ANALYSIS OF CASPASE-3 ENZYME MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA - AN IMMUNOHISTOCHEMICAL STUDY

A Dissertation submitted
in partial fulfilment of the requirements
for the degree of

MASTER OF DENTAL SURGERY

BRANCH – VI

ORAL PATHOLOGY AND MICROBIOLOGY

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY
CHENNAI- 600032

2015 – 2018

CERTIFICATE

This is to certify that the Dissertation entitled **“ANALYSIS OF CASPASE-3 ENZYME MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA - AN IMMUNOHISTOCHEMICAL STUDY"** by **Dr. K. SHARIFA** Post Graduate student **MDS Oral Maxillofacial Pathology and Microbiology**, Madha Dental College & Hospital-Chennai - 69. Submitted to Tamilnadu Dr. M.G.R. Medical University the MDS Degree Examination April 2018 is bonafide research work carried out by her under my super vision and guidance

Guided By

Dr. G. SIVAKUMAR, M.D.S.,

Professor and Head

Department of Oral Maxillofacial Pathology & Microbiology

Madha Dental College & Hospital,

Chennai - 600 069

MADHA DENTAL COLLEGE AND HOSPITAL

CHENNAI - 600 069.

ENDORSEMENT BY HEAD OF THE DEPARTMENT / HEAD OF THE INSTITUTION

This is to certify that the Dissertation entitled **"ANALYSIS OF CASPASE-3 ENZYME EXPRSSION IN ORAL SQUAMOUS CELL CARCINOMA-AN-IMMUNOHISTOCHEMICAL STUDY"** is a bonafide work done by **Dr. SHARIFA. K**, Post Graduate student (2015-2018) in the Department of Oral and Maxillofacial Pathology, under the guidance of **Dr. G. SIVAKUMAR, MDS.,** Professor, Department of Oral and Maxillofacial Pathology, Madha Dental College and Hospital, Chennai – 600 069.

Dr. G. SIVAKUMAR, M.D.S.
Professor and Head
Department of Oral Maxillofacial Pathology & Microbiology

Dr. M.C. SAINATH, M.D.S.
PGDCR, MBA
Principal

MADHA DENTAL COLLEGE AND HOSPITAL
CHENNAI - 600 069.

DECLARATION

I, **Dr. K. SHARIFA**, do hereby declare that the Dissertation titled **"ANALYSIS OF CASPASE-3 ENZYME MARKER EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA – AN IMMUNOHISTOCHEMICAL STUDY"** was done in the Department of Oral Maxillofacial Pathology & Microbiology, Madha Dental College & Hospital, Chennai - 600 069. I have utilized the facilities provided in the Madha Dental College for the study in partial fulfillment of the requirements for the degree of Master of Dental Surgery in the specialty of Oral Maxillofacial Pathology & Microbiology (Branch VI) Madha Dental College, Cheenai-69 during the course period **2015-2018** under the conceptualization and guidance of my dissertation guide, **Professor Dr. G. SIVAKUMAR, MDS.**

I declare that no part of the Dissertation will be utilized for gaining financial assistance for research or other promotions without obtaining prior permission from Department of Oral & Maxillo Facial Pathology, Madha Dental College & Hospital.

I also declare that no part of this work will be published either in the print or electronic media except with those who have been actively involved in this dissertation work and I firmly affirm that the right to preserve or publish this work rests solely with the prior permission of the Principal, Madha Dental College & Hospital,

Chennai 600 069, but with the vested right that I shall be cited as the author(s).

Signature of the PG student

ACKNOWLEDGEMENT

I would like to extend my sincere gratitude and thanks to our beloved **Chairman, Managing Director and Vice Chairperson** of Madha Dental College and Hospital, Kundrathur, Chennai -69 for their continuous support, help and encouragement during my post graduation to accomplish my academic carrier.

My sincere and heartfelt thanks to **Dr. M.C. SAINATH, M.D.S.,** our Principal and Professor of Orthodontics, Madha Dental College and Hospital, Chennai – 69, for his continuous and enormous support and for his constant encouragement and advice.

I express my deep sense of gratitude and great honour to my respected guide, Professor **Dr. G. SIVAKUMAR MDS.,** Head of Department of Oral Maxillofacial Pathology, Madha Dental College & Hospital, Chennai - 69, for his astute, conceptualization guidance, support and encouragement throughout my post graduate course and to bring this dissertation to a successful completion.

I owe my thanks and great honour to **Dr. R. SARANGARAJAN, M.D.S.,** Professor, Department of Oral Maxillofacial Pathology, Madha Dental College & Hospital, Chennai - 69, for helping me with his valuable and timely suggestions and encouragement.

I owe my thanks and great honour to **Dr. K.T. SHANMUGAM, M.D.S.,** Reader, Department of Oral Maxillofacial

Pathology, Madha Dental College & Hospital, Chennai - 69, for helping me with his valuable and timely suggestions and encouragement.

I owe my thanks and great honour to **Dr. M. SIVAKUMAR, M.D.S.,** Reader, Department of Oral Maxillofacial Pathology, Madha Dental College & Hospital, Chennai - 69, for helping me with his valuable and timely suggestions and encouragement.

I owe my thanks and great honour to **Dr. G.KESEVAN, M.D.S.,** Sr.Lecture, Department of Oral Maxillofacial Pathology, Madha Dental College & Hospital, Chennai - 69, for helping me with his valuable and timely suggestions and encouragement.

I owe my thanks and great honour to **Dr. R. RAGHINI, M.D.S.,** Sr. Lecture, Department of Oral Maxillofacial Pathology, Madha Dental College & Hospital, Chennai - 69, for helping me with her valuable and timely suggestions and encouragement.

I take this opportunity to express my gratitude to my friend’s colleagues and my family members for their valuable help and suggestions throughout this study.

I seek the blessing of the almighty God without whose benevolence the study would not have been possible.

TRIPARTITE AGREEMENT

This agreement herein after the “Agreement” is entered into on this..... day of January 2018 between the Madha Dental College and Hospital represented by its **Principal** having address at Madha Dental College and Hospital, Chennai-69, (hereafter referred to as , ’the college’)

And **Dr. G. SIVAKUMAR** aged 46 years working as professor and HOD at the College, having residence address at 25A, 3rd Cross Street, Shenoy Nagar,Chennai-600030, Tamilnadu (Herein after referred to as the ‘Principal Author’)

And **Dr. K. SHARIFA** aged 39 years currently studying as postgraduate student in Department of Oral Maxillofacial Pathology & Microbiology in Madha Dental College and Hospital (Herein after referred to as the ‘PG/Research student and Co- Author’).

Whereas the ‘PG/Research student as part of his curriculum undertakes to research **"CASPASE 3 EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMA"** for which purpose the PG/Principal author shall act as principal author and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG/Research student as to the extent possible as a Co-Author.

Whereas the parties, by this agreement have mutually agreed to the various issues including in particular the copyright and confidentiality issues that arise in this regard.

Now this agreement witnesses as follows:

1. The parties agree that all the Research material and ownership therein shall become the vested right of the college, including in particular all the copyright in the literature including the study, research and all other related papers.
2. To the extent that the college has legal right to do go, shall grant to license or assign the copyright do vested with it for medical and/or commercial usage of interested persons/entities subject to a reasonable terms/conditions including royalty as deemed by the college.
3. The royalty so received by the college shall be shared equally by all the parties
4. The PG/Research student and PG/Principal Author shall under no circumstances deal with the copyright, Confidential information and know – how generated during the course of research/study in any manner whatsoever, while shall sole vest with the manner whatsoever and for any purpose without the express written consent of the college.
5. All expenses pertaining to the research shall be decided upon by the principal investigator/Co-Author or borne sole by the PG/research student (Co-Author).

6. The college shall provide all infrastructure and access facilities within and in other institutes to the extent possible. This includes patient interactions, introductory letters, recommendation letters and such other acts required in this regard.
7. The principal author shall suitably guide the student Research right from selection of the Research Topic and Area till its completion. However the selection and conduct of research, topic and area research by the student researcher under guidance from the principal author shall be subject to the prior approval, recommendations and comments of the Ethical Committee of the college constituted for this purpose.
8. It is agreed that as regards other aspects not covered under this agreement, but which pertain to the research undertaken by the student Researcher, under guidance from the Principal author, the decision of the college shall be binding and final
9. If any dispute arises as to the matters related or connected to this agreement herein, it shall be referred to arbitration in accordance with the provisions of the Arbitration and Conciliation Act, 1996.

In witness whereof the parties hereinabove mentioned have on this the day month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its

Principal **PG Student**

Witnesses **Student Guide**

1.

2.

ABSTRACT

BACKGROUND

Oral cancer is the most common malignancy of head and neck region its ranking 6th position of all malignancy. The most commonly occuring sites are tongue, buccal mucosa, floor of the mouth, lip and gingiva. Smoking tobacco and pan chewing are the etiological factor for the occurence of oral squamous cell carcinoma. Inhibition or imbalance of apoptosis or programmed cell death leads to cancer. Two pathways are involved in apoptosis 1. Intrinsic or mitochondrial pathway 2. Extrinsic pathway. Caspase-3 enzymes or involved in the apoptosis.

AIM

To Study the Immunohistochemical expression of caspase-3 in oral squamous cell carcinoma.

MATERIALS AND METHODS

In our present study, totally 25 samples were included to detect immunohistochemical expression of caspase-3. Twenty, formalin fixed, paraffin embedded wax tissues histopathologically diagonsed oral squamous cell carcinoma were included as case (group I). Five normal buccal and gingival tissue formalin fixed, paraffin embedded wax tissues included as control (groupII). Tissues were sectioned 0.5micron thickness and stained with caspase-3 antibody. The results were obtained and statistically analyzed by using Mann-Whitney test.

RESULTS

Increased expression of caspase-3 was seen in epithelium and connective tissue of oral squamous cell carcinoma tissue. In most of the normal mucosa expression of caspase-3 was absent. The p value for epithelium and connective tissue was $p<0.008$ and $p<0.016$ respectively which was highly significant.

CONCLUSION

The results of our present study demonstrated that increased caspase-3expression was seen in oral squamous cell carcinoma tissues than that of the normal mucosa. These findings indicate the caspase-3 expression response to prognosis of the cancer and this has to be explored in a larger sample for further studies.

KEY WORDS

Apoptosis, Caspase-3, Oral cancer.

CONTENTS

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most frequent oral malignancy of head & neck accounting for 90% of all malignancies, [1, 2] and often present a clinical diagnostic challenge, particularly in its early stages of development. The incidence of OSCC differs widely in various parts of the world, and ranges from 2-10 per 1, 00,000 in a year.

The South Asian countries such as Srilanka, India, Pakistan and Bangladesh have shown a high incidence rate [3, 4]. Despite advances in prevention, diagnosis, and treatment, the 5-year survival rate has not improved over the past 30 years. [5] Surveys in India have shown that about 80% of oral carcinomas are preceded by oral potentially malignant oral disorders, most often, persistent leukoplakia (the most common potentially malignant lesion of the oral mucosa or oral sub mucous fibrosis (OSMF), which predominantly affects the people of South-East origin.[6]

The most common causes for the development of oral Squamous cell carcinoma were use of tobacco or betel quid and the regular consumption of alcoholic beverages. Infection of human papillomavirus (HPV) geno-types, and person eating less amount of fresh fruits and vegetables were also causative of occurence of oral Squamous cell carcinoma [7,8]. The mutagenic effects of tobacco, alcohol, betel quid or areca-nut are dependent upon dose, frequency and duration of use,

and are accelerated by the concurrent use of two or more of these agents [8].

Compare to women men were most commonly affected by oral squamous cell carcinoma because of high risk habits. The probability of developing oral Squamous cell carcinoma increases with the period of exposure to risk factors, and increasing age which adds the further dimension of age-related mutagenic and epigenetic changes.

The ventral surface of the tongue and the floor of the mouth are the most common sites affected by squamous cell carcinoma because they are lined by thin non-keratinized epithelium. Carcinogens readily penetrate this thin epithelium to reach the progenitor cell compartment, as well as carcinogens such as tobacco products and alcohol in solution, constantly accumulate in the floor of the mouth and bathe the tissues of the floor of the mouth and the ventral aspect of tongue [9]. The stage of advancement of oral Squamous cell carcinoma at the time of diagnosis is the most important prognostic factor [10].

Oral Squamous cell carcinoma is most frequently diagnosed late in the course of the disease because affected persons fail to seek timely professional advice, either because they do not understand the significance of early signs and symptoms, or because they are ignorant of the health implications. The course of oral Squamous cell carcinoma is unpredictable, but the TNM stage (T-tumor size, N-nod al

metastasis, M-distant metastasis) of the primary tumor correlates well with the survival rate [8].

In general, Squamous cell carcinomas of the posterior part of the oral cavity are more likely to metastasize to regional lymph nodes than squamous cell carcinomas of the anterior part of the oral cavity [7]. Small well-differentiated, low-grade oral Squamous cell carcinomas usually metastasize to regional lymph nodes only after invading connective tissue, muscle or bone. On the other hand, poorly-differentiated, high-grade oral Squamous cell carcinomas are biologically more aggressive and tend to metastasize to regional lymph nodes early in the course of the disease. [7]

In the last 30 years, the 5-year survival rate of patients with oral Squamous cell carcinoma has not improved despite advances in diagnostic techniques and improvements in treatment modalities. Indeed, the incidence and prevalence of oral SCC are increasing, particularly in younger persons [9].

Apoptosis or Programmed Cell Death (PCD) was first described by Kerr et al. (11) which are characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to neighbors or to extracellular matrix (12). Biochemical changes include chromosomal DNA cleavage in to internucleosomal fragments, phosphatidylserine externalization and a

number of intracellular substrate cleavages by specific proteolysis [13, 14].

Apoptosis (type I PCD) is the major type of cell death that occurs when DNA damage is irreparable two pathways initiate apoptosis, both of which activate the executioner caspases 3, 6, and 7. The first pathway is the intrinsic or mitochondrial, pathway where the mitochondria control the initiation of apoptosis.

Apoptotic stimuli induce the release of cytochrome c and other apoptotic regulators from the intermembrane space of mitochondria. In the cytosol, cytochrome c, APAF-1, ATP, and the initiator procaspase 9 forms the apoptosome, which affects the cleavage of effectors caspases. The Bcl-2 family of proteins regulates the mitochondrial permeability.

The second pathway is known as the extrinsic pathway, which is mediated by various death receptors on the cell surface. These receptors, once activated by specific ligands, initiate the recruitment of FAS-associated death domain protein and procaspases 8 and 10 to the death domain, which forms the death inducing signal complex and promotes the activation of caspase 8. [15]

Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain the cell populations in tissues.

Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents. [16]

Although there are a wide variety of stimuli and conditions, both physiological and pathological, that can trigger apoptosis, not all cells will necessarily die in response to the same stimulus. Irradiation or drugs used for cancer chemotherapy results in DNA damage of some cells, which can lead to apoptotic death through a *p53*-dependent pathway. Some hormones, such as corticosteroids, may lead to apoptotic death of some cells (e.g. thymocytes) although other cells are unaffected or even stimulated.

Some cells express Fas or TNF receptors that can lead to apoptosis via ligands binding and protein cross-linking. Other cells have a default death pathway that must be blocked by a survival factor such as a hormone or growth factor. There is also the issue of distinguishing apoptosis from necrosis, two processes that can occur independently, sequentially, as well as simultaneously.[17]

In some cases it's the type of stimuli and/or the degree of stimuli that determines the cells death by apoptosis or necrosis. At low doses, several of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis but these same stimuli can cause necrosis at higher doses. Finally, apoptosis is a coordinated and often energy-dependent process that involves the activation of a

group of cysteine proteases called “caspases” and a complex cascade of events that link the initiating stimuli to the final demise of the cell.

Caspases (**c**ysteinal**asp**artate-specific prote**ases)** are enzymes which utilize a catalytic cysteine to cleave their peptide substrates after specific aspartate residues. The first caspase was discovered in 1992 and because of its function was named interleukin-1-“converting enzyme (ICE) but was later renamed to caspase-1. In 1993, Ced-3 from *C. elegans* was found to be homologous to ICE and the corresponding human protein CPP32 (later named caspase-3) was found in 1994. The official caspase nomenclature was decided on in 1996 to alleviate the confusion that went along with the discovery of ten different caspases (some with multiple names). [18]

Caspases are divided into two main categories based on their function: apoptotic caspases and inflammatory caspases. The apoptotic caspases are further divided into two categories based on the time of entry into the apoptotic cascade: initiator caspases and effector caspases.

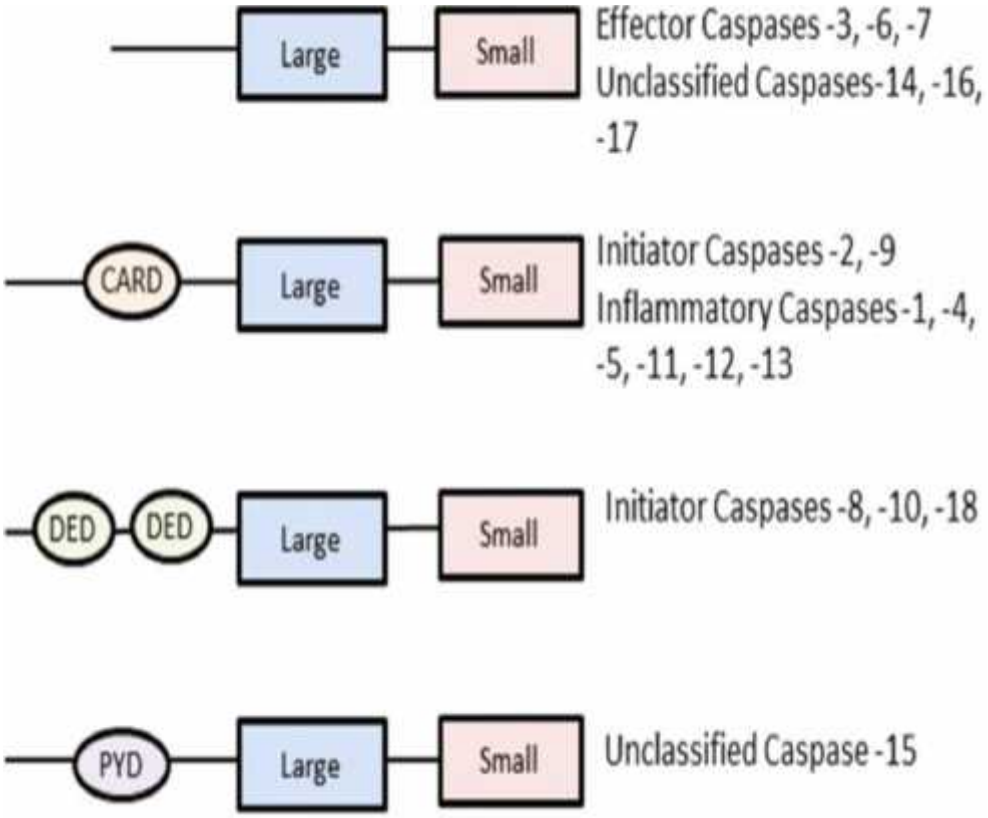


Fig1; Domain arrangement of mammalian caspases

The effectors caspases-3, -6, and -7, are found as inactive dimers in the cell. They are activated once an initiator caspase cleaves their intersubunit linkers. Because they do not require death scaffolds for dimer formation, their prodomains are short and lack the CARD (caspase activation and recruitment domain) or DED (death effectors domain), domains typical of initiator caspases. Their prodomains are, however, likely to be involved in targeting within the cell.[19]

The activation of caspases commits the cell to apoptosis. The main hallmarks of apoptosis include rounding of cells , retraction from neighbors, membrane blebbing to form vesicles called apoptotic bodies, nuclear fragmentation, chromatin condensation, hydrolysis of genomic

DNA to approximately 200 bp fragments, and translocation of phosphatidylserine (PS) to the external surface of cells as an “eat me” signal to phagocytes.

The apoptotic caspases are necessary for conferring all of these phenotypes. In addition to the systematic dismantling of the cell, caspases are also involved in producing “find-me” signals to cause chemo taxis of phagocytes to apoptotic cells [20]. The recruitment of phagocytes keeps the cells from releasing their contents into extracellular space and activating an immune response which could be harmful to the tissue.

When the number of apoptotic cells is too great for consumption by phagocytes, Secondary necrosis can occur. When this happens, the cell releases its contents into the extracellular space. However, immune cells are somehow able to recognize the cells undergoing apoptosis (and secondary necrosis) differently from the necrotic cells. This is likely due to the action of caspases. Caspases keep danger-associated molecular patterns (DAMPs) and alarming from being activated. [21] This can be thought of as a “tolerate me” signal.

Caspases are also involved in turning off transcription and translation. This keeps any infecting viral particles from replicating using the host’s machinery and they also fragment the Golgi, ER, and mitochondria. [22] Caspase-3 activity was found to be important for

differentiation of erythroblasts, skeletal muscle, bone marrow stromal stem cells, and neural stem cells.

Caspase-3 has several other non-apoptotic functions in nerve cells. In addition to neural cell differentiation, caspase-3 has also been implicated in neuronal migration and plasticity, axon pruning, and synapse elimination .Caspases have been shown to play a role in cell migration and invasion under certain circumstances. They can also induce the neighboring cells to proliferate and the replace dying cells in a process called apoptosis-induced proliferation. [23] These roles of caspases have implications for cancer such as moderate activation of caspases could, cause cancer to progress rather than regress.

Caspase-3 is a member of the cysteine protease family and plays a critical role in the regulation of programmed cell death (apoptosis). Caspase-3 expression has been extensively studied in many cancers and the positive correlation between its expression and a favorable prognosis has been reported in several cancers, suggesting its use as a prognostic marker for cancers. In oral squamous cell carcinomas (OSCCs), increased caspase-3 expression has been shown to inversely correlate with cell differentiation.[24]

AIM AND OBJECTIVES

- To evaluate the expression of caspase 3 enzyme marker in oral squamous cell carcinoma.
- To evaluate the expression of caspase3 enzyme marker in normal oral mucosa.
- To compare the expression of caspase3 enzyme marker in oral squamous cell carcinoma and normal oral mucosa.
- To aid in assessing the prognosis of the oral squamous cell carcinoma and its adjuvant role in therapy

MATERIALS AND METHODS

STUDY DESIGN:

A cross sectional study was performed to determine the expression of caspase3 enzyme marker in Oral squamous cell carcinoma tissue. These tissues were obtained from twenty (20) patients with lesions clinically and histopathologically confirmed of Oral squamous cell carcinoma and Five (5) healthy individuals comprised the control group.

STUDY SUBJECTS:

Twenty (20) cases of oral squamous cell carcinoma were selected and constituted as Group I. A predetermined clinical case sheet was used to record all the cases and a detailed case history including age, sex, occupation, past medical and dental history along with the history of habits and drugs were recorded.

This was followed by general examination and intra oral examination. Incisional biopsy of sufficient width and depth to ensure inclusion of connective tissue from the affected mucosa was done and transferred to 10% buffered formalin. Only lesions, which were diagnosed histo pathologically as squamous cell carcinoma, were included in the study.

Five (5) patients who reported to the outpatient department of Oral and Maxillofacial Surgery for removal of impacted third molars

constituted the control group Group II. Normal buccal and gingival mucosa adjacent to the site of surgery was biopsied. The tissues taken were immediately transferred to 10% buffered formalin for further processing and after adequate fixation; paraffin blocks of the tissues were made.

Informed consent was obtained from all the subjects enrolled in the study and Institutions of Ethical Committee permission was obtained.

IMMUNOHISTOCHEMISTRY PROCEDURE:

Armamentarium:

- Microtome
- Autoclave
- Hot air oven
- Coupling jars
- Measuring jar
- Refrigerator
- Weighing machine
- Cyclomixer
- APES coated slides [3 –Amino Propyl tri Ethoxy Silane]
- Slide carrier
- Slide warmer
- Aluminum foil
- Micropipettes
- Tooth forceps
- Electronic timer
- Beakers
- Rectangular steel trays with glass rods
- Sterile gauze
- Cover slips
- Light microscope

Reagents for IHC

1. Conc.HCL
2. Laxbro solution
3. Acetone
4. Citrate buffer [Ph-6]
5. TRIS buffer saline [Ph 7.2-7.4]
6. 3 %(H_2O_2)
7. Deionized distilled water
8. Haematoxylin
9. Eosin
10. Alcohol (70%)
11. Absolute alcohol
12. Xylene

Antibodies used:

1. Primary antibody

   Monoclonal mouse anti caspase 3(Cell Signalling)

2. Secondary antibody

   Citrate buffer

3. Chromogen DAB (3- Diaminobenzidine Tetra Hyrochooride)

PROCEDURE:

Pre made APES coated slides were used. Tissue section of 0.5 micron thickness were made in a rotary semi automatic microtome and the ribbon of tissue sections were transferred on to the APES [3 Amino Propyl tri –Ethoxy Silane] coated slide from the tissue floatation bath such that two tissue bits comes on to the slide with a space in between. One of the tissue sections was labeled positive [P] control and the other as negative [N] control.

IMMUNOHISTOCHEMISTRY PROCEDURE:

The slides with the tissue section were treated with three changes of xylene to remove paraffin wax and then they were put in descending grades of alcohol and later rehydrated with water. Then the slides were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 minutes. The slides were washed in two changes of distilled water and were dipped in 3 changes of Tri-Phosphate buffer saline (TBS) for 5 minutes each. The slides were then wiped carefully without touching the tissue section with gauze in order to remove excess TBS. Circles were drawn around the tissues, so that the

antibodies added later do not spread and are restricted to the circle. Blocking reagent was added to both the specimens and incubated for 5 minutes. The sections taken out were washed in three changes of cold TBS for 5 minutes in each. Then the slides were wiped carefully without touching the tissue section to remove excess TBS. The primary antibody, CASPASE 3 Mouse Monoclonal Antibody was added to [P] positive tissue on the slide and then TBS was added to the [N] negative tissue. The slides were incubated for 1 hour.

The sections taken out were washed in three changes of cold TBS for 5 minutes in each to remove the excess antibody. Then the slides were wiped carefully without touching the tissue section to remove excess TBS. Then a drop of biotnylated link from secondary antibody kit was added on both the sections and the slides were incubated for 20 minutes. Later slides were washed in three changes of cold TBS for 5 minutes each and were wiped carefully without touching the tissue section to remove excess TBS. Then a drop of Streptavidin- Horse Radish Peroxidase (HRP) from the secondary antibody kit was added on both the sections and the slides were incubated for 10 minutes. The sections were washed in three changes of cold TBS for 5 minutes in each and were wiped carefully without touching the tissue section to remove excess TBS.

Then a drop of freshly prepared DAB (3-Diaminobenzidine Tetra Hydrochloride – a substrate chromogen) was added on both sections and was then washed in running distilled water to remove excess DAB

and counter stained with hematoxylin. The slides were placed in a tray with tap water for bluing. Then the slides were transferred to 70% alcohol 100% alcohol and two changes of xylene. The tissue sections were then mounted with DPX and were then observed under the microscope. Throughout the procedure, care was taken not to dry the tissues.

POSITIVE CONTROL:

Tonsil carcinoma specimen tissues were fixed, processed, embedded, sectioned and stained in the same manner and was used as a positive control. One positive control tissue slide was included for each batch of staining.

IHC PROCEDURE FLOW CHART:

APES coated slides with 2 paraffin embedded tissues

↓

Placed in xylene thrice (5 minutes each)

↓

Placed in 100% isopropanol (5 minutes)

↓

Placed in 70% isopropanol (5 minutes)

↓

Washed in distilled water thrice (5 minutes)

↓

Placed in 3% hydrogen peroxide (20 minutes)

↓

Washed in distilled water thrice (5 minutes each)

↓

Kept in a citrate buffer Ph 6 and then autoclaved at 21°c with 121psi for Antigen retrieval followed by bench cooling for 40 minutes

↓

Washed in TBS thrice (5 minutes each)

↓

Blocking reagent was added to both the specimens and then incubated at room temperature in an enclosed hydrated container (5 minutes)

↓

Washed in TBS thrice (5 minutes each)

↓

Primary antibody was added to the study specimen and incubated in an enclosed hydrated container (1 hour)

↓

Washed in TBS thrice (5 minutes)

↓

Secondary antibody was added and incubated in an enclosed hydrated container (20 minutes)

↓

Washed in TBS thrice

↓

Streptavidin HRP was added and incubated in an enclosed hydrated container

(10 minutes)

↓

Washed in TBS thrice (5 minutes each)

↓

DAB was added and incubated in an enclosed hydrated container

(5minutes)

↓

Washed in TBS thrice (5 minutes)

↓

Stained with hematoxylin (1 minutes)

↓

Washed in running water

↓

Placed in ammonia (1 minute)

↓

Washed in running water

↓

Placed in 70% alcohol (5 minutes)

↓

Placed in 100% alcohol (5 minute)

↓

Placed in xylene twice (5 minute each)

↓

Slides were mounted using DPX

↓

Slides were observed under Light Microscope and graded

STATISTICAL ANALYSIS

The Normality tests Kolmogorov-Smirnov and Shapiro-Wilks tests results reveal that variablesdo NOT follow Normal distribution. Therefore to analyse the data Non parametric methods are applied. To compare values between cases and Controls Mann Whitney test is applied. To analyse the data SPSS (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp. Released 2013) is used. Significance level is fixed as 5% ($\alpha = 0.05$).

- Independent sample T-Test was done to compare mean values between Groups.
- Chi-Square test to compare proportions between Cases and Controls.
- Mann Whitney test was done to compare the staining intensity between the Groups (epithelium and connective tissue).

REVIEW OF LITERATURE

The name caspase is short for cysteinyl aspartate-specific protease. Caspases (cysteinal aspartate-specific proteases**)** are enzymes that utilize a catalytic cysteine to cleave their peptide substrates after specific aspartate residues. Caspases are the terminal proteases involved in apoptosis, as well as being involved in inflammation.[25]

The first caspase was discovered in 1992 and because of its function it was named as interleukin-1-"converting enzyme (ICE) [26, 27] but was later renamed to caspase-1. In 1993, Ced-3 from *C. elegans* was found to be homologous to ICE [28] and the corresponding human protein CPP32 (later named caspase-3) was found in 1994 [29].The official caspase nomenclature was decided on in 1996 to alleviate the confusion that went along with the discovery of ten different caspases and some with multiple names.

Caspases are expressed as proenzymes (zymogens) called procaspases, which then become activated to the mature caspase form. Procaspase structure can be divided into three domains such as an N-terminal prodomain, a large subunit, and a small subunit and their first step in maturation is dimerization. Then, proteolytic processing removes the prodomain and cleaves a loop called the intersubunit linker between the larger and smaller subunits.

.

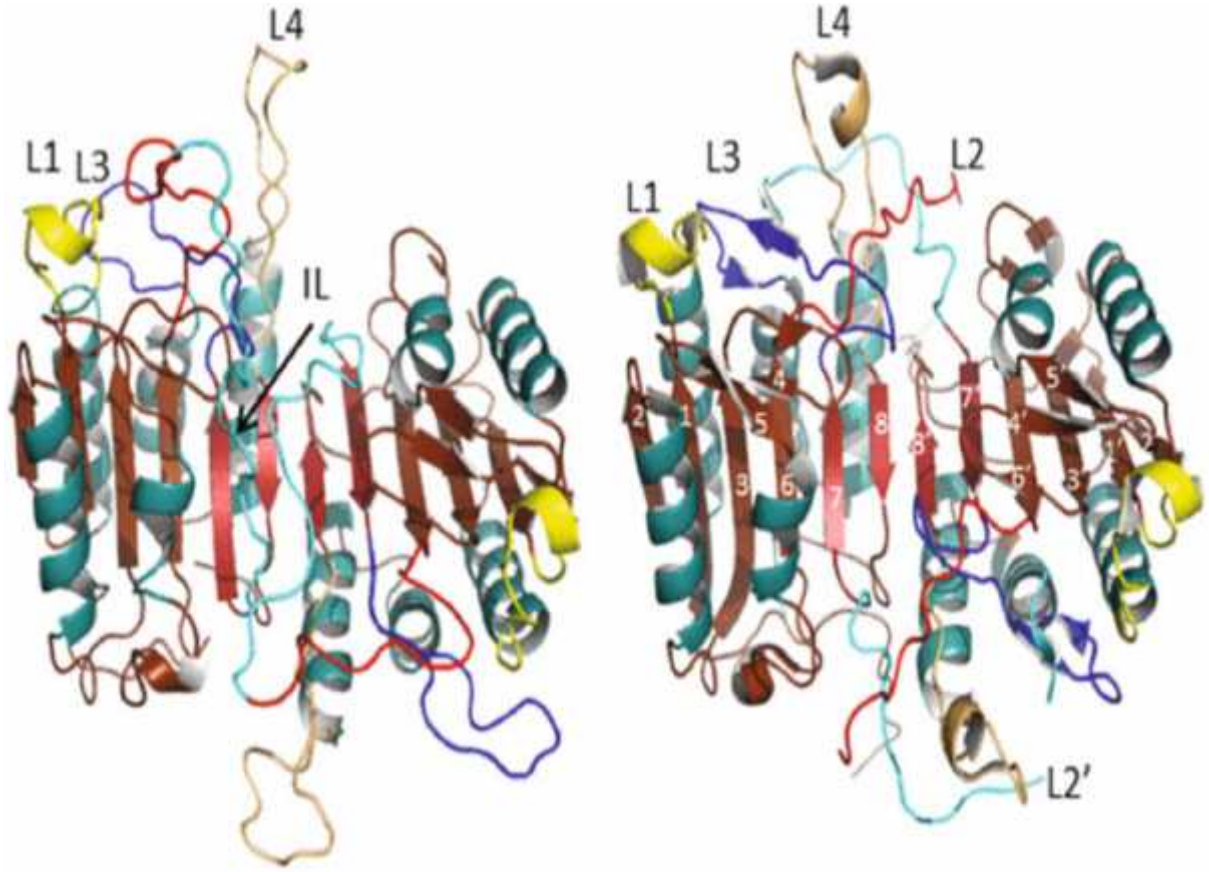


Fig 2.Procaspase-3 model and crystal structure of caspase-3. Active site loop coloring: *yellow*DL1, *red*DL2, *cyan*DL20, *blue*DL3, *tan*DL4

The secondary structure of mature caspases consist of six core - strands in a slightly twisted sheet in each monomer, with two main helices on one face (the “front”) of the protein and three helices on the other face (the “back”) of the protein (Fig 2). The first four core strands and helices 1–3 form the large subunit, whereas the last two core strands and helices 4–5 form the smaller subunit. The dimer interface consists of the final strand from each monomer, side by side in an anti parallel manner. The two monomers are related through a C2 axis of symmetry such that one monomer is “upside-down” compared to

the other monomer. Five loops are important for the formation of the active site and once the intersubunit linker is cleaved, the two halves of the cleaved linker are called L2 and L20. Active site loops L1, L2, L3, and L4 come from one monomer, and loop L20 comes from the other. The catalytic cysteine is a part of loop L2, while the catalytic histidine is part of a loop extending from the C terminal end of 3. [18] Caspases are not only required for apoptosis, but it also be required for regulating non apoptotic functions in certain cell types.[30]

.

Pei-Feng Liu et al conducted a study for the expression levels of cleaved caspase-3, caspase-8, and caspase-9 on tissue microarrays consisting of samples from 246 oral squamous cell carcinoma (OSCC) patients (tongue) by immunohistochemistry. The protein levels of cleaved caspase-3, caspase-8, and caspase-9 in tumor tissues were significantly higher when compared to those in adjacent normal tissues (all $p<0.001$).

The expression levels of caspase-8 in tumors were elevated in patients with lymph node invasion and moreover, positive expression of cleaved caspase-3 was associated with shorter disease-free survival (DFS) in OTSCC patients with moderate differentiation and lymph node invasion. Combination of either positively cleaved caspase-3 or higher caspase-3 expression or both was associated with poor DFS. They concluded that co-expression level of cleaved caspase-3 and caspase-3 might be a prognostic biomarker for OTSCC patients,

particular in those patients with certain tumor stages and cell differentiation status.[31]

Huang et al. Evaluated caspase 3 expressions by immunohistochemical analysis in two cohorts of human cancer patients, in which the first cohort, included 39 head and neck cancer patients treated with radiotherapy or chemo-radiotherapy while the second cohort included 48 advanced stage breast cancer patients. Results showed higher cleaved (activated) caspase 3 levels in the tumor samples and a higher rate of tumor recurrence; hence they concluded that elevated caspase 3 levels were seen in cancer patients.[32].

Ana Flavia et al conducted a retrospective study in 120 patients which included 20 oral leukoplakia cases (16 with and 4 without dysplasia) 20 actinic chelitis cases(15 with and 5 without dysplasia) 40 oral squamous cell carcinoma cases (20 intraoral squamous cell carcinomas and 20 lower lip Squamous cell carcinomas) and 40 inflammatory fibrous hyperplasia cases (20 intra oral inflammatory fibrous hyper plasia and 20 lower lip inflammatory fibrous hyperplasia) by immunohistochemical analysis with anticleaved caspase-3 antibody. Their results showed that cleaved caspase 3 levels were higher in intra oral Squamous cell carcinomas .Hence they concluded that caspase 3 levels were elevated in oral squamous cell carcinoma.[33]

Shuxia LI,Yanqi et al conducted a study to investigate the impact of survivin and caspase-3 on apoptosis and angiogenesis in oral cancer patients, which included a total of 16 oral leukoplakia cases of moderate epithelial dysplasia, 12 oral leukoplakia cases of severe epithelial dysplasia, 17 cases of moderately differentiated oral squamous cell carcinoma and 10 cases of normal oral mucosa. Immunohistochemical analysis was done to detect the expression levels of survivin, caspase-3, and caspase inhibitor factor VIII in lesions from each group and they revealed an increased expression level of survivin in oral cancer tissues when compared with the normal mucosa and also concluded that the expression of Caspase-3 was decreased during malignant transformation.(34)

Janakiraman etal conducted a study on RNA-binding protein Hu Antigen-R (HuR) that controls post-transcriptional gene regulation and undergoes stress-activated caspase-3 dependent cleavage in cancer cells. They said that the Caspases are defined as either tumor suppressors or oncogenes by executing cell death in normal cells or by undergoing severe suppression in some cancers cells and the Absence of caspase-3 was reported in multiple cancers including breast cancer, hepatocellular and prostate carcinomas. In addition to that, inactive caspase-3 was reported in both oral carcinomas and normal oral epithelial tissues and their results clearly showed that over expressed COX-2 inhibits the cleavage of caspase-3 and HuR in oral cancer cells.(35)

Hague A et al Conducted a study to determine alteration of caspase-3 expression during oral carcinogenesis.Caspase-3 expression in normal oral epithelium and oral squamous cell carcinoma were compared by including 39 samples of normal oral epithelium and 54 oral squamous cell carcinomas in their study and the results showed that Squamous cell carcinomas had more intense caspase-3 staining than normal epithelium. In the oral squamous cell carcinoma series, there was significantly more intense caspase 3 expression in nuclear and cytoplasmic staining with increasing STNMP stage.[36]

Coutinho-Camillo et al conducted a research study to analyze the expression profiles of 23 proteins that have been linked to the inhibition (Bcl-2, Bcl-x, Bcl-xL, Bcl-2-related protein A1, BAG-1, and survivin) and promotion (Bak, Bax, Bim/Bod, Bim-Long, Bad, Bid, PUMA, Apaf-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, Smac/DIABLO, and cytochrome c) of apoptosis in oral squamous cell carcinoma and they include Two-hundred and twenty nine cases of oral squamous cell carcinoma, by using immunohistochemical method analysis, results were quantified on an automated imaging system and their results showed that the caspase-3 was expressed higher in squamous cell carcinoma and it was correlated with lymphnode metastasis.[37]

Liang Pang et al evaluated the decreased mRNA expression of caspase-3, caspase-8 and caspase-9 in Human Oral Squamous Cell Carcinoma (HSC-3) oral cancer cells were analyzed by RT-PCR and

Western blot assays. After ursodeoxycholic acid (UDCA) treatment, the mRNA and protein expression of caspase-3, caspase-8 and caspase-9 were increased compare to the control cells, and these changes were increased in a concentration-dependent manner.[38]

Poomsawat et al investigated the expression of survivin and caspase 3 in 114 samples including oral squamous cell carcinoma, oral leukoplakia(with and without dysplasia) and normal oral mucosa and were immunohisto chemically analyzed and their results showed that the numbers of cytoplasmic and nuclear expression of caspase 3 in OSCC were significantly higher than that of normal mucosa and oral leukoplakia (with and without dysplasia) so they concluded that elevated expression of caspase 3 in oral squamous cell carcinoma indicates the advanced stage of oral cancer.[39]

Nagaraj et al conducted a research study on hypoxia mediated apoptosis in oral squamous cell carcinoma and showed that oral cancer cells from primary tumors and lymph node metastasis undergo apoptosis after 24 to 48 hours of hypoxia. He also revealed that during hypoxic growth, an increase in the caspase-3 proteolytic activity accompanied by the cleavage of PARP (poly ADP-ribose. polymerase) was observed in oral squamous cell carcinoma.[40]

Veeran varmal et al conducted a study in 112 samples which included 15 normal tissues and 97 diseased tissues (oral sub mucous fibrosis and oral squamous cell carcinoma associated with oral sub mucous fibrosis)

by immunohistochemical analysis using caspase 3 antibody. The percentage of positive cells were calculated using Image J software and the result showed that caspase-3 expression epithelial cells ranged from 11% to 38% in the normal oral mucosa and 0-8% in the oral squamous cell carcinoma, and hence they concluded that the decreased expression of caspase-3 in disease progression reflects its role in the malignant transformation.[41]

D Andresskis et al investigated caspase-3 and caspase-8 protein expression in 87 cases of primary tongue squamous cell carcinoma by immunohistochemical analysis and digital imaging analysis assay. Their results showed that expressions of caspase -3 proteins were observed 79 samples (90.5 %).[42]

Antonio Bascones –Martinez et al conducted a research study for the expression of p53, caspase-3 Bcl-2, and ki-67 in premalignant oral epithelium and to study its clonal behavior 41tumors samples with their adjacent non-tumor epithelia were analyzed by immunohistochemical using monoclonal antibodies that recognize p53, caspase-3, Bcl-2, and Ki-67 and they showed statistically significant expression of caspase 3 in tumors and its adjacent area[43]

SX Li et al was conducted a research study to investigate the expression of survivin and caspase 3 in oral squamous cell carcinoma and peritumoral tissues, by using ELISA and Western blot method which included 13 tumor samples, 13 peritumoral tissue of oral

squamous cell carcinoma and 10 normal tissue samples. Their results showed that no difference in expression of caspase 3 in peritumoral tissues and normal tissues, but it was evidently decreased in carcinoma tissues.(44)

M.V.Fiandalo et al found that during tumor genesis, significant loss or inactivation of caspase leads to impaired apoptosis induction, thereby causing a dramatic imbalance in the growth dynamics and hence resulting in aberrant growth of human cancers.(45)

Andrej Cör et al conducted a study, in 28 Head and Neck Squamous Cell Carcinoma (HNSCC) by immunohistochemical analysis and their results showed Pro-caspase 3 expression reduced in Head and Neck Squamous Cell Carcinoma.(46)

Atsushi Kurabayashi et al conducted a study in 76 esophageal squamous cell carcinoma by immunohistochemical analysis and their results showed that expression of caspase 3 was significantly associated with poor prognosis.(47)

Qian Hu et al, conducted a study by using immunohistochemical analysis, to observe the expression of cleaved caspase-3 in 367 human tumor samples (gastric cancer: 97 cases, ovarian cancer: 65 cases, cervical cancer: 104 cases; colorectal cancer: 101 cases) and they found that the patients with increased expression of cleaved caspase-3

had a significant shorter overall survival time when compared with decreased levels of expression.[48]

Heshiki et al conducted a research study for caspase 3 expressions in 30 patients who had received surgical treatment between April 2007 and March 2014. They included the primary carcinoma sites such as the tongue (*n*=10), maxillary gingiva (*n*=6), mandibular gingiva (*n*=5), floor of the mouth (*n*=3), and buccal mucosa (*n*=6) and their results showed that all the cases of oral squamous cell carcinomas demonstrated a positive expression of cleaved caspase-3.[49]

Manisha Shrivastava et al conducted a prospective pilot study based upon the expression of XIAP and caspase 3 proteins in 11s samples of oral squamous cell carcinoma. The results showed amplification of caspase-3 in 7 pure DNA samples and hence they concluded that the oral cancer cells could be recognized by expression of caspase 3 as well as it may have a further role in future target therapy in oral cancer patients.[50]

M Olsson et al review of primary breast tumor samples obtained from patients undergoing breast surgery found that in approximatel75% of the tumors as well as morphologically normal peritumoral tissue samples, caspase-3 transcripts and caspase-3 protein expression were lacking.[51]

Reiner et al studied the expression of caspase-3 in knockout mice, and concluded that a caspase is functionally deleted in a cancer cell line while it is also required for certain distinctive biochemical and morphological changes during apoptosis.[52]

Ding YP et al conducted a study for the expression of surviving and caspase-3 in 17 oral squamous cell carcinoma (OSCC), 28 oral leukoplakia with dysplasia and 10 normal oral mucosa cases .The results showed that Caspase-3 was expressed in all the normal mucosa samples but it was obviously down-regulated in dysplasia and oral squamous cell carcinoma.[53]

Abdel et al conducted a study in 25 lesional biopsy specimens, which included 15 Cutaneous Lichen Planus (CLP) , 10 Oral Lichen Planus (OLP)] and 10 control specimens [5 normal skin and 5 normal oral mucosa] by immunohistochemical analysis which revealed an increase caspase-3 protein expression in lichen planus lesions.[54]

Jer –shyung huang et al conducted a study to evaluated caspase-3 expression in tumor genesis and its prognosis in 185 buccal mucosa squamous cell carcinoma patients by immunohistochemical staining and their results showed that the expression levels of cleaved caspase-3 in 185 buccal mucosa squamous cell carcinoma tissues were significantly higher when compared to those in the tumor adjacent normal tissues.[55]

Yu-Xia zhang et al conducted a study for cell viability of tongue squamous cell carcinoma after the treatment of staurosporine, and their results showed that after treatment a time-dependent reduction of survivin and an activation of caspase-3 were observed.(56)

Zarin Zainul etal analyzed the expression of caspase 3,7,8,9 and 10 in oral squamous cell carcinoma by tissue micro array and their result showed that caspase 8 and 9 were prominently expressed while caspase 3,7and 10expressed causally.(57)

Tetsu shimane et al conducted a study in 119 oral squamous cell carcinoma cases by using immunohistochemical analysis , their results showed that apoptosis associated speck like protein appear in well differentiated cancer cells.(58)

Mutsuko Mukai et al conducted a study, in his study results showed that caspase-3 activation contributes to the stress-induced invasive capacity of these cancer cells.(59)

EA Slee et al showed in their review that the cells deficient in caspase-3 (either from CASP-3 null mice or due to a frame shift mutation, i.e. MCF-7 cells) clearly died in response to many pro-apoptotic stimuli.(60)

Claudia Malherios Coutinho-Camilo et al conducted a study in 229 tissues of oral squamous cell carcinoma by immunohistochemical analysis and their results showed that caspase 3 was expressed occasionally.(61)

Eswaran Devarajan et al conducted a study for the caspase 3 expressions in primary breast tumor samples and normal breast parenchyma samples by reverse transcriptase-polymerase, western blot and northern blot analysis and they found that 75% of the tumor and normal peritumoral tissues lacked caspase3 protein expression. Hence their results suggested that the loss of caspase3 expression represents an important role in the survival mechanism of breast cancer.(62)

Yuan –Feng Lin etal found in his research study that the Caspase-3 down regulation (CASP3/DR) in tumors conferred resistance to cancer therapy and was significantly correlated with a poor prognosis in cancer patients.(63)

Richard Jager et al in his reviewed that the caspase-3 was detected less in colon and stomach cancer, Non-Hodgkin lymphoma (NHL), and hepatocellular carcinoma and while it was elevated in acute myelogenous leukemia (AML) breast and prostate carcinoma. Hence they concluded that the expression increased with tumor progression.(64)

Pieter de Heer Elza et al conducted a randomized clinical trial on pre operated rectal biopsies and found that caspase 3 activity was significantly lower than the normal tissue and hence they concluded that caspase 3 activity was an important denominator for local recurrence.(65)

J.Kania etal conducted a study in gastric cancer, and they found that caspase 3 protein expression was undetectable in gastric cancer tissues when compare to normal mucosa.(66)

Ulla Toramen etal conducted a study to evaluate the caspase 3 level in non small cell lung carcinoma and they found that caspase expression was seen in tumors tissues and its appearance was not associated with apoptosis.(67)

RESULTS

A total of 25 patients were included in this study, comprising of 2 groups. Group I was the study group of 20 histologically confirmed oral squamous cell carcinoma patients. Group 2 was made of 5 normal patients whose gingival tissues were studied.

Age distribution in study groups

In Group I .age distribution among the patients ranged between 25 to 75 years with mean age of 55.60 yrs .In Group II, age ranged from 27 to 30 years with mean age of 25.60 yrs.(Table 1,Graph 1)

Gender distribution in study groups

Out of 20 patients in Group I consists of 15 were males and 5 were females with a male:female ratio of 3:1. In Group II, 3 were males and 2 were females, with a male:female ratio of 3:2 . (Table 2, Graph 2)

Site of biopsy in study groups

In Group I, biopsies were taken from buccal mucosa for 12 patients, alveolus or gingival for 4patients, from the tongue for 4 patients. In Group II all samples were taken from the gingival. (Table 3, Graph 3)

Comparison of intensity of expression of Caspase3 in between epithelium and connective tissue of Group 1 and Group 2

Epithelium:

Group 1 – Out of the 20 tissue samples, 7 (35%) showed mild staining intensity, 4 (20%) showed moderate intensity and 6 (30%) showed more intensity. Epithelial expression was not seen in 3 (15%) tissue samples.

Group 2 – Out of 5 tissue samples, 1(20%) sample showed mild epithelial staining and 4(80%) tissue samples was not showed any staining.

The difference in epithelial staining was statistically significant between Group 1 and Group 2

(P-0.046)(Table 4,Graph 4)

Connective tissue:

Group 1 – out of the 20 tissue samples,9(45%) tissue samples showed mild expression,3(15%) tissue samples showed moderate expression and 1(5%) tissue showed more intensity in staining. No expression was seen in 7 (35%) tissue samples.

Group 2 – No expression was seen in 5 tissue samples.

The difference in connective tissue staining was significantly between Group 1 and Group 2

(P-0.087) (Table 5, Graph 5)

Mann-Whitney Test to compare Epithelium grading between Cases and Controls:

To compare the staining intensity of epithelium in between oral squamous cell carcinoma and normal mucosa Mann –Whitney test was performed. The p value for epithelium grading was < 0.008 which was highly statistically significant. (Table6, Graph6)

Mann-Whitney Test to compare Connective tissue grading between Cases and Controls:

To compare the staining intensity of Connective tissue in between oral squamous cell carcinoma and normal mucosa Mann – Whitney test was performed. The p value for Connective tissue grading was <0.016 which was highly statistically significant. (Table7, Graph7)

TABLES AND GRAPHS

TABLE 1: AGE DISTRIBUTION BETWEEN GROUP1 AND GROUP2

Group	N	Mean Age	Std. Dev	p-Value
Case	20	55.60	12.098	<0.001
Control	5	25.60	2.793	

TABLE 1: AGE DISTRIBUTION BETWEEN GROUP1 AND GROUP2

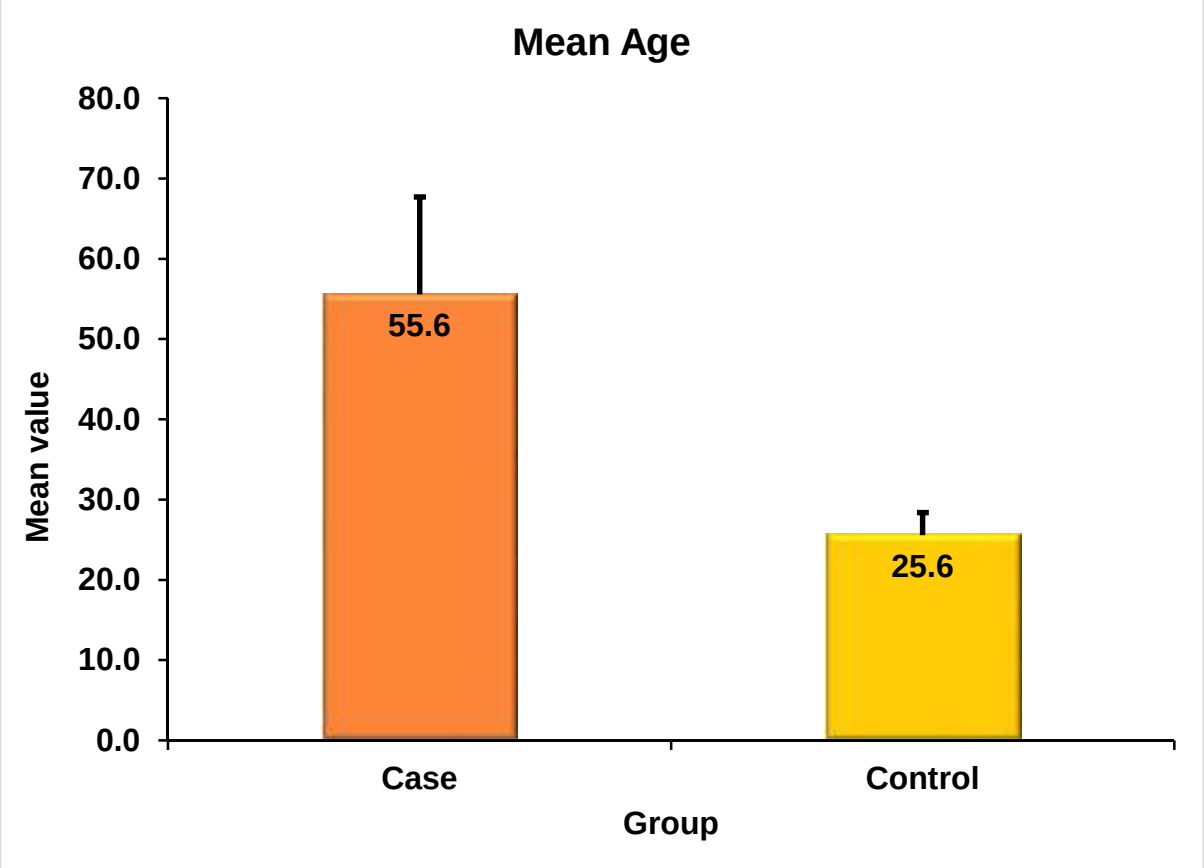


TABLE 2: GENDER DISTRIBUTION BETWEEN THE TWO STUDY GROUPS

Gender	Group						p-Value
	Case		Control		Total		
	N	%	N	%	N	%	
Female	5	25.0%	2	40.0%	7	28.0%	0.597
Male	15	75.0%	3	60.0%	18	72.0%	
Total	20	100.0%	5	100.0%	25	100.0%	

GRAPH 2: GENDER DISTRIBUTION BETWEEN THE TWO STUDY GROUPS

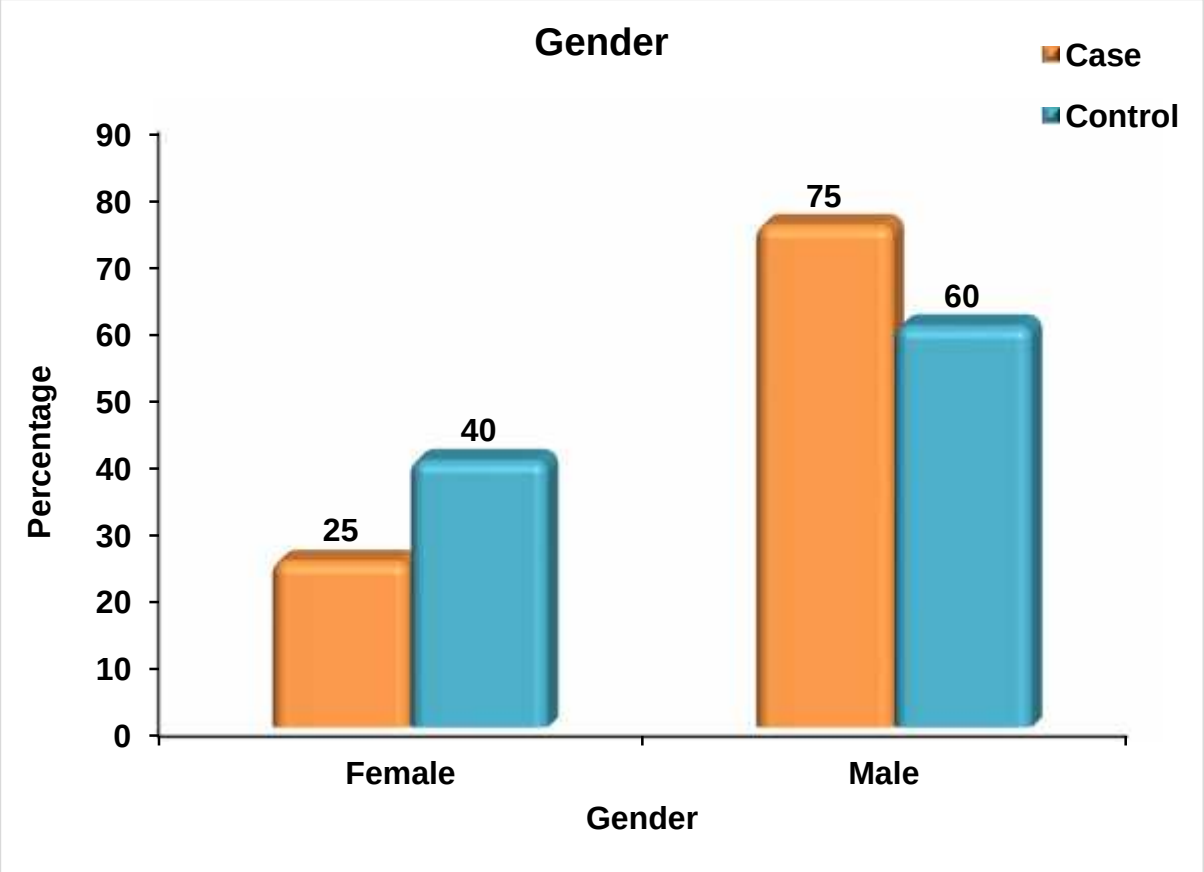


TABLE 3: SITE OF BIOPSY IN THE STUDY GROUPS

Site of biopsy	Group						p-Value
	Case		Control		Total		
	N	%	N	%	N	%	
Buccal Mucosa	10	50.0%	0	0.0%	10	40.0%	0.012
Floor of Mouth	2	10.0%	0	0.0%	2	8.0%	
Gingiva	4	20.0%	5	100.0%	9	36.0%	
Tongue	4	20.0%	0	0.0%	4	16.0%	
Total	20	100.0%	5	100.0%	25	100.0%	

GRAPH 3: SITE OF BIOPSY IN THE STUDY GROUPS

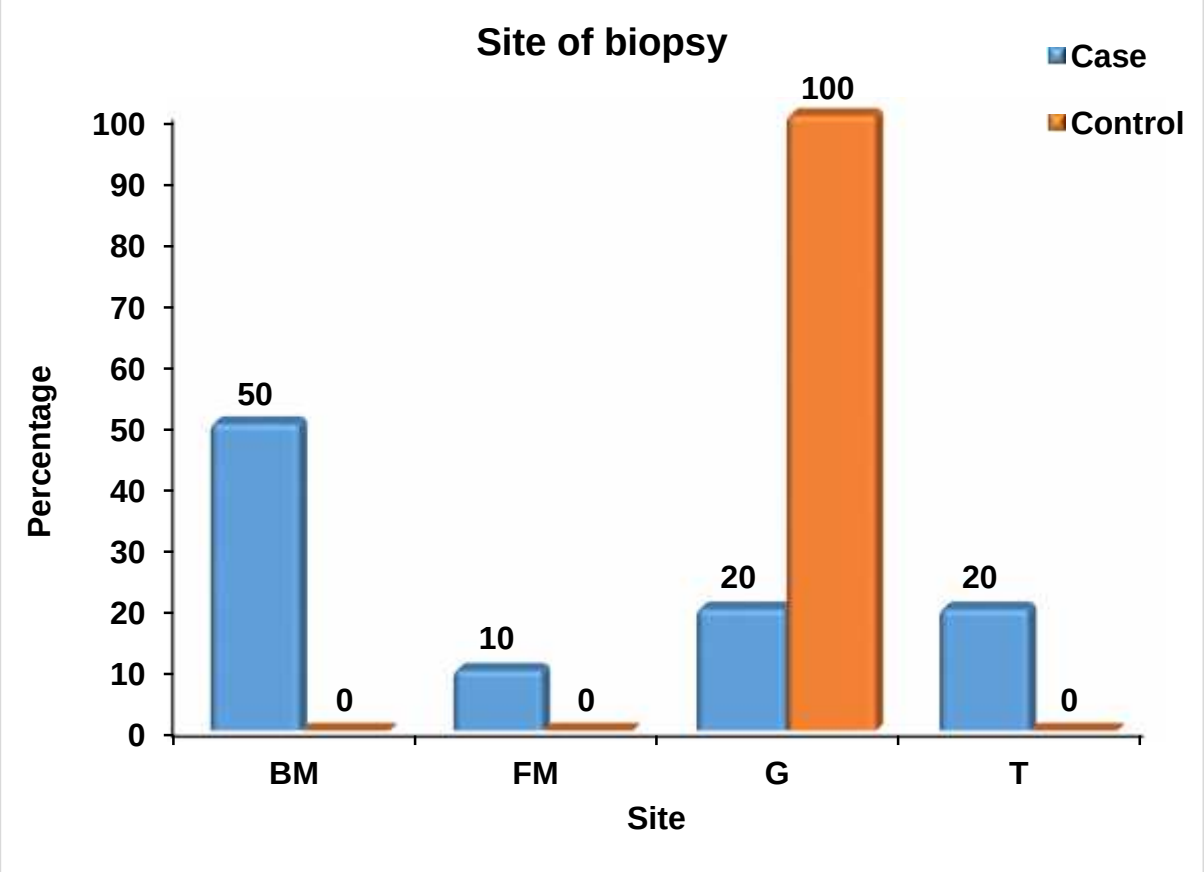


TABLE4: DISTRIBUTION OF EPITHELIUM INTENSITY OF STAIN IN GROUP1 AND GROUP2

Epithelium intensity	Group						p-Value
	Case		Control		Total		
	N	%	N	%	N	%	
INTENSE	6	30.0%	0	0.0%	6	24.0%	0.046
MILD	7	35.0%	1	20.0%	8	32.0%	
MODERATE	4	20.0%	0	0.0%	4	16.0%	
NOSTAIN	3	15.0%	4	80.0%	7	28.0%	
Total	20	100.0%	5	100.0%	25	100.0%	

GRAPH 4: DISTRIBUTION OF EPITHELIUM INTENSITY OF STAIN IN GROUP1 AND GROUP2

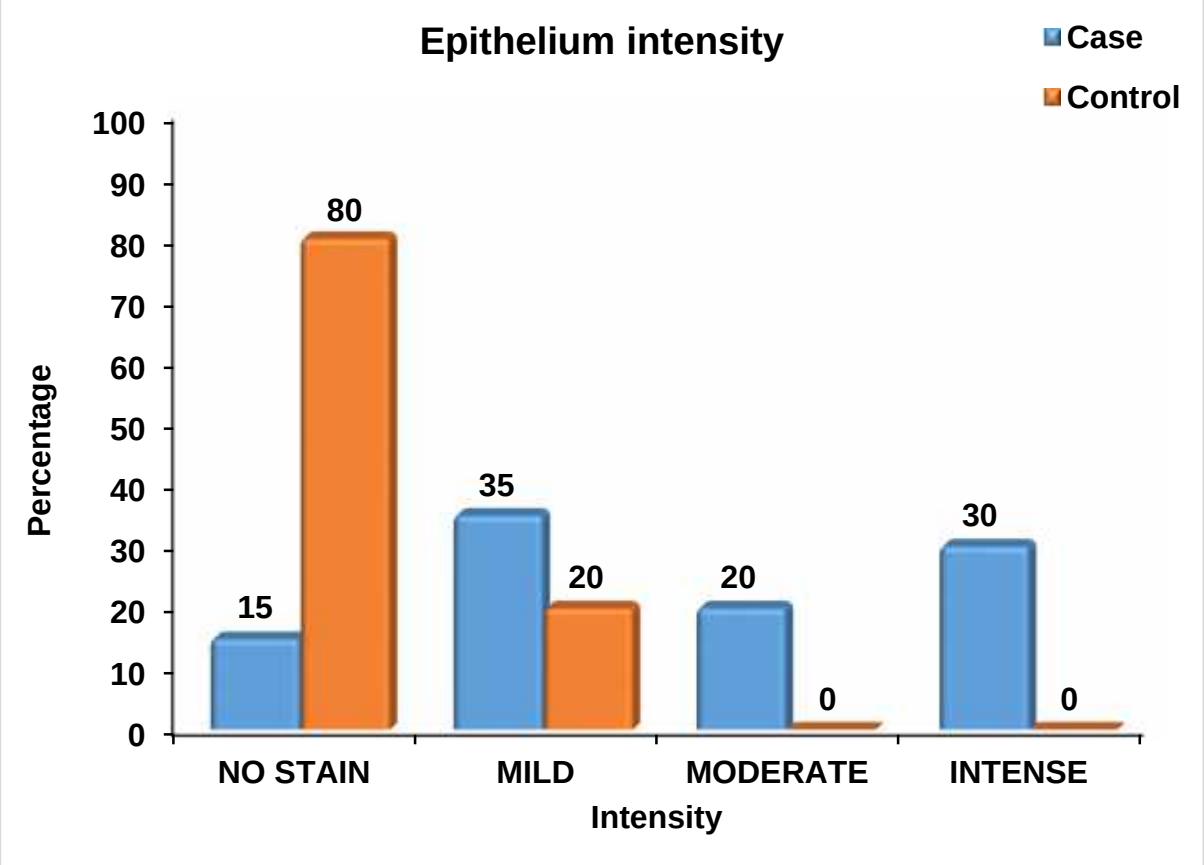


TABLE 5: DISTRIBUTION OF CONNECTIVE TISSUE INTENSITY OF STAIN IN GROUP1 AND GROUP2

Connective tissue intensity	Group						p-Value
	Case		Control		Total		
	N	%	N	%	N	%	
INTENSE	1	5.0%	0	0.0%	1	4.0%	0.087
MILD	9	45.0%	0	0.0%	9	36.0%	
MODERATE	3	15.0%	0	0.0%	3	12.0%	
NOSTAIN	7	35.0%	5	100.0%	12	48.0%	
Total	20	100.0%	5	100.0%	25	100.0%	

GRAPH 5: DISTRIBUTION OF CONNECTIVE TISSUE INTENSITY OF STAIN IN GROUP 1 AND GROUP 2

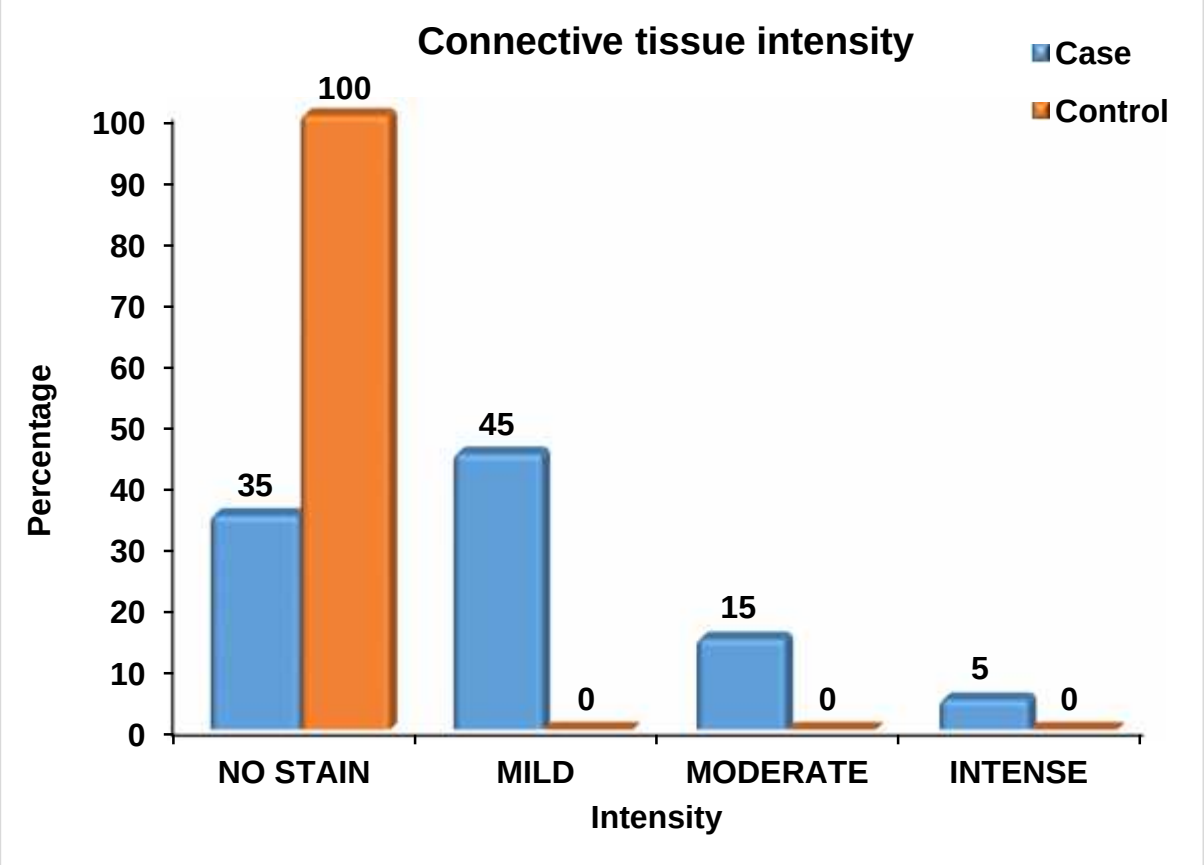


TABLE 6: DESCRIPTIVE STATISTICS FOR EPITHELIUM GRADING

		Group	
		Case	Control
Epithelium Grading	N	20	5
	Mean	1.650	.200
	Std. Dev	1.089	.447
	Median	1.5	.0
	1st Quartile	1.0	.0
	3rd Quartile	3.0	.0

MANN-WHITNEY TEST TO COMPARE EPITHELIUM GRADING BETWEEN CASES AND CONTROLS

Variable	Group	N	Mean Rank	P-Value
Epithelium Grading	Case	20	14.88	0.008
	Control	5	5.50	(Sig)

GRAPH 6: COMPARING EPITHELIUM GRADING BETWEEN CASES AND CONTROLS

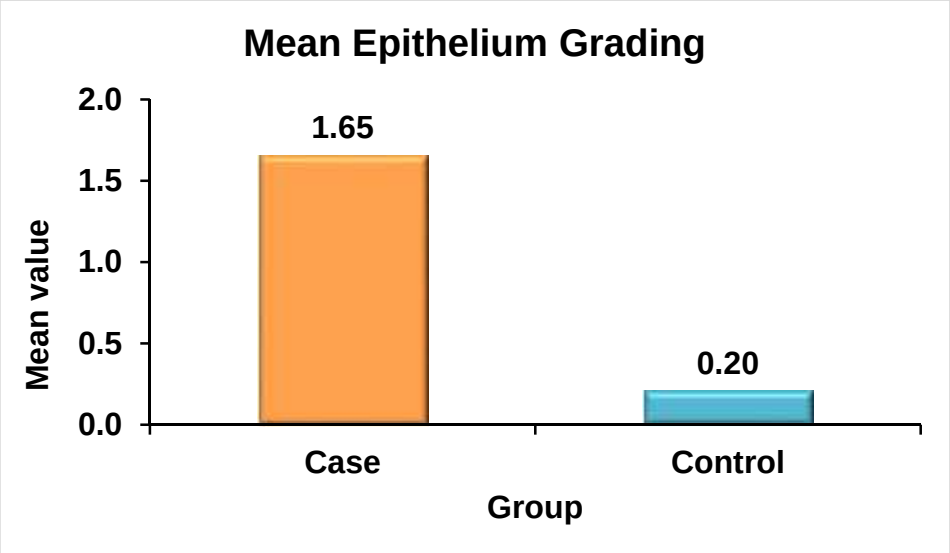


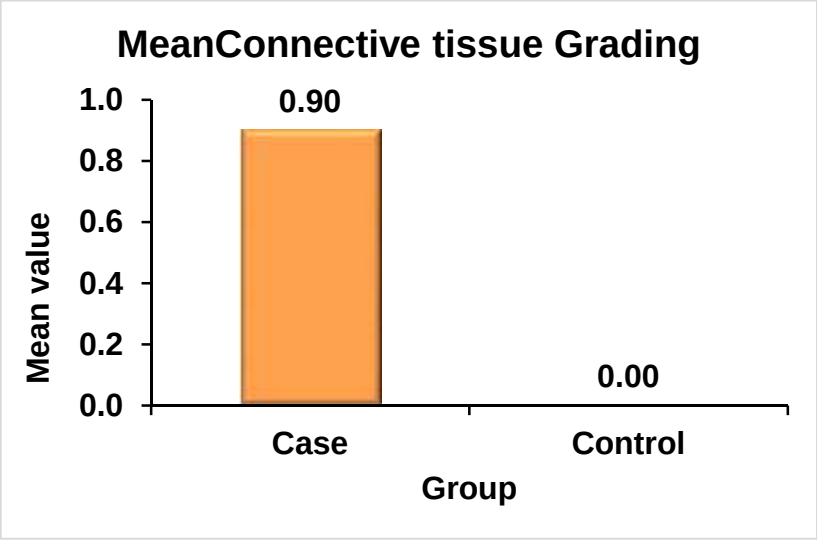
TABLE 7: DESCRIPTIVE STATISTICS FOR CONNECTIVE TISSUE GRADING

		Group	
		Case	**Control**
Connective tissue Grading	N	20	5
	Mean	.900	.000
	Std. Dev	.852	.000
	Median	1.0	.0
	1st Quartile	.0	.0
	3rd Quartile	1.0	.0

MANN-WHITNEY TEST TO COMPARE CONNECTIVE TISSUE GRADING BETWEEN CASES AND CONTROLS

Variable	**Group**	**N**	**Mean Rank**	**P-Value**
Connective tissue Grading	Case	20	14.63	0.016 (Sig)
	Control	5	6.50	

GRAPH7: COMPARING CONNECTIVE TISSUE GRADING BETWEEN CASES AND CONTROLS



PHOTOGRAPHS

POSITIVE CONTROL

Figure 3: Tonsil: H & E: 10 x

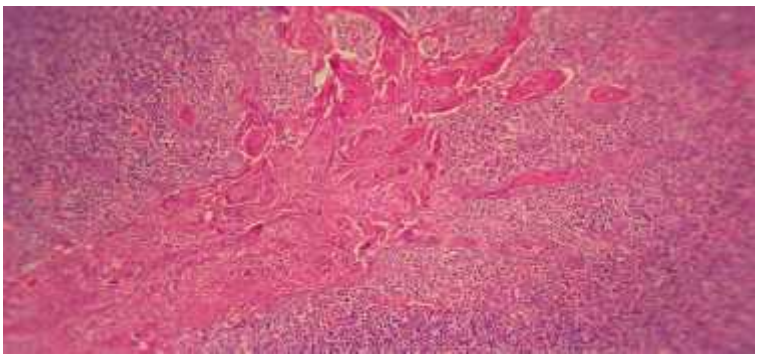


Figure 6: Tonsil: Caspase 3 stain: 10 x

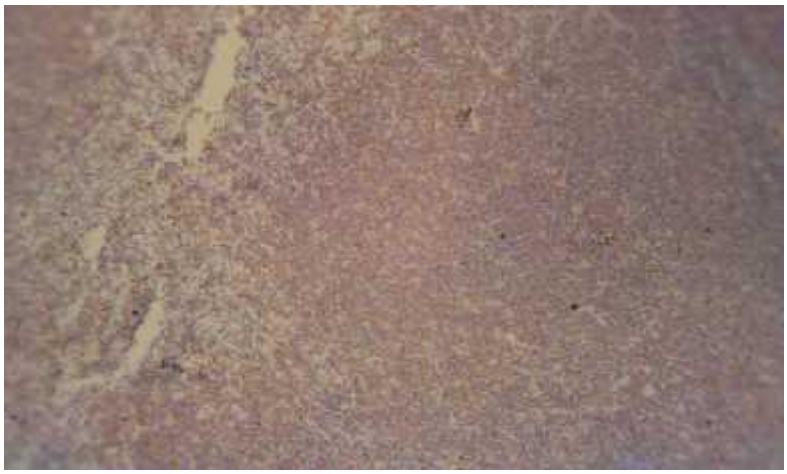
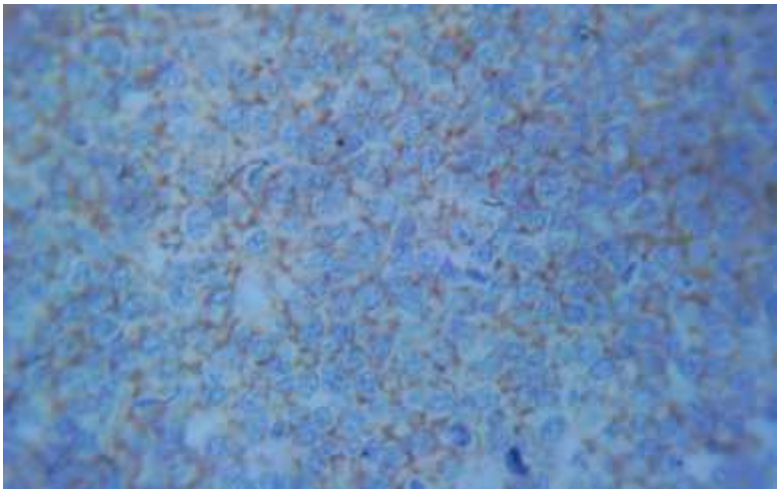


Figure 7: Tonsil: Caspase 3 stain: 40x



ORAL SQUAMOUS CELL CARCINOMA

Figure 8: H & E: 10x

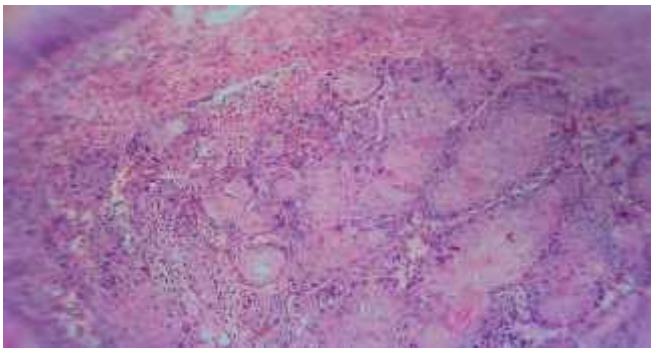


Figure 9: Caspase 3 stain 10 x

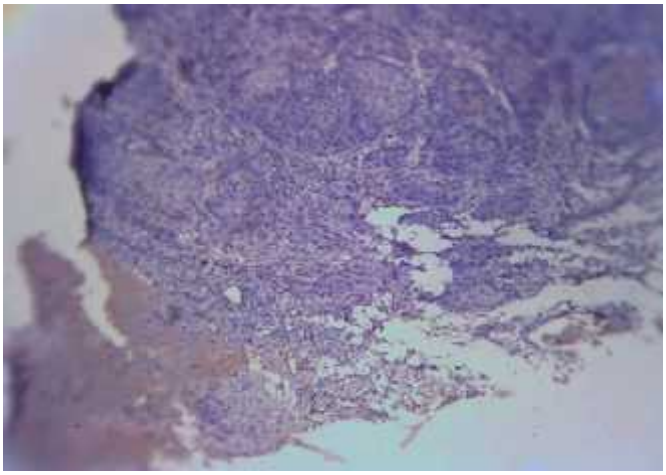
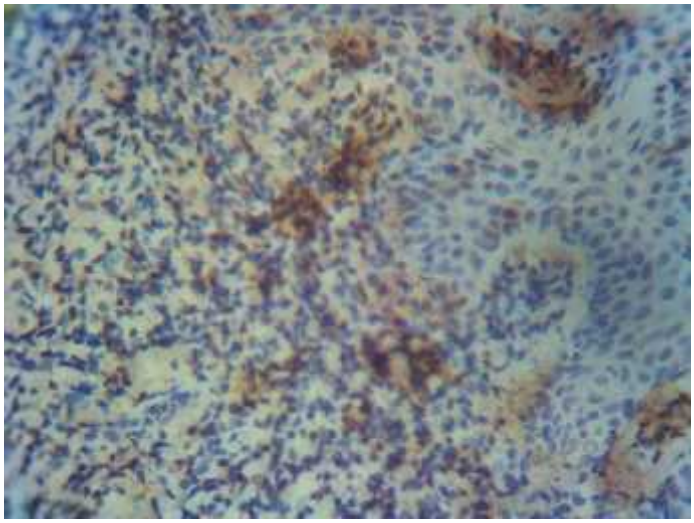


Figure 10: Caspase 3 stain 40 x



ORAL SQUAMOUS CELL CARCINOMA

Figure 11: H & E stain:10x

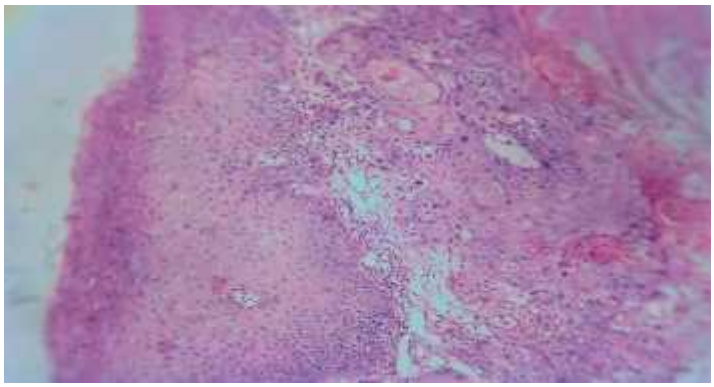
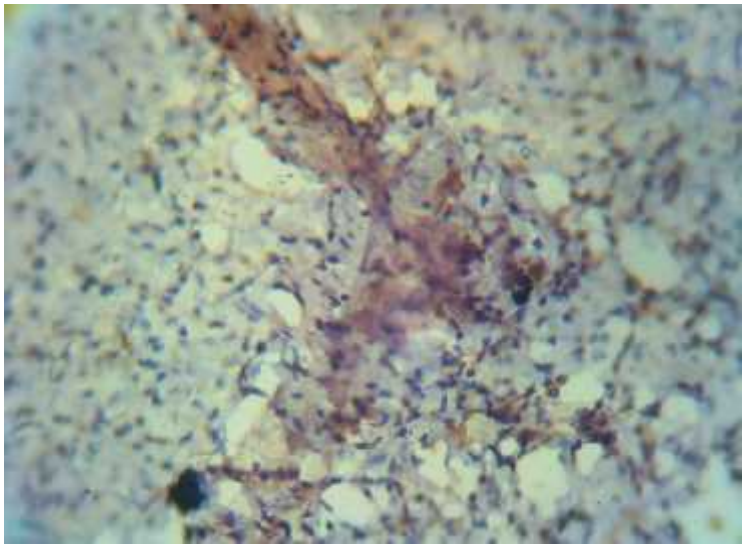


Figure 12: Caspase 3 stain: 10 x

Figure 13: Caspase 3stain:40 x



ORAL SQUAMOUS CELL CARCINOMA

Figure 14: H & E stain: 10 x

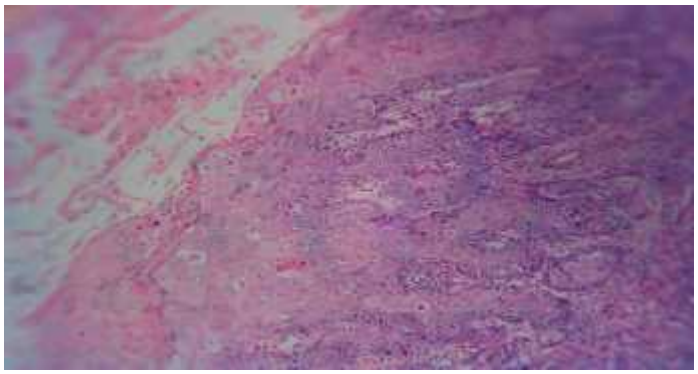
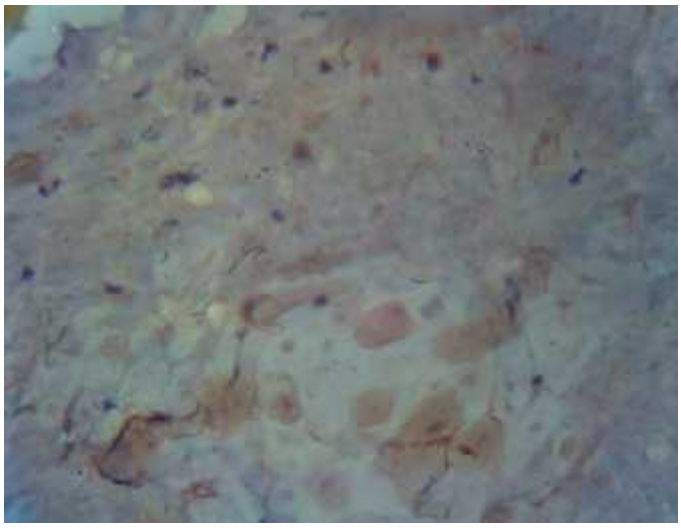


Figure15: Caspase 3 stain:10 x

Figure 16: Caspase 3 stain :40 x



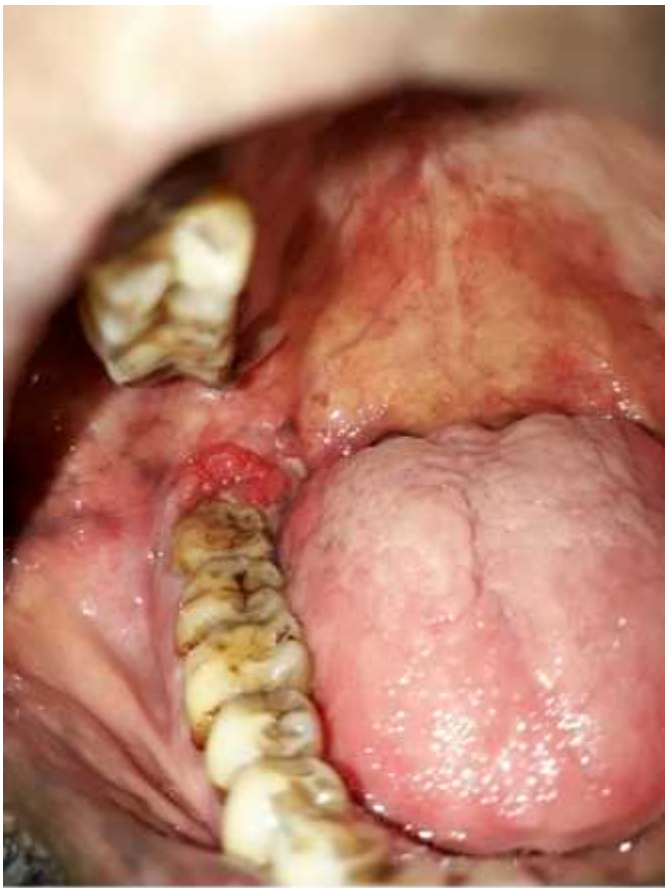


Fig 1: Oral Squamous Cell Carcinoma in the Alveolar Mucosa

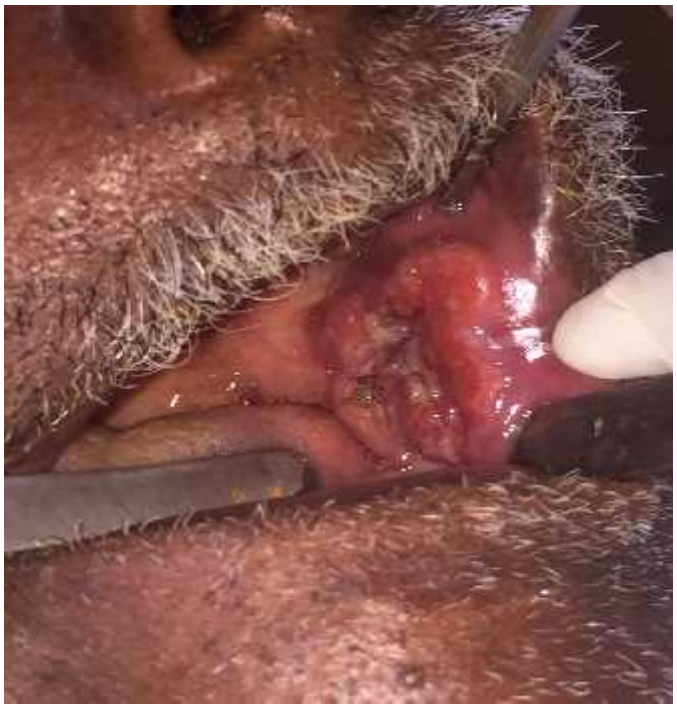


Fig 2: Oral Squamous Cell Carcinoma in the Buccal Mucosa

DISCUSSION

World 6th most common cancer is Oral Squamous Cell Carcinoma. In India oral cancer ranks first among all the cancers in males and it is the third most common cancer in females. (68) In head and neck region squamous cell carcinoma is the commonest type which has increased in the recent year. (69)

Oral squamous cell carcinoma is a multi factorial disease which is associated with tobacco chewing, alcohol, smoking, ultra violet radiation, human papilloma virus, candidal infection, nutritional deficiencies and genetic pre disposition. (70) Oral squamous cell carcinoma is more common in the later decades of the life, with increased risk as the age advances.

Approximately 94% of oral cancers are squamous cell carcinoma.(71) our present study had patients of age group between the 4th and 5th decades of life which was similar to studies of Fábio Ramôa PIRES et al, Mahindra Pratap singh et al, Preeti Sharma et al, R Shenoi et al, and Juliana Hintz Germanos Scheidt et al .(70,72,73,77,82) Takuji Tanaka et al and Liviu Feller et al in their studies they pointed out that oral squamous cell carcinoma occurs under 40years and 45 years respectively (75,68)

Oral squamous cell carcinoma shows male predilection with a male to female ratio of 3:1. In our present study males were 75% and females were 25% with a female and male ratio of 3:1, which was consistent with the below cited reports where Liviu Feller et al, Fábio Ramôa PIRES et al, and Arvind Krishamurthy et al, in their studies pointed out that males were affected more commonly when compared to females, because of indulgence into more habits [68, 70, 74].

Juliana Hintz Germanos Scheidt et al pointed out in his study that, among the persons who do not smoke and consume alcohol, women were affected most commonly in relation to increasing age when compared to men, because of the alteration of the choromogenic pattern. [82]

Arvind Krishnamurthy et al, Takuji Tanaka et al, and R.Shenoi et al in their studies showed that tobacco, betel quid and alcohol were the major carcinogens for the occurrence of oral squamous cell carcinoma. [74, 75, 77]. Takuji Tanaka et al also showed in their studies that human papilloma virus particularly HPV type 16 was an etiological factor, especially among persons who do not smoke or consume alcohol. [75]

Brad W.Neville et al stated that heavy smoking ,heavy alcohol usage, using tobacco, betel leaf with betel nut and slacked lime were the important etiological factors for the development of oral squamous cell carcinoma in India.[76] Similar observations were reported in our

present study, as most of the patients were smokers, alcoholics and pan chewers.

The most common sites of oral squamous cell carcinoma are tongue, floor of the mouth, hard palate, buccal mucosa, labial mucosa and retro molar area.

In our presnt study ,the most of the cases of oral squamous cell carcinoma were seen in the buccal mucosa, which was similar to the studies of Preethi Sharma et al and Vaidhehi narayan nayak et al.[73,79] In contrast to our present study, R Shenoi et al and Antoniades et al showed that, the mandibular alveolus and lip were the common sites respectively,[77,78] while Arvind Krishnamurthy et al, Juliana Hintz Germanos Scheidt et al and Liviu Feller et al showed in their study that the common site was tongue .[74,82,68]

Pindborg et al classified oral squamous cell carcinoma microscopically based on the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity. The grades are well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3). Our present study includes all categories of well, moderately and poorly differentiated oral squamous cell carcinoma cases. [80]

In our present study, we used Immunohistochemical methods to analyse caspase 3 expression in oral squamous cell carcinoma, which was similar to the methods used by Claudia Malheiros Coutinho-Camillo et al, Shuxia et al, Sopee Poomsawat et al, Ana Flavia Schueler de Assumpcao et al and Pei-Feng Liu et al in their studies.[31,33,34,39,61] In contrast to our study Sx li et al used ELISA and Western blot method[44] while Wataru Heshiki et al and Eswaran Devarajan et al included Immunohisto chemical analysis along with cell line culture and flow cytometry method to analyse caspase 3 expression.[49,62]

Caspase3 expression was seen as a brown end reaction in both cytoplasm and nucleus. In our present study, caspase 3 was expressed in the epithelium and connective tissue with varying degrees and was absent in most of the normal oral mucosa, which was similar to the studies done by Claudia Malheiros Coutinho-Camillo et al and Shuxia et al where they observed caspase 3expression in the cytoplasm of oral squamous cell carcinoma at varying degrees and absence in the normal mucosa.[34,39]

Ana Flavia Schueler de Assumpcao et al , Pei-Feng Liu et al, Hague et al and D Andressakis et al observed in their studies that the caspase 3expression was significantly higher in oral squamous cell carcinoma when compared to the normal mucosa.[33,39,36,42] while Qian Hu et al, Andrej cor et al and Vakkala M et al also observed in their

studies that the caspase 3 expression was higher in gastric cancer, head & neck cancer and breast cancer when compared to the normal tissues respectively.[48,46,81] Hence these findings indicate that decreased caspase 3 expression was associated with advanced clinical staging of the disease.

In contrast to our present study, Veeranvermal et al showed that the caspase3 expression was significantly lower in oral squamous cell carcinoma tissues when compared to the normal mucosa which was possibly due to the inhibition of caspase 3 synthesis by survivin, and this process lead to the development of oral squamous cell carcinoma by prevention of apoptosis.[41]

In our present study, expression of caspase 3 antigen was seen in oral squamous cell carcinoma tissues of both epithelium and connective tissue. In the oral squamous cell carcinoma group of 20 cases epithelium showed 85% of caspase 3 expressions while connective tissue showed 65% caspase3 expression. In control group, epithelium showed 20% caspase3 expression and the connective tissue showed negativity.

SUMMARY AND CONCLUSION

A total of 25 patients were included in our study, comprising of 20 patients of oral squamous cell carcinoma and 5 normal controls.

- In our study,the mean age of the patients with oral squamous cell carcinoma was 55.60 years ± 12.098 whereas for control group the mean age was 25.60 ± 2.793
- Our study comprised of 15 males and 5 females in oral squamous cell carcinoma, 3 males and 2 females in normal group.
- The site of biopsy in Oral Squamous Cell Carcinoma was the buccal mucosa(50%), tongue (20%), gingival (20%)and floor of the mouth (10%). In Normal mucosa tissues were obtained from gingiva.
- Immunohischemical study was done to demonstrate the expression of caspase 3 enzyme marker in oral squamous cell carcinoma and normal mucosa by using Monoclonal mouse anti caspase 3.
- Caspase 3enzyme marker expression in epithelium and connective tissue was higher in Oral Squamous Cell Carcinoma when compare to Normal tissue. The results were statistically significant.

The results of our present study demonstrated that increased caspase 3expression was seen in oral squamous cell carcinoma tissues than that of the normal mucosa. These findings indicate the caspase 3 expression response to prognosis of the cancer and this has to be explored in a larger sample for further studies.

BIBLIOGRAPHY

1. **Sciubba JJ**. Oral cancer and its detection-History taking and the diagnostic phase of management. J Am Dent Assoc 2001;132:12-8.
2. **Goshen E, Yahalom R, Talmi YP, Rotenberg G, Oksman Y, Zwas ST**. The role of gamma-PET in the evaluation of patients with recurrent Squamous cell cancer of the head and neck. Oral and Maxillofac Surg 2005; 34:386-90.
3. **Sankaranarayanan R**. Oral cancer in India: An epidemiological and clinical review. Oral Surg Oral Med Oral Pathol 1990; 69:325-30.
4. **Monique GC, Slootweg PJ.** Oral field cancerization: Carcinogen induced independent events or micro metastatic deposits. Cancer Epidemiol Biomarkers Prev 2000; 9:249-56
5. **Todd R, Donoff RB, Wong DT.** The molecular biology of oral carcinogenesis: Toward a tumor progression model. Oral Maxillofac. Surg 1997; 5:613-23.
6. **Sudha Vm, Hemavathy S** Role of bcl2 Oncoprotein in oral potentially malignant disorder of squamous cell carcinoma
7. **J. P. Shah and Z. Gil,** "Current Concepts in Management of Oral Cancer-Surgery," *Oral Oncology*, Vol. 45, No. 4, 2009, pp. 394-401.
8. **S. Petti**, "Lifestyle Risk Factors for Oral Cancer," *Oral Oncology*, Vol. 45, No. 4, 2009, pp. 340-350.

9. **B. W. Neville and T. A**. Day, “Oral Cancer and Precan-cerous Lesions,” *CA*: *A Cancer Journal for Clinicians*, Vol. 52, No. 4, 2002, pp. 195-215.
10. **C. Scully and J. Bagan**, “Oral Squamous Cell Carcinoma Overview,” *Oral Oncology*, Vol. 45, No. 4, 2009, pp. 301- 308.
11. **Kerr JFR, Wyllie AH, Currie AR (**1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.
12. **Nishida K, Yamaguchi O, Otsu K (**2008) Crosstalk between autophagy and apoptosis in heart disease. Circ. Res. 103, 343–351.
13. **Martin SJ, Green DR** (1995) Protease activation during apoptosis:
14. **Cohen GM, Sun XM, Fearnhead H et al.** (1994) Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. J. Immunol. 153, 507–516. eath by a thousand cuts? Cell 82, 349–352.
15. **Wu W, Liu P, Li J** (2012) Necroptosis: an emerging form of programmed cell death. Crit. Rev. Oncol. Hematol. 82, 249–258.
16. **Norbury CJ, Hickson ID.** Cellular responses to DNA damage. Annu Rev Pharmacol Toxicol 2001;41:367–401. [PubMed: 11264462]
17. **Hirsch T, Marchetti P, Susin SA et al** The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial

permeability transition determine the mode of cell death. Oncogene 1997;15:1573–81. [PubMed: 9380409]

18. **Christine.K Cade and .A Clay Clark** Caspase-Key players in Apoptosis
19. **Pop C, Chen YR, Smith B et al (2001)** Removal of the pro-domain does not affect the conformation of the procaspase-3 dimer. Biochemistry 40:14224–14235
20. **Chekeni FB, Elliott MR, Sandilos JK, Walk SF et al** (2010) Pannexin1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature 467:863–867
21. **Martin SJ, Henry CM, Cullen SP (**2012) A perspective on mammalian caspases as positive and negative regulators of inflammation. Mol Cell 46:387–397
22. **Lane JD, Lucocq J, Pryde J, Barr FA et al** (2002) Caspase mediated cleavage of the stacking protein GRASP 65 is required for Golgi fragmentation during apoptosis. J Cell Biol 156:495–509
23. **Miura M, Chen XD, Allen MR, Bi Y, Gronthos S et al** (2004) A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. J Clin Invest 114:1704–1713
24. **Wataru Heshiki, Kei Tomihara,Manabu Yamazaki et al** Constitutive Activation of Caspase 3in Non Apoptotic Oral Squamous Cell Carcinoma. J Cancer Sci Ther 2015, 7:2
25. **Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G et al** (1996) Human ICE/CED-3 protease nomenclature. Cell 87:171

26. **Cerretti DP, Kozlosky CJ, Mosley B, Nelson N et al** (1992) Molecular cloning of the interleukin-1 beta converting enzyme. Science 256:97–100

27. **Thornberry NA, Bull HG, Calaycay JR, Chapman KT et al** (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356:768–774

28. **MiuraM, Zhu H, Rotello R, Hartwieg EA, Yuan J** (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell 75:653–660

29. **Fernandes-Alnemri T, Litwack G, Alnemri ES** (1994) CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J Biol Chem 269:30761–30764

30. **U Fischer, RU Ja¨nicke and K Schulze-Osthoff** Many cuts to ruin: a comprehensive update of caspase substrates Cell Death and Differentiation (2003) 10, 76–100

31. **Pei-Feng Liu, Yu-Chang Hu, Bor-Hwang Kang et al** Expression levels of cleaved caspase-3 and caspase-3 in tumorigenesis and prognosis of oral tongue squamous cell carcinoma. PLoS ONE 12(7): e0180620. https://doi.org/10.1371/journal

32. **Qian Huang, Fang L, Xinjian Liu, Wenrong Li et al** Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. *Nat Med*. ; 17(7): 860–866. doi:10.1038/nm.2385

33. **Ana Flávia Schueler de Assumpção LEITE.** Immunoexpression of cleaved caspase-3 shows lower apoptotic area indices in lip carcinomas than in intraoral cancer www.scielo.br/jaos J Appl Oral Sci.2016 Jul-Aug:24(4):359-365.

34. **Shuxia Li, Yanqi Yang, Yanping Ding, Xiaofei Tang and Zheng Sun** Impacts of survivin and caspase-3 on apoptosis and angiogenesis in oral cancer DOI: 10.3892/ol.2017.6626

35. **Harinarayanan Janakiraman,Reniqua P.House,SudhaTalwar et al** Repression of caspase-3 and RNA-binding protein HuR cleavage by cyclooxygenase-2 promotes drug resistance in oral squamous cell carcinoma

36. **Hague A, Eveson JW, MacFarlane M, Huntley S, Janghra N, Thavaraj S**. Caspase-3 expression is reduced, in the absence of cleavage, in terminally differentiated normal oral epithelium but is increased in oral squamous cell carcinomas and correlates with tumour stage. J Pathol. 2004;204:175-82.

37. **Cláudia Malheiros Coutinho-Camillo,Silvia Vanessa Lourenço et al** Profile of apoptotic proteins in oral squamous cell carcinoma: A cluster analysis of 171 cases. Applied Cancer Research (2017) 37:2

38. **Liang Pang , Xin Zhao , Weiwei Liu , Jiang Deng et al** Anticancer Effect of Ursodeoxycholic Acid in Human Oral Squamous Carcinoma HSC-3 Cells through the Caspases.www.mdpi.com/journal/nutrients 2015 May: 7(5) 3200-3218.

39. **Sopee Poomsawat,Punyasingh, Jirapa,Vejchapipat, Paisarn** Overexpression of Survivin and Caspase 3 in Oral Carcinogenesis Applied Immunohistochemistry & Molecular Morphology: January 2014 - Volume 22 - Issue 1 - p 65–71

40. **Nagathihalli S Nagaraj, Nadarajah Vigneswaran and Wolfgang Zacharias** Hypoxia-mediated apoptosis in oral carcinoma cells occurs *via* two independent pathways. *Molecular Cancer* 2004, 3:38 doi:10.1186/1476-4598-3-38

41. **Veeran Veeravarmal, Ravi David Austin et al** Caspase-3 expression in normal oral epithelium, oral sub mucous fibrosis and oral squamous cell carcinoma. J Oral Maxillofacial Pathol 2016; 20:445-52.

42. **D Andressakis , A C Lazaris , E Tsiambas , N Kavantzas** .. Evaluation of caspase-3 and caspase-8 deregulation in tongue squamous cell carcinoma based on immunohistochemistry and computerised image analysis. Volume 122, Issue 11 November 2008 , pp. 1213-1218.

43. **Antonio Bascones-Martínez , Carlos Rodríguez et al** Evaluation of p53, Caspase-3, Bcl-2, and Ki-67 markers in oral squamous cell carcinoma and premalignant epithelium in a sample from Alava Province (Spain) Med Oral Patol Oral Cir Bucal. 2013 Nov 1;18 (6):e846-50.

44. **SX Li, L Chai, ZG Cai, LJ Jin, Y Chen, HR Wu, Z Sun** Expression of Survivin and Caspase 3 in Oral Squamous Cell

Carcinoma and Peritumoral Tissue. *Asian Pacific J Cancer Prev*, 13 (10), 5027-5031

45. **M.V. Fiandalo and N. Kyprianou** Caspase Control: Protagonists of Cancer Cell Apoptosis. Exp Oncol. 2012 October; 34(3): 165–175.
46. **Andrej Cör, Jože Pižem, Nina Gale** The expression of bcl-2 and pro-caspase 3 in head and neck squamous cell carcinoma. Zdrav Vestn 2002; 71: III-39–43
47. **Atsushi Kurabayashi, Mutsuo Furihata et al** Expression of Bax and Apoptosis-Related Proteins in Human Esophageal Squamous Cell Carcinoma Including Dysplasia. Departments of Pathology II (AK, MF, MM, YO) and Surgery II (AK, SS), and Vice President (SO), Kochi Medical School, Nankoku, Kochi, Japan..Mod Pathol.2001 Aug 14(8):741-7.
48. **Qian Hu, Jing Peng, Weiping Liu, Xiaoli He et al** Caspase-3 is associated with shortened overall survival in several cancer types. Elevated cleaved Int J Clin Exp Pathol 2014;7(8):5057-5070. .
49. **Wataru Heshiki, Kei Tomihara1, Manabu Yamazaki et al** J Constitutive Activation of Caspase-3 in Non-Apoptotic Oral Squamous Cell Carcinoma Cells Cancer Sci Ther 2015, 7:2
50. **Manisha Shrivastava, Rashmi Bathri, Shweta Mishra et al** World Journal of Pathology 2017, 6:9
51. **M Olsson and B Zhivotovsky** Cell Death and Differentiation (2011) 18, 1441–1449; doi:10.1038/cdd.2011.30; . Caspases and cancer

52. **Reiner U. Ja¨nicke, Michael L. Sprengart, Mas R et al** Caspase-3 Is Required for DNA Fragmentation and Morphological Changes Associated with Apoptosis.The Journal Of Biological Chemistry Vol. 273, No. 16, Issue of April 17, pp. 9357–9360, and 1998.

53. **Ding YP, Li SX, Wu HR, Zhang XY, Tang XF, Sun Z et al** Expression of survivin, caspase-3 in oral precancerous lesions and oral squamous-cell carcinoma. 2010 Feb; 45(2):85-8

54. **Abdel-Latif AM, Abuel-Ela HA, El-Shourbagy SH.** Increased caspase-3 and altered expression of apoptosis-associated proteins, Bcl-2 and Bax in lichen planus.lin Exp Dermatol. 2009 Apr;34(3):390-5. doi: 10.1111/j.1365-2230.2008.03029.x.

55. **Jer-Shyung Huang, Cheng-Mei Yang, Jyh-Seng Wang et al** Caspase-3 expression in tumorigenesis and prognosis of buccal mucosa squamous cell carcinoma. 2017, Vol. 8, (No. 48), pp: 84237-84247

56. **Yu-xia ZHANG, Shi-bin YU, Jing-ping OU-YANG et al** Effect of protein kinase C alpha, caspase-3, and survivin on apoptosis of oral cancer cells induced by staurosporine1Acta Pharmacologica Sinica 2005 Nov; 26 (11): 1365–1372.

57. **Zarin Zainul** Recent Immunohistochemical Approaches for Predicting Tumor Recurrence in Oral Squamous Cell Carcinoma (OSCC). Int J Oral-Med Sci 10(4):406-413, 2012.

58. **Tetsu Shimane, DDS, Hiroichi Kobayashi, DDS, PhD et al** Clinical significance of apoptosis-associated speck-like protein containing a caspase recruitment domain in oral squamous cell

carcinoma. Vol. 115 No. 6 June 2013(Oral Surg Oral Med Oral Pathol Oral Radiol2013;115:799-809)

59. **Mutsuko Mukai,Toshiyuki Kusama, Yukou Hamanaka et al** Cross Talk between Apoptosis and Invasion Signaling in Cancer Cells through Caspase-3 Activation. (Cancer Res 2005; 65(20): 9121-5)
60. **EA Slee, C Adrain1 and SJ Martin,** Serial killers: ordering caspase activation events in apoptosis. Cell Death and Differentiation (1999) 6, 1067 ± 1074.
61. **Cla´udia Malheiros Coutinho-Camillo,Silvia Vanessa Lourenc et al** Caspase Expression in Oral Squamous Cell Carcinoma DOI: 10.1002/hed.21602.
62. **Eswaran Devarajan, Aysegul A Sahin, Jack S Chen et al** Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance . Oncogene (2002) 21, 8843 ± 8851. doi:10.1038/sj.onc.1206044
63. **Yuan-Feng Lin, Tsung-Ching Lai Chih-Kang Chang et al** Targeting the XIAP/caspase-7 complex selectively kills caspase-3–deficient malignancies *J Clin Invest.* 2013; 123(9):3861–3875. Doi: 10.1172/JCI67951
64. **Richard Jäger and Ralf M. Zwacka** The Enigmatic Roles of Caspases in Tumor Development *Cancers* 2010, *2*, 1952-1979; doi: 10.3390/cancers2041952.

65. **Pieter de Heer,Elza C. de Bruin, Elma Klein-Kranenbarg et al**Caspase-3 Activity Predicts Local Recurrence in Rectal Cancer doi:10.1158/1078-0432.CCR-07-0343.

66. **J. Kania, Phd, S.J. Konturek, Md,K. Marlicz et al** Expression of Survivin and Caspase-3 in Gastric Cancer *Digestive Diseases and Sciences, Vol. 48, No. 2 (February 2003), pp. 266–271 (*C ° *2003)*

67. **Ulla Tormanen-Napankangas,Ylermi soini et al** Expression Of Caspases-3, -6 And -8 And Their Relation To Apoptosis In Non-Small Cell Lung Carcinoma *Int. J. Cancer:* **93,** 192–198 (2001)

68. **Liviu Feller, Johan Lemmer** Oral Squamous Cell Carcinoma: Epidemiology, Clinical Presentation and Treatment. *Journal of Cancer Therapy*, 2012, 3, 263-268

69. **Bo Wang, Shu Zhang and XU-Dong Wang** The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases.

70. **Fábio Ramôa PIRES, Amanda Barreto RAMOS et al** Northen American Journal of Medical Sciences Clinicopathological prognostic implicators of oral squamous cell carcinoma:Need to Understand and Revise Oral squamous cell carcinoma: clinicopathological features from 346 cases from a single Oral Pathology service during an 8-year period.

71. **Neville BW, Damm DD,Allen CM, Bouquot JE**. Oral maxillofacial pathology 2 edition.

72. **Mahendra Pratap Singh, Vijay Kumar And Sanjeev Misra** journal of Oral Biology and Craniofacial research Clinico-

epidemiological Study of oral squamous vell carcinoma; A tertiary care centre study in north india.

73. **Preeeti Sharma, Susmita Saxena , Pooja Aggarwal.** Trends in the epidermology of oral squamous cell carcinoma in western UP An institutional study Indian J Dent Res 2010:21;316-90.
74. **Arvind Krishnamurthy, Vijayalakshmi Ramshankar** Early Stage Oral Tongue Cancer among Non-Tobacco Users - An Increasing Trend Observed in a South Indian Patient Population Presenting at a Single Centre. *Asian Pac J Cancer Prev,* **14 (9)**, 5061-5065.
75. **Takuji Tanaka, Rikako Ishigamori** Understanding Carcinogenesis for Fighting Oral Cancer DOI.org 10.115/2011/603740
76. **Brad W. Neville, DDS;Terry A. Day, MD, FACS** Oral Cancer and Precancerous Lesions CA Cancer J Clin 2002;52:195-215.
77. **R Shenoi, V Devrukhar,Chaudri, BK Sharma,SB Sapre, A Chikhale** Demographic and clinical profile of oral squamous cell carcinoma patients: A retrospective study DOI10,4103/0019-509X.9891.
78. **Antoniades DZ, Styanidis K, PapanyotouP, Trigonidis G**, Squamous cell carcinoma of the lip in a northen Greek population. Evalution of prognostic factors on survival rate 5 years. Eur J Cancer B Oral Oncol 1995; 31:331-9.

79. **Vaidhehi Narayan Nayak, Mandana Donoghue, Selva mani M** Oral squamous cell carcinoma: A 5year s institutional study doi: 10.15713?ins.jmrps.28.

80. **Pindborg JJ, Reichart PA, Smith CJ, van der Wall I**. Histological typing of cancer and precancer of the oral mucosa. 2nd ed. Berlin: Springer-Verlag; 1997. p. 10–6.

81. **M Vakkala, P Pääkkö and Y Soini** Expression of caspases 3, 6 and 8 is increased in parallel with apoptosis and histological aggressiveness of the breast lesion. British Journal of Cancer (1999) 81**(**4), 592–599.

82. **Juliana Hintz Germanos Scheidt Liliane Soares et al** Characteristics of oral squamous cell carcinomain users or non users of tobacco and alcohol. Rev Odonto Cienc 2012;27(1):69-73

ANNEXURES

MADHA

INSTITUTIONAL ETHICAL COMMITTEE

MADHA DENTAL COLLEGE AND HOSPITAL
KUNDRATHUR, CHENNAI-600072.
Telephone no: 044-24780736, *Email id:* madhadentalcollege@gmail.com
Affiliated to The Tamil Nadu Dr. M.G.R. Medical University
DCI Recognition No: Lr.No.F.No. V.12017/75/2006-DE Dated: 01.11.2011

Ref. No.: 002/MDC/IEC/2015: **Date:** 22.12.2015

Title of the work: Caspase 3 expression in Oral Squamous Cell Carcinoma.-IHC

Principal Investigator: Dr. Sharifa. K
I year MDS

Department: Oral Maxillofacial Pathology and Microbiology

The request for an approval from the Institutional Ethical Committee (IEC) considered on the IEC meeting held on 14.09.2015 at the Principal's Chamber Madha Dental College and Hospital, Kundrathur, Chennai- 69 is granted subsequent to her modification letter dated 21.12.2015 and you are

" Advised to proceed with the study"

The Members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the Principal Investigator.

The principal investigator and their team are directed to adhere the guidelines given below:

1. You should get detailed informed consent from the patients/ participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of any change of study procedure, site and clearance.
4. You should not deviate from the area of work for which you have applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions. You should abide to the rules and regulations of the institution (s).
6. You should complete the work within the specific period and if any extension of time is required, you should apply for permission again and do the work.
7. You should submit the summary of the work to the ethical committee on completion of the work.
8. You should not claim your funds from the Institution while doing the work or on completion.
9. You should understand that the members of the IEC have the right to monitor the work with prior intimation.
10. Your work should be carried out under the direct supervision of your Guide/ Professor.

Secretary
Prof. Dr.M.C.Sainath. MDS;
Principal- Madha dental college and Hospital

Chairman
Prof. Dr. Gajendran, M.D;
Dean- Madha Medical college and Hospital

Subject:	Fw: [Urkund] 9% similarity - dr_ksharifa@yahoo.in
From:	Sharifa K (dr_ksharifa@yahoo.in)
To:	netwayprints@gmail.com;
Date:	Thursday, 11 January 2018 5:11 PM

--- On Fri, 5/1/18, <report@analysis.urkund.com> wrote:

> From: <report@analysis.urkund.com>
> Subject: [Urkund] 9% similarity - dr_ksharifa@yahoo.in
> To: dr_ksharifa@yahoo.in
> Date: Friday, 5 January, 2018, 11:39 AM
> Document sent by: dr_ksharifa@yahoo.in
> Document received: 1/5/2018 12:38:00
> PM
> Report generated 1/5/2018 12:39:35 PM
> by Urkund's system for automatic control.
>
> Student message:
>
> ---
>
> Document : FINAL THESIS.pdf
> [D34374432]
>
> IMPORTANT! The analysis contains 1
> warning(s).
>
> About 9% of this document consists of
> text similar to text found in 47 sources. The largest
> marking is 109 words long and is 89% similar to its primary
> source.
>
> PLEASE NOTE that the above figures do
> not automatically mean that there is plagiarism in the
> document. There may be good reasons as to why parts of a
> text also appear in other sources. For a reasonable
> suspicion of academic dishonesty to present itself, the
> analysis, possibly found sources and the original document
> need to be examined closely.
>
> Click here to open the analysis:
> https://secure.urkund.com/view/33873861-697743-128538
>
>
> Click here to download the document:
> https://secure.urkund.com/archive/download/34374432-730725-979105
>
>

CERTIFICATE - II

This is to certify that this dissertation work titled ……..

of the candidate …… with registration Number

……for the award of …… in the branch of

…….. . 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 …… percentage of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.