ANALYSIS OF CD44 EXPRESSION IN ORAL CANCER TISSUE

A THESIS SUBMITTED TO DEPARTMENT OF LIFE SCIENCES FOR PARTIAL FULFILLMENT OF THE M.Sc. DEGREE IN LIFE SCIENCE

By

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DEPARTMENT OF LIFESCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA

2014

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Date: 10/05/14

CERTIFICATE

This is to certify that the thesis entitled **"Analysis of CD44 expression in oral cancer tissue"** which is being submitted by Ms. Supriya Dehury, Roll No-412LS2036, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

SK Bhutia
Sujit K Bhutia

DECLARATION

I do hereby declare that the Project report entitled **"ANALYSIS OF CD44 EXPRESSION IN ORAL CANCER TISSUE"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bona fide and original research work carried out by me under the guidance and supervision of **Dr. Sujit Kumar Bhutia**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: 10th May 20114 Supriya Dehury

Place: NIT, Rourkela

ACKNOWLEDGEMENT

It gives me immense pleasure to express my deep sense of gratitude to the almighty **God** for His blessings. There goes a popular maxim "other things may change us, but we start and end with family." I would like to thank my **parents** for their numerous sacrifices and ever increasing unconditional love for me.

I avail this opportunity to extend my hearty indebtedness to my supervisor **Dr. Sujit Kumar Bhutia** for his invaluable guidance, motivation, constant inspiration and above all for her ever co-operating attitude that enabled me in bringing up this thesis in the present form.

I would also like to convey my deep regards to **Dr. Samir Kumar Patra**, Head of the Department of Lifescience for providing all kinds of possible help and advice during the course of this work.

I would also like to thank to **Prajna Paramita Naik, Niharika Sinha, , Durgesh Nandini Das, Prashanta Kumar Panda, Subhadip Mukhopadhyaya,** for their encouragement and guidance throughout the work.

I sincerely thanks to all the staff members of the Department of Lifescience and all my well wishers, classmates and friends for their inspiration, help and having faith on me.

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CONTENTS

LIST OF ABBREVIATIONS

Csc-cancer stem cell

Esc-Embryonic stem cell

Hsc-Haematopoetic stem cell

Mscs-Mesenchymal stem cell

Nscs-Neural stem cell

Oct-4- Octamer-binding transcription factor

SSEAs -Stage Specific Embryonic Antigens

ABCG2: ATP-binding cassette superfamily G member 2

Sca-1: Stem cell antigen 1

PSA-NCAM :Polysialic acid-neural cell adhesion molecule

p75 NTR: p75 Neurotrophin R

CD-Cluster Differention

IHC: Immunohistochemistry

EBV: Epstein-Barr virus

HPV: Human papillomavirus

LIST OF FIGURES

ABSTRACT

Oral cancer is the sixth most frequently occurring cancer worldwide. The recurrence of oral cancer purported to Cancer Stem cells. Prospective identification, characterization, and isolation of markers is an important aspect in the potential identification and isolation of CSCs. CD44, a hyaluronic acid receptor, is one of the most commonly studied surface markers, which is expressed by almost every tumour cell. The expression of CD44 in oral cancer tissue was analysed by using immunohistochemical technique. Three different groups of oral cancer tissue were taken i.e. well differentiated, moderately differentiated, & poorly differentiated. The poorly differentiated tissue have high metastasis properties so the expression of the marker is very high on those tissues as compare two other two. From the IHC study we found that the CD44 stem cell marker is highly expressed in poorly differentiated oral cancer tissue, moderately expressed in moderately differentiated tissue and the expression profile is relatively low in well differentiated oral cancer tissue.

Key Words: Cancer Stem Cells, Stem cell marker, CD44, Immunohistochemistry

CHAPTER-1

1. INTRODUCTION

Growth of cancerous tissue in oral cavity causes oral cancer. It is also called as mouth cancer. The term oral cancer refers to a diverse group of tumors growth from lip, cheek, floor of the mouth, hard and soft palate, tongue, pharynx and oral cavity. Oral cancer is the sixth most frequently occurring cancer worldwide.In United States, about 29 000 people suffer cancer every year. Oral cancer alone accounts for 270 000 new cases annually and 145 500 annually, the majority of which occur in developing countries. The mortality rates due to oral cancer have been increased significantly in developing countries in past few years (Day et al., 1984; WHO, 1992). The countries reported to be most affected by oral cancer are India, Pakistan, Bangladesh, Honk Kong, Singapore, and Phoillipines (Malaowalla et al., 1976). Squamous cell carcinoma (SCC) has been identified as the most common type of oral cancer till date. It alone accounts for about 90% of the total oral cancers (Walker et al., 2003). Although, several surgical techniques and adjuvant therapies have been developed for the diagnosis, the prognosis of patients with OSCC remains poor. The main reasons for serious delay in the diagnosis of the oral cancer are misdiagnosis, ineffective treatment, problems with access to healthcare professionals, and unawareness of the specific biomarkers of OSCC (Brouha et al 2005;Scott et al). Early detection of oral cancer is believed to be the more effective way to improve survival. These cancers are largely amendable to curative surgery or radiotherapy, with or without concurrent chemotherapy, when diagnosed at an early stage.

The etiology of tongue SCC is well described. Tobacco use, heavy alcohol consumption, poor nutrition, immunocompromised health states and viral infections have all been implicated in the carcinogenesis of squamous cell carcinoma of the tongue. Of these risk factors, tobacco and alcohol consumption are thought to account for more than 75% of oral tongue cancers.Oral cancer results from a variety of factors that operate over time and is dependent on each person's unique response to these factors. The 2 most important modifiable risk factors for oral cancer are tobacco and alcohol consumption. Up to 75% of oral cancers may be attributed to exposure to tobacco or alcohol. The most commonly implicated viruses in oral cancer transformation have been the human papillomavirus (HPV), herpes group viruses, and the adenoviruses. HPV and herpes have been the most thoroughly studied and are now considered to be the most likely "synergistic viruses" involved in human oral cancer. The herpes viruses most often linked to oral cancer are the Epstein-Barr virus

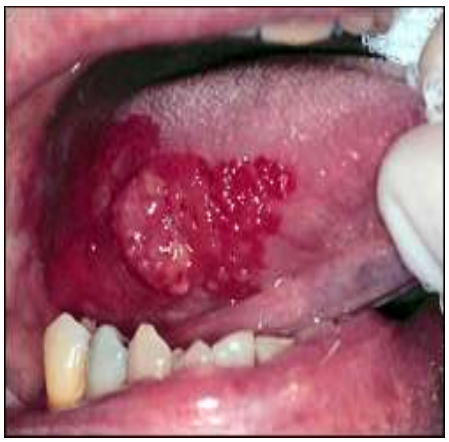
(EBV) and cytomegalovirus (CMV); both EBV DNA and CMV DNA have been demonstrated in oral carcinomas.

1.1 SYMPTOMS:

Common symptoms of oral cancer include:

- Patches inside mouth or on lips that are white, a mixture of red and white, or red White patches (leukoplakia) are the most common. White patches sometimes become Malignant.
- Mixed red and white patches (erythroleukoplakia) are more likely than white patches to become malignant. Red patches (erythroplakia) are brightly colored,
    - Bleeding in mouth.
    - Loose teeth
    - Difficulty in swallowing
    - Difficulty wearing dentures
    - A lump neck
    - Difficulty in moving tongue or jaws

LEUKOPLAKIA



ERYTHROPLAKIA

Fig1.Leukoplakia and Erythroplakia

1.2 RISK FACTORS

1.2.1. Tobacco

The risk of oral cancer and premalignant lesions increases with the amount of tobacco consumed. This increased risk holds for all types and uses of tobacco, whether it is smoked as a cigarette, cigar, pipe or bidi (a small, hand-rolled cigarette commonly used in Asia), or used smokeless as a chew, plug or snuff. Stopping smoking reduces the risk of oral cancer and premalignant lesions, although it may take 10 to 20 years for a former smoker's risk to reduce to that of a nonsmoker. Patients from South Asian countries may also chew betel quid or paan, a common habit in their culture. Betel quid is a carcinogenic complex mixture of plant components that frequently contains tobacco.

1.2.2 Alcohol

Alcohol consumption is also a strong risk factor for oral cancer and premalignant lesions. The risk increases with increased consumption and duration of use of alcohol. Typically, one 8-ounce glass of beer, one 4-ounce glass of wine and 1 ounce of spirits have equal amounts of alcohol. Again, the risk of oral cancer decreases when alcohol is no longer consumed. But it takes many years for a drinker's risk to reduce to that of someone who has never been a drinker. Tobacco and alcohol consumption work together synergistically, increasing the risk of oral cancer to more than 30 time that of those who do not smoke or drink. Heavy drinkers and smokers are also more likely to be diagnosed with late-stage disease. Ceasing to use tobacco and alcohol greatly reduces the risk of developing oral cancer and premalignant lesions.

1.2.3 Human Papilloma Virus

Human papilloma virus (HPV) is a strong risk factor for oral cancers, especially when the lingual and palatine tonsils, the soft palate and the base of the tongue are involved. There are more than 120 types of HPV, but only a few are high-risk factors for oral cancer, primarily HPV- 16 and HPV-18. Over 90% of HPV-positive oral cancers are HPV-16 positive. Since risk factors for HPV infection include having a large number of sexual partners and first intercourse at a younger age, changing sexual practices in our society may increase the effect of HPV infection on the development of oral cancers and premalignant

lesions, especially in younger adults. The combination of smoking and HPV infection and of alcohol and HPV infection may have an additive effect.

1.2.4 Diet and Vitamins

A diet rich in fruits and vegetables, particularly fruit, reduces the risk of oral cancer and premalignant lesions. Several studies have shown that higher levels of vitamin C or carotene consumption reduce the risk of oral cancer.

1.3 TREATMENT

Oral cancer treatment may include surgery, radiation therapy, and chemotherapy

1.3.1 Surgery

Surgery to remove the tumor in the mouth or throat is a common treatment for oral cancer.

1.3.2 Radiation Therapy

Radiation therapy also called radiotherapy is a type of local therapy. It affects cells only in the treated area. Radiation therapy is used alone for small tumors or for patients who cannot have surgery. It may be used before surgery to kill cancer cells and shrink the tumor. It also may be used after surgery to destroy cancer cells that may remain in the area. Radiation therapy uses high-energy rays to kill cancer cells. Doctors use two types of radiation therapy to treat oral cancer.

• **External radiation**: The radiation comes from a machine. Patients go to the hospital or clinic once or twice a day, generally 5 days a week for several weeks.

• **Internal radiation** (implant radiation): The radiation comes from radioactive material placed in Seeds, needles, or thin plastic tubes put directly in the tissue. The patient stays in the hospital. The Implants remain in place for several days. Usually they are removed before the patient goes home. Some people with oral cancer have both kinds of radiation therapy.

1.3.3 Chemotherapy

Chemotherapy (chemo) is a technique that involves the use of anti-cancer drugs that are administered into a vein or taken by mouth. These drugs enter the bloodstream and can reach cancer that has spread to organs beyond the head and neck.

It is used in several ways i.e.

- Chemo (typically combined with radiation therapy)
- Chemo (combined with radiation therapy, called adjuvant chemotherapy) - given after surgery to kill any small deposits of cancer cells that may have been left behind.
- Chemo (sometimes with radiation, called neoadjuvant or induction chemotherapy) used to try to shrink some larger cancers before surgery.
- Chemo (with or without radiation) can be used to treat cancers that are too large or have spread too far to be removed by surgery.

The chemo drugs used most often for cancers of the oral cavity and oropharynx are: Cisplatin, 5-fluorouracil (5-FU), Carboplatin, Paclitaxel (Taxol), Docetaxel (Taxotere), Methotrexate, Ifosfamide (Ifex), Bleomycin .Combining drugs shrink tumors more effectively. A commonly used combination is cisplatin and 5-FU which is more effective than either drug alone in shrinking cancers of the oral cavity and oropharynx. Chemo is often given at the same time as radiation (known as chemoradiation). Cisplatin alone is usually the preferred chemo drug when given along with radiation. Some doctors prefer to give the radiation and chemo before surgery. However, the side effects can be severe and may be too much for some patients.

CHAPTER-2

2. LITERATURE REVIEW

2.1 CANCER STEM CELL

Cancer stem cells are subset of cancer cells which have self renewal capacity and contain tumor. It is also called as tumor initiating cells (TIC). The cancer stem cells were first demonstrated in acute myelogenous leukemia (AML) in 1994 by John Dick and colleagues. Cancer stem cells have been characterized in many different types of human tumors like brain tumors, multiple myeloma, colon cancer, prostate cancer, head and neck cancer, melanoma, hepatocellular carcinoma (HCC), pancreatic cancer and lung cancer. Cancer stem cells originate from normal stem cells and it has multiple mutations. Cancer stem cells have de-differentiation and transdifferentiation process. The difference between "stem cells" and "cancer stem cell" is that "cancer stem cells" as "tumor initiating cells" have high tumorigenicity and self-renewal ability. Currently, three methods are most used to isolate cancer stem cells. These methods include flow cytometry sorting based on surface marker expression, sorting as side population (SP), and sphere culture.

There are three main characteristics to identify CSCs: (1) Differentiation, which provides the ability to give rise to a heterogeneous progeny, (2) Self-renewal capability that maintains an intact stem cell pool for expansion, (3) Homeostatic control that ensures an appropriate regulation between differentiation and self renewal according to the environmental stimuli and genetic constraints of each organ tissue, which accounts for the tissue specificity of CSCs. Cancer stem cells divide asymmetrically creating two different cell populations. One population retains the self-renewing properties of the parental cancer stem cell and the other population is tumor cell with ability to differentiate but without the ability to initiate tumor growth.

2.2 STEM CELL MARKER

Stem cells are capable of generating a huge number of mature cells through a sequential procedure of proliferation and differentiation, at the same time retaining its ability to self-renew to maintain the stem cell pool. In a study conducted by Judd et al (2011), the researchers have tried to get an insight into the OSCC disease. Stem cell markers are the type

of genes and the protein products are used to isolate and identify stem cell."Stem cell marker" should identify a multipotent single cell capable of recapitulating the heterogeneity of the tumor. It has self-renewal capacity; it should have the ability to divide and give rise to an exact copy of itself.

There are four different types of stem cell marker.

1. Embryonic stem cell marker

2. Hematopoietic stem cell marker

3. Mesenchymal/ stromal stem cell marker

4. Neural stem cell marker

2.2.1 Embryonic stem cell marker:

Embryonic stem cell is derived from Embryo. There are different types of ESC markers i.e.Oct-4, SSEA.

2.2.1.1 Oct-4 (octamer-binding transcription factor 4):

It identify as a DNA-binding protein that activates gene transcription via a cis-element which containing the octamer motif (Scholer et al., 1990). It is POU transcription factors which expressed in totipotent embryonic stem cell and germ cells (Rosner et al., 1990) . Oct-4 expression is associated with an undifferentiated phenotype and tumors cell An Oct-4 expression is required to sustain stem cell self-renewal capacity and pluripotency. Oct-4 is not only a master regulator of pluripotency but is also the first and most recognized marker used for the identification of totipotent ES cells.

2.2.1.2 SSEAs (Stage Specific Embryonic Antigens):

SSEAs are identified by three monoclonal antibodies associated with lacto- and globo-series glycolipid . There are different types of SSEAs i.e. SSEAs-1, SSEAs-3, SSEAs-4.SSEAs-1 is expressed on the surface of preimplantation-stage of the embryos and has been found on the surface of teratocarcinoma stem cells. The oviduct epithelium, endometrium and epididymis, as well as some areas of the brain and kidney tubules in adult mice have also

been shown to be reactive with SSEA-1 Abs. SSEA-3 and -4 are synthesized during oogenesis and are present in the membranes of oocytes, zygotes and early cleavage-stage embryos.

2.2.2 Hematopoietic stem cell markers

Hematopoietic Stem Cells are morphologically similar to white blood cells (WBCs). There are different types of HSC markers i.e. CD34, CD133, ABCG2, SCA-1**.**

2.2.2.1 CD34:

The cell surface CD34 has been expressed on a small fraction of human bone marrow cells. There are two different types of CD34 marker are there i.e. $CD34^+$ & $CD34^-$.The $CD34^+$-enriched cell population from marrow is responsible for most of the hematopoietic activity. Therefore CD34 has been considered to be the most critical marker for hematopoietic stem cells (HSCs). CD34 expression on primitive cells is down-regulated as they differentiate into mature cells. Although, CD34 plays a significant role in early hematopoiesis, Osawa et al. first demonstrated that CD34 HSCs could be negative. Human $CD34^-$ cells have low level of engraftment and hematopoietic capacity. $CD34^-$ cell population have repopulating activity in fetal sheep. Both murine and human $CD34^+$ cells may be derived from $CD34^-$ cells. The possibility of HSCs may be $CD34^+$ or $CD34^-$ and expression of CD34 more primitive stem cells. Almost all clinical and experimental protocols including ex vivo culture, gene therapy, and HSC transplantation are currently designed for cell populations enriched for $CD34^+$ cells.

2.2.2.2 CD133:

It is a glycosylated protein containing five transmembrane domains and is identify by the AC133 monoclonal Ab, which recognizes a $CD34^+$ subset of human HSCs (Yin, et al. 1997;) Recently ACD133 isoform has been cloned and identified the original surface the antigen which is recognized by the AC133 Ab. CD133 alternative to CD34 for HSC selection. According to multilineage capacity A $CD133^+$ enriched subset can be expanded in a similar as a $CD34^+$ enriched subset (Kobari, et al., 2001). Recent studies have evidence that CD133 expression is not limited to primitive blood cells, but defines unique cell populations in non-hematopoietic tissues. $CD133^+$ progenitor cells from peripheral blood which induced

to differentiate into endothelial cells in vitro. In human neural stem cells can be directly isolated by using an anti-CD133 Ab (Uchida, et al. 2000).

2.2.2.3 ABCG2

ABCG2 (ATP-binding cassette superfamily G member 2) is a determinant of the Hoechst-negative phenotype of side population (SP) cells.ABCG2 is a member of ABC transporters family and was first identified in a breast cancer cell line. It belongs to the half-transporter group and is unique as it is localized to the plasma membrane. The expression of ABCG2 appears greatest on $CD34^-$ cells and is down-regulated of CD34 on the cell surface. Down-regulation in ABCG2 expression is also observed in various committed hematopoietic progenitors. ABCG2 may therefore serve as a more promising marker than CD34 for primitive HSC isolation and characterization. The expression pattern of ABCG2 is not limited to HSC. ABCG2 expression exclusively characterizes the Hoechst SP phenotype in cells from diverse sources, including bone marrow, skeletal muscle and ES cells. The potential plasticity of SP cells has been demonstrated by cardiomyocytes and muscle regenerated from transplanted bone marrow- which derived SP cells. Exclusive expression of ABCG2 on SP cells suggests that ABCG2 is a potential marker for positive selection of pluripotent stem cells from various adult sources. ABCG2 have playing a functional role in developmental stem cell biology.

2.2.2.4 Sca-1

Sca-1 (stem cell antigen 1), is a phosphatidylinositol-anchored protein, is a member of the Ly-6 antigen family (Bunting, et al., 2002). Sca-1 is the most recognized HSC marker in mice. It is expressed on multipotent HSCs. An anti-Sca-1 Ab is frequently used negative selection and expressed the number of cell surface markers. The characteristic of differentiated cells of hematolymphoid lineages (Lin^-) is to identify and isolate murine HSCs. $Sca\text{-}1^+$ HSCs is found in the adult bone marrow, fetal liver and mobilized peripheral blood and spleen within the adult animal. Sca-1 also discovered several non-hematopoietic tissues, and can it is use in enrich progenitor cell populations. Sca-1 also involved in regulating both B cell and T cell activation (Codias et al., 1990).

2.2.3 Mesenchymal/ stromal stem cell marker

Mesenchymal stem cells (MSCs) are multipotent cell which derived progenitor cells. They have the capacity to differentiate into cells that compose adipose, bone, cartilage, and muscle tissue. Adult mesenchymal stem cells can be isolated from the stroma of the bone marrow.so it is also called as stromal cell. STRO-1 & CD44 comes under the mesenchymal stem cell marker.

2.2.3.1 CD44

CD44 is a ubiquitous multi-structural and multi-functional cell surface glycoprotein involved in cell-cell adhesion, cell-matrix interactions, and cell migration. In stem cells CD44 is widely expressed by many cell types and functions in cell adhesion, migration, homing, proliferation, survival and apoptosis. CD44 also plays critical roles in stemness regulation of cancer stem cells (CSCs). CD44 is expressed at the time of extracellular matrix formation in many sites during embryonic development. HA interacts with cells during embryonic development via the CD44 receptor, and these HACD44 interactions induce the activation of ERK to promote cell proliferation. CD44 also is expressed in various kinds of cancer cells and cancer stem cells. (Kenneth,2011), in his study of cell adhesion molecule CD44: its functional roles in prostate cancer proposed that CD44 is a cell adhesion glycoprotein and dysregulated CD44 expression characterizes most human cancers, including prostate cancer. From their experiment they conclude that CD44s reduces PCa growth and invasion in vitro, and possibly in vivo. And CD44v7-10 may be a target for chemosensitization, and plays a role in nutraceutical abrogation of tumor development.

2.2.3.2 STRO-1

It is a murine IgM monoclonal Ab. STRO-1, produced from an immunization with a population of human $CD34^+$ bone marrow cells which identify a cell surface antigen. It is express the stromal elements in human bone marrow. There are two dfferent types of population i.e. STRO-1^+/Glycophorin A^+ and 1^+/Glycophorin A^- (Simmons, et al.,1991) . From bone marrow cells, the frequency of fibroblast colony-forming cells (CFU-F) is enriched approximately 100-fold in the STRO-1^+/Glycophorin A^- population than in the STRO-1^+/Glycophorin A^+ population. A STRO-1^+ is a subset of marrow cells which is capable of differentiating into multiple mesenchymal lineages including hematopoiesis-

supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts and chondrocytes. STRO-1 is a valuable Ab for the identification, isolation and functional characterization of human bone marrow stromal cell.

2.2.4 Neural stem cell marker:

Neural stem cells are derived from neuron.

2.2.4.1 Nestin:

Nestin is a class VI intermediate filament protein. It is expressed predominantly in the central nervous system (CNS) (Frederiksen et al.,1988) Nestin has been the most extensively used marker to identify CNS stem cells within various areas of the developing nervous system.Although nestin does not form intermediate filaments by itself in vitro it co-assemble with vimentin or alpha-internexin to form and heterodimer . Heterodimer coiled-coil complexes which is a intermediate filaments. Its transient expression has been suggested to be a major step in the neural differentiation pathway. Nestin is also expressed in non-neural stem cell populations, such as pancreatic islet progenitors and hematopoietic progenitors

2.2.4.2PSA-NCAM (Polysialic acid-neural cell adhesion molecule):

It regulates the expression of neural cell adhesion molecule (NCAM). The isoforms of this marker have a critical role in neural developmental processes. The embryonic form of NCAM, PSA-NCAM, is highly polysialylated and expressed in the developing nervous system (Kiss et al., 2001). PSA-NCAM is related to synaptic rearrangement and plasticity. In the adult tissue, PSA-NCAM expression is restricted to regions that retain plasticity. A neuronal-restricted precursor is identify its high expression of PSA-NCAM and undergo self-renewal and differentiate into multiple neuronal phenotypes . PSA-NCAM^{+} neonatal brain precursors are restricted to a glial fate and thyroid hormone and modulate them into an oligodendrocyte fate. NCAM adhesiveness Polysialic acid modification significantly decreases and therefore, it is originally suggested that PSA-NCAM is a purely anti-adhesive factor that modulates cell-cell interactions in promoting brain plasticity. PSA-NCAM interacts with secreted signaling molecules to perform an instructive role in development.

2.2.4.3 p75 Neurotrophin R (NTR):

p75 NTR is also named as low affinity nerve growth factor (NGF) . It is a type I receptor transmembrane protein that belongs to the tumor necrosis factor. It binds to NGF, BDNF, NT-3 and NT-4 equally (with low affinity).When p75NTR activate in the presence of Trk, enhances response to neurotrophin. TrkC receptors working together with p75 NTR serve the critical functions during the development of the nervous system (Hapner et al., 1998). Neural crest stem cells (NCSCs) have isolate based on their surface expression of p75NTR (Morrison et al.,1999).Freshly isolated $p75NTR^{+}$ NCSCs from peripheral nerve tissues can self-renew and generate neurons and glia both in vitro and in vivo. In addition, neuroepithelial-derived $p75NTR^{+}$ cells are also able to differentiate into neurons, smooth muscle and Schwann cells in culture. Recently, p75 NTR has been used as a marker to identify mesenchymal precursors as well as hepatic stellate cells (Cassiman, et al. 2001).

2.3 CD44 in oral cancer

`Georgolios et al. (2006), in his review focused on the role of CD44 adhesion molecule in carcinogenesis of the oral cavity with special emphasis on its potential use for diagnosis and prognosis of cancer of oral cavity.It is widely accepted today that the development of squamous cell tumors, including oral squamous cell carcinoma, is final outcome of multistep process characterized by progressive genetic alterations and gradual transition to malignancy (Alho et al.,1989).The early mutational events at genomic level are reflected in differential protein expression and subsequent molecular abnormalities at cellular level, in this way from normal to histopathologically established malignancy. The carcinogenesis is related to the dysregulation of cell adhesion and cell proliferation while alteration of angiogenetic function is a substantial part of tumor growth (Bartolazzi et al.,1996).The degree of glucosylation can affect the ligand binding features of the protein and alter its function. Observations indicate that changes in glucosylation of CD44 can affect its interaction with hyaluronic acid (HA). So this posttranslational modification may provide important regulatory mechanism for CD44 functioning (Georgolios et al.,2006). Over expression of CD44 is shown to be linked with poor outcome in squamous cell carcinomas of the oropharynx, hypopharynx, and larynx, whereas evidence concerning the oral cavity is slightly less assuring.[13] It has been speculated that CD44 can be used as a cancer stem cell marker, meaning that tumor cells expressing CD44 are capable of forming new tumors.

CHAPTER 3

3.1 Objectives

- **To analyse the CD44 expression in different oral cancer tissue samples**

CHAPTER 4

4. MATERIALS AND METHODS

Three different groups of oral cancer cell tissues from different patients were take for immunohistochemical studies.i.e. Well differentiated, moderately differentiated, and poorly differentiated.

4.1 MATERIALS

4.1.1 CHEMICALS:

1. xylene
2. 100% ethanol
3. 90% ethanol
4. 70% ethanol
5. Mayers Hematoxylin
6. CD44 Primary antibody
7. Polymer HRP
8. DAB chromogen

4.1.2 MISCELLANEOUS

1. Paraffin block
2. Slide
3. Cover slip
4. Paper bond
5. Brush
6. Oven
7. Microtome
8. Water bath
9. Inverted Microscope

4.2 Methods

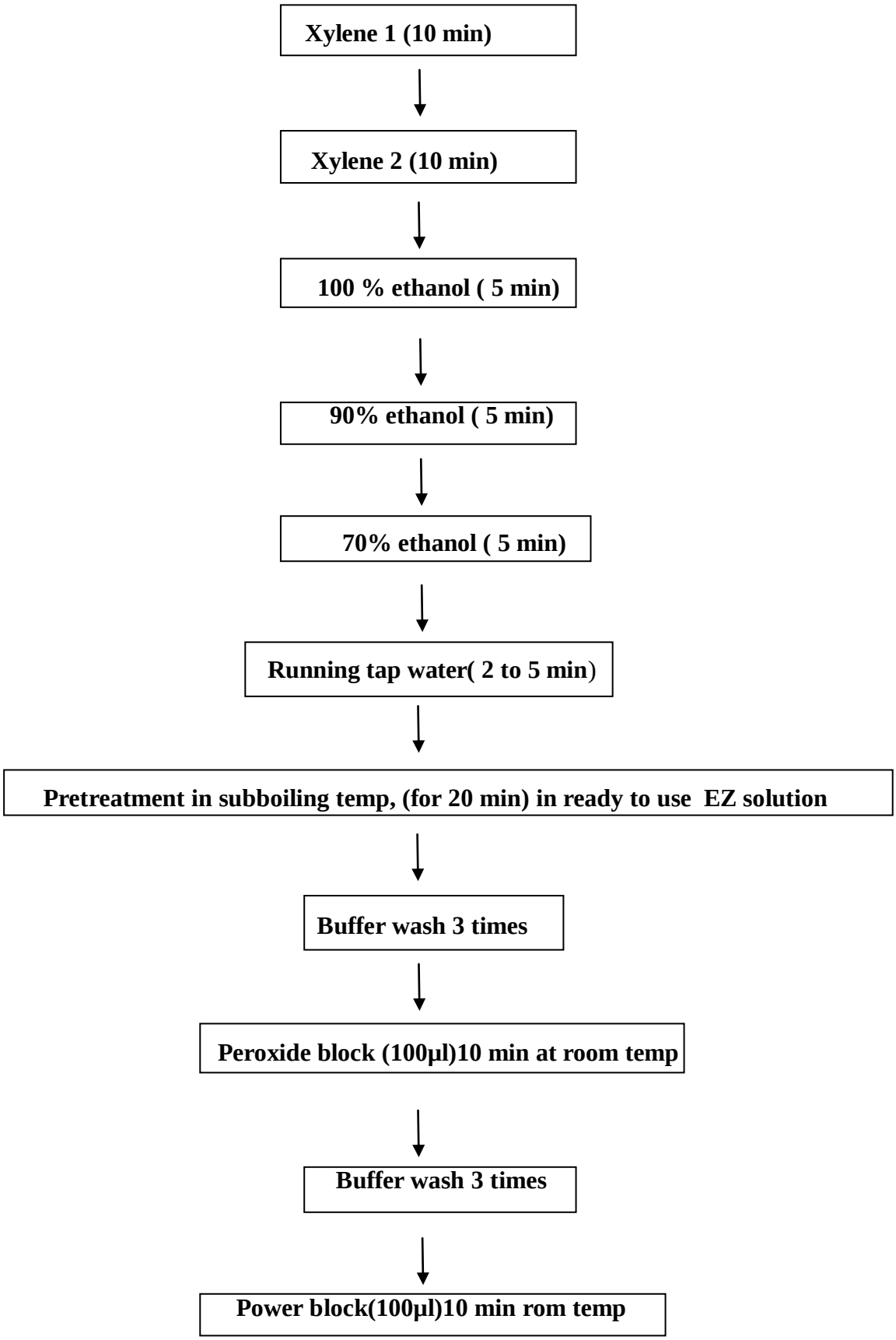
(A)Three different Groups (Well differentiated, moderately differentiated, poorly differentiated) tissue block was collected from Tridevi hospital kolkata.

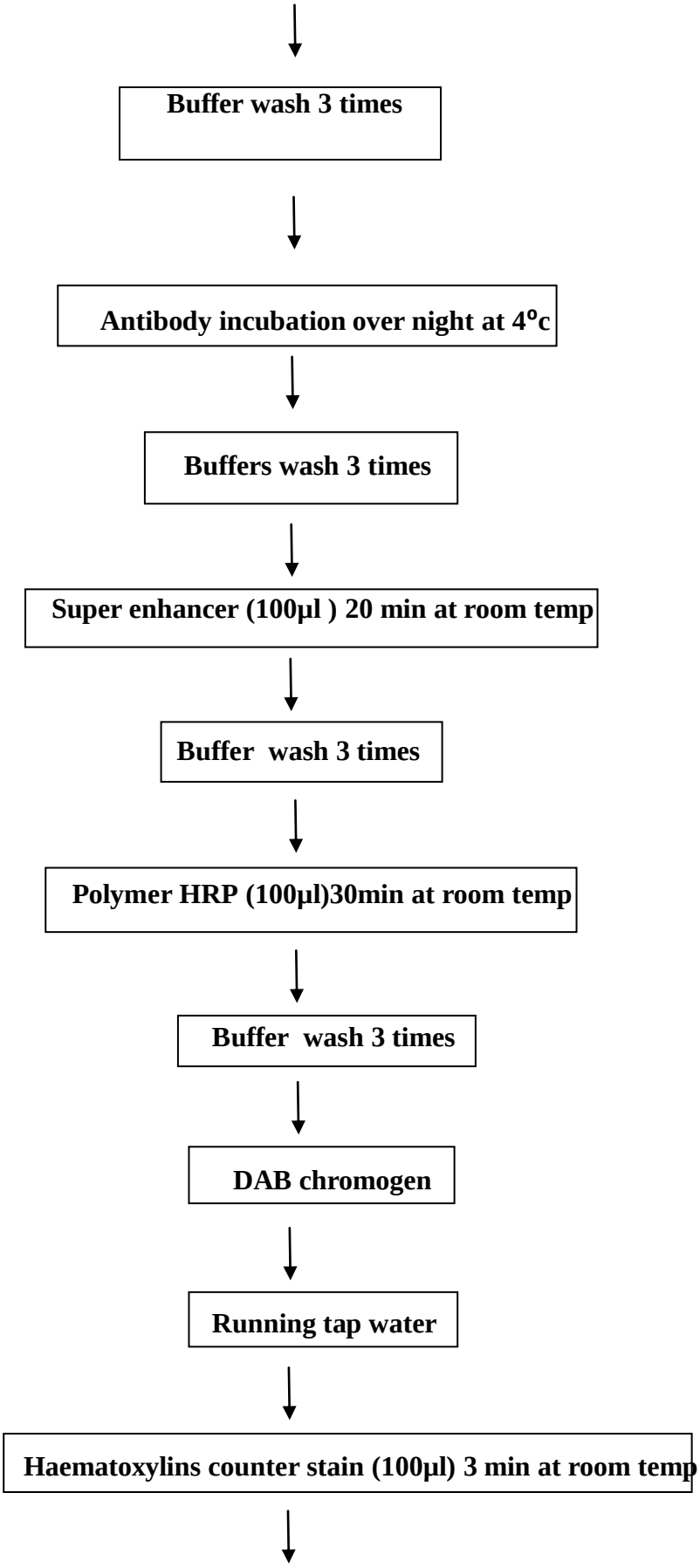
(B) 0.5 micron of thin section was prepared using microtome.

(C)Immunohistochemistry was done by following a standard protocol

The slide was baked for 1 hour at 60°C temperature. Then wax was removed by xylene -1 & xylene-2 solution for 10 mins. After that It is kept in 100 % ethanol for 5 min, in 90% ethanol for 5 min, 70% ethanol for 5 min sequentially .After that the slide was washed in with running tap water for 2 to 5 mins. To expose the antigen the tissue was Pre-treated in sub-boiling temperature for 20 min in EZ ready to use solution. Buffer wash is done 3 times. after that it is kept in Peroxide block(100µl)for 10 min at room temp subsequently it was wash with PBS buffer 3 times .Then it is kept in Power block(100µl)10 min room temp followed by 3 times PBS buffer wash. Then the tissue was incubated with primary CD44 antibody for overnight at 4°c followed by 3 times PBS buffer wash. Then the tissue was incubated in Super enhancer (100µl) for 20 min at room temp and washed with PBS Buffer 3 times.Tissue was kept in kept in Polymer HRP (100µl) for 30min at room te wamp and it was again washed in PBS buffer 3 times. 1 drop of liquid DAB (brown chromogen) was mixed in 1 ml stable DAB buffer. Then 100µl DAB chromogen solution was added to the tissue and incubated for 5min at room temp. Then the tissue was washed in running tap water. The ttissue was counter stained with Hematoxylin counter stain (100µl) for 3 min at room temp. Again kept the slide in 70% ethanol for 5mins, 90% ethanol for 5 mins, 100% ethanol for 5 mins. Finally the dehydrated slide was kept in xylene-2 for 10 min .Then the tissue were observed in inverted microscope to check the expression of CD44.

4.3 PROTOCOL FOR IMMUNOHISTOCHEMISTRY

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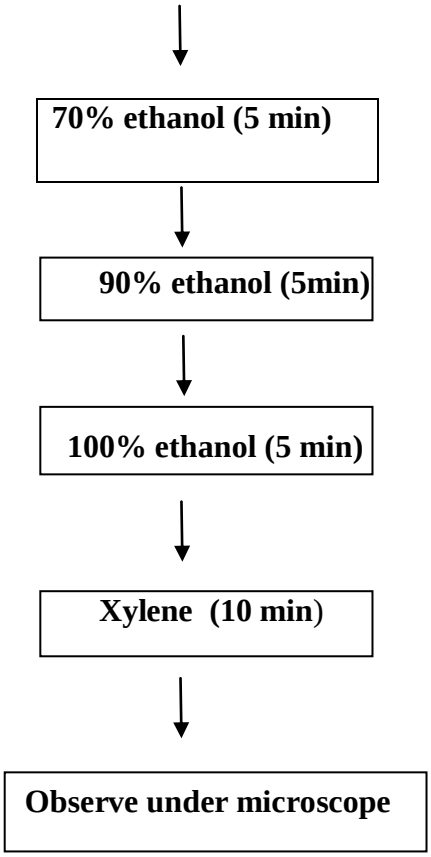
Buffer wash 3 times
DAB chromogen
Running tap water
Haematoxylins counter stain (100µl) 3 min at room temp

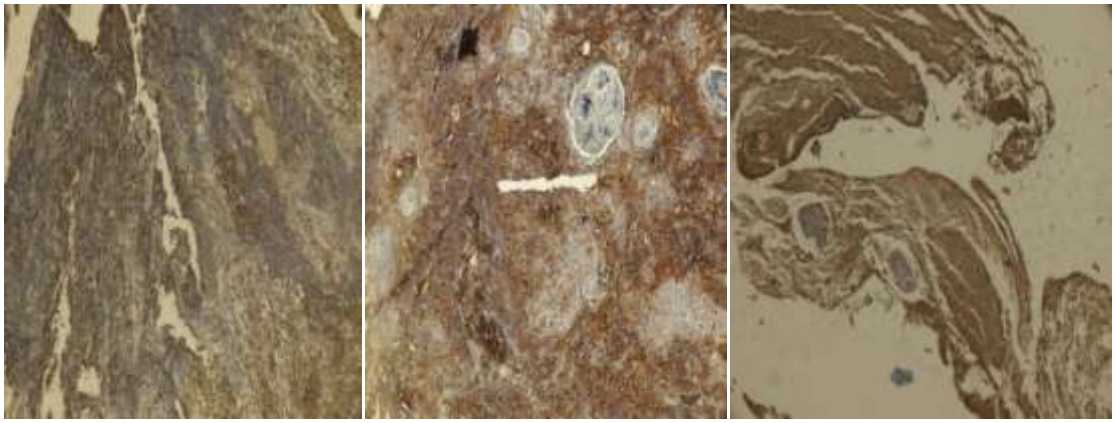

70% ethanol (5 min)
90% ethanol (5min)
100% ethanol (5 min)
Xylene (10 min)
Observe under microscope

CHAPTER-5

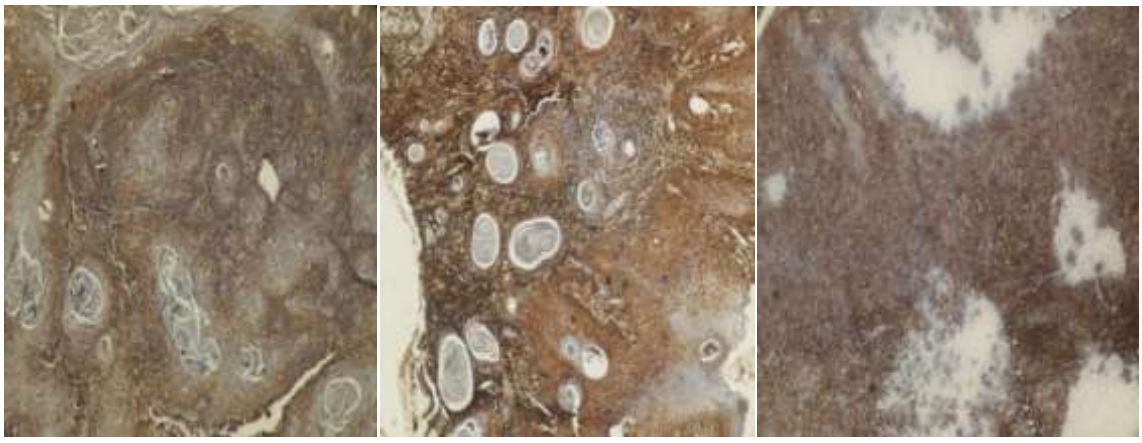
5. RESULT AND DISCUSSION

Fig. 2: CD 44 expression in well differentiated oral cancer tissue Samples from 9 patients

Patient-1 **patient-2** **patient-3**

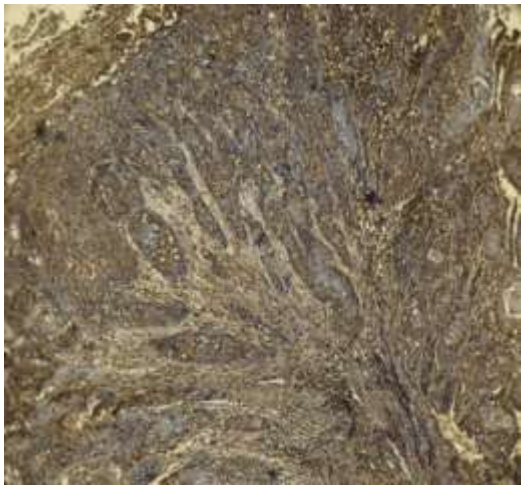


Patient-4 **Patient-5** **Patient-6**

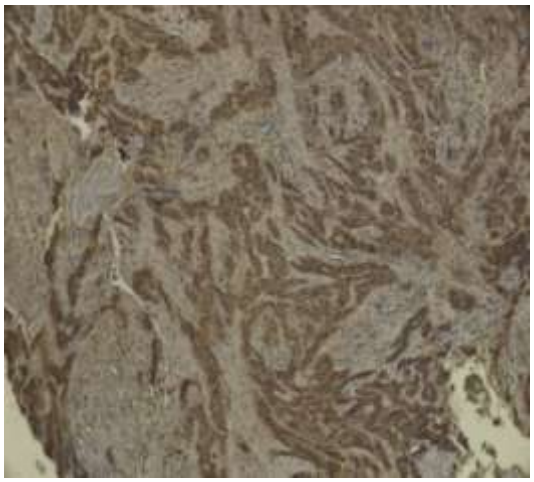


Patient-7 **Patient-8** **Patient-9**

Fig.3: CD44 expression in moderately differentiated oral cancer tissue Samples from 5 patients

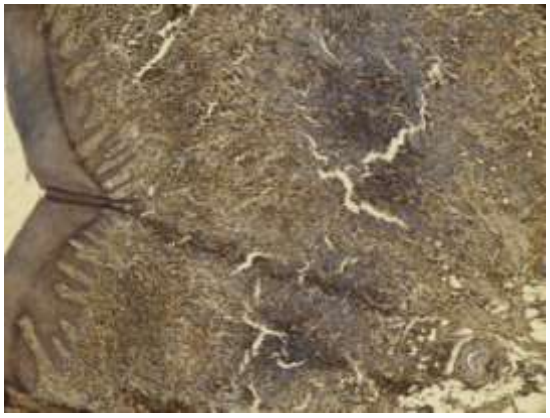


Patient-10

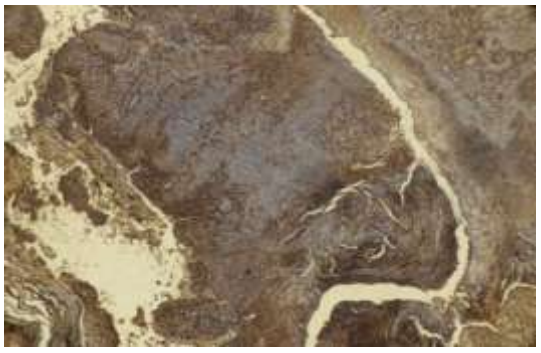


Patient-11

Patient-12

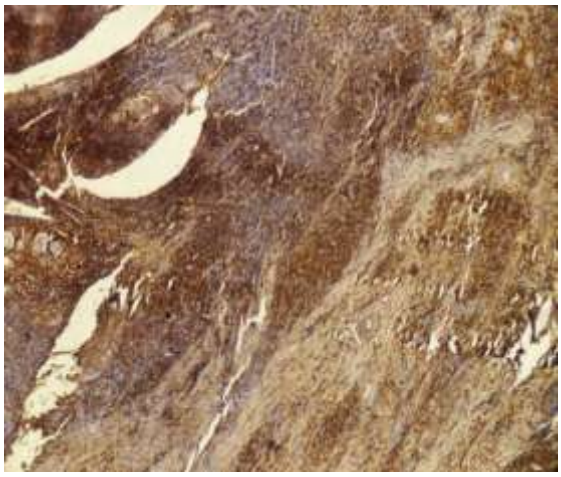


Patient-13

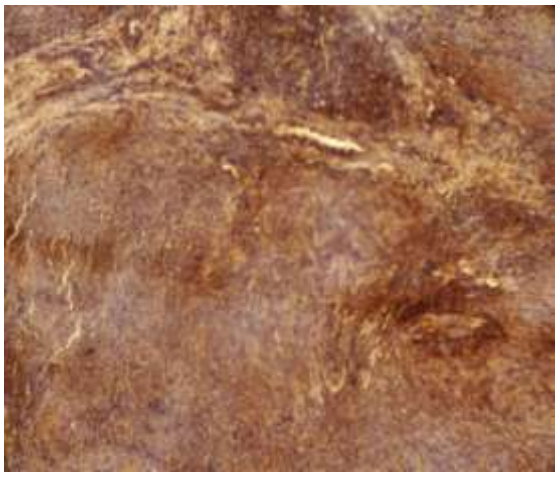


Patient 14

Fig.4 CD44 expression in poorly differentiated oral cancer tissue samples of 2 patients



Patient-15



Patient-16

CD44 is a marker of stem cells and cancer stem cells that helps stem cell migration, homing, and differentiation, while in cancer, CD44 promotes metastasis, self-renewal, drug resistance and apoptosis resistance. For the 16 cases we studied, we observed the immunostaining degree for CD44 of the 3 different differentiation grades of OSCC. All cases of well-differentiated carcinomas presented the lowest immunostaining degree, the moderately ones presented a little higher immunostaining degree and the poorly differentiated were shown to present highest immunostaining degree. From the above study, we can assert that as poorly differentiated oral cancer tissue has high metastasis properties, high proliferation rate, highly invasive capacity as compare to the other two groups, the expression of CD44 stem cell marker is more in this groups which renders them to acquire the unique properties involved in invasion and metastasis. The well differentiated and moderately differentiated tissue has not very much aggressive & metastasis properties like poorly dif ferentiated tissue. So, in both case the CD44 expression is as low compare to the other two groups of tissues. Elevated expression of CD44 is also reported to be associated with poor outcome in other malignancies such as hematological malignancies, colorectal cancer, and gastric cancer.

CHAPTER-5

5.1 CONCLUSION

Knowledge concerning biological activity of CD44 and its isoforms is not fully elucidated. However, as far as this glucoprotein is highly expressed in squamous epithelium, oral squamous cell carcinoma is expected to be one of most prevalent field for further investigation. The progress in study of adhesion molecules promise to be rapid during following years. Many opportunities for novel and more effective prognostic, diagnostic and therapeutic approaches involving CD44 are opening now. From the IHC study we concluded that the CD44 stem cell marker is highly expressed in poorly differentiated oral cancer tissue, moderately expressed in moderately differentiated tissue and the expression profile is relatively low in well differentiated oral cancer tissue**.C**D44 can be used as a prognostic marker in oral cancer.

CHAPTER 6

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