Molecular Prognostication of Oral Cavity Squamous Cell Carcinoma by Tissue Microarray Analysis

by

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Abstract

Aims

Oral cavity squamous cell carcinoma (OCSCC) is a molecularly heterogeneous disease, which is thought to account for differences in treatment response between patients who have otherwise similar characteristics. Cancer biomarkers provide a means of molecular classification, which can be of diagnostic and prognostic utility. This study aims to investigate the prognostic value of a panel of established biomarkers in OCSCC.

Methods

Using a prospectively collected dataset, patients with OCSCC diagnosed and treated during 1998-2010 were identified for study inclusion. Formalin-fixed paraffin embedded tumors from these patients were obtained for the construction tissue microarrays (TMAs), which were stained by immunohistochemistry with p16, p53, Bcl-xL, EGFR, Ki67, pancytokeratin, and DAPI. Each biomarker was measured using quantitative immunofluorescence, within tumor nuclear and cytoplasmic compartments, relative to normal control tissue controls. Biomarker expression levels were correlated with patient survival outcomes using univariate and multivariate analyses.

Results

A total of 254 OCSCC patients were identified of which 187 had adequate tissue for TMA construction. All the included patients had been treated with surgical modalities. Negative Ki67 expression was associated with improved disease specific survival ($p = 0.02$). High EGFR or high p53 expression were associated with significantly lower survival outcomes ($p = 0.02$ and 0.034 respectively). P16 and Bcl-xL levels were not predictive of survival.

Conclusions

OCSCC patients with negative Ki67 have significantly higher survival outcomes, while high EGFR or high p53 have significantly lower survival outcomes. These biomarkers may be predictive of more aggressive pathology and/or treatment resistance in OCSCC.

Dedication

The completion of this undertaking could not have been possible without the support of people whom I want to acknowledge here. I proudly dedicate my master's thesis work to my loving family. My gratitude and special thanks to the role model parents in my life, my parents, Jawza and Fahad Alenazi, for their support, encouragement, and unconditional love. A very special dedication goes to my brother, Abdulaziz, who passed away in January 2011 before commencing his dream of becoming a doctor. Your words will always ring in my ears, and you will be forever with me, and as you always called me "our doctor", here I am. I hope to make you proud of me one day as I always will remain proud of you.

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Glossary of Terms

Abbreviation	Explanation
OCSCC	Oral cavity squamous cell carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
EGFR	Epidermal growth factor receptor
Bcl-xL	B-cell lymphoma extra large
PCK	Pancytokeratin
TMA	Tissue Microarray
TNM staging	Tumor, lymph node, metastasis
HPV	Human Papilloma Virus
pRB	Retinoblastoma protein
TGF	Transforming growth factor
EGF	Epidermal growth factor
PCR	Polymerase chain reaction
IHC	Immunohistochemistery
HIER	Heat-induced enzymatic retrieval
PIER	Proteolytic-induced enzymatic retrieval
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
OS	Overall survival
DSS	Disease-specific survival

Chapter One: Introduction

1.1 Background

Head and neck squamous cell carcinoma is a devastating disease, which is ranked sixth among common cancer types (1). Cancer of the oral cavity is the most common type of head and neck squamous cell carcinoma (2). The main risk and etiologic factors for this malignancy are: alcohol consumption, tobacco use, and more recently, human papilloma virus (HPV) infection (2). These- causative agents induce genetic alterations modifying essential molecular processes that result in tumorigenesis.

Current treatment recommendations are dependent on the anatomic extent of the disease, such as the TNM staging system. The traditional modes of treatment, such as surgery or radiation, are effective in early stage disease, but combining these modalities and the inclusion of adjuvant chemotherapy result in the best 5-year survival in patients with Stages III and IV disease (3). These invasive and toxic treatments can, however, be harmful to the patient, causing devastating side effects and significantly affecting the patient's quality of life in survivorship (1,2).

There is a paucity of research investigating therapies based on the molecular pathways involved in oral cavity squamous cell carcinoma (OCSCC). Biomarkers have been found to be useful in predicting survival outcomes of OCSCC patients, but despite the available data, biologic markers have not been used in cancer management. Biomarker profiling provides a method of classifying OCSCC that is reflective of the ongoing molecular processes. Combined biomarker profiles and the classical anatomic staging may allow for better prediction of clinical behaviour and selection of treatment protocols, which may, in turn, improve patient survival and quality of life. Identifying patient cohorts with the assistance of biomarkers that have excellent

treatment responses would allow for de-escalation of regimens, reducing the toxicity and associated morbidity of the treatment. Alternatively, patient cohorts that have poor responses to treatments could benefit from alteration in treatment philosophies and the introduction of innovative treatment options, such as biologic agents or immunotherapy. The biomarkers known to have clinical importance in head and neck cancer include HPV/p16, EGFR, p53, Bcl-xL, Ki67.

Our study aims to establish a biomolecular profile (HPV/p16, EGRF, Bcl-xL, p53, and Ki67) for OCSCC patients. This profile will be combined with and contrasted to the TNM staging with regards to survival outcomes for the patients, taking into account the treatment they underwent. In so doing, this research could add a new staging system for the OCSCC that is more accurate than the traditional one (TNM) in determining the optimal treatment plan for each patient.

This introductory chapter will divide the topic into three main parts. The first part will cover the normal cell cycle and the cancer development in the cell, the second part will elaborate on the clinical and treatment aspects of the OCSCC, and the third part will discuss the biomarkers contributing to OCSCC and their prognostic application.

1.2 Cell Cycle and Cancer Development

A cell cycle is a group of controlled events inside the cell that leads to cell division and result in the production of two daughter cells that have identical sets of chromosomes to the original cell. These events are coordinated through four different cell stages, starting with the G1 (Gap1) stage where the cell growth starts. This is followed by the S (synthesis of DNA), the G2 (Gap2), and the M (mitosis) stages. The cell starts to divide in M stage, and the cell chromosomes separate (4,5). This process is normally required in order to make new cells, which

replace aged or dead ones. The cell cycle is maintained in a normal cell by different molecules, regulating and driving the cell from one stage to another. Any mutation introduced in the regulatory molecules will affect its function and may cause cancer development (4). In broad terms, cancer is defined as a group of diseases in which abnormal cells start proliferating and dividing without control, leading to tumor growth (6).

Cycline dependent kinases (CDKs) and Cyclin are the main regulators of the cell cycle, and their complex together drives the cell through all of the stages (4,8). Leland Hartwell, Tim Hunt, and Paul Nurse are the scientist who discovered the fundamental mechanism of controlling the cell cycle in 2001, and they were awarded the Nobel Prize in physiology and medicine. The complex of CDK and cycline is considered the "turn-on" of the cell cycle. In order to have this complex active, the cell needs growth factors to act on the cell membrane receptors and activate the cascade of CDK-Cycline complex. The neighboring cells in the tissue are mainly the secretors of these growth factors, acting through cell-to-cell coordination in tissue hemostasis (4). These mechanisms are precisely controlled in normal cells, to provide the human body with the appropriate number of cells depending on its need, and to prevent proliferation of unwanted cells.

The human body consists of cells that are frequently replaced through cell division and proliferation via the cell cycle. Mitosis is a tightly controlled process by means of biologic molecules that act at all the cell cycle's stages. These molecules will stop the division, correct genetic errors, or induce programmed cell death (i.e., apoptosis) to prevent the development of cancer cells. Apoptosis was first described in 1972. This biologic process eliminates damaged cells and consists of a cascade of events that result in cell death (7). Dysfunction of apoptosis is one of the leading causes of the human cancers (4,7,8) and most of anti-cancer drugs

conceptually destroy cancer cells by stimulating apoptosis. Different molecules are involved in the regulation of apoptosis, either as enhancers or inhibitors and p53 and Bcl2 are two of those molecules that work in opposition to each other to maintain apoptosis hemostasis (4,7,8,9).

The molecules that control the cell cycle and the proliferation of cell are, in turn, regulated and modified by certain genes and their products. The two most important types of genes that contribute to cancer development in the cell are tumor suppressor genes and oncogenes (8). The Rat Sarcoma (RAS) was the first identified human oncogene, while the retinoblastoma gene (RB) is the first identified tumor suppressor gene (4). RB is located on chromosome 13, and its functional protein is called pRB (8,11). The main function of pRB is to protect cell's DNA from mutations, which influence apoptosis and control cell's proliferation and differentiation (11). Previous studies have shown that mutations in tumor suppressors and oncogenes will affect their functions, and that will lead to abnormal cell cycle and eventually to tumor growth (8,12-14). Activation of the oncogenes and inhibition of the tumor suppressor genes will initiate the cancer development process, which is seen in many types of cancers (8,11). Recent studies have reported that in order to develop a tumor, the cell needs to have many mutations in almost all of its molecular levels triggered by genome instability, so it is a multistep mutation fashion (4,13).

The multistep nature of the development of cancer from a mutation in a normal cell to tumor outgrowth is fairly complicated (4). Hanahan and Weinberg summarized the hallmarks of cancer according to six basic principles (15):

> Sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Underlying these hallmarks is genome instability, which generates the genetic

> diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions. Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer. (15:1)

Furthermore, Burrell et al. (13) reported,

> Recent studies have revealed extensive genetic diversity both between and within tumors. This heterogeneity affects key cancer pathways, driving phenotypic variation, and poses a significant challenge to personalized cancer medicine. A major cause of genetic heterogeneity in cancer is genomic instability. This instability leads to an increased mutation rate and can shape the evolution of the cancer genome through a plethora of mechanisms. By understanding these mechanisms we can gain insight into the common pathways of tumor evolution that could support the development of future therapeutic strategies. (13: 342)

In the case of OCSCC, many types of regulatory molecules and mutations have been identified to have either direct or indirect associations with the tumor development and treatment response **(**9,16,17**)**. These molecules are HPV/p16, EGFR, Bcl-xL, p53, and Ki67.

1.3 The Oral Cavity

1.3.1 Anatomy

The oral cavity is oval shaped and extends from the lips up to anterior pillars of the tonsils (18). It is divided into nine anatomical sub sites (18-20) as provided in the following list.

1. **Anterior two-thirds of the tongue**: the oral tongue is divided into tip, lateral borders, dorsum, and the undersurface. The posterior one-third of the tongue is part of the oropharynx.
2. **Floor of the mouth:** the area between the gingivae and undersurface of the tongue.

3. **Upper alveolar ridge:** structures covered by the gum (gingivae).
4. **Lower alveolar ridge and mandible:** structures covered by the gum (gingivae).
5. **Buccal (cheek) mucosa:** line the inner surface of cheeks and lip.
6. **Retromolar trigone:** a triangular area of mucosa, covering the ascending ramus of the mandible.
7. **Upper lip**
8. **Lower lip**
9. **Hard palate:** it is the roof of the oral cavity.

All these structures are lined by a mucous membrane (i.e., oral mucosa), which provides protection, sensation, and secretions. The oral mucosa is composed of stratified squamous epithelium over the connective tissue called lamina propria (21). All of the oral cavity anatomic sites have the risk to develop squamous cell cancer within the mucosa.

The structures of oral cavity are mainly supplied by blood via branches from facial, maxillary, and lingual arteries, which are branches of external carotid artery. The venous drainage is through the facial, lingual, and maxillary vein into the internal jugular vein. The primary lymphatic drainage is to the parotid, submental, and submandibular nodes. These nodes, in turn, drain the deep cervical lymph nodes and, ultimately, into jugular lymphatic trunk (22). The oral cavity is supplied with sensory and motor innervation through the trigeminal (V2, V3), and hypoglossal nerves (22).

1.3.2 Physiology

The oral cavity is intimately associated with swallowing, speech production, and breathing. Swallowing is a complex function that was historically divided into four phases. These include the (a) oral preparatory phase, (b) oral phase, (c) pharyngeal phase, and

(d) esophageal phase. The oral cavity is essential for the proper functioning of the first three phases and is essential for normal swallowing. The oral tongue and lips are the most important structures for normal speech production, but the whole oral cavity is necessary because it acts as resonance chamber. The oral tongue is the main component of taste sensation, as it contains the majority of the taste buds (18,20).

1.4 Oral Cavity Cancers

Head and neck cancers represent five percent reported malignancies each year in the world and nearly 30 percent of these cancers occur in the oral cavity (3,23). In India and many other developing countries in Asia, oral cancers remain among the most common malignancies and cancer-related death (24). There are no accurate documentations for the incidence of oral cavity cancers because they are usually combined with those of the oropharynx. The types of oral cavity cancers include squamous cell carcinoma, salivary glands cancers, sarcomas, melanomas, lymphomas, and metastatic cancers (25,26), but OCSCC is the most common.

1.5 Oral Cavity Squamous Cell Carcinoma

This cancer originates in the epithelial cells of the mucosal lining of the oral cavity (27). It is a devastating malignancy that robs the affected patients of quantity and quality of life.

1.5.1 Epidemiology

Formerly, OCSCC was believed to be a cancer of older ages, particularly in men, as they are more prone to smoking and alcohol use than younger age groups and their female counterparts (10). In recent years, however, many reports have shown an increase in the incidence of OCSCC in patients younger than 40 years of age (10), and this may be due to HPV infections and its endemic era.

The most commonly affected site is the oral tongue, accounting for 30 percent of all oral cavity cancer patients (25,26,28). In Southeast Asia, where OCSCC represents one of the most common cancers, the most commonly affected site is the buccal mucosa. This could be related to tobacco smokeless chewing, while it represents around 10 percent of overall oral cavity sites in North American populations (26). According to GLOBOCAN 2008, "An estimated 263,900 new cases and 128,000 deaths from oral cavity cancer occurred in 2008 worldwide", with most of them in developing countries.

The prevalence of OCSCC, especially the tongue, is higher among blacks compared to whites of the same regions in the United States (29). In all the countries, the approximate male to female ratio is 3:1 (29). This might be because men have been more likely to use tobacco and alcohol in the past, but in recent years, the rate of OCSCC is increasing among the females, especially in European countries, which could be reflective of the ongoing tobacco epidemic (27,30).

Regarding oral cavity cancer, the Canadian Cancer Statistics Publication (20) estimated cases for 2013 and reported, "4,100 Canadians will be diagnosed with oral cancers, approximately 2,800 men and 1,350 women. In addition, around 1,150 Canadians will die from oral cancers, approximately 778 men and 380 women" (20).

According to a previous study done at Alberta University (3), 859 patients were diagnosed to have OCSCC in Alberta between 1998 and 2010. Among these, 311 patients were diagnosed at late Stage III and Stage IV.

1.5.2 Histopathology

Ninety-five percent of oral cavity cancers are squamous cell carcinomas, and according to the Canadian Cancer Society, approximately 300,000 OCSCC cases are diagnosed each year

in the world (20). These cancers are further subdivided histologically into: (a) well differentiated, (b) moderately differentiated, and (c) poorly differentiated.

1.5.3 Risk Factors

Smoking, alcohol consumption, smokeless tobacco (snuff or chewing tobacco), and more recently Human Papilloma Virus infection (HPV) are the most common risk factors for OCSCC. Tobacco smoking and alcohol consumption are the leading causative factors for the OCSCC in North America (26). Using alcohol and tobacco together increases the risk of developing oral cancer more than using either one alone because of their synergistic effect (7,10,20). "Men who both smoke and drink are nearly 38 times more likely to develop head and neck cancers than men who do neither." (10)

Cigarettes contain many carcinogenic chemicals that contribute to the development of cancers. Among these chemicals, *N*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and polycyclic aromatic hydrocarbons (PAHs) are the likely carcinogens involved in OCSCC (7). Smoking can lead to inactivation of p53 and activation of RAS, thus promoting the development of cancers, including OCSCC (7).

Ethanol found in alcoholic beverages is considered one of the most common carcinogenics affecting humans today, especially in causing head and neck squamous cell carcinoma (10,31,32). Acetaldehyde, a metabolite of ethanol, forms DNA adducts that interfere with cell's DNA integrity and stability. These latter make the cell prone to mutations and at a high risk to develop cancers (33). Moreover, patients who drink alcohol and smoke show p53 mutations in their tumor specimens more than those who only smoke (10).

The new cases of OCSCC related to HPV infections have increased dramatically in young adults in the United States and Europe. The reason for this is unclear, but maybe reflects

changes in sexual behaviour, especially with regard to oral sex (33,34). The prevalence of HPV infection increases with age (23). A review done by Marur et al. (23) showed that the prevalence of oral HPV infection was 3-5% in adolescents and 5-10% in adults. The role of HPV as a carcinogen in head and neck squamous cell carcinoma, particularly types 16 and 18, is established more clearly in cases of oropharyngeal carcinomas (22,33). More recently, marijuana smoking has been linked to the progression of HPV-positive head and neck squamous cell carcinoma, including OCSCC, as it has an immunomodulatory effect (33).

There are some situations where the risk of OCSCC will slightly increase, like in the case of weakened immunity, graft versus host disease, periodontal disease, fanconi anemia, dyskeratosis, irritation from dentures, and lichen planus (20,33,34). Mouthwash use is a controversial risk factor, with some studies suggesting that there could be a link to oral cavity cancer development, especially the ones that have high alcohol content (20,34).

1.5.4 Signs and Symptoms

The most common signs and symptoms of OCSCC are (18):

- Non-healing ulcer either on the side of the tongue, floor of the mouth, or even inside the cheeks
- White patches (leukoplakia), red patches (erythroplakia) or mixed red and white patches (eryhtroleukoplakia)
- Neck mass
- Intra oral mass
- Bleeding inside the mouth with no specific reason
- Halitosis
- Referred ear pain
- Vague mouth pain
- Numbness sensation in the tongue
- Lose of teeth
- Pain with swallowing
- Change in the speech
- Difficulty in the mouth opening (trismus)
- Difficulty with breathing
- Loss of appetite
- Loss of weight

1.5.5 Diagnosis

OCSCC diagnosis is established based on the patient's signs and symptoms, medical history, physical examination, investigations, and tissue biopsy. All patients suspected of having OCSCC should undergo a detailed scrutiny of their medical history, along with a thorough physical examination by a specialist.

The medical history should focus the signs and symptoms listed above and the risk factors, in particular, tobacco and alcohol consumption, family history of oral cancers, and any record of previous oral cancer diagnosis. A complete history, including a review of systems, past medical history, social and family history, is essential in planning the proper therapy. Head and neck cancer patients tend to have a prolonged history of tobacco and alcohol abuse and consequently suffer from cardiac, pulmonary, and liver disease.

A complete physical and thorough head and neck examination should be routinely performed on all patients. Visualization of all the surfaces of the upper aerodigestive tract to identify second primaries is warranted due to the "field cancerization" phenomenon. The primary tumor should be thoroughly assessed by visualization and palpation to judge the extent of spread. The mandibular range of motion and cranial nerve function are also assessed. Fiberoptic examination is extremely helpful in evaluating the three dimensionality superior and inferior extent of the tumor, especially in patients with trismus. Systemic palpation of all the levels of the neck is always performed, and size, location, or fixation of nodes is noted. The patient's dentition is also evaluated, as restoration or extraction may be required before the initiation of the treatment.

The diagnostic investigations include, but are not limited to:

- Oral cavity X-ray or panoramic X-ray;

- Ultrasound (US) on the neck area: to assess any lymph node or major vessels involvement;
- Computed Tomography (CT) Scan with contrast: to determine the tumor dimensions and extensions;
- Magnetic Resonance imaging (MRI) with contrast: to assess the adjacent soft tissues and tumor deeply invading in oral cavity, as indicated;
- PET/CT scans of the head, neck, chest, abdomen, and pelvis are used to assess the local, regional, and distal metastatic disease; and
- Tissue diagnosis can be obtained through incisional or fine needle biopsies of the oral lesion or the neck metastasis respectively (20,25,26,35).

1.5.6 Staging

Currently, the staging of oral cancers is based on the anatomical system (TNM). Description of the tumor falls under T, N refers to the lymph nodes involved, while M signifies distant metastasis. The TNM staging is then combined into an overall stage that confers prognosis and guides treatment recommendations (26,36) (see Tables 1-1 and 1-2).

Table 1-1. *TMN Stages of Oral Cancer*

Stage	Levels
T-Stage	Tx: primary tumor cannot be assessed.
	T0: no evidence of primary tumor.
	Tis: carcinoma in situ.
	T1: tumor 2 cm or less in greatest dimension.
	T2: tumor is greater than 2 cm, but less than 4 cm in greatest dimension.
	T3: tumor is greater than 4 cm.
	T4a: tumor invades the cortical bone, into deep extrinsic muscles of tongue, maxillary sinus, or skin of the face.
	T4b tumor invades masticator space, pterygoid plates or skull base, or encases internal carotid artery (20).
N-Stage	Nx: lymph node status cannot be assessed.
	N0: no evidence of lymph node involvement.
	N1: single ipsilateral lymph node 3 cm or less in greatest dimension.
	N2: single ipsilateral lymph node greater than 3 cm but less than 6 cm in greatest dimension (N2a); multiple ipsilateral lymph nodes, none of which is more than 6 cm in greatest dimension (N2b); bilateral or contralateral lymph node, but less than 6 cm in greatest dimension (N2c).
	N3: lymph node involved is greater than 6 cm in greatest dimension.
M-Stage	Mx: presence of metastasis cannot be assessed.
	M0: no evidence of metastasis.
	M1: distant metastasis present.

Table 1-2. The Overall OCSCC Staging (18,26)

Stage	T-Level	N-Level	M-Level
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1	N1	
	T2	N1	
	T3	N1	
Stage IV A	T4	N0	M0
	T4	N1	M0
	Any T	N2	M0
Stage IV B	Any T	N3	M0
Stage IV C	Any T	Any N	M1

Stages I and II are considered as early stages of OCSCC, while Stages III and IV are regarded as late stages of oral cancer, which require extensive treatment. Unfortunately, most of the patients are diagnosed at late stages (26).

1.5.7 Treatment

The anatomical complexity of the oral cavity, along with its anatomical relationship to other head and neck subsites, makes treatment of oral cavity cancer highly challenging and complex (26). A team that includes a head and neck surgeon, reconstructive surgeon, radiation oncologist, medical oncologist, prosthodontist, and speech therapist offers the patient the best opportunity for a comprehensive treatment plan (26). Deciding on the optimal regimen for the individual patient depends on the multiple factors—the most important of which are the type of treatment needed for the primary tumor, neck, mandible, the reconstructive options, the general

medical condition of the patient, and his or her preference. The availability of facilities, expertise, and social support also plays a role (20).

Surgery and radiation therapy, alone or combined, are the mainstays of treatment for OCSCC. Chemotherapy has been used recently to augment the effectiveness of radiation. Generally, OCSCC Stage I and Stage II can be treated with surgery alone, while Stages III and IV are treated with combined therapy (i.e., surgery followed by radiation, with the possible addition of chemotherapy) (2,3,10). The recent advent of new reconstructive techniques allows the performance of more complex surgery with improved survival and post treatment function. Combined treatment is toxic and can give rise to many adverse effects, causing severe cosmetic and functional morbidity, which are detrimental to the patient's quality of life. This is also true even in cases where patients have been successfully treated (37,38). In summary, "The ultimate goal of oral cavity cancer treatment is to eliminate the cancer, preserve or restore form and function, minimize treatment complications and to prevent any subsequent new primary cancer" (26).

1.5.8 Prognosis

Over the past three decades, patient's quality of life has been improved, but the 5-year-survival rate is still ranging between 50-60 percent (26). In the United States, the period of time between 1973 and 1999 has shown decline in the overall prognosis of oral cavity cancers based on a 5-year survival rate, unlike those of nasopharyngeal, oropharyngeal, and hypopharyngeal carcinomas, which showed a better prognosis (33). Recurrence of the cancer or even developing the second primary cancer is a big challenge in the OCSCC after definitive treatment (10). Recurrence happens in twenty to forty percent of the treated patients, and this occurs mostly during the first 24 months after the treatment. Within five years of definitive treatment, up to 50

percent of treated patients die of cancer recurrence or due to its late complications after the treatment (38). Overall prognosis varies between the patients according to the tumor stage and adverse pathologic features, such as angiolymphatic invasion, perineural invasion, depth of invasion, and the presence of extracapsular spread (27).

1.5.9 Follow Up

A study by Sasaki et al. (39) has showed that the cases of recurrence were detected in the first three years after treatment, and most of these were in the first year, accounting for 86.3%. Nowadays, the routine follow-up visits for patients with OCSCC after definitive treatment is based on clinical practice rather than studies (39). The aim of these visits is to detect any recurrence of the primary cancer or the development of secondary cancers as a consequence of treatment. Most of the recommendations suggest scheduling close visits for the patient during the first three years post-treatment; each visit should include precise examinations of the oral cavity and neck area, and a short medical history of any new complaints. In addition, neck US and CT scan examinations should be performed as needed. Thereafter, the follow-up visits should be performed up to five years post treatment.

1.6 Clinical Trials

A new clinical trial study titled “Efficacy of Optically-guided Surgery in the Management of Early-Staged Oral Cancer” is currently going on. This collaboration study is between the University of British Colombia, Terry Fox Research Institute, and British Colombia Cancer Agency (40). This study aims to use tissue auto fluorescence in the screening and diagnosis of pre-cancer lesions in the oral cavity. Apart from this, more importantly, the use of this tool in the surgical treatment of oral cancers is expected to result in low (i.e., 2-years) recurrence rate of the disease by accurate removal of the tumor. The study started in January 2013 and was estimated

to be complete in June 2015. Another interventional study sponsored by the Dana-Farber Cancer Institute is currently under investigations (41). This study is investigating the effect of Ponatinib, an oral anti-cancer drug intended to prevent the growth of cancer cells. In addition, Ponatinib is found to have a potential effect on inhibition of lung squamous cancer cells, as the latter shows FGFR Kinase alterations. FGFR Kinases are a family of proteins that were found to be mutated only in cancer cells, particularly squamous cell cancers. Using Ponatinib can inhibit the mutations in those kinases. The study is in Phase II trial and expected to be completed in December 2014. In December 2009, results from one clinical trial were published; this trial has studied the use of Cetuximab, a monoclonal antibody, on advanced-stage head and neck squamous cell carcinomas treated with radiotherapy, including tongue squamous cell carcinoma. The results suggested that using of Cetuximab plus radiotherapy in patients with Stages III and IV head and neck cancers improves the overall survival compared to radiotherapy alone. Furthermore, adding Cetuximab to the treatment regimen causes less toxic effects compared to the current treatment with combined chemoradiation. Cetuximab (Erbitux) is a monoclonal antibody that inhibits the EGFR, thusly increasing the response to radiation in advanced head and neck squamous cell carcinomas (42).

1.7 Biomarkers of Oral Cavity Squamous Cell Carcinoma

There is a broad consensus that attests to the fact that sustained proliferation signaling is a key feature of malignant cell transformation (15,16). Proliferation signaling rates and mechanisms are different between various tumor types and are also patient specific (43.44). These variations are caused by different molecular expressions (4,13) and are responsible for the differing treatment response in some patients (45,46). Understanding these variations and

interactions will facilitate the development of new drugs and treatment tools and allow us to develop patient-specific treatment, based on patient-specific molecular profiles (4).

Treatment of OCSCC ideally should take into account both patient characteristics and expression of the molecules involved in the tumor (4). Current studies have shown some of the different biomarkers involved in OCSCC development, such as HPV/p16, EGFR, Bcl-xL, p53, and Ki67 (9,16,17,47). In addition, multiple other genes and molecules are being discovered to have impact on OCSCC development and treatment response (48). Although the actions of these biomarkers are not fully understood, they are found to have a significant impact on determining the patient's response to the treatment and prognosis (9,14,16). In the next sections, I will discuss the functions of the above-mentioned biomarkers and their roles in determining the treatment outcome of OCSCC patients.

1.8 History of the Biomarkers in OCSCC

A biomarker is a molecular, or genetic component, that can be objectively assessed to show the presence or progress of a certain disease. As genetic research has grown, various biomarkers have been studied, resulting in an increased understanding of processes involved in tumor initiation and progression. Prior to such deeper understanding of the biological and pathological role of biomarkers in cancer research, these biomarkers were merely used as prognostic indicators for patients with head and neck squamous cell carcinoma. Nowadays, such biomarkers can aid in establishing a more coherent patient care plan, such as early cancer diagnosis, better cancer staging, suitable anti-cancer treatment, and post-treatment plan. (14,33)

1.8.1 Human Papilloma Virus (HPV)

HPV is a double-stranded DNA virus belonging to the family of Papilloma virus that contains more than 120 viruses. Most of HPV infection manifests as a benign papilloma (wart) in

different places in the body. Among these viruses, there are at least 30 strains (especially HPV16 and 18) that are known to cause different types of cancer involving the cervix, penis, anus, oropharynx, hypopharynx, and oral cavity. HPV infection is responsible for almost all cases of cervical cancer. In the United States, it is believed that infection with HPV will exceed smoking as a causative factor for oropharyngeal and oral cancers. HPV16 has been linked to a high risk of developing oropharyngeal and OCSCC (17,49-52). In the United States, 60% of oropharyngeal SCC cases are associated with HPV16 infection, most commonly in lingual and palatine tonsils (22).

Typically, 30 to 40 subtypes of HPV are transmitted by unprotected sexual intercourse and ultimately lead to invasive cancers (22,49,50). HPV induces cancer through integration of their viral sequences in the cellular genome (4,50). Proto-oncogenes found in HPV, such as E6 and E7, can promote cell proliferation, disarrange cell cycle, and cause tumor growth through many sequences of genetic alterations in the normal cell genome. Furthermore, E6 and E7 also inhibit p53 and RB genes respectively, two main involved pathways in HNSCC development, through the upregulation of p16 (4,23,50,53). Overall, the role of HPV in head and neck squamous cell carcinoma is mainly carcinogenic, especially in absence of alcohol and tobacco use (23,53).

Different studies in literature suggested that HPV-positive head and neck SCC cases have less tumor invasion, lower recurrence rate, and better overall survival compared to those with HPV-negative tumors (27,55-57). In 2010, Marur et al. suggested that “HPV 16 is a prognostic marker for enhanced overall and disease-free survival, but its use as a predictive marker has not yet been proven.” (23:Abstract)

As HPV infection is recognized as a main risk factor in a variety of cancers, prevention has become very important. Nowadays, the ways of prevention include protected sexual contact and the use of vaccine. Different vaccines against the wild types of HPV have been approved for clinical practice, and many others are under investigation (33). It is recommended to start giving HPV vaccine as a routine vaccination for young women as a means of prevention against cervical cancer. Besides its importance in the prophylaxis, the new direction of using these vaccines is to introduce them in the treatment of the cancers that are caused by the virus, including HNSCC (33).

1.8.2 p53

p53 is a tumor suppressor gene. It is particularly important in cases of severe DNA damage in stressed cells, as it arrests the cell cycle mainly at G1 or S to allow for repair; however, if the damage is severe enough, it induces apoptosis and eliminates damaged cells from further division (8,14). Apoptosis is considered to be one of the main mechanisms in normal cells to prevent cancer development (4,58,59). P53 also works as an RB gene enhancer by maintaining its dephosphorylation state (i.e., active state) through increased regulation of p21. Consequently, p21 in turn prevents formation of Cyclin/ CDK complex, particularly Cyclin/ CDK4, which is responsible for the phosphorylation of RB (i.e., inactive state) (8). Activation of RB gene prevents E2F from taking the cell cycle forward (4,7,8). The p53 gene is considered one of the most effective tumor suppressor genes, and this is due to its important role in cell's genome stabilization to prevent mutations (7,8,60). P53 gene mutation contributes to inactivation of apoptosis and dysregulation of cell cycle. In addition, p53 mutations have been associated with tobacco smoking and a poorer prognosis (7,23).

1.8.3 p16

P16 is a cyclin- dependent kinase inhibitor that mainly inhibits CDK4 AND CDK6 and is considered a tumor suppressor. It prevents pRB phosphorylation, which eventually leads to cell cycle inhibition. P16 acts through the cell cycle as a regulator and plays important role in controlling cell cycle progression from G1 to S phase. P16 is encoded by the CDKN2A gene, which is frequently mutated in many types of cancer, including head and neck squamous cell carcinoma, and leads to p16 overexpression (4,47). P16 overexpression is associated with high-risk human papilloma virus infection in head and neck squamous cell carcinoma. Nowadays, p16 is used as a surrogate marker of HPV-positive head and neck tumors (1,52,61,62).

1.8.4 Ki67

Ki67 is a nuclear protein encoded by the MKI 67 gene (4). This protein plays an essential part in cell proliferation and in the clinical course of cancer (4,63). Presence of Ki67 in the active cell phases G1, S, G2, and M makes it a good biomarker for the cell proliferation in neoplastic tissue (63,64).

1.8.5 Bcl-xL

Bcl-xL is the abbreviation for B cell lymphoma extra-large protein; it presents exclusively in mitochondria membrane. Bcl-xL belongs to Bcl2 family, which works as an antiapoptotic protein by direct inhibition of Bax, the key activator of apoptosis. Over expression of the Bcl-xL gene has been identified in many types of cancer (1,65-67). In OCSCC, Bcl-xL is associated with resistance to radiotherapy treatment (68).

1.8.6 EGFR

EGFR is the abbreviation that stands for Epidermal Growth Factor Receptor, which is a glycoprotein receptor located at the cell membrane of normal cell, and it belongs to the family of

ErbB of tyrosine kinase receptor. This receptor binds to specific extracellular protein ligands, including transforming growth factor-a [TGF-a] and epidermal growth factor [EGF] (69). The complex of EGFR with its ligands generates autophosphorylation of receptor tyrosine kinase and intercellular signal transductions activation, which is responsible for cell cycle activation toward cellular proliferation and survival (13,69,70). EGFR is overexpressed in many types of cancer, including head and neck squamous cell carcinoma, though it varies between the subsites, and it is associated with poor prognosis and outcome (1,47,69,71). Mutations in EGFR will lead to inactivation of apoptosis and evolution of tumor (72), while the deletion mutations results in EGFR activation without the ligand stimulus (73). EGFR over activation is responsible for a major part in chemo and radiation therapy resistance in cancer cells. Cigarette smoking makes the EGFR overexpressed in oral cells, which is responsible for apoptosis inactivation and an increase in the proliferation in those cells, leading to eventual development of OCSCC (7).

1.9 Current Studies

"Oral cancers typically display an array of genetic alterations, many of which are likely to be secondary events in tumor development and the result of clonal evolution" (51: 913). There is a discrepancy in the literature in regards to the precise causative mechanism of OCSCC. Some molecules expressed in tumor are thought to be direct causative agents, while others are found to have indirect actions. The literature was also inconsistent due to the heterogeneity of studies with regards to the differing treatment protocols, study populations, methodology, tumor stages, and primary sites (55,74).

This lack of agreement in the literature is illustrated by the reported role of HPV OCSCC (93). Nemes et al. (75) have shown that oncogenic HPV types do not directly cause OCSCC. Equally, Branka et al. (51) showed a low prevalence of HPV in OCSCC. On the other hand, Lee

et al. (55) revealed that HPV infection prevalence, particularly HPV16, is higher in patients with early oral tongue cancer, T1 and T2, than among those in the control group. In addition, they found that infection with HPV16 is associated with deep tumor invasion. DA Silva et al. (76) likewise demonstrated, by using a PCR assay, that HPV infection prevalence is high (74%) in oral tongue cancers. Dahlgren et al. (77) have challenged this finding by reporting a low rate (2%) of HPV in a sample of 85 oral cancers, and they showed an insignificant relation between HPV infection and oral tongue cancer. A large study done by Syrjanen (61) involving 766 oral cavity cancers also showed a very low incidence of HPV infections (4%). Lastly, a meta-analysis done by Karemier et al. (74) on 5,046 patients involved in 60 different studies of head and neck cancers showed an overall HPV prevalence on HNSCC of 25.9%; the prevalence was significantly higher in oropharyngeal SCC compared to oral SCC. In addition, the most accurate method to detect HPV remains controversial, with most studies performing PCR and in-situ hybridization (23), while IHC technique has gained more popularity in the recent years with good detection results.

In sum, HPV infection plays a role in OCSCC, but its prevalence varies between the studies in the literature (51,61,75,78,79). HPV-related cancers have favourable outcomes and good responses to the treatment, mainly surgery and radiation (23).

1.10 Predictive Role and Prognostication of the Biomarkers in OCSCC

The roles of the molecules involved in OCSCC in the prediction of survival after treatment have not yet been fully investigated or understood.

1.10.1 Role of p16

Overall, there are few studies in the literature demonstrating the correlation between p16 overexpression and prognosis in patients with OCSCC. The majority of the studies have shown

the effect of p16 expression on the survival in those patients with oropharyngeal squamous cell carcinoma after treatment or have studied its effect over all HNSCC subsites with nothing specific for oral tumors (57,80,81).

Chandarana et al (17) have studied the relationship between p16 and EGFR expression with survival in patients with oropharyngeal and OCSCC who were treated with surgical resections. They utilized tissue from 49 patients with OCSCC and 36 patients with oropharyngeal SCC. Tissue was imbedded and immunostained using a tissue microarray. As a result, p16 was positive in 13% of patients with OCSCC. By contrast, with oropharyngeal SCC, 57% of the patients were p16 positive. The authors, therefore, could not assess the effect of p16 expression as a biomarker in the oral cavity on the survival outcome because of its low prevalence (13%). Hence, the authors stated p16 is less likely to be a surrogate marker for HPV status in oral cavity and emphasized that clinical nodal status, extra capsular spread, and perinural invasion are indicators of unfavorable prognosis in OCSCC. Lau et al. (57) showed that p16 and HPV-positive HNSCC patients responded better to treatment with Cisplatin and radiation than those who were negative, in terms of overall survival, disease-free survival, and locoregional recurrence rates, but this study is not OCSCC specific**.**

1.10.2 Role of EGFR

Chandarana et al. (17) showed that EGFR expression in OCSCC was positive in 63% of the patients after surgery. The authors did not find any effect of EGFR expression on the patients' survival outcomes; they showed no difference between EGFR positive and negative patients. This study had a small sample size (85 patients) which could explain why both p16 and EGFR expression on OCSCC patients showed no effect on survival outcomes. The same result was reached by Samid et al. (85) when they studied the effect on survival outcomes of EGFR

expression in OCSCC patients treated with curative surgery and post-operative radiotherapy. They found no association between EGFR expression and overall survival among 165 patients. Another study has shown that EGFR expression in OCSCC is one component of many others responsible for resistance to irradiation (68).

A study done by McIntyre et al. (73) examined the frequency of EGFR mutation expression in the form of EGFRvIII in OCSCC using a real-time PCR assay. The study showed a rare expression for the mutation in the examined specimens (2%) among 50 patients. The study concluded that anti-EGFR therapies are not suitable for targeting cancer in OCSCC as a first line treatment. In contrast, Smith et al. (14) found that high expression of EGFR may be correlated to increased radiation response in oral cavity and oropharyngeal SCC patients treated with total surgical resections and post-operative radiotherapy. Furthermore, in their cohort study, they demonstrated that high expression of EGFR is important to determine the loco-regional disease recurrence and that EGFR was a better outcome predictor than classical TNM staging. Although the results of their study showed novel information, the investigators admitted that the sample size of their study was too small (56 patients) and in need of larger samples in future studies to verify their results.

In general, overexpression of EGFR in head and neck cancers is not found equally in all the subsites (1). Some studies suggested that EGFR is associated with poor prognosis among HNSCC patients treated with surgery and post-operative radiotherapy, while others showed no association especially in case of OCSCC. (1,17,82,85)**.**

1.10.3 Role of Bcl-xL

Mallick et al. (9) studied the expression of the Bcl-xL protein as a prognosis predictor on oral cancer patients treated with curative radiotherapy. The study involved 39 patients treated

with curative radiation. Expression of the Bcl-xL protein in the biopsy was examined by using immunohistochemistry, and patients were monitored after curative treatment. Their results showed a high expression of Bcl-xL is correlated to poor prognosis (low survival rate) and with aggressive tumor behaviour. On the other hand, low Bcl-xL expression is associated with better overall prognosis. The authors raised a key observation that Bcl-xL protein is considered an inhibitor of radiation-induced apoptosis in oral cancers. Furthermore, the authors concluded their study by emphasizing the importance of Bcl-xL protein along with clinical parameters on stratifying oral cancer patients who are likely to get cured by radiotherapy. In addition, the authors stressed that radiation treatment in those showing low Bcl-xL should be reduced to avoid unwanted side effects.

1.10.4 Role of Ki67

There was a lack of consensus between the studies that investigated the effect of Ki67 expression on OCSCC survival. Some studies have revealed that Ki67 is a leading factor in neck metastasis and cancer recurrence (86-89, as cited in 16). On the other hand, other reports stated that there is no association between the two (90, 91, as cited in 16). Klimowicz et al. (16) have attributed this discrepancy to the difference between the approaches used to identify Ki67 expression in those studies. Klimowicz et al. (16) studied the relation between different measures of Ki67 expression and survival in OCSCC patients; all of the patients were treated with primary surgery, and some of them received adjuvant radiotherapy based on the surgical pathology. Their study was the first one to report that over expression of Ki67 in OCSCC patients is associated with improving survival rate. This was observed mainly in those patients who were treated with surgery and radiation. According to the authors, this is explained by the fact that expression of Ki67 in cancer cells is associated with a high proliferation rate, which makes those cells very

sensitive to DNA damage-inducing radiation. Furthermore, low expression of Ki67 in OCSCC specimens was associated with poor response to radiotherapy.

1.10.5 Role of p53

Mallick et al. (9) have analyzed the effect of p53-altered expression in oral cancer cells. The first observation during this study was that high p53 protein expression is higher in females than their male counterparts. Secondly, p53 intensity has shown strong relation with smokers versus non-smokers. After definitive radiotherapy, patients who showed high p53 expression in their pre-treatment biopsies did not exhibit any difference versus those who were not. Indeed, this study showed no significant impact of p53 on overall survival post treatment. In contrast, other studies in literature have reported that high p53 expression is associated with poor a prognosis in oral cancer patients after radiotherapy treatment (8). Tandon et al. (62) investigated the presence of mutant p53 as a prognostic factor in oral cavity, oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma. An important observation was raised by the authors who said, “The prognostic value of p53 in patients with squamous cell carcinoma of the head and neck is inconclusive” (62: #). They attributed the discrepancy between the studies that examined p53 as a prognostic factor in the literature to the heterogeneity in study-level and patient characteristics. Furthermore, the authors stressed the importance of p53 expression in HNSCC and the need for future studies to examine every separate anatomical subsite.

1.11 Limitations in the Literature

There were several limitations to some studies in the literature. The studies tended to be heterogeneous with small sample sizes, included other head and neck sites, and utilized various methods to analyze biopsies for target molecules in OCSCC. Certain studies investigated the

effects of one molecule in a chosen treatment modality, while others investigated several molecules on one treatment.

1.12 The Study

There is no literature examining OCSCC survival that takes into account both differences in treatment and molecular profiles. This study addresses this important question and may, therefore, provide novel insight into the optimal treatment of OCSCC by molecular triaging of patients into the most appropriate protocols. Although this study is a retrospective, it would represent the highest level of clinical evidence for this study question, as randomized controlled trials comparing surgical versus non-surgical modalities in head and neck cancer have been shown to be unattainable given the nature of the treatment differences.

1.12.1 Hypothesis

I hypothesized that patients with certain biomarker profiles would have better response to surgical treatment protocols with improved survival.

1.12.2 Objectives and Research Plan

1) To establish biomolecular profiles (HPV/p16, EGFR, Bcl-xL, p53, and Ki67) for OCSCC patients.
2) To determine survival outcomes of OCSCC patients according to biomolecular profile and treatment regimens.

1.12.3 Inclusion Criteria and Methods

1) Patients diagnosed and treated at the University of Alberta for OCSCC between 1998 and 2010.
2) Patients greater than the age of 18.
3) Patients not meeting these basic criteria were excluded.

1.12.4 Anticipated Results

It was anticipated that patients who are treated with triple modality (surgery and chemoradiation) would have the best survival outcomes. Within all treatment groups, I would also expect those who are positive for p16 (HPV) to have improved outcomes. Based on other reports, I predicted tumors with low levels of Bcl-xL, p53, and EGFR, and high levels of Ki67 would be associated with superior outcomes. I anticipated a subset of patients with particular combinations of biomarker levels, not yet described, would have superior outcomes with particular treatment combinations.

Chapter Two: Methods and Materials

2.1 Patients Identifications

Institutional Research Ethics Board approval from the University of Alberta Health Research Ethics Board was obtained prior to commencement of the project. Demographic, pathological, and survival data of the patients diagnosed with OCSCC and treated at the University of Alberta, Edmonton, Canada, from 1998 to 2010, approximately 584 patients, were retrieved from the Alberta Cancer Board, and some added data were obtained by a comprehensive chart review. The study's inclusion criteria for this cohort were including patients above age of 18 with histologically confirmed OCSCC from different TNM stages. The vast majority of the study patients were treated primarily with surgical resection of their tumors along with neck dissection. Further adjuvant treatment, including radiation, chemotherapy, or the combination of both, was determined by the final pathological results.

2.2 Tissue Microarray Construction

Tissue microarray (TMA) is a method where tissues or cell components are re-located from multiple pre-existing donor paraffin wax blocks and assembled in one recipient paraffin wax TMA block. The main concept of using tissue microarray (TMA) technique is applying miniaturization and a high throughput approach to the analysis of tissues (94). Fortuna, Furmanski, and Wan first described this method in 1987. It allows analysis of multiple tissues taken from different patients on one slide, which ensures a high level of analysis quality, especially in immunohistochemistry staining procedure (94). It is used usually to examine the molecular expression in any tissue sample from a disease or tumor (95). Formerly, the main application of TMA was in cancer research, but nowadays it has been widely used in different

pathological purposes (94). Not like in the past, which was mainly strict to solid tumors, TMA can be used now in different types of tumors (94).

TMA technique is gaining momentum in today's practice, particularly in analysis of protein expression. This technique offers many advantages that would facilitate the standardization of IHC staining protocols and eliminate the variability in the staining results between the different tissue sections (94). However, TMA as a technique has been criticized for using tiny cores "biopsies," ranging between 0.5 and 1 mm, from donor blocks to construct one TMA block and that could result in missing the area of interest, tumor areas mostly, and affect the final staining results. To solve this issue, TMA experts use multiple cores from the same tissue section, three cores on average, to ensure that the area of interest is being represented in the recipient TMA block.

In this study, pre-treatment pathological specimens from the study population were retrieved and marked for tissue microarray processing (TMA) with the assistance of the study's pathologist, Dr. Lakshmi Puttagunta. Marked specimens were used to construct TMA blocks, by taking two cores of 1.5 mm from each donor block, with the assistance of the histopathology lab technicians in Li Ka Shing Centre at University of Alberta by using the Tissue Microarray Master tool. In this project, seven TMA recipient blocks have been constructed from 187 OCSCC donor blocks, and I included six controls cores in each TMA block: three positive and three negative for the Oropharyngeal Squamous Cell Carcinoma.

2.2.1 The steps of constructing the TMA blocks from multiple donor blocks in the laboratory

1. Precise selection of the donor tissue blocks and their corresponding H&E stained slides from University of Alberta tissue bank, and then retrieving their clinicopathological data from the University of Alberta hospital archive.
2. Reviewing the H&E slides with the pathologist to identify and mark the tumor area in each slide.
3. Marking the same area in the corresponding block.
4. Using Tissue Microarray Master tool to punch each block twice and taking two needle core biopsies of 1.5 mm each.
5. Transferring multiple cores from multiple patients to one TMA block into ready-made holes.
6. After completing the TMA block, an Excel worksheet was created to fill up the patients' data on the same order that their cores had been assembled in the TMA block, to identify them easily by knowing the location of each core.
7. Covering the constructed block with paraffin wax and baking the block in an oven at 60 degree-Celsius overnight.
8. Suctioning the block into thin slices, 5 micrometer each, by using microtome and fixing them into microscope glass slide, to prepare the slide for IHC staining; it was scanned under the digital fluorescent microscope, Aperio Scanscope, to identify the expressed OCSCC markers.

Figure 2-1. Donor IHC slide and paraffin blocks used to construct the TMA blocks and the suctioned TMA slide

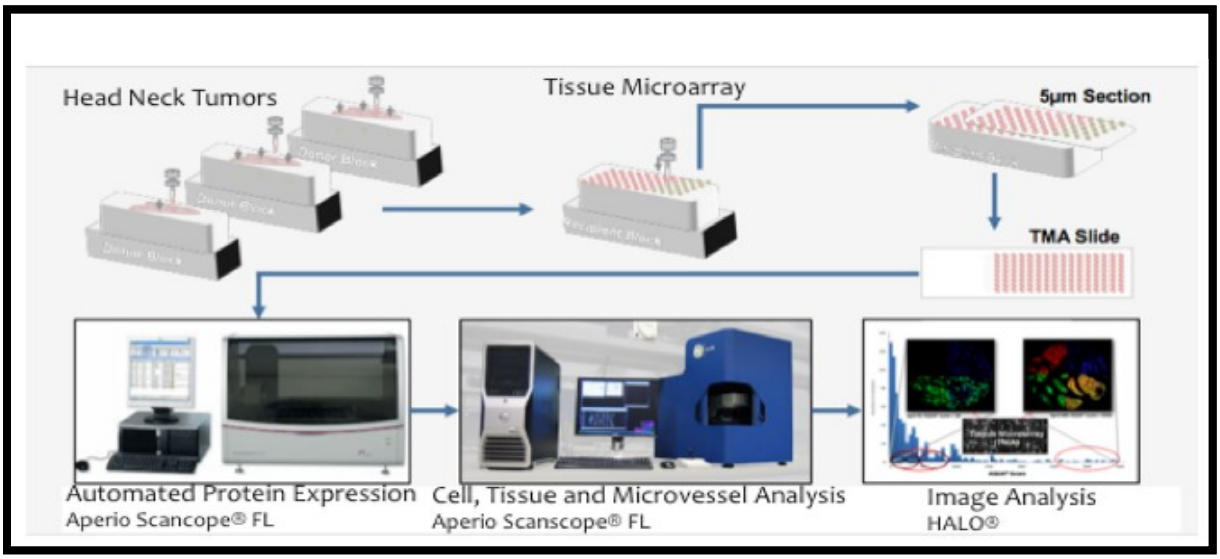


Figure 2-2. Illustration of TMA construction steps.

Note: Summary of the steps that have been followed in the project from using donor paraffin blocks to construct the TMA blocks that were suctioned on slides for IHC staining going through visualizing the molecular expression of a certain molecule in cytoplasmic and nuclear compartment on a digital image using Aperio Scanscope FL, and then measuring the intensity of the molecular expression using HALO software.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) is basically utilizing tissue sections to show the location and distribution of specific proteins by applying highly specific antibodies that have the affinity to bind these proteins to accomplish the staining procedure (96). The tumor tissues utilized in

this study were pre-treatment specimens taken from OCSCC patients and stored in FFPE blocks. These donor blocks were used to assemble TMA blocks. It is possible to use microtome to suction one TMA block into TMA slides up to 300 times with average thickness of 5 micrometer per slide. I used TMA slides to do IHC staining and examine the expression of the marker of interest in the slide.

Before I proceeded with the IHC staining to identify the expressed marker, I prepared the slide through two essential steps in IHC, deparaffinization and antigen retrieval, which can ensure a reliable staining result and ease the interaction between the antibody molecule and the specific antigen. All the steps of IHC were followed as recommended by Abcam, the supplier of the antibodies utilized in the study.

2.3.1 Deparaffinization and Rehydration

This step is fundamental when treating paraffin-embedded tissue sections to remove the paraffin, which can interfere with IHC staining and produce a poor staining result (96).

Method

Wash with Xylene 2 times, 3 min each.

Wash with xylene 1:1 100% ethanol once for 3 min.

Wash with 100% ethanol twice for 3 min each.

Wash with 95% ethanol once for 3 min.

Wash with 70% ethanol once for 3 min.

Wash with 50% ethanol once for 3 min.

Rinse in cold tap water for 10 min, while you heat up the antigen retrieval solution.

Once deparaffinized, slides should not be allowed to dry to prevent non-specific binding and eventually high background staining.

2.3.2 Antigen retrieval

During formalin fixation process of storing cancer tissues in blocks to save the tissue's structure and antigenicity, methylene bridges can be formed and mask the antigenic epitopes in the tissues. Therefore, an antigen retrieval step is required before proceeding with IHC staining in most occasions. There are two methods to perform antigen retrieval in tissue sections: (a) heat-induced epitope retrieval (HIER) and (b) proteolytic (enzymatic) induced epitope retrieval (PIER). Using either one depends on trials of optimization and which works best for the specimens; optimization of the reagents is always advised. Enzymatic retrieval method can damage the morphology of the tissue sections due to its destructive features and ultimately impact adversely on the staining results (96). In the lab, I and my assistants performed this step by following a heat-mediated epitope method using a rice cooker with EDTA PH 8.0 buffer.

Method

Place the slide rack in 1 mM EDTA PH 8.0 and boil it in a rice cooker for 22 min.

After boiling the slide, let cool to room temperature over 10-15 minutes.

2.3.3 IHC Staining

Immunohistochemistry (IHC) is a procedure that shows the expression of a target biomarker in a tissue section, by utilizing specific antibodies that have the affinity to bind specific antigens and visualize it (96). This procedure is highly useful in cancer diagnosis, progression, and optimizing the treatment. There are two different detection systems that can locate and identify the cell compartments where the antigen of interest exists and shows a positive signal. The first system is demonstrated using a fluorescent dye, which is essentially a fluorophore that is conjugated to the secondary antibody. It has the ability to re-emit fluorescent light at the area where the secondary antibody binds to the primary antibody, which is on the

other side bound to the antigen. The light signal of the positive staining can be then captured using a manual fluorescent microscope or digital imaging system (96). The other detection system is based on a chromogenic reaction, where a chromogenic dye is usually used to react with an enzyme conjugated secondary antibody and yield coloured-products that indicate a positive staining. Both systems were applied in the study, the fluorescence IHC and chromogenic IHC; the latter was used in detecting the p16 positive cells. The other four antigens in this study were identified using the fluorescent dyes.

Method

1. Permeabilize cells by washing the slides with 1X PBS + Triton 100-X (0.1%) for 10 minutes
2. Block with 10% normal serum + 0.1% Triton 100-X + 1% BSA in 1X PBS at RT for 1 h
3. Use PAP, Hydrophobic pen, to mark and surround the whole tissue area
4. Apply primary Ab diluted in 1%BSA in 1X PBS for 1 h at RT, or incubate overnight in fridge at 4°C.
5. Wash three times, 5 min each, with 0.1% Triton 100-X in 1X PBS.
6. Apply secondary Ab diluted in 1%BSA in 1X PBS at RT for 1 h.
7. Wash three times, 5 min each with 0.1% Triton 100-X in 1X PBS
8. Dry the slides with a piece of tissue then mount the slide using mounting media, prolong gold anti-fade with DAPI (around 60-100 ul) on a cover slide, and cover it. Do this slowly and avoid bubble formation under the thin glass cover.
9. Keep the slides in a dark box.

- It is recommended to make the blocking and washing solutions fresh before you use them. Sometimes, the solution can be stored at 4°C in the fridge up to one week; however, the risk of bacteria/fungal growth is always there.
- Using mounting media in the last step would ensure preserving the stained tissue morphology and retard photobleaching over a long period of time, in addition to enhancing the image quality. In the lab, I used the mounting media mixed with DAPI, the nuclear counterstain that has specific binding affinity to the nucleus components, DNA and RNA, with very low affinity to the cytoplasmic compartments; however, some researchers prefer not to use the mounting media mixed with DAPI to avoid the high background non- specific staining in the sections.
- In the case of p16 chromogenic IHC staining, the above-mentioned steps are followed exactly the same, with two additional steps added to the routine protocol. The first additional step is blocking the endogenous peroxidase activity in the tissue specimen by incubating the slide with Hydrogen Peroxide (H_2O_2) for 10 minutes before applying the secondary antibody that is conjugated to Hoarse Radish Peroxidase (HRP). This step will ensure that no non-specific staining will show in the sample, and only the specific primary antibody is binding to the secondary antibody without any other antigen that might express a peroxidase activity in the tissue sample. The second added step takes place before mounting the slide with the mounting media, where DAB (3, 3'-diaminobenzidine) is added to the tissue to react with the Hoarse Radish Peroxidase enzyme and produces a brown coloured precipitate in the area where p16 is expressed in the cell.

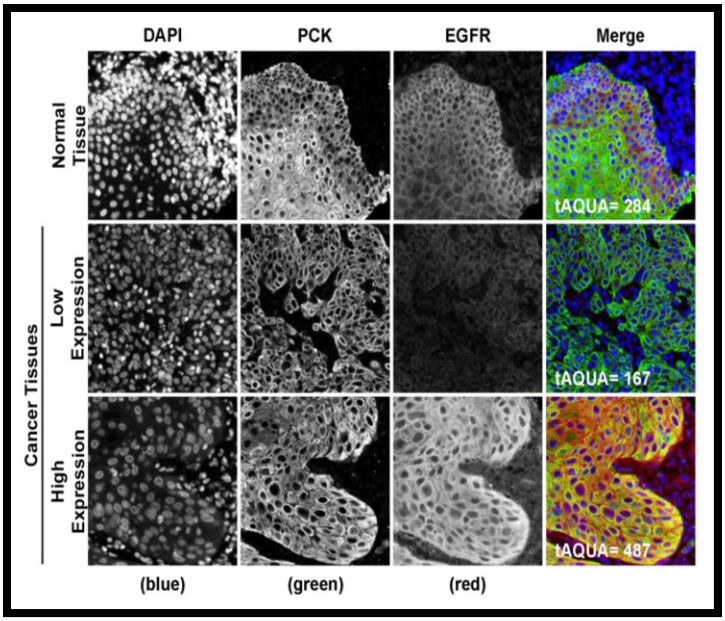


Figure 2-3. Fluorescent IHC digital image produced by Aperio Scan scope.

Note: The example shows EGFR expression in the normal and cancer tissue in both cytoplasmic and nuclear compartment, pink stain. DAPI stain was used to visualize the nuclear expression of EGFR, as it stains the nuclei, blue stain. Pan cytokeratin (PCK) was mainly utilized to show the cancer compartment in the SCC tissue, green stain.

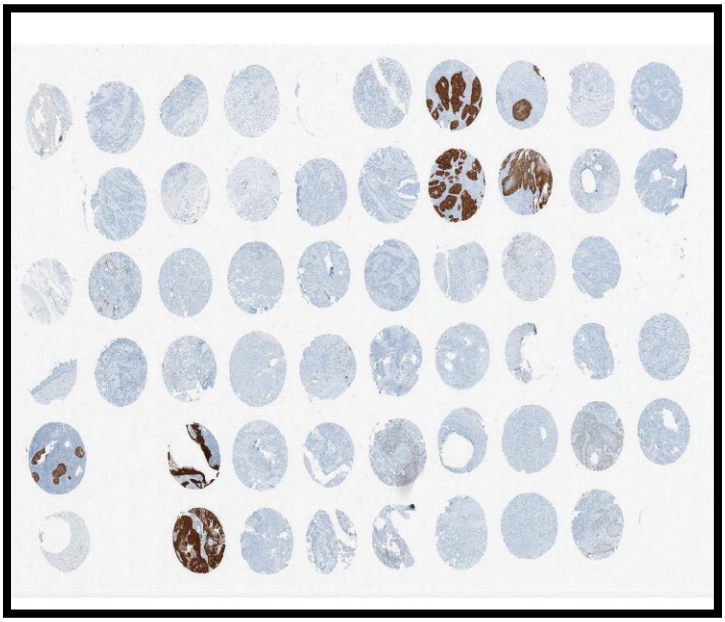


Figure 2-4. Chromogenic IHC of p16.

Note: Chromogenic IHC staining performed to show p16 expression, with standard Aperio Scanscope image showing positive (brown precipitate) and negative (faded blue) expression of the molecule.

2.4 Antibodies, Fluorescent, and Chromogenic Dyes

Abcam supplied the lab with all the primary and secondary antibodies, fluorophore, and chromogenic dyes used in the experiment. Antibody optimization was done at the early stage of

the experiment for all of the five antigens, based on the supplier's dilution recommendations for paraffin-embedded sections for immunohistochemistry staining.

Table 2-1. *Primary Antibodies Concentration*

Primary Antibody	Dilution
Rabbit Monoclonal Ab to p53	1/1000
Rabbit Monoclonal Ab CDKN2/p16/INK 4a	1/100
Rabbit Monoclonal Ab to EGFR	1/200
Rabbit Polyclonal Ab to Ki67	1/400
Rabbit Polyclonal Ab to Bcl-xL	1/1000

The primary antibodies are diluted in 1% BSA in PBS with 0.1% Triton 100-X is added. Because all five primary antibodies have been raised in rabbit species, Goat Anti-rabbit Alexa Fluro 647 that is conjugated to a fluorophore, was used as the secondary antibody to show the binding of the primary antibody with the antigen of interest as a light stained area. The secondary antibody was used in different concentrations diluted in 1% BSA in PBS and 0.1% Triton 100-X based on which primary Ab had been used. DAB also was used as a chromogenic dye to detect the reaction between the HRP conjugated secondary antibody and the anti-p16 primary antibody in the nucleus where p16 positively existed.

2.5 Detergents and Solutions

Table 2-2. *Detergents and Solutions Composition*

Solution	Recipe
Xylene	Stock solution
100% ETOH	Stock solution
Xylene 1:1 100%ETOH	To make 100 ml, add 50 ml Xylene to 50 ml 100%ETOH
95% ETOH	To make 100 ml, add 95 ml of 100% ETOH to 5 ml dd H_2O
70% ETOH	To make 100 ml, add 70 ml of 100% ETOH to 30 ml dd H_2O
50% ETOH	To make 100 ml, add 50 ml of 100% ETOH to 50 ml dd H_2O
1X EDTA, Antigen retrieval solution	To make 500 ml, add 450 dd H2O to 50 ml of 10X EDTA stock solution
1X PBS	Stock Solution, or by adding 10 ml of 10X PBS to 100 ml MQH2O
1% BSA in 1X PBS	To make 500 ml, add 5 g BSA to 500 mL PBS
5% BSA in 1X PBS	To make 500 ml, add 25 g to 500 ml PBS
0.1% Triton in 1X PBS	To make 1 L, add 5 ml Triton 100X to 995 1X PBS
10X EDTA PH 8.0	Stock Solution

The solutions presented in Table 2-2 were used to wash the TMA slides for deparaffinization after being suctioned from the TMA blocks and, in the following two steps, antigen retrieval to expose the epitope sites and immunohistochemistry staining.

2.6 Digital Imaging of the TMA slides

After getting the TMA slide stained and prepared for identifying the type and quantity of the expressed marker, it was scanned using a new digital florescent slide scanner, Aperio Fluorescence Scanscope. This innovative scanner provides a high-resolution scanning and produces a high-quality digital image. The scanscope was provided to the lab by Lecia Biosystems, and it came with a full package of software that enabled scanning, sharing, and compressing the slides (97). Moreover, I was able to set up different software on the scanscope

that helped in quantifying the expressed markers and worked simultaneously to analyze the components of the tissue sections. Using this digital fluorescence microscope would save more time and effort compared to manual scopes, and it ensures a high-standardized analysis of tissues sections, which ensures minimizing the variability between the different slides measured for a specific antigen of interest.

2.7 Controls

Although TMA procedure has been in use for decades, interalaboratory and interlaboratory variations in the IHC staining results are still a major issue (94). This is perhaps due to the lack of the consensus in IHC staining protocols between different labs. Therefore, including positive controls where a tissue is known to have the antigen of interest, and negative controls where a tissue does not normally have this antigen expressed in the same quantity, has become a standard procedure in IHC protocols. Using negative and quantifiable positive controls tissue sections in all IHC incubations will improve the procedure quality, as described in many previous studies, and can immediately discover the variations in the staining results (94,96). I have included three positive oropharyngeal squamous cell carcinoma control cores, as well as three normal oropharyngeal squamous epithelium cores as a negative control to each TMA block. Also, I considered testing the reliability of the detection procedure and protocols by performing a reagents control with only using secondary antibody. Moreover, I considered testing the non-specific background staining by scanning each TMA slide before every staining experiment just to make sure that the cores did not display any natural fluorescence. The purpose of these measurements was to validate the staining results of the experiment.

2.8 Quantitative Immunohistochemistry and Scoring System

This step was performed in the lab using HALO software, which is a quantification tool that can objectively assess the nuclear and cytoplasmic molecular expressions intensity of a fluorescent digital image. This software works simultaneously with the Aperio scanscope and can be integrated with the e-slide manager, the slide viewer software, to interpret the staining result of the digital images. By assigning the simple sitting, I classified the intensity signal of up to three different dyes on one slide to negative, weak positive, moderate positive, and strong positive signals. Then, the data can be pulled into an Excel spreadsheet and used in any statistical software for further analysis and correlation.

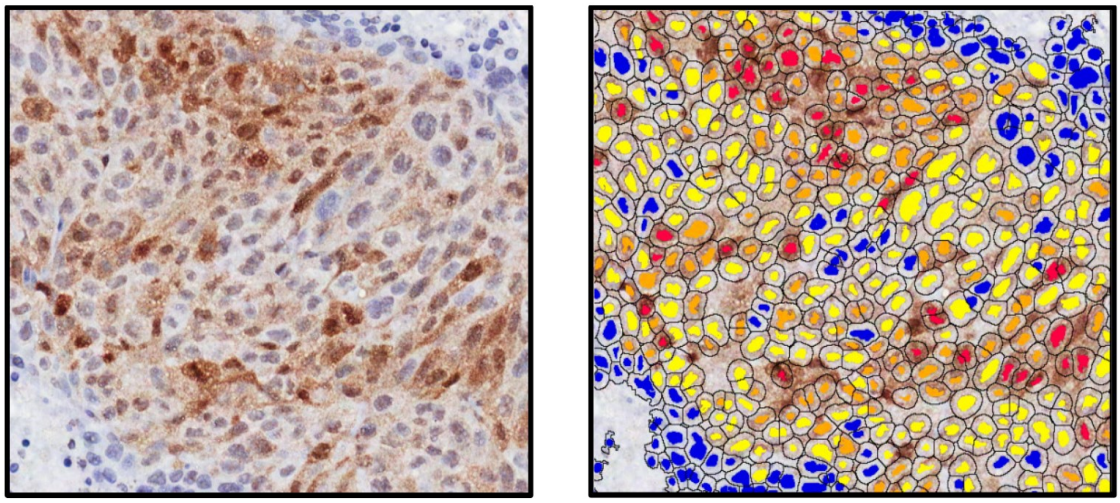


Figure 2-5. Digital IHC and markup imaging by the digital scanscope.

Note: The digital image produced by Aperio Scanscope after analyzing the TMA IHC slide for the antigen (molecule) of interest. The left-side image: the cytoplasmic IHC staining of the tumor. The right-side image: the markup digital image by HALO showing negative expression of the molecule (coloured in blue), weak expression (coloured yellow), moderate expression (colored orange), and strong expression (colored red).
(Source: *HALO User Guide* by Indica Lab, date, Corrales, NM)

A histogram representation showing the intensity of the molecule expression and the scoring system criteria used in the study for each TMA slide for each different molecule is

presented in Appendix A. The blue line represents the median expression of a given molecule in normal tissue (negative control), and the red line represents the median expression of the same molecule in the cancer tissue. The expression is considered negative if it is below the median expression of the molecule in the normal tissue and positive if it is above that median line. High positive versus low positive expression of any given molecule was calculated based on the median expression of that molecule in the cancer tissue, with tissue samples that showed molecular expression above the median of the positive cancer cells labelled as high positive, and tissue samples that showed molecular expression below the positive cancer cells median line were labelled as low positive.

2.9 Statistical analysis

All statistical analyses were done with SPSS version 24.0. A 2-tailed *p* value of 0.05 or less was considered statistically significant. Patient clinical and pathological data with tumor characteristics are represented in Tables 2-1 and 2-2. Semi-quantitative scoring and expression of p16 was assigned based on tumor proportion staining of the tissue suctions according to American College of Pathologists guidelines, with cut-off value of 75% positive staining at least for the tissue suctions to be considered positive for p16 with the help of the pathologist in the study (L.P). The expression of the other four molecules (p53, Ki67, EGFR, and Bcl-xL) and their scoring was assigned based on the intensity of the expression to negative, low positive, and high positive. The median of the negative control sample was the cut-off between whatever was considered positive and negative, while the median of the positive cores in each TMA block was the cut-off to consider low versus high positive expression of each of the molecule. The Kaplan-Meier survival curves were used to compare the intensities of different biomarkers' expression with the

OS and DSS. The Log Rank and Breslow statistical tests were used to compare the different survival curves within and between categories of discrete variables. Only patients who had sufficient specimen for immunostaining (more than 20% tissue) were considered for further analysis.

2.10 Technical Challenges

Although utilizing these highly technical tools and software tools would help accelerate the analysis process of the tissue slides and make it more standardized throughout the experiment, there are some technical limitations that could affect the work and impact the final result. In the TMA block construction step, it is known that during needle punch of the area of interest to get cores to array them in the recipient block, handling a decalcified donor block is more difficult than their non-decalcified counterpart. Moreover, decalcified specimens are anticipated to show some different staining results attributable to the harsh acidic chemical reagents that are routinely used for the decalcification process of bone-containing sections to remove the calcium deposits and make the sections suitable with the embedded media to save it in a block. However, I identified those decalcified samples in this cohort in advance on the assumption that it may produce contrastive results. Additionally, before the scanning step, the TMA slide should be prepared in a way that makes it clean, with no air bubbles under the cover slips, to prevent any false negative or positive signals, as the scan scope is too sensitive to dirty slides with air bubbles beneath the coverslips.

Chapter Three: Results

3.1 Patients and Tumor Characteristics

The cohort consisted of 187 patients who were eligible to proceed with the analysis. Their pre-treatment biopsies and/or surgical specimens along with their demographic data were retrieved from the Alberta Cancer Registry. The patients in this cohort have undergone either single or combined modality of treatment according to their TNM staging with curative intent. The clinicopathological data of the cohort are illustrated in Table 3-1. After constructing the TMA blocks, and before commencing the IHC staining, the pathologist in the study (L.P.) re-evaluated the cores and made certain each had tumor cells in it by examining the corresponding TMA H&E slides under microscope. The results of IHC staining with regards to the molecular expression are presented in Table 3-2.

Table 3-1. *Clinicopathological Characteristics for OCSCC*

Participant Demographics	Number of Responses	% (*N* =187)
Age: Mean (*SD*)	15	56.3
Gender		
Male	112	59.9
Female	65	40.1
Smoking history:		
Non-Smoker	67	35.8%
Smoker	120	64.2%
Treatment		
Surgery	86	46.0%
Surgery+RT	73	39%
Surgery + CRT	20	10.7%
CRT	2	1.1%
RTx6(3.2%)		
Subsites		
Tongue	91	48.7%
Hard Palate	1	0.5%
Lip	2	1.1%
Floor of mouth	47	25.1%
Alveolar ridge	17	9.1%
Retromolar Trigon	29	15.5%
Pathological Stage		
Early	98	52.4%
Advanced	89	47.6%

Table 3-2. *Molecular Expression Characteristics*

Characteristic	Number of Respondents	% (*N* = 187)
p16		
Negative	180	96.3%
Positive	7	3.7%
EGFR		
Negative	22	11.8%
Positive	165	88.2%
p53		
Negative	85	45.5%
Positive	102	54.5%
Ki67		
Negative	42	22.5%)
Positive	145	77.5%
Bcl-xL		
Negative	20	10.7%
Positive	167	89.3%

3.2 Single Molecular Expression

3.2.1 p16 survival analysis

IHC staining for p16 in the clinical settings is performed using the chromogenic technique of staining. With 3,3'-diaminobenzidine (DAB) compound primarily used to stain the nucleic acids and visualize the positive protein expression in the nuclei (Figures 3-1 and 3-2). Kaplan-Meier survival curves were utilized in the study to show the relationship between the intensity of p16 expression, whether positive or negative, and patient's survival outcomes expressed in five years overall and disease specific survival rates. Log Rank and Breslow statistical tests are the two main tests that are widely used for comparison of survival curves with their *p* value results to show the significance of association if present.

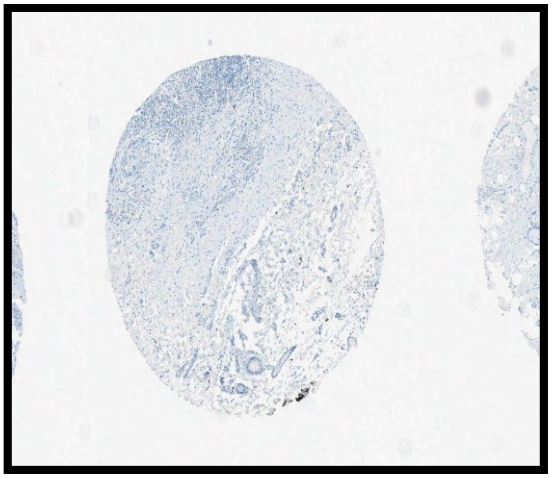


Figure 3-1. Negative p16 expression on chromogenic IHC staining of cancer cells.

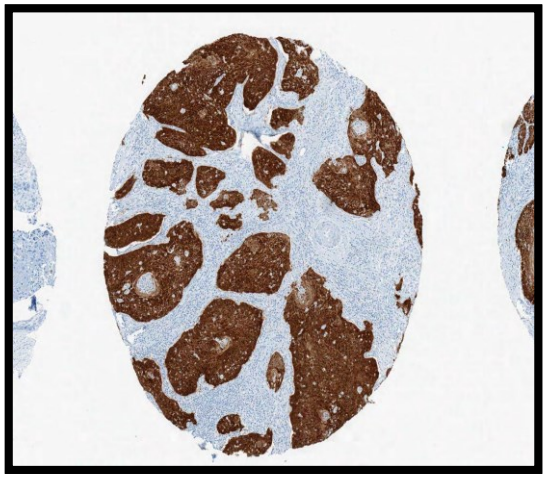


Figure 3-2. Positive p16 expression on chromogenic IHC staining of cancer cells.

P16 positivity prevalence was only 3.7% in OCSCC patients of this cohort. For overall survival curves of p16, there was no significant difference between the negative and positive expression of the molecule. Although, the negative p16 cohort had an improved survival rate trend compared to p16 positive cohort towards the end of the 5-year period, I cannot conclude that p16 negative has a favourable prognosis over p16 positive expression in OCSCC patients when I know that the majority of the samples (180 out of 187) showed negative p16 staining (Figure 3-3).

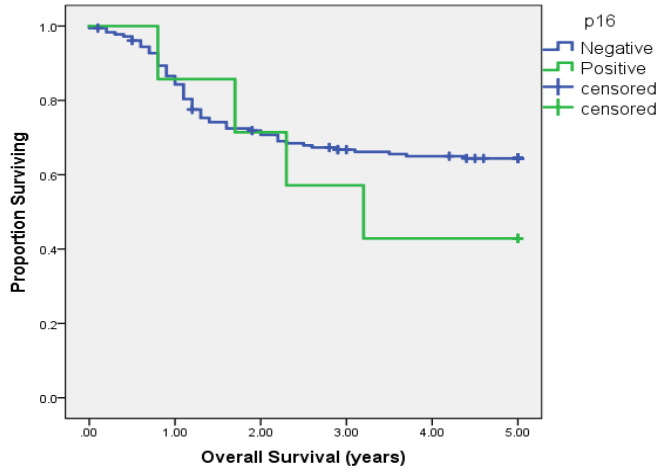


Figure 3-3. Kaplan-Meier overall survival curve of p16.

Note: Kaplan-Meier survival curves of OCSCC patients showing the overall survival rate in regards to p16 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule with no statistically significant association. $p > 0.05$

For further analysis of p16 overall survival curves, patients were dichotomized to early and advanced stage of the disease, and I examined the difference between the negative and positive expression of the molecule in each stage (Figures 3-4 and 3-5). In an early stage of OCSCC, patients with a positive p16 had an improved survival outcome compared to patients with a negative p16. Three out of 98 patients in the early stage of the disease had positive p16 staining, and their survival rate during the 5-year period was up to 100%; however, that was statistically insignificant. In an advanced stage of OCSCC, negative p16 expression patients had a better survival outcomes compared to the patients with positive p16, especially at the end of the 5-year period, with *p* value close to the significance level with 0.073 in Log Rank test, which gave the same weight to all death events regardless of the time at which the event occurred. Again, the conclusion cannot be established on whether the negative or positive p16 has a favourable prognosis over the other when I know that there were only four patients out of 89 who had a positive p16 staining, and the rest were negative in advanced stages.

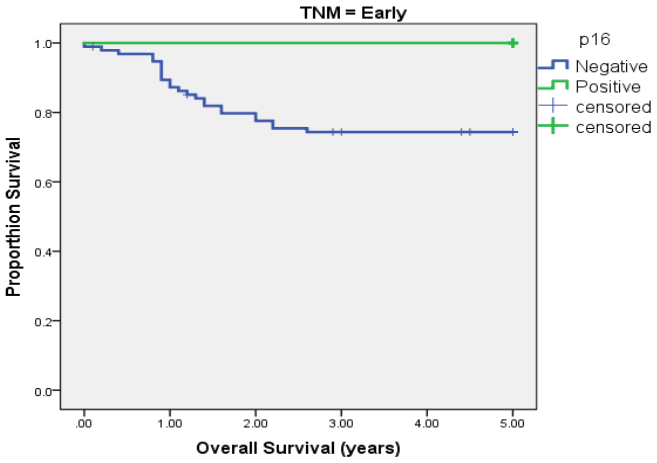


Figure 3-4. Kaplan-Meier overall survival curve of p16 in early stage.

Note: Kaplan-Meier survival curves of OCSCC patients in early stage of the disease showing the overall survival rate in regards to p16 expression. Patients with positive expression of the molecule had improved survival rates compared to patients with negative expression of the molecule with no statistically significant association. $p > 0.05$

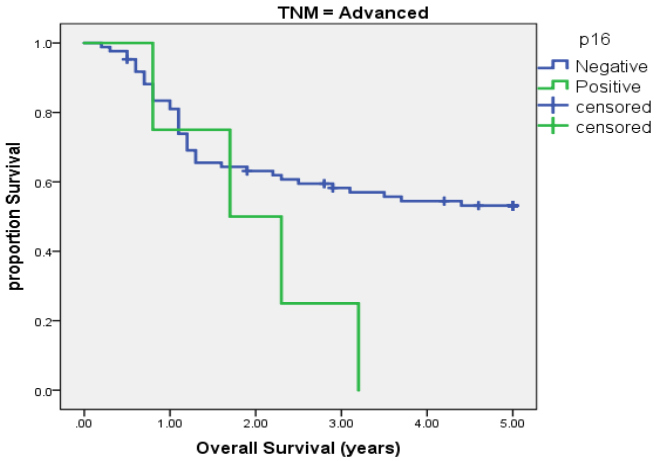


Figure 3-5. Kaplan-Meier overall survival curve of p16 in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC patients in advanced stage of the disease showing the overall survival rate in regards to p16 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule with no statistically significant association. $p = 0.073$

For disease-specific survival (DSS) curves of p16, patients with positive expression of the molecule had slightly improved survival outcomes overall (Figure 3-6). When dichotomized to early versus advanced stages of the disease, patients with positive expression of p16 still showed an improved survival compared to patients with negative p16 in early stages of OCSCC, while in the advanced stages, negative p16 still showed a favourable prognosis, without any statistically significant associations in both stages between the different curves (Figures 3-7 and 3-8).

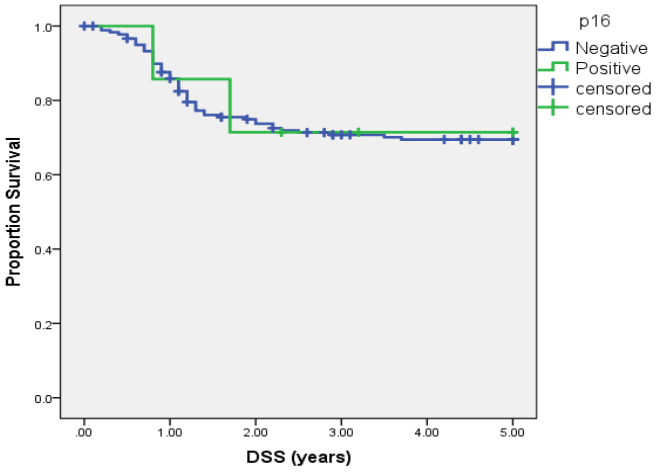


Figure 3-6. Kaplan-Meier DSS of p16

Note: Kaplan-Meier survival curves of OCSCC patients showing the disease specific survival (DSS) rate in regards to p16 expression. Patients with positive expression of the molecule had improved survival rates compared to patients with negative expression of the molecule towards the end of the 5-year period, with no statistically significant association. $p > 0.05$

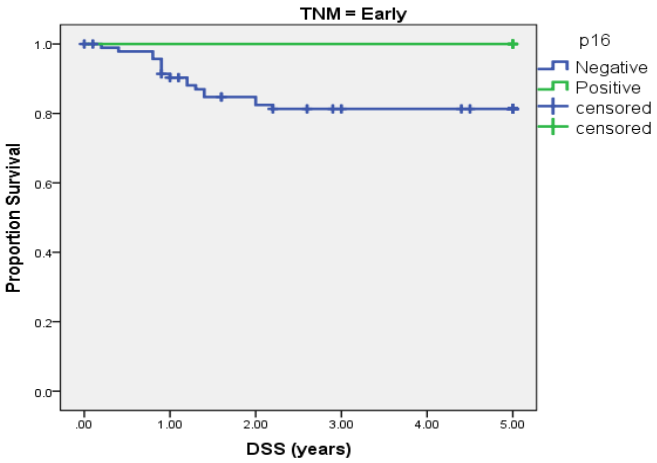


Figure 3-7. Kaplan-Meier DSS of p16 in early stage.

Note: Kaplan-Meier survival curves of OCSCC patients in early stage of the disease, showing the disease specific survival (DSS) rate in regards to p16 expression. Patients with positive expression of the molecule had improved survival rates compared to patients with negative expression of the molecule, with no statistically significant association. $p > 0.05$

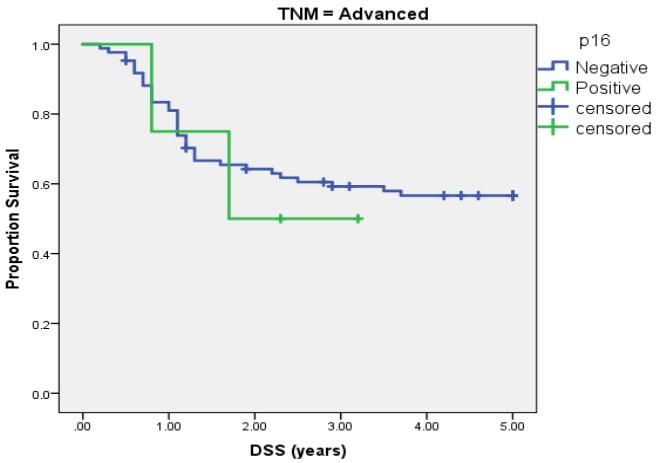


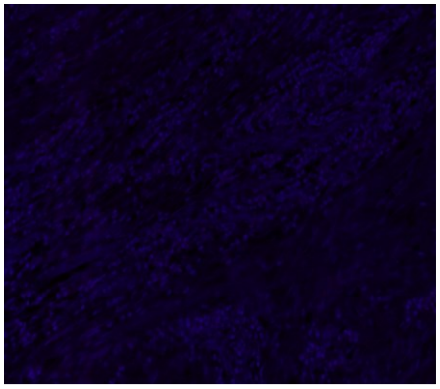
Figure 3-8. Kaplan-Meier DSS of p16 in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC patients in advanced stage of the disease, showing the disease specific survival (DSS) rate in regards to p16 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

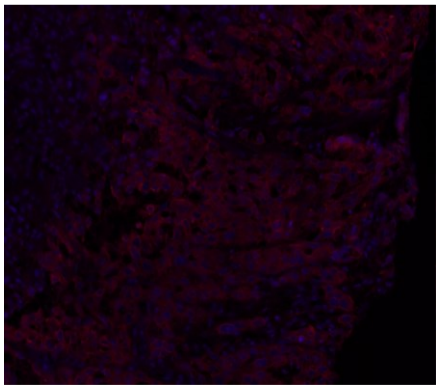
3.2.2 EGFR survival analysis

IHC staining for EGFR in the clinical settings was performed using fluorescence technique of staining. EGFR primarily exists in the cytoplasmic compartment of the cell, and visualizing the positive protein expression in the cytoplasm requires using primary and secondary antibodies as described above in the IHC-staining technique section (Figures 3-9a and 3-9b). PCK stain was performed in the study only in conjunction with EGFR to show the tumor compartment in the tissue just as a confirmation step to show that the TMA tissue sections have SCC (Figures 3-9c and 3-9d). Kaplan-Meier survival curves were utilized in the study to show the relationship between the different intensities of EGFR expression, whether high positive, low positive or negative, and patients' survival outcomes expressed in five years overall and disease-specific survival rates. Log Rank and Breslow statistical tests are the two main tests that are widely used for comparison of survival curves with their *p* value results to show the significance of association if present.

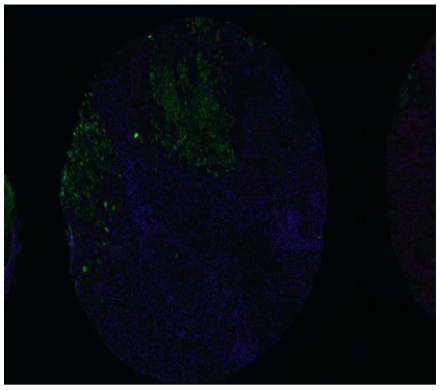
EGFR positivity prevalence was 88.2% in OCSCC patients of this cohort (165 patients of 187). For overall survival curves of EGFR, there was no statistically significant association between the negative and low/high positive expression of the molecule that affected survival. Although, the negative EGFR cohort had trends of improved survival rates compared to EGFR positive cohort throughout the period of the five years with close to significant level of *p* value (0.085 in Log Rank test between negative and high expression) (Figure 3-10). For further analysis of EGFR overall survival curves, patients were dichotomized to early and advanced stages of the disease, and I examined the difference between the negative and positive expression of the molecule in each stage (Figures 3-11 and 3-12).



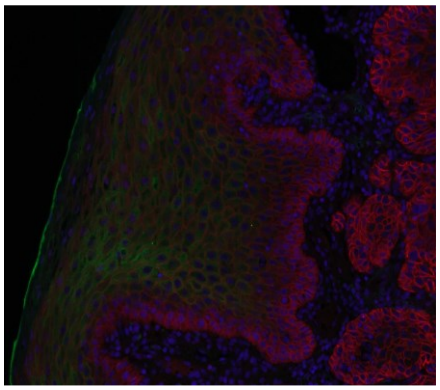
(a)



(b)



(c)



(d)

Figure 3-9. EGFR IHC staining intensities.

Note: EGFR fluorescence IHC staining of OCSCC tissue: a) Negative EGFR expression in the cytoplasmic compartment, b) Positive EGFR expression in the cytoplasmic compartment. c) Negative cytoplasmic expression of EGFR in a positive PCK staining background, d) Positive EGFR expression in a positive PCK staining background. PCK staining was performed to identify the squamous cell carcinoma component in the tissue section.

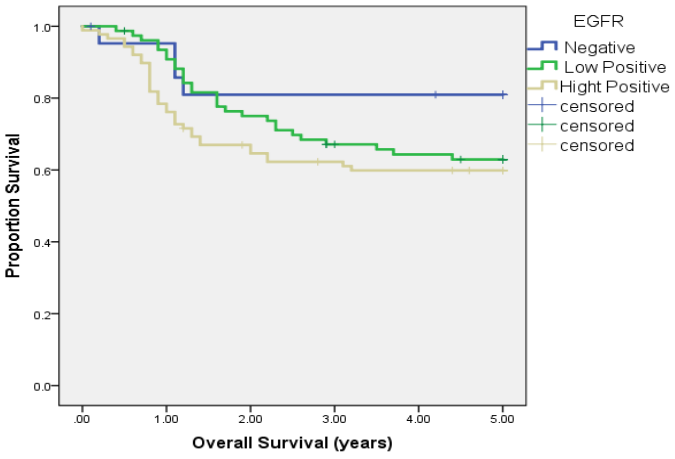


Figure 3-10. Kaplan-Meier overall survival curve of EGFR.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of EGFR expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, and patients with low-positive expression had a slightly improved survival rates compared to patients with high positive expression of EGFR, without any association being a statistically significant. $p > 0.05$

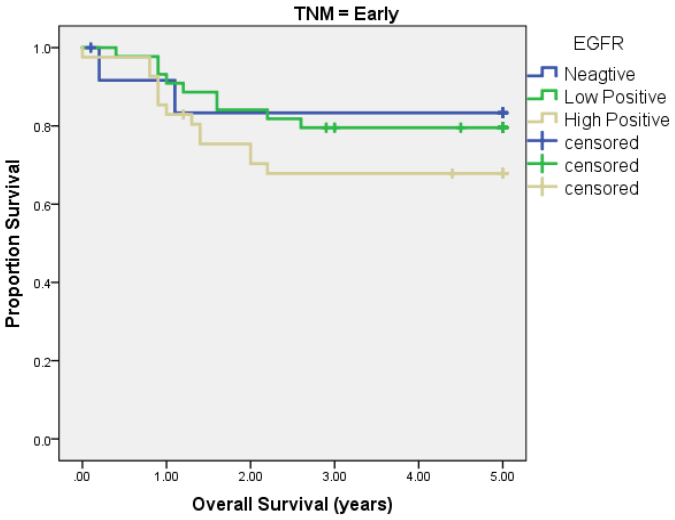


Figure 3-11. Kaplan-Meier overall survival curve of EGFR in early stages.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of EGFR expression in the early stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, and patients with low-positive expression had a slightly improved survival rate compared to patients with high-positive expression of EGFR, without any association being a statistically significant. $p > 0.05$

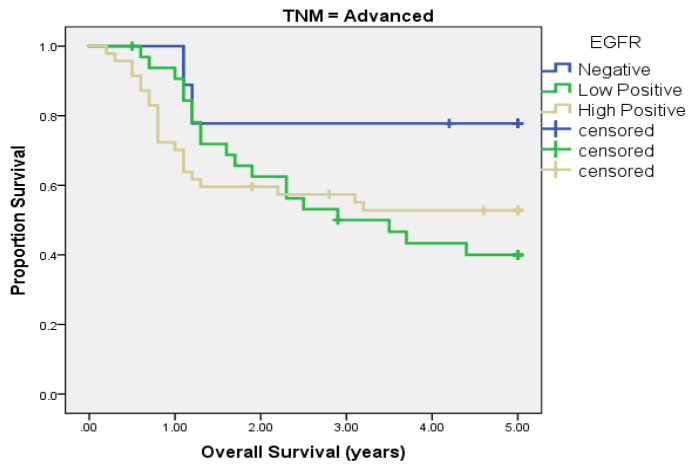


Figure 3-12. Kaplan-Meier overall survival curve of EGFR in advanced stages.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of EGFR expression in the advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule; however, patients with low-positive expression had a slightly poor survival rate compared to patients with high-positive expression of EGFR, without any association being a statistically significant. $p > 0.05$

In early stage of OCSCC, patients with negative EGFR had improved survival outcome compared to patients with positive EGFR, with statistically insignificant association. In an advanced stage of OCSCC, negative EGFR expression similarly had better survival outcomes compared to the patients with positive EGFR during the 5-year period, with *p* value of insignificant level in both Log Rank and Breslow tests. The conclusion cannot be established if the negative EGFR has a favourable prognosis over the positive expression or not in any given disease's stage.

For disease-specific survival (DSS) curves of EGFR, patients with positive expression of the molecule had poorer survival outcomes compared to patients with negative expression of the molecule, with *p* value of 0.023 and 0.029 in Log Rank and Breslow tests respectively. Moreover, patients with high positive expression of EGFR had poorer survival outcomes compared to patients with low-positive EGFR expression profiles, *p* value 0.038 (Figure 3-13). Overall, 88 patients out of 165 positive EGFR patients showed high positive expression. Among these, 36 patients were treated with surgery followed by radiation, and 35 patients had surgery

alone, whereas only nine patients underwent triple modality of treatment surgery with chemoradiation. Of the 77 patients with low EGFR expression who showed improved DSS, 43 of them had been treated with surgery alone, 25 patients with combined surgery and radiation, and only nine patients had surgery followed by chemoradiation. When dichotomized to early versus advanced stages of the disease, patients with low-positive expression of EGFR had significantly improved DSS compared to high-positive expression of the molecule in early stage of OCSCC, with $p = 0.004$ and $p = 0.006$ in Log Rank and Breslow tests respectively (Figure 3-14). While the relationship between the negative and low-positive EGFR was unclear, in the advanced stages, negative EGFR still showed a favourable prognosis over positive EGFR profiles, without any statistically significant associations between the different intensity curves (Figure 3-15).

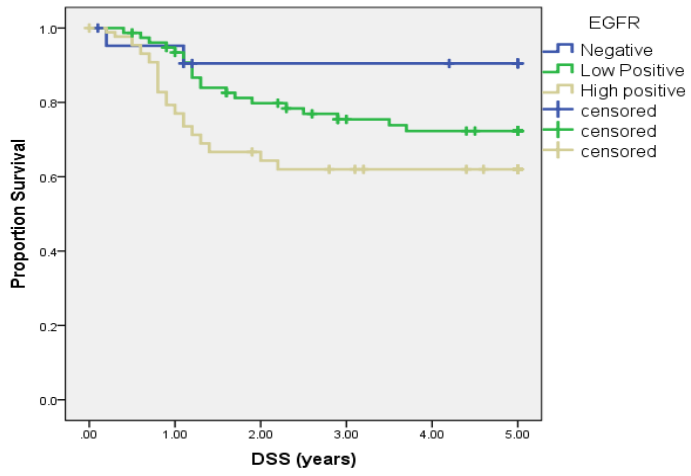


Figure 3-13. Kaplan-Meier DSS curve of EGFR.

Note: Kaplan-Meier survival curves of OCSCC showing the disease specific survival rate (DSS) in regards with different intensities of EGFR expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, and patients with low positive expression had a slightly improved survival rates compared to patients with high positive expression of EGFR, with both associations being statistically significant. $p = 0.023$ and 0.038 respectively.

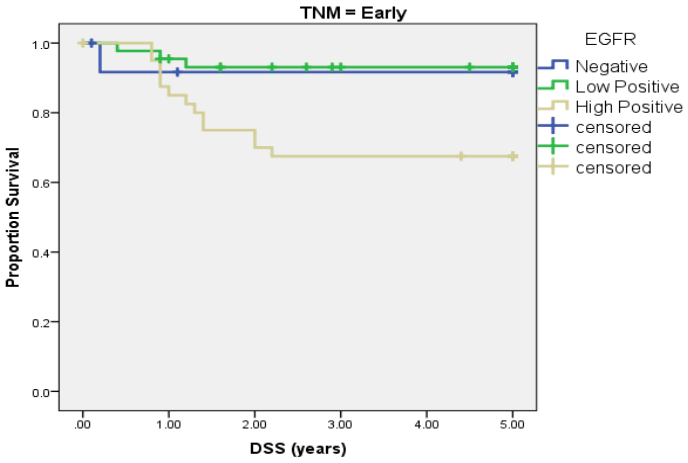


Figure 3-14. Kaplan-Meier DSS curve of EGFR in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease specific survival rate (DSS) in regards with different intensities of EGFR expression in early stage of the disease. Patients with low-positive expression of the molecule had improved survival rates compared to patients with high-positive expression of the molecule—a statistically significant association. $p = 0.004$

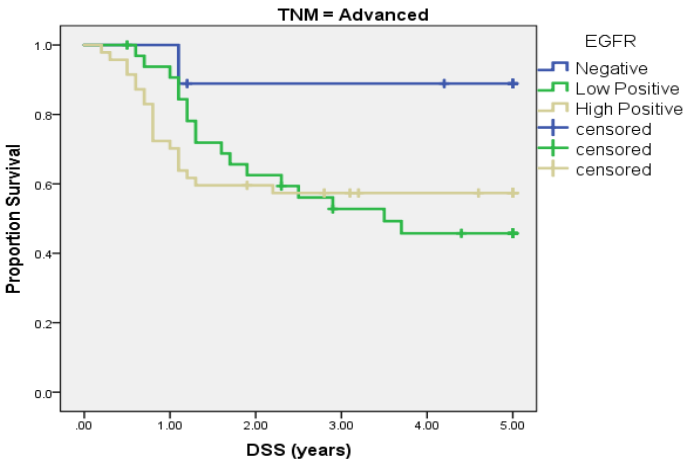


Figure 3-15. Kaplan-Meier DSS curve of EGFR in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease specific survival rate (DSS) in regards with different intensities of EGFR expression in advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with low and high-positive expression of the molecule, with no statistically significant association. $p > 0.05$

3.2.3 Ki67 survival analysis

IHC staining for Ki67 in the clinical settings is performed using fluorescence technique of staining. Ki67 primarily exists in the nuclear compartment of the cell, and visualizing the positive protein expression in the nucleus requires using primary and secondary antibodies for the protein and special nuclear counterstain (DAPI) to capture the conjugation between the primary antibody with the antigen of interest in the nucleus as described above in the IHC staining technique section (Figure 3-16 and 3-17). Kaplan-Meier survival curves were utilized in

the study to show the relationship between the different intensities of Ki67 expression, whether high positive, low positive, or negative, and patients' survival outcomes expressed in five years overall and disease-specific survival rates. Log Rank and Breslow statistical tests are the two main tests that are widely used for comparison of survival curves, with their *p* value results to show the significance of association if present.

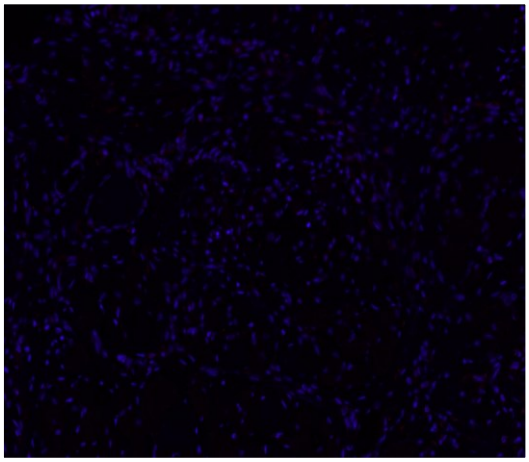


Figure 3-16. Negative Ki67 expression on IHC staining.

Note: Negative expression of Ki67 on fluorescence IHC staining of the oral cavity squamous cell carcinoma tissue sections.

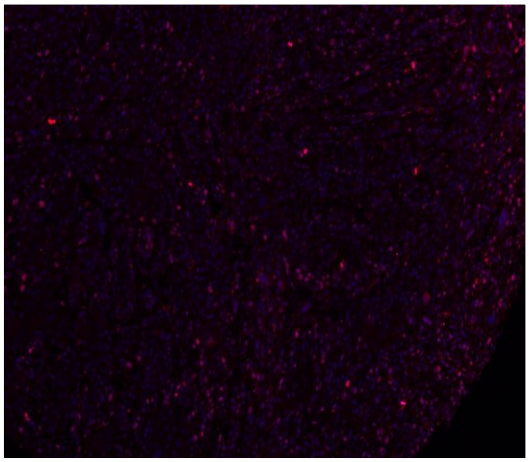


Figure 3-17. Positive Ki67 expression on IHC staining.

Note: Positive expression of Ki67 on fluorescence IHC staining of the oral cavity squamous cell carcinoma tissue sections.

Ki67 positivity prevalence was 77.5% in OCSCC patients of this cohort (145 patients of 187). For overall survival curves of Ki67, there was no statistically significant association between the negative and low/high positive expression of the molecule that affected survivals.

Although, negative Ki67 cohort had improved survival rates compared to Ki67 positive cohort more prominent towards the end of the 5-year period, it was statistically insignificant (Figure 3-18). For further analysis of Ki67 overall survival curves, patients were dichotomized to early and advanced stage of the disease, and I examined the difference between the negative and positive expression of the molecule in each stage (Figures 3-19 and 3-20). In the early stage of OCSCC, patient with negative Ki67 had improved survival outcomes compared to patients with positive Ki67 (particularly low-positive profiles), with statistically insignificant association ($p = 0.067$ and 0.053 in Log Rank and Breslow tests respectively). In the advanced stage of OCSCC, negative Ki76 expression had no effect on the overall survival, with no clear pattern between the three different intensities of the molecule expression throughout the 5-year period with p values of insignificant level in both Log Rank and Breslow tests.

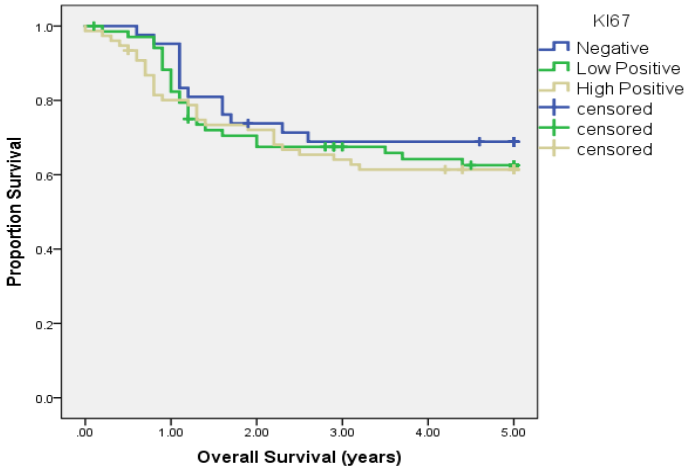


Figure 3-18. Kaplan-Meier overall survival of Ki67.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Ki67 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significance association. $p > 0.05$

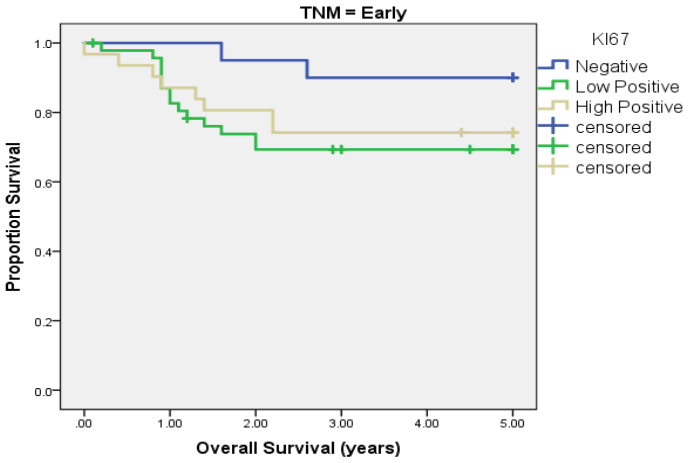


Figure 3-19. Kaplan-Meier overall survival of Ki67 in early stages.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Ki67 expression in early stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule; however, that remains a statistically insignificance association. $p = 0.067$

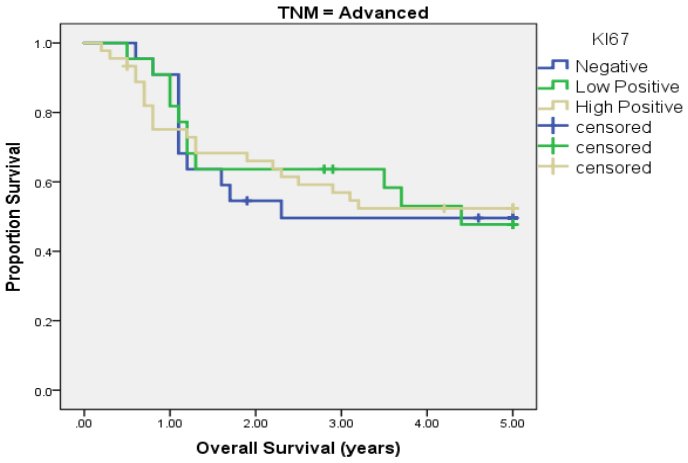


Figure 3-20. Kaplan-Meier overall survival of Ki67 in advanced stages.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Ki67 expression in an advanced stage of the disease. Patients with negative expression of the molecule had similar survival outcomes compared to patients with positive expression of the molecule, with no statistically significance association. $p > 0.05$

For disease-specific survival (DSS) curves of Ki67, patients with positive expression of the molecule had poorer survival outcomes compared to patients with negative expression of the molecule, with insignificant *p* value in Log Rank and Breslow tests (Figure 3-21). When dichotomized to early versus advanced stages of the disease, patients with negative expression of Ki67 had significantly improved DSS compared to low and high expression of the molecule in early stage OCSCC ($p = 0.018$ and 0.037 respectively), with an almost 100% survival rate among the patients in the 5-year period (Figure 3-22). Those patients with negative Ki67 in early

disease stage have been identified to be 20 patients; 17 of them underwent primary surgical interventions without any adjeovant therapies, and three patients had surgical resections followed by radiation. While the relationship between the negative and positive Ki67 was unclear in the advanced stages, negative Ki67 showed no favourable prognosis over positive Ki67 profiles, without any statistically significant associations between the different intensity curves (Figure 3-23).

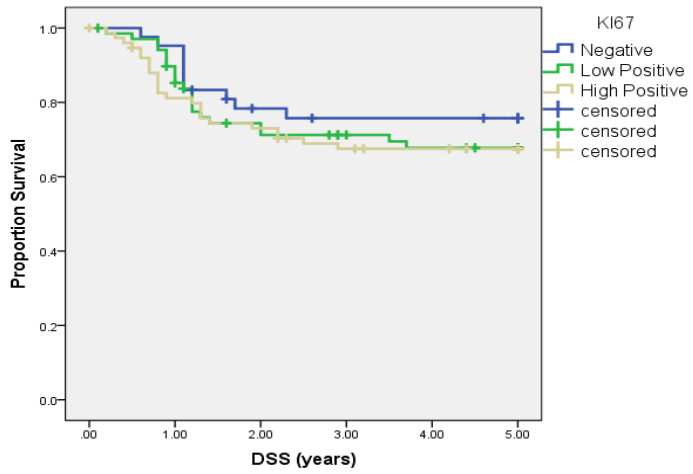


Figure 3-21. Kaplan-Meier DSS curve of Ki67.

Note: Kaplan-Meier survival curves of OCSCC showing the disease specific survival rate (DSS) in regards with different intensities of Ki67 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significance association. $p > 0.05$

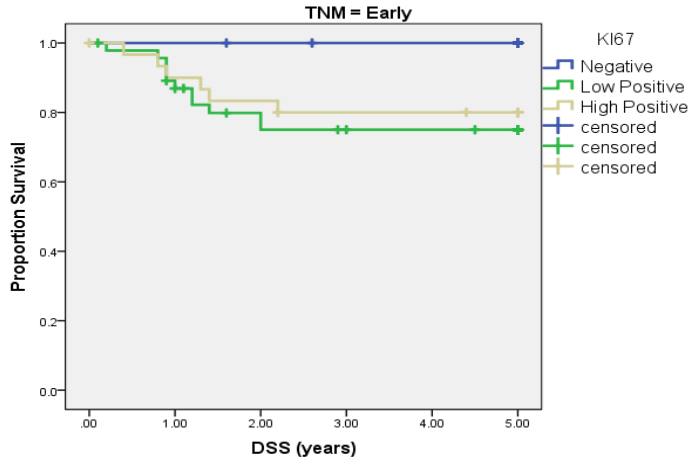


Figure 3-22. Kaplan-Meier DSS curve of Ki67 in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Ki67 expression. Patients with negative expression of the molecule had statistically significant improved survival rates compared to patients with positive expression of the molecule. $p = 0.018$

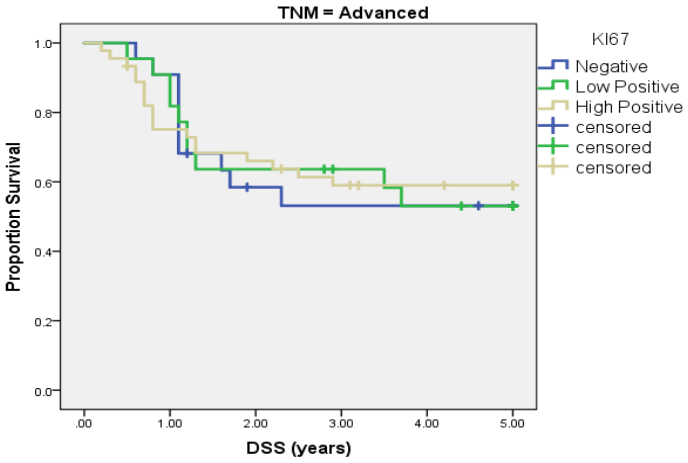


Figure 3-23. Kaplan-Meier DSS curve of Ki67 in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Ki67 expression in advanced stage of the disease. Patients with negative expression of the molecule showed no favourable survival outcomes compared to patients with positive expression of the molecule. $p > 0.05$

3.2.4 p53 survival analysis

IHC staining for p53 in the clinical settings was performed using fluorescence technique of staining. The p53 gene primarily exists in the nuclear compartment of the cell, and visualizing the positive protein expression in the nucleus requires using primary and secondary antibodies for the protein and special nuclear counterstain (DAPI) to capture the conjugation between the primary antibody with the antigen of interest in the nucleus, as described in the IHC staining technique section (Figures 3-24 and 3-25). Kaplan-Meier survival curves were utilized in the study to show the relationship between the different intensities of p53 expression, whether high positive, low positive or negative, and patients survival outcomes expressed in five years overall and disease-specific survival rates. Log Rank and Breslow statistical tests are the two main tests that are widely used for comparison of survival curves with their p value results to show the significance of association if present.

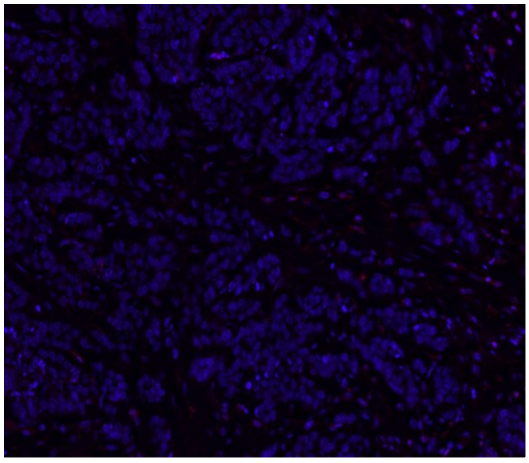


Figure 3-24. Negative p53 expression on IHC staining.

Note: Negative p53 expression on fluorescence IHC staining of oral cavity squamous cell carcinoma tissue sections.

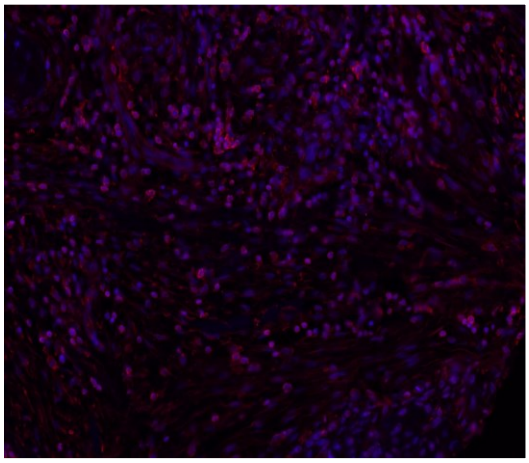


Figure 3-25. Positive p53 expression on IHC staining.

Note: Positive p53 expression on fluorescence IHC staining of oral cavity squamous cell carcinoma tissue sections

p53 positivity prevalence was 54.5% in OCSCC patients of this cohort (102 patients of 187). For overall survival curves of p53, the negative expression cohort had significantly improved survival rates compared to p53 positive cohort being more prominent towards the end of the 5-year period, with *p* value of 0.049 and 0.025 in Log Rank and Breslow tests respectively (Figure 3-26). For further analysis of p53 overall survival curves, patients were dichotomized to early and advanced stage of the disease, and I examined the difference between the negative and positive expression of the molecule in each stage (Figures 3-27 and 3-28). In early stage OCSCC, patients with negative p53 had no difference in the survival outcome compared to patients with positive p53, with statistically insignificant association. In advanced stage OCSCC,

negative p53 expression had improved overall survival compared to the high-positive expression of the molecule throughout the 5-year period, with *p* values of 0.046 in Breslow test. Breslow tests imply more weight on the death events that have occurred in early point in the 5-year survival time.

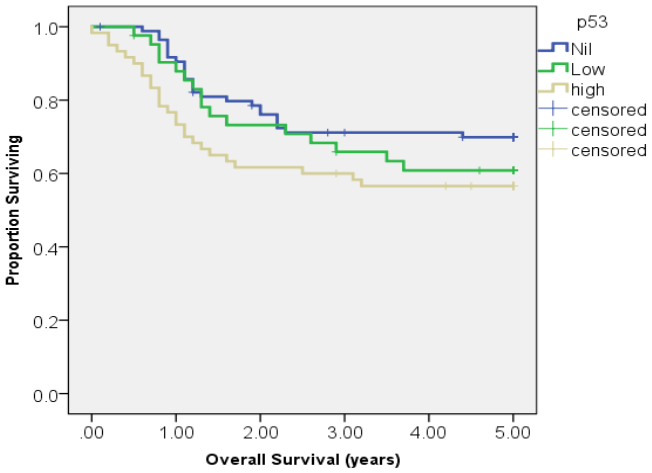


Figure 3-26. Kaplan-Meier overall survival curve of p53.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of p53 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with high positive expression of the molecule, with statistically significance association. $p = 0.049$

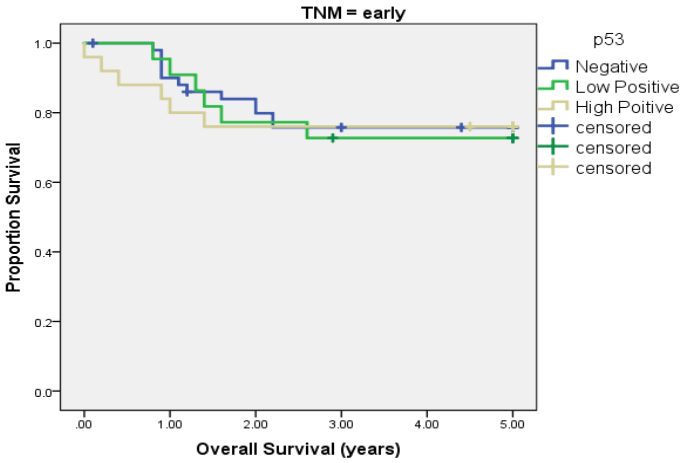


Figure 3-27. Kaplan-Meier overall survival curve of p53 in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of p53 expression in the early stage of the disease. Patients with negative expression of the molecule had no difference survival outcomes compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

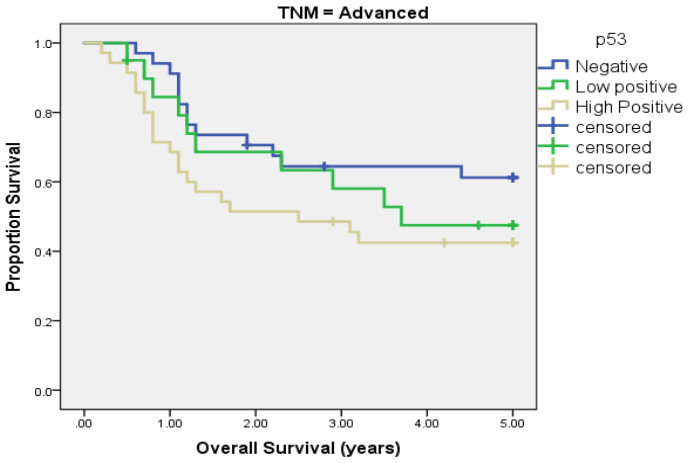


Figure 3-28. Kaplan-Meier overall survival curve of p53 in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of p53 expression in advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with high-positive expression of the molecule, with statistically significant association. $p = 0.046$

For disease-specific survival (DSS) curves of p53, patients with negative expression of the molecule had significantly improved survival outcomes compared to patients with high-positive expression of the molecule with *p* value in Log Rank and Breslow tests of 0.034 and 0.019 respectively (Figure 3-29). When dichotomized to early versus advanced stages of the disease, patients with negative expression of p53 had significantly improved DSS compared to low and high expression of the molecule in advanced stage of OCSCC, with $p = 0.044$ and 0.030 in Log Rank and Breslow tests respectively (Figure 3-31). While the relationship between the negative and positive p53 was unclear in the early stages, negative p53 showed no favourable prognosis over positive p53 profiles without any statistically significant associations between the different intensity curves (Figure 3-30).

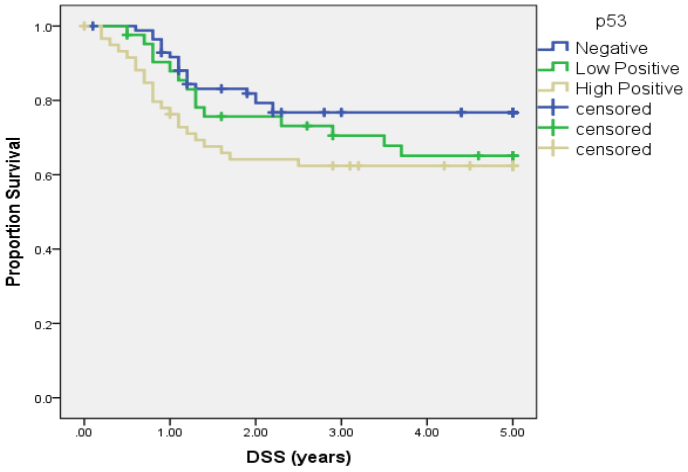


Figure 3-29. Kaplan-Meier DSS curve of p53.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of p53 expression. Patients with negative expression of the molecule had improved survival rates compared to patients with high-positive expression of the molecule with statistically significant association. $p = 0.034$

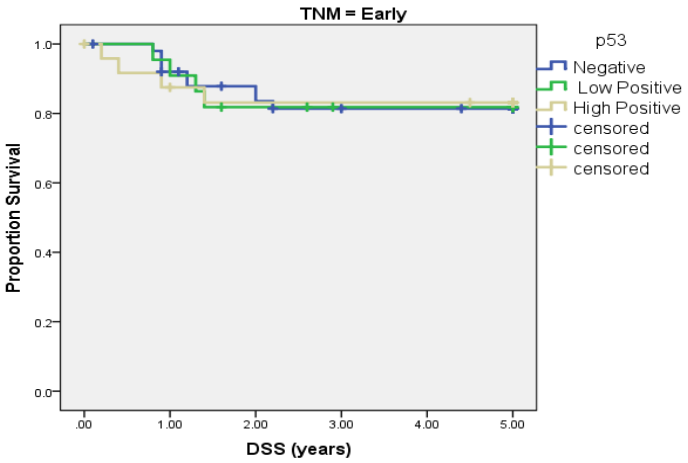


Figure 3-30. Kaplan-Meier DSS curve of p53 in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of p53 expression in early stage of the disease. Patients with negative expression of the molecule had no difference in the survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

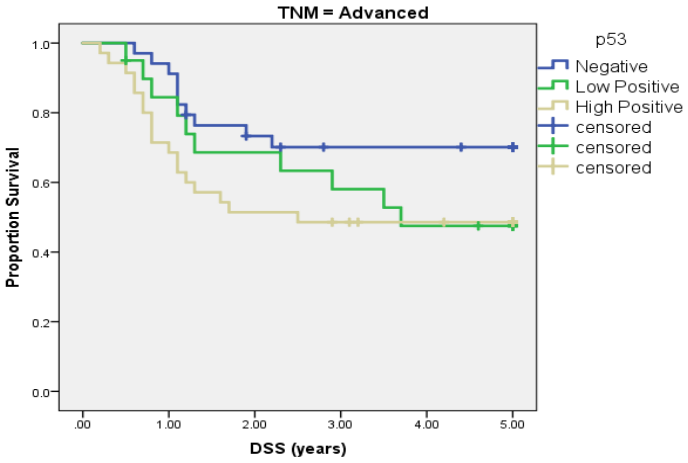


Figure 3-31. Kaplan-Meier DSS curve of p53 in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of p53 expression in advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with high-positive expression of the molecule, with statistically significant association. $p = 0.044$

3.2.5 Bcl-xL survival analysis

IHC staining for Bcl-xL in the clinical settings was performed using the fluorescence technique of staining. Bcl-xL primarily exists in the cytoplasmic compartment of the cell, and visualizing the positive protein expression requires using primary and secondary antibodies for the antigen to capture the conjugation between the primary antibody with the antigen of interest in the cytoplasm as described in the IHC staining technique section (Figures 3-32 and 3-33). Kaplan-Meier survival curves were utilized in the study to show the relationship between the different intensities of Bcl-xL expression, whether high positive, low positive, or negative, and patients' survival outcomes expressed in five years overall and disease-specific survival rates. Log Rank and Breslow statistical tests are the two main tests that are widely used for comparison of survival curves, with their *p* value results to show the significance of association if present.

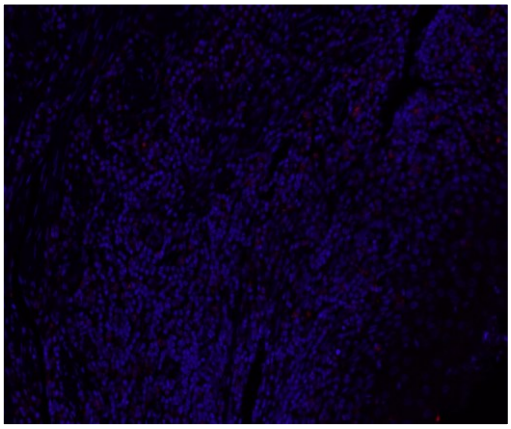


Figure 3-32. Negative Bcl-xL expression on IHC staining.

Note: Negative Bcl-xL expression on fluorescence IHC staining of oral cavity squamous cell carcinoma tissue sections.

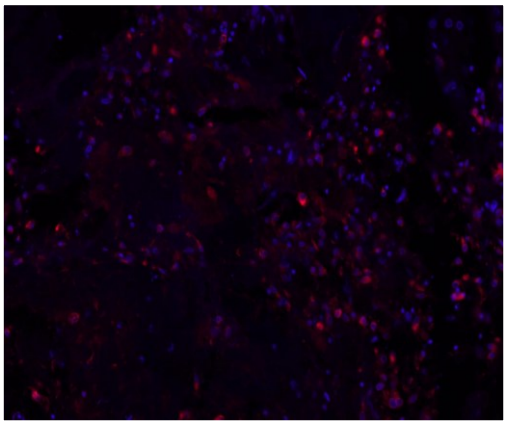


Figure 3-33. Positive Bcl-xL expression on IHC staining.

Note: Positive Bcl-xL expression on fluorescence IHC staining of oral cavity squamous cell carcinoma tissue sections.

Bcl-xL positivity prevalence was 89.3% in OCSCC patients of this cohort (167 patients of 187). For overall survival curves of Bcl-xL, negative expression cohort had clearly improved survival rates compared to Bcl-xL positive cohort in the 5-year period. Being positive for Bcl-xL expression appeared to be associated with poorer survival outcomes in general; a more detailed analysis showed that low-positive expression had slightly improved survival compared to high-positive expression. Nonetheless, the *p* value in Log Rank and Breslow tests was insignificant for all the above-mentioned associations (Figure 3-34). For further analysis of Bcl-xL overall survival curves, patients were dichotomized to early and advanced stage of the disease, and I examined the difference between the negative and positive expression of the molecule in each stage (Figures 3-35 and 3-36). In both early and advanced stage of OCSCC, patients with negative Bcl-xL had improved survival outcomes compared to patients with positive Bcl-xL, with statistically insignificant association in both stages.

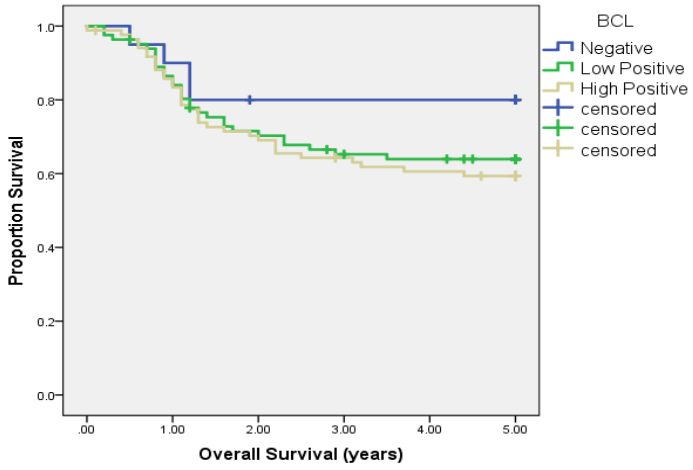


Figure 3-34. Kaplan-Meier overall survival curve of Bcl-xL.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Bcl-xL expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

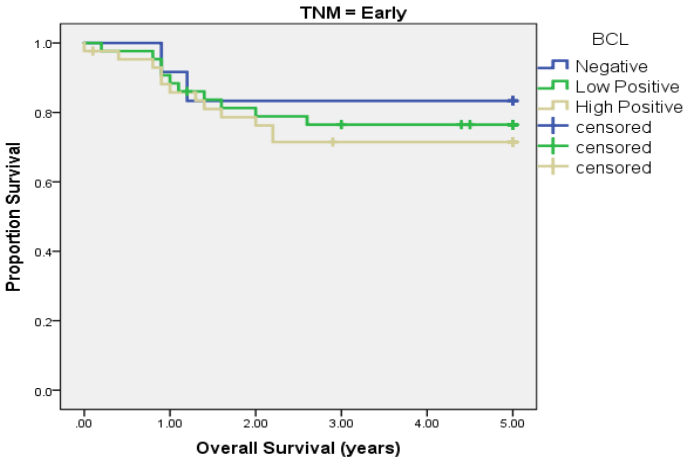


Figure 3-35. Kaplan-Meier overall survival curve of Bcl-xL in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Bcl-xL expression in early stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

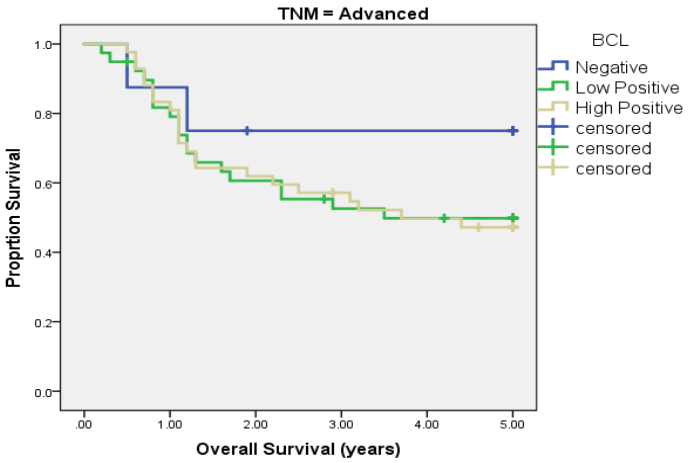


Figure 3-36. Kaplan-Meier overall survival curve of Bcl-xL in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC showing the overall survival rate in regards to different intensities of Bcl-xL expression in advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

For disease-specific survival (DSS) curves of Bcl-xL, patient with negative expression of the molecule had improved survival outcomes compared to patients with high positive expression of the molecule with p value close to the significant level in Log Rank and Breslow tests of 0.072 and 0.084 respectively (Figure 3-37). When dichotomized to early versus advanced stages of the disease, patients with negative expression of Bcl-xL still had improved DSS compared to low and high expression of the molecule in early and advanced stage of the disease, with insignificant *p* values (Figures 3-38 and 3-39). While the relationship between the low and high positive of Bcl-xL was unclear, low positive expression profiles had slightly improved

prognosis over high positive profiles in early stage of the disease and worse prognosis in the advanced stages without any statistically significant associations.

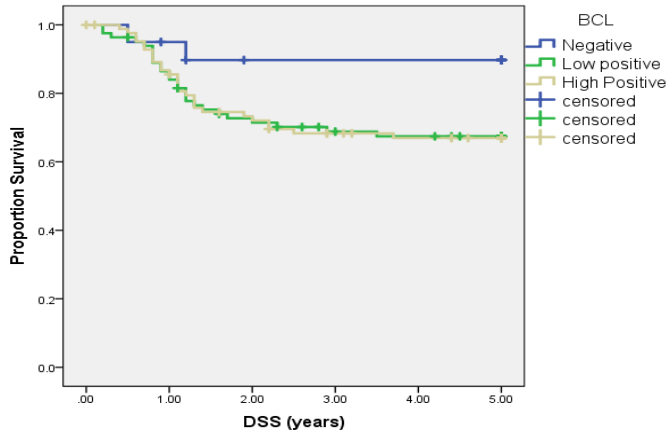


Figure 3-37. Kaplan-Meier DSS curve of Bcl-xL.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of Bcl-xL expression. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p = 0.074$

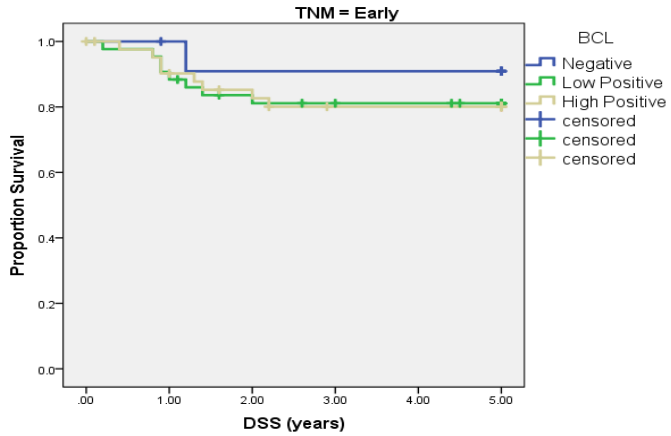


Figure 3-38. Kaplan-Meier DSS curve of Bcl-xL in early stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of Bcl-xL expression in early stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$

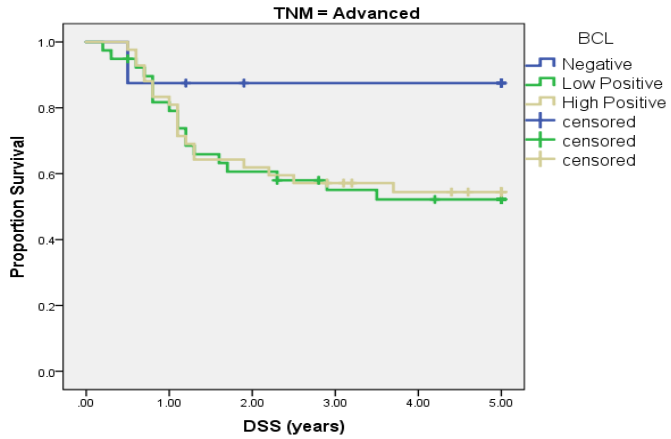


Figure 3-39. Kaplan-Meier DSS curve of Bcl-xL in advanced stage.

Note: Kaplan-Meier survival curves of OCSCC showing the disease-specific survival rate (DSS) in regards with different intensities of Bcl-xL expression in advanced stage of the disease. Patients with negative expression of the molecule had improved survival rates compared to patients with positive expression of the molecule, with no statistically significant association. $p > 0.05$.

Chapter Four: Discussion and Conclusion

Oral cavity squamous cell carcinoma (OCSCC) represents more than 50% of head and neck squamous cell cancers. Despite the advances in the treatment protocols of treating the disease, the 5-year disease-specific survival has remained between 50% and 60% (3,98). A multimodality treatment approach with surgery and combination of chemotherapy and radiation is the standard care of treatment of locally advanced OCSCC. Previous reports have shown that combined therapy, although, has a huge impact on the quality of life and is superior in treating patients with advanced stage OCSCC, with improved survival outcomes compared to a single modality of treatment (3). Clinical parameters of both patient and tumor, which mainly include tumor stage based on TNM anatomical staging, lymphovascular invasion, extracapsular extension, and the physical and health status of the patient, guide the decision of the type of the treatment that the patient requires, but without taking into the account the ongoing molecular changes and the biomarkers involved in the disease to help in tailoring the treatment plan and customize it based on the biomarkers' profiles of each patient (17).

In more recent reports, biological markers have been found useful in predicting OCSCC patient outcomes and response to the different modalities of treatment after completing the prescribed treatment regimen. Among these markers, p16, p53, Ki67, Bcl-xL, and EGFR have been investigated the most in OCSCC and have been shown to aid in predicting the cancer behaviour and aggressiveness, which may help in escalation or de-escalation of the treatment protocols to cure patients with the best possible quality of life (17). Given their better understood part in head and neck squamous cell carcinoma in general, the aforementioned biomarkers' expressions have been assessed in this study by IHC technique in naïve specimens and correlated

to the OCSCC patients' overall survival and DSS after the different modalities of treatment, with attention being paid to the different stages of the disease.

A p16 overexpression is associated with high-risk human papilloma virus infection in head and neck squamous cell carcinoma, particularly oropharyngeal subsites. Nowadays, p16 is used as a surrogate marker of HPV-related head and neck tumors. P16 is a cyclin-dependent kinase inhibitor that prevents retinoblastoma tumor suppressor gene phosphorylation by upregulating oncoprotien E7 and, thus, lead to an abrupt sequence in the cell cycle (1,52,53,61,62). HPV prevalence in oral cavity cancer is not clear; however, it is believed to be between 3% and 12% (17,56). The association between p16 overexpression and OCSCC survival outcomes has yet to be investigated. Most of the reports in the literature have demonstrated the correlation between p16 and patient survivals in oropharyngeal SCC, mainly with convincing results of the superior prognosis of p16 positive expression translated into improved 5-year overall and disease-specific survival of oropharyngeal SCC patients after treatment (17).

The results of this study showed that the prevalence of p16 positivity in the cohort (187 patients considered a large cohort compared to the studies in the literature) was 3.7%, which fit in the range of the prevalence in the literature. Results from the study with this cohort suggests that patients with negative expression of p16 had a trend of improved overall survival compared to patients with positive p16 when the stage of the disease was omitted (p = 0.362 and 0.526). When stratified to early and advanced stages, patients with positive p16 had improved survival outcomes compared to patients with negative expression (p = 0.345 and 0.347) in the early stage of the disease, which is in keeping with most of the reports in the literature. Negative expression of p16 still had a better survival trend in advanced stages of the disease (p = 0.073 and 0.222). The p16 positive cohort had also improved DSS rates compared to negative expression (p =

0.952 and 0.904) for the stages analysed simultaneously. Stratification of the cohort to early and advanced stages revealed improved DSS, with p16 positive cohort compared to negative expression (p =0.431 and 0.432) in early stages; however, patients with advanced OCSCC stages had slightly improved DSS when they demonstrated negative p16 compared to the subjects with positive p16 (p = 0.747 and 0.831).

Ki67 presents predominantly in the active cell phases of G1, S, G2, and M, which makes it a good biomarker for the cell proliferation in neoplastic tissue. Ki67 expression in normal cells is low in phase G1 and early S phase, and increases dramatically before the start of cell division, prior to M phase (16, 63, 64). As mentioned at the beginning of this dissertation, there was a lack of consensus between the different reports in the literature that investigated the correlation between Ki67 expression in OCSCC and patient survival outcomes after treatment (99). Klimowicz et al.'s (16) study was the most prominent, as they pronounced that over expression of Ki67 in OCSCC patients is associated with improved survival rate. This was observed mainly in those patients who were treated with surgery and radiation. The results of this study showed that the prevalence of Ki67 positivity in the cohort was 77.5%, using IHC technique of protein staining and quantitatively measuring protein expression using HALO digital software.

Results of the study with this cohort suggest that patients with a negative expression of Ki67 had a trend of improved overall survival compared to patients with low-positive Ki67 (p = 0.460 and 0.429) and with high positive expression of the molecule (p = 0.376 and 0.332) when the stage of the disease was omitted. When stratified to early and advanced stages, patient with negative Ki67 had improved survival outcomes compared to patients with low expression (p = 0.067 and 0.053) and with high expression of the molecule (p = 0.151 and 0.13) in the early stage of the disease, which was in keeping with most of the reports in the literature. Negative

expression of Ki67 still had no clear improved survival trend in advanced stages of the disease. The Ki67 negative cohort had also improved DSS rates compared to low-positive expression (p = 0.381 and 0.389) and to high-positive expression (p = 0.316 and 0.282) for the stages analysed simultaneously.

Stratification of the cohort to early and advanced stages revealed improved DSS with Ki67 negative cohort compared to low-positive expression (p = 0.018 in both statistical tests) and with high-positive expression (p = 0.37 and 0.38) in early stages of the disease. However, patients with advanced OCSCC stages had no difference between the negative and the different intensities of the positive Ki67 in DSS, with no significant p values. I identified those patients with negative expression of Ki67, who had 100% DSS survival rate, and who were in the early stage of OCSCC to be 20 out of 98 patients. Tracking down their modalities of treatment they had undergone showed that 17 patients had been treated with surgery alone, and three patients had surgery followed by radiation. According to these findings, I may conclude that patients with a high expression of Ki67 may be a subject to escalate treatment protocols, even if they present in early stages of the disease, while negative expression of Ki67 might be a favourable prognostic factor in which patients may be offered less aggressive treatment modalities that ensure them a better quality of life.

p53 is a tumor suppressor gene and the main regulator of the apoptosis, one of the vital mechanisms against cancer development in normal cells. The p53 gene is found to be mutated and over expressed in more than 50% of head and neck SCC including oral cavity subsites (9,92). The correlation between p53 expression and OCSCC patient outcomes in the literature has not been defined clearly. Some reports have shown that overexpression of p53 is associated with poorer responses to treatment, especially after radiotherapy, while some other studies have

yielded no correlation whatsoever of the molecule with the disease prognosis (8,100).

The results of this study showed that the prevalence of p53 positivity in the sample was 54.5% using the IHC technique of protein staining and quantitatively measuring protein expression using HALO digital software. The results from this cohort suggest that patients with a high-positive expression of p53 had poor overall survival compared to patients with negative expression profiles in both statistical tests ($p = 0.049$ and 0.025) when the stage of the disease was omitted. When stratified to early and advanced stages, patients with high-positive expression of p53 had no survival difference compared to negative expression profiles ($p = 0.871$ and 0.748) in the early stage of the disease. High expression of p53 had worse overall survival compared to patients with negative p53 in advanced stages of the disease ($p = 0.072$ and 0.046). The p53 high-positive cohort also had poor DSS rates compared to negative expression profiles ($p = 0.034$ and 0.019) for the stages analysed simultaneously.

Stratification of the cohort into early and advanced stages revealed no difference in DSS between high-positive expression and negative p53 ($p = 0.955$ and 0.957) in early stages of the disease; however, in advanced OCSCC stages, patients with high-positive expression had worse DSS compared to patients with negative expression of the p53 ($p = 0.044$ and 0.030). I identified 62 patients in this cohort with high-positive p53; 23 patients had been treated with surgery alone, 27 patients had surgery followed by radiation, nine patients had triple modality of treatment surgery followed by chemoradiation, and three patients had only radiation. According to these observations, I may conclude that patients with high-positive expression of p53 may have a poor prognosis, and they might be subjects to escalate treatment protocols and treat them with triple treatment modality, especially if they present with an advanced stage of the disease. Alternatively, a more innovative treatment protocol in treating them could even be considered,

such as immunotherapy or targeted gene therapy.

EGFR is one of the key regulators for cell cycle activation toward cellular proliferation and cell survival (13,69,70). EGFR is overexpressed in many types of cancer, including head and neck squamous cell carcinoma, although it is mainly correlated to oropharyngeal subsites more than oral cavity; it has been found to be associated with poor prognosis and outcomes (1,47,69,71). Overexpression in EGFR leads to inactivation of apoptosis, increase of disease recurrence rate, tumor invasiveness, and increase in cellular proliferation and survival in epithelial tumors (72). In the literature, most of the reports that investigated the association between EGFR overexpression and head and neck SCC patient outcomes have included more than one anatomical subsite of head and neck in their studies, which makes no pure literature for OCSCC relationship to EGFR (83). Smid et al. (85) have investigated the expression of EGFR and OCSCC response to treatment with surgery followed by radiation in their retrospective study of 165 OCSCC patients, and they found no association between overall survival and EGFR overexpression. However, they concluded that high EGFR patients may benefit from reduction of postoperative radiation time compared to patients with low EGFR expression. Some reports have shown that overexpression of EGFR is associated with worsened survivals and response to surgical treatment along with shortened local recurrence time compared to low EGFR expression (17).

The results of this study showed that the prevalence of EGFR positivity in our cohort was 88.2% using the IHC technique of protein staining and quantitatively measuring protein expression using HALO digital software. This cohort suggests that patients with high-positive expression of EGFR had poor overall survival compared to patients with negative-expression profiles ($p = 0.085$ and 0.093) when the stage of the disease was omitted. When stratified to early

and advanced stages, patients with high-positive expression of EGFR had still worsened overall survival compared to negative-expression profiles (p = 0.34 and 0.38) in the early stage of the disease. Also, high expression of EGFR had poorer overall survival compared to patients with negative EGFR in advanced stages of the disease (p = 0.165 and 0.141). The EGFR high-positive cohort had significantly worse DSS rates compared to negative-expression profiles (p = 0.023 and 0.029) for the stages analysed simultaneously.

Stratification of the cohort to early and advanced stages revealed poor outcomes in DSS with high-positive expression correlated to negative or low expression (p = 0.14 and 0.17) in early stages of the disease. Moreover, in advanced OCSCC stages, patients with high-positive expression had still persistent worse DSS compared to patients with negative expression of EGFR, with close to the significance level in both statistical tests (p = 0.09 and 0.08). Eighty-four patients were identified with high-positive EGFR expression, of which 34 had been treated with surgery alone, 36 had surgery followed by radiation, seven had triple modality of treatment with surgery and chemoradiation, five patients had received radiation only, and two patients had chemoradiation with no surgical intervention. According to these observations that keep with what I knew beforehand about EGFR overexpression in terms of increased tumor proliferation, invasiveness, and high risk of local recurrence, I may support the argument that patients with a high-positive expression of EGFR may have a poor prognosis, and they might be subject to escalated treatment protocols. They could be treated with triple treatment modality, especially if they present with an advanced stage of the disease or even consider more innovative treatment protocol in treating them, such as immunotherapy or targeted gene therapy.

Bcl-xL is an antiapoptotic protein and a member of Bcl2 family that inhibits activation of Bax protein and eventually deactivates apoptosis process. Bcl-xL has been detected in head and

neck squamous cell carcinoma, and different reports showed that the molecule can be linked to radiotherapy resistance and poor treatment responses (9,67,68). The results of this study showed that the prevalence of Bcl-xL positivity in the cohort was unsurprisingly high. This level was up to 89.3% using IHC technique of protein staining and quantitatively measuring protein expression using HALO digital software, as most of tumor cells overly express Bcl-xL in their cytoplasm. As suggested by the results obtained from this cohort, patients with a high-positive expression of Bcl-xL had poor overall survival in early and advanced stages, without statistically significant results ($p = 0.126$ and 0.158) compared to patients with negative expression profiles. The Bcl-xL high-positive cohort also had poor DSS rates in both early and advanced stage of the disease compared to negative-expression profiles, with close to significance level p values in both statistical tests used to compare Kaplan-Meier survival curves in this study, Log Rank and Breslow tests ($p = 0.072$ and 0.084). According to these observations, I may conclude that patients with a high-positive expression of Bcl-xL may have a poor prognosis, and they might be subjects to escalate treatment protocols and treat them with triple treatment modality, even when they present in early stages of the disease process or even consider more innovative treatment protocol in treating them, such as immunotherapy or targeted gene therapy.

This study is distinguished in many ways from the existing studies in the literature. The OCSCC cohort sample size was relatively large. In this study, I examined the association between five different molecules that have been found to be overexpressed in OCSCC and patient survival outcomes after treatment. This study reflected one homogenous cohort at the same time, using TMA techniques that ensure experiment standardization. Moreover, the cohort had been treated with different treatment strategies according to each patient's tumor stage and clinical parameters, which makes it more interesting when I stratify the outcomes to the different

treatment strategies and track the different responses in the cohort to assess the need to escalate or deescalate the treatment regimen based on the molecular profiles. In addition, I analysed the survival rates in two survival curves, overall and disease-specific survival. Also, I included all patients in all OCSCC tumor stages, early and advanced, and analysed each stage differently to make it a more homogenous cohort.

There are multiple limitations that I can identify in this study, and this includes the retrospective nature of the study, which makes it a subject for recall bias and confounding. However, the large sample size of this cohort helps in making the results more reliable and in identifying the risk (the molecular expression) that makes the outcome (i.e., survival rate) after treatment worse or better. Using TMA cores might be considered one of the limitations in the study, as this technique might miss the cancer areas in the whole tissue sections upon constructing the TMA blocks. To overcome this potential limitation, I utilized two cores of 1.5 mm each from the pre-marked cancer tissue in the pre-treatment FFPE blocks from each patient to be certain that the tumor was included in one of these two cores. Moreover, the pathologist in this study (L.P) has reviewed all the cores after constructing them on H&E slides to confirm the tumor presence in each core; cores that showed no tumor or has less than 20% tissue sample were excluded from further analysis. Finally, very few pre-treatment FFPE donor blocks were decalcified by using a chemical decalcifier to dissolve the calcium deposits in the tissue sections. This technique is usually performed in histopathology labs to eliminate the calcium components of the bone tissue for better tissue saving in FFPE blocks, which accounts for interfering with the IHC staining process and makes it subject to poor staining and eventually makes for unreliable results. There were seven decalcified blocks in this cohort, which

represented seven patients out of 187. This information may not have any significant impact on the results at the end of the study given the small number of these decalcified samples.

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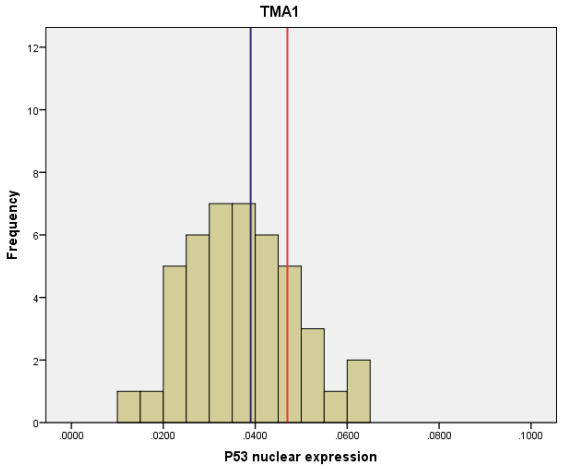
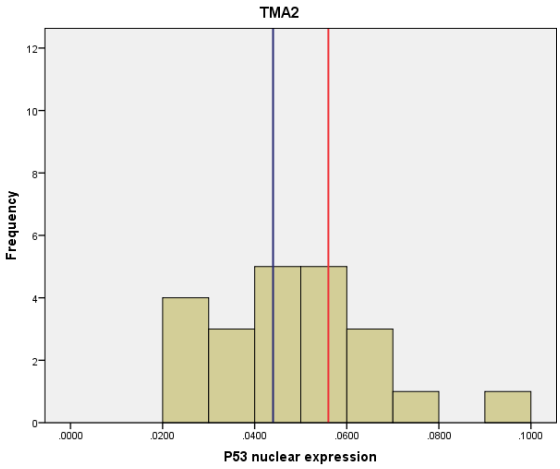
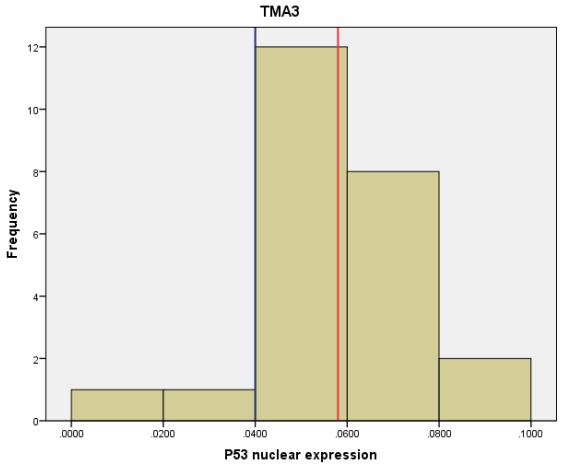
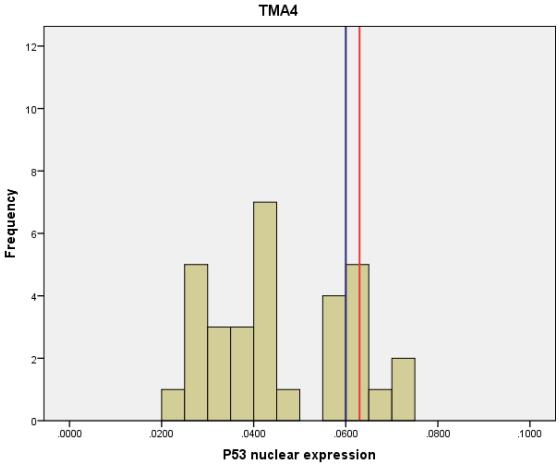
Appendix A: Molecular Expression Intensities by HALO Software and Expression Scoring

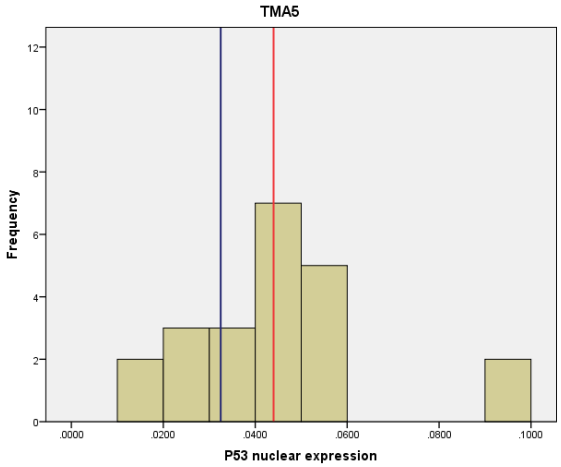
The 16 graphs in this appendix are a histogram representation showing the intensity of the molecule expression and the scoring system criteria used in the study for each TMA slide for each different molecule. The blue line represents the median expression of a given molecule in normal tissue (negative control) and the red line represents the median expression of the same molecule in the cancer tissue. The expression considered negative if it is below the median expression of the molecule in the normal tissue, and positive if it is above that median line. High positive versus low positive expression of any given molecule was calculated based on the median expression of that molecule in the cancer tissue, tissue samples that showed molecular expression above the median of the positive cancer cells labelled as high positive and tissue samples that showed molecular expression below the positive cancer cells median line was labelled as low positive.

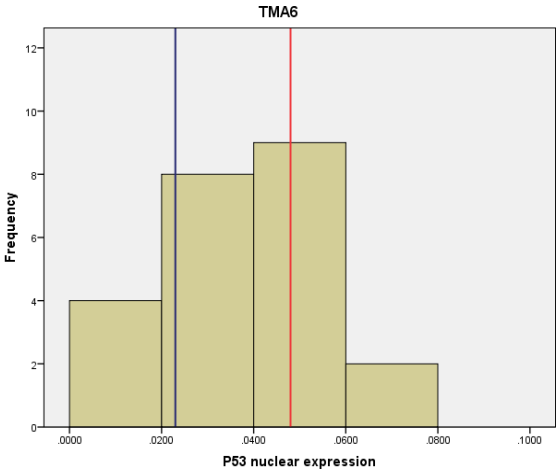

TMA1
Frequency
P53 nuclear expression

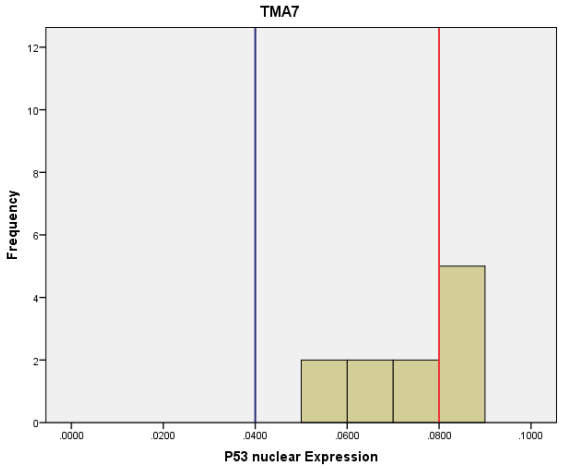

TMA2
Frequency
P53 nuclear expression

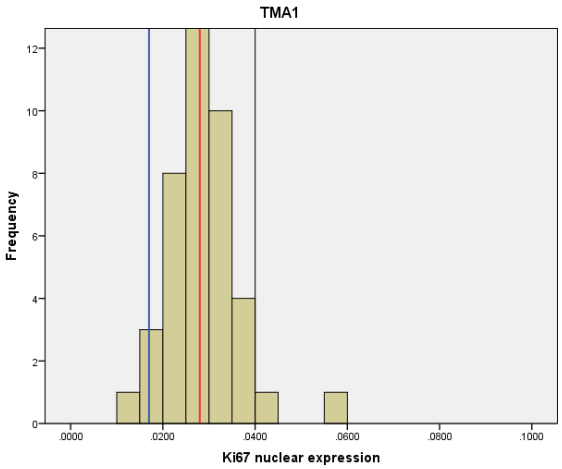

TMA3
Frequency
P53 nuclear expression

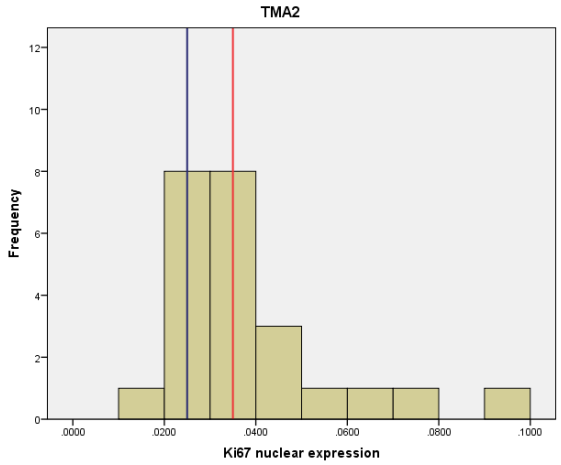

TMA4
Frequency
P53 nuclear expression

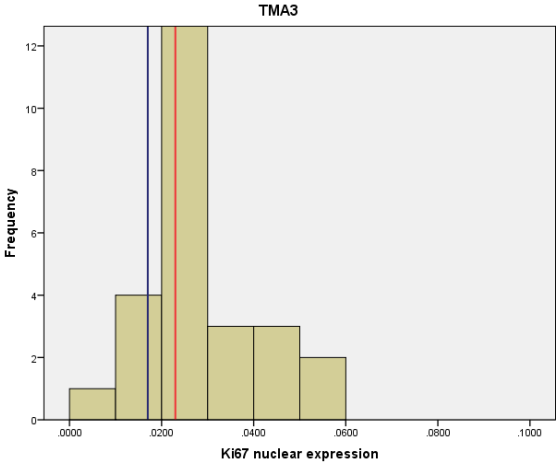

TMA5
Frequency
P53 nuclear expression

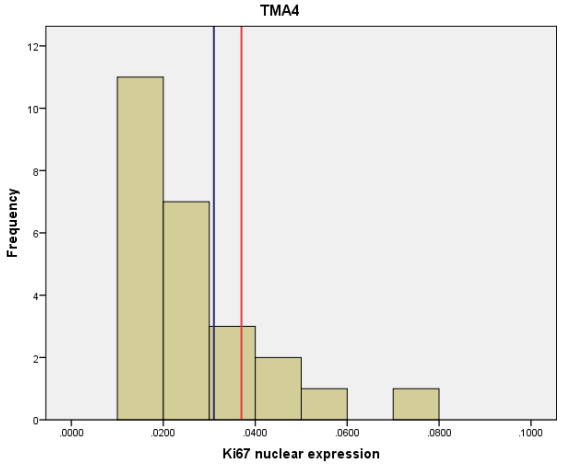

TMA6
Frequency
P53 nuclear expression

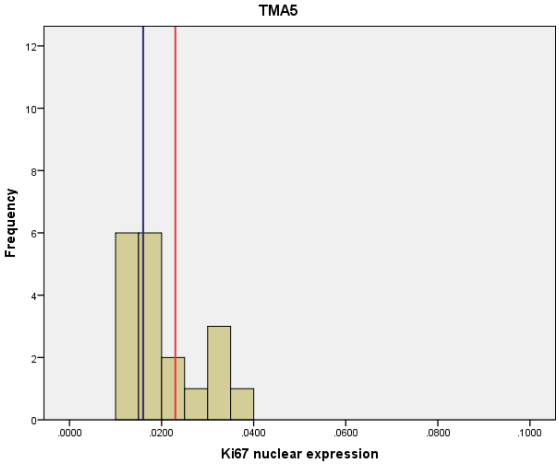

TMA7
Frequency
P53 nuclear Expression

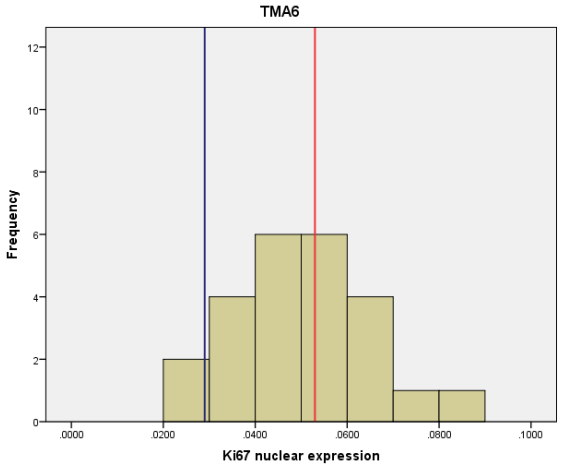

TMA1
Frequency
Ki67 nuclear expression

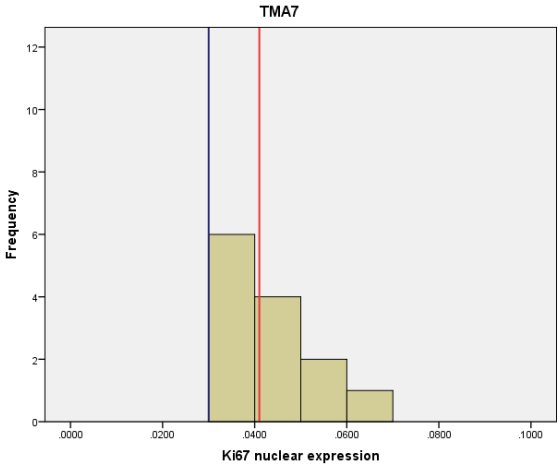

TMA2
Frequency
Ki67 nuclear expression

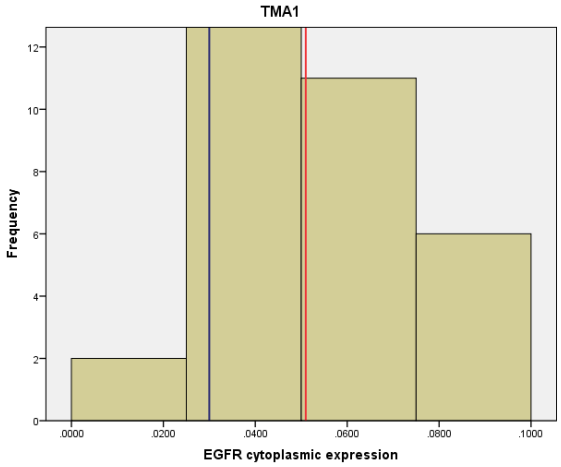

TMA3
Frequency
Ki67 nuclear expression

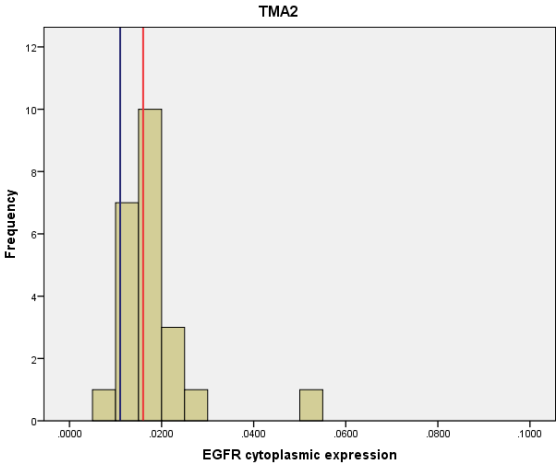

TMA4
Frequency
Ki67 nuclear expression

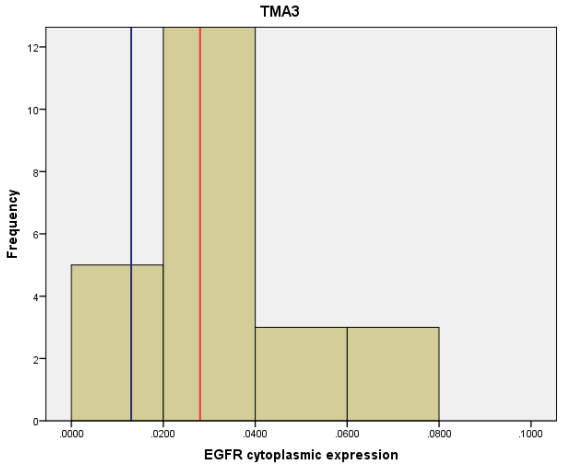

TMA5
Frequency
Ki67 nuclear expression

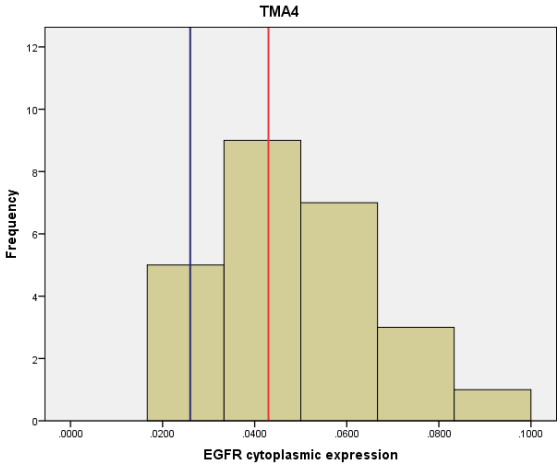

TMA6
Frequency
Ki67 nuclear expression

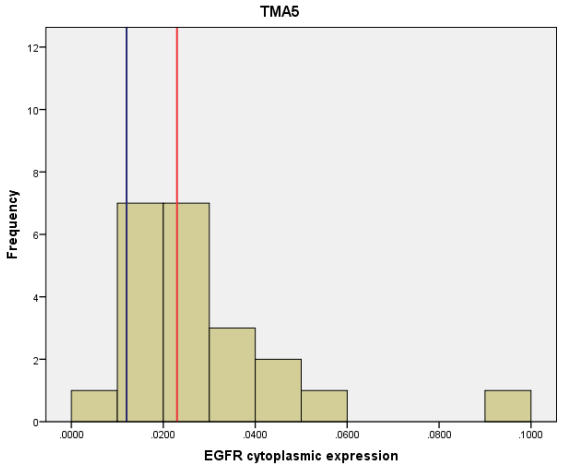

TMA7
Frequency
Ki67 nuclear expression

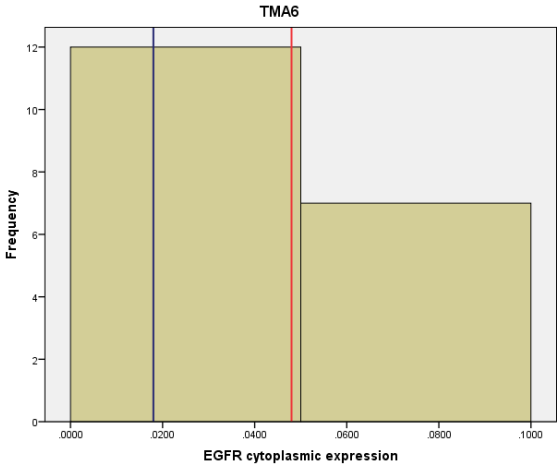

TMA1
Frequency
EGFR cytoplasmic expression

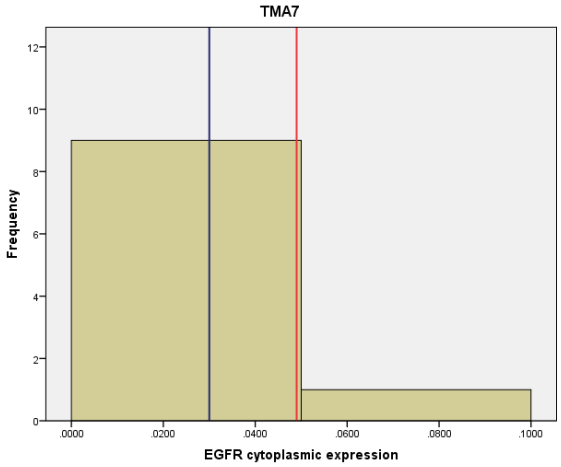

TMA2
Frequency
EGFR cytoplasmic expression

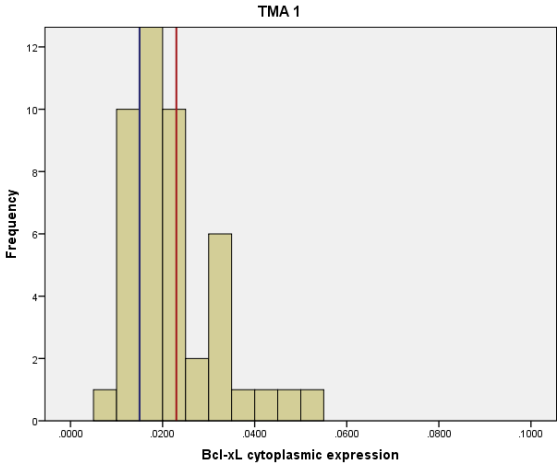

TMA3
Frequency
EGFR cytoplasmic expression

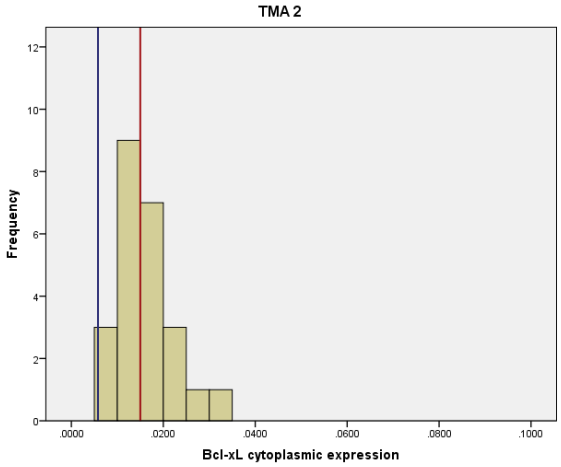

TMA4
Frequency
EGFR cytoplasmic expression

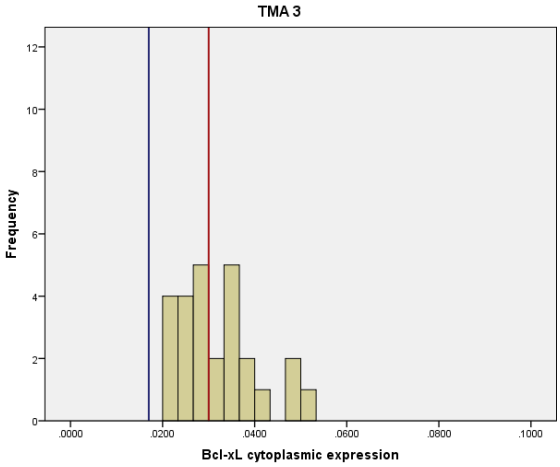

TMA5
Frequency
EGFR cytoplasmic expression

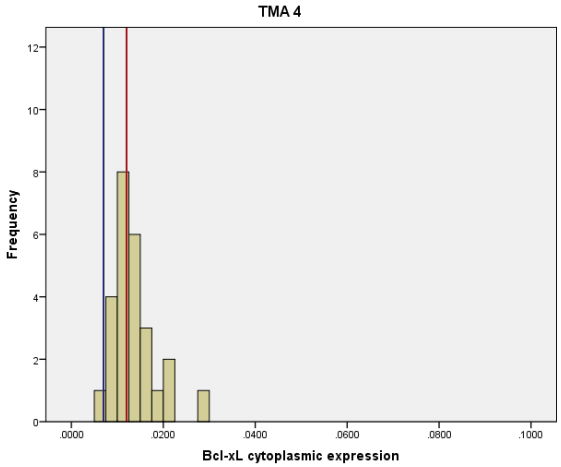

TMA6
Frequency
EGFR cytoplasmic expression

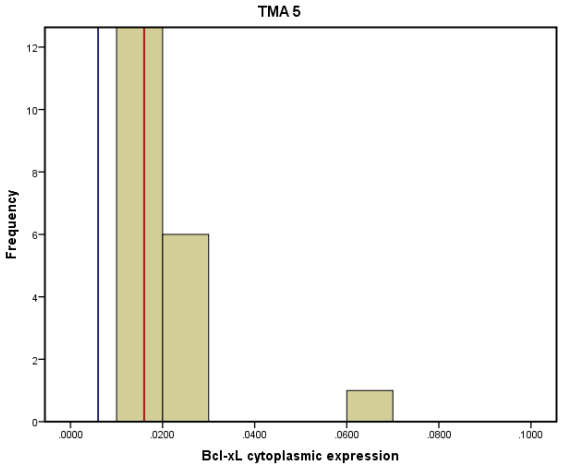

TMA7
Frequency
EGFR cytoplasmic expression

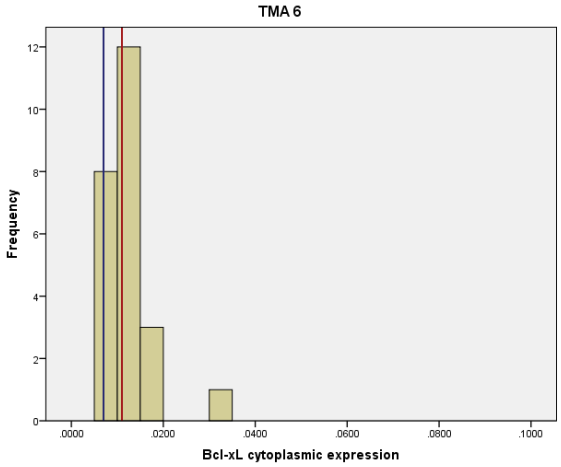

TMA 1
Frequency
Bcl-xL cytoplasmic expression

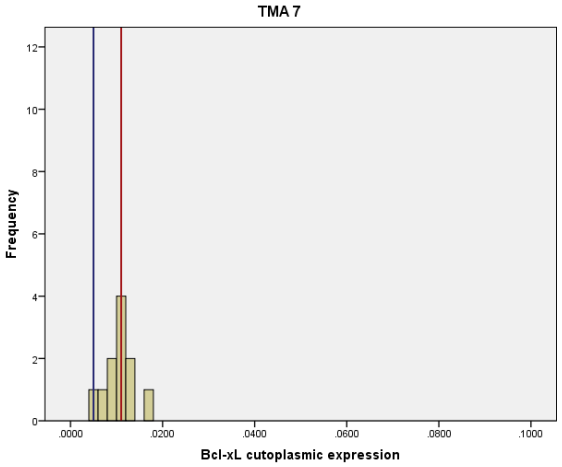

TMA 2
Frequency
Bcl-xL cytoplasmic expression


TMA 3
Frequency
Bcl-xL cytoplasmic expression


TMA 4
Frequency
Bcl-xL cytoplasmic expression


TMA 5
Frequency
Bcl-xL cytoplasmic expression


TMA 6
Frequency
Bcl-xL cytoplasmic expression


TMA 7
Frequency
Bcl-xL cutoplasmic expression