EXPRESSION OF ALDEHYDE DEHYDROGENASE 1 (ALDH 1) AND CD144 IN NON-HABIT ASSOCIATED ORAL SQUAMOUS CELL CARCINOMA

Dissertation submitted to

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In partial fulfilment for the Degree of

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BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

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CERTIFICATE

This is to certify that this dissertation titled **"EXPRESSION OF ALDEHYDE DEHYDROGENASE 1 (ALDH 1) AND CD144 INNON-HABIT ASSOCIATED ORAL SQUAMOUS CELL CARCINOMA"** is a bonafide dissertation performed by **Dr PREETHA S** under our guidance during the post graduate period 2017-2020

This dissertation is submitted to **THE TAMIL NADU Dr.M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for thedegree of **MASTER, OF DENTAL SURGERY** in **ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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ENDORSEMENT BY THE HEAD OF THE DEPARTMENT AND HEAD OF THE INSTITUTION

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ABSTRACT

Background:

Cancer stem cells are tumour cells that have the capacity of self-renewal, the potential to develop into any cell in the overall tumour population and the proliferative ability to drive continued expansion of the population of malignant cells.

Aldehyde dehydrogenase 1 (ALDH1) is a cell surface marker for cancer stem cells.ALDH1 is involved in the conversion of retinol to retinoic acid and catalyses the oxidation of intercellular aldehyde metabolites into carboxylic acid. Vascular Endothelial-Cadherin(VE-Cadherin;CD144) is an adhesion molecule that promotes cell-to-cell interaction. CD144 also controls the cohesion and organization of the intercellular junctions. Overexpression level of VE-Cadherin enhances the cancer neovascularization, growth, and progression.

Aim and Objectives:

To evaluate the expression of ALDH 1 and CD144 in patients with non-habit associated OralSquamous Cell Carcinoma by Immunohistochemistry(IHC).

Materials and Methods:

Immunohistochemical detection of ALDH1 was done using polyclonal antibody and Poly Excel HRP/DAB^{TM}chromogen detection system on 37formalin fixed paraffin embedded tissue samples, which included non-habit

associated Oral Squamous Cell Carcinoma(OSCC; n=20) and the expression was compared with that of normal mucosa(n=17). Immunohistochemical detection of CD144 was done using polyclonal antibody and Poly ExcelHRP/DAB chromogen detection system on 37formalin fixed paraffin embedded tissue samples,which included non-habit associated Oral Squamous Cell Carcinoma(OSCC; n=20) and the expression was compared with that of normal mucosa(n=17).

Results:

The positivity of CD144 expression was higher in OSCC when compared with normal mucosa.The connective tissue also showed positively stained vascular channels, cells around the vascular channels and keratin pearls.ALDH1 positivity was expressed more in normal mucosa when compared to OSCC.The connective tissue showed ALDH1 positively stained blood vessels, keratin pearls and muscle.

Conclusion:

When comparing the expression of CD144 and ALDH1 between the two groups, CD144 was increased in OSCC (Group I) and decreased in normal mucosa (Group II). ALDH1 was increased in normal mucosa and decreased in OSCC. The decreased expression of ALDH1 in OSCC can be due to the lower levels of acetaldehyde during alcohol metabolism in non-alcoholics and the biological behaviour difference between smokers and alcoholics when compared to non-smokers and non-alcoholics.

Key words: ALDH1, CD144, OSCC

CONTENTS

Introduction

Oral cancer is the eleventh most common cancer in the world. In 2012, the estimated new cases of oral cancer globally were 300,000 and 145,000 deaths. In India, 20/100,000 population are affected by oral cancer. Oral cancers include cancers of the mucosal lip, tongue, gum, floor of the mouth, palate, and mouth (International Classification of Diseases, 10th revision)[1,2].Oral squamous cell carcinoma (OSCC) is the most common neoplasm of the oral cavity and constitutes 90% of all oral malignancies[3].

Development of oral cancer is influenced by genetic and epigenetic factors. Tobacco is the main risk factor associated with oral cancer. The non-habit associated risk factors for oral cancer include dental factors, diet and nutrition, viruses, radiation, ethnicity, familial and genetic predisposition, immunosuppression , syphilis.[4,5]. Oral cancer can show a biologically more aggressive phenotype in smokers and alcohol drinkers. In patients not exposed to smoking and alcohol, OSCC tends to be well or moderately differentiated, while in exposed individuals, a lower degree of cell differentiation has been observed[6]. The risk of tumour recurrence is lower and survival, prognosis is also better in non-smokers and non-alcoholics[6]. Response to radiotherapy also tends to be better in patients who are non-smokers or who quit the habit during treatment[6] also tumour suppressor protein p53 expression associated with a poor prognosis of SCC of the head and neck, is greater in those who are smokers and alcohol drinkers[6].

Cancer stem cells (CSCs) are a small subpopulation of tumour cells with capabilities of self-renewal, differentiation and tumorigenicity. Stem cells reside in special tissue niche and contribute to progression of tumor[6]. CSC markers include CD34, CD44, CD123, CD133, Oct4, SOX2, Nanog, c-kit, ABCG2 and ALDH. Identification and characterization of CSCs in malignant tumour niches can help in tailoring personalized treatment for aggressive tumour phenotypes[8].

Aldehyde dehydrogenase 1 (ALDH1), is a detoxifying enzyme which is responsible for the oxidation of intracellular aldehydes. It contributes to early stem cell differentiation by catalysing the oxidation of exogenous and endogenous aldehydes and the oxidation of retinal to retinoic acid. ALDH1 isoforms are widely distributed and the highest expression is observed in the liver and kidney. The cellular distribution of ALDH1 include cytoplasm, mitochondria and endoplasmic reticulum. ALDH1 positivity correlates with the number of cells undergoing epithelial-mesenchymal transition. Studies have identified this marker in CSC in lung, pancreatic, prostrate tumours and head and neck squamous cell carcinoma[9].

Cell-to-cell adhesion plays a dynamic and fundamental role in the development and maintenance of multi-cellular organisms. Cadherins are a family of calcium-dependent transmembrane proteins involved in cell-to-cell adhesion and are expressed in stratified squamous epithelial cells. They have been implicated in the development and progression of carcinomas of

epithelial origin[10].Vascular endothelial-cadherin (VE-cadherin; CD144, is an adhesion molecule involved in cell-to-cell interaction. VE-cadherin has been demonstrated both in tumour endothelial cells and highly aggressive melanoma cells. Overexpression of VE-cadherin is associated with cancer neovascularization, growth, and progression[11].

This study was done to assess the expression of ALDH1 and CD144 in non-habit associated Oral Squamous Cell Carcinoma (OSCC).

Aim and Objectives

AIM:

To evaluate the expression of ALDH 1 and CD144 in formalin fixed paraffin embedded tissues of non-habit associated Oral Squamous Cell Carcinoma.

OBJECTIVE:

1. Tostudy ALDH1 expression in formalin fixed paraffin embedded tissues of non-habit associated Oral Squamous Cell Carcinoma using polyclonal ALDH1 primary antibody and PolyExcel HRP/DAB detection kit by Immunohistochemistry.
2. To study ALDH1 expression in formalin fixed paraffin embedded tissues of normal mucosa using polyclonal ALDH1 primary antibody and Poly Excel HRP/DAB detection kit by Immunohistochemistry.
3. To studyCD144 expression in formalin fixed paraffin embedded tissues of non-habit associated Oral Squamous Cell Carcinoma using polyclonal CD144 primary antibody and PolyExcel HRP/DAB detection kit by Immunohistochemistry.
4. To studyCD144 expression in formalin fixed paraffin embedded tissues of normal mucosa using polyclonal CD144 primary antibody and Poly Excel HRP/DAB detection kit by Immunohistochemistry.
5. To compare the expression of ALDH1 and CD144 in non-habit associated Oral squamous cell carcinoma and normal mucosa.

Materials and Methods

STUDY DESIGN:

This study was done to evaluate the expression of ALDH1 and CD144 in Formalin Fixed Paraffin Embedded tissues of non-habit associated Oral Squamous Cell Carcinoma and normal mucosa.

STUDY GROUP:

Group I:Formalin fixed paraffin embedded non-habit associated Oral Squamous Cell Carcinoma

Group II:Formalin fixed paraffin embedded normal mucosa- Control (Clinically healthy individuals)

SAMPLE SIZE:

Group I: 20 Formalin fixed paraffin embedded non-habit associated Oral squamous cell carcinoma

Group II:17Formalin fixed paraffin embedded normal mucosa

TECHNIQUE:

Immunohistochemistry using polyclonal ALDH1 primary antibody, polyclonal CD144 primary antibody and Poly Excel HRP/DAB detection kit

STUDY SETTING:

This study was done at Ragas Dental College and Hospital and approved by the Institutional Ethics Committee of Ragas Dental College and Hospital, Chennai.

ARMAMENTARIUM USED:

- Microtome
- Autoclave
- Hot air oven
- Slide warmer
- Coplin jars
- Measuring jar
- Weighing machine
- APES (3 amino propyl triethoxysilane) coated slides
- Slide box
- 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
7) Disodium hydrogen phosphate dihydrate
8) Potassium dihydrogen orthophosphate
9) Tris EDTA (Ethylene Diamine Tetra Acetate) buffer
10) Distilled water
11) 1 N sodium hydroxide

ANTIBODIES USED:

Primary antibody

1. Anti-ALDH1A1 rabbit polyclonal antibody, CAT NO: E-AB-33427(Elabscience)™
2. Anti-CD144 rabbit polyclonal antibody, CAT NO: E-AB-33688(Elabscience)™

Secondary antibody: Poly Excel-HRP/DAB IHC Detection system (PathInsitu)™

APES (3 Amino propyl tri ethoxysilane) coating:

1. Slides first dipped in couplin jar containing acetone for 2 minutes
2. Dipped in APES for 5 minutes

3. Dipped in two changes of distilled water for 2 minutes each
4. Slides left to dry

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 study group and control.
2) Tissue samples of normal mucosa and non-habit associated 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.

IMMUNOHISTOCHEMICAL STAINING OF ALDH1:

Tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred onto APES 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 as the positive. The slides with tissue sections were treated with two 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 were placed in microwave oven for antigen retrieval at 100°C for 30 seconds. Slides were then treated with 3 % hydrogen peroxide for 10minutes to quench endogenous

peroxidase activity of cells that would result in non-specific staining. The slides were wiped carefully without touching the tissue section. The sections were incubated at room temperature with rabbitpolyclonal primary ALDH1 antibody (Elabscience)™. The primary antibody was detected usingPoly Excel HRP/DAB IHC Detection system™. The sections weretreated with target binder for 20 minutes at roomtemperaturefollowed by incubation withPoly Excel HRP™ reagent for 15 min at room temperature. After two washes with wash buffer, 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 hematoxylin, 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 breast carcinoma that was previously known to be positive for ALDH1 was used as positive control. 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 (3 minutes each)
3. Placed in 100% isopropanol (5 minutes)
4. Placed in 90% isopropanol (5 minutes)

5. Washed in distilled water (2 minutes each)
6. Keep in Tris EDTA buffer at pH 9 in microwave oven at 100°C for 30 seconds for antigen retrieval
7. Cooling of solution done for 20 minutes
8. Slides were transferred to TRIS wash buffer.
9. Placed in 3% hydrogen peroxide (10 minutes)
10. Washed in TRIS wash buffer (5 minutes)
11. Primary antibody added and incubated (overnight)
12. Washed in TRIS wash buffer (2-3 minutes)
13. Poly excel target binder reagent added and incubated (10 minutes)
14. Washed in TRIS wash buffer(2-3 minutes)
15. Poly Excel HRP added and incubated (15 minutes)
16. Washed slides in Tris wash buffer (2-3 minutes)
17. DAB added and incubated in an enclosed in hydrated container (10 minutes)
18. Washed in Tris was buffer (2-3 minutes)
19. Stained with Harris Hematoxylin (10 minutes)
20. Washed in tap water
21. Placed in 70% alcohol (1 minute)
22. Placed in 100% alcohol (1 minute)
23. Placed in xylene (1 dip)
24. Slides were mounted using DPX
25. Slides were observed under the LM and graded

IMMUNOHISTOCHEMICAL STAINING OF CD144:

Tissue sections of 5 micron thickness were made in a rotary manual microtome. The ribbons of tissue section were transferred ontoAPES 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 with tissue sections were treated with two changes of xylene to remove paraffin wax. They were put in descending grades of alcohol and then rehydrated withwater. 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 were 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. The slides were wiped carefully without touching the tissue section. The sections were incubated at room temperature with rabbitpolyclonal primary CD144 antibody (Elabscience)^{TM}. Primary antibody was detected using Poly Excel HRP/DAB IHC Detection system^{TM}.The sections weretreated with target binder for 20 min at room temperature followed by incubation withPoly Excel HRP reagent^{TM} for 15 min at room temperature. After two washes with wash buffer, 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 hematoxylin, 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 lungthat was previously known to be positive for CD144 was used as positive control. 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 (3 minutes each)
3. Placed in 100% isopropanol (5 minutes)
4. Placed in 90% isopropanol (5 minutes)
5. Washed in distilled water (2 minutes each)
6. Keep in Tris EDTA buffer at pH 9 in microwave oven at 100°C for 30 seconds for antigen retrieval
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20. Washed in tap water
21. Placed in 70% alcohol (1 minute)
22. Placed in 100% alcohol (1 minute)
23. Placed in xylene (1 dip)
24. Slides were mounted using DPX
25. Slides were observed under the LM and graded

CRITERIA FOR EVALUATION OF STAINING

Evaluation for IHC

- Grade 0– Negative (-)
- Grade 1 – Mild (+)
- Grade 2 – Moderate(++)
- Grade 3 – Intense (+++)

Each case was evaluated by two blinded observers independently with respect to positive control.

Statistical analysis:

Chi-square test was used to investigate the association between the staining intensity of cells for ALDH1 and CD144. Kappa analysis was done to evaluate the inter-observer agreement. Statistical significance was determined for a p-value < 0.05 for all tests. The statistical analyses were carried out by using the SPSS^{TM} software version 25 (Statistical Package for Social Sciences)

Review of Literature

ORAL CANCER

Oral cancer is the eleventh most common cancer in the world and accounts for an estimated 702,000 prevalent cases over a period of five years (old and new cases). Oral cancers include cancers of the mucosal lip, tongue, gum, floor of the mouth, palate, and mouth, corresponding to the International Classification of Diseases, 10th revision [ICD-10], codes C00, C02, C03, C04, C05, and C06, respectively. Oral cancer in low- and middle-income countries (LMICs) accounts for two-thirds of the global incidence and half of those cases are in South Asia. India accounts for one-fifth of all oral cancer cases and one-fourth of all oral cancer deaths. The incidence rates are the highest in India and lowest in Belarus. Globally, the Age-Standardised Incidence Rate per 100,000 populations for oral cancer in men and women is 5.5 and 2.5respectively.

In South and Southeast Asia, buccal mucosa is the most common site for oral cancer and in all other regions, tongue is the most common site. The mortality rates of oral cancer are influenced by its incidence, access to treatment, and variations in site distribution. In countries like India, Papua New Guinea, Taiwan, China where chewing of betel quid with tobacco or without tobacco or areca nut chewing is common, the incidence rate and mortality of oral cancer is high; as well as in Eastern Europe, France, and parts of South America (Brazil and Uruguay), where tobacco smoking and alcohol consumption are high.

Five-year survival is approximately 65 percent in the United States, less than 35 percent in India and ranges between 32 and 54 percent in China, the Republic of Korea, Pakistan, Singapore, and Thailand. The five-year survival for early, localized cancers exceeds80 percent and when regional lymph nodes are involved it falls to less than 20 percent[1].

RISK FACTORS OF ORAL CANCER

Oral Squamous Cell Carcinoma (OSCC) is the most common epithelial malignancy of the oral cavity. OSCCs and their variants constitute over 90% of oral malignancies[11].Development of oral cancer is influenced by genetic and epigenetic factors. Smoking and alcohol are the main risk factors associated with oral cancer and the combinations of these factors enhance the carcinogenic effect. The effect of these factors has been well established. However, approximately 15-20% of oral cancer occurs in patients without the traditional risk factors. The non-habit associated risk factors of oral cancer include dental factors, diet and nutrition, viruses, radiation, ethnicity, familial and genetic predisposition, oral thrush, immunosuppression, use of mouthwash, syphilis, occupational risks, and mate. Identification of these non-traditional factors is important for the diagnosis of long standing ulcer or small tumour size.[4,5]

DENTAL FACTORS

Poor oral hygiene, chronic mechanical trauma from sharp and fractured teeth due to caries and trauma, chronic ulceration from an ill-fitting denture

and loose anchoring attachments promote neoplasm in the presence of other risk factors. A retrospective analysis by **Ranganathan *et al (2015)***observed non-tobacco, non-areca nut using and non-alcohol drinking patients (NTND) with OSCCand that the prevalence of OSCC is on the decrease in non-glossal sites[13].**Randhawa *et al(2008)*** describes a young female patient with squamous cellcarcinoma of posterolateral border of tongue which was not associated with any deleterious habits usually associated with oral cancer[4,5].

DIET AND NUTRITION

International Agency for Research on Cancer (IARC) affirms that low intake of fruits and vegetables predisposes to increased risk of cancer development and high consumption of fruits and vegetables is associated with a reduction of 40–50 percent in the risk of oral cancer[1,4]. Reduced risk of oral and pharyngeal cancer is associated with carrots, fresh tomatoes, and green pepper consumption.Vegetables and fruits contain micronutrients, dietary fibers and phytochemicals and reduce the risk of malignant neoplasia of the oral cavity, larynx, and pharynx by regulation of the expression and activity of transcription factors, inflammatory mediators,growth factors and cell cycle intermediates. Vitamins A (retinol), C (Ascorbic Acid, AA), and E (α-tocopherol); carotenoids (β-carotene); potassium; and selenium decrease the risk of oral cancer development.Antioxidantslike β-carotene, retinol, retinoids, vitamin C (AA), and vitamin E (α-tocopherol) reduce free radical reactions which can cause DNA mutations, changes in enzymatic activity, and

lipid peroxidation of cellular membranes. The interplay of cultural risk factors and dietary factorsinfluence development of oral cancer and precancer.

De Podesta *et al (2019)* evaluated the association between minimally processed food consumption and the risk of HNC in Brazil and concluded that consumption of a heathy diet rich in fruits and vegetables was associated with a reduced risk of HNC[14].

Chuang *et al(2012)* investigated the association between diet and head and neck cancer and observed that higher dietary pattern scores, reflecting high fruit/vegetable and low red meat intake, were associated with reduced HNC risk whereas intake of red meat and processed meat were associated with increased risk of head and neck cancer[15].

VIRUSES

Human papilloma virus(HPV)and Herpes simplex virus (HSV) and Epstein– Barr virus (EBV), have been established as causative agents of oral cancer. Viruses hijack host cellular apparatus, modify the DNA and the chromosomal structures and induce proliferative changes in the cells[12]. Viral infections of latent or chronic nature usually induce malignant transformation by interfering with the host's cell cycle machinery.

HPV are DNA viruses and are epitheliotropic. They cause benign proliferative lesions such as papillomas, condylomaacuminatum, verruca vulgaris, and focal epithelial hyperplasia (Heck's disease). Some HPV types

like HPV 16, 18, 31, 33, 35, and 39 are called as 'high-risk' types and are associated with OSCC and oral premalignant lesions. HPV encodes oncoproteins E6 and E7. The E6 and E7 proteins bind and destroy p53 and Rb tumour suppressor genes, respectively, thereby disrupting the cell cycle with loss of control on DNA replication, DNA repair, and apoptosis[4]. Patients with HPV positive cancers are associated with increased risk of cervical lymphadenopathy than those of HPV negative HNC and the association between HPV infection and HNSCC is independent of tobacco and alcohol use[16].

HSV-1 is involved in ocular and oral infections and HSV-2 is involved in genital infections. HSV is involved in the induction of heat shock proteins, host cell shutoff process, stimulation of other viruses like HPV and chromosomal rearrangements[5].Molecular detectionof Herpes Simplex Virus (HSV1, HSV2) in Oral Squamous Cell Carcinoma at Khartoum was done by **Osman *et al (2017)***and asignificant increase of occurrence of HSV-1 and oral squamous cell carcinoma in the mandible was observed[17].EBV causes nasopharyngeal carcinoma, Burkitt's lymphoma, post-transplant lymphoma and gastric carcinoma.**Chaturvedi*et al(2013***) identified EBV DNA and EBV-encoded small messenger RNA,through PCR and *in-situ* hybridization[5].

RADIATION

Ultraviolet radiation (UVR) causes DNA damage, mutagenesis, immunosuppression, and interaction with viruses such as human papilloma

virus (HPV) and impede the repair of UVR-induced DNA damage in HPV-infected cells[18]. Exposure to low doses of radiation (less than 50 mSv/year) early in life increases the susceptibility to damage from high-dose radiation exposure later in life. **Raygoza *et al (2019)***evaluated micronuclei in oral mucosa of individuals exposed to ionizing radiation and observed that medical and nursing staff from radiology centers presented with higher genetic damage compared to individuals who were not exposed to radiation[19].

MATE

Maté, is a tea-like beverage which is consumed in South America and in parts of Europe.This has been shown to cause oral and pharyngeal cancers. The proposed pathogenesis for mate's carcinogenicity include thermal injury, solvent for other chemical carcinogens, and presence of tannins and N-nitroso compounds[4].

MOUTHWASH

The risk of mouthwash causing oral cancer is influenced by the frequency and duration of use and its alcohol which is used as a solvent for other ingredients or as a preservative content. But,there is no cause–effect relationship between mouthwash and oral cancer[4].**Boffeta *et al (2016)*** conducted a pooled analysis of mouthwash use and cancer of the head and neck and found an association between long term frequent use of mouthwash and increased risk of head and neck cancer[20]. A similar result was observed by

Wilson *et al (2016)*whoassessed the association of mouthwash use with cancers of the oral cavity, oropharynx, hypopharynx and larynx[21].

IMMUNOSUPPRESSION

Immunosuppressed individuals are more prone to develop oral cancers. Human immunodeficiency virus (HIV)infected patients are predisposed to developing Kaposi's sarcoma and lymphomas, although not to OSCC.Immunosuppressed organ transplant patients have been shown to develop lip cancers and the possible reason was attributed to increased exposure to radiation[4].

OCCUPATIONAL RISK

Exposure to excessive solar radiation/ultraviolet (UV) light is known to cause lip cancers. UV rays also causes actinic cheilitis which may transform to OSCCs. Sulfur dioxide, asbestos, pesticide exposures, and mists from strong inorganic acids and burning of fossil fuels has been known to cause cancers of posterior mouth, pharynx, and larynx. Increased risk for the development of salivary gland carcinomas have been reported in people who work at manufacturing of rubber products, plumbing (exposure of metals), and woodworking in an automobile industry[4].

SYPHILIS

Tertiary syphilis had been known to predispose to the development of oral cancer along with other risk factors such as tobacco and alcohol.

However, tertiary syphilis is rare as the infection is diagnosed and treated before the onset of tertiary stage[4].

GENETIC FACTORS

A study by **Copper *et al(1995)***, followed up first-degree relatives of 105 head and neck cancer patients and found that 31 of these subjects developed cancers of respiratory tract and upper aerodigestive tract.Butpopulation-based studies to determine the genetic or familial disposition to oral cancers are limited by the coexisting risk factors like smoking and alcohol.Certain individuals inherit the susceptibility of inability to metabolize carcinogens or procarcinogens and/or an impaired ability to repair the DNA damage. Genetic polymorphisms in the genes coding for the enzymes (P450 enzymes and XMEs) which are responsible for tobacco carcinogen metabolism play an important role in genetic predisposition to tobacco-induced head and neck cancers.

Polymorphisms in alcohol-metabolizing enzymes also contribute to development of oral cancers. Individuals with the fast-metabolizing version (allele) of alcohol dehydrogenase have a greater risk of developing oral cancer in the presence of alcoholic beverage consumption than those with the slow-metabolizing forms[1].

FUNGAL INFECTIONS

Candida albicans which are opportunistic pathogens have been implicated in the pathogenesis of oral premalignant lesions. Superficial fungal hyphae of Candida albicans have been foundsuperimposed on leukoplakia, especially nodular leukoplakia, many of which have undergone malignant transformation. Candida infection can coexist or be associated with other risk factors like irondeficiency and in chronic smokers which may prove synergistic in the development of oral cancer[4].

ORAL SQUAMOUS CELL CARCINOMA IN TOBACCO AND ALCOHOL

The primary factor (90%) for the development of OSCC is tobacco usage[6].According to the International agency for research on cancer (IARC), cigarette smoke contains more than 5,000 chemicals and 62 carcinogens that has been recognized as a risk factor for cancer. Carcinogenic components include nitrogen oxide, isoprene, butadiene, benzene, formaldehyde, acetaldehyde, acrolein, arsenic, cadmium, ethyleneoxide, 2-naphthylamines, nitromethane, eruption, radioactive polonium, metals, nitrosamine, and polycyclic aromatic hydrocarbons (PAH). Among all, PAH and nitrosamine are the two most important components with carcinogenic properties[22].

TOBACCO AND GENETIC CHANGES

Alteration in the expression of oncogenes, tumour suppressors, DNA repair mediators, and apoptosis-related genes are involved in the development and pathogenesis of cancers.Toxic and carcinogenic agents in cigarettes alter the expression of genes through several mechanisms, such as point mutations, deletions, translocations and gene recombination. TP53is commonly deregulated in many human cancers.PAH, in cigarette, increases the frequency of thymine and guanine replacement in p53 and Nicotine-derived nitrosamine ketone (NNK), elevate substitution of guanine to adenine in exon 5 of p53 gene.Cigarette smoke is a rich source offree radicals and reactive oxygen species (ROS) and each pack of cigarettes produces approximately 5 × 104 free radicals, which eventually cause a wide range of cell damages, such as inactivation of enzymes, lipid peroxidation, and protein/lipoprotein oxidation[22].

TOBACCO AND EPIGENETIC CHANGES

Epigenetic changes include DNA methylation, histone modifications, histone and nucleosome changes, and gene regulation by microRNA which alter the chromatin structure.Nicotine change the expression of DNA methyl transferases, such as DNMT1, DNMT3a, and DNMT3b, causing demethylation of SNCG (synuclein-gamma) oncogene.Smoking tobacco is also associated with methylated tumour suppressor gene CDKN2A (p16)[22].

TOBACCO AND GROWTH SIGNALING PATHWAYS

Nicotine, as a major component of tobacco, binds to nicotinic acetylcholine receptors (nAchR) , epidermal growth factor receptor (EGFR) and beta-adrenergic receptor (AR-β)which induce signal transduction pathways, such as MAPK, AKT and PKC , which in turn inhibit apoptosis, stimulate cell proliferation and induce angiogenesis[22].

To evaluate the clinicopathologic characteristics of patients without the traditional risk factors associated with oral cancer, **Kruse *et al(2010)*** conducted a study with 278 patients who were newly diagnosed,previously untreated with OSCC and those who were treated between 1999 and 2008 with a minimum follow up time of 12 months and without the risk factors of alcohol and tobacco use.It was reported that out of the 278 patients who were involved in the study,67.2% were women and the mean age was 70 years. The most common tumour sites were mandibular alveolar ridge (22 patients) and maxilla (18 patients). Fifteen patients experienced a recurrence, and 10 patients developed lymph node metastases during thefollow-up period. It was concluded that the group of patients with no tobacco and alcohol use tends toward a higher proportion of females, a higher proportion of patients over 70 years, and a higher number of oral maxillary SCC[23].

Lo *et al(2003)*reported that in individuals exposed to risk factors (chewing tobacco, smoking and alcohol), the lesion developed a mean of 12 years earlier than in those not exposed.**Dahlstrom *et al(2008)*** and **Harris *et***

al(2010) observed that the group of non- smokers and non-alcohol drinkers had a low mean age and that women were more affected in comparison[24].

In patients not exposed to the traditional risk factors, the lesions develop primarily in the oral cavity, especially in the anterior tongue, alveolar ridge and gingiva. In individuals associated with smoking and alcohol consumption, the tumours occur mostly in the larynx, hypopharynx, posterior tongue, retro- molar trigone and mouth floor[6].

Mayne *et al(2009)* reported that in patients were treated for oral, pharynx and larynx carcinoma, the smokers and alcohol drinkers showed a worse prognosis and **Girod *et al(2009)***observed that female smokers with a diagnosis of oral and oropharynx cancer showed worse prognosis[25,26]. **Ide *et al(2008)*** and **Fortin *et al(2009)*** also demonstrated that smokers and alcohol drinkers, showed survival rates and local control of the disease that were inferior to that in patients not exposed to these risk factors[27,28]. **Do *et al(2003)***and **Sassi *et al (2010)***reported that smokers and alcohol drinkers had a significantly greater risk of developing a second primary tumour in comparison to non-smokers, particularly if they continued the habit after diagnosis of the lesion[29,30].**Chen *et al (2011)*** in his study reported that non-smokers showed a better prognosis after radiotherapy than smokers who continued their habit[31].

Farshadpour *et al(2008)* and **Hsieh *et al(2001)*** observed increased expression of p53 mutation in smokers and alcohol drinkers[32,33]. In addition to

higher rates of mutation of protein p53, head and neck SCC from smokers showed percentage of infection by HPV lower, loss of heterozygosity in 3p, 4q, and 11q13 and the greater number of chromosome losses[21]. **Faustino *et al(2008)*** and **Kyzas *et al(2005)*** found no correlation between the immunoreactivity of vascular endothelial growth factor (VEGF) with smoking and alcohol consumption in patients with oral SCC[34,35].

CANCER STEM CELLS

Stem cells are characterized by the capacity for self-renewal and the ability to differentiate into diverse cell types[7]. Based on their origin, stem cells are categorized either as embryonic stem cells (ESCs) or as postnatal stem cells/somatic stem cells/adult stem cells (ASCs).

Characteristics

1. Totipotency: generate all types of cells including germ cells (ESCs).
2. Pluripotency: generate all types of cells except cells of the embryonic membrane.
3. Multipotency: differentiate into more than one mature cell (MSC).
4. Self-renewal: divide without differentiation and create everlasting supply.
5. Plasticity: MSCs have plasticity and can undergo differentiation. The trigger for plasticity is stress or tissue injury which upregulates the stem cells and releases chemoattractants and growth factors[36].

Cancer stem cells (CSCs) are a small subpopulation cells within the tumours with capabilities of self-renewal, differentiation and tumourigenicity[7]. **Kelly *et al(2007)*** observed that as 25% of cancer cells may have the properties of CSCs[37].(Figure 1)[7] CSCs play a role in tumour growth, tumour initiation, angiogenesis, reorganization of the extracellular matrix, metastasis anddruginducedresistance[38].

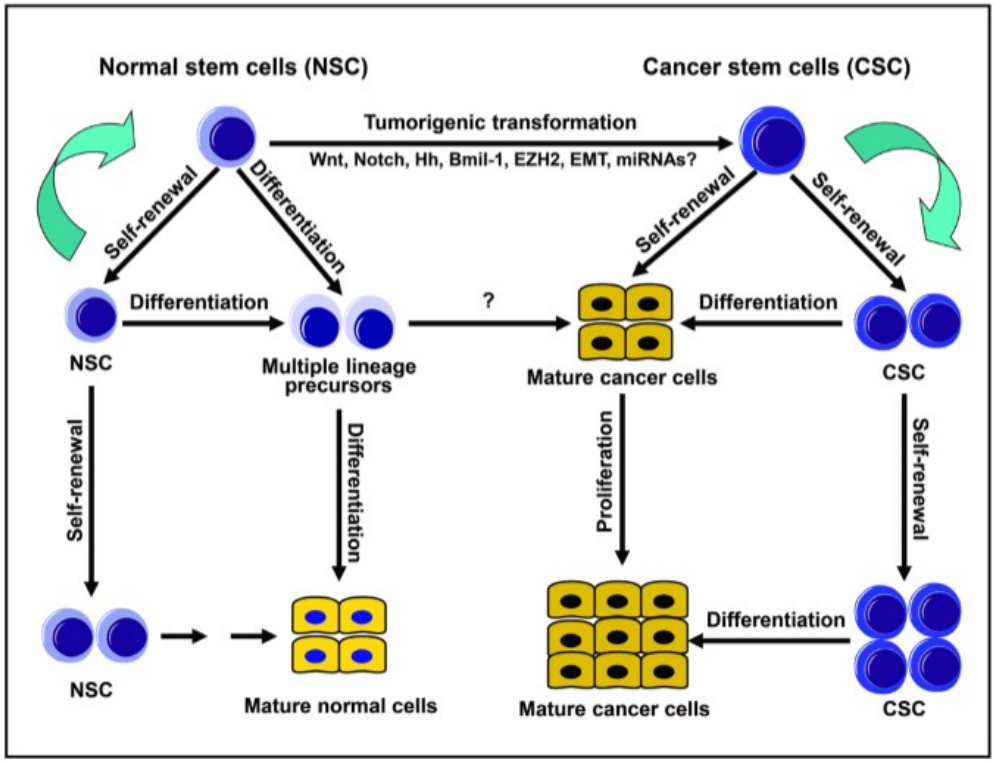


FIGURE 1. NSC: normal stem cells; CSC: cancer stem cells; Hh: hedgehog; Bmil-1: polycomb complex protein; EZH2: enhancer of zeste homolog 2; miRNAs: microRNAs.

Expression of cell surface markers such as CD44, CD24, CD29, CD90, CD133, epithelial- specific antigen (ESA), and Aldehyde Dehydrogenase1 (ALDH1) have been used toisolateand enrich CSCs from different tumours ***(*Al-Hajj *et a;2003)***[39], Singh et al(2003)[40], **Ginestier*et al(2007)***[41].The expression of CSC surface markers is tissue type-specific, even tumour subtype-specific. **Yu *et al (2010)*** observed that CD44+CD24−/low lineage and ALDH+ were characterized for breast CSCs; CD133+ for colon, brain and lung; CD34+CD8− for leukemia; CD44+ for head and neck; CD90+ for liver; CD44+/CD24+/ESA+ for pancreas CSCs[42]. Solid tumours like head and neck squamous cell carcinoma are histologically heterogeneous and contain various types of cells like tumour cells, stromal cells and inflammatory cells[43]. Identification and characterization of these CSCs in malignant diseases help to selectively inhibit or eradicate CSCs[8].The isolation of cells by *in vitro* and *in vivo* self-renewal assays can be done by determination of ALDH activity, ability to efflux vital dyes, ability to form tumour spheres *in-vitro* and xenograft assays[43].

ALDEHYDE DEHYDROGENASE 1(ALDH1)

Aldehydes are widely found in nature and occurs in common things like plants, smog and smoke. The majority of aldehydes *in-vivo* are found as physiologically derived intermediates in the metabolism of other compounds. The effect of aldehyde includes cytotoxicity, mutagenecity, genotoxicity and carcinogenicity. Aldehyde dehydrogenases are involved in the oxidation of

aldehyde to carboxylic acid.ALDH is an NADP dependent enzyme and found in liver, stomach, kidney, eye and brain.

STRUCTURE

The active form of the enzyme is a dimer, consisting of two identical 452 residue subunits with overalldimensions of 90x60x40 Å. Each subunit contains an NADP binding, a catalytic and an arm-like bridging domain. The subunits in dimer are related by a pseudo two-fold symmetry. The dimer is stabilized by a total of 62 intra molecular hydrogen bonds.

ALDH POLYMORPHISM

Two main ALDH enzymes metabolize the acetaldehyde produced during ethanol oxidation ***(*Crabb *et al. 2004;* Hurley *et al. 2002)***ALDH1, is found in the fluid filling cytosoland is encoded by the ALDH1A1 gene, and ALDH2, which is found in the mitochondria is encoded by the ALDH2 gene. The ALDH1A1 gene extends over about 52 kb on chromosome 9, and ALDH2 extends over 43 kb on chromosome 12. Both genes have a similar structure with 13 exons. Moreover, the proteins they encode are 70 percent identical in sequence and are very similar in structure ***(*Hurley *et al. 2002).***

The cytosolic ALDH1 enzyme also contributes to the elimination of acetaldehyde, helping to control acetaldehyde levels even in people with the ALDH2 allele. Several promoter polymorphisms in the ALDH1A1 gene affect gene expression *in vitro****(*Spence *et al. 2003).***These alleles only occur at low

frequencies. **Ehlers *et al. (2004)*** observed in Southwest California Indians that people carrying an ALDH1A1 2 allele had lower rates of alcohol dependence and lower maximum number of drinks ever consumed in a 24-hour period. In contrast, **Hansell *et al. (2005)*** found that in an Australian community-based sample, ALDH1 enzyme activity in blood cells was not associated with alcoholism or the reaction to alcohol[44,45].

deMoraes *et al (2017)*analyzed the expression of CD24, CD44, CD133, ALDH1, CD29 (integrin-β1), and Ki-67 in squamous cell carcinoma of the oral cavity and oropharynx. Fifty-two tumours and 21 metastatic lymph nodes were evaluated by using immunohistochemistry and found that seven of 52 cases (13.5%) showed positive cytoplasmic staining of aldehyde dehydrogenase 1; integrin-β1 was expressed in 45 of 50 cases (90%); 30 of 52 cases (57.7%) had positive membranous staining of CD44; CD24 was expressed in 44 of 50 cases (88%); and three of 52 cases (5.8%) stained positively for membranous CD133. Median proliferation rate, measured by Ki-67, was 37.1% for tumours. Five-year cancer-specific survival rates for the CD44-negative and CD44-positive groups were 74% and 38%, respectively.The tumourigenecity of CD44 + cells in head and neck squamous cell carcinoma appeared to increase in cells which coexpress ALDH and that absence of ALDH1 was associated with tumours occurring in the oral cavity[43].

Custódio*et al(2018*) showed that ALDH1 cytoplasmic staining was invariable amongst the grades of epithelial dysplasia and between actinic

cheilitis (AC) and lip squamous cell carcinoma[46].**Clay *et al (2010)***collected six primary HNSCCs and isolated cells with high and low ALDH activity (ALDHhigh/ALDHlow). ALDHhigh and ALDHlow populations were implanted into NOD/ SCID mice and monitored for tumour development. He observed that ALDHhigh cells represented a small percentage of the tumour cells (1% to 7.8%). ALDHhigh cells formed tumours from as few as 500 cells in 24/45 implantations, whereas only 3/37 implantations of ALDH low cells formed tumours[47].**Tamatani *et al (2018)*** suggested that there was a significant association between ALDH1 expression, invasion, metastasis and disease-free survival rate[48]. **Wu *et al (2017)***demonstrated that ALDH1 was mainly expressed in the cytoplasm and its expression were significantly higher in OSCC than in normal oral mucosa tissue.

CD144(VE-CADHERIN)

Cadherins are calcium-dependent transmembrane proteins that are evolutionarily conserved and have two or more extracellular domains. Since many related molecules were cloned, cadherins constitute a superfamily and the original cadherins are now called as “classiccadherins”. Approximately twenty members of cadherins are included in the classic cadherin family depending on their domain structures.

STRUCTURE

The classic cadherins are subdivided into type I and type II. Type I cadherin contains a His-Arg-Val sequence in the N-terminal EC domain, and

other classic cadherins that do not contain the sequence are grouped into type II cadherin. The type I cadherin includes epithelial-cadherin (E-cadherin, CDH1), neural- cadherin (N-cadherin, CDH2), placental-cadherin (P-cadherin, CDH3). Vascular endothelial-cadherin (VE-cadherin, CDH5), osteoblast-cadherin (OB-cadherin, CDH11) belong to the type II cadherins[10].

Through its cytosolic tail,classical cadherins such as E- or N-cadherins and VE-cadherin recruits catenins. These accessory molecules, mainly β-and p120-catenins, bridge cadherin multimers to the actin cytoskeleton via actin binding proteins, among which are α-catenin, vinculin, and eplin. It has been recently observed that β-catenin dephosphorylation, together with VE-cadherin mobility, contribute to endothelial cell–cell junction stabilization. However, the role of β-catenin in the endothelial barrier remains complex, as this multifaceted protein is also an essential mediator of the Wntsignaling cascade, operating as a transcription factor in the nucleus. Thus, β-catenin may exert broader effects on gene expression and vascular plasticity, including barrier function[50,51].

FUNCTION

In addition to its adhesive functions, VE-cadherin regulates various cellular processes such as cell proliferation and apoptosis and modulates vascular endothelial growthfactor receptor functions. Consequently, VE-cadherin is essential during embryonic angiogenesis[51,52].

VE-CADHERIN EXPRESSION IN ORAL SQUAMOUS CELL CARCINOMAS

VE-Cadherin expressed by endothelial cells at cell-cell junction is one of the important adhesion molecules and the integrity of the endothelium is dependent on the. Its expression is correlated to the formation of Vasculogenic Mimicry (VM) channels in highly aggressive tumours and the tumour plasticity allows VM to occur. Four methods have been proposed for the migration of cancer cells through the endothelium.

- Cancer cells migrate through the endothelial cell body.
- Cancer cells induce endothelial cell apoptosis
- Cancer cells migrate through endothelial cell-cell junction without permanently destroying the endothelial cell layer.
- Cancer cells push the endothelial cells to deeper extracellular matrix to migrate

Elevated expression of VE-Cadherin is found in melanoma and breast cancer. The expression of VE-Cadherin by aggressive melanoma cells, serve as a vasculogenic switch. In triple-negative breast cancer, CD133+ cells cancer stem cells express higher levels of VE-Cadherin, to promote endothelial migration.Multi-factors may regulate the E-cadherin repression in oral carcinoma cells. Although germ-line mutation with the loss of heterozygosity is rare ***(*Saito *et al., 1998)***[53], epigenetic aberrations, including the promoter hypermethylation and expression of transcription repressors,

were commonly observed in an aggressive subset of OSCCs. The hypermethylation was detected in 35-85% of OSCCs ***(*Viswanathan *et al., 2003***[54],**Yeh *et al., 2002***[55]***)*** and prompts carcinoma cells to develop invasive tumours (**Nakayama *et al., 2001***[56]). **Kudo *et al. (2004)*** reported that the hypermethylation was observed in oral carcinoma cells at the invasive front but not in non-invasive areas[57].**Irani *et al(2018)*** evaluated the expression of CD144 in Oral squamous cell carcinoma and concluded that it plays an important role in angiogenesis and vasculogenic mimicry in OSCC , and therefore , it is involved in tumour progression and metastasis[11]. Another study by the same author (**Irani *et al., 2017)***, in Mucoepidermoid Carcinoma revealed that CD144 is associated with Vascular Mimicry (VM) formation, epithelial mesenchymal transition and microvessel count in OSCC[58].

Results

SAMPLE CHARACTERISTICS:

The study sample comprised of 37 formalin fixed paraffin embedded archival blocks. They were categorized into two groups. Group I (n=20) comprising of non- habit associated Oral Squamous Cell Carcinoma, Group II (n=17) comprising of normal mucosa. All the samples were analyzed for immunohistochemical expression of ALDH1 and CD144.

Distribution of age (Table 1 & Graph 1):

The age of patients was divided into 3 groups: 21 – 40 years, 41 – 60 years and those above 61 years. Group I consisted of 2 (10%) case in the age group of 21-40 years, 10 (50%) cases in the age group of 41-60 and 8 (40%) cases above 61 years. Group II consisted of 12 (70.5%) cases in the age group of 21-40 years, 2 (11.7%) cases in the age group of 41-60 and 3(17.6%) cases above 61 years. A significant difference was found with respect to age in the study groups (**p=0.000**)

Distribution of gender in the study groups (Table 2 & Graph 2):

In group I, 11(55%) were males and 9(45%) were females. In group II, 6(35.3%) were males and 11(64.7%) were females. However, no significant difference was found with respect to gender in the study groups (p=0.230)

Distribution of site of lesion in the study groups (Table 3 & Graph 3):

In Group I, 12(60%) from the tongue, 5 (25%) of cases from the buccal mucosa, 1 (5%) from palate, alveolar mucosa and commissure of the lip, no cases (0%) from the retromolar region and gingiva were taken. In Group II, 2 (11.7%) of cases from the retromolar region, 6(35.2%) cases from the buccal mucosa, 1 (5.8%) case from the alveolar mucosa, 8(47%) from gingiva and no cases (0%) from the tongue , palate and commissure of the lip were taken. A significant difference was found with respect to site of the lesion among the study groups (**p=0.000**)

Comparison of CD144 staining intensity of basal layer in the study groups (Table 4 & Graph):

In Group I, 19(95%) of the cases showed no staining in the basal layer, no case showed mild or intense staining, but 1(5%) case showed moderate staining in the basal layer. In Group II, 15(88%) of the cases showed no staining in the basal layer, 1(6%) showed mild and moderate staining while no cases showed intense staining. No significant difference was found with respect to CD144 staining intensity of basal layer among the study groups (p=0.744)

Comparison of CD144 staining intensity of supra basal layer in the study groups (Table 5 & Graph 5):

In Group I, 12 (60%) of cases showed no staining in the suprabasal layer, 7 (35%) of cases showed mild staining, 1 (5%) case showed moderate stain, while no cases showed intense staining. In Group II, 10 (58.8%) of cases showed no staining, 6 (35.2%) cases showed mild staining, 1 (5.8%) case showed moderate stain and no cases showed intense staining. No significant difference was found with respect to CD144 staining intensity of supra basal layer among the study groups**.** (p=0.999)

Comparison of CD144 staining intensity of connective tissue in the study groups (Table 6 & Graph 6):

In Group I, 15 (75%) of cases showed no staining in the connective tissue, 3 (15%) of cases showed mild staining, 1 (5%) case showed moderate stain and 1(5%) case showed intense staining. In Group II, 11 (64.7.%) of cases showed no staining, 2 (11.7%) cases showed mild staining, 3 (17.6%) case showed moderate stain and 1 (5.8%) case showed intense staining. No significant difference was found with respect to CD144 staining intensity of connective tissue among the study groups (p=0.663)

Comparison of ALDH1 staining intensity of basal layer in the study groups (Table 7 & Graph 7)

In Group I, all the cases;20 (100%) showed negative staining. In Group II, 16 (94.1%) of cases showed no staining of the basal layer, no cases (0%) showed neither mild nor intense staining, while 1 (5.8%) showed moderate staining. No significant difference was found with respect to ALDH1 staining intensity of basal layer among the study groups (p=0.750)

Comparison of ALDH1 staining intensity of supra basal layer in the study groups (Table 8 & Graph 8)

In Group I, 14 (70%) of cases showed negative staining, 6 (30%) of cases showed mild staining and no case showed moderate or intense staining of the supra basal layer. In Group II, 6 (35.2%) cases showed no staining, 10 (50.8%) cases showed mild staining, 1 (5.8%) showed moderate staining and no cases showed intense staining. Significant difference was found with respect to ALDH1 staining intensity of supra basal layer among the study groups (p=0.000)

Comparison of ALDH1 staining intensity of connective tissue in the study groups (Table 9 & Graph 9)

In Group I, 17 (85%) of cases showed negative staining, 3 (15%) of cases showed mild staining, no cases showed moderate or intense staining. In group II, 11(64.7%) of cases showed negative staining, 5(29.4%) of cases

showed mild staining, 1 (5.8%) showed intense staining and no cases showed moderate staining. No significant difference was found with respect to ALDH1 staining intensity of supra basal layer among the study groups (p=0.572)

Comparison of percentage of staining between CD144 and ALDH1 in group I (Table 10& Graph 10)

CD144 showed 10(50%) of positivity and 10(50%) were negative. 35% were positive for ALDH1 and 65% were negative. Significant difference was present between the percentage of staining between CD144 and ALDH1 in group I (p=0.337)

Comparison of percentage of staining between CD144 and ALDH1 in group II (Table 11& Graph 11)

CD144 showed 41.2% positivity and 58.8% were negative, while ALDH1 showed 64.7% positivity and 35.3% were negative. There was no significant difference was present between percentage of staining between CD144 and ALDH1 in group II (p=0.169)

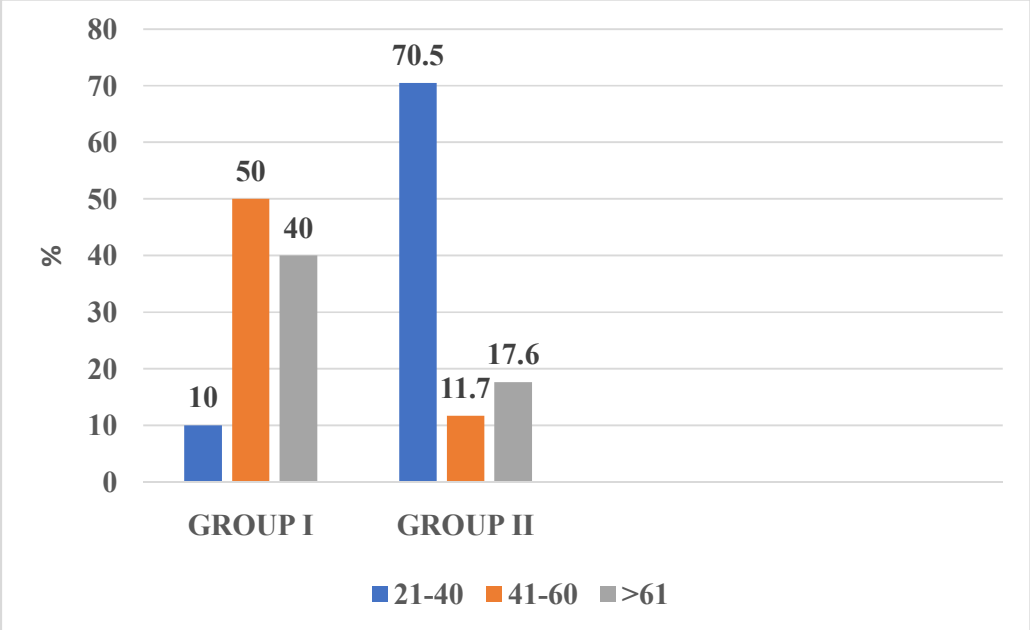
Tables and Graphs

TABLE 1: DISTRIBUTION OF AGE (N=37)

AGE GROUPS IN YEARS	GROUP I (n=20)	GROUP II (n=17)	pVALUE
21-40	2(10%)	12(70.5%)	0.000*
41-60	10(50%)	2(11.7%)	
>61	8(40%)	3(17.6%)	

*p value<0.05 is significant

GRAPH 1: DISTRIBUTION OF AGE (N=37)



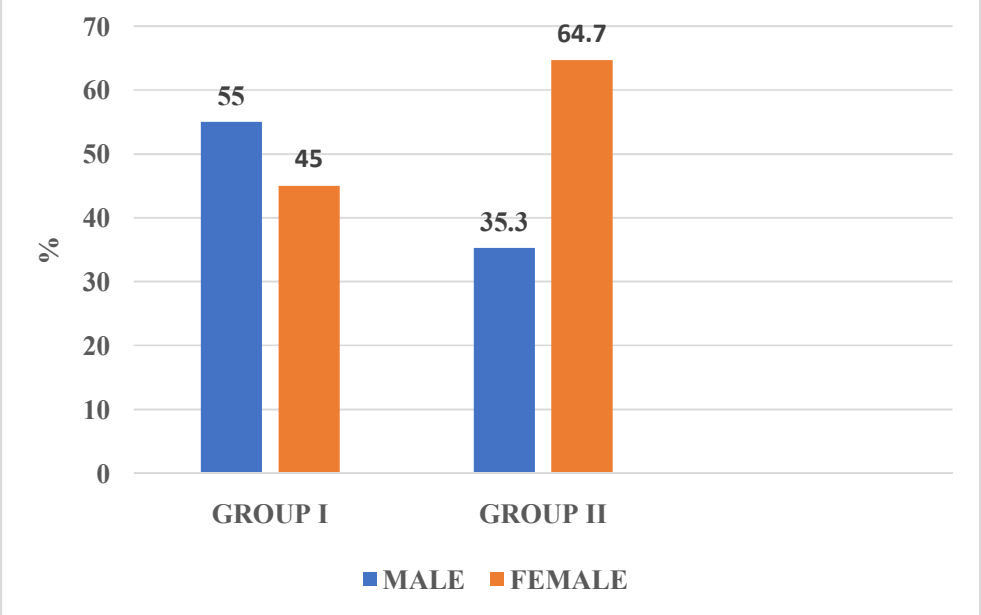
GROUP I – Non- habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

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

GENDER	GROUP I (n=20)	GROUP II (n=17)	p VALUE
MALE	11(55%)	6(35.3%)	0.230
FEMALE	9(45%)	11(64.7%)	

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



GROUP I – Non- habit associated Oral Squamous Cell Carcinoma

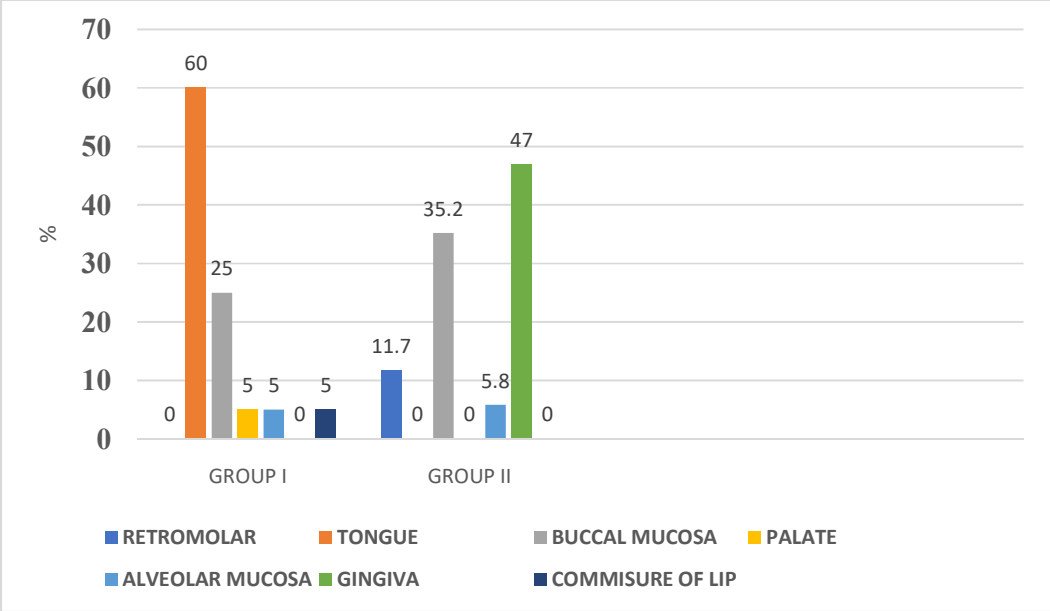
GROUP II – Normal mucosa

TABLE 3: DISTRIBUTION OF SITE OF LESION INTHE STUDY GROUPS (N=37)

SITE OF LESION	GROUP I (n=20)	GROUP II (n=17)	p Value
Retromolar region	0 (0%)	2 (11.7%)	0.000*
Tongue	12 (60%)	0 (0%)	
Buccal mucosa	5 (25%)	6 (35.2%)	
Palate	1 (5%)	0 (0%)	
Alveolar mucosa	1 (5%)	1 (5.8%)	
Gingiva	0 (0%)	8 (47.0%)	
Commisure of lip	1 (5%)	0 (0%)	

*p value<0.05 is significant

GRAPH 3: DISTRIBUTION OF SITE OF LESION IN THE STUDYGROUPS (N=37)



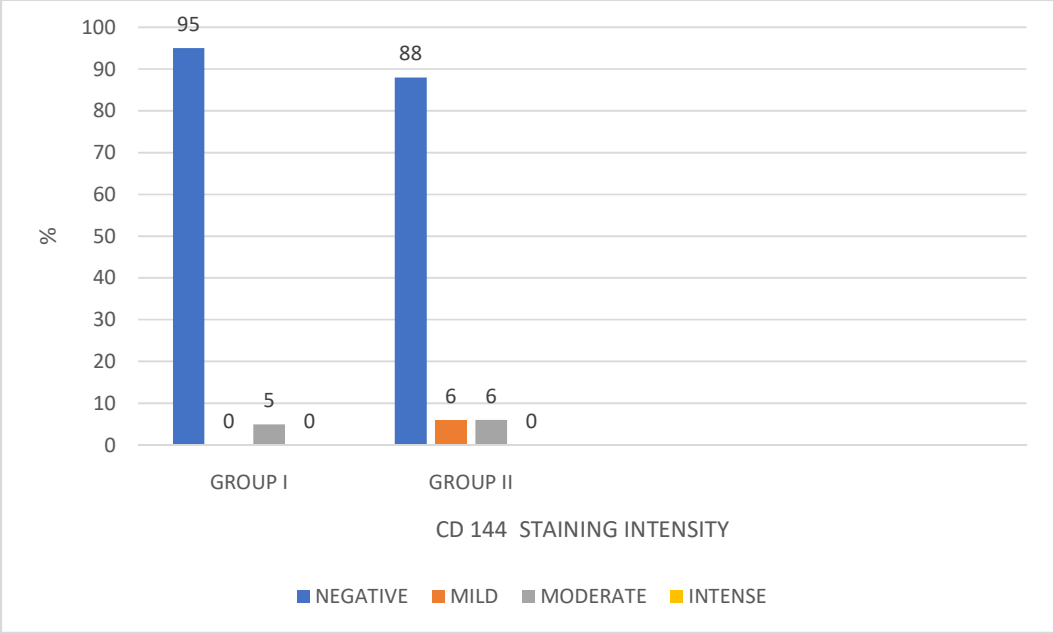
GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

TABLE 4: COMPARISON OF CD144 STAINING INTENSITY OF BASAL LAYER IN THE STUDY GROUPS (N=37)

CD144 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	19(95%)	15 (88%)	0.744
MILD	0 (0%)	1 (6%)	
MODERATE	1 (5%)	1 (6%)	
INTENSE	0 (0%)	0 (0%)	

GRAPH 4: COMPARISON OF CD144 STAINING INTENSITY OF BASAL LAYER IN THE STUDY GROUPS (N=37)



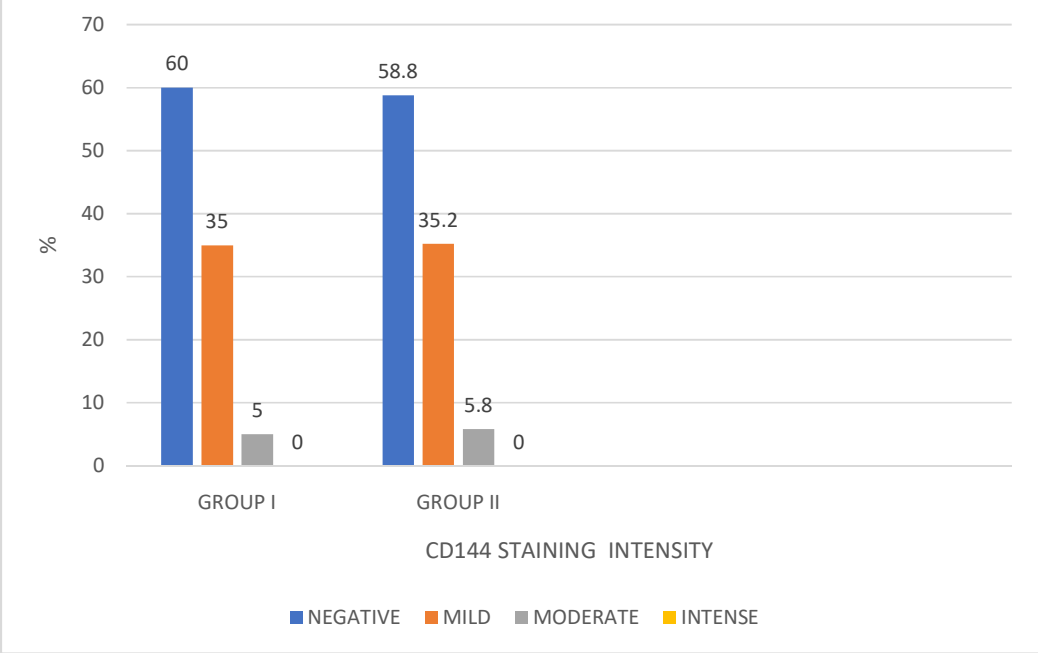
GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

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

CD144 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	12 (60%)	10 (58.8%)	0.999
MILD	7 (35%)	6 (35.2)	
MODERATE	1 (5%)	1 (5.8%)	
INTENSE	0 (0%)	0 (0%)	

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



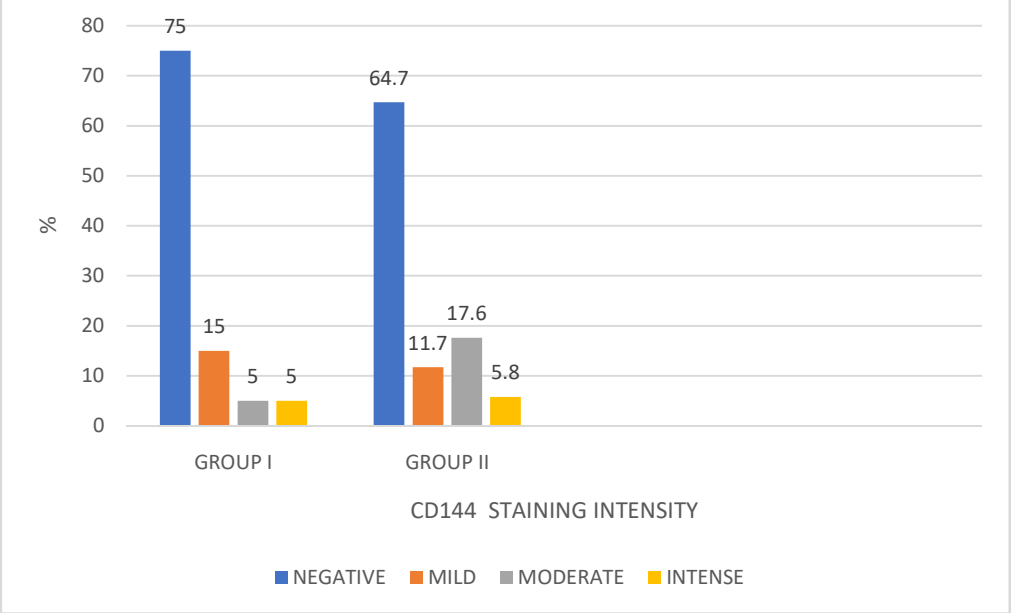
GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

TABLE 6: COMPARISON OF CD144 STAINING INTENSITY OF CONNECTIVE TISSUE IN THE STUDY GROUPS (N=37)

CD144 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	15 (75%)	11 (64.7%)	0.663
MILD	3 (15%)	2 (11.7%)	
MODERATE	1 (5%)	3 (17.6%)	
INTENSE	1(5%)	1 (5.8%)	

GRAPH 6: COMPARISON OF CD144 STAINING INTENSITY OF CONNECTIVE TISSUE IN THE STUDY GROUPS (N=37)



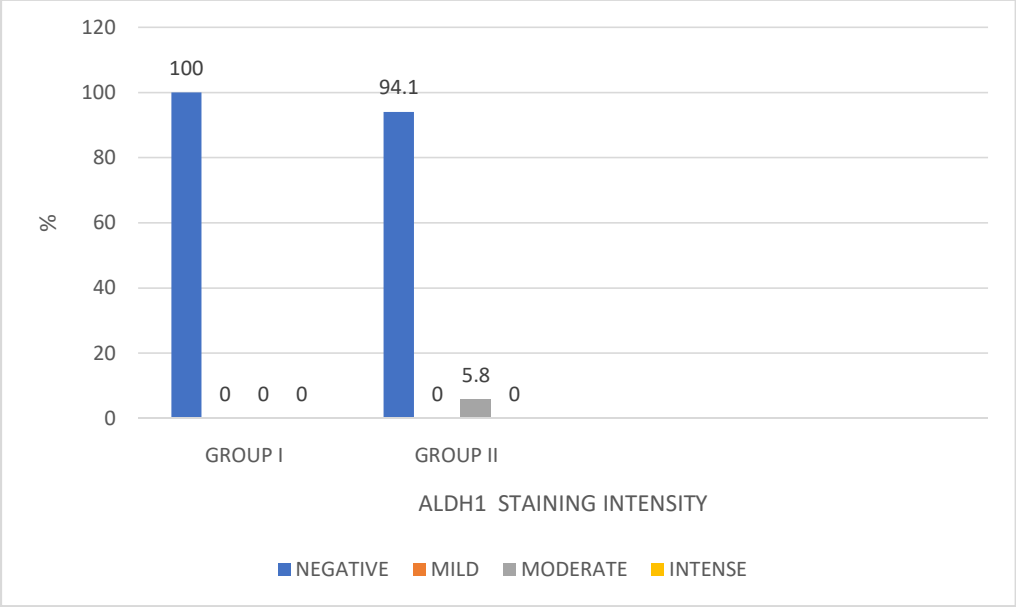
GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

TABLE 7: COMPARISON OF ALDH1STAINING INTENSITY OF BASAL LAYER IN THE STUDY GROUPS (N=37)

ALDH1 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	20 (100%)	16 (94.1%)	0.750
MILD	0 (0%)	0(0%)	
MODERATE	0 (0%)	1(5.8%)	
INTENSE	0(0%)	0(0%)	

GRAPH 7: COMPARISON OF ALDH1STAINING INTENSITY OF BASAL LAYER IN THE STUDY GROUPS (N=37)



GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

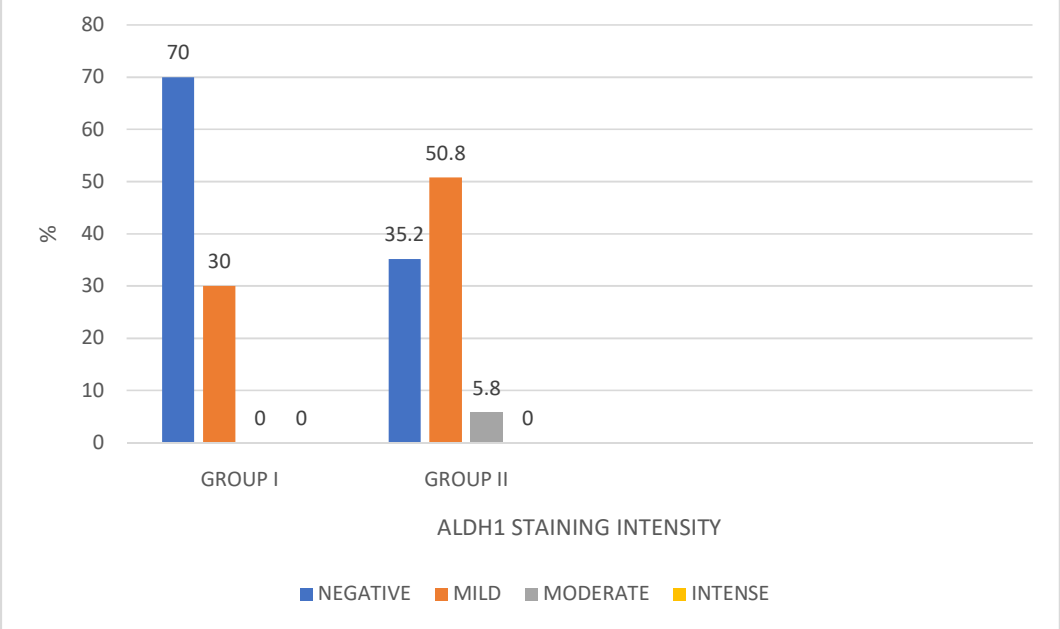
GROUP II – Normal mucosa

TABLE 8: COMPARISON OF ALDH1 STAINING INTENSITY OF SUPRA BASAL LAYER IN THE STUDY GROUPS (N=37)

ALDH1 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	14 (70%)	6(35.2 %)	0.000*
MILD	6 (30%)	10(50.8%)	
MODERATE	0(0%)	1(5.8%)	
INTENSE	0(0%)	0(0%)	

*p value<0.05 is significant

GRAPH 8: COMPARISON OF ALDH1 STAINING INTENSITY OF SUPRA BASAL LAYER IN THE STUDY GROUPS (N=37)



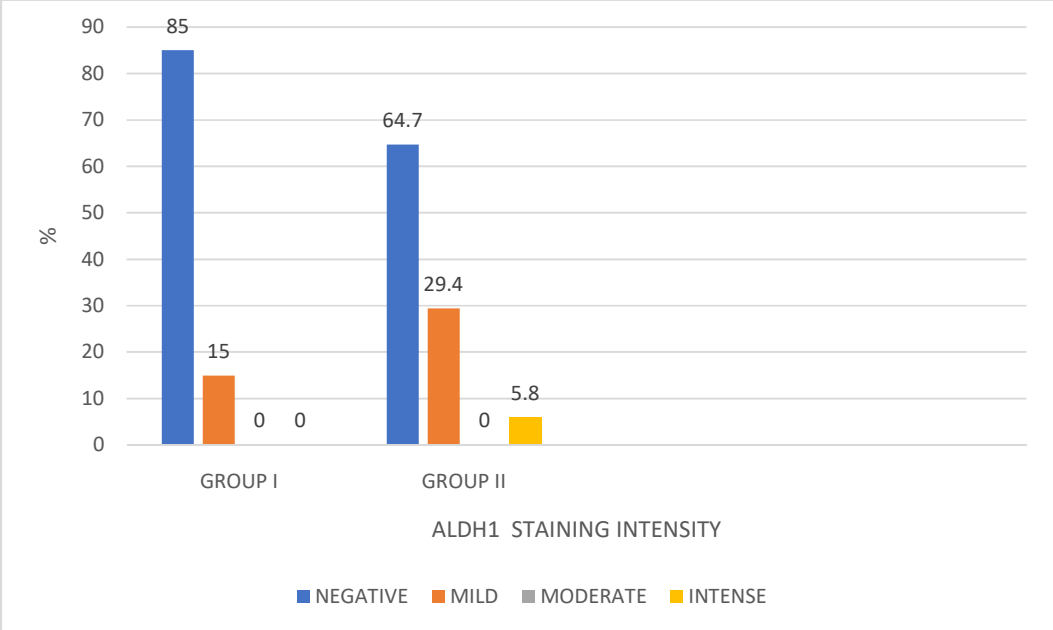
GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

TABLE 9: COMPARISON OF ALDH1 STAINING INTENSITY OF CONNECTIVE TISSUE IN THE STUDY GROUPS (N=37)

ALDH1 STAINING INTENSITY	GROUP I (n=20)	GROUP II (n=17)	p VALUE
NEGATIVE	17 (85%)	11(64.7%)	0.572
MILD	3 (15%)	5 (29.4%)	
MODERATE	0(0%)	0(0%)	
INTENSE	0(0%)	1(5.8%)	

GRAPH 9: COMPARISON OF ALDH1 STAINING INTENSITY OF CONNECTIVE TISSUE IN THE STUDY GROUPS (N=37)



GROUP I – Non-habit associated Oral Squamous Cell Carcinoma

GROUP II – Normal mucosa

TABLE 10: COMPARISON OF PERCENTAGE OF STAINING BETWEEN CD144 AND ALDH1 IN GROUP I(N=40)

	CD144 (n=20)	ALDH1 (n=20)	p VALUE
PRESENT	10 (50%)	7 (35%)	0.337*
ABSENT	10 (50%)	13(65%)	

*p value<0.05 significant

GRAPH 10: COMPARISON OF PERCENTAGE OF STAINING BETWEEN CD144 AND ALDH1 IN GROUP I(N=40)

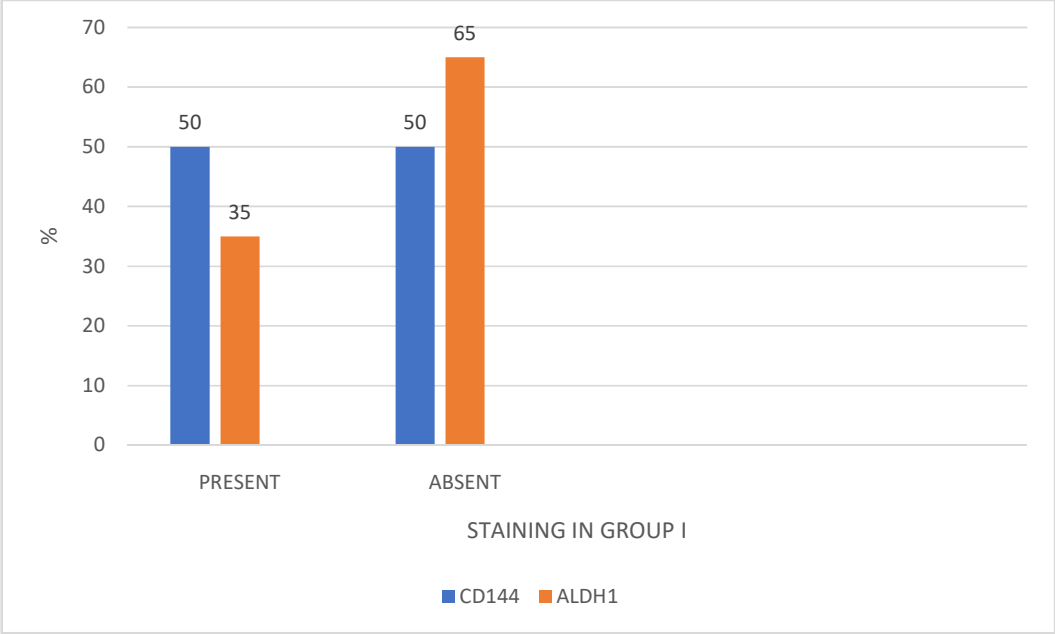
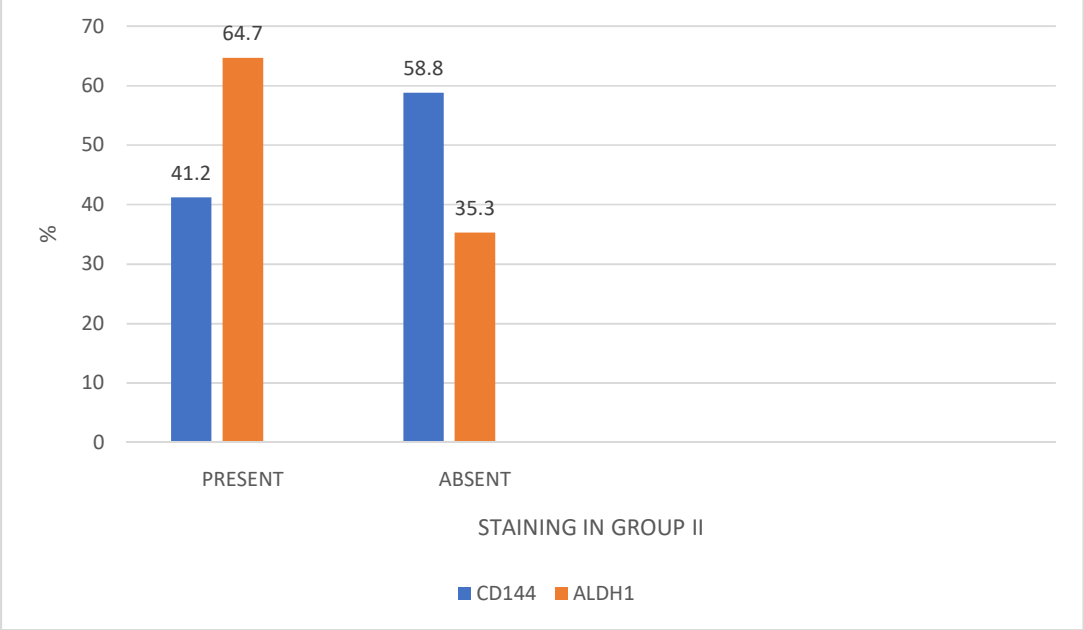


TABLE 11: COMPARISON OF PERCENTAGE OF STAINING BETWEEN CD144 AND ALDH1 IN GROUP II(N=34)

	CD144 (n=17)	ALDH1 (n=17)	p VALUE
PRESENT	7 (41.2%)	11 (64.7%)	0.169
ABSENT	10 (58.8%)	6(35.3%)	

GRAPH 11: COMPARISON OF PERCENTAGE OF STAINING BETWEEN CD144 AND ALDH1 IN GROUP II(N=34)

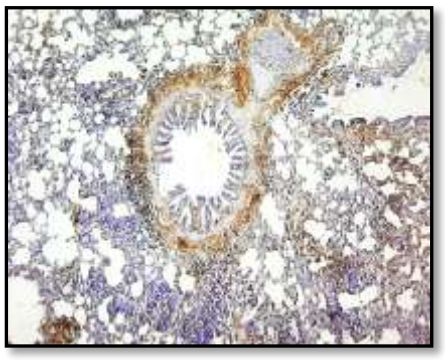


Photographs

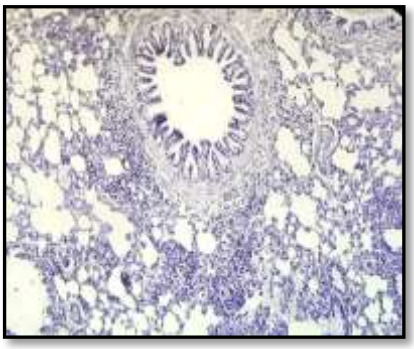
ARMAMENTARIUM

ANTIBODY KIT

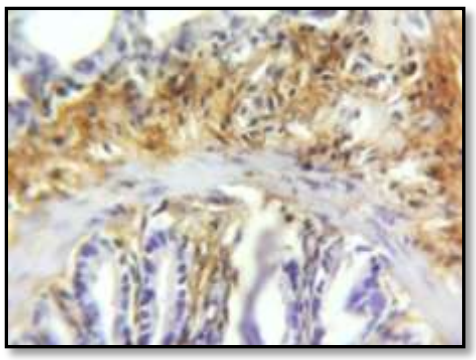
CD144 EXPRESSION IN CONTROL (RAT LUNG)



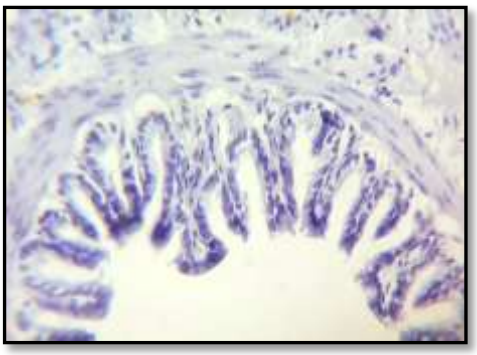
POSITIVE CONTROL 10X



NEGATIVE CONTROL 10X

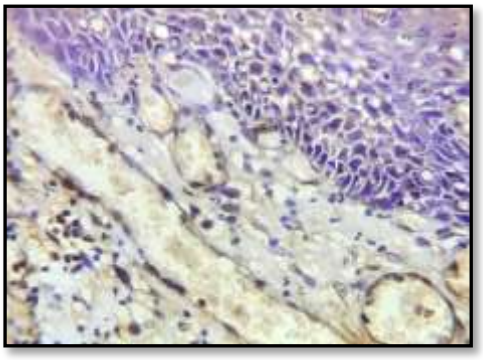


POSITIVE CONTROL 40X



NEGATIVE CONTROL 40X

CD144 EXPRESSION IN NON-HABIT ASSOCIATED OSCC



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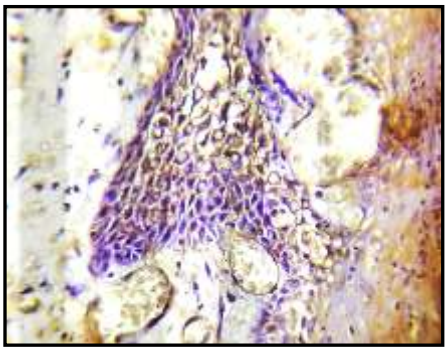
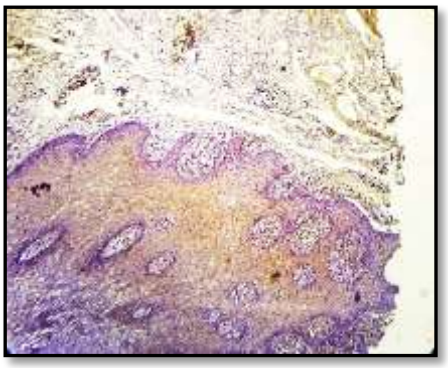


40X

CD144 EXPRESSION IN NORMAL MUCOSA



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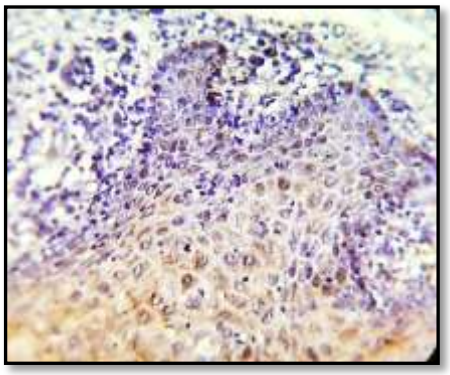
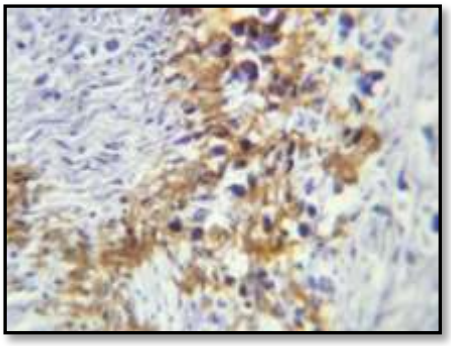
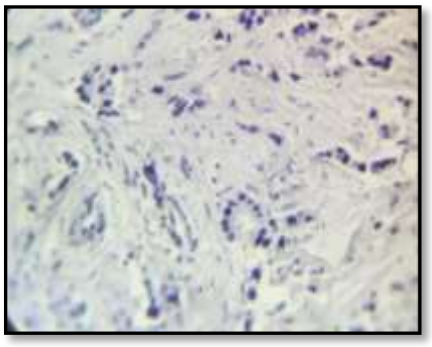


40X

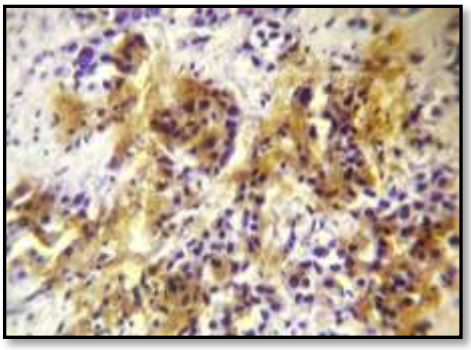
ALDH1 EXPRESSION IN CONTROL (BREAST CANCER)



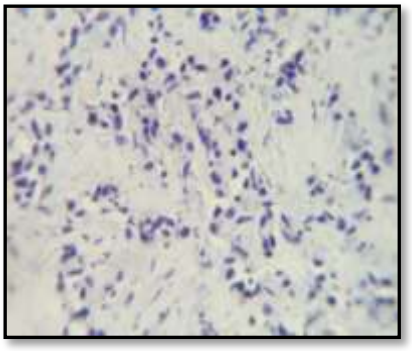
POSITIVE CONTROL 10X



NEGATIVE CONTROL 10X

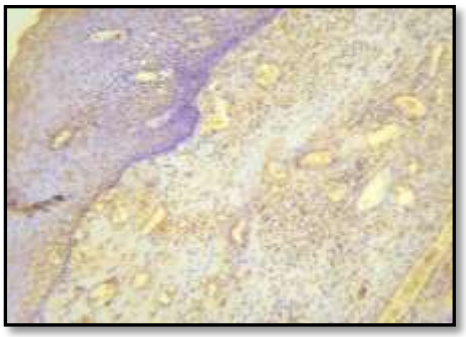


POSITIVE CONTROL 40X



NEGATIVE CONTROL 40X

ALDH1 EXPRESSION IN NON-HABIT ASSOCIATED OSCC



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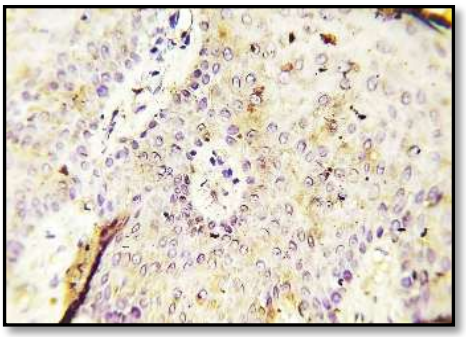

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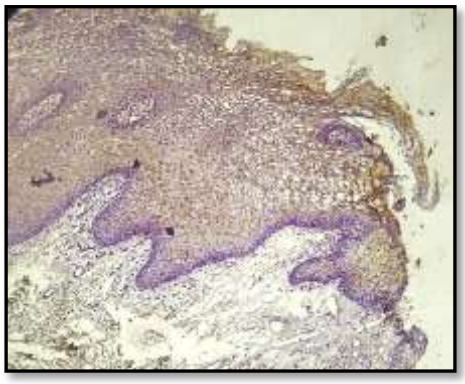
ALDH1 EXPRESSION IN NORMAL MUCOSA



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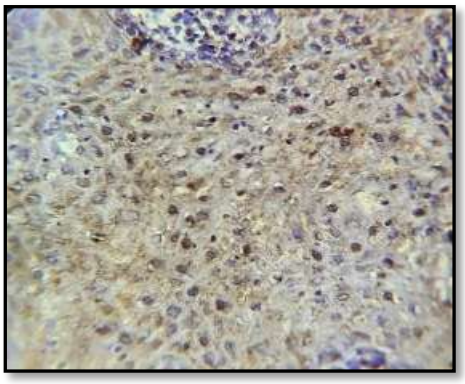

40X

Discussion

Cancer Stem Cells (CSC) which have been identified in oral squamous cell carcinoma have the ability for perpetual self-renewal, proliferation and produce downstream progenitor cells and cancer cells that drive tumour growth[59]. The resistance of CSC to conventional antineoplastic therapies contribute to poor prognosis and recurrence of cancers. The intact tumour stem cell niche can contribute to recurrence.The study of CSC markers is important to understand carcinogenesis,personalized treatment, manage recurrence and prognosis[60,61].

Aldehyde dehydrogenase 1 (ALDH1)has been implicated in cancer pathogenesis. It is a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid and plays a role in early differentiation of stem cells[62].ALDH1 is located in the cytoplasm and mitochondria. It has been identified in various cancers including glioblastoma and breast cancer, in which it was determined as a predictive marker of worse prognosis[63]. CD144 which is Vascular Endothelial (VE)-cadherin, an endothelial-specific cell-cell adhesion protein is responsible for sustaining intercellular adherens junctions in the vascular endothelium and modulate endothelial permeability[64].

This study was done to assess the immuohistochemical expression of ALDH1 and CD144 in non-habit associated Oral Squamous Cell Carcinoma and compare it with that of normal mucosa.

In this study, in Group I(non-habit associated Oral Squamous Cell Carcinoma)10% of cases were in the age group of 21-40 years, 50%in the age

group of 41-60 and 40%above 61 years. With respect to normal mucosa in Group II, 70.5%werein the age groupof 21-40 years, 11.7%in the age group of 41-60 and 17.6% above 61 years(p-0.00).

The present study demonstrated a male predominance (55%) in OSCC which is in contrast to Group 2, which had a female predominance (64.7%)(p-0.230). The results were concurrent with that of the study by **Gotz *et al*** whose results showed a malepredominance in OSCC study group(76.3%)[63].

In Group I, tongue was the frequent site of OSCC (60%) followed by 25% of cases from the buccal mucosa, 5% of cases from the palate, alveolar mucosa and commissure of the lip.This finding was concurrent with the study of **Pires FR *et al*** who reported that the most common site of OSCC was the lateral border of tongue[65].This is also was consistent with the study by **Chen YJ *et al*** in which 35% of OSCC was reported on the lateral border of tongue[66]. In Group II, 11.7% of cases were taken from the retromolar region, 35.2% cases from the buccal mucosa, 5.8% case from the alveolar mucosa and 47% from the gingiva.

STAINING INTENSITY OF CD144

On comparing CD144staining intensity in the basal layer, between normal mucosa (Group II), non-habit associated OSCC (Group I) did not express CD144 (19/20 cases).In the supra basal layer,there was no significant difference in the staining intensity among the study groups. In the connective tissue intense staining was expressed in one case in both OSCC (1/20) and in

normal mucosa (1/20). The connective tissue also showed positively stained vascular channels, cells around the vascular channels and keratin pearls. A similar observation was made by**Irani *et al,*** who studied VE-Cadherin expression in Mucoepidermoid carcinoma (MEC) and found evidence of VE-Cadherin positivity invascular channels and detached cells around the vessels[52].**Tang *et al*** , found that Vasculogenic Mimicry (VM) can be inhibited by targeting VE-Cadherin in esophageal cancers[67].

In the present study, CD144 expression was higher in OSCC (50%) when compared with that of normal mucosa (41.2%). This is in accordance with the study by **Irani *et al,*** who found a higher expression of VE-Cadherin in Oral Squamous Cell Carcinoma. He also suggested that there was a definite relationship between VE-cadherin expression levels, vasculogenic mimicry (VM) formation, Epithelial Mesenchymal Transition (EMT) and CSCs in OSCC[58]. This could possibly be due to*P.gingivalis* which causes proteolytic disruption and cleavage of adherence junction proteins resulting in detachment of endothelial cells[68].

VE-Cadherin is expressed by CSCs and is associated with Vasculogenic Mimicry (VM) which plays an important role in tumour progression and metastasis. Overexpression of VE-Cadherin is associated with poor prognosis in cancers such as melanoma and breast cancer.Endothelial cells at the cell-cell junction express VE-Cadherin and is one of the key adhesion molecules for maintaining the integrity of the endothelium. So,

cancer cells affect VE-Cadherin at early stages of transmigration and provide an easier route for migration.**Hendrix *et al,*** demonstrated melanoma cells expressing VE Cadherin exclusively associated with endothelial cells[69]. However, VE-Cadherin was not expressed by endothelial cells along the borders withcancer cells. This suggests that the endothelium may detach and/or be displaced by cancer cells.

STAINING INTENSITY OF ALDH1

On comparing the staining intensity of ALDH1,in the basal layer among the study groups, normal mucosa expressed CD144 (5.8%), but OSCC tissues did not express ALDH1. There was a statistically significant difference (p value- 0.00) in the staining intensity in thesuprabasal layer among the study groups. In the connective tissue, blood vessels, keratin pearls and muscle tissue were stained. **Tamatani*et al***, reported that ALDH1 expression was absent in keratin pearls and that the positive expression of ALDH1 was mainly localized in the invasive front and that the ALDH1 positive cells were scattered[48]. This could be because**Tamatani *et al*** used monoclonal rabbit anti-human ALDH1 in contrast to our study where polyclonal rabbit anti-human ALDH1 was used.

When compared to normal mucosa (64.7%), the ALDH1 expression in OSCC (35%)was low. A similar finding was reported by **de Moraes *et al***whoshowed absence of ALDH1 in tumours occurring in the oral cavity[43].In contrast**Wu *et al,*** who examined ALDH1 levels in Oral Squamous Cell

Carcinoma found that ALDH1 expression was significantly higher in OSCC than in normal mucosa[49].

ALDH1 expression in OSCC correlates with the number of cells undergoing epithelial mesenchymal transition and metastasis. When oral dysplastic epithelium is positivefor ALDH1, the risk of malignant transformation is higher. It has been suggested that the risk is better predicted by the presence of ALDH1 expression rather than histological grading[46].

However, the present study exhibited higher percentage of positive ALDH1 expression in normal mucosa than in OSCC. Use of tobacco constitutes a primary factor for the development of OSCC. Alcohol consumption is associated with cell hyper proliferation, production of metabolites with carcinogenic action, such as acetaldehyde,induction of enzymes that activate procarcinogens and reduction of retinoic acid. Synergistic effect of exposure to tobacco and alcohol is associated with a higher risk of developing cancer. ALDH1 catalyzes the conversion of acetaldehyde to acetic acid. Since, the OSCC samples examined in the present study were non-tobacco users and non-alcoholics, the low levels of ALDH1 expression can be possibly due to the lower levels of acetaldehyde during alcohol metabolism[70]. It can also be due a distinct pathological pattern and biological behavioural differencebetween smokers and alcoholics when compared to non-smokers and non-alcoholics[6].

CSCs represents the critical subset within the tumour mass in perpetuating the tumour, even aftereffective therapy and leads to tumour aggression. Thus, CSC markersare important to understand carcinogenesis and to plan appropriate therapy.

Although both ALDH1 and CD144 are cancer stem cell markers, there was altered expression pattern of these markers in basal, suprabasal and connective tissue of non-habit associated Oral Squamous Cell Carcinoma compared to normal mucosa.

When comparing the expression of CD144 and ALDH1 between the two groups, CD144 was increased in OSCC (Group I) and decreased in normal mucosa (Group II). ALDH1 was increased in normal mucosa and decreased in OSCC. The increased expression of CD144 in OSCC can be attributed to *P.gingivalis,*which causes proteolytic disruption and cleavage of adherence junction proteins resulting in detachment of endothelial cells.The connective tissue also showed positively stained vascular channels, cells around the vascular channels and keratin pearls suggesting that CD144 is associated with Vasculogenic Mimicry. The decreased expression of ALDH1 in OSCC can be due to the lower levels of acetaldehyde during alcohol metabolism in non-alcoholics and the biological behaviour difference between smokers and alcoholics when compared to non-smokers and non-alcoholics. However, a conclusive evidence on the efficacy of these CSC markers can be determined by a larger sample size.