ESTIMATION OF DIPEPTIDYL PEPTIDASE IV IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS UNDERGOING RADIOTHERAPY

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Your dissertational study titled "ESTIMATION OF DIPEPTIDYL PEPTIDASE IV IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS UNDERGOING RADIOTHERAPY" presented before the ethical committee on 16th Dec. 2016 has been discussed by the committee members and has been approved.

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ABBREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
DPP IV	Dipeptidyl Peptidase IV
CD 26	Cluster of Differentiation Protein 26
ADAbp	Adenosine Deaminase binding protein
WHO	World Health Organization
IARC	International Agency for Research on Cancer
PCD	Programmed Cell Death
ELISA	Enzyme Linked Immunosorbent Assay
IL/U	International Unit / Litre
ATM	Ataxia Telengiectasia Mutated gene
APP	Amyloid Precursor Protein
TNF α	Tumor Necrosis Factor – alpha
SP	Serine Protease
GHRF	Growth Hormone Releasing Factor
TGF – ß1	Transforming Growth Factor – Beta 1
WDSCC	Well Differentiated Squamous Cell Carcinoma
MDSCC	Moderately Differentiated Squamous Cell Carcinoma
PDSCC	Poorly Differentiated Squamous Cell Carcinoma

Introduction

INTRODUCTION

Oral cancer is one of the most common malignancy in India and is the major form of cancer worldwide. It contributes about 30-40% of all cancers. In India, the standardized incidence rate of oral cancer patients is about 12.6 per 100,000 population and prevalence is also very high. It is 4 times higher than in other countries. The important etiologic agents of oral cancer in India are tobacco, chewing with betel quid or tobacco smoking and alcohol consumption. Oral squamous cell carcinoma (OSCC) makes upto 90 % of all oral cancers (**Ganesan and Kumar, 2014)**.

Biomarkers is defined by WHO as "any substance, structure or process that can be measured in the body or its product and influence or predict the incidence of outcome or disease" **(Strimbu and Travel, 2010).**

Biomarkers have featured prominently in tests designed to aid in medical decision making, such as establishing the diagnosis, determining prognosis, and assessing the effects of treatment. An ideal marker for cancer diagnosis and surveillance is one that is non-invasive and reproducible, with high sensitivity and specificity. The classic path to cancer biomarker discovery involves measuring differential levels of proteins in the blood or tissue of interest **(Nazarian et al., 2014).**

In the case of cancer, proteases are one such class, as several of its members have been implicated in promoting both tumor progression and suppression. It has been suggested that the cumulative exopeptidase activity in blood can provide accurate class discrimination between patients with tumors and controls without cancer **(Nazarian et al., 2014).** Dipeptidyl peptidase IV (DPP IV) is one among the proteases family.

DPP IV was first discovered in 1966 by Hopsu – Havu and Glenner and rediscovered by Schulz and Alfred Barth in 1974. Dipeptidyl peptidase 4 (DPP4/DPPIV/CD26 (Cluster of differentiation 26) or ADAbp (adenosine deaminase binding protein) is a 220 kDa homodimeric, type II transmembrane glycoprotein and a cell surface protease belonging to the prolyl oligopeptidase family. A soluble form is also found in plasma. DPP4/CD26 was originally characterized as a T cell differentiation antigen and is expressed on various cell types **(Kang, 2014)**.

The protein encoded by DPP IV gene is an antigenic enzyme expressed on surface of most cell types and is associated with immune regulation, signal transduction and apoptosis. It is an intrinsic membrane glycoprotein and a serine exopeptidase that cleaves x-proline dipeptides from N- terminus of polypeptides **(Chen, 2006).**

The progressive loss of cellular and molecular regulatory mechanisms that occurs during carcinogenesis promotes alterations on key cellular processes, which ultimately determine the raise of malignant phenotypes displaying: autonomous cell growth, irresponsiveness to growth inhibitory signals, evasion of apoptosis, increased telomerase activity, sustained angiogenesis, tissue invasion and metastasis.

Most of these alterations are related to abnormal cell signaling circuits, with over expressed or constitutively expressed oncogenes, or tumor suppressor genes with null or decreased expression. Particularly, most of those circuits are triggered by molecules secreted by the tumor or by its microenvironment. It is known that DPP-IV participates in peptide-mediated growth regulation and differentiation and in the regulation of extracellular matrix interactions.

The regulation of the DPP-IV-mediated proteolysis could have marked effects on the availability of growth promoting or inhibitory factors in a given microenvironment. Therefore, the loss or lack of DPP-IV expression, and its expression or that of its ligands in the tumor neighboring cells which can be crucial for the progression and metastasis events in several tumor types. The evidences of such events are multifactorial, and their interpretations depend on the properties of the carcinogenesis affected areas.

DPP-IV expression is found to be decreased in several cancers, such as: melanoma, lung cancer, prostate cancer, oral cancer, colorectal cancer and endometrial adenocarcinoma.

The DPP IV expression is found to be increased in some cancer types, such as: primary lung tumors, ovarian carcinoma, thyroid carcinoma, dermal basal cell carcinoma, esophageal adenocarcinoma, B-cells chronic leukemia and certain types of T cell cancers (T-cell lymphoblastic lymphoma, anaplastic large cell lymphomas and T-cell acute lymphoblastic leukemia).

This change in expression of DPP IV in different cancer is due to its significant role in apoptotic activity. Apoptosis is defined as programmed cell death. It is clear that apoptosis has to be tightly regulated since decreased or increased cell death may lead to pathology, including developmental defects, autoimmune diseases, neuro-degeneration or malignancy. We examined this enzyme activity in the plasma of patients with OSCC, in order to determine whether serum enzyme activities are decreased or increased **(Arrebola et al., 2014).**

Studies have proved that there was a decrease in serum and plasma levels of DPP IV in oral cancer patients compared to normal healthy individuals. Only limited studies exist to compare the levels of DPP IV in OSCC patients of blood. There is scarcity of literature for the studies on plasma levels of DPP IV in oral cancer patients. Moreover, no study has been done to assess the plasma levels of DPP IV OSCC patients during treatment. Therefore, the present study has made an attempt to evaluate the levels of DPP IV in blood of OSCC patients undergoing radiation therapy without surgery and in patients undergoing radiation therapy after surgery and to compare with that of the healthy controls and to establish the diagnostic efficacy of DPP IV levels in OSCC patients.

Aims and Objectives

AIM

To estimate the levels of DPP IV enzyme in blood of OSCC patients undergoing only radiation therapy, OSCC patients undergoing radiation therapy after surgery and in control group.

OBJECTIVES OF THE STUDY

- To estimate the levels of DPP IV enzyme in blood of OSCC patients (group I – undergoing only radiation therapy) before(IA) and during(IB) radiation therapy and to compare the level of DPP IV activity in group IA & IB
- To estimate the levels of DPP IV enzyme in blood of OSCC patients (group II – undergoing radiation therapy after surgery) before(IIA) and during(IIB) radiation therapy and to compare the levels of DPP IV activity in group IIA & IIB
- To determine the levels of DPP IV enzyme in blood of healthy individuals (group III) during 1st day (IIIA) and 21st day(IIIB) and to compare the level of DPPIV activity in group IIIA & IIIB
- To compare the levels of DPP IV activity in blood of group IA, IIA & IIIA
- To compare the levels of DPP IV activity in blood of group IB, IIB & IIIB
- To compare the level of DPP IV activity within groups and between groups namely IA, IB, IIA, IIB, IIIA & IIIB

Review of Literature

ORAL CANCER

Cancer is the major cause of morbidity and mortality all over the world. It is one of the main causes of death in all countries with its relative position varying with age and sex. The sixth most common cancer in the world is oral and oropharyngeal carcinomas (**Shenoi et al., 2012)**. The incidence of oral cancer is highest in India, South and Southeast Asian countries. In India, oral squamous cell carcinoma (OSCC) constitutes about 90-95% of all the oral cancers. The lifetime risk for mortality due to cancer in India for both males and females is estimated to be 61% **(Varshitha, 2015).**

A number of etiological factors has been attributed to the high incidence of oral cancer in India. The common causes for oral cancer are tobacco consumption habit, either as smokeless tobacco or smoking and alcohol consumption. The other causes for oral cancer are positive family history of oral cancer, viral infections like HPV and poor oral hygiene **(Varshitha, 2015).**

However, not all the people who follow these habits develop OSCC. There may be a few genetic characteristics specific to an individual or certain other environmental factors which may either offer protection against OSCC, or may predispose to or even promote OSCC **(Feller and Lemmer, 2012).**

According to World Health Organization (WHO), in developing countries, in males, carcinoma of oral cavity is the sixth commonest cancer after lung, prostate, colorectal, stomach and bladder cancer, while in females, it is the tenth commonest site of cancer after breast, colorectal, lung, stomach, uterus, cervix, ovary, bladder and liver **(Mehrotra** and **Yadav, 2006).**

The international agency for research on cancer (IARC) confirmed that smoking of various forms of tobacco is carcinogenic in humans. There is increased exposure to carcinogenic agents such as tobacco-specific nitrosamines released from tobacco chewing with betel quid and to nitrosamines derived from areca nut alkaloids Furthermore, apoptosis is important in carcinogenesis and suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of cancers.

APOPTOSIS

Programmed cell death (PCD) in physiological and pathological processes is known as apoptosis. It is derived from Ancient Greek word meaning "falling off" **(Green and Douglas, 2011)**.

Apoptosis, or programmed cell death (PCD), is an important counterpart to mitosis for the regulation of cell numbers during development, in homeostatic cell turnover in the adult **(Wyllie et al., 1980).**

It is a biochemical events which leads to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and mRNA decay. Between 50 and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 to 30 billion cells die a day **(Karam and Jose, 2009).**

Apoptosis is a highly regulated and controlled process that confers advantages during an organism's lifecycle. For example, the separation of fingers and toes in a developing human embryo occurs because cells between the digits undergo apoptosis.

Apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out into surrounding cells and cause damage to the neighboring cells**(Albert et al., 2008)** (Fig1).

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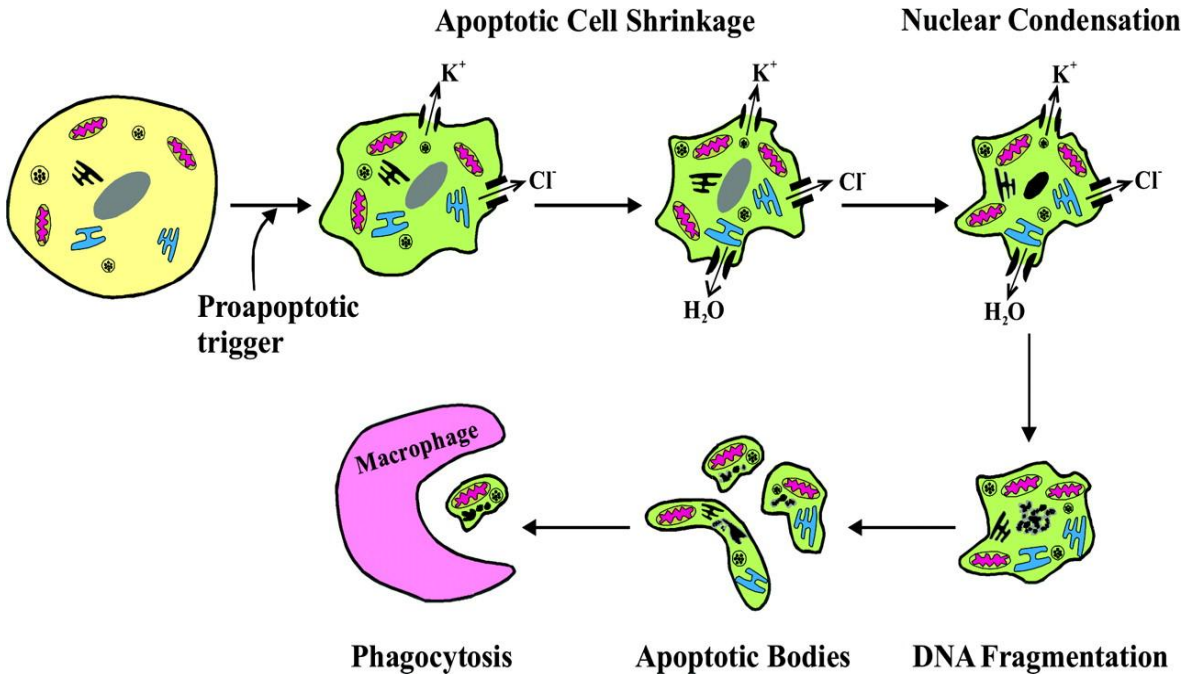

Figure 1: Apoptosis showing characteristic cell changes, formation of apoptotic bodies and phagocytosis

Apoptosis is a highly regulated process. Apoptosis can be initiated through one of two pathways. In the *intrinsic pathway* the cell kills itself because it senses cell stress, while in the *extrinsic pathway* the cell kills itself because of signals from other cells.

An increased understanding of apoptosis makes anti-apoptosis engineering possible, which is an approach used to inhibit apoptosis for the purpose of therapeutic applications in the treatment of the disease associated with increased apoptosis.

WHY DO CELLS UNDERGO APOPTOSIS?

Many cells in the human body have the built-in ability to undergo apoptosis (in the same way that they have the built-in ability to copy their DNA or break down fuels). Basically, apoptosis is a general and convenient way to remove cells that should no longer be part of the organism **(Albert et al., 2008).**

- Some cells need to be "deleted" during development – for instance, to whittle an intricate structure like a hand out of a larger block of tissue.
- Some cells are abnormal and could hurt the rest of the organism if they survive, such as cells with viral infections or DNA damage.
- Cells in an adult organism may be eliminated to maintain balance – to make way for new cells or remove cells needed only for temporary tasks.

SIGNIFICANCE OF APOPTOSIS

During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues **(Meier et al., 2000).**

In the human body about100, 000 cells are produced every second by mitosis and a similar number die by apoptosis.

In development and Morphogenesis

- During limb formation separate digits evolve by death of interdigital mesenchymal tissue
- Ablation of cells no longer needed such as the amphibian tadpole tail during metamorphosis
- Demise of cells allows formation of hollow structures
- Formation of reproductive organs (Müllerian duct - uterus, deleted in males; Wolffian duct- male organs, deleted in females)
- Massive cell death occurs during early development of the nervous system

In homeostasis

Immune system has its role in maintaining homeostasis in the process of apoptosis: several millions of B and T cells are generated everyday and the majority (> 95percent) of those die during maturation or by activation induced cell death (AICD) of peripheral immune cells.

In deletion of damaged and dangerous cells

- Cells with severely damaged DNA that cannot be repaired are usually removed by apoptosis
- Inappropriate mitogenic signaling that is in conflict with the environmental or cellular status of the cell usually results in cell cycle arrest or apoptosis
- Autoreactive cells of the immune system are deleted by apoptosis
- Elimination of infected cells **(Gewies, 2003).**

MECHANISMS OF APOPTOSIS

Apoptosis is a tightly regulated and efficient cell death program involving multiple factors. Every cell contains an intrinsic mechanism which signals death or survival and any imbalance in these signals can result in apoptosis.

Understanding the mechanisms of apoptosis is crucial and helps in the understanding of the pathogenesis of conditions as a result of disordered apoptosis. This in turn, may help in the development of drugs that target certain apoptotic genes or pathways.

Caspases take major and a central role in apoptotic mechanism. The term caspases is derived from cysteine-dependent aspartate-specific proteases. Caspases are central to the mechanism of apoptosis as they are both the initiators and executioners.

There are three pathways by which caspases can be activated.

The two commonly described initiation pathways are the intrinsic or mitochondrial and extrinsic or death receptor pathways of apoptosis. Both pathways eventually lead to a common pathway or the execution phase of apoptosis. (Fig 2)

A third less well-known initiation pathway is known as intrinsic endoplasmic reticulum pathway **(Rebecca and Wong, 2011).**

The *intrinsic pathway* is activated by intracellular signals generated when cells are stressed and depends on the release of proteins from the intermembrane space of mitochondria.

The *extrinsic pathway* is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex.

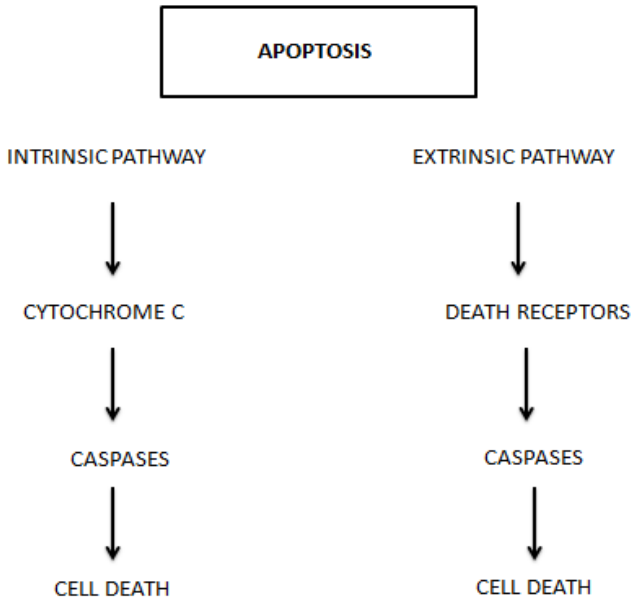

Figure 2: Mechanism of apoptosis showing intrinsic and extrinsic pathway

A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration, for example, damage to the membrane, can trigger the release of intracellular apoptotic signals by a damaged cell.

Before the actual process of cell death is precipitated by enzymes, apoptotic signals must cause regulatory proteins to initiate the apoptosis pathway. This step allows signals to cause cell death, or the process have to be stopped, if the cells no longer need to die.

PHYSIOLOGIC APOPTOSIS

The role of apoptosis in normal physiology is as significant as that of its counterpart, mitosis. It demonstrates a complementary but opposite role to cell proliferation in the regulation of various cell populations. It is estimated that to maintain homeostasis in the adult human body, around 10 billion cells are made each day just to balance which are undergoing apoptosis **(Renehan et al., 2001)** and that number can increase significantly when there is increased apoptosis during normal development and aging or during disease.

Apoptosis is critically important during various developmental processes. Apoptosis is also necessary to get rid of pathogen-invaded cells in the body and it's a vital component of wound healing, which involves in the removal of inflammatory cells and the evolution of granulation tissue **(Greenhalgh, 1998).** Dysregulation of apoptosis

during wound healing can lead to pathologic forms of healing such as excessive scarring and fibrosis.

Apoptosis is also needed to eliminate activated or auto-aggressive immune cells during maturation in the central lymphoid organs (bone marrow and thymus) or in peripheral tissues **(Osborne, 1996).** Furthermore, as organisms grow older, some cells begin to deteriorate at a faster rate and are eliminated via apoptosis. One theory states that oxidative stress plays a primary role in the pathophysiology of age-induced apoptosis via accumulated free-radical damage to mitochondrial DNA **(Harman, 1992).**

It is clear that apoptosis has to be tightly regulated since too little or too much cell death may lead to pathology, including developmental defects, autoimmune diseases, neuro-degeneration, or cancer.

PATHOLOGIC APOPTOSIS

Abnormalities in cell death regulation can be a significant component of diseases such as cancer, autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neuro-degenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease and Amyotrophic Lateral Sclerosis. Decreased apoptosis may be seen in some conditions, whereas excessive apoptosis may be seen in others.

Cancer is an example where the normal mechanisms of cell cycle regulation are dysfunctional, with either an over proliferation of cells and/or decreased removal of cells **(King and Cidlowski, 1998).** In fact, suppression of apoptosis during carcinogenesis is

thought to play a central role in the development and progression of some cancers **(Kerr et al., 1994).**

Tumor cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins such as Bcl-2 or by the down-regulation or mutation of pro-apoptotic proteins such as Bax. The expression of both Bcl-2 and Bax is regulated by the *p53* tumor suppressor gene **(Miyashita et al., 1994).** Certain forms of human B cell lymphoma have over expression of Bcl-2, and this is one of the first and strongest lines of evidence that failure of cell death contributes to cancer. Another method of apoptosis suppression in cancer involves evasion of immune surveillance **(Smyth et al., 2001).**

Certain immune cells (T cells and natural killer cells) normally destroy tumor cells by the death-receptor pathway. In order to evade immune destruction, some tumor cells will diminish the response of the death receptor pathway to FasL produced by T cells. This has been shown to occur in a variety of ways including down-regulation of the Fas receptor on tumor cells. In fact, some tumor cells are capable of a Fas ligand-mediated "counterattack" that results in apoptotic depletion of activated tumor infiltrating lymphocytes **(Koyama et al., 2001).**

Alterations of various cell signaling pathways can result in dysregulation of apoptosis which leads to cancer. The *p53* tumor suppressor gene is a transcription factor that regulates the cell cycle and is the most widely mutated gene in human tumorigenesis. The critical role of *p53* is evident by the fact that it is mutated in over 50% of all human cancers. *p53* can activate DNA repair proteins when DNA has sustained damage, can hold the cell cycle at the G1/S regulation point on DNA damage recognition, and can

initiate apoptosis if the DNA damage proves to be irreparable **(Pietenpol and Stewart, 2002)**. If the *p53* gene is damaged, then tumor suppression is severely reduced. The *p53* gene can be damaged by radiation, various chemicals, and viruses such as the Human papillomavirus (HPV). People who inherit only one functional copy of this gene will most likely develop Li–Fraumeni syndrome, which is characterized by the development of tumors in early adulthood **(Varley et al., 1997).**

The ataxia telangiectasia-mutated gene (ATM) has also been shown to be involved in tumorigenesis via the ATM/*p53* signaling pathway. The ATM gene encodes a protein kinase that acts as a tumor suppressor. ATM activation, via ionizing radiation damage to DNA, stimulates DNA repair and blocks cell cycle progression. One mechanism through which this occurs is ATM dependent phosphorylation of *p53* **(Kurz and Lees-Miller, 2004).**

In addition to cancer, too little apoptosis can also result in diseases such as autoimmune lymphoproliferative syndrome (ALPS) **(Worth et al., 2006).** This occurs when there is insufficient apoptosis of auto-aggressive T cells, resulting in multiple autoimmune diseases. An over proliferation of B cells occurs as well, resulting in excess immunoglobulin production, leading to autoimmunity. Some of the common diseases of ALPS include hemolytic anemia, immune-mediated thrombocytopenia, and autoimmune neutropenia.

Excessive apoptosis may also be a feature of some conditions such as autoimmune diseases, neurodegenerative diseases and ischemia-associated injury. Autoimmune deficiency syndrome (AIDS) is an example of an autoimmune disease that results from

infection with the human immunodeficiency virus (HIV). This virus infects CD4+ T cells by binding to the CD4 receptor.

Alzheimer's disease is a neurodegenerative condition that is thought to be caused by mutations in certain proteins such as APP (amyloid precursor protein) and presenilins. Presenilins are thought to be involved in the processing of APP to amyloid β. This condition is associated with the deposition of amyloid β in extracellular deposits known as plaques and amyloid β is thought to be neurotoxic when found in aggregated plaque form. Amyloid β is thought to induce apoptosis by causing oxidative stress or by triggering increased Fas ligand expressions in neurons and glia. It may also activate microglia, which would result in TNFα secretion and activation of the TNF-R1, leading to apoptosis.

Excessive apoptosis is also thought to play an important role in various ischemia-associated injuries. One example is myocardial ischemia caused by an insufficient blood supply, leading to a decrease in oxygen delivery to, and subsequent death of the cardiomyocytes. Although necrosis does occur, over expression of BAX has been detected in ischemic myocardial tissue and therapy aimed at reducing apoptosis has shown some success in reducing the degree of tissue damage. One hypothesis is that the damage produced by ischemia is capable of initiating apoptosis but if ischemia is prolonged, necrosis occurs. If energy production is restored, as with reperfusion, the apoptotic cascade that was initiated by ischemia may proceed. Although the extent to which apoptosis is involved in myocardial ischemia remains to be clarified, there is clear evidence that supports a role for this mode of cell death **(Worth et al., 2006).**

REGULATION OF APOPTOSIS

Apoptosis involves a cascade of complex events which include the delivery of external signals through defined receptor complexes, the well-regulated expression of a number of genes, and the execution of apoptosis by proteases and endonucleases. A large number of genes and proteins have been implicated in the control of apoptosis. Adverse assortment of triggers activates the cascade, which is subject to tight homeostatic regulation by a number of regulators or modulators of the death pathway. The "point of no return" in apoptosis is reached when caspases become enzymatically active in cleaving target proteins. When there is disruption in the balance of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, the result is dysregulated apoptosis in the affected cells. This can be due to an over expression of one or more anti-apoptotic proteins or an under expression of one or more pro-apoptotic proteins or a combination of both.

APOPTOSIS AND ORAL TUMORIGENESIS

Apoptosis prevents the development of aneuploidy and other genetic abnormalities that are associated with the development and progression of precancerous lesions (Fig 3).

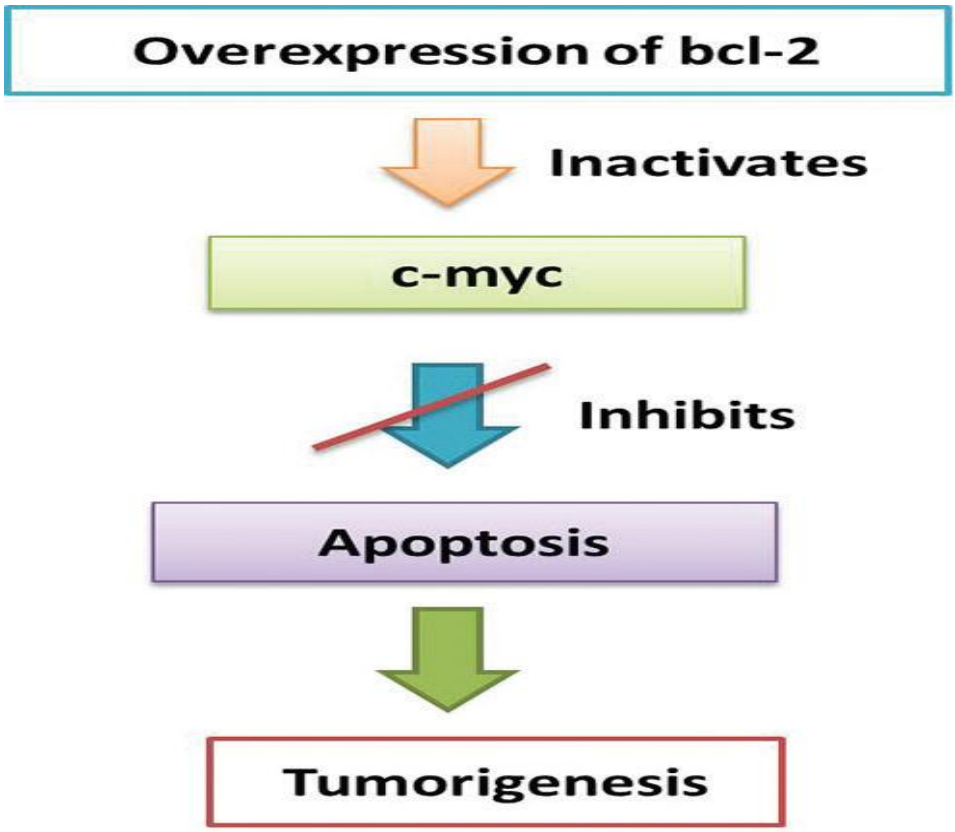

Figure 3: Collaboration of BCL- 2 and c-Myc in tumorigenesis

Damaged normal cells due to hypoxia or anticancer therapy are eliminated by apoptosis, failure to which leads to the development of cancer. Cancer cells may evade apoptosis by inactivation of apoptosis- inducing genes or by enhancement of antiapoptosis genes. TP53 gene induces arrest of cell cycle and apoptosis. It exerts its effects at multiple stages of cancer progression, implying that there is a strong selection for tumor cells to inactive TP53. This is shown by the frequency with which it is mutated in human cancers.

A number of apoptosis- inducing proteins, such as Bax and BAD, may be inactivated in tumors. Bax was shown to be down-regulated in oral carcinoma cells. Tumors also increase the expression of proteins that inhibit apoptosis. Bcl-XL, an antiapoptotic protein, was found to be over expressed in oral cancer cells and could confer resistance to multiple chemotherapeutic agents in several squamous cell carcinoma cell lines. (Fig 4).

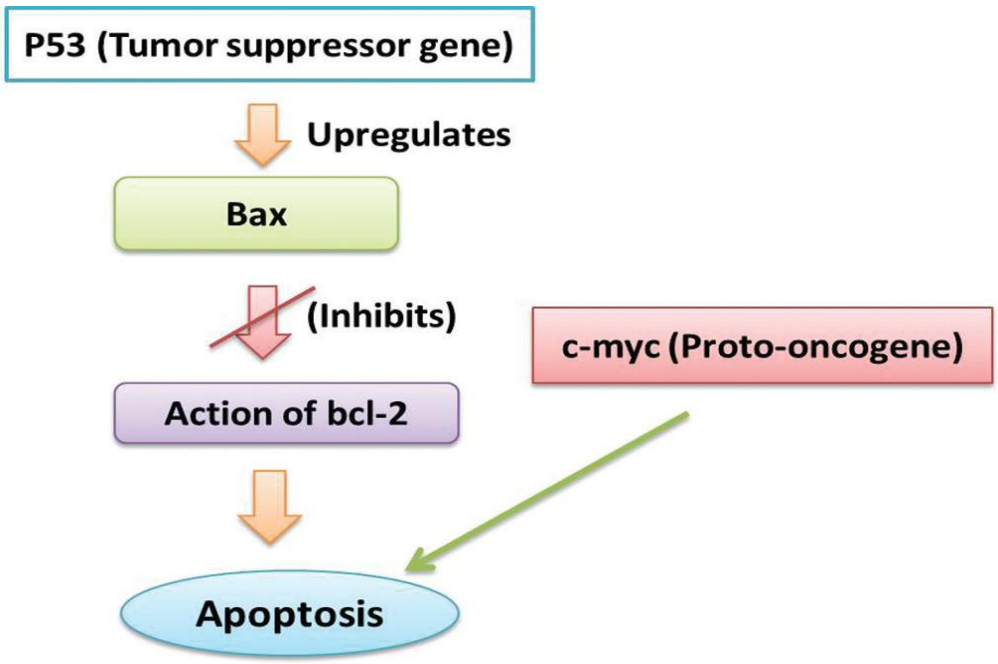

Figure 4**:** Role of p53, Bax, BCL- 2 and c-Myc in apoptosis

BIOMARKER IN ORAL CANCER

Biomarkers is defined by WHO as "any substance, structure or process that can be measured in the body or its product and influence or predict the incidence of outcome or disease" **(Strimbu and Travel, 2010).**

Biomarkers have featured prominently in tests designed to aid in medical decision making, such as establishing a diagnosis, determining prognosis, and assessing the effects of treatment. An ideal marker for cancer diagnosis and surveillance is one that is noninvasive and reproducible, with high sensitivity and specificity. The classic path to cancer biomarker discovery involves measuring differential levels of proteins in the blood or tissue of interest **(Nazarian et al., 2014).**

In the case of cancer, proteases are one such class, as several of its members have been implicated in promoting both tumor progression and suppression. Proteases are involved in a myriad of physiological cellular processes, including growth, differentiation, nutrition, protein turnover, migration and diapedesis, fertilization and zygote implantation, programmed cell death, and others. They also mediate physiopathological events such as: cancer, neurodegenerative, respiratory and cardiovascular disorders, parasitic infestations, viral and fungal infections. Hence, the proteases systems have to be tightly controlled by effective metabolic mechanisms, with proteases inhibitors as one of the key mechanisms. Since proteases are crucial mediators in the replication and infectivity of several pathogens in man, plants and animals, the development of specific and efficacious inhibitors for potential therapeutic application has emerged as an active research field. They have been found as effective therapeutic tools in cancer, the human

immunodeficiency syndrome (AIDS), inflammation, cardiovascular and respiratory diseases, Alzheimer's disease, and type 2 diabetes mellitus

DIPEPTIDYL PEPTIDASE IV AS A BIOMARKER IN ORAL CANCER

Particularly, the serine proteases (SP) comprise the best characterized family of proteases due to exhaustive studies conducted in the last 50 years with kinetic, chemical, physical and genetic techniques. A remarkable example is dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5), also known as cluster of differentiation protein 26 (CD26), a SP belonging to the prolyl-oligopeptidases with a cell surface expression pattern. It bears a wide anatomic distribution, with its highest specific activity in the kidney **(Itou et al., 2013).** Besides, a soluble isoform is present in several body fluids.

DPP-IV selectively removes the amino terminal dipeptide from peptides having proline or alanine in the second position. Various cytokines, growth factors and some neuropeptides bear this structural motif, contributes to their respective biological activities and their protection against unspecific proleolysis.

The DPP-IV exists as a cell surface protein and is characterized by its ubiquity, being found in humans, in epithelial cells in the liver, intestines and kidneys. A soluble form is also found in body fluids, and its expression is regulated by B and T lymphocytes. The highest specific enzymatic activity of this protease is found in the seminal fluid and the kidney **(Yu et al., 2010).**

FUNCTIONS OF DPP IV

The protein encoded by the *DPP4* gene is an antigenic enzyme expressed on the surface of most cell types and is associated with immune regulation, signal transduction and apoptosis.

It is an intrinsic membrane glycoprotein and serine exopeptidase that cleaves X-proline dipeptides from N-terminus of polypeptides.

It is rather indiscriminate enzyme for which a diverse range of substrates are known. The substrates of CD26/DPP IV are proline or alanine – containing peptides and include growth factors, chemokines, neuropeptides and vasoactive peptides (**Chen, 2006**)

DPP4 plays a major role in glucose metabolism. It is responsible for the degradation of incretins such as GLP-1. Furthermore, it appears to work as a suppressor in the development of cancer and tumours

CD26 / DPP IV plays an important role in tumor biology, and is useful as a marker for various cancers, with its levels either on cell surface or in serum increased in some neoplasms and decreased in others(**Havre et al., 2008**).

A new class of oral hypoglycemic called DPP IV inhibitors work by inhibiting the action of this enzyme, thereby prolonging incretin effect invivo (**Rosenstock and Zinman, 2007**).

STUDIES ANALYSING DPP IV IN SYSTEMIC DISEASE

Kobayashi et al., 2002 in their study stated that serum levels of CD26 and its specific DPP IV activity were significantly decreased in patients with systemic lupus erythematosus (SLE) and were inversely correlated with SLE disease activity index score, but not with clinical variables of SLE. More recently, **Valizadeh et al., 2018** also found CD26 expression on SLE patients is decreased significantly than that of healthy controls.

Detel et al., 2012 in their study proved that enzymatic activity levels of DPP IV in plasma were significantly decreased in rheumatoid arthritis (RA) patients who were diagnosed according to American college of Rheumatology criteria and found statistically significant result compared to that of control group.

L**ee et al., 2013** in their study found that **s**erum DPP IV level and enzymatic activity is higher in patients with type 2 diabetes mellitus and active glucose control shows decreased DPP IV expression on T cells. Insulin resistance was associated with the increase in expression and release of DPP IV, insulin and Tumor necrosis factor – α can stimulate DPP IV release from adipocytes. In addition, recent finding that DPP IV inhibitor therapy plays an important role in disease control of type 2 diabetes patients

Crohn's disease and ulcerative colitis are categorized as inflammatory bowel disease (IBD) and are associated with increased risk of colon cancer as the progressive inflammatory condition affects the entire gastrointestinal tract (GIT) and colonic mucosa. **Abrahami et al., 2018** in their study found that sera from IBD patients contain lower levels of circulating DPP IV activity, while membrane expression of DPP IV on T cells

isolated from IBD patients is higher than healthy controls which indicates that DPP IV might play a significant role in perpetuating the inflammatory response associated with IBD.

STUDIES ANALYSING DPP IV IN AUTOIMMUNE DISEASE

DPP IV activity is found only in basic isoforms but not in acidic isoforms. A shift to acidic isoforms has been observed during HIV infection. $CD4^+$ cells in HIV patients have intrinsic defect in their ability to recognize and respond to antigens before a reduction of total number of $CD4^+$ cells occurs in these patients. The memory function to recall antigens is a property of $CD4^+$ T cells expressing DPP IV. It is the only type of T helper cells that is known to proliferate in response to soluble antigens. There is a decrease in DPP IV positive T cells in HIV-1 infected individuals prior to general reduction in number of $CD4^+$ cells which indicates the importance of immune-modulating role DPP IV.

STUDIES ANALYSING DPP IV IN OTHER MALIGNANCIES

The exact role of DPP IV in various cancers remains to be elucidated, partly due to its variable expression on these tumors. In general, it is strongly expressed on some cancers, while being absent or present at low levels in others. Furthermore, given the plethora of its biological functions, including its ability to associate with several key proteins and its cleavage of a number of soluble factors to regulate their function, it is likely that DPP IV

effect on tumor biology is at least partly mediated by the effect of these biological functions on specific tumor types.

Houghton et al., 1988 in their study found that DPP IV is highly expressed in normal melanocytes, but not in melanoma cells, suggesting DPP IV expression is lost in malignant transformation. Loss of DPP IV was also associated with development of specific chromosome abnormalities. DPP IV also induces MMP-9 expression in cancer cells, which may facilitate metastasis. DPP IV also inactivates circulating growth hormone releasing factor (GHRF), so decreased DPP IV may result in cancer growth by increased GHRF.

Kotani et al., 1992 in their study observed that CD26/DPPIV was expressed in nearly all cases of thyroid follicular and papillary carcinoma, suggesting its potential usefulness as a marker for distinguishing thyroid cancer from benign tumors. Decreased levels of CD26/DPPIV were detected in undifferentiated areas compared with the differentiated areas, suggesting that it may play a role in regulating tumor aggressiveness and serve as a marker of disease prognosis.

Darmoul et al., 1992 in their study observed that CD26/DPPIV mRNA level in colon cancer was low in undifferentiated cells, but increased as differentiation progressed.

Carbone et al., 1994 did a study in non-hodgkin's lymphoma, DPP IV expression is found mainly in aggressive subtypes, such as T-lymphoblastic lymphoma (LBL) and anaplastic large cell lymphoma. DPP IV expression in LBL was associated with a worse survival. Loss of DPP IV appears to be characteristic of cutaneous T-cell lymphoma (CTCL) and has been suggested as a useful diagnostic marker.

Gaetaniello et al., 1998 in their study evaluated that all hepatocellular carcinomas displayed loss of DPP IV activity or an altered distribution of CD26/DPPIV activity.

Klobusicka and Babusikova, 1998 in their study analysed 30 patients with the diagnosis of T- acute lymphoblastic leukemia and found increased CD26 expression and DPP IV activity. **De Andrade et al., 2009** in their study found similar findings that DPP IV activity in plasma of patients with acute leukemia is elevated.

Dang et al., 2003 in their study found that DPP IV expression is associated with a more aggressive clinical course in T-cell large granular lymphocyte leukemia (T-LGLL). T-LGLL patients with low expression of DPP IV had more indolent course, while high expression developed recurrent infections due to neutropenia.

Khin et al., 2003 in their study found that CD26/DPPIV expressed on normal endometrial glandular cells, but its expression on endometrial adenocarcinoma was down-regulated with increasing grade of neoplasm. Therefore, down-regulation of CD26/DPPIV expression was correlated with neoplastic transformation and tumor progression.

Wesley et al., 2004 in their study found that CD26 expression in lung cancer appears to be dependent on the specific histologic subtype. When the expression of CD26/DPPIV was compared at the mRNA and protein levels in non-small-cell lung cancer cell lines and normal bronchial epithelial cells, CD26/DPPIV was detected in normal epithelial cells, but was reduced or not detectable in NSCLC cell lines. CD26/DPPIV from lung cancer tissue consists of more basic molecular forms than that from normal lung tissue,

suggesting that the molecular properties of CD26 in the two types of lung tissues are different.

Inamoto et al., 2007 in their study stated that DPP IV was shown to have highest activity among cell surface aminopeptides in human mesothelial cells and found that mesothelial cells cultured in presence of malignant ascites from ovarian carcinoma patients exhibited an increase in DPP IV activity. DPP IV is expressed at high level on the surface of malignant mesothelioma cells.

Eric-Nikolic et al., 2011 did a study on serum DPP IV activity on breast carcinoma and found decreased DPP IV activity in patients with breast carcinoma when compared with control groups.

STUDIES ANALYSING DPP IV IN ORAL CANCER

Fukasawa et al., 1982 did a study on serum DPP IV activity in oral cancer patients and found that patient with OSCC had significantly lower enzyme activity compared with normal individuals. Patient with high grade carcinoma showed a more significant reduction of serum enzyme activity.

Urade et al., 1989 measured serum DPP IV activity in 51 patients with oral squamous cell carcinoma before treatment. DPP IV activity in the sera of cancer patients were significantly decreased, compared with those in the sera of healthy subjects. When DPP IV activities in the sera of cancer patients taken at intervals were determined, it appeared that the activities had changed dynamically, reflecting the clinical status. They were

increased in tumor regression by cancer therapy including irradiation, chemotherapy, and tumor excision, but were decreased in disease progression. The increased activities were maintained in patients with a fair prognosis, but they remained low in patients with poor prognosis.

Uematsu et al., 2004 did a study on oral cancer patients and found that CD26 / DPPIV activity in serum and expression on peripheral blood T lymphocytes are decreased in patients with oral cancers compared to normal controls. To better understand the biochemical mechanism involved in CD26/DPPIV down-regulation, the effect of cytokines produced by the squamous cell carcinoma cell line was studied in peripheral blood T cells. The factor present in KB culture media which was responsible for CD26/DPPIV down-regulation in T cells, and decreased activity in serum, was identified as tumor growth factor beta 1 (TGF-beta 1).

Pro and Dang, 2004 did a study on role of DPP IV in oral cancer and stated that decreased expression of DPP IV was observed in oral cancer and DPP IV also have a role in tumor cell migration and metastasis, by binding to collagen fibronectin and by interacting with extracellular matrix components, CD26 affects immune regulation by recruiting activated lymphoid cells to sites of inflammation or tumor. Moreover, binding of CD26/DPPIV to extracellular matrix proteins can influence tumor growth by regulating tumor adhesion, migration, and metastasis.

Pranoti et al., 2010 did a study on estimation of DPP IV in oral squamous cell carcinoma patients undergoing radiotherapy based on different stages in 50 patients and found that serum and saliva of OSCC patients showed significantly lower level of DPP

IV than the control group. In study group, well differentiated carcinoma had highest mean level of DPP IV activity than moderately and poorly differentiated carcinoma. There was an increase in DPP IV levels in OSCC patients after radiotherapy. They concluded that DPP IV activity can be used as prognostic indicator in OSCC patients.

Kandekar et al., 2014 did study on quantitative analysis of DPP IV in 31 patients with different stages of OSCC and found significantly lower level of DPP IV in study group compared with the control group. Among study group, well differentiated carcinoma had highest mean serum DPP IV activity than moderately and poorly differentiated carcinoma. They concluded that DPP IV activity in serum can be used as biochemical marker in the diagnosis of OSCC.

Materials and Methods

SOURCE OF DATA

Data was collected from patients visiting the cancer centre in Madurai. Informed consent (Annexure I) was obtained from all the patients before collecting the samples. The study was performed after obtaining approval from the institutional ethical committee.

METHOD OF COLLECTION OF DATA

The sample for the present study comprised of 60 patients of both genders with an age distribution between 30 and 75 years. The study samples were divided into three groups which follows as;

Study group 1: Patients who were histopathologically diagnosed with OSCC and undergoing radiation therapy alone (n = 20)

Study group 2: Patients who were histopathologically diagnosed with OSCC and undergoing radiation therapy after surgery (n = 20)

Study group3: Normal healthy individuals with clinically normal oral mucosa (n = 20)

- Blood samples were collected twice from each patient in group 1 and 2, during 1st day before radiation therapy (IA, IIA) and at the end of third week (21st day) during radiation therapy (IB, IIB). In group 3 blood samples were collected in the 1st day (IIIA) and 21st day (IIIB).

INCLUSION CRITERIA

- ✓ Histopathologically diagnosed cases of OSCC were included in the study group
- ✓ Patients undergoing treatment such as radiotherapy and surgery

EXCLUSION CRITERIA

- ✓ Patients with any other systemic disorders
- ✓ Patients undergoing chemotherapy

METHODOLOGY

After obtaining an informed consent (Annexure-I), patients from three groups were subjected to thorough clinical examination. Each patient's complete medical, dental history and clinical photographs were recorded. Blood samples were collected from each patient in all three groups

MATERIALS REQUIRED

- ✓ Disposable syringe & needle
- ✓ Heparinised tubes for collecting blood
- ✓ Plain test tubes (5ml)
- ✓ Cooling centrifuge
- ✓ Micropipettes with plastic disposable pipette tips
- ✓ Cuvettes
- ✓ Stirrer
- ✓ Dipeptidyl peptidase IV (DPP IV) enzyme kit
- ✓ Distilled water
- ✓ Beaker
- ✓ Incubator shaker
- ✓ Measuring cylinder
- ✓ Test tube stand
- ✓ ELISA plate
- ✓ Multimode reader
- ✓ Auto analyzer

SAMPLE COLLECTION – BLOOD

- ✓ Under aseptic precautions, 5ml of venous blood was collected from antecubital vein of all the subjects using sterile disposable syringe
- ✓ Haemolysed and lipemic samples were avoided

PROCESSING OF BLOOD SAMPLES

- ✓ 5 ml of whole blood was transferred to a heparinised tube.
- ✓ Samples were then centrifuged in a cooling centrifuge and the supernatant was stored at - 80^0C until analysis of DPP IV by Gly pro –p- nitroaniline toluene sulfonate was done

ESTIMATION OF DIPEPTIDYL PEPTIDASE IV

CALCULATION:

$$\text{DPP IV activity} = \frac{\text{A serum sample} - \text{A blank of serum sample}}{\text{A standard} - \text{A blank of standard}} \times 100 \text{ IU/L}$$

For estimation of DPP IV enzyme activity in plasma, we need substrate, blank and standard solutions. Preparation of substrate, blank and standard solution has different procedures which are as follows.

SUBSTRATE PREPARATION:

- ✓ Gly-pro-p-nitroaniline toluene sulfonate of molecular weight 464.49g/mol is used for substrate preparation
- ✓ For substrate, Gly-pro-p-nitroaniline toluene sulfonate is prepared at a concentration of 20 mmol using Tris-HCL buffer at pH 7.5.

BLANK PREPARATION:

- ✓ For blank preparation, 180µl of Tris-HCL buffer at pH 7.5 is added to 820µl of distilled water to make final volume for 1000µl to nullify the enzymatic reaction
- ✓ Blank solution contain substrate solution but do not contain plasma sample

STANDARD PREPARATION:

- ✓ P-Nitroaniline ($C_6H_6N_2O_2$) of molecular weight – 138.13g/mol is used for standard preparation
- ✓ Standard stock concentration = mg/ml in Tris-HCL buffer at pH 7.5. Eg – 1mg/ml (1mg of p-nitroaniline is added to 1ml of Tris-HCL buffer)
- ✓ 20µl of standard solution is added to 180µl of Tris-HCL buffer at pH 7.5
- ✓ This mixture is incubated at $37^{O}C$ for one hour
- ✓ To the mixture add 800µl of sodium acetate buffer
- ✓ Standard solution do not contain substrate and plasma sample in it

PROCEDURE:

- ✓ 10µl of plasma sample is added to 20µl of substrate solution
- ✓ To this mixture 170µl of Tris-HCL buffer at pH 7.5 is added in the wells of ELISA plate
- ✓ Incubate this mixture at $37^{O}C$ for one hour in an incubator shaker

- ✓ After one hour 800µl of sodium acetate buffer at pH 4.5 is added to the mixture in the ELISA plate
- ✓ Read the enzyme activity at 405nm wavelength by placing the ELISA plate in multimode reader (SYNERGY/HTX – BIOTEK COMPANY - USA) using Gen5 2.09 software application, by doing end point assay (by stopping the reaction using sodium acetate buffer solution – to prevent production of further products)
- ✓ Obtained values were substituted in the above formula to estimate the DPP IV enzyme activity
- ✓ For blank and standard calculation the procedures above mentioned are same with minute changes as follows
- ✓ For blank calculation, plasma sample is not added instead 20µl of substrate is added to 180µl of Tris-HCL buffer at pH 7.5
- ✓ For standard calculation instead of plasma sample and substrate, 20µl of p-Nitroaniline is added to 180µl of Tris-HCL buffer at pH 7.5

STATISTICAL METHODS

All the parameters were tabulated for statistical significance using Statistical Package for Social Science (SPSS) software. The difference in levels of DPP IV activity in blood of OSCC patients undergoing only radiation therapy, OSCC patients undergoing radiation therapy after surgery and control group were statistically analyzed using paired T test. Multiple groups were analyzed by one way ANOVA test and intergroup comparisons were made using Post-hoc test.

Figure 5: Materials used for sample collection and analysis

Figure 6: Blood samples

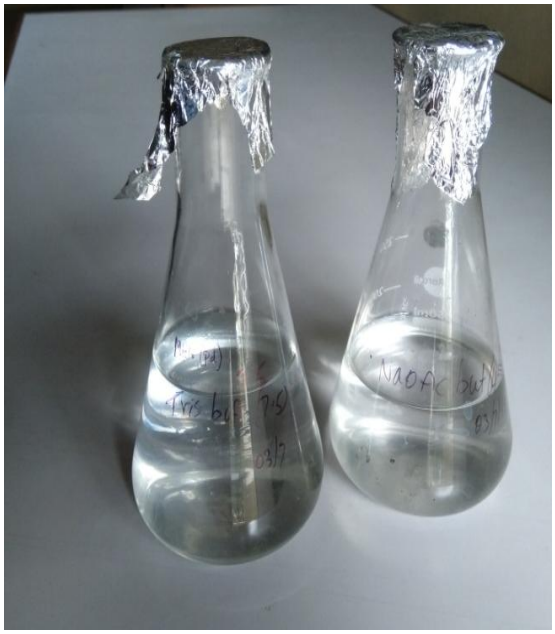

Figure 7: Buffers

Figure 8: Photograph showing reagents used for Dipeptidyl peptidase IV (DPP IV) enzyme estimation

Figure 9: Placement of tubes for centrifuging

Figure 10: ELISA Plate

Figure 11: Incubator shaker

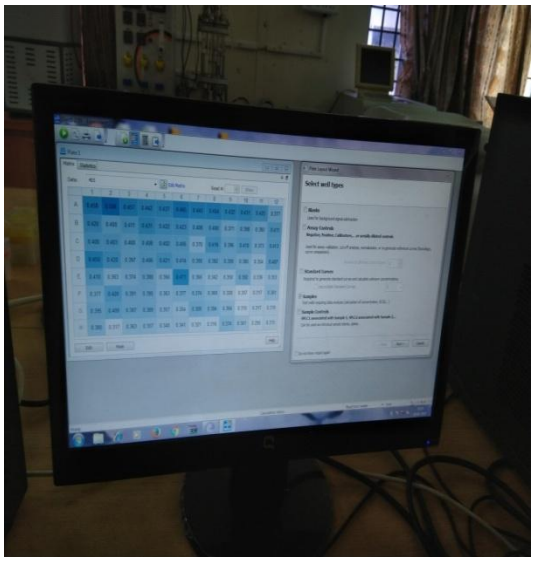

Figure 12: Photograph showing multimode reader (SYNERGY/HTX – BIOTEK COMPANY - USA) which reads at 405nm using Gen5 2.09

Results

Table – 1
DPP IV enzyme levels in blood of oral squamous cell carcinoma patients undergoing only radiation therapy (Group I)

GROUP	N	MEAN (IU/L)	STANDARD DEVIATION	MINIMUM (IU/L)	MAXIMUM (IU/L)
CASES – BEFORE RADIATION (Group IA)	20	27.4430	1.73625	24.36	30.81
CASES – DURING RADIATION (Group IB)	20	36.4685	2.04783	32.92	39.39

IU/L – International unit / Litre

The mean value of DPP IV level in blood of OSCC patients undergoing only radiation therapy in cases before and during radiation therapy were 27.4430 IU/L and 36.4685 IU/L respectively

Table – 2
Comparison of DPP IV enzyme levels in blood of oral squamous cell carcinoma patients undergoing only radiation therapy

Comparison groups	"t" value	P value
Group IA & IB	-34.316	<0.001

T test was used for the comparison of mean blood levels of DPP IV enzyme in OSCC patients undergoing only radiation therapy in cases before and during radiation therapy and the difference was found to be highly significant ($P<0.01$)

Table – 3

DPP IV enzyme levels in blood of oral squamous cell carcinoma patients undergoing radiation therapy after surgery (Group II)

GROUP	N	MEAN (IU/L)	STANDARD DEVIATION	MINIMUM (IU/L)	MAXIMUM (IU/L)
CASES – BEFORE RADIATION (Group IIA)	20	33.1770	2.26866	28.73	36.89
CASES – DURING RADIATION (Group IIB)	20	42.1025	2.47970	36.82	45.27

IU/L – International unit / Litre

The mean value of DPP IV level in blood of OSCC patients undergoing radiation therapy after surgery in cases before and during radiation therapy were 33.1770 IU/L and 42.0125 IU/L respectively

Table - 4

Comparison of DPP IV enzyme levels in blood of oral squamous cell carcinoma patients undergoing radiation therapy after surgery

Comparison groups	"t" value	P value
GROUP IIA & IIB	-73.026	<0.001

T test was used for the comparison of mean blood levels of DPP IV enzyme in OSCC patients undergoing radiation therapy after surgery in cases before and during radiation therapy and the difference was found to be highly significant ($P<0.01$)

Table - 5

DPP IV enzyme levels in blood of control group during 1st day and 21st day (Group III)

GROUP	N	MEAN (IU/L)	STANDARD DEVIATION	MINIMUM (IU/L)	MAXIMUM (IU/L)
CONTROLS 1ST DAY (Group IIIA)	20	61.7290	2.04307	58.69	65.81
CONTROLS 21ST DAY (Group IIIB)	20	62.3370	2.03852	58.9	66.12

IU/L – International unit / Litre

The mean value of DPP IV level in blood of control group during 1st day and 21st day were 61.7290 IU/L and 62.3370 IU/L respectively

Table – 6

Comparison of DPP IV enzyme levels in blood of control group

Comparison groups	"t" value	P value
Group IIIA & IIIB	-10.955	<0.001

T test was used for the comparison of mean blood levels of DPP IV enzyme in control group during 1st day and 21st day and the difference was found to be highly significant (P<0.01)

Table – 7

Comparison of DPP IV enzyme levels in blood between Group IA (OSCC - Before radiation), IIA (OSCC - Before radiation after surgery) & IIIA (Control – 1st day)

PARAMETERS	F	P value
Group IA, IIA & IIIA	1640.492	<0.001

One way ANOVA test was done to compare the DPP IV enzyme levels in blood of OSCC patients undergoing only radiation therapy, OSCC patients undergoing radiation therapy after surgery in cases before radiation therapy and in control group during 1st day and the difference was found to be statistically highly significant (P<0.01).

Table – 8

Comparison of DPP IV enzyme levels in blood between Group IB (OSCC - During radiation), IIB (OSCC - During radiation after surgery) & IIIB (Control – 21st day)

PARAMETERS	F	P value
Group IB, IIB & IIIB	765.863	<0.001

One way ANOVA test was done to compare the DPP IV enzyme levels in blood of OSCC patients undergoing only radiation therapy, OSCC patients undergoing radiation therapy after surgery in cases during radiation therapy and in control group during 21st day and the difference was found to be statistically highly significant (P<0.01).

Table – 9

Comparison of DPP IV enzyme levels in blood within groups and between groups

PARAMETERS	F	P value
Between groups and within groups	985.691	<0.001

One way ANOVA test was done to compare the DPP IV enzyme levels in blood of OSCC patients undergoing only radiation therapy, OSCC patients undergoing radiation therapy after surgery in cases before and during radiation therapy and in control group during 1st day and 21st day and the difference was found to be statistically highly significant (P<0.01)

Table – 10

Multiple comparison of DPP IV enzyme levels in blood of all groups

GROUP	IA	IB	IIA	IIB	IIIA	IIIB
IA	-	<0.001	<0.001	<0.001	<0.001	<0.001
IB	<0.001	-	<0.001	<0.001	<0.001	<0.001
IIA	<0.001	<0.001	-	<0.001	<0.001	<0.001
IIB	<0.001	<0.001	<0.001	-	<0.001	<0.001
IIIA	<0.001	<0.001	<0.001	<0.001	-	<0.1
IIIB	<0.001	<0.001	<0.001	<0.001	<0.1	-

Post Hoc test was done for multiple comparisons of DPP IV enzyme levels in blood of all groups and the difference was found to be highly significant between all groups except between group IIIA & IIIB. However we found statistically significant value in T test whereas Post Hoc test showed no significance between group IIIA & IIIB, this may be due to biological variability.

Discussion

Cancer is an event occurring at the genetic level and apoptosis is an important event in which its failure results in carcinogenesis. Viruses, chemicals, irradiation and the genetic makeup of the individual are the multiple factors that play a role in carcinogenesis. In the past three decades, the science of molecular biology was revolutionized by the rapid advancement of the complementing fields of genomics and bioinformatics. This revolution had a profound effect on cancer research. Indeed, new molecular characteristics of cancer cells are discovered almost daily. Recently, a number of enzymes from the peptidase/protease class were found to be highly expressed in several types of tumors and it plays an important role in the pathophysiology of tumor cells. DPP IV has a role in the onset and progression of several cancer types. DPP IV gene is an antigenic enzyme which is expressed on the surface of most cell types and is associated with immune regulation, signal transduction and apoptosis. However, Inamoto et al., demonstrated that the blockage of DDPIV reduced several cancer-related processes in the human renal cell carcinoma **(Dahan et al., 2014).**

DPP IV/CD26 suppresses the malignant phenotype, possibly by degrading or inactivating growth factors and chemokines necessary for growth and survival of cancer cells and by modulating the extracellular microenvironment through its interaction with extracellular matrix component (**Beckenkamp et al., 2015).** The higher rates of apoptosis of the CD26 (DPP IV) transfectants was explained by the finding that CD95 (Fas/Apo-1) was upregulated in mutants without DPP IV activity in comparison with transfectants with DPP IV activity **(Morimoto et al., 1994).** CD95 is a member of the nerve growth factor/tumor necrosis factor receptor family that mediates apoptosis **(Trauth et al., 1989).**

In addition to its expression and involvement in cancer, DPP IV is one of the few proline-specific proteases that are able to cleave proline-associated peptide bonds, as the unique cyclic structure of proline serves as a structure regulation element which limits the susceptibility for nonspecific enzymatic degradation. Thus, a high level of expression in cancer cells, combined with high substrate specificity, indicated that DPP IV may be a potential target molecule for the delivery of chemotherapeutic drugs and sparked the interest in developing a DPP IV-cleavable anticancer prodrug **(Dahan et al., 2014).**

The functional activity of pure porcine DPP IV was assessed against three Gly-X dipeptide chromogenic compounds, GPpNA, a well-known standard DPP IV substrate, as well as GFpNA and GR-pNA, two dipeptide analogues that do not contain proline in the P1 position **(Dahan et al., 2014).**

Involvement of DPP IV enzyme activity in many systemic diseases such as SLE, rheumatoid arthritis, diabetes, ulcerative colitis, etc. Recent studies have also shown association between DPP IV and other malignancies like colon cancer, melanoma, non-hodgkin's lymphoma, ovarian carcinoma, breast carcinoma, hepatocellular carcinoma, etc. Oral cancer has been a major concern worldwide, as it accounts for the sixth most common malignancy in the world **(Arrebola et al., 2014).**

The present study was carried out in 40 histopathologically diagnosed patients of OSCC who are undergoing only radiation therapy and in patients undergoing radiation therapy after surgery and 20 normal healthy individuals. Blood samples were collected from all the subjects of three groups. The samples were centrifuged and analyzed for the DPP IV enzyme activity.

DPP IV enzyme level was evaluated by Gly-pro-p-nitroaniline toluene sulfonate using spectrophotometric method.

In the present study, all individuals in the study group were in the age range of 36-75 years. Gender distribution was 26(65%) males and 14(35%) females. Most commonly affected site was tongue (26/40cases) followed by buccal mucosa (9/40 cases) and retromolar trigone (5/40). Among the OSCC cases 21 (52.5%) were affected with moderately differentiated squamous cell carcinoma (MDSCC), 11 (27.5%) with well differentiated squamous cell carcinoma (WDSCC) and 8 (20%) with poorly differentiated squamous cell carcinoma (PDSCC). The results were discussed under the following headings:

- ✓ DPP IV enzyme activity in blood of OSCC subjects in group IA (before radiation – undergoing only radiation therapy) & IB (during radiation – undergoing only radiation therapy)
- ✓ DPP IV enzyme activity in blood of OSCC subjects in group IIA (before radiation – undergoing radiation therapy after surgery) & IIB (during radiation – undergoing radiation therapy after surgery)
- ✓ DPP IV enzyme activity in blood of group IIIA(control - 1st day) & IIIB(control -21st day)
- ✓ Comparison of DPP IV enzyme levels in blood between group IA, IIA & IIIA

- ✓ Comparison of DPP IV enzyme levels in blood between group IB, IIB & IIIB
- ✓ Comparison within groups and between groups of DPP IV enzyme activity in blood namely IA, IB, IIA, IIB, IIIA & IIIB

I. DPP IV ENZYME ACTIVITY IN BLOOD OF OSCC SUBJECTS IN GROUP IA (BEFORE RADIATION – UNDERGOING ONLY RADIATION THERAPY) & IB (DURING RADIATION – UNDERGOING ONLY RADIATION THERAPY)

In the present study, the mean DPP IV levels in blood sample of OSCC patients in group IA & IB were 27.4430 IU/L and 36.4685 IU/L respectively. This difference between before and during radiation therapy was found to be statistically highly significant ($P<0.01$) and there is an increase in DPP IV activity during radiation therapy in OSCC patients undergoing only radiation therapy.

Using the keywords: Dipeptidyl peptidase IV, blood, serum, plasma, oral cancer, oral squamous cell carcinoma, radiation therapy in Google and PubMed search, only one study was available that estimated the levels of DPP IV in blood sample of oral squamous cell carcinoma patients before and during radiation therapy using spectrophotometric method (**Pranoti et al., 2010**)

Pranoti et al., 2010 observed DPP IV level in various grades of OSCC and also estimated the DPP IV value before and during radiation therapy in blood and saliva. He found that mean DPP IV activity was significantly lower in study group compared with control group. His study showed that well differentiated squamous cell carcinoma has

highest mean DPP IV activity compared with moderately differentiated and poorly differentiated squamous cell carcinoma, suggesting that mean DPP IV activity could be

used for histological staging of squamous cell carcinoma. He also found that there was definite increase in serum DPP IV activity in patients undergoing radiation therapy i.e. after 1 month (4000rads) in all three study groups. Our findings were in agreement with that of study done by **Pranoti et al., 2010.**

Uematsu et al., 2004 did a study on oral cancer patients and found that CD26 / DPP IV activity in serum and expression on peripheral blood T lymphocytes are decreased in patients with oral cancers compared to normal controls. He discussed that DPP IV activity in blood were potentially useful as prognostic markers for head and neck carcinomas and added that Transforming Growth Factor – Beta 1 (TGF-$ß_1$) is produced by OSCC as well as keratinocytes, suggesting that OSCC – derived TGF- $ß_1$ could biologically influence healthy cells and tissues, particularly T cell activation and expression of DPP IV. TGF- $ß_1$ potently induces G1-phase cell cycle arrest by inhibiting cyclin D and cyclin E associated kinase complexes. Down regulation of kinase activity is mediated by induction of cyclin dependent kinase inhibitor p15, which blocks CDK4 and CDK6 kinases and leads to binding of p27 to the CDK2-cyclin E complex. Therefore, mature TGF- $ß_1$ produced by cancer cells down regulates DPP IV expression in T cells concomitant with suppression of T cell growth by maintaining p27 expression, which leads to cell cycle arrest in G1, resulting in decreased serum DPP IV activity in oral cancer patients.

II. DPP IV ENZYME ACTIVITY IN BLOOD OF OSCC SUBJECTS IN GROUP IIA (BEFORE RADIATION – UNDERGOING RADIATION THERAPY AFTER SURGERY) & IIB (DURING RADIATION – UNDERGOING RADIATION THERAPYAFTERSURGERY)

In the present study, the mean DPP IV levels in blood sample of OSCC patients in group IIA & IIB were 33.1770 IU/L and 42.0125 IU/L respectively. This difference between

before and during radiation therapy after surgery was found to be statistically highly significant ($P<0.01$) and there is an increase in DPP IV activity during radiation therapy in OSCC patients after surgery.

Using the keywords: Dipeptidyl peptidase IV, blood, serum, plasma, oral squamous cell carcinoma, surgery and radiation therapy, oral cancer in Google and PubMed search, yielded no results. As no other published study was available to compare the levels of DPP IV in OSCC patients undergoing radiation therapy after surgery, the statistical significance of our findings could not be compared.

III. DPP IV ENZYME ACTIVITY IN BLOOD OF GROUP IIIA (CONTROL - 1ST DAY) & IIIB (CONTROL -21ST DAY)

In the present study, the mean DPP IV levels in blood sample of control group IIIA & IIIB were 61.7290 IU/L and 62.3370 IU/L respectively. This difference between before and during radiation therapy was found to be statistically highly significant using T test ($P<0.01$) and there is a mild increase in DPP IV activity during 21st day. However, Post

Hoc test showed no significance between control group during 1st day and 21st day, this may be due to biological variability.

Using the keywords: Dipeptidyl peptidase IV, blood, serum, plasma, healthy individuals, 1st day and 21st day in Google and PubMed search, yielded no results. As no other published study was available to compare the levels of DPP IV in control group during 1st day and 21st day, the statistical significance of our findings could not be compared.

IV. COMPARISON OF DPP IV ENZYME LEVELS IN BLOOD BETWEEN GROUP IA, IIA & IIIA

In the present study, the mean DPP IV levels in blood sample of group IA, IIA and IIIA were 27.4430 IU/L, 33.1770 IU/L and 61.7290 IU/L respectively. This difference between group IA (only radiation therapy – before radiation), II A (radiation therapy after surgery – before radiation) and IIIA (control – 1st day) was found to be statistically highly significant using T test ($P<0.01$) and there was increased DPP IV activity in control group compared with OSCC cases.

Using the keywords: Dipeptidyl peptidase IV, blood, serum, plasma, healthy individuals, OSCC, before radiation after surgery in Google and PubMed search, yielded no results. As no other published study was available to compare the levels of DPP IV before radiation in OSCC patients undergoing only radiation therapy, in OSCC patients undergoing radiation therapy after surgery and in control group during 1st day, the statistical significance of our findings could not be compared.

V. COMPARISON OF DPP IV ENZYME LEVELS IN BLOOD BETWEEN GROUP IB, IIB & IIIB

In the present study, the mean DPP IV levels in blood sample of group IB, IIB and IIIB were 36.4685 IU/L, .1025 IU/L and 62.3370 IU/L respectively. This difference group IB (only radiation therapy – during radiation), II B (radiation therapy after surgery – during radiation) and IIIB (control – 21st day) was found to be statistically highly significant using T test ($P<0.01$) and there was increased DPP IV activity in control group compared with OSCC cases.

Using the keywords: Dipeptidyl peptidase IV, blood, serum, plasma, healthy individuals, OSCC, during radiation after surgery, during radiation in Google and PubMed search, yielded no results. As no other published study was available to compare the levels of DPP IV during radiation in OSCC patients undergoing only radiation therapy, in OSCC patients undergoing radiation therapy after surgery and in control group during 21st day, the statistical significance of our findings could not be compared.

VI. COMPARISON WITHIN GROUPS AND BETWEEN GROUPS OF DPP IV ENZYME ACTIVITY IN BLOOD NAMELY IA, IB, IIA, IIB, IIIA & IIIB

In the present study the mean value of DPP IV level in blood sample of OSCC patients undergoing only radiation therapy in cases before and during radiation therapy were 27.4430 IU/L and 36.4685 IU/L, OSCC patients undergoing radiation therapy after surgery in cases before and during radiation therapy were 33.1770 IU/L and 42.0125

IU/L and in controls during 1st day and 21st day were 61.7290 IU/L and 62.3370 IU/L respectively. This difference between group and within group was found to be statistically highly significant (P<0.01).

Our findings were in agreement with several studies that have found significantly decreased levels of DPP IV in oral cancer groups compared with that of healthy individuals (**Fukasawa et al., 1982, Urade et al., 1989, Uematsu et al., 2004**, **Pro and Dang, 2004, Kandekar et al., 2014)**.

Pranoti et al., 2010 did a study on estimation of DPP IV in oral squamous cell carcinoma patients undergoing radiotherapy based on different stages on 50 patients and found that serum and saliva of OSCC patients showed significantly lower level of DPP IV than the control group and observed that there was an increase in DPP IV levels in OSCC patients after radiotherapy. Our findings were also in agreement with the study done by **Pranoti et al., 2010.**

There were certain limitations in the present study- smaller sample size, cases and controls were not age/sex matched. A major issue was the use of numerous method for DPP IV estimation. So, the values were seem to vary extremely between laboratories; correct reporting on used methods in the previously published articles is more important for the reproducibility and comparability of results.

Although the present study showed significant results, the scope for further research remains open, as there is a paucity of similar literature for comparison with the present

study. The results should be viewed as a pilot for extending studies with larger sample sizes, with various clinical stages and histopathological grades of OSCC for more accurate result. Only then, these markers can be used for prediction of malignancy, for early detection and prevention of cancer and for preventive measures in clinical setting and to determine the prognosis of OSCC.

Summary and Conclusion

The present study was conducted in 20 histopathologically confirmed cases of OSCC undergoing only radiation therapy, 20 histopathologically confirmed cases of OSCC undergoing radiation therapy after surgery and 20 healthy controls. Blood were collected from all subjects before radiation therapy and during radiation therapy and control group during the 1st day and 21st day and analyzed for DPP IV enzyme activity by spectrophotometry method. The values were tabulated and results were analyzed using Paired T test, One Way ANOVA test and Post Hoc test.

The analysis showed the following results:

- ✓ A highly significant difference ($p<0.01$) was observed between mean levels of DPP IV activity in blood of OSCC subjects in group IA (before radiation – undergoing only radiation therapy) & IB (during radiation – undergoing only radiation therapy)
- ✓ In group I, the levels of DPP IV were found to be raised in group IB when compared to IA.
- ✓ A highly significant difference ($p<0.01$) was observed between mean levels of DPP IV activity in blood of OSCC subjects in group IIA (before radiation – undergoing radiation therapy after surgery) & IIB (during radiation – undergoing radiation therapy after surgery
- ✓ In group II, the levels of DPP IV were found to be raised in group IIB when compared to IIA.
- ✓ A highly significant difference ($P<0.01$) was observed between mean levels of DPP IV activity in blood of control during 1st day (IIIA) and 21st day (IIIB)

- ✓ A highly significant difference (P<0.01) was observed between mean levels of DPP IV activity in blood of group IA, IIA & IIIA and the levels of DPP IV were found to be raised in group IIIA
- ✓ A highly significant difference (P<0.01) was observed between mean levels of DPP IV activity in blood of group IB, IIB & IIIB and the levels of DPP IV were found to be raised in group IIIB with p<0.01
- ✓ A highly significant difference was observed between mean levels of DPP IV activity in blood of group IA, IB, IIA, IIB, IIIA & IIIB and were found to be statistically highly significant with p<0.01 using T test, however Post Hoc test shows no significance between group IIIA & IIIB and this may be due to biological variability. DPP IV levels were found to be high in control group compared with OSCC patients. Among OSCC patients the DPP IV levels were high in patients undergoing radiation therapy after surgery and DPP IV levels were found to be raised in OSCC patients during radiation therapy compared with before radiation therapy.

In this study, DPP IV enzyme levels were found to be decreased in OSCC patients before radiation therapy and DPP IV enzyme levels were found to be increased during radiation therapy. Since DPP IV apoptotic enzymes are increased during treatment, it can be used as a valid, convenient and reliable diagnostic measuring biomarker. Hence it can be suggested that estimation of blood DPP IV using spectrophotometry method can be used as a biochemical marker in diagnosis as well as in prognosis of oral squamous cell carcinoma.

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Annexures

ANNEXURE 1

INFORMED CONSENT FORM

ESTIMATION OF DIPEPTIDYL PEPTIDASE IV IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS UNDERGOING RADIOTHERAPY

Name: Age/Sex: Date:

I, _____ aged _____ have been informed about the study:

1. I agree to give my personal details like name, age, sex, address, previous dental history and details required for the study to the best of my knowledge.
2. I will cooperate with the dentist for my intra oral examination or extra oral examination.
3. I permit the dentist to take blood samples required for the study.
4. If am unable to participate into study for reasons unknown. I can withdraw from the study.

   In my full consciousness and presence of mind, after understanding all the procedure in my own language, I am willing and give my consent to participate in this study.

Signature / Thumb impression

ஆராய்ச்சி ஒப்புகை படிவம்

வாய் புற்று நோயினால் பாதிக்கப்பட்ட நோயாளிகளின் இரத்தத்தில் டைபெப்டிட்யல் பெப்டிடேஸ் IV அளவினை ஆராய்ந்து அறிதல்.

பெயர் .....

வயது .....

பாலினம் .....

என் பெயர், வயது, பாலினம், நான் மேற்கொள்ளும் சிகிச்சை பற்றிய முழு விவரங்களையும் கொடுக்க நான் முழு மனதுடன் ஒப்புக்கொள்கிறேன். என்னுடைய வாயின் முன் பகுதி மற்றும் வெளிப்பகுதியை மருத்துவர் பரிசோதனை செய்ய ஒத்துழைக்கிறேன். மேற்கண்ட ஆராய்ச்சிக்காக என் இரத்தம் எடுக்க அனுமதி அளிக்கிறேன் .

ஆராய்ச்சி சம்மந்தப்பட்ட விவரங்களை முழுமையாக புரிந்து கொண்ட பிறகு என் முழு மனதுடன் இந்த ஆராய்ச்சியில் பங்கு கொள்ள சம்மதிக்கிறேன்.

இப்படிக்கு,

இடம்:

தேதி:

ANNEXURE 2

A. DPP IV ENZYME LEVELS IN BLOOD OF OSCC SUBJECTS UNDERGOING ONLY RADIATION THERAPY IN CASES BEFORE AND DURING RADIATION THERAPY

S.NO	BEFORE RADIATION THERAPY (IU/L)	DURING RADIATION THERAPY(IU/L)
1	28.62	39.39
2	30.81	42.1
3	26.54	37.25
4	24.36	35.28
5	25.31	36.4
6	29.58	38.27
7	28.62	37.2
8	26.3	34.61
9	27.49	35.7
10	28.6	37.1
11	27.24	35.24
12	28.3	36.84
13	24.6	32.92
14	26.35	34.81
15	25.94	33.72
16	28.47	36.89
17	29.26	37.26
18	27.63	36.48
19	25.94	34.67
20	28.9	37.24
Mean	27.4430	36.4685

B. DPP IV ENZYME LEVELS IN BLOOD OF OSCC SUBJECTS UNDERGOING RADIATION THERAPY AFTER SURGERY IN CASES BEFORE AND DURING RADIATION THERAPY

S.NO	BEFORE RADIATION THERAPY(IU/L)	DURING RADIATION THERAPY(IU/L)
1	31.52	40.7
2	34.1	43.51
3	32.62	41.2
4	28.73	36.82
5	31.26	40.35
6	33.47	43.3
7	32.34	41.58
8	36.42	45.27
9	34.7	42.91
10	29.58	37.29
11	35.86	44.7
12	33.29	41.93
13	31.69	40.38
14	36.89	45.17
15	30.58	39.62
16	33.21	42.57
17	35.49	44.93
18	34.68	44.22
19	31.82	40.99
20	35.29	44.61
Mean	33.1770	42.1025

C. DPP IV ENZYME LEVELS IN BLOOD OF CONTROL GROUP COLLECTED DURING 1st DAY AND 21st DAY

S.NO	BEFORE RADIATION THERAPY(IU/L)	DURING RADIATION THERAPY(IU/L)
1	60.83	61.59
2	62.64	62.94
3	59.97	60.8
4	65.81	66.12
5	63.34	63.96
6	61.38	62.1
7	58.84	58.9
8	63.5	64.27
9	64.28	64.81
10	61.47	62.16
11	58.69	59.3
12	60.87	61.23
13	64.81	65.1
14	62.15	63.22
15	60.56	61.11
16	58.97	59.46
17	62.12	62.98
18	63.41	64.21
19	61.56	62.24
20	59.38	60.24
Mean	61.7290	62.3370