EXPRESSION OF CD24 AND CD144 IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS ASSOCIATED WITH HABITS

Dissertation submitted to
THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY

In partial fulfilment for the Degree of
MASTER OF DENTAL SURGERY

BRANCH VI
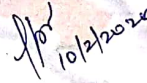**ORAL PATHOLOGY AND MICROBIOLOGY**

MAY 2020

CERTIFICATE

This is to certify that this dissertation titled "EXPRESSION OF CD24 AND CD144 IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS ASSOCIATED WITH HABITS" is a bonafide dissertation performed by **Dr VISHNU PRIYA V** under our guidance during the post graduate period 2017-2020.

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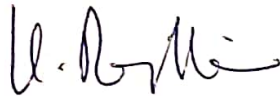
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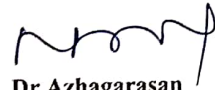Chennai.

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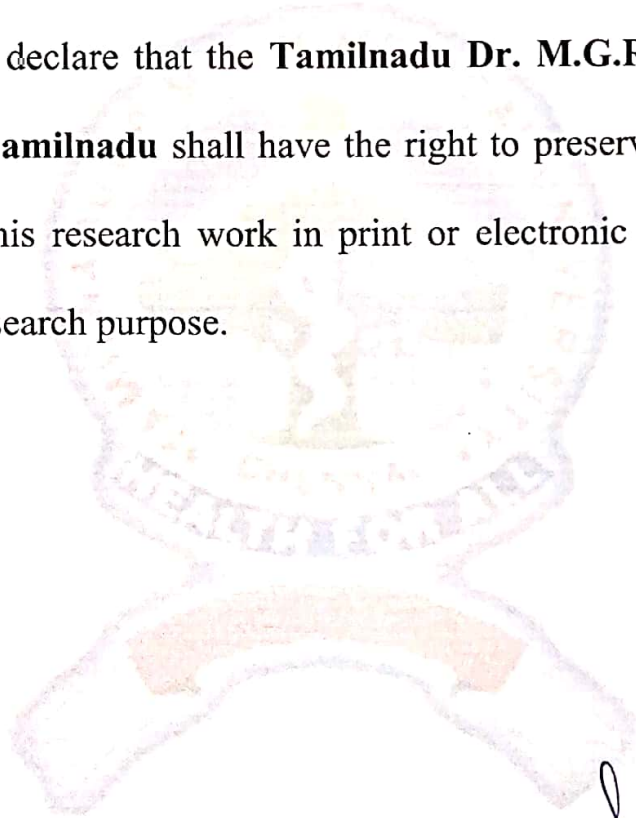RAGAS DENTAL COLLEGE AND HOSPITAL
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ABSTRACT

Background:

Oral cancer is a serious and growing burden in many parts of the world. It is the eleventh most common cancer in the world. There is a wide variation in the incidence and mortality globally due to significant variations in exposure to behavioral and environmental risk factors linked to oral cavity and pharynx. In India tobacco and excessive alcohol consumption are well established risk factors for oral cancer [2].

Ninety percent of oral cancers are OSCC histologically. Pathogenesis of OSCC involves cytogenetic changes and epigenetic processes that brings about modification in progression of the cell cycle, DNA repair mechanisms, cell differentiation and apoptosis. This may occur due to mutation, exposure to a variety of biological factors, such as HPV, carcinogens or errors in the DNA repair process [3]. The 5-year survival rate after diagnosis of OSCC remains low in spite of significant advances in treatment due to advanced stage at diagnosis, tumor recurrence and lack of markers for early detection [4].

It has been suggested that tumor growth and propagation may be driven by "cancer stem cells" (CSCs). CD24 is one of the most common stem cell marker implicated in tumor progression and metastasis [5]. CD24 is a small, heavily glycosylated, mucin-like cell surface protein that is expressed in many human malignancies [6]. It functions in cell-cell and cell-matrix interactions an also identified as an alternate ligand for P-selectin, an adhesion receptor on platelets and endothelial cells. CD24 mediates signal transduction by recruiting Src family protein tyrosine kinases (PTKs) via membrane rafts, and activates the mitogen-activated protein kinase pathway, which involves B- and T-cell development and apoptosis, cell binding and granulocyte oxidative burst. High levels of CD24 in tumor tissue are linked to tumor progression, suggesting that CD24 might be a

diagnostic biomarker and therapeutic target in human head and neck squamous cell carcinoma (HNSCC) [7].

Metastasis begins with the invasion of tumor cells through the walls of small blood vessels or lymph vessels. Vascular‑like channels were formed in melanoma which functions as tumor blood vessels. This phenomenon was called vasculogenic mimicry (VM). VM indicates a poor prognosis. Vascular endothelial‑cadherin (VE-cadherin), an adhesive protein, is a major determinant of endothelial cell contact integrity and regulation of its activity or its presence at cell contacts is an essential step that controls the permeability of the blood vessel wall for cells and substances. Overexpression level of VE-cadherin enhances the cancer neovascularization, growth and progression [9].

Hypothesis:

There is no difference in the expression of CD24 and CD144 in normal mucosa and oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

Aim:

To evaluate the expression of CD24 and CD144 in oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

Objectives:

To ascertain the expression of CD24 and CD144 using anti-CD24 rabbit polyclonal primary antibody, VE-cadherin rabbit polyclonal primary antibody and secondary polyexcel HRP/DAB detection kit by immunohistochemistry on formalin fixed paraffin embedded tissue sections of:

- Oral squamous cell carcinoma associated with the habit of smoking/chewing tobacco and areca nut.

- Normal mucosa.

- To compare the expression of CD24 and CD144 in normal mucosa and OSCC.

Materials and Methods:

The study material comprised of 37(N=37) formalin - fixed, paraffin embedded archival tissue specimens. The samples were divided into 2 groups namely: Group I, Group II.

- Group I: Oral squamous cell carcinoma tissues(n=20)

- Group II: Normal oral mucosal tissues(n=17)

Results:

Group I consisted of 8(40%) cases in the age group of 25 – 50 years and 12(60%) cases in the age group of above 50 years. Group II consisted of 2(11.8%) cases below 20 years, 12(70.6%) cases in 25 – 50 years and 3(17.6%) cases above 50 years. In group I, 18(90%) were males and 2(10%) were females. In group II, 6(35.3%) were males and 11(64.7%) were females. In group - I (oral squamous cell carcinoma), 13(65%) had the habit of chewing tobacco, 2 (10%) cases had habit of chewing betel nut and alcohol consumption, 3(15%) had the habit of cigarette smoking with the habit of chewing tobacco and betel nut, 1(5%) had the habit of smoking cigarette and 1(5%) had the habit of smoking beedi. In group - II (normal mucosa), 17(100%) case had no habit history. In group - I of 20(100%), the site of biopsy of 9(45%) cases was buccal mucosa, 5(25%) cases was tongue, 2(10%) cases was palatal mucosa and 1(5%) cases was vestibular mucosa. In group - II 6(35. 3%) cases was buccal mucosa, 8(47.1%) cases was gingiva and 2 (11.8%) cases was pericoronal flap. In group - I, the staining intensity of CD144 in basal cell layer was mild in 2(10%) cases and absent in 18(90%) cases. In group - II, mild expression of CD144 in basal cell layer was seen

in 3(17.6%) of cases and absent in 14(82.4%) cases. In group - I, the staining intensity of CD144 in supra basal cell layer was mild in 4(20%) cases, moderate in 7(35%) cases and absent in 9(45%) cases. In group - II, mild expression of CD144 in supra basal cell layer was seen in 10(58.8%) cases, moderate in 4(23.5%) cases and absent in 3(17.6%) cases. The difference was statistically significant (**p=0.045**). In group - I, the staining intensity of CD144 in connective tissue was mild in 10(50%) cases, moderate in 5(25%) cases, intense in 3(15%) cases and absent in 2(10%) cases. In group - II, mild expression of CD144 in connective tissue was seen in 9(52.9%) cases, moderate in 3(17.6%) cases, intense in 1(5.9%) cases and absent in 4(23.5%) cases. In group - I, the staining intensity of CD24 in basal cell layer was mild in 1(5%) case and absent in rest 19(95%) cases. In group - II, mild expression of CD24 on basal cell layer was seen in 2(11.8%) cases and absent in rest 15(88.2%) cases. In group - I, the staining intensity of CD24 in supra basal cell layer was mild in 11(55%) cases, moderate in 1(5%) case and absent in 8(40%) cases. In group - II, mild expression of CD24 in supra basal cell layer was seen in 11(64.7%) cases, moderate in 1(5.9%) cases and absent in 5(29.4%) cases. In group - I, the staining intensity of CD24 in connective tissue was mild in 6(30%) cases, moderate in 10(50%) cases, intense in 2(10%) cases and absent in 2(10%) cases. In group - II, mild expression of CD24 in connective tissue was seen in 8(47.1%) cases, moderately expressed in 1(5.9%) case, intense in 1(5.9%) cases and absent in 7(41.2%) cases. This was statistically significant (**p=0.014**). In oral squamous cell carcinoma cases, 2(10%) cases showed mild expression of CD144 and 1(5%) case showed mild expression of CD24 in basal cell layer. In oral squamous cell carcinoma cases, 4(20%) cases showed mild expression and 7(35%) showed moderate expression of CD144 in suprabasal cell layer while 11(55%) cases showed mild expression and 1(5%) case showed moderate expression of CD24 in basal cell layer. A statistically significant difference was found between the staining

intensity in the suprabasal cell layer of CD144 and CD24 among oral squamous cell carcinoma cases (**p=0.020**).

Conclusion:

Increased CD144 and CD24 expression was seen in OSCC compared to normal controls. The expression of CD144 was higher in OSCC cases associated with the habit of chewing tobacco and increased CD24 expression was observed in OSCC cases associated with the habit of smoking cigarette and chewing tobacco. CD144 was intense in vascular channels and cells around the vascular channels indicating neoangiogenesis in OSCC. Higher expression of CD24 in the connective tissue could be indicative of invasive front. Our results suggest CD144 and CD24 expression correlates to angiogenesis and vascular mimicry.

Keywords: CD24, CD144, OSCC, CSCs, VM

CONTENTS

Introduction

Oral cancer is a serious and growing burden in many parts of the world. It is the eleventh most common cancer in the world and in India alone over 100,000 cases are registered every year [1]. There is a wide variation in the incidence and mortality globally due to significant variations in exposure to behavioral and environmental risk factors linked to oral cavity and pharynx. In India tobacco and excessive alcohol consumption are well established risk factors for oral cancer [2].

Ninety percentage of oral cancers are OSCC histologically. Development of OSCC is a highly complex multifactorial process that occurs when epithelial cells are affected by various genetic alterations. Pathogenesis of OSCC involves cytogenetic changes and epigenetic processes that brings about modification in progression of the cell cycle, DNA repair mechanisms, cell differentiation and apoptosis. This may occur due to mutation, exposure to a variety of biological factors, such as HPV, carcinogens or errors in the DNA repair process [3]. The 5-year survival rate after diagnosis of OSCC remains low in spite of significant advances in treatment due to advanced stage at diagnosis, tumor recurrence and lack of markers for early detection[4].

It has been suggested that tumor growth and propagation may be driven by "cancer stem cells" (CSCs). CD24 is one of the most common stem cell marker implicated in tumor progression and metastasis [5]. CD24 is a small, heavily glycosylated, mucin-like cell surface protein that is expressed

1

in many human malignancies [6]. It functions in cell-cell and cell-matrix interactions an also identified as an alternate ligand for P-selectin, an adhesion receptor on platelets and endothelial cells. CD24 mediates signal transduction by recruiting Src family protein tyrosine kinases (PTKs) via membrane rafts, and activates the mitogen-activated protein kinase pathway, which involves B- and T-cell development and apoptosis, cell binding and granulocyte oxidative burst. High levels of CD24 in tumor tissue are linked to tumor progression, suggesting that CD24 might be a diagnostic biomarker and therapeutic target in human head and neck squamous cell carcinoma (HNSCC) [7].

Metastasis begins with the invasion of tumor cells through the walls of small blood vessels or lymph vessels. Vascular-like channels were formed in melanoma which functions as tumor blood vessels. This phenomenon was called vasculogenic mimicry (VM). VM indicates a poor prognosis. Vascular endothelial-cadherin (VE-cadherin), an adhesive protein, is a major determinant of endothelial cell contact integrity and regulation of its activity or its presence at cell contacts is an essential step that controls the permeability of the blood vessel wall for cells and substances. Overexpression level of VE-cadherin enhances the cancer neovascularization, growth and progression [9]. This study was done to assess the expression of CD24 and CD144 in oral squamous cell carcinoma patients associated with the habits of smoking/chewing tobacco and areca nut.

Aim and Objectives

Hypothesis:

There is no difference in the expression of CD24 and CD144 in normal mucosa and oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

Aim:

To evaluate the expression of CD24 and CD144 in oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

Objectives:

To ascertain the expression of CD24 and CD144 using anti-CD24 rabbit polyclonal primary antibody, VE-cadherin rabbit polyclonal primary antibody and secondary polyexcel HRP/DAB detection kit by immunohistochemistry on formalin fixed paraffin embedded tissue sections of:

- Oral squamous cell carcinoma associated with the habit of smoking/chewing tobacco and areca nut.

- Normal mucosa.

- To compare the expression of CD24 and CD144 in normal mucosa and OSCC.

Materials and Methods

STUDY GROUP:

- **Group I:** Archival tissues of oral squamous cell carcinoma patients associated with the habit of smoking cigarette only, smoking beedi only, tobacco chewing only, betel nut chewing with alcohol consumption and with cigarette, tobacco, betel nut chewing along with alcohol consumption.

- **Group II:** Archival oral mucosal tissues of apparently healthy individuals.

SAMPLE SIZE:

- **Group I:** Formalin - fixed, paraffin embedded archival tissues of oral squamous cell carcinoma patients. (n=20)

- **Group II:** Formalin - fixed, paraffin embedded archival oral mucosal tissues of apparently healthy individuals (control). (n=17)

TECHNIQUE: Immunohistochemistry

ANTIBODIES USED:

- • Primary antibody

1. Anti-CD24 Rabbit Polyclonal Antibody, CAT NO- E - AB - 52318(Elabscience)

2. VE - Cadherin Rabbit Polyclonal Antibody, CAT NO- E - AB - 33688(Elabscience)

•   Secondary antibody - POLYEXCEL HRP/DAB Detection System (Pathn Situ)

ARMAMENTARIUM

• Microtome

• Autoclave

• Hot air oven

• Slide warmer

• Coupling jars

• Measuring jar

• Weighing machine

• APES coated slides

• Slide box

• Aluminium foil

• Micro-pipettes

• Toothed forceps

• Electronic timer

• Beakers

• Rectangular steel tray with glass rods

• Sterile gauze

• Cover slips

• Light microscope

REAGENTS USED:

1) Xylene

2) Absolute alcohol (Isopropyl alcohol)

3) Harris Hematoxylin

4) 1% acid alcohol

5) Eosin

6) APES (3 amino propyl triethoxysilane)

7) 1 N sodium hydroxide

8) 1 N Hydrochloric acid

9) Tris EDTA buffer

10) 3% Hydrogen peroxide

11) Phosphate buffered Saline (PBS)

12) Distilled water

13) Ammonium hydroxide

PROCEDURE:

1. A detailed case history including patient's age, gender, past medical and dental history, history of drug intake, deleterious habits and trauma was taken from records for control and study group.

2. Tissue samples of normal oral mucosa and oral mucosa of Squamous Cell Carcinoma were taken from the archival blocks.

3. From the Formalin Fixed Paraffin Embedded tissues, 5 micron thick sections were cut and used for Immunohistochemical (IHC) staining.

4. Positive control for CD24 was a section of human tonsil tissue and positive control for CD144 was a section of rat lung tissue.

APES (3 Amino propyl tri ethoxy silane) COATING:

Slides first dipped in couplin jar containing acetone for 2 minutes

↓

Dipped in APES for 5 minutes

↓

Dipped in two changes of distilled water for 2 minutes each

↓

Slides left to dry

IMMUNOHISTOCHEMICAL STAINING OF CD24:

After the slides were dried, tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slides from the tissue float bath such that two tissue bits come on to each slide with a gap in between. One of the tissue sections towards the frosted end of the slide was labelled negative and the tissue section away from the frosted side is the positive. The slides were warmed using slide warmer. The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Circles were drawn using a diamond marker around the tissues, so that the antibodies added later are

restricted to the circle. The slides were transferred to TRIS EDTA buffer of pH 9 and placed in microwave oven for antigen retrieval at 100°C for 30 seconds. Slides were then treated with 3 % hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity of cells that would result in non-specific staining. Then, the slides were dipped in phosphate buffered saline for 10 minutes. The slides were wiped carefully without touching the tissue section. The sections were incubated at room temperature with Anti-CD24 Rabbit Polyclonal Antibody (Elabscience). Primary antibody was detected using Polymer-HRP/DAB IHC Detection system (PathnSitu). After thorough washing with phosphate buffered saline at pH 7.4, sections were treated with target binder for 20 min at room temperature followed by incubation with Polymer-HRP reagent for 15 min at room temperature.

After three washes with PBS, substrate DAB was applied to the sections for 10 min in the dark. Slides were then washed in distilled water to remove excess chromogen and counterstained with haematoxylin, blueing done with ammonium hydroxide and dehydrated with ethanol and xylene and mounted permanently with DPX. The slides were then observed under the Light Microscope (LM).

POSITIVE AND NEGATIVE CONTROL:

Section of human tonsil tissue that was previously known to be positive for CD24 was used as positive control. Negative control sections were processed by omitting primary antibody[10].

IMMUNOHISTOCHEMICAL STAINING OF CD144:

After the slides were dried, tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto the APES coated slides from the tissue float bath such that two tissue bits come on to each slide with a gap in between. One of the tissue sections towards the frosted end of the slide was labelled negative and the tissue section away from the frosted side is the positive. The slides were warmed using slide warmer. The slides with tissue sections were treated with three changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated with water. Circles were drawn using a diamond marker around the tissues, so that the antibodies added later are restricted to the circle. The slides were transferred to TRIS EDTA buffer of pH 9 and placed in microwave oven for antigen retrieval at 100°C for 30 seconds. Slides were then treated with 3 % hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity of cells that would result in non-specific staining. Then, the slides were dipped in phosphate buffered saline for 10 minutes. The slides were wiped carefully without touching the tissue

section. The sections were incubated at room temperature with VE – Cadherin Rabbit Polyclonal Antibody (Elabscience). Primary antibody was detected using Polymer-HRP/DAB IHC Detection system (PathnSitu). After thorough washing with phosphate buffered saline at pH 7.4, sections were treated with target binder for 20 min at room temperature followed by incubation with Polymer-HRP reagent for 15 min at room temperature. After three washes with PBS, substrate DAB was applied to the sections for 10 min in the dark. Slides were then washed in distilled water to remove excess chromogen and counterstained with haematoxylin, blueing done with ammonium hydroxide and dehydrated with ethanol and xylene and mounted permanently with DPX. The slides were then observed under the Light Microscope (LM).

POSITIVE AND NEGATIVE CONTROL:

Section of rat lung tissue was used as positive control for CD144 as advised by the manufacturer. Negative control sections were processed by omitting primary antibody.

STEPS INVOLVED:

1. APES coated slides with 2 paraffin embedded tissue placed in warming table

2. Placed in xylene twice (2 minutes each)

3. Placed in 100% isopropanol (5 minutes)

4. Placed in 90% isopropanol (5 minutes)

5. Placed in 70% isopropanol (5 minutes)

6. Washed in distilled water (2 minutes each)

7. Keep in Tris EDTA buffer at pH 9 in microwave oven at 100°C for 30 seconds for antigen retrieval

8. Cooling of solution done for 20 minutes

9. Slides were transferred to distilled water

10. Placed in 3% hydrogen peroxide (10 minutes)

11. Washed in phosphate buffer saline (2-3 minutes)

12. Primary antibody added and incubated (overnight)

13. Washed in phosphate buffer saline (2-3 minutes)

14. Poly excel target binder reagent added and incubated (20 minutes)

15. Washed in PBS buffer (2-3 minutes)

16. Polymer-HRP added and incubated (15 minutes)

17. Washed slides in PBS buffer (2-3 minutes)

18. DAB added and incubated in an enclosed in hydrated container (10 minutes)

19. Washed in PBS buffer (2-3 minutes)

20. Stained with Harris Hematoxylin (10 minutes)

21. Washed in tap water

22. Dipped in ammonium hydroxide

23. Placed in 70% alcohol (2 minute)

24. Placed in 100% alcohol (2 minute)

25. Placed in xylene (1 dip)

26. Slides to be mounted using DPX

27. Slides to be observed under the LM and graded

CRITERIA FOR EVALUATION OF STAINING:

- CD24 and CD144 expression was evaluated as brown membranous staining in tumor nests & stromal cells (fibroblasts).

- The cells were then assessed for staining intensity.

INTENSITY OF STAINING:

The staining intensity was analysed in the study groups. Each case was graded as (-) nil or absence of stain, (+) mild, (++) moderate and (+++) intense staining, based on the intensity of staining taken up by the tissue as

observed by two blinded observers independently with respect to positive control. Observer 1 was a trained post graduate student and observer 2 was a geneticist who has more than 15 years experience in the field of immunohistochemistry.

TISSUE LOCALIZATION:

Epithelial cells that exhibited brown membranous staining were counted as positive for expression of CD24 and CD144. The sections were initially scanned at low power. For sections that showed heterogeneous staining, the predominant pattern of staining in basal, suprabasal cell layer was taken into account for scoring.

CELLULAR LOCALIZATION OF STAIN:

CD24 and CD144 expression was seen as brown membranous or cytoplasmic staining. The stained slides were screened, examined systematically for CD24 and CD144 expression in membrane, cytoplasm and walls of the epithelial cell.

STATISTICAL TEST USED:

The staining intensity between each group was evaluated using Chi-squared test. For inter-observer reliability, kappa statistics was done ($p \leq 0.05$). Data analysis to be done using *SPSS software version 21*.

Review of Literature

ORAL CANCER

Oral cancer is a serious and growing burden in many parts of the world. It is the eleventh most common cancer in the world and in India alone over 100,000 cases are registered every year. **Warnakulasuriya S (2009)** reported that the ratio of males to females diagnosed with oral cancer has declined and is currently 1.5:1 for oral cancer and 2.8:1 for oropharyngeal cancer. The risk of developing oral cancer occur in people aged 50 years or over[1].

Rivera C (2015) referred oral cancer as a squamous cell carcinoma (OSCC), because 90% of cancers are histologically originated in the squamous cells[8].

SQUAMOUS CELL CARCINOMA:

Squamous cell carcinoma is defined as "a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges"[11].

Kademani D (2007) reported the most common sites of OCSCC are the dorsal and lateral borders of the tongue (40%), the floor of the mouth (30%), the retromolar trigone, the buccal mucosa, and the maxillary and mandibular gingiva[12].

ETIOLOGY AND RISK FACTORS:

Incidence of OSCC is sharply increasing globally due to significant variations in exposure to behavioral and environmental risk factors linked to oral cavity and pharynx. The development of oral or head and neck squamous cell carcinoma (HNSCC) is influenced by genetic and epigenetic factors namely tobacco, alcohol, viruses, radiation, ethnicity,diet and nutrition, familial and genetic predisposition, oral thrush, syphilis, dental factors, occupational risks,immunosuppression and use of mouthwash[13]. In a study by **Thavarajah R (2017)** showed that tobacco use with or without areca nut is the driver of the high incidence of OSCC in India[14].

TOBACCO:

The tobacco - specific nitrosamines (TSNs) namely 4-(nitrosomethylamino) 1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) and aromatic hydrocarbon benz-pyrene are the most important carcinogens in tobacco smoke. Animal studies by **Warnakulsuriya S (1999)** have shown that NNK, NNN and their metabolites called pre-carcinogens, which suffer coordinated alterations by oxidative enzymes, so that the final product becomes poor in electrons that make them extremely reactive being capable of promoting mutations by complex mechanisms by covalently bind with deoxyribonucleic acid (DNA) of

keratinocyte stem cells and form DNA adducts which is responsible for critical mutations involved in DNA replication[1].

Taghavi N (2014) showed that approximately 2-fold increase in the risk of recurrence and 5-fold increase in the risk for disease - related death in association with smoking[4].

Kumar M, Nanavati R, Modi TG *et al* (2019) stated that the genetic polymorphisms in the genes coding for the enzymes involved in metabolism of DNA replication are suspected to play a major role in the genetic predisposition to tobacco-induced head and neck cancers[13]. Snuff consumption expose the oral epithelium to free radicals of oxygen and nitrogen that can affect antioxidant defense mechanisms. Smokeless tobacco is placed in contact with the mucous membranes inside the oral cavity where the nicotine is absorbed to provide the desired effect. Elevated levels of these free radicals are found in oral precancer and cancer[8].

ALCOHOL:

In a study by **Gupta B, Bray F, Kumar N *et al* (2017)** stated that tobacco chewing and smoking and excessive alcohol consumption are well established risk factors for oral cancer in India and having them both has a synergic effect increases the risk of oral cancer[2].In a review by **Kumar M (2019)** reported that certain substances that are carcinogenic to humans have been seen in alcoholic beverages like N-nitroso compounds, mycotoxins,

urethane and inorganic arsenic. Themajor metabolite of alcohol is acetaldehyde which causes interferes with the DNA synthesis and repair and also induces sister chromatid exchanges and specific gene mutations[13]. 80% of alcohol dependent patients are reported to smoke cigarettes and nicotine dependence appears more severe in smokers with a history of alcohol dependence[15].

CANCER STEM CELLS:

Five year survival rate after diagnosis of OSCC remains low because most lesions are not diagnosed in the initial stages[16].

Kazi R, Sayed S, Dwivedi R (2019) explained that the tumor growth and propagation is often dependent on a rare subset of cells known as "cancer stem cells" (CSCs). The general term "stem cells" includes several different types of cells. The first distinction to be made is between (a) normal stem cells (SC), which are responsible for the development and maintenance of all of the tissues of the body, and (b) their diseased counterpart, called cancer stem cells (CSC), that have lost the close growth control that is a property of normal stem cells as shown in the figure 1[64].The American Association for Cancer Research Workshop on Cancer Stem Cells defined "cancer stem cells" as a cell within a tumor that possesses the capacity to self-renew and to generate heterogeneous lineages of cancer cells that comprise the tumor[5].

Al hajj M, Wicha MS, Hernandez A *et al* **(2003)** were the first to identify cancer stem cells in solid tumors[17]. **Allegra E and Trapasso S (1997)** were the first to isolate cancer stem cells in acute myeloid leukemia[18].
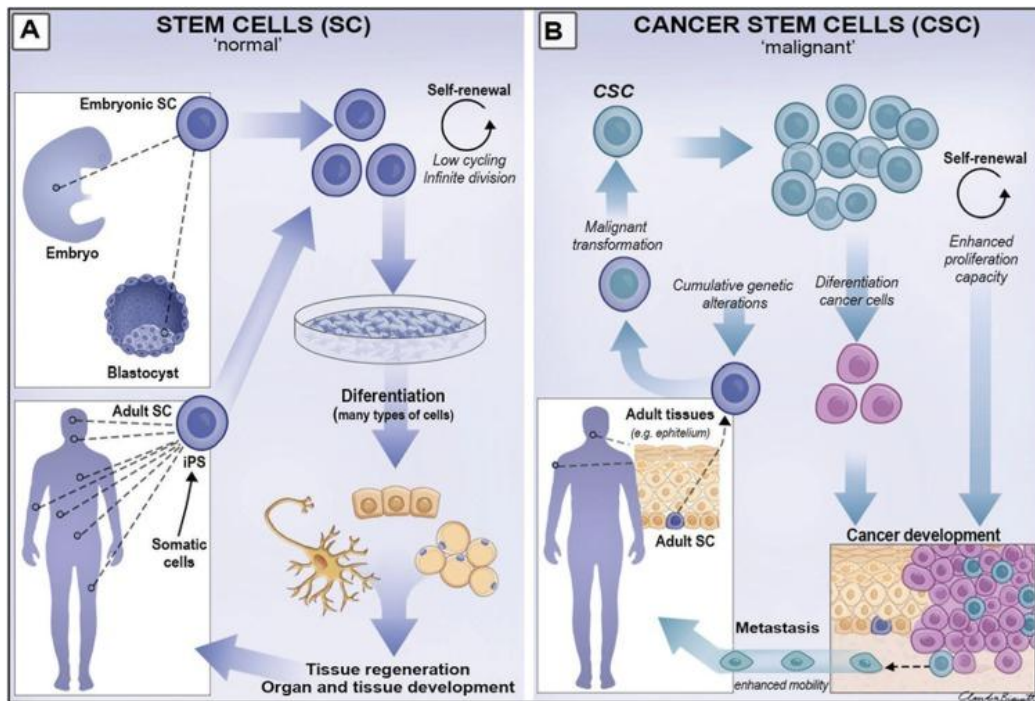
FIGURE 1: Schematic view of normal stem cells (A) and cancer stem cells (B). A shows different sources of normal SCs, their biological properties of indefinite division through self-renewal and generation of differentiated cells under appropriate conditions; while embryonic stem cells are totipotent, adult stem cells are unipotent but can regain totipotent properties under in vitro conditions, originating the induced pluripotent stem cells (iPSCs). In B, adult epithelial SCs can undergo malignant transformation after cumulative genetic alterations caused by carcinogens, generating CSCs. These CSCs retain the biological properties of the self-renewal and generation of differentiated (cancer) cells, leading to cancer development and further metastasis [64].

SOURCE: Oral cancer stem cells-properties and consequences. Journal of Applied Oral Sciences.

CANCER STEM CELLS IN OSCC:

Sridharan G (2014) explained that the oral CSCs express high ATP binding cassette (ABC) transporters that can actively efflux drugs and shield them from adverse effects of chemotherapeutic insult and these cells also possess a unique mechanism to resist cell death including modified anti-apoptotic machinery, increased pump activity and decreased cell division. Hypoxia inducing factor are found to be overexpressed in CSC which may be responsible for some aspect of radiation resistance in head and neck squamous cell carcinoma [18]. **Baillie R (2017)** stated that CSCs are highly tumorigenic compared to the other cancer cells and are believed to be largely responsible for the biological characteristics of cancer, namely, rapid growth, invasion, and metastasis[19]. For 40 years, the overall 5-year survival of oral cavity squamous cell carcinoma (OCSCC) has remained at 50%.

CHARACTERISATION OF CANCER STEM CELLS:

There are three main characteristics of Cancer Stem Cells (CSCs).

1. The cell must show potent tumor initiation in that it can regenerate the tumor which it was derived from a limited number of cells.

2. The cells should demonstrate self-renewal in vivo, which is practically observed through regrowth of phenotypically indistinguishable and heterogeneous tumors following serial transplantation of reisolated CSCs in secondary and tertiary recipients.

3. The cells must show a differentiation capacity, allowing them to give rise to a heterogeneous progeny, which represents a phenocopy of the original tumor. Purified cancer stem cells are potently tumorigenic and demonstrate self-renewing abilities and some differentiative capacity[20].

CD24:

The clusters of differentiations (CDs) are the surface markers in different cells which induce the signaling pathway for the communication of cells with one other. Ectopic expression of CD markers has been observed in different cancer cells. CD24 is one of the CD molecules which has recently gained new interest in cancer research which is implicated for the detection of tumor progression and metastasis[5].

CD24 is a 27-amino-acid, single-chain, protein that is O - and N-glycosylated. It is bound to the extracellular matrix and the extracellular membrane by a glycosylphosphatidylinositol (GPI) anchor as cell adhesion molecule[21]. Its molecular weight ranging from 30 to 70kDa[22]. Due to the presence of carbohydrate structures it is also called heat stable antigen. CD24

gene is located on chromosome 6q21. CD24 increases proliferation and adhesion of tumor cells to fibronectin, collagen, and lamnin[23]. The increased expression of CD24 enhances tumor growth and metastatic potential because of its role as a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets[24].

Sano A, Kato H, Sakurai S *et al* **(2009)** CD24 was traditionally used as a marker for pre-B lymphocytes and also functions the regulation of B-cell apoptosis, leukocyte signal transduction and leukocyte adhesion[25]. **Oliveira LR, Oliveira-Costa JP, Araujo IM** *et al* **(2011)** described CD24 as B-cell marker, and its expression is associated with B-cell development. Both positive and negative CD24 expression is used in combination with other markers to identify putative CSCs in tumors[26].

Modur V, Oliveira LR, (2016) in his study on cisplatin treatment in HNSCC predicted CD24 is an attractive marker to explore the possibility of it predicting cisplatin treatment response in HNSCC[27].

ROLE OF CD24 IN CANCER CELLS:

CD24 plays an important role in the carcinogenesis of various human malignancies such as retinoblastoma, glioma, laryngeal squamous cell carcinoma, nasopharyngeal carcinoma, small cell lung cancer, breast cancer, renal cell, hepatocellular cancer, gallbladder carcinoma, pancreatic adenocarcinoma, colorectal epithelial ovarian cancer and bladder

carcinoma[28]. One of the most known mechanisms is binding of CD24 to its ligand, P-selectin which is expressed on activated endothelial cells and platelets. Binding of tumor cells expressing CD24 to P selectin on the platelets or endothelial cells facilitates their rolling and dissemination on these cells. CD24 can engage in both cis and trans interactions. To mediate rolling, CD24 must act in trans as a cell adhesion point for the migrating tumour cells to interact with P selectin expressed on endothelial cells[29].

Another known CD24 cancer-related mechanisms is the association of CD24 with signaling factors as Src kinase in lipid rafts microdomains. Src kinase launches other mechanisms that potentially can be involved in tumorigenesis such as activation of STAT3 cytoplasmic transcription which transcripts the carcinogenesis related genes such as survivin, matrix metalloproteinase-7 (MMP-7) and Cyclin D1[30].

In a study on CD24 and tumor growth, **Tanaka T, Terai Y, Kogata Y** *et al* **(2019)** isolated CD24 in suppression subtractive hybridization screens to identify genes whose expression is upregulated in metastatic breast and pancreatic carcinoma cells. CD24 expression stimulates tumor cell proliferation, can promote tumor cell binding to P-selectin, fibronectin, and other extracellular matrix components, and also stimulates cell motility and invasion. These properties are highly relevant for tumor growth and progression and suggest that CD24 is a pleiotropic stimulator of these processes[29].

CD24 EXPRESSION IN CANCER CELLS:

CD24 expression is associated with cell adhesion, proliferation, growth, invasion, and metastasis and apoptosis inhibition of tumor cells [21].

In a study by **Oliveira LR, Araujo IM (2011)** the down-regulation of CD24 has been shown to inhibit proliferation and inducing apoptosis in malignant cells of colorectal cancers[26]. **de Moraes FP, Lourenço SV, Ianez RC *et al* (2016)** showedhigh levels of CD24 are linked to tumor progression, suggesting that CD24 might be a diagnostic biomarker and therapeutic target in human head and neck squamous cell carcinoma (HNSCC)[7].

Huang L, Zhao X (2016) found that high level of CD24 expression detected by immunohistochemistry (IHC) has been found in association with poor prognosis in human neoplasm[24]. **Lim SC (2018)** determined that the expression of CD24 is often correlated with poor prognosis in ovarian cancer. The staining pattern of CD24 and the degree of positivity constitute an important molecular marker for various epithelial neoplasms, which define the malignant transformation and predict lymph node metastasis[31].The association between CD24 and oral squamous cell carcinoma is not very much explored.

EPITHELIAL MESENCHYMAL TRANSITION:

Epithelial mesenchymal transition (EMT) is the process by which epithelial cells adopt a mesenchymal phenotype or fibroblast-like properties. The epithelial cells undergoing EMT involve reorganizing their cytoskeleton, stretching out, and breaking connections with their neighbors. After the transition, those cells dissolve the extracellular matrix that restrains them and start spreading to the surrounding tissue.

EMT AND ANGIOGENESIS:

Angiogenesis is an important part of the vascular phase in tumor growth and metastasis. Inflammation and hypoxia are the dynamic forces of angiogenesis and altered metabolism.

Lee HJ, Choe G, Jheon S *et al* (1971) proposed that tumor forms new vasculature from existing blood vessels. **Maniotis (1999)** indicated that the vascular like channels which function as tumor blood vessels were formed in melanoma. This phenomenon was called "vasculogenic mimicry" (VM) which facilitates tumor growth and cancer metastasis. VM indicates a poor prognosis in oral squamous cell carcinoma (OSCC)[36].

In a study by **Cortegoso AV, Laureano NK, Silva AD *et al* (2017)** on cell proliferative markers stated that OSCC uses the glycolytic and oxidative

metabolism to feed tumor genesis through mechanisms which are coupled between regions of cancer cell and TME cells[37]. Some markers for elements in OSCC tumor microenvironment are CD144, E-cadherin, cytokeratin, CD33, CD144, ALDH, N-cadherin, vimentin, α-SMA, integrin α6, CD4+, CD25+, FoxP3+ (T regulatory cells), CD8+ and CD34+ (myeloid precursor cells)[8].

CADHERINS:

Cadherins ("calcium-dependent adhesion") are a type of cell adhesion molecule (CAM) that is important in the formation of adherens junctions to bind cells with each other. Cell-cell adhesion is mediated by extracellular cadherin domains, whereas the intracellular cytoplasmic tail associates with numerous adaptor and signaling proteins, collectively referred to as the cadherin adhesome. They are defined by the typical extracellular cadherin domains (EC-domain). EC-domain mediates adhesion via homophilic, Ca^{2+}-dependent interactions. Most of the cadherins typically possess 5 extracellular cadherin domains. Based on sequence comparison they can be divided into different subfamilies of which 2 are the classical type I cadherins such as E- N- P- and C-cadherin, and the type II cadherins, which lack the HAV motif, a classical cadherin binding motif in the EC1 domains of type I cadherins. VE-cadherin belongs to the type II cadherins.

CD144:

Vascular endothelial-cadherin (VE- cadherin) also called as CD144 or cadherin 5, an adhesive protein, promotes cell-to-cell interaction[38]. This protein cannot be found in blood cells or hemopoietic stem cells, and, like a signature for the endothelium. It is expressed during development, when cells become committed to the endothelial lineage. VE-cadherin dimerizes laterally in cis and makes head-to-head contacts in-trans, via the most amino-terminal repeats promoting cell-to-cell adhesion. VE-cadherin is the major determinant of endothelial cell contact integrity and regulation of its activity or its presence at cell contacts is an essential step that controls the permeability of the blood vessel wall for cells and substances [39].

CD144 IN CANCER:

VE-cadherin is an important gene for both VM and endothelial-lined vessels. Hypoxia has an important role in VM. Cancer cells that form VM channels can express VE-cadherin which is an important marker for VM. VE-cadherin expression is regulated by hypoxia-inducible factors. The cancer cells lining the VM vessels secrete matrix metalloproteinase and express VE-cadherin and laminin to promote the formation of VM[40].

Irani S and Dehghan A (2019)showed that under hypoxic conditions elevated expression level of VE-cadherin has been implicated in the cancer neovascularization, growth, and progression of OSCC and VE cadherin

expression level is suggested as a metastatic biomarker for OSCC[40].

Breier G, Grosser M, Rezaei M (2014) proved that VE-cadherin is also present in tumor endothelium and application of VE - cadherin-specific antibodies in experimental tumors was able to block angiogenesis and tumor growth[41].

A study by **Tang NN, Zhu H, Zhang HJ *et al* (2014)** on esophageal cancer found that VM formation can be inhibited by targeting VE-cadherin[42].

Results

SAMPLE CHARACTERISTICS:

The study population comprised of 37 cases taken from the formalin fixed paraffin embedded archival blocks. They were categorized into two groups. Group - I (n=20) comprising of Oral squamous cell carcinoma samples and Group - II (n=17) comprising of normal mucosa tissue. All the samples were analyzed for immunohistochemical expression of CD144 and CD24.

INTEROBSERVER VARIATION IN STAINING INTENSITY:

The intensity of staining taken up by the tissue was observed by two blinded observers independently with respect to positive control. Observer 1 was a trained post graduate student and observer 2 was a geneticist who has more than 15 years experience in the field of immunohistochemistry. The overall Kappa value for the inter observer variation of CD144 was 0.94 and CD24 was 0.89.

DISTRIBUTION OF AGE IN THE STUDY GROUPS (TABLE 1 & GRAPH 1):

The age of patients were divided into 3 groups: <25 years, 25 – 50 years and those above 50 years. Group - I consisted of 8(40%) cases in the age group of 25 – 50 years and 12(60%) cases in the age group of above 50 years. Group- II consisted of 2(11.8%) cases below 20 years, 12(70.6%) cases in

25 – 50 years and 3(17.6%) cases above 50 years. No statistically significant difference was found with respect to age in the study groups (**p=0.18**).

DISTRIBUTION OF GENDER IN THE STUDY GROUPS (TABLE 2 & GRAPH 2):

In group - I, 18(90%) were males and 2(10%) were females. In group - II, 6(35.3%) were males and 11(64.7%) were females. A statistically significant difference was found with respect to gender among the study groups **(p=0.001).**

DISTRIBUTION OF HABITS IN THE STUDY GROUPS (TABLE 3 & GRAPH 3):

Based on the prevalence of habits in the study groups, they were categorized in to six groups. They were those with

1. No habits

2. Habit of tobacco chewing

3. Habit of betel nut chewing and alcohol consumption

4. Habit of Cigarette smoking with tobacco and betel nut chewing and alcohol consumption

5. Habit of cigarette smoking only

6. Habit of beedi smoking

In group - I (oral squamous cell carcinoma), 13(65%) had the habit of chewing tobacco, 2(10%) cases had habit of chewing betel nut and alcohol consumption, 3(15%) had the habit of cigarette smoking with the habit of chewing tobacco and betel nut, 1(5%) had the habit of smoking cigarette and 1(5%) had the habit of smoking beedi. In group - II (normal mucosa), 17(100%) case had no habit history. A statistically significant difference was found with respect to habits in the study groups (**p=0.00**).

DISTRIBUTION OF SITE OF BIOPSY IN THE STUDY GROUPS (TABLE 4 & GRAPH 4)

In group - I of 20(100%), the site of biopsy of 9(45%) cases was buccal mucosa, 5(25%) cases was tongue, 2(10%) cases was palatal mucosa and 1(5%) cases was vestibular mucosa. In group – II, 6(35.3%) case was buccal mucosa, 8(47.1%) cases was gingiva and 2(11.8%) cases was pericoronal flap. A statistically significant difference was found with respect to site of biopsy among the study groups (**p=0.003**).

DISTRIBUTION OF STAINING INTENSITY OF CD144 IN BASAL CELL LAYER OF THE STUDY GROUPS (TABLE 5 & GRAPH 5):

In group - I, the staining intensity of CD144 in basal cell layer was mild in 2(10%) cases and absent in 18(90%) cases. In group - II, mild expression of CD144 in basal cell layer was seen in 3(17.6%) of cases and absent in 14(82.4%) cases. No statistically significant difference was found

with respect to staining intensity of CD144 in basal cell layer of the study groups (**p=0.498**).

DISTRIBUTION OF STAINING INTENSITY OF CD144 IN SUPRA BASAL CELL LAYER OF THE STUDY GROUPS (TABLE 6 & GRAPH 6):

In group - I, the staining intensity of CD144 in supra basal cell layer was mild in 4(20%) cases, moderate in 7(35%) cases and absent in 9(45%) cases. In group - II, mild expression of CD144 in supra basal cell layer was seen in 10(58.8%) cases, moderate in 4(23.5%) cases and absent in 3(17.6%) cases. A statistically significant difference was found with respect to staining intensity of CD144 in supra basal cell layer of the study groups (**p=0.045**).

DISTRIBUTION OF STAINING INTENSITY OF CD144 IN CONNECTIVE TISSUE OF THE STUDY GROUPS (TABLE 7 & GRAPH 7):

In group - I, the staining intensity of CD144 in connective tissue was mild in 10(50%) cases, moderate in 5(25%) cases, intense in 3(15%) cases and absent in 2(10%) cases. In group - II, mild expression of CD144 in connective tissue was seen in 9(52.9%) cases, moderate in 3(17.6%) cases, intense in 1(5.9%) cases and absent in 4(23.5%) cases. No statistically significant difference was found with respect to staining intensity of CD144 in the connective tissue of the study groups (**p=0.575**).

DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE BASAL CELL LAYER OF THE STUDY GROUPS (TABLE 8 & GRAPH 8):

In group - I, the staining intensity of CD24 in basal cell layer was mild in 1(5%) case and absent in rest 19(95%) cases. In group - II, mild expression of CD24 on basal cell layer was seen in 2(11.8%) cases and absent in rest 15(88.2%) cases. No statistically significant difference was found with respect to staining intensity of CD24 in the basal cell layer of the study groups (**p=0.452**).

DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE SUPRA BASAL CELL LAYER OF THE STUDY GROUPS (TABLE 9 & GRAPH 9):

In group - I, the staining intensity of CD24 in supra basal cell layer was mild in 11(55%) cases, moderate in 1(5%) case and absent in 8(40%) cases. In group - II, mild expression of CD24 in supra basal cell layer was seen in 11(64.7%) cases, moderate in 1(5.9%) cases and absent in 5(29.4%) cases. No statistically significant difference was found with respect to staining intensity of CD24 in the supra basal cell layer of the study groups (**p=0.798**).

DISTRIBUTION OF STAINING INTENSITY OF CD24 IN CONNECTIVE TISSUE OF THE STUDY GROUPS (TABLE 10 & GRAPH 10):

In group - I, the staining intensity of CD24 in connective tissue was mild in 6(30%) cases, moderate in 10(50%) cases, intense in 2(10%) cases and absent in 2(10%) cases. In group - II, mild expression of CD24 in connective tissue was seen in 8(47.1%) cases, moderately expressed in 1(5.9%) case, intense in 1(5.9%) cases and absent in 7(41.2%) cases. A statistically significant difference was found with respect to staining intensity of CD24 in the connective tissue of the study groups (**p=0.014**).

DISTRIBUTION OF BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (TABLE 11 & GRAPH 11):

In cases with oral squamous cell carcinoma, 1(7.7%) case showed mild expression of CD144 in basal cell layer among cases with the habit of chewing tobacco and smoking beedi. A statistically significant difference was found with respect to basal cell layer staining intensity of CD144 in habits among group I (**p=0.045**).

DISTRIBUTION OF SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (TABLE 12 & GRAPH 12):

In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression and 1(50%) case showed moderate expression of CD144 in supra basal cell layer among cases with the habit of chewing betel nut and consuming alcohol. 3(23.1%) cases showed mild expression and 4(30.8%)

showed moderate expression of CD144 in supra basal cell layer among cases with the habit of chewing tobacco.1(33.3 %) case showed moderate expression of CD144 in supra basal cell layer among cases with the habit of smoking and chewing tobacco with alcohol consumption. No statistical significant difference was found with respect to supra basal cell layer staining intensity of CD144 in habits among study group I (**p=0.641**).

DISTRIBUTION OF CONNECTIVE TISSUE STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (TABLE 13 & GRAPH 13):

In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression and 1(50%) case showed intense expression of CD144 in connective tissue among cases with the habit of chewing betel nut and consuming alcohol. 6(46.2%) cases showed mild expression 3(23.1%) showed moderate expression and 2(15.4%) showed intense expression of CD144 in connective tissue among cases with the habit of chewing tobacco. 3(100 %) cases showed mild expression of CD144 in connective tissue among cases with the habit of smoking and chewing tobacco with alcohol consumption. No statistical significant difference was found with respect to connective tissue staining intensity of CD144 in habits among study group I (**p=0.464**).

DISTRIBUTION OF BASAL CELL LAYER STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (TABLE 14 & GRAPH 14):

In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression of CD24 in basal cell layer among cases with the habit of chewing betel nut and consuming alcohol. A statistically significant difference was found with respect to basal cell layer staining intensity of CD24 in habits among study group I (**p=0.050**).

DISTRIBUTION OF SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (TABLE 15 & GRAPH 15):

In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression of CD24 in supra basal cell layer among cases with the habit of chewing betel nut and consuming alcohol.7(53.8%) cases showed mild expression of CD24 in supra basal cell layer among cases with the habit of chewing tobacco. 2(66.7%) cases showed mild expression and 1(33.3 %) case showed moderate expression of CD24 in supra basal cell layer among cases with the habit of smoking and chewing tobacco with alcohol consumption. No statistically significant difference was found with respect to supra basal cell layer staining intensity of CD24 in habits among group I (**p=0.314**).

DISTRIBUTION OF CONNECTIVE TISSUE STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (TABLE 16 & GRAPH 16):

In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression and 1(50%) case showed moderate expression of CD24 in connective tissue among cases with the habit of chewing betel nut and consuming alcohol. 5(38.5%) cases showed mild expression, 5(38.5%) showed moderate expression and 1(7.7%) showed intense expression of CD144 in connective tissue among cases with the habit of chewing tobacco. 3(100%) cases showed mild expression of CD144 in connective tissue layer among cases with the habit of smoking and chewing tobacco with alcohol consumption. No statistically significant difference was found with respect to connective tissue staining intensity of CD144 in habits among group I (**p=0.254**).

COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP I (TABLE 17 & GRAPH 17):

In oral squamous cell carcinoma cases, 2(10%) cases showed mild expression of CD144 and 1(5%) case showed mild expression of CD24 in basal cell layer. No statistically significant difference was found between the staining intensity in the basal cell layer of CD144 and CD24 among the study group I (**p=0.548**).

COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP I (TABLE 18 & GRAPH 18):

In oral squamous cell carcinoma cases, 4(20%) cases showed mild expression and 7(35%) showed moderate expression of CD144 in supra basal cell layer while 11(55%) cases showed mild expression and 1(5%) case showed moderate expression of CD24 in basal cell layer. A statistically significant difference was found between the staining intensity in the supra basal cell layer of CD144 and CD24 among oral squamous cell carcinoma cases (**p=0.020**).

COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP I (TABLE 19 & GRAPH 19):

In oral squamous cell carcinoma cases, 10(50%) cases showed mild expression, 5(25%) showed moderate expression and 3(15%) cases showed intense expression of CD144 in connective tissue while 6(30%) cases showed mild expression, 10(50%) showed moderate expression and 2(10%) cases showed intense expression of CD24 in connective tissue. No statistically significant difference was found between the staining intensity in the connective tissue of CD144 and CD24 among the study group I (**p=0.413**).

**COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP II (TABLE 20 & GRAPH 20):**

In cases with normal mucosa, 3(17.6%) cases showed mild expression of CD144 and 2(11.8%) cases showed mild expression of CD24 in basal cell

layer. No statistically significant difference was found between the staining intensity in the basal cell layer of CD144 and CD24 among the study group II (**p=0.628**).

COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP II (TABLE 21 & GRAPH 21):

In cases with normal mucosa, 10(58.8%) cases showed mild expression and 4(23.5%) showed moderate expression of CD144 in supra basal cell layer while 11(64.7%) cases showed mild expression and 1(5.9%) case showed moderate expression of CD24 in basal cell layer. No statistically significant difference was found between the staining intensity in the supra basal cell layer of CD144 and CD24 among group II (**p=0.309**)

COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP II (TABLE 22 & GRAPH 22):

In cases with normal mucosa, 9(52.9%) cases showed mild expression, 3(17.6%) showed moderate expression and 1(5.9%) case showed intense expression of CD144 in connective tissue while 8(47.1%) cases showed mild expression, 1(5.9%) showed moderate expression and 1(5.9%) showed intense expression of CD24 in connective tissue. No statistically significant difference

was found between the staining intensity in the connective tissue of CD144 and CD24 among group II (**p=0.598**).

Tables and Graphs

TABLE 1: DISTRIBUTION OF AGE IN THE STUDY GROUPS (N=37)

AGE GROUPS IN YEARS	GROUP I (n=20)	GROUP II (n=17)	p-value
<25 YEARS	0(0%)	2 (11.8%)	
25 – 50 YEARS	8(40%)	12(70.6%)	0.18
>50 YEARS	12(60%)	3(17.6%)	

GRAPH 1: DISTRIBUTION OF AGE IN THE STUDY GROUPS (N=37)
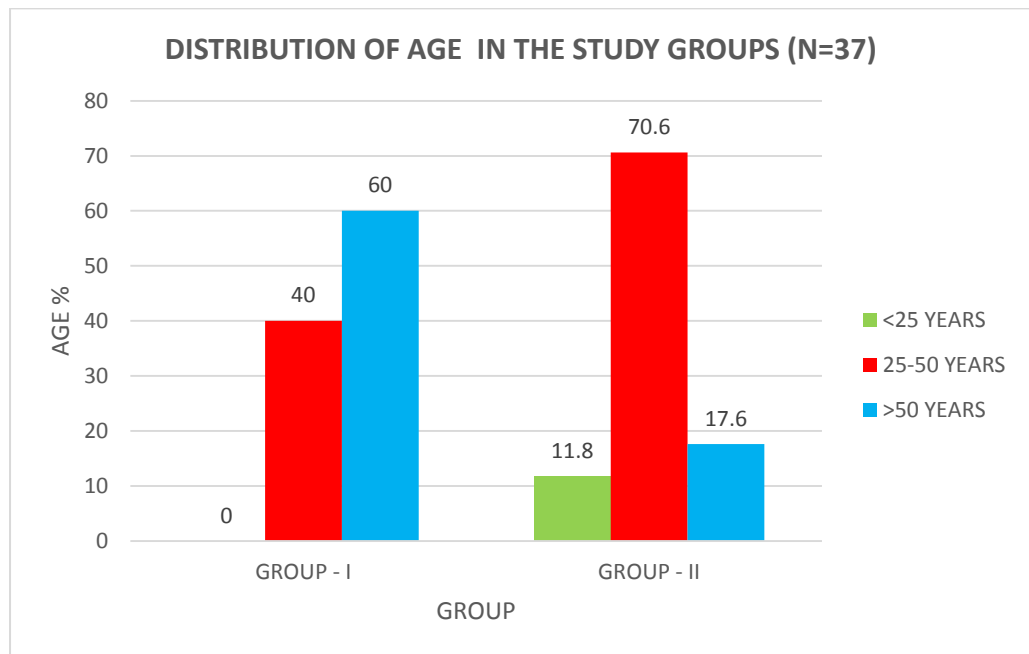
GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS (N=37)

GENDER	GROUP I (n=20)	GROUP II (n=17)	p-value
MALE	18 (90%)	6 (35.3%)	**0.001***
FEMALE	2 (10%)	11 (64.7%)	

*p$\leq$0.05 is significant.

GRAPH 2: DISTRIBUTION OF GENDER IN THE STUDY GROUPS (N=37)
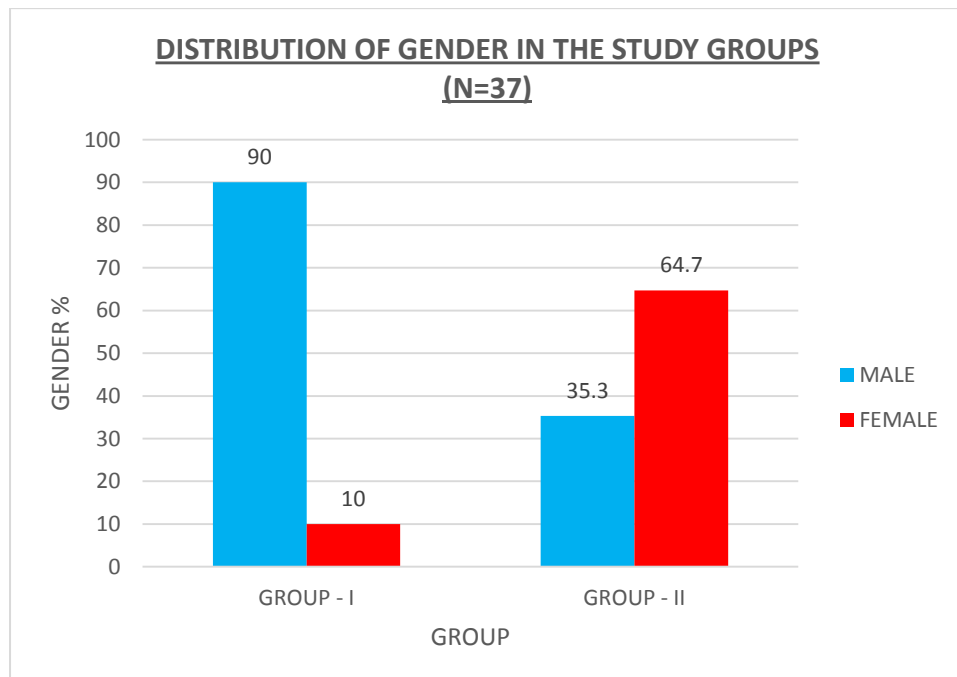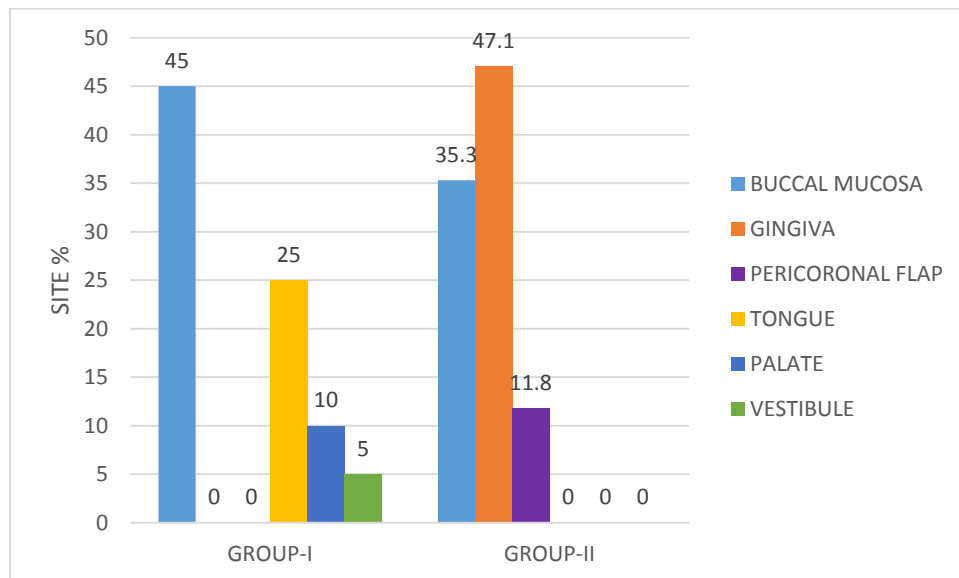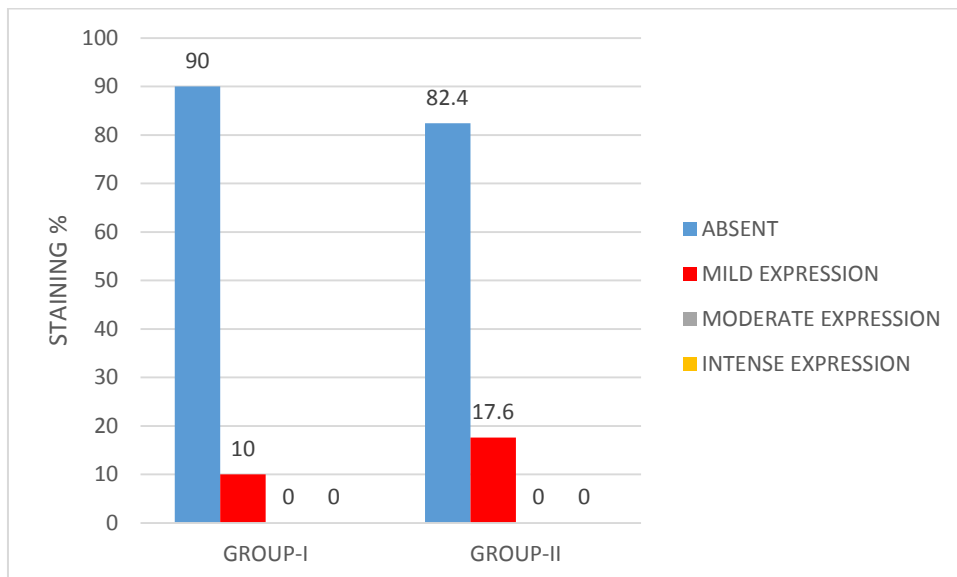

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS (N=37)

HABITS	GROUP – I (n=20)	GROUP – II (n=17)	p -value
1.NO HABITS	0(0%)	17(100%)	
2.BETEL NUT CHEWING WITH ALCOHOL CONSUMPTION	2(10%)	0(0%)	
3.TOBACCO CHEWING	13(6%)	0(0%)	
4.CIGARETTE+ TOBACCO+ BETEL NUT + ALCOHOL CONSUMPTION	3(15%)	0(0%)	0.00*
5.CIGARETTE SMOKING	1(5%)	0(0%)	
6.BEEDI SMOKING	1(5%)	0(0%)	

*p≤0.05 is significant.

GRAPH 3: DISTRIBUTION OF HABITS IN THE STUDY GROUPS (N=37)
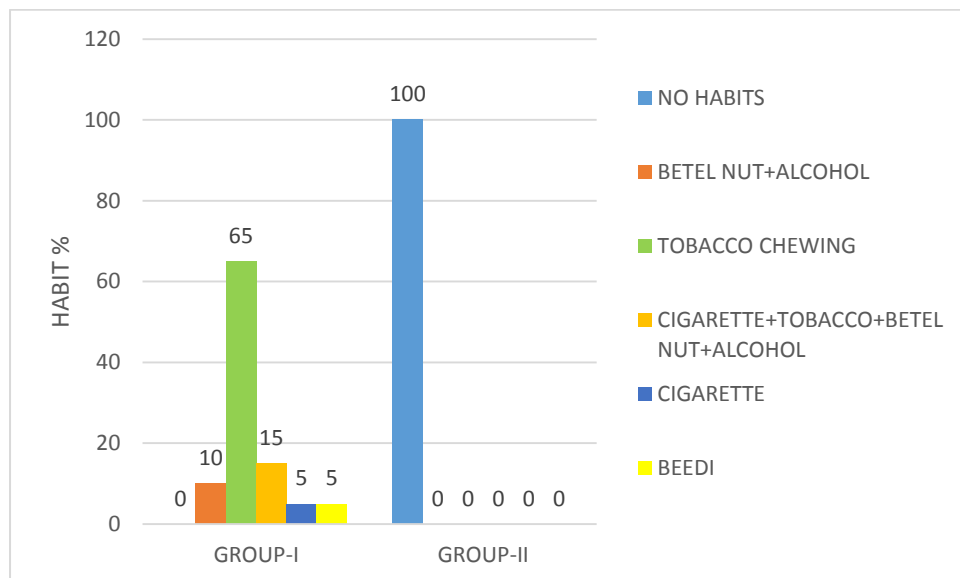
GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 4: DISTRIBUTION OF SITE OF BIOPSY IN THE STUDY GROUPS (N=37)

SITE OF BIOPSY	GROUP – I (n=20)	GROUP – II (n=17)	p-value
BUCCAL MUCOSA	9(45%)	6(35.3%)	
GINGIVA	0(0%)	8(47.1%)	
PERICORONAL FLAP	0(0%)	2(11.8%)	
TONGUE	5(25%)	0(0%)	0.003*
PALATE	2(10%)	0(0%)	
VESTIBULE	1(5%)	0(0%)	

*p≤0.05 is significant.

GRAPH 4: DISTRIBUTION OF SITE OF BIOPSY IN THE STUDY GROUPS (N=37)

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

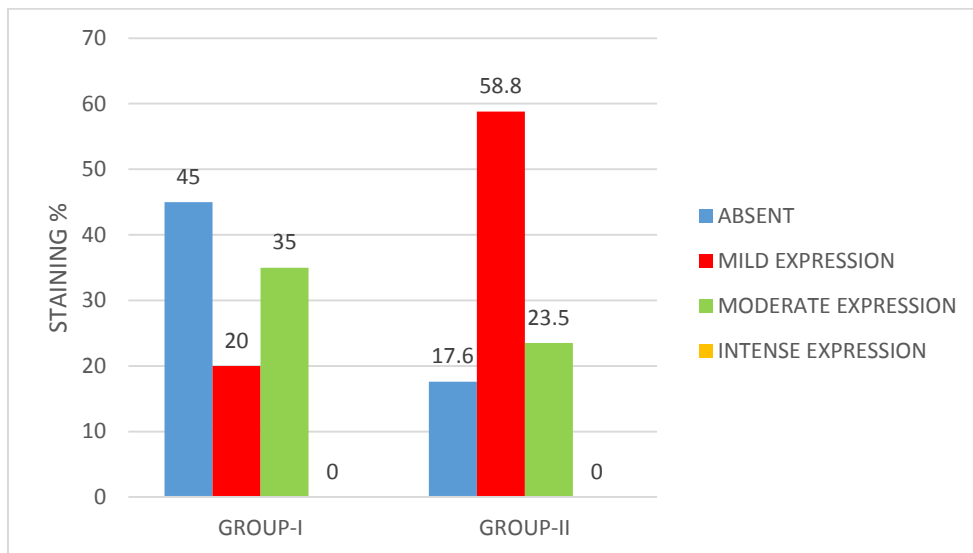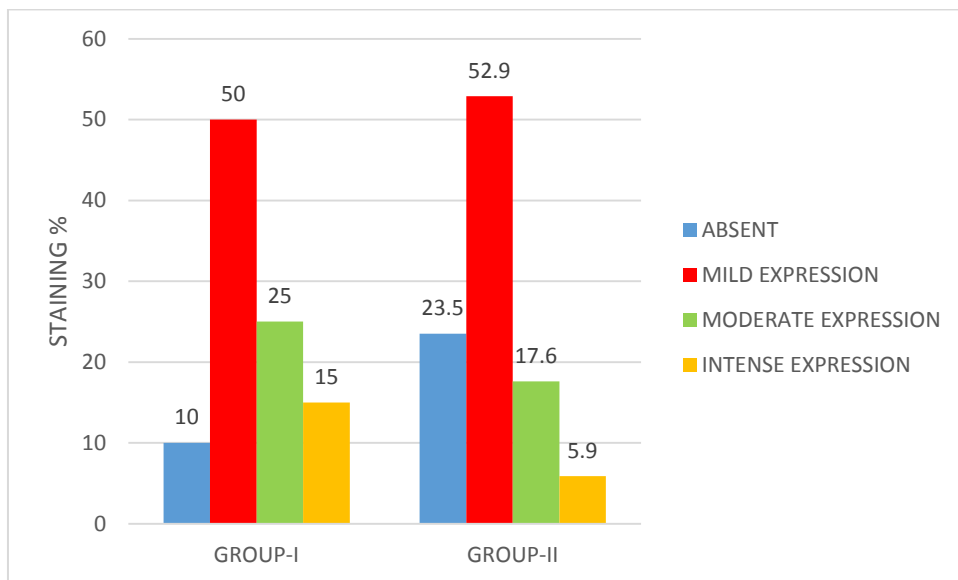**GROUP – II: NORMAL MUCOSA (n=17)**

TABLE 5: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	18 (90%)	14 (82.4%)	
MILD EXPRESSION	2 (10%)	3 (17.6%)	0.498
MODERATE EXPRESSION	0(0%)	0(0%)	
INTENSE EXPRESSION	0(0%)	0(0%)	

GRAPH 5: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 6: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN SUPRABASAL CELL LAYER OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	9 (45%)	3 (17.6%)	
MILD EXPRESSION	4 (20%)	10 (58.8%)	0.045*
MODERATE EXPRESSION	7 (35%)	4 (23.5%)	
INTENSE EXPRESSION	0(0%)	0(0%)	

*p≤0.05 is significant.

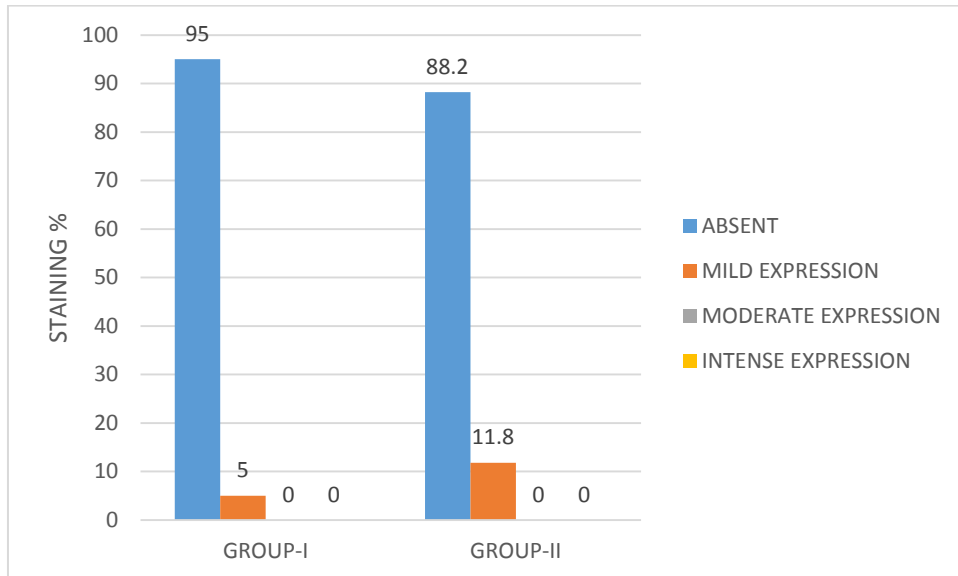**GRAPH 6: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN SUPRA BASAL CELL LAYER OF THE STUDY GROUPS (N=37)**

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 7: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN CONNECTIVE TISSUE OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	2 (10%)	4 (23.5%)	
MILD EXPRESSION	10(50%)	9(52.9%)	
MODERATE EXPRESSION	5 (25%)	3 (17.6%)	0.575
INTENSE EXPRESSION	3 (15%)	1 (5.9%)	

GRAPH 7: DISTRIBUTION OF STAINING INTENSITY OF CD144 IN CONNECTIVE TISSUE OF THE STUDY GROUPS (N=37)
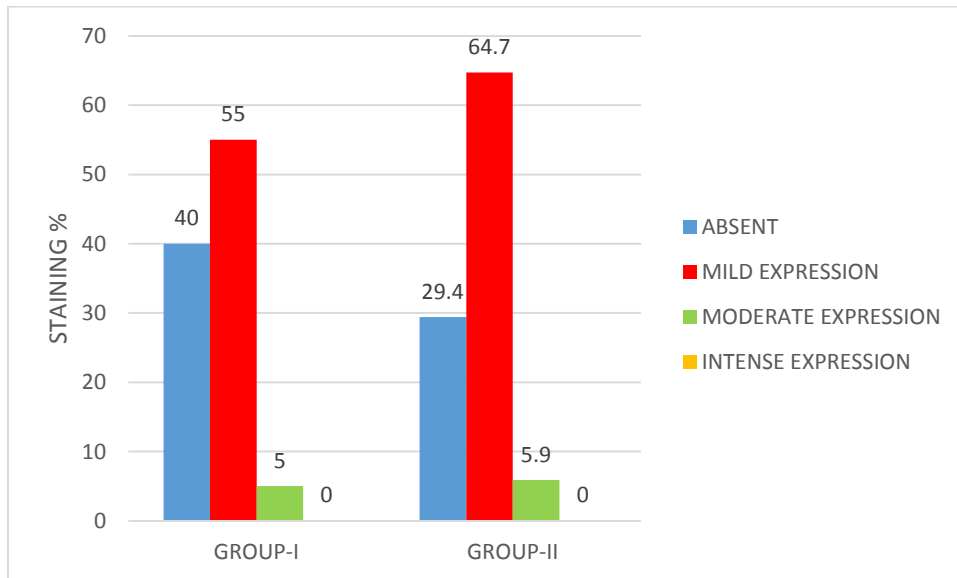

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 8: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	19 (95%)	15 (88.2%)	
MILD EXPRESSION	1 (5%)	2 (11.8%)	0.452
MODERATE EXPRESSION	0(0%)	0(0%)	
INTENSE EXPRESSION	0(0%)	0(0%)	

GRAPH 8: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

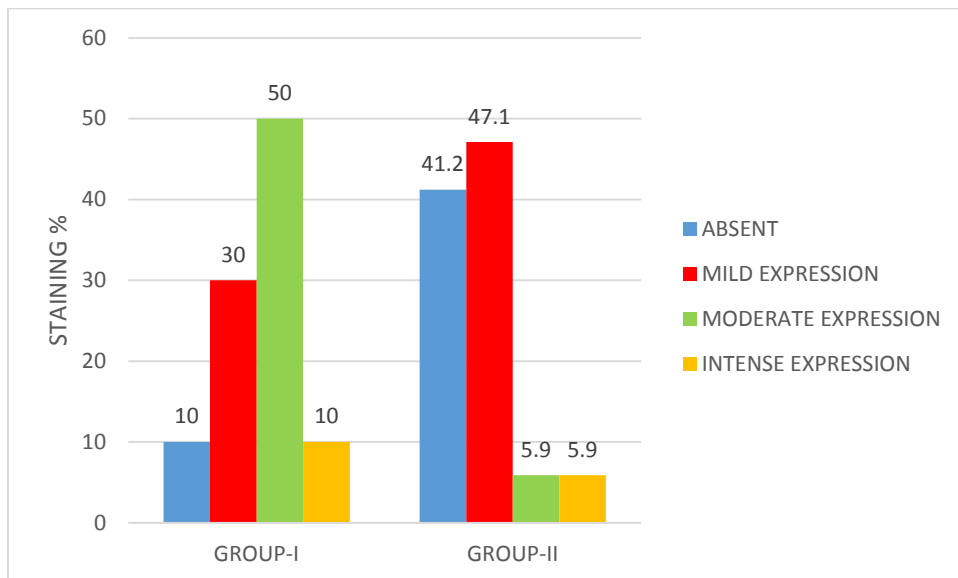**GROUP – II: NORMAL MUCOSA (n=17)**

TABLE 9: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE SUPRA BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	8 (40%)	5 (29.4%)	
MILD EXPRESSION	11 (55%)	11 (64.7%)	0.798
MODERATE EXPRESSION	1 (5%)	1 (5.9%)	
INTENSE EXPRESSION	0(0%)	0(0%)	

GRAPH 9: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE SUPRA BASAL CELL LAYER OF THE STUDY GROUPS (N=37)

**GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)**

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 10: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE CONNECTIVE TISSUE OF THE STUDY GROUPS (N=37)

	GROUP – I (n=20)	GROUP – II (n=17)	p-value
ABSENT	2 (10%)	7 (41.2%)	
MILD EXPRESSION	6 (30%)	8 (47.1%)	0.014*
MODERATE EXPRESSION	10 (50%)	1 (5.9%)	
INTENSE EXPRESSION	2 (10%)	1 (5.9%)	

*p≤0.05 is significant.

GRAPH 10: DISTRIBUTION OF STAINING INTENSITY OF CD24 IN THE CONNECTIVE TISSUE OF THE STUDY GROUPS (N=37)

GROUP – I: ORAL SQUAMOUS CELL CARCINOMA (n=20)

GROUP – II: NORMAL MUCOSA (n=17)

TABLE 11: BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)

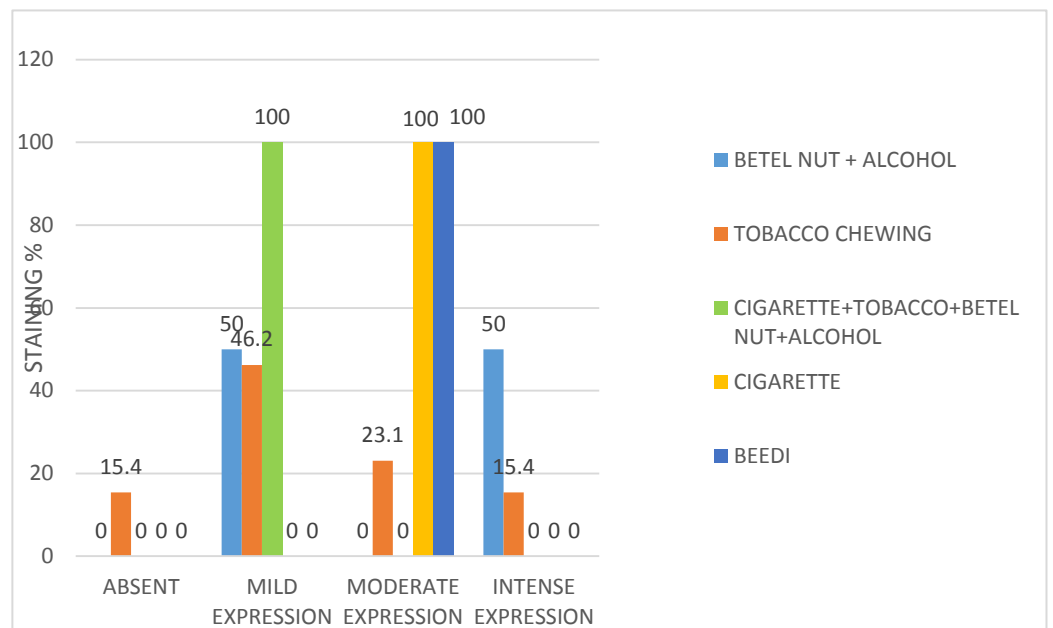|	BETEL NUT + ALCOHOL	TOBACCO CHEWING	CIGARETTE+ TOBACCO+ BETEL NUT +ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	2 (100%)	12 (92.3%)	3 (100%)	1(100%)	0(0%)	
MILD EXPRESSION	0(0%)	1 (7.7%)	0(0%)	0(0%)	1(100%)	
MODERATE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0.045*
INTENSE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	

*p≤0.05 is significant.

GRAPH 11: BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)
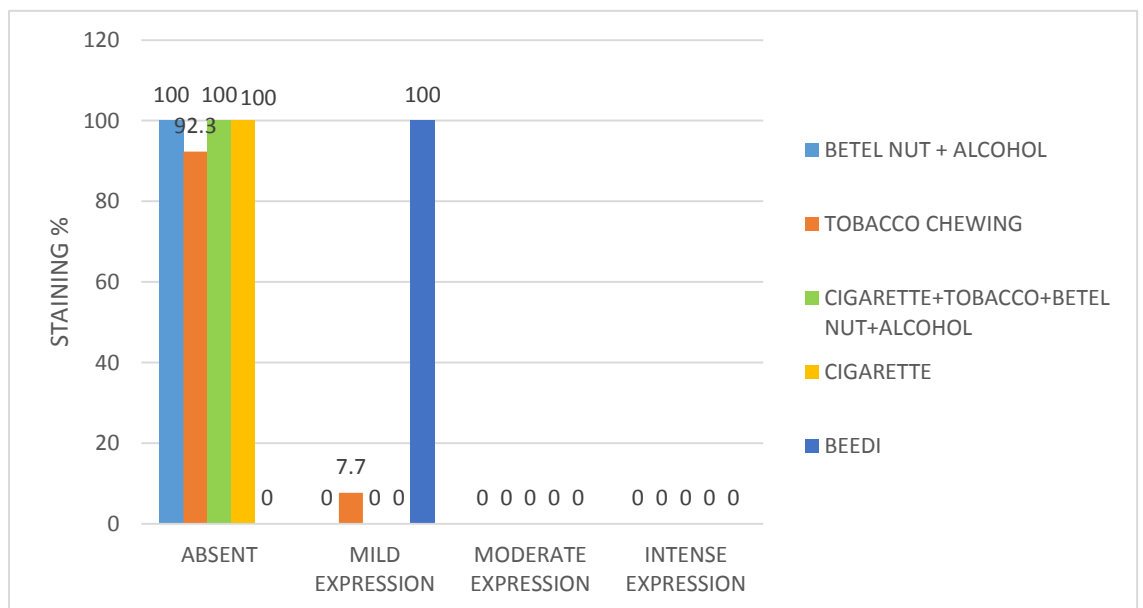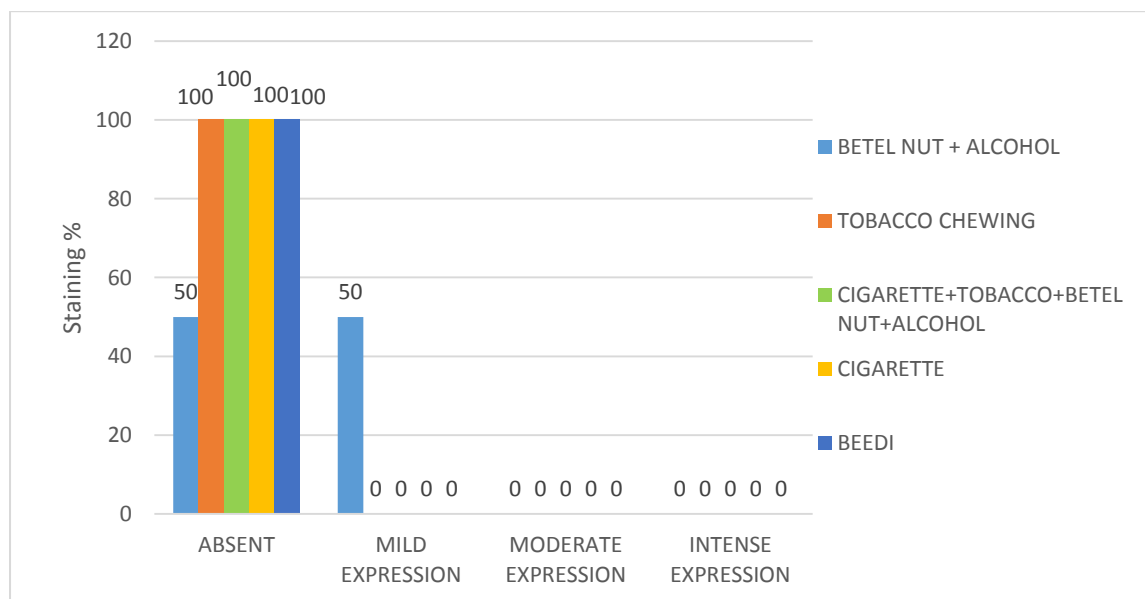
TABLE 12: SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)

	BETEL NUT+ ALCOHOL	TOBACCO CHEWING	CIGARETTE+ TOBACCO + BETEL NUT +ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	0(0%)	6 (46.2%)	2 (66.7%)	1 (100%)	0(0%)	
MILD EXPRESSION	1 (50%)	3 (23.1%)	0(0%)	0(0%)	0(0%)	
MODERATE EXPRESSION	1 (50%)	4 (30.8%)	1 (33.3%)	0(0%)	1 (100%)	0.641
INTENSE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	

GRAPH 12: SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)
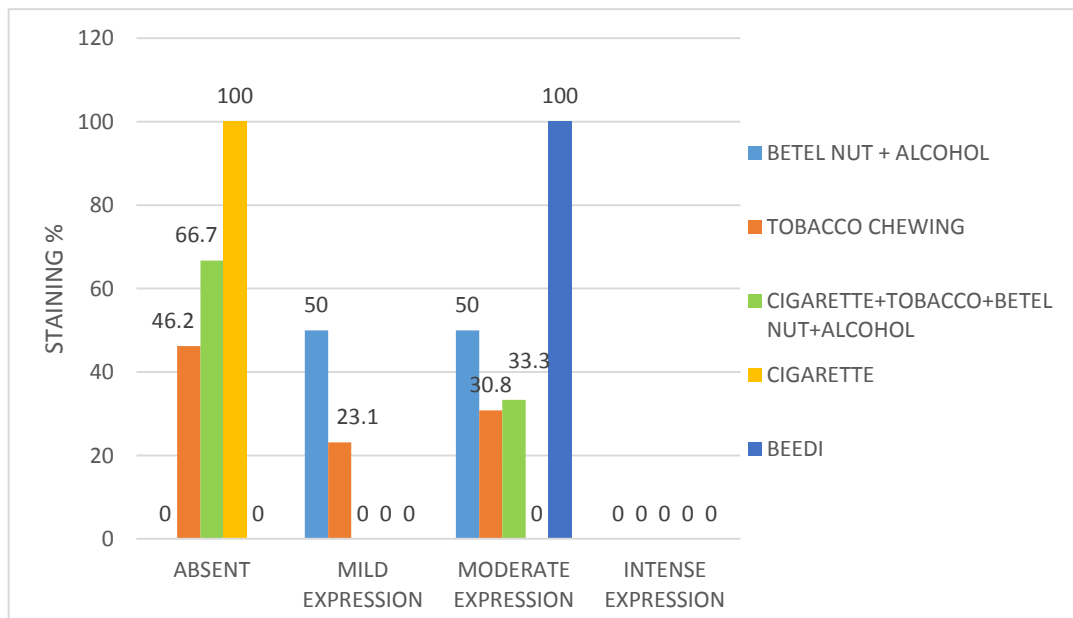
TABLE 13: CONNECTIVE TISSUE STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)

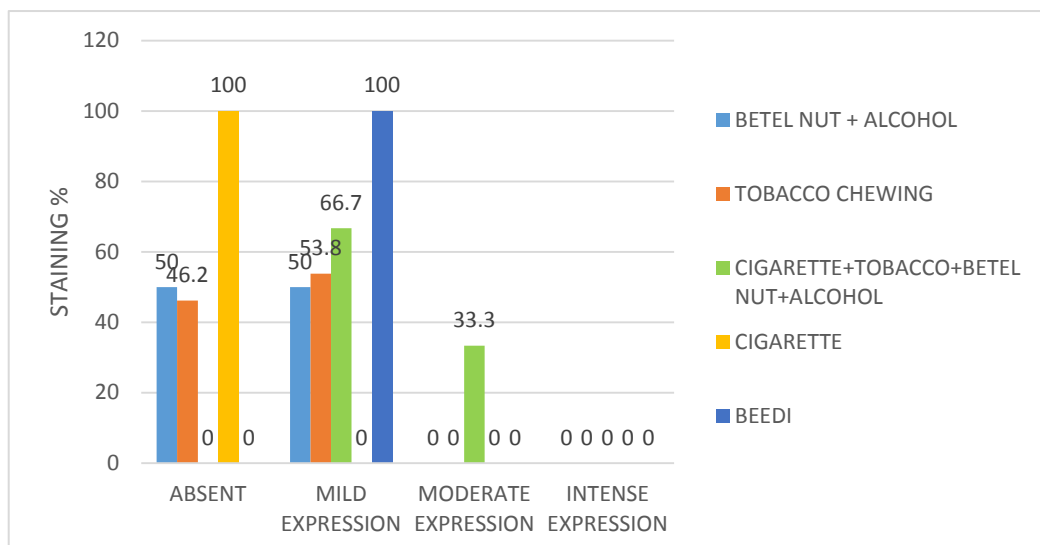|	BETEL NUT + ALCOHOL	TOBACCO CHEWING	CIGARETTE + TOBACCO + BETEL NUT+ ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	0(0%)	2 (15.4%)	0(0%)	0(0%)	0 (0%)	
MILD EXPRESSION	1 (50%)	6 (46.2%)	3 (100%)	0(0%)	0 (0%)	
MODERATE EXPRESSION	0(0%)	3 (23.1%)	0(0%)	1 (100%)	1 (100%)	0.464
INTENSE EXPRESSION	1 (50%)	2 (15.4%)	0(0%)	0(0%)	0 (0%)	

GRAPH 13: CONNECTIVE TISSUE STAINING INTENSITY OF CD144 BY HABITS IN GROUP I (n=20)

TABLE 14: BASAL CELL LAYER STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (n=20)

	BETEL NUT + ALCOHOL	TOBACCO CHEWING	CIGARETTE + TOBACCO+ BETEL NUT+ ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	1 (50%)	13 (100%)	3 (100%)	1 (100%)	1 (100%)	
MILD EXPRESSION	1 (50%)	0(0%)	0(0%)	0(0%)	0(0%)	
MODERATE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0.050*
INTENSE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	

*p$\leq$0.05 is significant.

GRAPH 14: BASAL CELL LAYER STAINING INTENSITY OF CD24

BY HABITS IN GROUP I (n=20)

TABLE 15: SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (n=20)

	BETEL NUT + ALCOHOL	TOBACCO CHEWING	CIGARETTE+ TOBACCO+ BETEL NUT+ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	1 (50%)	6 (46.2%)	0(0%)	1 (100%)	0(0%)	
MILD EXPRESSION	1 (50%)	7 (53.8%)	2 (66.7%)	0(0%)	1 (100%)	
MODERATE EXPRESSION	0(0%)	0(0%)	1 (33.3%)	0(0%)	0(0%)	0.314
INTENSE EXPRESSION	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	

GRAPH 15: SUPRA BASAL CELL LAYER STAINING INTENSITY OF CD24 BY HABITS IN GROUP I (n=20)

**TABLE 16: CONNECTIVE TISSUE STAINING INTENSITY
OF CD24 BY HABITS IN GROUP II (n=20)**

	BETEL NUT + ALCOHOL	TOBACCO CHEWING	CIGARETTE + TOBACCO + BETEL NUT+ ALCOHOL	CIGARETTE	BEEDI	p-value
ABSENT	0(0%)	2 (15.4%)	0(0%)	0(0%)	0(0%)	
MILD EXPRESSION	1 (50%)	5 (38.5%)	0(0%)	0(0%)	0(0%)	
MODERATE EXPRESSION	1 (50%)	5 (38.5%)	3 (100%)	0(0%)	1 (100%)	0.254
INTENSE EXPRESSION	0(0%)	1 (7.7%)	0(0%)	1 (100%)	0(0%)	

**GRAPH 16: CONNECTIVE TISSUE STAINING INTENSITY
OF CD24 BY HABITS IN GROUP II (n=20)**
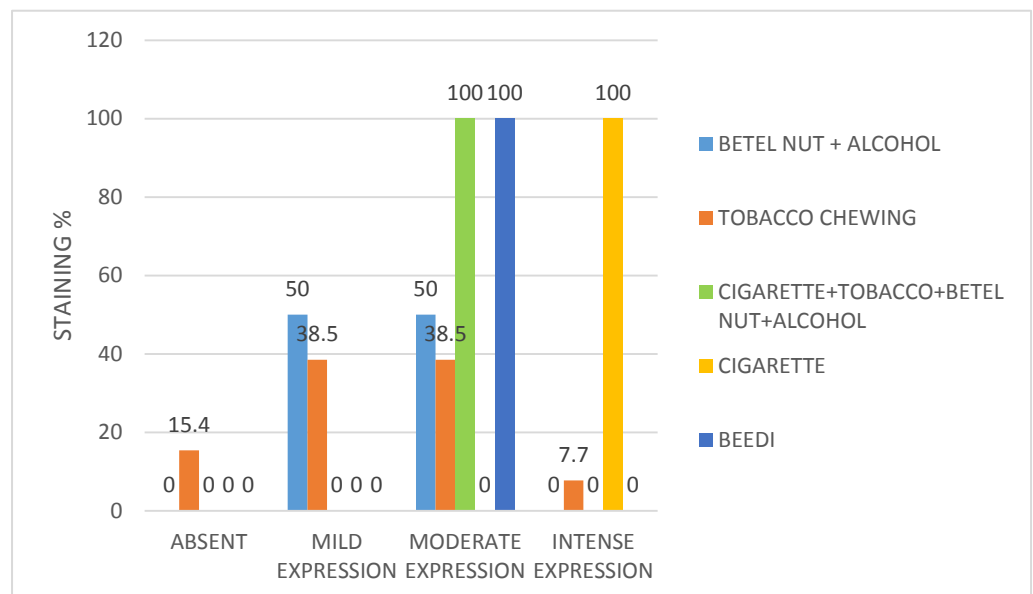
TABLE 17: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP – I

	CD144	CD24	p- value
NEGATIVE	18 (90%)	19 (95%)	
MILD	2 (10%)	1 (5%)	0.548
MODERATE	0(0%)	0(0%)	
INTENSE	0(0%)	0(0%)	

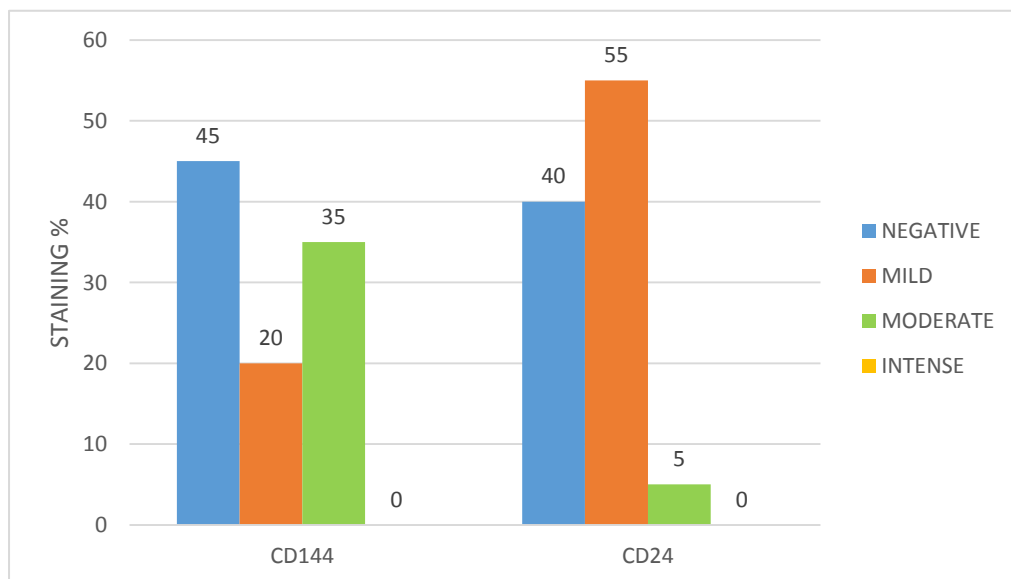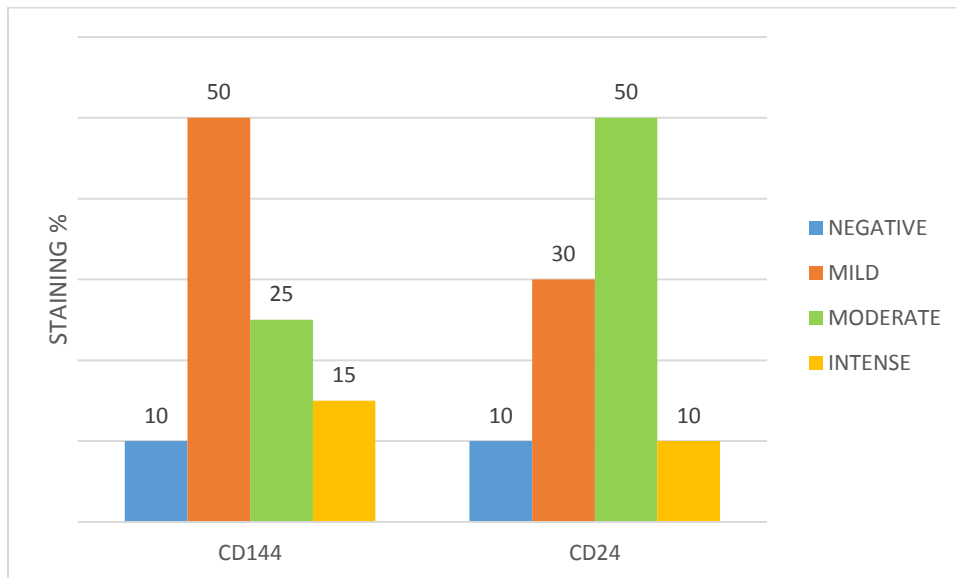**GRAPH 17: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP – I**
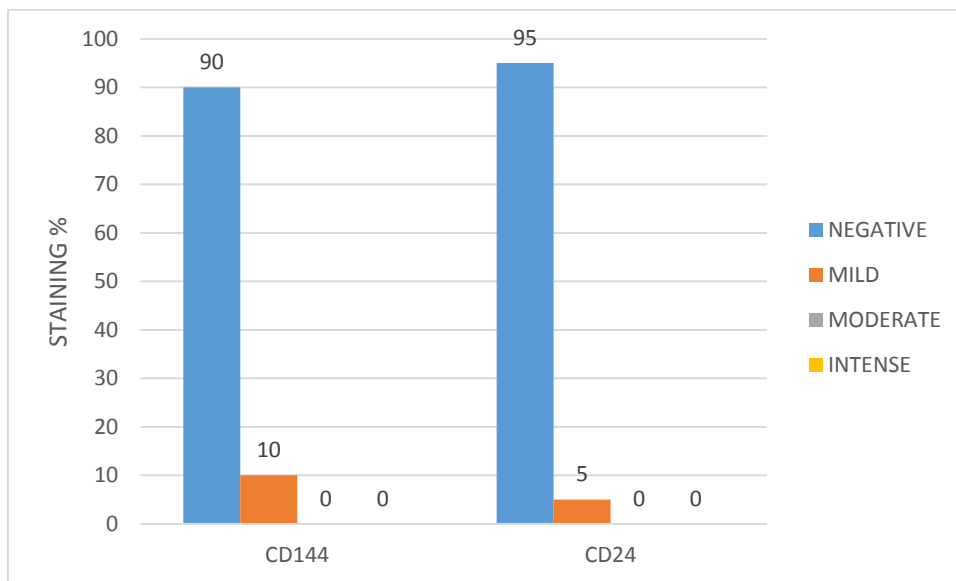
TABLE 18: COMPARISON OF CD144 vs CD24 STAINING
INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP – I

	CD144	**CD24**	**p-value**
NEGATIVE	9 (45%)	8 (40%)	
MILD	4 (20%)	11 (55%)	
MODERATE	7 (35%)	1 (5%)	0.020*
INTENSE	0(0%)	0(0%)	

*p≤0.05 is significant.

GRAPH 18: COMPARISON OF CD144 vs CD24 STAINING
INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP – I

TABLE 19: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP – I

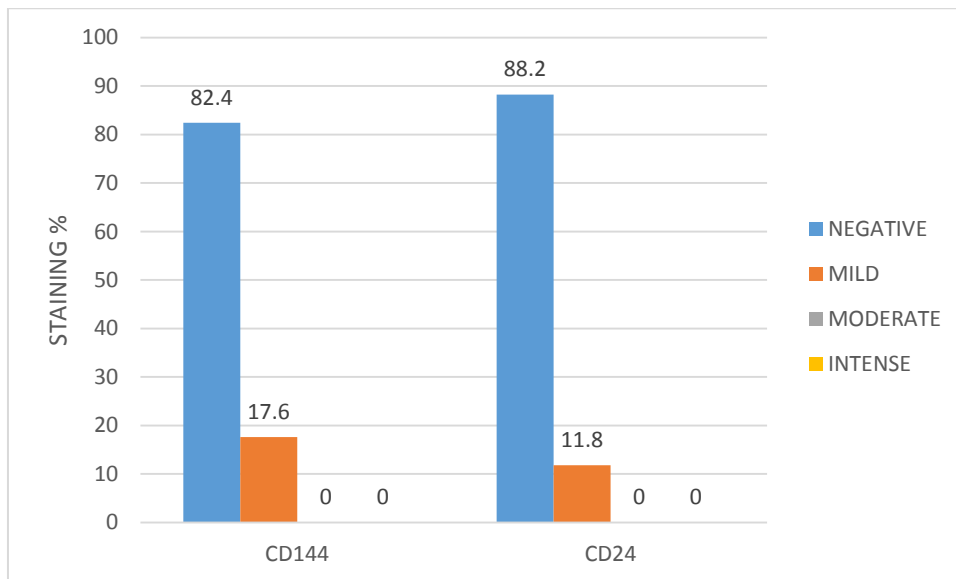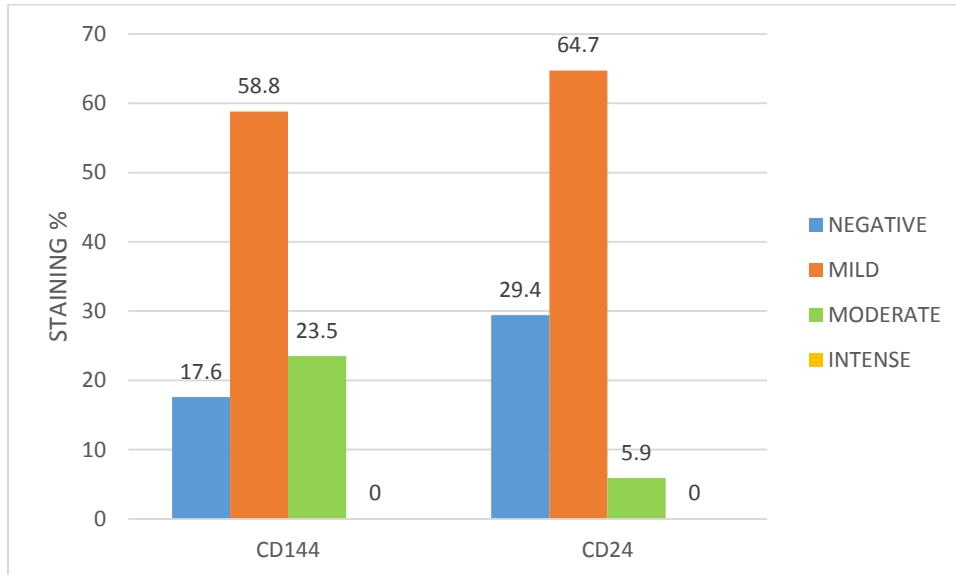|	**CD144**	**CD24**	**p-value**
NEGATIVE	2 (10%)	2 (10%)	0.413
MILD	10 (50%)	6 (30%)	
MODERATE	5 (25%)	10 (50%)	
INTENSE	3 (15%)	2 (10%)	

GRAPH 19: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP – I

TABLE 20: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP – II

	CD144	**CD24**	**p-value**
NEGATIVE	14 (82.4%)	15 (88.2%)	
MILD	3 (17.6%)	2 (11.8%)	
MODERATE	0(0%)	0(0%)	0.628
INTENSE	0(0%)	0(0%)	

GRAPH 20: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE BASAL CELL LAYER OF GROUP – II

TABLE 21: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP – II

	CD144	CD24	p-value
NEGATIVE	3 (17.6%)	5 (29.4%)	0.309
MILD	10 (58.8%)	11 (64.7%)	
MODERATE	4 (23.5%)	1 (5.9%)	
INTENSE	0(0%)	0(0%)	

GRAPH 21: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE SUPRA BASAL CELL LAYER OF GROUP – II
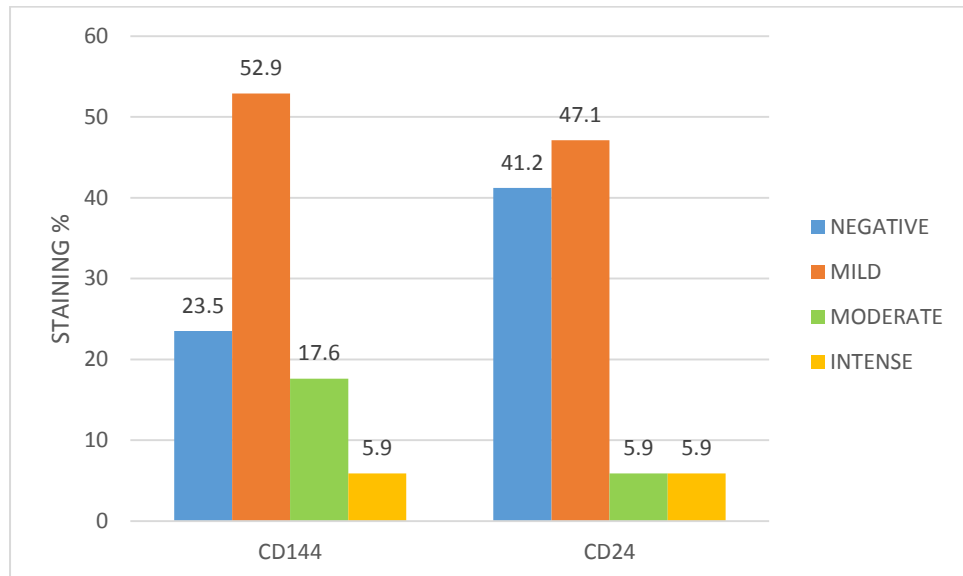
TABLE 22: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP – II

	CD144	**CD24**	**p-value**
NEGATIVE	4 (23.5%)	7 (41.2%)	
MILD	9 (52.9%)	8 (47.1%)	
MODERATE	3 (17.6%)	1 (5.9%)	0.598
INTENSE	1 (5.9%)	1 (5.9%)	

GRAPH 22: COMPARISON OF CD144 vs CD24 STAINING INTENSITY IN THE CONNECTIVE TISSUE OF GROUP – II

Photographs

PRIMARY ANTIBODY

CD144
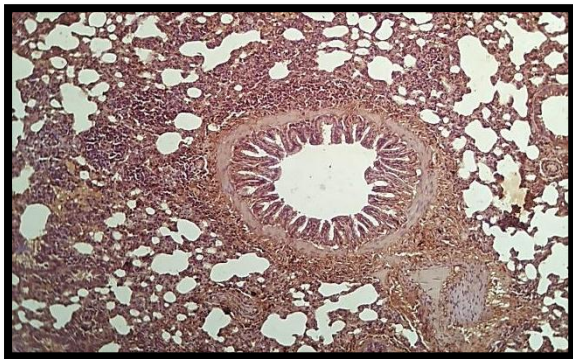

CD24

SECONDARY ANTIBODY

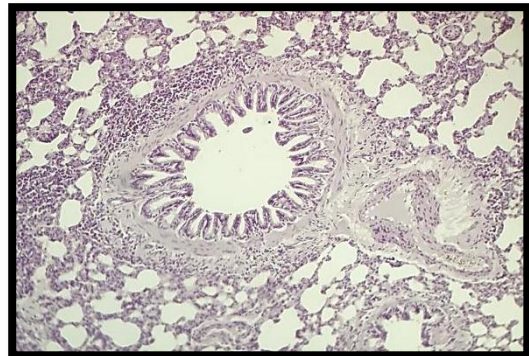**ARMAMENTARIUM**

RAT LUNG - CD144 POSITIVE CONTROL

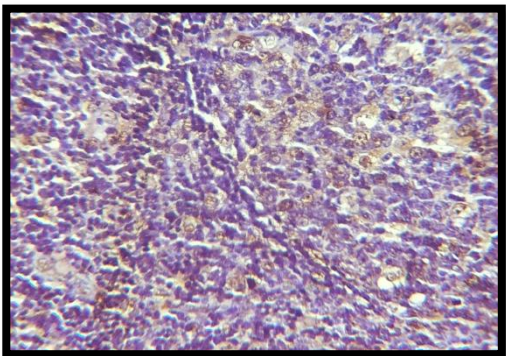**POSITIVE**   **NEGATIVE**

10 x

10 x
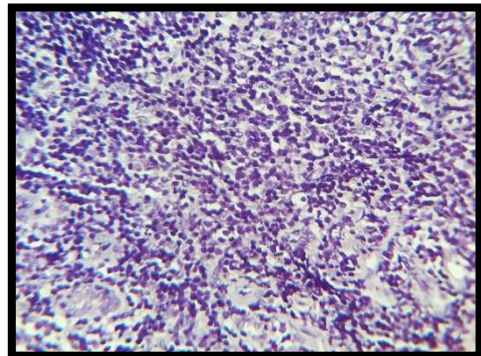
HUMAN TONSIL – CD24 POSITIVE CONTROL

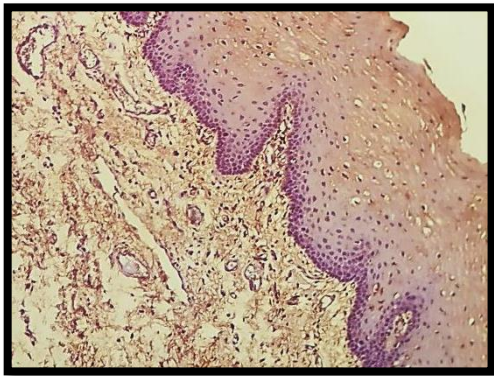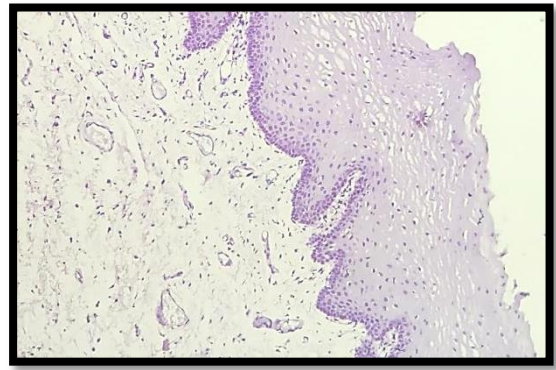**POSITIVE**   **NEGATIVE**

10 x

10 x

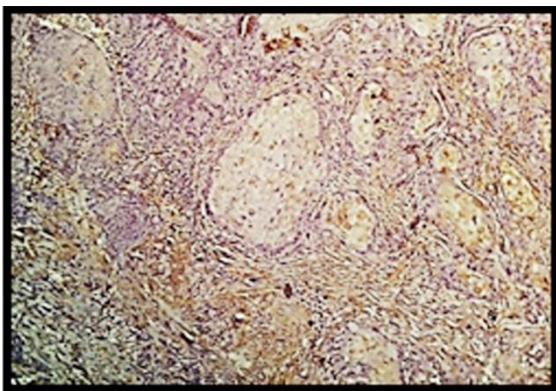**CD144-NORMAL MUCOSA**
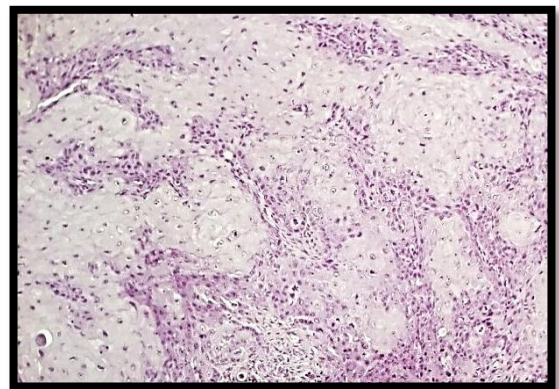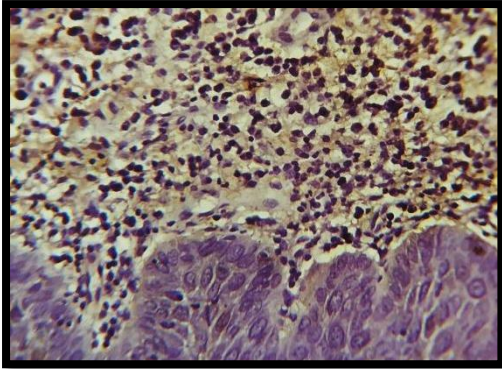
POSITIVE

NEGATIVE

10 x

10 x

CD144-ORAL SQUAMOUS CELL CARCINOMA
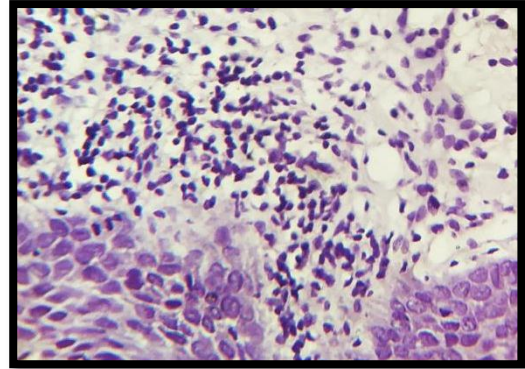
POSITIVE

NEGATIVE

10 x

10 x

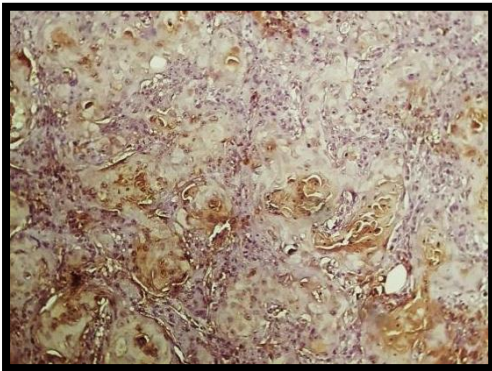## CD24-NORMAL MUCOSA
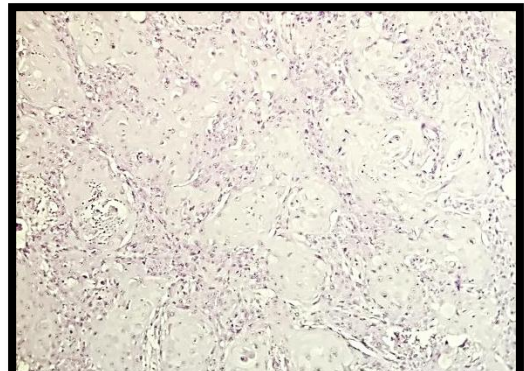
POSITIVE

NEGATIVE

10 x

10 x

CD24-ORAL SQUAMOUS CELL CARCINOMA

POSITIVE

NEGATIVE

10 x

10 x

Discussion

Oral cancer is associated with deleterious oral habits such as tobacco chewing or smoking. Tobacco use with or without areca nut is the driver of the high incidence of OSCC in India. The labial and buccal mucosa are the more commonly involved sites[14].

Tumour initiation and clonal proliferation are dependent on a subset of cells known as cancer stem cells (CSC) that are responsible for rapid growth, invasion, and metastasis[19]. One approach to isolate and target CSCs is through identification using specific immunohistochemical markers [45].

CD24 and CD144 are CSC marker whose higher expressions is linked to cancer neovascularization, growth and assess progression to invasion and metastasis in various tumours [7, 9]. This study was done to analyse the expression of CD24 and CD144 through immunohistochemistry in the FFPE tissues of OSCC patients who had habits such as tobacco chewing, betel nut chewing and in clinically normal mucosa.

In the present study, patients were divided into 3 groups: <25 years, 25 - 50 years and those above 50 years. 60% of the OSCC cases were above 50 years (Table 1). The distribution of cases between group -I and group -II was not statistically significant (p = 0.18). This results was in parallel with the study by **Silva EM, Freitas VM (2018)**, **Mehrotra R, Yadav S (2006)** and **Tandon A, Bordoloi B, Jaiswal R** *et al* **(2018)** who reported that oral squamous cell carcinoma was more frequently seen involving the elderly

population of India [49,61,62]. However, the results were in contrast to the findings by **Hashmiet AA, Hussain ZF, Hashmi SK *et al* (2018)**, who studied the distribution of OSCC in Indian population had stated that majority of the cases (50%) in their study were below the age of 50 years [48]. The difference could be due to the fact in the study setting, though at the same period of reporting.

The gender distribution among the study groups, showed a male predominance pattern in OSCC (Table 2). This results were inconsistent with the studies done by **Monterioet LS, Delgado ML, Ricardo S *et al* (2018), Linquistet D, Tarjan M, Tot T *et al* (2016)** and **Abdulla R, Hussain ZF, Hashmi SK (2012)** whose results showed 71%, 80%, and 64% of males in their study groups, respectively [50,51,47]. Oral squamous cell carcinoma affects men more than womenwhich would be attributed to relatively higher exposure to risk factors such as tobacco chewing, tobacco smoking, betel nut chewing with or without accompanied by alcohol consumption by men[1].

The oral deleterious habits such as betel nut chewing, tobacco chewing, and cigarette with tobacco chewing, betel nut chewing and alcohol consumption, cigarette smoking and beedi smoking were studied. The habit of chewing tobacco was found to be more dominant among OSCC cases (Table 3). The results are in agreement with study done by **Warnakulsuriya S (2009), Kumar M , Nanavati R ,Modi TG *et al* (2016)** and **Carreras C, Gay-Escoda C (2015)***,* who showed tobacco in the form of chewing and

smoking increases the risk of OSCC[1,13,52]. Nitric oxide present in the tobacco inhibits DNA repair mechanisms, which aggravates the oxidative DNA damage in cells, which is related to carcinogenesis[63]. This proves tobacco consumption, as a greater risk factor for exposing the oral epithelium to free radicals of affects the antioxidant defence mechanism.

The most common site involving the OSCC to be the buccal mucosa in this study was both genders (Table 4), this result was in agreement with the findings of **Warnakulsuriya S (2009)** and **Ranganathan K, Rooban T, Rao UM (2015).** Their study results shows that the most common site of OSCC among their population was buccal mucosa contributed by betel quid/tobacco chewing habits [1, 59]. In a study by **Thavarajah R, Ranganathan K (2017)** on trends in oral squamous cell carcinoma, showed the tobacco use with or without areca nut is the driver of the high incidence of OSCC in India with labial and buccal mucosa being the most commonly affected sites in the oral cavity region [14].

All the cases enrolled in the present study showed CD144 staining, except two normal mucosal tissue sections from the third molar region. This could have been due to highly exposed to *P. gingivalis* causing proteolytic disruption and cleavage of adherence junction proteins resulting in the detachment of the endothelial cells [55].

CD144 is a known vascular channel marker that has been used extensively to study the neo angiogenesis in the vascular mimicry in association with VEGF [39]. Most of the studies pertaining to CD144 has been previously reported in the OSCC connective tissue, especially in association with VEGF. Similarly, CD24 is a known B-cell marker expressed in several neoplasms like retinoblastoma, glioma, laryngeal squamous cell, nasopharyngeal carcinoma, small cell lung, breast cancer, renal cell, hepatocellular, gallbladder carcinoma, pancreatic adenocarcinoma, colorectal, epithelial ovarian cancer and bladder carcinoma[28]. However there are very few studies highlighting its association in OSCC. Recent studies indicate that CD24 is a CSC marker and has been associated with OSCC. This study explored CD144 and CD24 expression in the epithelial part of OSCC as well as the connective tissue front. As this is possibly the first study to explore the CD 144 and CD24 expression of CSCs inside the OSCC lesional tissue, there is no previous literature to support or refute the findings of this study.

The epithelium is divided into basal and supra basal layers. CD144 expression in basal layer of group-I was 5% and group -II was 11.8% the difference was however not statistically significant (**p=0.452**). In supra basal layer, CD144 expression in group-I was 55.5% and group -II was 82.3%. The difference of which was statistically significant (Table 5, 6).

In connective tissue, 90% (n=20) cases of group I and 76.4% (n=17) of cases expressed CD144. The staining intensity of CD144 was not statistically significant in the connective tissue of the study groups (Table 7). Connective tissue showed positively stained vascular channels and cells around the channels. This finding was found to be consistent with the study done by **Irani S, Dehghan A (2017),** who observed that cases of mucoepidermoid carcinoma had a high expression of VE-cadherin in the vasculogenic like networks and in the detached cells around the vessels[9]. The cancer cells lining the VM vessels secrete matrix metalloproteinase and express VE-cadherin, laminin to promote the formation of Vasculogenic mimicry (VM). VM is a tumor blood supply system that takes place independently of angiogenesis or endothelial cells, and is associated with poor survival rate in cancer patients [40].

Breier G, Grosser M, Rezaei M *et al* (2014) proved that VE-cadherin is also present in tumor endothelium and the application of VE - cadherin-specific antibodies in breast carcinoma, it was possible to block angiogenesis and tumor growth [41]. A study by **Tang NN, Zhu H, Zhang HJ *et al*, (2014)** on esophageal cancer found that VM formation can be inhibited by targeting VE cadherin [42]. **Hendrix MJ, Seftor EA, Meltzer PS *et al* (2001),** demonstrated melanoma cells expressing VE cadherin exclusively associated with the endothelial cells [38]. **Bartolome RA, Torres S, de Val SI *et al*(2017)** studied CD144 expression in breast carcinoma and melanoma and concluded

that overexpression of VE-cadherin in cancers such as melanoma and breast cancer is associated with poor prognosis [56].

Staining of CD144 was more prominent in the connective tissue compared with supra basal and basal layer in tobacco chewers (Table 11,12,13). This result was consistent with the findings by **Cooke JP (2015)** who demonstrated nicotine induced angiogenesis in lung cancer model and proposed that there was a 5 fold increase in capillary density within the tumor tissue which had helped accelerate the rate of tumor growth in the nicotine group [57].

All the cases in the present study showed CD24 staining with both membranous and cytoplasmic expression. In OSCC, the staining was comparatively more intense in connective tissue (90%) when compared to the supra basal (60%) and basal cell layer (5%) (Table 8, 9, 10). Connective tissue showed extensive staining of CD24 in the keratin pearls which was concurrent with the results of **Sano A, Kato H, Sakurai S *et al* (2009)** who had demonstrated the higher expression of CD24 in sites of keratin pearl formation in esophageal squamous cell carcinoma[25]. The shift from the membranous CD24 localization to the cytoplasm found in well-differentiated tumors could reflect the transition of epithelial cells to a more invasive phenotype [6]. In normal mucosa, the staining was moderate in supra basal cell layer and connective tissue and moderate in basal cell layer. Our results were consistent with studies by **Kristiansen G, Winzer KJ, Mayoroma E *et al***

(2003) and Sagiv R (2015) who observed CD24 expression in ovarian cancer and colorectal cancer and stated that CD24 was highly expressed in tumour epithelia and barely expressed in normal tissue [46]. **Jaggupilli A, Elkord E (2012)** studied the pattern of expression of CD24 in cases of renal cell carcinoma and demonstrated that there was an increase in CD24 expression which had correlated with aggressive behaviour such as invasion and metastasis in such cases[23]. **Lee HJ, Choe G, Jheon S *et al* (2010)** studied CD24 expression in non-small cell lung carcinoma and stated that tumours expressing high intensity of CD24 tended to have a higher risk of disease progression [36].

When the expression of CD24 in OSCC cases associated with habits was compared, staining of CD24 was more prominent in the connective tissue compared with supra basal and basal layer in tobacco chewers (Table 14, 15, 16). This results correlated with the findings by **Turker S, Guven C, Sener A *et al* (2018)** where the relationship of nicotine with cancer stem cells based on CD24 expression was studied and described that in the presence of nicotine and in its metabolites, the number of CSCs was associated with an increase in the CD24 cell population. This correlated with the results of our study, where 73% expression of CD24 was seen in the tissues of OSCC with the habit of chewing tobacco [58].

In OSCC, CD144 and CD24 showed similar staining in basal cell layer while CD144 showed higher expression in supra basal cell layer and connective tissue (Table 17, 18, 19). In normal tissues, CD144 showed higher expression in all the three layers (Table 20, 21, 22).

Summary and Conclusion

- In this study we had a total of 37 samples which are divided into two groups, group I, had 20 oral squamous cell carcinoma and group II comprised of 17 normal mucosa tissues.

- All the tissue sections were analysed for CD144 and CD24 expression that was stained by immunohistochemistry using anti-CD24 rabbit polyclonal primary antibody, VE-cadherin rabbit polyclonal primary antibody and secondary polyexcel HRP/DAB detection kit.

- In group - I, 18(90%) were males and 2(10%) were females. In group - II, 6(35.3%) were males and 11(64.7%) were females. A statistically significant difference was found with respect to gender among the study groups **(p=0.001).**

- In group - I (oral squamous cell carcinoma), 13(65%) had the habit of chewing tobacco, 2 (10%) cases had habit of chewing betel nut and alcohol consumption, 3(15%) had the habit of cigarette smoking with the habit of chewing tobacco and betel nut, 1(5%) had the habit of smoking cigarette and 1(5%) had the habit of smoking beedi. In group - II (normal mucosa), 17(100%) case had no habit history. A statistically significant difference (p=0.00) was found with respect to habits in the study groups.

- In group - I of 20(100%), the site of biopsy of 9(45%) cases was buccal mucosa, 5(25%) cases was tongue, 2(10%) cases was palatal

mucosa and 1(5%) cases was vestibular mucosa. In group - II 6 (35.3%) cases were buccal mucosa, 8(47.1%) cases was gingiva and 2 (11.8%) cases was pericoronal flap. A statistically significant difference (p=0.003) was found with respect to site of biopsy among the study groups.

- In group - I, the staining intensity of CD144 in supra basal cell layer was mild in 4(20%) cases, moderate in 7(35%) cases and absent in 9(45%) cases. In group - II, mild expression of CD144 in supra basal cell layer was seen in 10(58.8%) cases, moderate in 4(23.5%) cases and absent in 3(17.6%) cases. The difference was statistically significant difference (p=0.045) in the staining intensity of CD144 in supra basal cell layer.

- In group - I, the staining intensity of CD24 in connective tissue was mild in 6(30%) cases, moderate in 10(50%) cases, intense in 2(10%) cases and absent in 2(10%) cases. In group - II, mild expression of CD24 in connective tissue was seen in 8(47.1%) cases, moderately expressed in 1(5.9%) case, intense in 1(5.9%) cases and absent in 7(41.2%) cases. A statistically significant difference (p=0.014) was found with respect to staining intensity of CD24 in the connective tissue of the study groups.

- In cases with oral squamous cell carcinoma, 1(7.7%) case showed mild expression of CD144 in basal cell layer among cases with the habit of chewing tobacco and smoking beedi. A statistically significant difference(p=0.045) was found with respect to basal cell layer staining intensity of CD144 in habit of chewing tobacco and smoking beedi among group I

- In cases with oral squamous cell carcinoma, 1(50%) case showed mild expression of CD24 in basal cell layer among cases with the habit of chewing betel nut and consuming alcohol. A statistically significant difference(p=0.050) was found with respect to basal cell layer staining intensity of CD24 in habit of chewing betel nut and alcohol consumption among study group I

- In oral squamous cell carcinoma cases, 4(20%) cases showed mild expression and 7(35%) showed moderate expression of CD144 in supra basal cell layer while 11(55%) cases showed mild expression and 1(5%) case showed moderate expression of CD24 in basal cell layer. A statistically significant difference(p=0.020) was found between the staining intensity in the supra basal cell layer of CD144 and CD24 among oral squamous cell carcinoma cases

CD144 is a vascular mimicry marker and CD24 is a stem cell marker in the epithelium. There is increased intensity of CD144 and CD24 staining indicating an increased expression in epithelial and connective tissue component of OSCC as compared to normal controls. Studies on CD24 and CD144 have focussed on its expression in the connective tissue component and not in the epithelium. A statistical significant positive association was present between CD144 and CD24 expression **(p=0.020)**. Both markers could be a used to indicate cancer stem cells in the epithelium.

This study indicates that CD144 and CD24 are expressed in epithelium and seem to have a correlation with the type of habit. Also, this expression in connective tissue could be useful to assess the carcinogen induced changes of the tissue with respect to angiogenesis.

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Annexures

ANNEXURE- I

INSTITUTIONAL ETHICAL COMMITTEE

RAGAS DENTAL COLLEGE & HOSPITAL

(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai - 600 032

2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA
Tele : (044) 2453 0002 - 06. Principal (Dir) 2453 0001 Fax : (044) 24530009

TO WHOM SO EVER IT MAY CONCERN

Date: 20.12.2019

Place: Chennai

From

The Institutional Review Board

Ragas Dental College and Hospital

Uthandi, Chennai – 600119

The Project titled **"EXPRESSION OF CD24 AND CD144 IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS ASSOCIATED WITH HABITS "** submitted by **Dr.Vishnu Priya.V** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.

Dr.N.S .Azhagarasan,MDS

Member secretary,

The Institutional Review Board

Ragas Dental College and Hospital

Uthandi, Chennai – 600119

<u>**ANNEXURE – II**</u>

<u>**DISSERTATION PROTOCOL**</u>

<u>**TITLE**</u>

Expression of CD24 and CD144 in oral squamous cell carcinoma patients associated with habits.

<u>**NAME AND DESIGNATION OF THE PRINCIPAL INVESTIGATOR:**</u>

Dr Vishnu priya. V

Post Graduate student- Oral Pathology and Microbiology,

Ragas dental college and Hospital,Chennai

<u>**NAME OF HOD & STAFF IN CHARGE:**</u>

Dr K. Ranganathan, MDS, MS (Ohio), PhD

Dr. Rooban.T, MDS

<u>**DEPARTMENT WHERE THE PROJECT IS TO BE CARRIED OUT:**</u>

Department of Oral Pathology and Microbiology,

Ragas dental college and Hospital,

Chennai.

<u>**BACKGROUND**</u>

Metastasis is cause for the majority of the cancer deaths in humans and can be categorized into a series of steps. During the initial stage of haematogenous metastasis, cells leave the primary tumour nodule and enter the vascular endothelium. VE-cadherin also known as CD 144 is a vascular endothelial cadherin expressed in endothelial cells. Aberrant expression of VE-cadherin has been documented in sarcoma or highly aggressive melanoma cells. It controls the cohesion and organisation of the intercellular junctions. CD24 is a glycosylated glycosylphosphatidylinositol anchored adhesion molecule, which has co-stimulatory role in B and T cells. Thus, CD 24 and CD 144 was involved in metastasis and intercellular adhesion.

HYPOTHESIS:

There is no difference in the expression of CD24 and CD144 in normal mucosa and oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

AIM:

To evaluate the expression of CD24 and CD144 in oral squamous cell carcinoma patients associated with the habit of smoking/chewing tobacco and areca nut.

OBJECTIVES:

To ascertain the expression of CD24 and CD144 using anti-CD24 rabbit polyclonal primary antibody, VE-cadherin rabbit polyclonal primary antibody and secondary polyexcel HRP/DAB detection kit by immunohistochemistry on formalin fixed paraffin embedded tissue sections of:

- Oral squamous cell carcinoma associated with the habit of smoking/chewing tobacco and areca nut.
- Normal mucosa
- To compare the expression of CD24 and CD144 in normal mucosa and OSCC

MATERIALS AND METHODS:

- Study setting : Department of Oral Pathology and Microbiology,Ragas dental college and Hospital,Chennai
- Study group:

  Group I: Archival tissues of oral squamous cell carcinoma patients associated with the habit of smoking cigarette only, smoking

beedi only, tobacco chewing only, betel nut chewing with alcohol consumption and with cigarette, tobacco, betel nut chewing along with alcohol consumption.

Group II: Archival oral mucosal tissues of apparently healthy individuals

- To evaluate the immunohistochemical expression of CD24 and CD144 in the formalin - fixed, paraffin embedded tissue sections of oral squamous cell carcinoma and normal oral mucosa using anti-CD24 rabbit polyclonal primary antibody, VE-cadherin rabbit polyclonal primary antibody and secondary polyexcel HRP/DAB detection kit.

- Statistics to be used: The propotion of CD24 and CD144 expression between each group is to be compared and analysed using Chi square test.

Detailed budget plan: Rs 85,000

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Signature of principal investigator

Signature of Head of Department

Permission Granted   **YES / NO**

Modifications / comments

ANNEXURE – III

URKUND

Urkund Analysis Result

Analysed Document:	VISHNU PRIYA FULL THESIS WITH BIBLIOGRAPHY.pdf (D63622932)
Submitted:	2/8/2020 10:07:00 AM
Submitted By:	vino.priya1994@gmail.com
Significance:	6 %

Sources included in the report:

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merged 2.pdf (D63622617)
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6770277/
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https://www.spandidos-publications.com/10.3892/ol.2015.3598
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0168900
https://mdanderson.elsevierpure.com/en/publications/expression-of-stem-cell-markers-in-oral-cavity-and-oropharynx-squ
https://www.spandidos-publications.com/10.3892/ol.2018.9311?text=fulltext

Instances where selected sources appear:

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ANNEXURE – IV

PRIMARY ANTIBODY DATA SHEET

Tel:240-252-7368(USA)
Fax:240-252-7376(USA)
www.elabscience.com
E-mail:techsupport@elabscience.com
Elabscience Biotechnology Inc.

Elabscience®

CD24 Polyclonal Antibody

Catalog No.	E-AB-52318	**Reactivity**	H
Storage	Store at -20℃. Avoid freeze/thaw cycles.	**Host**	Rabbit
Applications	IHC, ELISA	**Isotype**	IgG

Note: Centrifuge before opening to ensure complete recovery of vial contents.

Images
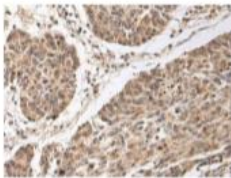
Immunohistochemistry of paraffin-embedded Human colorectal cancer tissue using CD24 Polyclonal Antibody at dilution of 1:200

Immunogen Information

Immunogen	Synthetic peptide of human CD24
Gene Accession	NP037362
Swissprot	P25063
Synonyms	CD 24,CD24,CD24 molecule,CD24,CD24A,FLJ22950,FLJ43543,HSA,MGC75043,Nectadrin

Product Information

Buffer	TRIS EDTA pH 9.0, PBS Buffer pH 7.4
Purify	Affinity purification
Dilution	IHC 1:100-1:300, ELISA 1:5000-1:10000

Background

This gene encodes a sialoglycoprotein that is expressed on mature granulocytes and in many B cells. The encoded protein is anchored via a glycosylphosphatidylinositol (GPI) link to the cell surface. Modulates B- cell activation responses. Signaling could be triggered by the binding of a lectin-like ligand to the CD24 carbohydrates, and transduced by the release of second messengers derived from the GPI-anchor. Promotes AG- dependent proliferation of B-cells, and prevents their terminal differentiation into antibody-forming cells.

Elabscience®

VE-Cadherin Polyclonal Antibody

Catalog No.	E-AB-33688	**Reactivity**	H,M,R
Storage	Store at -20℃. Avoid freeze/thaw cycles.	**Host**	Rabbit
Applications	WB,IHC-p,ELISA	**Isotype**	IgG

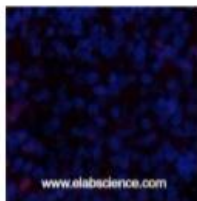**Note:** Centrifuge before opening to ensure complete recovery of vial contents.

Images
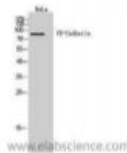
Western Blot analysis of Hela cells using VE-Cadherin Polyclonal Antibody at dilution of 1:500.

Immunohistochemistry of paraffin-embedded Rat lung tissue using VE-Cadherin Polyclonal Antibody at dilution of 1:200.

Immunofluorescence analysis of Rat spleen tissue using VE-Cadherin Polyclonal Antibody at dilution of 1:200.

Immunogen Information

Immunogen	Synthesized peptide derived from the Internal region of human VE-Cadherin.
Swissprot	P33151
Synonyms	CDH5,Cadherin-5,7B4 antigen,Vascular endothelial cadherin,VE-cadherin,CD144

Product Information

Calculated MW	88kDa
Observed MW	86kDa
Buffer	TRIS EDTA pH 9.0, PBS Buffer pH 7.4
Purify	Affinity purification
Dilution	WB 1:500-2000, IHC 1:100-300, ELISA 1:10000-20000

Background

This gene is a classical cadherin from the cadherin superfamily and is located in a six-cadherin cluster in a region on the long arm of chromosome 16 that is involved in loss of heterozygosity events in breast and prostate cancer. The encoded protein is a calcium-dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail.

Functioning as a classic cadherin by imparting to cells the ability to adhere in a homophilic manner, the protein may play an important role in endothelial cell biology through control of the cohesion and organization of the intercellular junctions. An alternative splice variant has been described but its full length sequence has not been determined.

ANNEXURE – V

SECONDARY ANTIBODY DATA SHEET

	Rev: A Release Date: 03/13/2014 IVD
PathnSitu	

PolyExcel HRP/DAB Detection System
Universal kit for Mouse and Rabbit Primary Antibodies

Intended Use: For In Vitro diagnostic use

PolyExcel detection system is intended to use with primary antibodies raised against **mouse** and **rabbit** for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues or cell preparations.

Summary and Explanation: PathnSitu's highly sensitive and specific PolyExcel two step detection system is non-biotin, micro-polymer based detection system which significantly reduce or shows no back ground on tissues containing high levels of avidin, biotin ex: Kidney, Liver and lymphoid tissues. This system is based on an HRP labeled polymer, which is conjugated with secondary antibodies.

Principal of procedure: Incubating the specimen for 5–10 minutes with H_2O_2 quenches any endogenous peroxidase activity. The specimen is then incubated with respective diluted mouse or rabbit primary antibody, followed by incubation with the PolyExcel Target Binder for 10 minutes then followed by a PolyExcel HRP labeled polymer using recommended 10minutes incubation. Staining is completed by a 5–10 minute incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen which results in a brown-colored precipitate at the antigen site (DAB is a potential carcinogen; Please take appropriate precautions).

Kit Contents:

PathnSitu PolyExcel detection kit supplied as 3 pack sizes. Details below:

Description	Cat#/Pack Size	Kit Contents
PolyExcel HRP/DAB Detection System	PEH2-6ml	PolyExcel H2O2 PolyExcel Target Binder PolyExcel PolyHRP PolyExcel Stunn DAB Substrate Buffer PolyExcel Stunn DAB Substrate Chromogen
	PEH2-50ml	
	PEH2-100ml	

Materials required but not supplied:
1. Positive charged slides (PathnSitu Cat# PS011-72)
2. Control Tissues
3. Xylene
4. Isopropyl alcohol
5. DI Water
6. Hematoxylin
7. Cover glass
8. Mounting media
9. Antigen retrieval buffers (PathnSitu Cat# PS007, PS008, PS009)
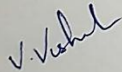10. Immuno wash Buffer (PathnSitu Cat# PS006)

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ANNEXURE-VI

DEPARTMENT DECLARATION FORM

DEPARTMENT DECLARATION FORM

The study titled "**EXPRESSION OF CD24 AND CD144 IN ORAL SQUAMOUS CELL CARCINOMA PATIENTS ASSOCIATED WITH HABITS** " have been done under the guidance of the staffs of Department of Oral Pathology and Microbiology during my post-graduation during 2017- 2020. The same has been submitted as a part of the syllabus MDS degree programme in Oral pathology and Microbiology of the TamilNadu Dr M.G.R. Medical University, Chennai.

I shall publish in full or part of this work in any media only with the prior written approval of the head of the department.

Dr Vishnu Priya V

Post-graduation, 2017- 2020

Department of Oral Pathology and Microbiology,

Ragas Dental College and Hospital, Chennai

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ANNEXURE VII

ABBREVIATIONS

CD	-	Cluster of Differentiation
OSCC	-	Oral Squamous Cell Carcinoma
CSC	-	Cancer Stem Cell
DN	-	Deoxyribonucleic Acid
HNSCC	-	Human head and neck squamous cell carcinoma
VM	-	Vasculogenic mimicry
VE	-	Vascular endothelial
IHC	-	Immunohistochemistry
IRB	-	Institutional Review Board
PBS	-	Phosphate buffered Saline
HRP	-	Horse Radish Peroxidase
DAB	-	3,3'-Diaminobenzidine
TBS	-	Tris Buffer Saline
EDTA	-	Ethylene Diamine Tetra Acetic acid
APES	-	3 amino propyl triethoxysilane

DPX	-	Distyrene Plasticizer Xylene
H&E	-	Hematoxylin and Eosin
LM	-	Light Microscopy
SPT	-	Second Primary Tumor
SPSS	-	Statistical Package for Social Sciences
TSNs	-	Tobacco - Specific Nitrosamines
NNN	-	N'-nitrosonornicotine
SC	-	Stem Cells
SRC	-	Sarcoma
ABC	-	ATP Binding Cassette
GPI	-	Glycosylphosphatidylinositol
STAT3	-	Signal transducer and activator of transcription *3*
HPV	-	Human Papilloma Virus
EGFR	-	Epidermal Growth Factor Receptor
ALDH	-	Aldehyde Dehydrogenase
MMP	-	Matrix metalloproteinase
MET	-	Mesenchymal-Epithelial Transition
CD144	-	Cluster of Differentiation 144
BMP	-	Bone Morphogenic Protein

ESC   -   Embryonic Stem Cell

ROS   -   Reactive oxygen species

CAFs   -   Cancer Associated Fibroblast

OPMD   -   Oral Premalignant Disorder

EMT   -   Epithelial Mesenchymal Transition