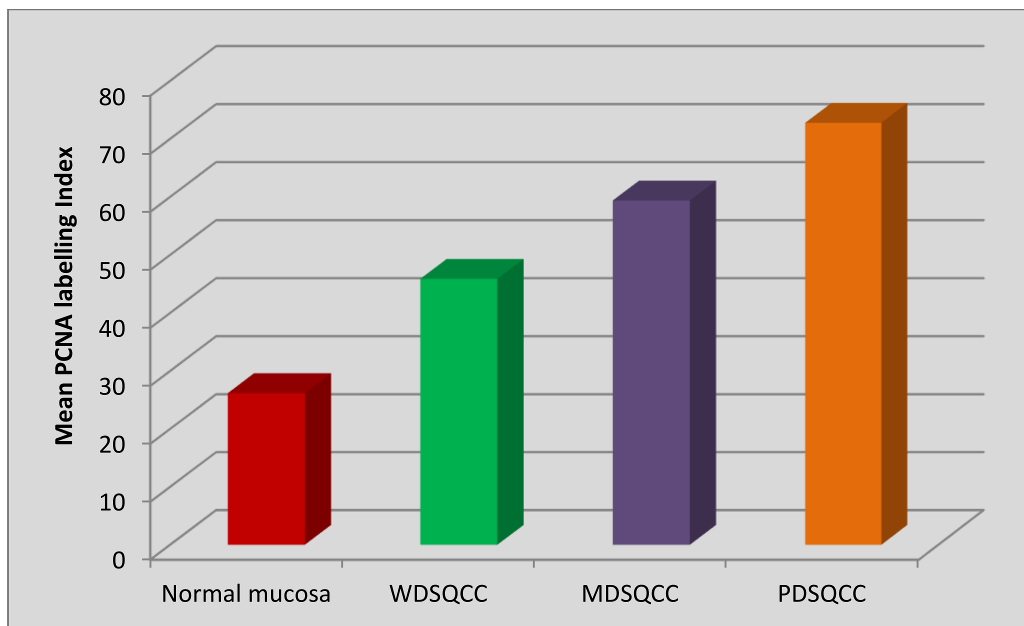
GRAPHS



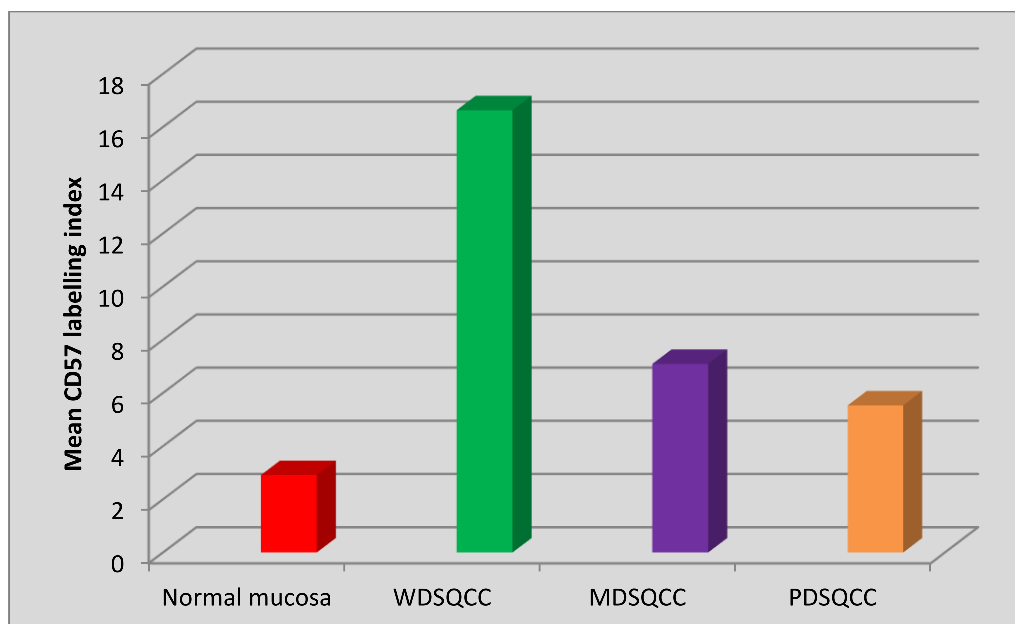
Graph-1: Mean age distribution in different grades of OSCC



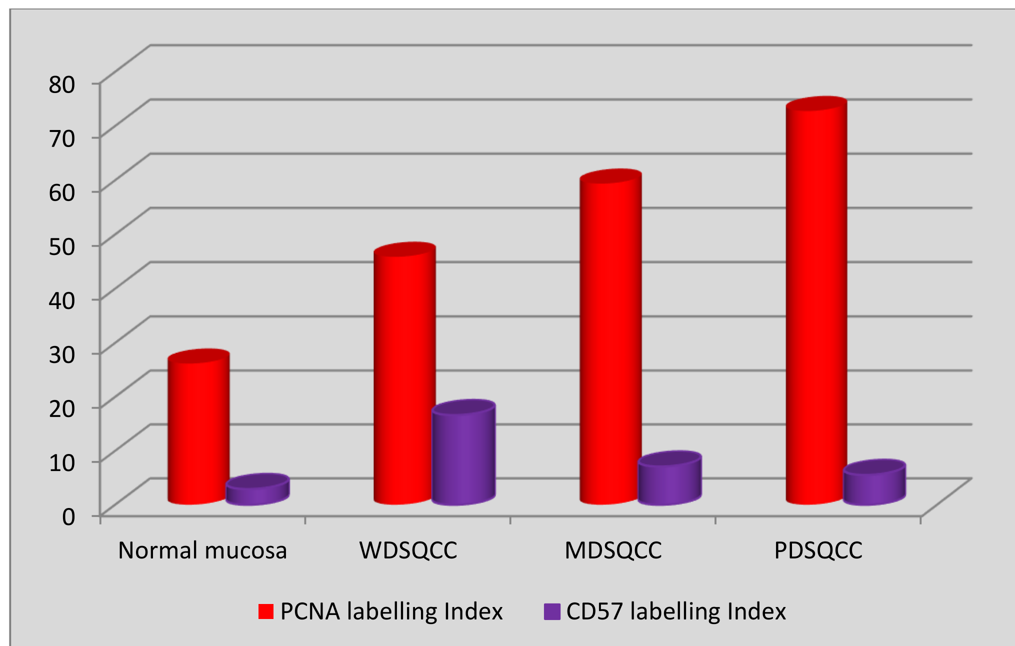
Graph-2: Ratio of sex in different grades of OSCC



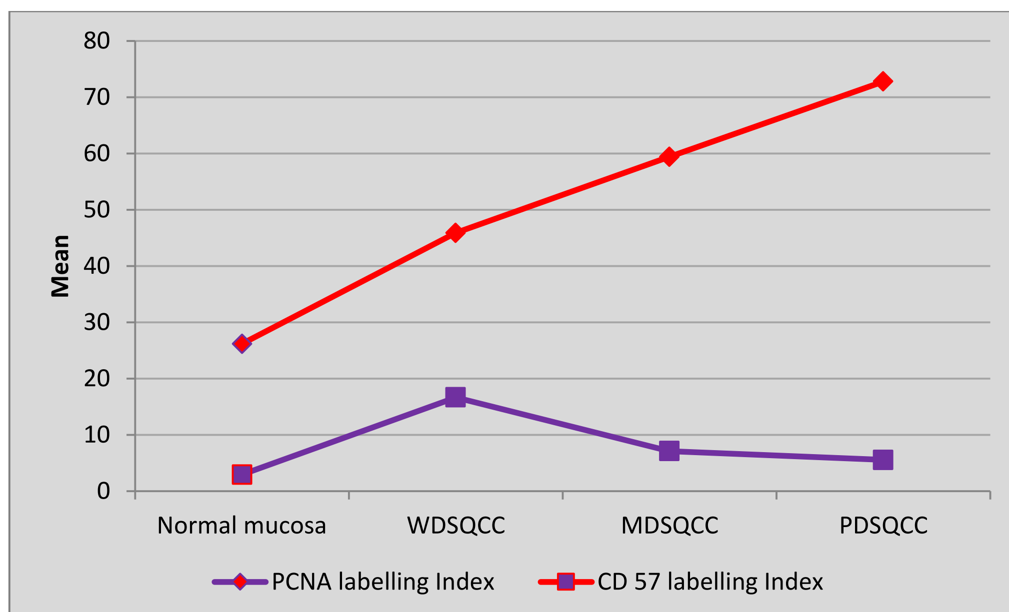
Graph-3: Comparison of mean labelling index of PCNA between the groups



Graph-4: Comparison of mean labelling index of CD57 between the groups.



Graph-5: Comparative correlation of PCNA labelling index with CD57 labelling index within the groups



Graph-6: Correlation of PCNA labelling index with CD57 labelling index between the groups

DISCUSSION

DISCUSSION

Oral cancer is the most common malignancy and it is the sixth most common cancer in the world[85]. The development of oral cancer is a multistep process that requires around four to seven events for the genetic changes to occur. These genetic alterations involve the classic hallmarks of malignancy and also undergo immortalization. As a result, the cells undergo behavioral and metabolic changes leading to uncontrolled proliferation[7].

Early diagnosis is vital as it reduces the severity and complications of the diseases but it also helps in planning of the treatment and prognosis. Recent advances in genomics and proteomics identify the diseases by using gene or protein profiles. These advanced techniques use a multiple panel of markers which helps in accurate identification of diseased status of the individual. Recently, an immunohistochemical study also involves the uses of multiple markers as an aid in the accurate diagnosis of the diseases[84].

PCNA is a characteristic marker for cell proliferation. These markers are helpful in identifying the aggressive nature of the lesion. These markers play a vital role in DNA replication, chromatin remodeling, control of cell cycle and DNA repair. Mutation of the PCNA gene is seen in various types of cancers[76]. Lee JJ et al (2005) showed that PCNA was a good indicator for the aggressive nature and the malignant potential of the lesion[86]. Tsuji et al (1992) showed that proliferation of tumour cells was an important predictor of the aggressiveness of the lesion and PCNA is an important protein which is expressed during the

process of proliferation and immunohistochemical detection of this protein helps to evaluate the proliferative capacity of tumour in oral squamous cell carcinoma[87].

Only few markers are useful in predicting the prognosis of the disease. Nagpal M (2016) concluded that the proliferative markers cannot be used as an independent marker for evaluating the prognosis of the diseases as these markers are useful indicators of the clinical aggressive nature of the lesion[88].

CD8+ cytotoxic lymphocytes mainly acts on the virus infected cells and tumour cells. Natural killer cell which is a subset of small population of T–cell, is an important effectors innate and adaptive immunity. These natural killer cells act without any prior activation. These cells identify the tumour cells by the lack of expression of class I major histocompatibility complex (MHC) and kill them by either secreting interferon-gamma or through perforin-granzyme activity[58]. CD 57 expression is most prominently seen in the highly matured NK cell. CD57 exhibit more potent immune response functions and inhibit the tumour development [80]. According to Carlos Alberto et al (2002), high density of CD57 cell infiltration alone was not considered as an independent prognostic marker for head and neck squamous cell carcinoma.[9].

Hence, in the present study, we use both PCNA and CD57 and analyzed the expression in different grades of oral squamous cell carcinoma. After a thorough data search, this study is the first documented study where PCNA and CD57 were used to find out the aggressiveness of the lesion and the immune status of the individual.

In the present study, we evaluated the immunohistochemical expression of PCNA in 10 cases each of well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and poorly differentiated squamous cell carcinoma. Normal oral mucosa was considered as the control group. PCNA positive cells were identified by the light brown granular staining of nucleus. PCNA index was calculated according to the criteria given by Poosarla et al (2015)[78]. The PCNA index was 26.19±1.25, 45.88±2.20, 59.38±1.04 and 72.77±4.35 for normal mucosa, WDSQCC, MDSQCC and PDSQCC respectively. Higher expression of PCNA was found in poorly differentiated squamous cell carcinoma. The intensity of staining and the number of positive PCNA cells were increased gradually from Group I to Group IV. This result was in accordance with the studies conducted by Abdul Khadir et al (2010)[79], Madan M et al (2015)[14], Poosarla CS et al (2015)[78], Roopavathi Keshav et al (2015)[89], Ahamed et al (2017)[82] (Table 1, 2, 3, 4, 5, 6 & Graph 3).

In 1993, Shin DM et al., studied the PCNA expression in oral squamous cell carcinoma. They showed that the PCNA was increased to about four to ten times in squamous cell carcinoma when compared to the normal epithelium[90].

Girod et al (1994) in their study found that there is a steady increase of PCNA expression from normal mucosa to well differentiated squamous cell carcinoma followed by moderately differentiated squamous cell carcinoma[91]. Zain et al (1995) opined that PCNA is good marker in differentiating normal epithelium and dysplastic epithelium[12].

Abdul Khadir et al (2010) from their study suggested that the expression of PCNA was a good indicator for the aggressiveness of the lesion[79]. Madan M et al (2015) showed that PCNA was a good marker in differentiating normal epithelium from oral squamous cell carcinoma[14]. Poosarla CS et al (2015) showed that proliferation of cells were increased from normal to premalignant lesions and to oral squamous cell carcinoma and they concluded that PCNA marker was useful in predicting the aggressiveness of the lesion[78]. Keshav R et al (2015) studied the expression of PCNA in oral submucous fibrosis and concluded that PCNA was an indicator of the biological behavior of the lesion[89]. Ahamed et al (2017) opined that proliferation of cells was more in poorly differentiated followed by moderately differentiated squamous cell carcinoma and well differentiated squamous cell carcinoma[81]. The current study also showed a steady increase of PCNA expression when the grades of the oral squamous cell carcinoma is increased.

In the present study, the expression of CD57 labelling index was 2.91±0.82, 16.63±2.33, 7.09±1.41 and 5.53±1.20 in normal mucosa, WDSQCC, MDSQCC and PDSQCC respectively. It was found that the CD57 expression was increased from the normal mucosa to well differentiated squamous cell carcinoma. According to Karpathiou et al (2017), in head and neck tumours, there is a dense infiltration of cytotoxic T lymphocytes, NK cells and dendritic cells. These cells are increased in number in order to kill the tumour cells[92]. Therefore, in well differentiated SQCC the CD57 expression was increased. In moderately differentiated and poorly differentiated SQCC, the CD57 expression was decreased compared to well differentiated SQCC but increased in comparison with the normal mucosa. This clearly indicates that rich infiltration of natural

killer cells is associated with increased survival of the patient. Lack of these cells in moderately differentiated and poorly differentiated squamous cell carcinoma indicates the poor survival rate.

Fang et al (2017) opined that a high CD57 expression in the early stage of the diseases and found that strong CD57 expression in oral squamous cell carcinoma could be an independent marker for longer survival[81]. Agarwal R et al (2016) opined that CD57 mean labelling index was higher in alive patients (10.67) than dead patients (3.67). This study concluded that higher CD57 labelling index had a significant correlation with the status of the life[2]. Taghavi et al (2015) indicated that high CD57 expression was associated with longer overall survival of the patient[80]. The present study also showed a high CD57 expression in well differentiated squamous cell carcinoma and lower CD57 expression in moderately differentiated and poorly differentiated squamous cell carcinomas that are associated with the decreased survival of the patient.

Iida et al (2014) showed that increased $CD57^+$ T-cells infiltration in the tumour microenvironment was a potent prognostic marker for OSCC[77]. Zancope et al (2010) showed that infiltration of NK cells (CD57) and $CD8^+$ cells in the tumour microenvironment reflected a favorable cytotoxic immune response against malignant cells[75]. These findings were in accordance with the present study interpreting that high CD57 expression in well differentiated squamous cell carcinoma will have a better prognosis whereas low expression of CD57 in moderately differentiated followed by poorly differentiated squamous cell carcinoma have poor prognosis. (Tables 7, 8, 9, 10, 11, 12 & Graph 4)

In the present study, correlation of PCNA and CD57 labelling index within the groups are not significant (Table 13 & Graph 5) But correlation of PCNA and CD57 was found to be significant between the groups (Table 14 & Graph 6). PCNA and CD57 are found to be good indicators of aggressive nature of the lesion and the immune status of the patient respectively. The combination of PCNA and CD57 were found to be effective in identifying the patients with good or poor survival rate and thereby it helps in planning of the treatment modalities.

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

The current study was performed in the department of oral pathology and microbiology at Sree Mookambika Institute of Dental Sciences in Kulasekharam. In the present study, a total of 40 samples were included. 10 samples each of WDSQCC, MDSQCC and PDSQCC constituted the study group whereas 10 samples of normal mucosa made up the control group.

In this study, PCNA and CD57 markers were used. PCNA is a well-known proliferative marker used to identify the aggressiveness or the biological behavior of the lesion. CD57 was used to determine the immune status of the patient.

The primary aim of this study was to assess the expression of PCNA and CD57 in different grades of oral squamous cell carcinoma. An immunohistochemical study was carried out by using the primary antibodies anti-PCNA and anti-CD57 according to the manufacture's protocol. Positively stained cells were counted in 1000 tumour cells and the labelling index of PCNA and CD57 were determined. The results were analyzed by using Anova (Post hoc) followed by Dunnet t-test between the groups. Comparison of PCNA and CD57 was done by Pearson correlation coefficient test.

Highly significant increase in the PCNA labelling index was seen from normal mucosa to WDSQCC, followed by MDSQCC and PDSQCC. Maximum proliferative index was noted in PDSQCC. Similarly, highly significant decrease in CD57 labelling index was seen from WDSQCC followed by MDSQCC and PDSQCC. Maximum CD57 index was noted in WDSQCC.

On correlation with Pearson correlation coefficient test, PCNA and CD57 were found to be correlated between the groups. This clearly indicates that PCNA and CD57 are related to each other in different grades of oral squamous cell carcinoma.

It was concluded that as the grades of oral squamous cell carcinoma increases, the aggressive nature of the lesion increases and the immune status of the patient is decreased. Hence forth, the combination of PCNA and CD57 can be used as valuable markers to detect the malignant potential of the lesion and also to determine the survival rate of the patient wherein which it helps in the stratification of patient for planning of treatment modalities.

Scope for future:

Curing of cancer can be possible with the advancement of cancer immunotherapy. Recently, introduction of monoclonal antibodies (mAbs), adoptive cancer therapy, cancer vaccines and immune checkpoint therapy have replaced the traditional cancer therapy. Recognition and stratification of the patient will provide a key factor for the success of treatment. Early identification of the disease will also improve the survival rate of the patient.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Martinez-Lara I, Gonzalez-Moles MA, Ruiz-Avila I, Bravo M, Ramos MC, Fernandez-Martinez JA. Proliferating cell nuclear antigen (PCNA) as a marker of dysplasia in oral mucosa. Acta Stomatol Belg 1996;93(1):29-32.
2. Agarwal R, Chaudhary M, Bohra S, Bajaj S. Evaluation of natural killer cell (CD57) as a prognostic marker in oral squamous cell carcinoma: An immunohistochemistry study. J Oral Maxillofac Pathol 2016;20(2):173-7.
3. Mallath MK, Taylor DG, Badwe RA, Rath GK, Shanta V, Pramesh CS et al. The growing burden of cancer in India: epidemiology and social context. Lancet Oncol 2014;15(6):e205-12.
4. Gomez SL, Noone AM, Lichtensztajn DY, Scoppa S, Gibson JT, Liu L et al. Cancer incidence trends among Asian American populations in the United States, 1990-2008. J Natl Cancer Inst 2013;105(15):1096-110.
5. Alberts B. Molecular biology of the cell. 5th ed. New York: Garland Science Taylor & Francis; 2008
6. Weinberg R. The biology of cancer. 2nd ed. New York Garland Science; 2013.
7. Merlo LM, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. Nat Rev Cancer 2006;6(12):924.
8. Ingber DE. Cancer as a disease of epithelial-mesenchymal interactions and extracellular matrix regulation. Differentiation 2002;70(9-10):547-60.
9. Fraga CA, De Oliveira MV, Domingos PL, Botelho AC, Guimarães AL, Teixeira-Carvalho A et al. Infiltrating CD57+ Inflammatory Cells in Head and

Neck Squamous Cell Carcinoma: Clinico pathological Analysis and Prognostic Significance. Appl Immunohistochem Mol Morphol 2012;20(3):285-90.

10. Abo T, Balch CM. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J Immunol 1981;127(3):102-9.
11. Knapp W, Rieber P, Dorken B, Schmidt RE, Stein H, vd Borne AE. Towards a better definition of human leucocyte surface molecules. Immunol Today 1989; 10(8):253-8.
12. Zain RV, Sakamoto F, Shrestha P, Mori M. Proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - an aid to conventional histological grading. Malaysian J Pathol 1995;17(1):23-30.
13. Chiang CP, Lang MJ, Liu BY, Wang, Leu JS, Hahn LJ, Kuo MY. Expression of proliferating cell nuclear antigen (PCNA) in oral submucous fibrosis, oralepithelial hyperkeratosis and oral epithelial dysplasia in Taiwan. Oral Oncol 2000; 36(4):353-59.
14. Madan M, Chandra S, Raj V, Madan R. Evaluation of cell proliferation in malignant and potentially malignant oral lesions.J Oral Maxillofac Pathol 2015; 19(3):297-305.
15. Attar E, Dey S, Hablas A, Seifeldin IA, Ramadan M, Rozek LS, Soliman AS. Head and Neck Cancer in a Developing Country: A Population-Based Perspective across 8 Years. Oral Oncol 2010;46(8):591-96.
16. Bagan J, Sarrion G, Jimenez Y. Oral Cancer: Clinical Features, Oral Oncol 2010; 46:414-7.

17. Suciu M, Morariu SH, Ormenisan A, Grigoras RI, Bostan RH, Mocanu S et al. Oral squamous cell carcinoma of the maxilla, a second malignancy after a right ethmoido-maxillary chondrosarcoma. Rom J Morphol Embryol 2014; 55(3suppl):1247-51
18. Bommer U, Vine KL. Cancer biology: Molecular and genetic basis. 1st ed. Sydney: Cancer Council Australia; 2014.
19. Chaurasia BD, Human anatomy for dental students. 4th ed. India: CBS Publishers & Distributors; 2004.
20. Landis SH, Murray T, Bolden S, Wingo PA, Cancer statistics,1999.CA Cancer J Clin 1999;49(1):8-31.
21. Varshita A. Prevalence of oral cancer in India. J Pharm Sci Res 2015;7(10): 845-8.
22. Nevile BW, Damn DD, Allen CM, Bouquot JE. Oral and maxillofacial pathology. 2nd ed. WB Saunders Company. London.2015
23. Kawajiri K, Fujii-Kuriyama Y. P450 and human cancer. Jpn J Cancer Res 1991; 82(12):1325-35.
24. Warnakulasuriya KA, Ralhan R. Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco - a review. J Oral Pathol Med 2007; 36(2):63-77.
25. Mehta FS, Gupta PC, Daftary DK, Pindborg JJ, Choksi SK. An epidemiologic study of oral cancer and precancerous conditions among101,761 villagers in Maharashtra, India. Int J Cancer 1972; 10:134-41.

26. Kumar M, Nanavati R, Modi TG, Dobariya C. Oral cancer: Etiology and risk factors: A review. J Can Res Ther 2016;12:458-63.
27. Yadav MR. S. Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations. Indian Journal of Cancer 2006:43(2):60-6.
28. Evstifeeva TV, Zaridze DG. Nass use, cigarette smoking, alcohol consumption and risk of oral and oesophageal precancer. Eur J Cancer Oral Oncol 1992; 28B(1): 29-35.
29. Jeng JH, Chang MC, Hahn LJ. Role of areca nut in betel quid-associated chemical carcinogenesis: Current awareness and future perspectives. Oral Oncol 2001; 37(6):477-92.
30. Negri E, Franceschi S, Bosetti C, Levi F, Conti E, Parpinel M , La Vecchia C. Selected micronutrients and oral and pharyngeal cancer. Int J Cancer 2000;86(1) :122-7
31. Sankaranarayanan R, Mathew B, Varghese C, Sudhakaran PR, Menon V, Jayadeep A et al. Chemoprevention of oral leukoplakia with vitamin A and beta carotene: an assessment. Oral Oncol 1997;33(4):231-6.
32. Stryker WS, Kaplan LA, Stein EA, Stampfer MJ, Sober A, Willett WC. The relation of diet, cigarette smoking, and alcohol consumption to plasma beta-carotene and alpha-tocopherol levels. Am J Epidemiol 1988;127(2):283-96.
33. Markopoulos AK, Current aspects on Oral Squamous Cell Carcinoma. Open Dent J 2012;6:126-130.
34. Williams HK. Molecular pathogenesis of oral squamous carcinoma. Mol Pathol 2000;53(4):165-72.

35. Todd R, Donoff RB, Wong DT. The molecular biology of oral cancer: toward a tumour progression model. J Oral Maxillofac Surg 1997;55:613-23.

36. Hunter T, Pines J. Cyclins and cancer II: cyclin d and cdk inhibitors come of age. Cell 1994;79(4):573-82.

37. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of head and neck cancer. Cancer Res. 1993;53:3579-84.

38. Sugerman PB, Savage NW. Current concepts in oral cancer. Aust Dent J 1999;44:147-56.

39. Callender T, El-Nagger AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG. PRAD-1(CCND)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer 1994;74(1):152-8.

40. Bagutti C, Speight PM, Watt FM. Comparison of integrin, cadherin and catenin expressions in squamous cell carcinomas in the oral cavity. J Pathol 1998;186(1):8-16.

41. Garzino-Demo P, Carrozo M, Trusolino L, Savoia P, Gandolfo S, Marchisio PC. Altered expression of alpha 6 integrin sub unit in oral squamous cell carcinoma and oral potentially malignant lesions. Oral Oncol 1998;34(3):204-10.

42. Shinohara M, Nakamura S, Sasaki M, Kurahara S, Ikebe T, Harada T, Shirasuna K. Expression of integrins in squamous cell carcinoma of the oral cavity. Correlations with tumour invasion and metastasis. Am J Clin Pathol 1999; 111:75-88.

43. Miller JF, Sadelain M, The journey from discoveries in fundamental immunology to cancer immunotherapy, Cancer cell 2015:27(4):439-49.
44. Kaja SL, Kiran SS, Kattapagari KK, Chitturi RT, Chowdary SD, Reddy BV. A review on tumor immunology. J Oro fac Sci 2017;9:7-15.
45. Zamai L, Ponti C, Mirandola P, Gobbi G, Papa S, Galeotti L, Cocco L, Vitale M. NK cells and cancer. J Immunol 2007;178(7):4011-6.
46. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008; 27(45):5932-43.
47. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22:329-60.
48. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: From immunosurveillance to tumor escape. Nat Immunol 2002;3(11):991-8.
49. Silverman S Jr, Gorsky M, Lozada F. Oral leukoplakia and malignant transformation. A follow-up study of 257 patients. Cancer 1984; 53(3):563-8.
50. Walsh T, Liu JL, Brocklehurst P, Glenny AM, Lingen M, Kerr AR et al. Clinical assessment to screen for the detection of oral cavity cancer and potentially malignant disorders in apparently healthy adults. Cochrane Database Syst Rev 2013;11(11):1-24.
51. Bhargava A, Saigal S, Chalishazar M. Histopathological grading systems in oral squamous cell carcinoma: A review. J Int Oral Health 2010;2(4): 1-10.
52. Doshi NP, Shah SA, Patel KB, Jhabuawala MF. Histological grading of oral cancer: A comparison of different systems and their relation to lymph node metastasis. Nat J comm med 2011; 2(1):136-42.

53. Garg P, Karjodkar F. “Catch them before it Becomes Too Late” Oral Cancer Detection. Report of Two Cases and Review of Diagnostic AIDS in Cancer Detection. Int J Prev Med 2012; 3:737-41.

54. Carreras-Torras C, Gay-Escoda C. Techniques for early diagnosis of oral squamous cell carcinoma: Systematic review. Med Oral Patol Oral Cir Bucal 2015;20 (3):e305-15.

55. Suvarna KS, Layton C, Bancroft DJ. Bancrofts theory and practice of histological techniques.7th ed. China: Elsevier; 2013.

56. Lavanya R, Mamatha B, Waghray S, Chaitanya NC, Reddy MP, Bau DG. Role of tumour markers in oral cancer: An overview. BJMMR 2016;15(7):1-9

57. Malathi T. Tumor markers-An overview. Indian J Clin Biochem 2007;22(2): 17-31.

58. Kumar V, Abbas AK, Aster JC. Robbins basic pathology, Disease of the Immune system, 10th ed, Canada; Elsevier: 2018.

59. Mitsumoto Y, Oka S, Sakuma H, Inazawa J, Kawasaki T. Cloning and chromosomal mapping of human glucuronyl transferase involved in biosynthesis of the HNK-1 carbohydrate epitope. Genomics 2000; 65(2):166-73.

60. Focosi D, Bestagno M, Burrone O, Petrini M. CD57 + T lymphocytes and functional immune deficiency. J Leukoc Biol. 2010; 87(1):107-16.

61. Nielsen CM, White MJ, Goodier MR, Riley EM. Functional significance of CD57 expression on human NK cells and relevance to diseases. Frontiers in immunology 2013; 4(422):1-8.

62. Ménard C, Blay JY, Borg C, Michiels S, Ghiringhelli F, Robert C et al. Natural killer cell IFN-gamma levels predict long-term survival with imatinibmesylate therapy in gastrointestinal stromal tumor-bearing patients. Cancer Res 2009; 69(8):3563-9.

63. Turkseven MR, Oygur T. Evaluation of natural killer cell defense in oral squamous cell carcinoma. Oral Oncol 2010; 46(5):e34-7.

64. Sorskaar D, Forre O, Lie SO. Increased natural-killer cell-activity and numbers of Leu-7 and Leu-11b (Cd 16)-positive cells in bone-marrow of children in remission from acute lymphoblastic-leukemia. Scand J Immunol 1989;29(1):65-72.

65. Ortac R, Aktas S, Diniz G, Erbay A, Vergin C. Prognostic role of natural killer cells in pediatric mixed cellularity and nodular sclerosing Hodgkin's disease. Anal Quant Cytol Histol 2002; 24(5):249-53.

66. Vaquero J, Zurita M, Aguayo C, Coca S. Apoptosis is not correlated with the presence of CD57+ NK-cells in brain metastases. Acta neurochirurgica 2003;145(9):773-6.

67. Lv L, Pan K, Li XD, She KL, Zhao JJ, Wang W et al. The accumulation and prognosis value of tumor infiltrating IL-17 producing cells in esophageal squamous cell carcinoma. PLoS One 2011; 6(3):e18219.

68. Villegas FR, Coca S, Villarrubia VG, Jiménez R, Chillón MJ, Jareno J, Zuil M, Callol L. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002; 35(1):23-8.

69. Strzalka W, Ziemienowicz A. Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Ann Bot 2011;107: 1127-40.

70. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. Nature 1987;326(6112):515-7.
71. Park SY, Jeong MS, Han CW, Yu HS, Jang SB. Structural and functional insight into proliferating cell nuclear antigen. J Microbiol Biotechnol 2016; 26(4):637-47.
72. Kurokawa H, Yoshihiro Y, Ishibashi H, Kajiyama M. Immunohistochemical study of PCNA in oral squamous cell carcinoma, J Soc Oral tumors 1995; 7(1):9-14
73. Myong H, Kim JM, lee JH, Ok YJ, Paeng JY, Yun PY. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. Int J Oral Maxillofac Surg 2006; 35(11):1005-10
74. Watanabe S, Watanabe R, Oton-Leite AF, Alencar R, Oliveira JC, Leles CR, Batista AC, Mendonca EF. Analysis of cell proliferation and pattern of invasion ain oral squamous cell carcinoma. J Oral Sci 2010;52(3):417-24
75. Zancope E, Costa N, Junqueira-Kipnis AP, Valadares MC, Silva TA, Leles CR et al. Differential infiltration of CD8+ and NK cells in lip and oral cavity squamous cell carcinoma. J Oral Pathol Med 2010; 39:162-7.
76. Kato K, Kawashiri S, Yoshizawa K, Kitahara H, Okamune A, Sugiura S, Noguchi N, Yamamoto E. Expression form of p53 and PCNA at the invasive front in oral squamous cell carcinoma: correlation with clinicopathologial features and prognosis. J Oral Pathol Med 2011;40:693-8.

77. Iida M, Takayama E, Naganawa K, Mitsudo K, Adachi M, Baba J, Fujimoto-Muto M. Increase of Peripheral Blood CD57+T-Cells in Patients with Oral Squamous Cell Carcinoma. Anticancer Res 2014; 34: 5729-34.

78. Poosarla CS, Ramesh M, Ramesh K, Gudiseva S, Bala S, Sundar M. Proliferating cell nuclear antigen in premalignancy and oral squamous cell carcinoma. J Clin Dia Res 2015;9(6):39-4.

79. Abdulkadir SN, Ali NR, Alchalabi NJ. Pathological Study of Oral Squamous Cell Carcinoma by Application of P53 and PCNA (Immuno histochemical Approach). Int J Curr Microbiol App Sci 2016;5(4):91-100.

80. Taghavi N, Bagheri S, Akbarzadeh A. Prognostic implication of CD57, CD16, and TGF-β expression in oral squamous cell carcinoma. J Oral Pathol Med 2016;45:58-62.

81. Fang J, Li X, Ma D, Liu X, Chen Y, Wang Y et al. Prognostic significance of tumor infiltrating immune cells in oral squamous cell carcinoma. BMC Cancer 2017;17(1):375.

82. Ahmed S, Desai D, Chandrappa PR, Pandit S, Shetty J, Suresh. A comparative study of AgNOR with PCNA immunohistochemical staining in oral squamous cell carcinoma. Int J Curr Res 2017;9(5): 49930-34.

83. Maciel TAO, Serpa MS, Mafra RP, Goes Gonzaga AK, De Souza LB, Pinto LP. Immunohistochemical Analysis of Natural Killer Cells and CD8+ T Lymphocytes in Lower Lip Squamous Cell Carcinoma. J Clin Dia Res 2017;11(12):22-5

84. Stelin S, Ramakrishan H, Talwar A, Arun KV, Kumar TS. Immunohistological analysis of CD1a+ langerhans cells and CD57+ natural killer cells in healthy

and diseased human gingival tissue: A comparative study. J Indian Soc Periodontol 2009;13(3):150-54.

85. Feller L, Lemmer J. Oral Squamous Cell Carcinoma: Epidemiology, Clinical Presentation and Treatment. Journal of Cancer therapy 2012;3:263-8.
86. Lee JJ, Kuo MY, Cheng SJ, Chiang CP, Jeng JH, Chang HH et al. Higher expressions of p53 and proliferating cell nuclear antigen(PCNA) in atrophic oral lichen planus and patients with areca quid chewing. Oral Surg Oral Med Oral Pathol Endod 2005; 99: 471-8.
87. Suji T, Sasaki K, Kimura Y, Yamada K, Mori M, Shinozaki F. Measurement of PCNA and its clinical applications in oral cancers. Int J Oral Maxillofac Surg 1992;21:369-72.
88. Nagpal M, Singh S, Singh P, Chauhan P, Zaidi MA. Tumor markers: A diagnostic tool. Natl J Maxillofac Surg 2016;7:17-20.
89. Keshav R, Narayanappa U. Expression of Proliferating Cell Nuclear Antigen in Oral Submucous fibrosis, J Clin Dia Res 2015;9(5):20-23.
90. Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN. Sequential increases in PCNA expression in head and neck tumourigenesis - a potential biomarker. J Natl Cancer 1993;85:971-8.
91. Girod SC, Pape HD, Gerhard RF. Krueger p53 and PCNA expression in carcinogenesis of oropharyngeal mucosa. Oral Oncology 1994; 30(6):419.
92. Karpathiou G, Casteillo F, Giroult JB, Forest F, Fournel P, Monaya A et al. Prognostic impact of microenvironment in laryngeal and pharyngeal squamous cell carcinoma: Immune cell subtypes, immune-suppressive pathways and clinicopathologic characteristics. Onco target 2017;8(12):19310-22.

ANNEXURE

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

KULASEKHARAM, KANYAKUMARI DIST., TAMIL NADU, INDIA.

INSTITUTIONAL RESEARCH COMMITTEE

Certificate

This is to certify that the research project protocol, ***Ref no. 07/09/2017*** titled, ***"Comparative analysis of CD 57 and Proliferating cell nuclear antigen (PCNA) expression in Oral squamous cell carcinoma – an immunohistochemical study"*** submitted by ***Dr. C. S. Ani Simila, II Year MDS, Department of Oral Pathology and Microbiology*** has been approved by the Institutional Research Committee at its meeting held on ***26th September 2017.***

Convener
Dr. T. Sreelal

Secretary
Dr. Pradeesh Sathyan

INSTITUTIONAL HUMAN ETHICS COMMITTEE

SREE MOOKAMBIKA INSTITUTE OF MEDICAL SCIENCES,
KULASEKHARAM, TAMILNADU

Communication of Decision of the Institutional Human Ethics Committee(IHEC)

SMIMS/IHEC No: 2 /Protocol no: 4 / 2017

Protocol title: COMPARATIVE ANALYSIS OF CD 57 AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY.
Principal Investigator: Dr.C.S.Ani Simila
Name& Address of Institution: Department of Oral Pathology and Microbiology Sree Mookambika Institute of Dental Sciences [✓] New review [ ] Revised review [ ] Expedited review
Date of review (D/M/Y): 04-12-2017 Date of previous review , if revised application:
Decision of the IHEC: [✓] Recommended [ ] Recommended with suggestions [ ] Revision [ ] Rejected
Suggestions/ Reasons/ Remarks:
Recommended for a period of : One year

Please note*

- Inform IHEC immediately in case of any Adverse events and Serious adverse events.
- Inform IHEC in case of any change of study procedure, site and investigator
- This permission is only for period mentioned above. Annual report to be submitted to IHEC.
- Members of IHEC have right to monitor the trial with prior intimation.

Reneeyagangadharan

Signature of Member Secretary (IHEC)

SREE MOOKAMBIKA INSTITUTE OF DENTAL SCIENCES

PADANILAM, KULASEKHARAM

DEPARTMENT OF ORAL PATHOLOGY & MICROBIOLOGY

CASE RECORD SHEET

OP No : **HP No :**

NAME : **AGE:** **SEX :**

ADDRESS : **PHONE NO :**

CLINICAL DETAILS

SITE :

SIZE :

APPEARANCE :

DURATION :

ADVERSE HABITS :

PROVISIONAL DIAGNOSIS :

HISTOPATHOLOGICAL REPORT :

FINAL DIAGNOSIS :

Comparative analysis of CD57 and PCNA expression in Oral squamous cell carcinoma - An immunohistochemical study

DATA ENTRY SHEET

Specimen number : Date:

Specimen category : a) Normal ☐

b) Well differentiated squamous cell carcinoma ☐

c) Moderately differentiated squamous cell carcinoma ☐

d) Poorly differentiated squamous cell carcinoma ☐

ESTIMATION OF CD57

PARAMETERS	HIGH FIELD MAGNIFICATION (40x)		
	Observation 1	Observation 2	Average
Positively stained cells			

CD57 labelling index = $\frac{Total\ number\ of\ positively\ stained\ cells}{1000} x100$

ESTIMATION OF PCNA

PARAMETERS	HIGH FIELD MAGNIFICATION (40x)		
	Observation 1	Observation 2	Average
Positively stained cells			

PCNA labelling index = $\frac{Total\ number\ of\ positively\ stained\ cells}{1000} x100$

COMPARATIVE ANALYSIS OF CD57 AND PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA-AN IMMUNOHISTOCHEMICAL STUDY

DATA ANALYSIS SHEET

Groups	Specimen No	DEMOGRAPHIC DETAILS				EXPERIMENTAL DATA	
		Age	Sex	Site	Habits	PCNA labelling Index	CD57 labelling index
Normal Mucosa	1	39	M	Gingiva	-	24.2	2.8
	2	50	M	Right buccal mucosa	-	26.4	1.7
	3	42	F	Gingiva	-	27.8	2.3
	4	53	M	Left buccal mucosa	-	25.6	2.9
	5	38	M	Gingiva	-	28.1	1.6
	6	28	M	Pericoronal tissue	-	25.3	3.2
	7	36	F	Gingiva	-	24.8	3.5
	8	34	F	Gingiva	-	26.5	4.1
	9	64	F	Left alveolar region	-	27	3.7
	10	45	M	Gingiva	-	26.2	3.3

Groups	Specimen No	DEMOGRAPHIC DETAILS				EXPERIMENTAL DATA	
		Age	Sex	Site	Habits	PCNA labelling Index	CD57 labelling index
WDSQCC	1	60	M	Right buccal vestibule	Tobacco chewing	47.2	20.1
	2	50	F	Left buccal mucosa	Tobacco chewing	43.2	15.2
	3	68	M	Left buccal mucosa	Betel nut chewing	46.4	19.5
	4	39	M	Left lateral border tongue	Tobacco chewing	44.7	12.4
	5	72	M	Lower buccal mucosa	Tobacco chewing	49.8	17.2
	6	60	F	Right buccal mucosa	Pan chewing	45.2	16
	7	70	M	Right buccal mucosa	Tobacco chewing	48.2	18.1
	8	47	M	Right buccal mucosa	Tobacco chewing	42.4	14.5
	9	55	M	Left dorsal surface of the tongue	Pan chewing	45.7	15.8
	10	48	M	Left buccal mucosa	Betel nut chewing	46	17.5

Groups	Specimen No	DEMOGRAPHIC DETAILS				EXPERIMENTAL DATA	
		Age	Sex	Site	Habits	PCNA labelling Index	CD57 labelling index
MDSQCC	1	55	M	Right buccal mucosa	Tobacco chewing	58.6	6.7
	2	60	M	Right lateral border tongue	No habits	60.4	10.2
	3	55	F	Right buccal mucosa	Tobacco chewing	59.3	7.5
	4	75	M	Right labial mucosa	Pan chewing	58.5	5.7
	5	53	M	Right buccal mucosa	Tobacco chewing	59.8	6.1
	6	64	F	Left buccal mucosa	Tobacco chewing	58.4	6.7
	7	63	M	Left buccal mucosa	Tobacco chewing	58.1	7.6
	8	58	F	Right buccal mucosa	Tobacco chewing	61.3	5.1
	9	57	F	Right buccalmcosa	Tobacco chewing	60.3	7.5
	10	74	M	Right lateral border tongue	No habits	59.1	7.8

Groups	Specimen No	DEMOGRAPHIC DETAILS				EXPERIMENTAL DATA	
		Age	Sex	Site	Habits	PCNA labelling Index	CD57 labelling index
PDSQCC	1	55	F	Right buccal mucosa	Tobacco chewing	72.7	7.3
	2	35	M	Right lateral border tongue	Tobacco chewing	76.3	3.9
	3	53	M	Right buccal mucosa	Tobacco chewing	80.5	4.4
	4	52	F	Left buccal mucosa	Betel nut chewing	68	4.7
	5	60	F	left buccal mucosa	Tobacco chewing	75.8	5.3
	6	56	M	Right buccal mucosa	Tobacco chewing	73.5	6.5
	7	70	F	Left lateral border tongue	No habits	71.9	4.2
	8	55	M	Right buccal mucosa	Tobacco chewing	69.5	5.9
	9	67	M	Left buccal mucosa	Tobacco chewing	74	6.1
	10	54	F	Right buccal mucosa	Tobacco chewing	65.5	7