CHAPTER 1

INTRODUCTION

Cancer is defined as uncontrolled tissue growth in susceptible patients, resulting from an imbalance between cell division and apoptosis (Ponder, 2001). With more than 10 million new cases and six million deaths reported each year globally [World Health Organization (WHO), 2009], cancer is a major cause of mortality.

According to the World Health Organisation International Statistic Classification of Disease, tenth edition (ICD-10), OC includes cancer of the lip, tongue, gum and alveolus, floor of the mouth and palate (ICD-10: C00-06). The yearly estimated incidence of OC is around 275,000 cases (Parkin et. al., 2002), where two thirds of these cases more frequently occur in developing countries such as India (Parkin et. al., 2002, Muwonge et. al., 2008).

In Peninsular Malaysia, oral cancer (OC) incidence was not listed among the ten most common cancers in the Malaysian population in 2006 (Zainal et. al., 2006). However from the ethnic distribution characteristics, the incidence of OC ranked 6th and 4th for Indian males and Indian females respectively. The Indian male population showed highest incidence of tongue cancer with an incidence rate at 4.5 per 100,000. The Indian female population presented the highest incidence of mouth cancer with a rate of 11.5 per 100,000 population. According to age distribution characteristics, OC highest incidence was reported in patients above 70 years with an incidence rate at 44.2

per 100,000 for Indian males. The highest incidence in Indian female patients was reported at age 60 to 69 years at 21.8 per 100,000 populations (Zainal et. al., 2006, Lim et. al., 2008).

OC is uncommon in young people below 45 years of age and only 4 to 6% of oral cancer was reportedly in young patients (Ribeiro et.al., 2009). In the USA, 95% of OC occurs in those more than 40 years old (National Institutes for Health (NIH), 2008) while in the UK, it is reported at 85% in people aged 50 years and above. The median age at diagnosis of oral cancer is below 60 years, but the incidence in younger people (40 years old) keeps increasing. According to the Malaysian National Cancer Registry data from 2003 to 2006, the age specific incidence of oral cancer in Peninsular Malaysia demonstrated an exponential increase after the age of 40 years (Zainal et. al., 2006, Lim et. al., 2008).

Lifestyle risk factors for oral squamous cell carcinoma (OSCC) are commonly associated with the use of alcohol, betel quid and tobacco (Jose et. al., 2008). The main risk factors associated with this disease in the west are tobacco smoking and alcohol consumption, whereas in India and Southeast Asia, OC is closely associated with betel quid (BQ) chewing habit (Nair et al., 1999). Among the Malaysian population, the Indians were identified as having a higher OC risk compared to Malays, Chinese and other ethnic groups. This trend in the Indians is due to quid chewing habit (Zain, 2001). Besides the above risk factors, geographic variation and the socio-cultural lifestyles of a population also play important roles in the determination of the aetiology and pathogenesis of OC.

OC is often detected in the late stages (Stage III and IV) when the chances of survival are poor. Therefore, improvement in early detection of OCs, especially during early stages (Stage I and II) is needed for better outcome to treatment of oral cancer patients. The search for molecular markers for early detection is currently on-going world-wide. One of the methods used is an advanced molecular cytogenetic approach to detect the chromosomal copy number alterations (CNAs) in cancer cell. In addition, there is a need to understand the basis of cancer and its diagnosis where the detection and mapping of these DNA copy number changes are important (Pinkel et. al., 1998).

Cytogenetics is a key in the diagnosis of many types of hematologic malignancy. Conventional cytogenetics is defined as the study of the structure of chromosome material which includes routine analysis of Giemsa (G)-Banded technique known as karyotyping. This technique however has its limitation as it is hard to identify cytogenetic aberrations because of the complexity of genetic changes (Sultan et al., 2003).

More recently, molecular cytogenetics, an advanced technology platform of conventional cytogenetics has been developed and introduced to allow for the detection of chromosomal imbalances in cells. The advanced technology platform was introduced in 1980s with the detection of chromosomal imbalance by fluorescently labelled probes.

Fluorescence in-situ Hybridization (FISH) is the first molecular cytogenetic technique that has been introduced to detect and localize the presence or absence of specific DNA sequences on chromosomes (Sieben et al., 2007). FISH has the advantage of high resolution, but is best suited for the confirmation of known microdeletion and

microduplication syndromes in patients presenting with suggestive phenotypes due to the limited number of chromosomal loci that can be simultaneously analyzed (Anat et. al., 2006).

The second generation of molecular cytogenetic technique is the introduction of Comparative Genomic Hybridization (CGH). CGH, a genome-wide profiling technique, can reveal non-random pattern of chromosomal alterations in cancers (Lin et. al., 2002) and it can analyze gains and losses of parts and whole chromosomes from a single experiment by mapping on normal reference metaphase cells (Takeno et al., 2009).

Recently, the introduction of a novel high-throughput technology such as arrayCGH, single nucleotide polymorphism array (SNP array) and gene expression (GE) arrays are available to study genome-wide alterations. Array studies can allow the detection of chromosomal alterations at high resolution and investigate the association between copy number changes and expression (Kloth et. al., 2007). ArrayCGH technique can identify novel genes or sets of genes and can be used for advance screening or the development of anti-cancer target towards individualised cancer treatment (Manning et. al., 2007).

Hence, the rationale to use this arrayCGH technique as a tool for detection of chromosomal alterations in OC. By applying this technique, known and unknown genes could be detected and with this, possible genes that are involved in tumour progression, which includes suppressor gene and oncogenes, would be characterized and detected according to their region in chromosomes.

CHAPTER 2

AIM AND OBJECTIVES

2.1 Aim

The aim of this study is to identify chromosomal alterations using arrayCGH leading to the identification of genes involved in the development of OSCC. Among the genes identified, selected gene will be validated to confirm the arrayCGH technique.

2.2 Specific Objectives

- To detect chromosomal alterations in OSCC by using arrayCGH
- To determine the relationship between selected regions with socio-demographic characteristics (gender, ethnicity and habits) and selected clinicopathologic parameters (TNM stage, tumour size (T), lymph node status (N), tumour grade and tumour site)
- To identify genes involved in OSCC by arrayCGH and selected genes function
- To validate one of the identified genes using quantitative Real Time PCR (q-PCR) technique

2.3 Rationale of the Study

Most of the chromosomal alteration studies have been done in western countries and Japan and the aetiological agents and risk factors are different from Southeast Asian countries including Malaysia. There are only a few studies describing the genetic alterations in OC for the South and Southeast Asian region. OC in Malaysia is mainly due to the habit of betel quid chewing (Zain 2001).

Hence, the rationale for conducting chromosomal alteration studies for OC in Malaysia is as follow: In the Malaysian scenario, to date, there is no published literature on chromosomal alterations among the Indian and indigenous population. Few preliminary studies (unpublished reports) are currently carried out on OC in Malaysia. Despite existing evidences regarding chromosomal alterations and their role in oral carcinogenesis, reported in other regions, no report has yet been published in the Malaysian population. There were many studies on detection of chromosomal alteration of OSCC being conducted in nearby countries using molecular cytogenetics, especially in India, but the results may not be applicable to the Malaysian population, as different countries have different habits and lifestyles.

Therefore, this study was carried out to determine the chromosomal alterations and its effects on OC risk by evaluating selected chromosomal alterations and gene detected to clinicopathological parameters. A new set of genes and pathway associated with OC may be discovered leading to possible biomarkers for improvement in prognosis as different individual may have different sets of genes that are important in the development of OC.

CHAPTER 3

LITERATURE REVIEW

3.1 Cancer

Cancer is defined as uncontrolled tissue growth in susceptible patients, resulting from an imbalance between cell division and apoptosis (Ponder, 2001). According to Anatoly (2005), cancer, is an active "gain-of-function" process where the cells acquire many new functions, including the ability to adapt to multiple changing environment, to avoid the body's protective mechanisms, as well as having the ability to recruit the surrounding normal tissue. Cancer is a disease caused by somatic and inherited mutations in genes called oncogenes and tumour suppressor genes (WHO, 2009).

Cancer occurs as a result of development of abnormal cells that divide uncontrollably and have the ability to destroy normal body tissue and spread throughout the body via the blood and lymphatic systems (NIH, 2009). Under the normal condition, new cells will be produced to replace old and damaged cells. However, when cancer occurs, DNA of a cell can be damaged or changed causing mutations, thus affecting cell growth. A tumour can be classified either as benign or malignant. Benign tumours do not spread to other parts of the body. Malignant tumours can spread to nearby tissues (local invasion) and other parts of the body (metastasis). According to the American National Cancer Institute (NCI, 2009), cancer can be categorized to five main types namely carcinomas, sarcomas, lymphomas and myelomas, leukaemias and central nervous system cancers. Carcinomas are the most common types of cancers that begin

from the cells that cover internal and external surfaces (skin). The example of sites involved with this type of cancer is lung, breast, colon and mouth. **Sarcomas** are cancers that originate from the supportive tissues of the body which comprise bone, cartilage, fat, muscle, blood vessels and connective tissues. Lymphomas and myelomas are cancers that originate from lymph nodes and cells in the human immune systems. Leukaemias are cancers of immature blood cells that grow in the bone marrow and have the probability to accumulate in large amounts in the bloodstream. Lastly, central nervous system cancers are cancers that originate in the tissues of the brain and spinal cord.

3.2 Epidemiology of Oral Cancer (OC)

3.2.1 OC Subsites

Oral cancer (OC) includes cancers of the lips, tongue and mouth, and the latter includes cheek, palate and gum [ICD-10: C01-06]. The grouping of the OC sites in the WHO International Statistical Classification of Diseases (ICD-10) is more specific to the anatomic site. The ICD-10 codes, C00 represent the lip excluding the skin part of the lip, C01-02 is the tongue site, C03 is gum site, C04 represent cancers in the floor of the mouth, C05 for palate and C06 for non-specific sites such as the buccal mucosa. The classification of OC excludes cancer of the salivary glands and tonsil (C07 –C09), oropharynx (C10), nasopharynx (C11), sinus and hypophyrynx (C12-C13) and ill defined sites in the lip, oral and oropharynx (C14). The majority of OCs are carcinomas where more than 90% are squamous cell carcinomas (Adisa, 2009).

3.2.2 Epidemiological Terminologies

Before the epidemiology of OC is described, the terms related to the terminology of epidemiology should be understood. Epidemiology measurements include *prevalence, incidence, mortality and survival. Prevalence* is defined as the number of affected persons present in a population at the time divided by sum of persons in the population at that time (Gordio, 2006). *Incidence* can be described as the rate of new cases of a disease to occur during a specific time per 100,000 potential populations at risk (Gordio, 2006). *Mortality* can be defined as the frequency of cancer deaths per 100,000 persons per year. *Survival rate* is the proportion of persons with cancer who survive after diagnosis (usually a five year interval).

To determine cancer incidence among population in countries, the term Age Standardized Rate (ASR) is used to compare the cancer incidence between the different ethnicity, gender among cancer cases or comparison of cancer rates between countries (Lim et. al., 2008). Crude Incidence Rate (CR) is a measurement that has been used widely to estimate new cancer incidence, divided by the number of population at risk at the same period which is usually expressed per 100,000 (Zainal et. al., 2006).

3.2.3 Prevalence and Incidence of OC

To date, there are 67,792 new cancer cases diagnosed among Malaysians in peninsular Malaysia from 2003 to 2005 and 21,773 cancer cases in the year 2006 registered in the Malaysian National Cancer Registry (Zainal et. al., (2006), Lim et. al., (2008)).

3.2.3.1 Global Scenario

Worldwide, oral and pharyngeal cancer, grouped together, constitates one of the most common cancers in the world (Warnakulasuriya, 2009), however its incidence varies between developed and developing countries (Peterson, 2009). There is an estimate of 390,000 new cases of OC occur each year, and half of the cases occur in Asia and developing countries [Cancer Research United Kingdom (UK), 2009]. The areas with high incidence of OC are found in the South and Southeast Asia which includes Sri Lanka, India, Pakistan and Taiwan. Parts of Western Europe (France), and Eastern Europe (Hungary, Slovakia and Slovenia), a few Latin American countries (Brazil, Uruguay and Puerto Rico) and the Pacific regions such as Papua Guinea and Melanesia are other areas associated with a high incidence of OC (Warnakulasuriya, 2009).

In most countries around the world, OC affects more men than women with a ratio of 1.5:1. OC among males in developing countries ranked as the sixth most common cancer after lung, prostate, colocrectal, stomach, and bladder cancer and is the tenth most common cancer among females (Warnakulasuriya, 2009). OC incidence increases by age and majority of cases occur in people aged 50 years and above (Warnakulasuriya, 2009). However, there has been an increase in OC incidence in young individuals who are less than 45 years of age (Llewellyn et. al., 2001). OC mortality rates among males were seen to decrease significantly in most countries which include, over the past decades those from Europe and Asia, but the rates continue to increase in several Eastern European countries which include Hungary and Slovakia (Mayne et. al., 2006, Garavello et. al., 2010, Jemal et. al., 2011).

3.2.3.2 Malaysian Scenario

The prevalence of OC was first reported in 1976 and involved peninsular Malaysia. It is estimated that only 0.01% of the 9076 subjects that were examined in 1973/74 had oral cancer (Ramanathan and Lakshimi, 1976). In 1993/94, the prevalence for OC was found to be 0.04% out of 11,697 patients examined in Malaysia (Peninsular Malaysia, Sabah and Sarawak) (Zain et. al., 1997).

Lim et. al., (2008) reported that mouth cancer (C03-C06) ranked twenty second among cancers in males and fifteen among females during the period of 2003-2005. OC is more predominant among the Indian females than males in peninsular Malaysia (Zainal et. al., 2006). In Malaysia, Indians are commonly affected with this disease (Zainal et. al., 2006, Lim et. al., 2008). Mouth cancer ranked as the fourth most common cancer among Indian females and tongue cancer as the sixth most common cancer in Indian males (Zainal et. al., 2006).

The incidence of Indian males affected with tongue cancer in Malaysia (ASR 4.4) was lower compared to other developing countries such as India where the ASR in Trivandrum, was 9.3 per 100,000, and in Madras was 6.6 (Lim et. al., 2008). Among Indian females in Malaysia, the ASR rate was 14.5 per 100,000 which is much higher than the ASR for Indian females in Bangalore (ASR 7.5), Madras (ASR 5.4), and Singapore Indians (ASR 5.1) (Lim et. al., 2008). In Malaysia, the age specific incidence for OC showed an exponential increase after the age of 40 (Zainal et. al., 2006, Lim et. al., 2008). Besides Indians, indigenous people in Sabah and Sarawak had a high occurrence of precancerous (15.4%) and cancerous lesion (1.9%) (Zain et. al., 1997).

3.3 Clinical Presentation of OC

OC may appear as white, red, ulcerated or exophytic oral lesions and are identified mostly on the lower buccal mucosa, the alveolar mucosa and the lower mucobuccal fold (Shah et. al., 2003). Early SCC often presents as a white patch (leukoplakia), red patch (erythroplakia), or a mixed red and white lesion (erythroleukoplakia). Superficial ulceration of the mucosal surface may develop and as the lesion grows, many cause an exophytic mass with fungating or papillary surface. It may also have an endophytic growth pattern that is characterized by a depressed, ulcerated surface with a raised, rolled border (Neville and Day, 2002a). Oral cancer has a varied clinical presentation as per described by Zain et. al., (2002).

3.4 Histopathology of OC

Over 90% of OCs are diagnosed histopathologically as OSCC that arises from the covering and lining epithelium of the oral cavity (Neville and Day, 2002a). OSCC is a malignant neoplasm showing varying degree of differentiation (Adisa, 2009). Histologically, OSCC can be classified into several grading schemes based on keratinisation (differentiation) and nuclear atypia (Bhargava et. al., 2010). Thus, OSCC can be divided into well, moderate and poorly differentiated types.

3.4.1 Well Differentiated

The well-differentiated SCC represents the normal squamous epithelium with extensive keratinisation and keratin pearl formation (Bhargava et. al., 2010). It is also known as grade 1 or low-grade. The well differentiated tumour is similar to normal squamous epithelium where the surface displays full thickness cytological changes with central keratinisation appearing within many of the islands (Fleming, 2007). In this category, the histological features of malignancy are minimal.

3.4.2 Moderately Differentiated

Moderately differentiated SCC show less keratinisation and more nuclear pleomorphism and mitotic activity (Bhargava et. al., 2010). It is also known as grade 2 or intermediate grade tumour. This type of tumour is graded in between poorly differentiated and well differentiated. Areas with moderate differentiation show more voluminous cytoplasm and individual cell keratinisation (Fleming, 2007).

3.4.3 Poorly Differentiated

The poorly-differentiated tumours show pleomorphic cells with minimal keratinisation and prominent mitotic activity with plenty of atypical mitotic figures (Bhargava et. al., 2010). It is termed grade 3 or high grade tumour where keratin formation is rare or absent and intercellular bridges are unclear. Most of the cells are polygonal, hyperchromatic with a higher nuclear cytoplasmic ratio (Fleming, 2007).

3.5 Clinicopathological Parameters in OC

Several clinicopathological parameters have been associated with prognosis (recurrence and survival) of OC. Prognosis may vary with parameters such as the tumour primary site, lymph node status (N), tumour size (T), tumour thickness, TNM staging and the status of the surgical margins (Bagan and Scully, 2008). Anatomical differences in oral cancer sites with variation in the richness of the blood supply, and lymphatic drainage, may also contribute to the difference in prognosis (Garzino-Demo et. al., 2006).

In oral cancer, the tumour-node-metastasis (TNM) staging system has been used to predict prognosis and determine the choice of treatment. The first edition of the TNM staging system was reported by Pierre Denoix in the early 1940s. This was later adapted by the International Union Against Cancer (UICC). The system was then modified and compiled as the first edition of the official TNM staging system in 1968, with the involvement of 23 body sites (Patel and Shah, 2005). TNM is an anatomic staging system that describes the anatomic extent of the primary tumour and the involvement of regional lymph nodes and distant metastasis (Sobin, 2003). As decades pass by, the classification was restructured and improved by both the UICC and American Joint Committee on Cancer (AJCC) to enable better assessment of tumours and understand the biological behaviour of numerous tumours in the anatomic area.

For many decades, the AJCC-UICC TNM staging system has been used for staging head and neck cancer widely. The staging system were T representing tumour size which include the extend of primary tumour, described both as clinical (T) and pathologic (pT) categories, N status (absence/presence and extend of regional lymph

node metastasis) described both as clinical (N) and pathologic (pN), M parameter which present the absence/presence of distant metastasis described as clinical (M) and pathologic (pM). For assessment of tumour margins which is also one of the major pathological parameter, the measurement was determined based on the criteria suggested by Batsakis, (1999) defined as negative when the tumour was ≥5 mm away from the inked surgical margin. Conversely, all the others were considered as being positive when the surgical margin is < 5 mm (Sutton et. al., 2003, Woolgar and Triantafyllou, 2009, Vered et. al., 2010).

3.6 Risk Habits

According to the NCI (2009), risk factor is anything that increases the chance of developing a disease. In OC, the practise of tobacco smoking, chewing of tobacco/betel quid and drinking alcohol are well known recognized risk factors worldwide (Ahmed et. al., 1997, Muwonge et. al., 2008, Madani et. al., 2010).

Other risk factors that play important roles in OC pathogenesis includes dietary habits i.e. the socio-cultural lifestyles of a population (Zain, 2001), human papilloma virus (HPV), genetic susceptibility (Urmila et. al., 1999), exposure to other carcinogenic agents such as sunlight, paint fumes, wood dust, asbestos and other environmental factors. It was reported that over a third of cancer deaths worldwide are associated with risk factors such as tobacco smoking, alcohol use, diets low in fruits and vegetables (Danaei et. al., 2005). In the Asian regions, OC is mainly associated with betel quid chewing and smoking while in western countries, OC is mainly related to cigarette smoking and heavy alcohol consumption (La Vecchia et. al., 1997).

3.6.1 Betel Quid Chewing

Areca nut (*Areca catechu*) is the fourth most widely used addictive substance in the world with more than 10% of the world's population usage (Boucher and Mannan, 2002, Gupta and Warnakulasuriya, 2002). There is an estimate of 600 million betel quid users globally (Gupta and Warnakulasuriya, 2002). The habit of chewing is commonly practised among Bangladeshi, Indian, Pakistani, Sri Lankan (Gupta and Ray, 2003) and Taiwanese (Su-Chen et. al., 2003). In Asian regions, OC is widely associated with chewing betel quid (BQ) (Manjari et. al., 1999, Gupta and Ray, 2004, Muwonge et. al., 2008). In Taiwan, the prevalence of BQ chewing is as high as 16.9% with 31% in men and 2.4% in women (Guh et. al., 2007).

There are many different ways to practise BQ chewing. For example, in South Asia, people chew the fresh, dried, or cured areca nuts with slaked lime, betel leaf (Piper *betle* vine), and tobacco leaves (Gupta and Warnakulasuriya, (2002), IARC, (2004)). However, in the Taiwan population, the people chew fresh unripe betel quid combined with P. *betle* (inflorescence or leaf) and lime but without tobacco leaves (Lan et. al., 2007). During betel quid chewing, four main areca alkaloids namely arecoline, arecaidine, guvacine, and guvacoline are absorbed (Gupta and Warnakulasuriya, 2002). These areca alkaloids acted as inhibitors of γ-aminobutyric acid receptor that have physiologic and metabolic effects on the human body systems including lung, gut, brain, cardiovascular system, and pancreas (Gupta and Warnakulasuriya, 2002).

BQ containing tobacco will increase exposure to tobacco specific nitrosamines (TSNA) and generate reactive oxygen species (ROS) during multistage carcinogenesis in the oral cavity (Mehrotra and Yadav, 2006). The production and release of ROS also

occurs under alkaline conditions due to the presence of slaked lime during antioxidation of areca nut polyphenols in the chewer's saliva (Manjari et. al., 1999). The ROS will cause tumorigenesis by being directly involved in tumour initiation process, inducing gene mutation (Mehrotra and Yadav, 2006). Previous studies (Wu et. al., 2006, Hsiao et. al., 2007, Zhang and Reichart, 2007) showed that betel quid chewing is not only linked to the development of oral and esophageal cancer, but also to other diseases such as hepatocellular carcinoma, liver cirrhosis, obesity, type II diabetes, chronic kidney disease, hypertension, hyperlipidemia, and metabolic syndrome (Chang et. al., 2006, Yen et. al., 2006, Guh et. al., 2007, Kang et. al., 2007, Lin et. al., 2008).

3.6.2 Smoking Habits

Tobacco consumption via smoking is the major risk factor for cancers of the oral cavity and pharynx in central Europe (Warnakulasuriya et. al., 2005). The risk of developing OC from this habit is estimated at 38 times as in heavy smokers who consumed +40 cigarettes/day as compared to abstainers (Blot et. al., 1988). The odds ratio for young people with OCs in southern England was significantly raised amongst males who had started smoking before the age of 16 years (Llewellyn et. al., 2004). Smoking is a dominant risk factor of OC in the United States (USA) where the development of cancer occurs at the upper aerodigestive tract (Bosetti et. al., 2008). Other developing countries such as Spain indicated the third highest incidence rates of oral cavity cancer in males and the trends are rising since 1983 up to now (Varela-Lema et. al., 2010).

According to Madani et. al., (2010) in his study in India, daily smokers have about a three fold increased risk compared those who never smoked. This is supported by the findings of Thomas et. al., (2007) in Papua New Guinea. It was reported by Muwonge et. al., (2008) that bidi smoking is an independent risk factor for cancer practised in Indians, which concurred with another previous study (Znaor et. al., 2003). Warnakulasuriya et. al., (2005) found an increased risk of OC for bidi smokers compared to those who never smoked, whereas there is no significant pattern of risk found for cigarette smokers. This result concurred with other studies by Rahman et al. (2003) and Subapriya et. al., (2007). According to Malson et. al., (2001 & 2002), the nicotine concentration in bidi is 21.2 mg/g as compared to commercial filtered cigarette (16.3 mg/g) and unfiltered cigarette (13.5 mg/g).

Therefore, it may be suggested that a higher content of nicotine in bidi contributed to higher risk of OC. Bidi in comparison to US cigarettes, contains a much higher concentration of several toxic agents such as hydrogen cyanide, carbon monoxide, ammonia, volatile phenols and carcinogenic hydrocarbons (Pednekar et. al., 2009). Smoking bidi is affordable to majority of Indian population rather than cigarette smoking that explained high risk of oral cancer in India (Madani et. al., 2010).

3.6.3 Alcohol Consumption

People who drink alcohol heavily are more likely to increase the risk of OC. Both smoking and drinking alcohol will synergistically increase risk of OC (Mehrotra and Yadav, 2006). Alcohol may act as an enhancer where the carcinogens will penetrate into target tissues while acetaldehyde which is the alcohol metabolite has been identified as a tumour promoter (Blot, 1992). In Japan, cancer in the floor of the mouth

and the oropharynx are the most common site of head and neck cancer due to alcohol habit among Japanese men (Zheng et. al., 1999). Alcohol drinking habit is shown to be associated with the increased risk of oral cancer in many western countries (Newell, 2003).

3.6.4 Human Papilloma Virus (HPV)

The Human Papilloma Virus (HPV) is a DNA virus with 7900 base pairs arranged in double stranded circular genome (Shah et. al., 2003). Different types of HPVs are classified according to their sequence of homology and this include HPVs types 6, 11, 13 and 32 (Shah et. al., 2003). HPV types 16, 18, 31, 33, 35 and 39 are commonly found associated with oral premalignant lesions and SCC (Zur Hausen, 1991). D'Souza et. al., (2007) reported that HPV is one of the major risk factor for developing oropharyngeal carcinoma. In his study, he demonstrated that HPV-16 existed in 72% of 100 pathology specimens and found that patients who developed HPV had an odds ratio of developing oropharyngeal cancer of 32.2.

Haddad (2007) reported that HPV-associated oral carcinoma occurs in a higher proportion of men, at a younger age and at a more advanced stage than non-HPV associated oral carcinoma. A study conducted by Saini et. al., (2011) indicated that infection with high-risk HPV is one of the contributing factors for OSCCs where HPV 16 was the predominant type found in Malaysian patients. The study also found a significant correlation between HPV and poorly differentiated carcinoma compared to well-differentiated OSCC. Saini et. al., (2010) also suggested that there was low risk of HPV infection in the oral cavity of women with cervical cancer.

3.6.5 Dietary Habits

From previous case control studies, researchers have suggested that diets high in animal fat and low in fruits and vegetables may increase the chances of having OC (Winn et. al., 1984, Singh 1991, Block 1992, Voelker 1995). β-Carotene is an antioxidant that can be found in plants and fruits. It is a precursor of vitamin A and its role to decrease the free radical damage in the body (Gaby et. al., 1991). Lack of vitamin A in the body can affect the cell differentiation, and lead to metaplasia in cells (Stacewicz-Sapuntzakis, 1997). From previous studies (Kaugars, 1996, Oral Cancer Foundation, 2011), there is evidence that low intake of β-carotene is associated with increase in breast, ovarian, cervical, gastric, lung and OCs. A low blood level of β-carotene is suggested to be associated with smoking and alcohol habits in OC (Stryker et. al., 1988, Nagao et. al., 2000).

A low intake of ascorbic acid has been reported to increase the risk for few types of cancers including stomach, oral cavity, esophagus, larynx, and cervix (Block, 1991, Mirvish, 1986). Ascorbic acid or well known as vitamin C is an antioxidant that gives essential nutrient for human body. It can prevent cancer growth by destabilizing a tumour's ability to grow under non-oxygen condition and prevent the DNA damage (Gao et. al., 2007). In another study that has been done by Gridley (1992) which involved more than 2000 cases, the use of vitamin E supplements were suggested to reduce the risk of oral and pharyngeal cancer.

3.6.6 Genetic Susceptibility

Variations in genetic susceptibility may be important in the aetiology of OC as equal to other factors such as tobacco and alcohol (Wang et. al., 2003). Some previous studies have found that individuals with polymorphism of *GSTM1, GSTT1* and *CYP1A1* showed increased risk of oral cancer, particularly with a low dose of cigarette smoking (Anatharaman et. al., 2007, Singh et. al., 2008). *CYP1A1, GSTM1* and *GSTT1* are the xenobiotic metabolizing enzymes that are widely reported to be associated with the increase of oral cancer (Anantharaman et. al., 2007). Conversely, a study by Amtha et. Al., (2009), from five different hospitals in Jakarta suggested that genetic polymorphisms of *GSTM1, GSTT1* and *CYP1A1* may not be risk factors for OC in the Jakarta population.

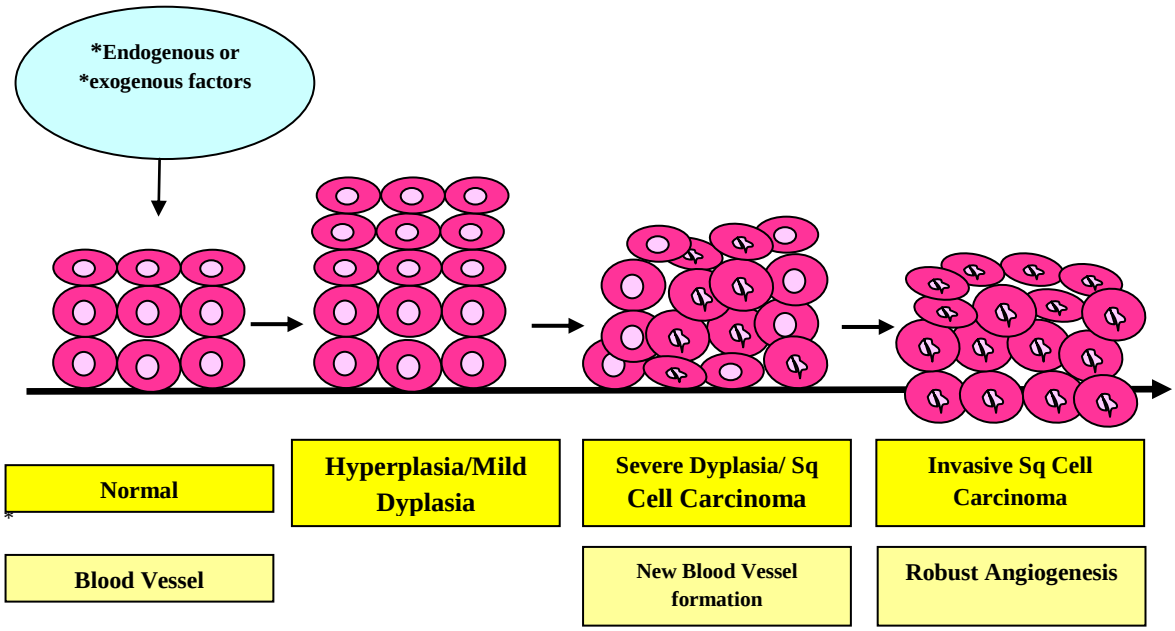
3.7 Carcinogenesis

Carcinogenesis is a multistage process of accumulation of gene defects that determine the characteristic traits of the cancer which involved the six hallmarks of cancers (Anatoly, 2005). During carcinogenesis, four main stages are involved in cancer development. It includes initiation, promotion, progression and malignant conversion (Fearon and Vogelstein, 1990). The development of cancer is a multistep and complex process that requires the accumulation of multiple genetic alterations, influenced by a patient's genetic predisposition and by the exposure to environmental carcinogens (Califano et. al., 1996) including tobacco, alcohol, chronic inflammation, and viral infection. Exposure to such carcinogens can lead to genetic damage.

Accumulation of genetic alterations as mentioned earlier can lead to change from normal oral mucosa to potentially malignant disorders and later to invasive carcinoma. These alterations include mutation and amplified activation of oncogenes and inactivation of tumour suppressor genes (TSG) which lead to the inhibition of cell proliferation. Following alterations of both oncogenes and TSG genes, tumour cells acquire autonomous self-sufficient growth and evade growth-inhibitory signals, resulting in uncontrolled tumour growth (Hanahan and Weinberg, 2000). Therefore, the tumour cells will escape programmed cell death and replicate continuously through the immortalization process. As tumour cells grow, invade, and metastasize, the formation of new blood vessel is critical. Hence, like tumour, cells are able to develop a blood supply by stimulating endothelial cell proliferation and develop new blood vessel formation itself (Choi and Myers, 2008).

During carcinogenesis, there is selective disruption of this process. Pro-angiogenic factors predominate, which is an essential part of solid tumour formation. (Hanahan and Weinberg, 2000). The subsequent progression of oral cancer includes tissue invasion and metastasis **(Figure 3.1)**.

The transition of normal epithelium to invasive cancer is accompanied by the process involving genetic alteration during multistep carcinogenesis, growth regulation, apoptosis, immortalization, angiogenesis, invasion, and metastases. As a conclusion, oral cancer develops through a series of histopathological stages: through mild (low grade), moderate, and severe (high grade) dysplasia to carcinoma *in situ* and finally invasive disease (Tsantoulis et. al., 2007).



*Exogenous factors = Carcinogens, diet, habits, environmental pollutants, viral infection

*Endogenous factors = DNA repair, programmed cell death, cell cycle, growth factors, cell signalling

Figure 3.1: Theoretical model of carcinogenesis in the oral cavity based on multistep carcinogenesis. (Adapted from: Tsantoulis et. al., 2007 and Choi and Myers, 2008).

3.8 Hallmarks of Cancer

The hallmarks of cancer consist of six changes namely self sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

3.8.1 Self Sufficiency in Growth Signals

In normal cell, Growth Signal (GS) is a tool required for cell division and growth. It will bind onto the transmembrane receptors and activate molecules interaction in cell. But, in cancer, the cell is overexpressed and generated a lot of uncontrolled artificial growth signals (Fedi et. al., 1997) and changes the stimulation of producing normal tissue (Hanahan and Weinberg, 2000).

3.8.2 Insensitivity to Antigrowth Signals

The normal cells differentiate during the post-mitotic stage where the cells carry a specific role beneficial to the organism. The function of the anti-growth signals is to block the proliferation process of damaged cells. But in cancer cells, the anti growth signals are ignored, as a consequence, it prevents normal function and full cell maturity; thus, there is continuous replication of cancer cells (Abbott et. al., 2006).

3.8.3 Evading Apoptosis

Apoptosis is programmed cell death that can occur in multicellular organisms. In a normal cell, receptors for intra and extracellular apoptosis signal are expressed to detect abnormalities such as DNA damage and signalling imbalances (Abbott et. al., 2006, Hanahan and Weinberg, 2000).

3.8.4 Limitless Replicative Potential

Early work by Hayflick, (1965) proposed that cells in culture have a fixed limit replicative potential to 50-70 divisions, after which they are not able to divide. This limitation will prevent cells from spreading all around the system. However, cancer cells are able to adapt with the surrounding microenvironment and become immortal (Abbott et. al., 2006). The cells are able to divide uncontrollably.

This mechanism arises from the loss of 50 to 100 bp of telomeric DNA of each chromosome duplication during each cell cycle due to inability of DNA polymerase to replicate the chromosomes completely. The DNA is exposed and the genetic material exchanges its configuration, causing the cell to die. While in cancer cell, the overproduction of telomerase enzyme would occur and extend the telomeric DNA length through the homologous recombination process. In normal cells, this process is prevented by mismatched repair.

3.8.5 Sustained Angiogenesis

Angiogenesis is a process where the new blood vessels are regulated after the formation of tissue. Angiogenesis is regulated by a multiple of signals which involves inducers and inhibitors (Folkman, 2002). Overproduction, lack of both of inhibitors or inducers may cause the formation of cancerous cell. To determine the angiogenesis threshold of inhibitors to inducers ratio, *angiogenic switch* is used as a landmark (Fiddler et. al., 2001).

3.8.6 Tissue Invasion and Metastasis

Invasion and metastasis are complex processes where the biochemical determination remained incompletely understood (Hanahan and Weinberg, 2000). Metastasis is the final stage of cancer development in which cancer cells spread to new locations in the body. Cancer cells can develop through mutation, transcriptional repression or proteolysis. Once cancer developed, it will influence the regulatory signals. Protease production also can be increased if the cancer spread, causing destruction inherent in the invasion of tissue (Hanahan and Weinberg, 2000, Abbott et. al.2006).

3.9 Genes Involved in OC

A gene is the basic unit of heredity in a living organism. To form cancerous environment, only a minority of the critical genes that are commonly mutated in the major cancer types are involved (Shah et. al., 2003). Mutated genes can cause overproduction, underproduction of protein, or alterations of proteins that will affect the functions of the proteins in the human body (Dixon and Kopras, 2004).

3.9.1 Oncogenes

An oncogene is a gene that helps a normal cell turns into a cancer cell, when mutated or expressed at high levels (Croce, 2008). Most oncogenes require additional steps such as mutation or environmental factors to be activated. In the human body, oncogenes arise from the normal gene called proto-oncogenes. Oncogenes are genes that are able to increase malignant potential in cells and major oncogenes that implicated other cancer types also contribute to the progression of OC. A huge amount of these genes promote unscheduled, aberrant proliferation, override the Growth (G), Synthesis (S) and Mitosis (M) checkpoints of the cell cycle, prevent apoptosis and cause the cellular survival under unfavorable conditions (Tsantoulis et. al., 2007).

Several oncogenes related to oral carcinogenesis are proto-oncogenes such as epidermal growth factor receptor (*EGFR*), members of the ras gene family (H, K and N-ras), *c-myc, int-2, hst-1, PRAD-1,* cyclin family (*CCND1*), *bcl-1* and recent findings which include vascular endothelial growth factor (*VEGF*) and matrix metalloproteinases (MMPs family) (Todd et. al., 1997, Tsantoulis et. al., 2007).

The epidermal growth factor receptor, *EGFR*, also known as *ErbB1* or *Her-1*, stimulate the proliferation of epithelial cells. Mutations of genes encoding growth factor receptors can result in an increased number of receptors or production of a continuous ligand-independent mitogenic signal (Todd e. al., 1997, Tsantoulis et. al., 2007). According to Grandis et. al., (1993a and 1993b), three mechanisms have been suggested to activate the *EGFR* gene in carcinogenesis: firstly, the deletion or mutations in the N-terminal ligand-binding domain such as occur in the viral oncogene *v-erb8*, second is the overexpression of the *EGFR* gene concurrent with the continuous presence of *EGF* or *TGF-cr*; and lastly the deletion in the C-terminus of the receptor that prevents downregulation of the receptor after ligand binding.

Patridge et. al., (1988) and Grandis et. al., (1993) showed that gene amplification and increased numbers of *EGF* receptors in OCs are associated with tumor grading. *EGFR* over-expression was also reported to be an independent prognostic marker of survival in BQ chewers (Chen et. al., 2003a). Other reports by Salomon et. al., (1995) also suggested that the over-expression of *EGFR* and *Her-2* are associated with higher grades or reduced survival in a variety of cancers, including breast, colorectal, and head and neck cancers.

The frequency of oncogene mutation namely ras genes, is estimated to be approximately 0–10% in the USA (Xu et. al., 1998), Europe (Kiaris et. al., 1995) and Japan (Matsuda et. al., 1996, Sakata et. al., 1996). In India, the *H-ras* and *K-ras* mutations may be present in 28–35% of tumors studied (Saranath et. al., 1991, Das et. al., 2000). Interestingly, a recent study has shown significant risk (odds ratio 1.6) associated with *H-ras* gene polymorphism in the Indian population (Sathyan et. al., 2006).

Abundant expression of *cyclin D* is also a common (36–66%) feature of OC (Koontongkaew et. al., 2000, Miyamoto et. al., 2003) and pre-malignant lesions (Rousseau et. al., 2001). According to Miyamoto et. al., (2003), Cyclin D over-expression or, specifically, *CCND1* gene amplification may predict worse prognosis and cause a greater risk of occult cervical lymph node metastasis in low stage tumors (Myo et. al., 2005). Cyclin A overexpression, which is closely associated with the presence of S-phase cells, has also been observed immunohistochemically (Kushner et. al., 1999, Chen et. al., 2003b) and was most prevalent in advanced tumors.

Similarly, *Cyclin B* was overexpressed in 37% of tongue tumours (Hassan et. al., 2007) and in OC in general (Kushner et. al., 1999). The *Cyclin D1* gene is frequently overexpressed in OCs as a result of amplification of the 11q13 region and the association of tumour grade on this region might increase the risk of OC and might be related to cancer prognosis (Kushner et. al., 1999).

3.9.2 Tumour Suppressor Genes (TSGs)

TSGs play roles as anti-proliferation signals and proteins that suppress mitosis and cell growth and react as a transcription factor in a condition of DNA damage and cellular stress (Kleinsmith, 2006). TSGs are usually entrusted with the regulation of discrete checkpoints during cell cycle progression and with the monitoring of DNA replication and mitosis. Cellular stress and a variety of insults can activate tumour suppressor pathways to arrest the cell cycle (Tsantoulis et. al., 2007).

The retinoblastoma protein and its associated molecular network are frequent found and is predicted to act as an early targets in many tumours. The retinoblastoma

tumour suppressor gene (*Rb*) encodes a nuclear phosphoprotein that is found in a mutated form in human tumours and plays an important role in the control of cell division and differentiation of oral squamous epithelium (Girod, 1999). Lack of immunohistochemical pRb expression was found in approximately 70% of oral tumors (Pande et. al., 1998, Koontongkaew et.al., 2000) and 64% of premalignant lesions. Soni et. al., (2005) reported that 84% of premalignant lesions and 90% of OSCC show altered expression of at least one of the components of the pRb network.

Runt-related transcription factor 3 (*RUNX3*) is one of the TSG in cancer and appear to be an important component of the transforming growth factor-beta (*TGF-β*) - induced tumour suppression pathway (Ito, 2004). Takaaki et. al., (2009) in their study on head and neck SCC suggested that *RUNX3* play an oncogenic role in cancer development with its involvement in the development of oral mucosa through the growth factor signalling pathway. Another TSG reported to be associated with OC is the *PRTFDC1* gene. This gene is located at chromosome 10p12. Suzuki et. al., (2007), suggested that epigenetic silencing of *PRTFDC1* by hypermethylation of CpG island may lead to a loss of *PRTFDC1* function and may be involved in oral carcinogenesis.

Other TSG related to OC is the *p53* gene. It is inactivated during oral carcinogenesis. The p53 protein is able to enforce cell cycle arrest or apoptosis under replication stress, thus causing the proliferation of potentially malignant cells (Tsantoulis et. al., 2007). Loss-of-heterozygosity (LOH) in the chromosome region of 17p13 that hosts the *TP53* gene is very common in oral cancer (Huang et. al., 1999, Lin et. al., 2002). The prognostic value of the *p53* status in OC is uncertain and many studies have not found any impact on patient survival (Xie et. al., 1999, Gonzales Moles et. al., 2001). Nevertheless, the expression of *p53* may predict poor prognosis in the

subset of patients with low stage and node negative disease (De Vecinte et. al., 2004). Overexpression was reported to be related with use of tobacco and alcohol consumption in both carcinomas and pre-malignant lesions in OC (Scully et. al., 2000).

Besides earlier mentioned genes that are involved in OC, the *CDKN2A* locus that encodes $p16^{INK4A}$ gene located on chromosome 9p21 is one of the most vulnerable areas of the genome in OC (Tsantoulis et. al., 2007). Lack of immunohistochemical p16 expression can be found in up to 83% of oral tumors (Reed et. al., 1996, Pande et. al., 1998) and up to 60% of pre-malignant lesions. **Figure 3.2 and Table 3.2** illustrated possible oncogenes and TSGs that are involved in oral carcinogenesis.

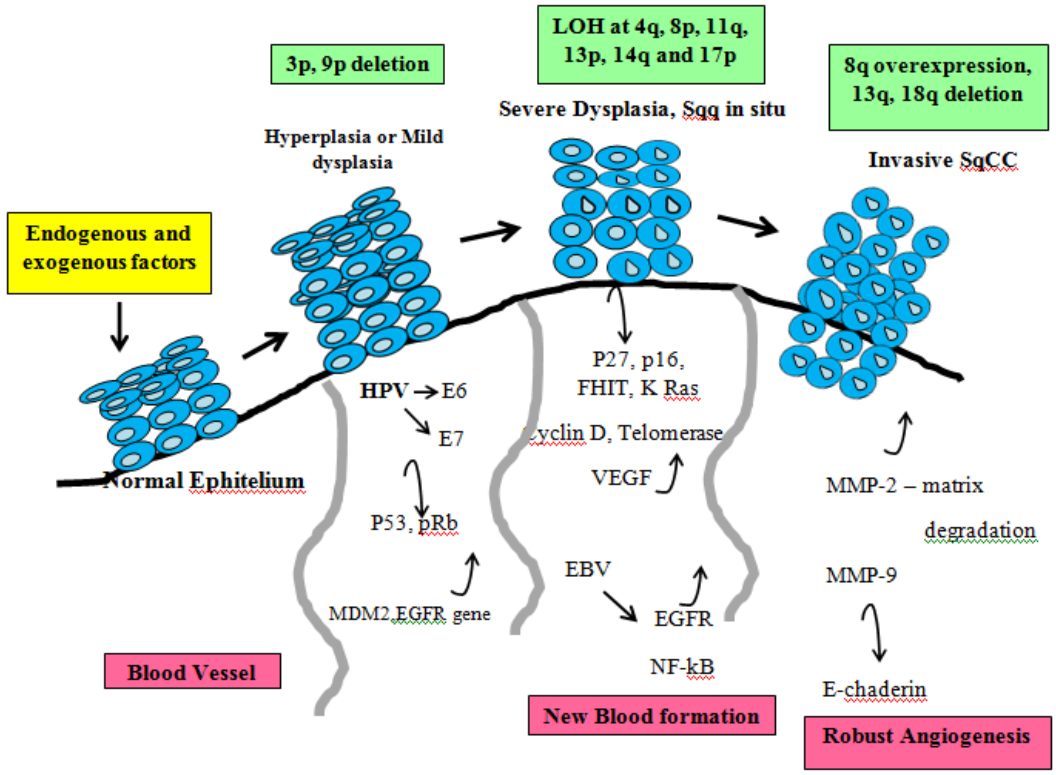


Figure 3.2: Possible oncogenes and tumour suppressor genes involved in oral cancer as the tumour progress from normal to squamous cell carcinoma (Adapted and modified from Todd et. al., 1997, Tsantoulis et. al., 2007, and Choi and Myers, 2008).

Table 3.1: Summary of oncogenes and TSGs involved in oral carcinogenesis reported in multiple studies

Type	Genes	Chromosome Location	References
Oncogene	*EGFR*	7p11.2	Grandis et. al., (1993), Salomon et. al., (1995), Todd e. al., (1997), Chen et. al., (2003). Tsantoulis et. al., (2007)
	H-Ras, K-Ras	11p15.5, 12p12.1	Saranath et. al., (1991), Kiaris et. al., (1995), Matsuda et. al., (1996), Sakata et. al., (1996), Xu et. al., (1998), Das et. al., (2000), Sathyan et. al., (2006)
	CCND1	11q13.3	Kushner et. al., (1999), Koontongkaew et. al., (2000), Chen et. al., (2003), Miyamoto et. al., (2003), Myo et. al., (2005), Hassan et. al., (2007)
TSG	*pRb*	13q14.2	Pande et. al., (1998), Girod, (1999), Koontongkaew et. al., (2000), Soni et. al., (2005)
	RUNX3	1p36.11	Ito, (2004), Takaaki et. al., (2009)
	TGF-β	19q13.2	Ito, (2004)
	PRTFDC1	10p12	Suzuki et. al., (2007)
	p53	17p13	Huang et. al., (1999), Xie et. al., (1999), Scully et. al., (2000), Gonzales Moles et. al., (2001), .Lin et. al., (2002), de Vecinte et. al., (2004), Tsaitoulis et. al., (2007)
	p16	9p21	Reed et. al., (1996), Pande et. al., (1998), Tsaitoulis et. al., (2007), Choi and Myers, (2008).

3.10 Cytogenetics Study

3.10.1 Human Chromosomes

The word chromosome means ‘coloured body’ referring to its ability to adapt with histological stains more effectively compared to other cell structures. It is composed of a long DNA compacted with protein and RNA (Dorian and Bruce, 2008). A chromosome is a single piece of coiled DNA containing genetic information, many genes, regulatory elements and other nucleotide sequences (Dorion and Bruce, 2008). In a normal human, there are 23 pairs of chromosomes including one pair of sex chromosomes (X and Y) and 22 pairs of autosomal chromosomes.

3.10.2 Definition of Cytogenetics

Cytogenetics is defined as the study of the number and structure of chromosomes (Dorion and Bruce, 2008). Cytogenetics was introduced as the study of cells and genetics. Cytogenetics is one of the genetic fields that focus on the study of the structure and function of the cell especially the chromosomes. It is the study of the structure of chromosome material which includes routine analysis of Giemsa (G)-Banded technique well known as karyotyping. G-banding has been the standard method for identifying numerical and structural chromosome aberrations (Brondum et. al., 1995).

Karyotyping however has its limitations. It can only detect large changes in the chromosome such as large deletions and insertions of base pairs, translocations, inversions and duplications. It cannot detect single nucleotide changes, deletions, or

insertions. Cytogenetics is useful in the diagnosis of many types of hematologic malignancies. It is difficult to identify clonal cytogenetic aberrations using conventional karyotype analysis because of the complexity of genetic changes (Cingoz et. al., 2003). With current technological and introduction of molecular cytogenetics, it is possible to get more specific and accurate results.

3.10.3 Molecular Cytogenetics and Cytogenetics Techniques

Molecular cytogenetics is a combination of molecular biology and cytogenetic study. It was designed to improve the diagnostics in cancer and genetic diseases (Chiara et. al., 2002, Tomoka et. al., 2006, Manning et. al., 2007). Molecular cytogenetics techniques involve the use of FISH, CGH and arrayCGH which focuses on gene detection. All these techniques use the same principle in which DNA probes are labelled with different fluorescent colours tags in order to visualize specific regions of the genome. These new technologies are advantageous to cytogeneticists worldwide in their search for prognostic markers and therapeutic targets for cancer.

3.10.3.1 Fluorescence *in situ* Hybridization (FISH)

FISH is a molecular cytogenetic technique that is used to detect the presence or absence of specific DNA sequences on chromosomes (Sieben et. al., 2006) or more specifically to detect chromosome aberrations at specific regions (Tomoko et. al., 2006). This technique is the first molecular cytogenetic technique introduced and is a revolution in the clinical identification of chromosome abnormalities (Jauch et. al., 1990).

In FISH, DNA probe is labelled with fluorescent agents to the target DNA on the chromosome either at metaphase or interphase phase during the cell cycle. It will detect small regions on the chromosomes that contain specific genes (Tomoko et. al., 2006). Clinically, FISH can be used in multiple disciplines for diagnosis, prognosis, and evaluating the stage of remission cancer (Chiara et. al., 2002).

3.10.3.2 Conventional Comparative Genomic Hybridization (CGH)

FISH based screening methods mentioned above have a common feature, which is shared with standard G-banding chromosome analysis, which is the dependence on the presence of metaphase in neoplastic cells (Chiara et. al., 2002). Whenever the mitotic rate of the neoplastic cells is poor, it was difficult to analyse using this technique. Therefore, the next generation of molecular cytogenetic technique which is called CGH is introduced. This technique is based on the *in situ* hybridisation of differentially labelled total genomic tumour DNA and normal reference DNA to normal human metaphase slide (Saeed et. al., 2007). CGH can detect copy number change without the need of cell culture (Kallionemi et. al., 1992).

CGH will detect sequence copy number differences present in tumour cells by characterizing the gains and losses in chromosomes (Chiara et. al., 2002). CGH is able to detect different grade of tumour more specifically (Saeed et. al., 2007) but do have limitation in detecting amplification and deletion at low resolution of only 10 to 20MB (Qing et. al 2005). Other findings also found that the results from CGH analysis were not consistent as it was not always linearly distributed (Piper et. al., 1995) and only reveal relative copy numbers (Du Manoir et. al., 1995). Differentially labelled tumour DNA and control normal DNA are co-hybridized to a metaphase chromosome spread

producing an average fluorescence ratio profile at approximately 20 Mbp resolution (Gebhart, 2004). While CGH provides a profile of the entire genome, the resolution is limited and therefore it is difficult to determine the identity of the specific gene that is altered. CGH study is often used in conjunction with FISH in order to fine map alterations to the gene level, in other words, FISH can be used to validate as results arising from CGH.

Current cytogenetic research focuses on the use of array technology which can detect genomic alteration at a higher resolution. Furthermore, the technique for array CGH is made simpler where in conventional CGH, there is a need to karyotype the chromosomes during analysis, but in arrayCGH, the arrangement of the chromosomes will be done automatically.

3.10.3.3 Array Comparative Genomic Hybridization (arrayCGH)

At first, conventional CGH that include metaphase analysis is considered as the most significant technical development in molecular cytogenetics in terms of further understanding of the cancer genome. But, with the development of multiple microarray technology nowadays, cytogenetic research also introduced its latest development which include arrayCGH in which the study is based on copy number variations. High resolution arrays allow for the detection of amplification and deletion boundaries in a single experiment. This type of array has been instrumental in the analysis of specific chromosomal regions (Michels et. al., 2007). This technology has been applied to analyze cell lines and tumours from lymphoma, bladder, breast, prostate, and kidney (Garnis et.al., 2004a, Carvalho et. al., 2004, Ahn et. al., 2010).

Array is a technology that used advanced genomic approach by targeting loci selected from the entire genome as regions of interest compared to FISH and CGH (Vissers et. al., 2003). ArrayCGH has provided a wealth of new information on copy number changes in cancer on a genome-wide level and also been utilized in cancer classification (Oh et. al., 2010). It also allowed highly accurate localization of specific genetic alterations that might be associated with tumour progression, response to therapy, or patient outcome and can detect genes involved that are likely to contribute to cancer development (Wicker et. al., 2007).

Chromosomal alterations are known to have a role in the initiation and progression of OSCC (Lisa, 2004). By applying arrayCGH, any alteration at a given size might also be detected and thus is more sensitive (Bradley et. al., 2007). ArrayCGH is rapidly gaining acceptance as a fast and efficient method of identifying chromosomal aberrations at a higher resolution than conventional non-array methods (Albertson and Pinkel, 2003). Furthermore, DNA arrayCGH profiling reduces noise and increases the sensitivity to detect genomic alterations at higher resolutions compared to conventional CGH (Qing et. al., 2005).

However, there are limitations of arrayCGH technique. Balanced chromosome rearrangements, such as balanced translocations and inversions cannot be identified using arrayCGH as these chromosomal rearrangements do not result in any loss or gain of chromosome material (www.rarechromo.org). Besides, it will also not detect some types of polyploidy and cannot detect mutation caused by point (single base pair) changes in the DNA.

3.11 Chromosomal Alterations and OC

Genetic alterations are a key feature of cancer cells, and typically target biological processes and pathways that contribute to cancer carcinogenesis. ArrayCGH has been widely used to detect chromosomal aberrations in cancers from the breast (Daigo et. al., 2001), gastric (Tay et. al., 2003), bladder (Veltman et. al., 2003, Takeno et. al., 2009), and ovarian (Schraml et. al., 2003).

3.11.1 Studies on CGH

Few early studies on OC using CGH have been described. Rosin et. al., (2002) reported that alterations in OSCC were mainly detected on 17q21–tel, 20q, 11q13, 3q27–29, and the X chromosome. Among these, gains of *EGFR* at 7p, *FGF4/FGF3*, *CCND1* and *EMS1* at 11q13, and *AIB1* at 20q were significantly associated with lymph node metastasis (N). Another study conducted by Garnis et. al., (2003) on 20 microdissected OSCC showed multiple and recurrent segments of copy number changes that include the detection of the *FHIT* gene; novel segments of copy decrease at 3p22, 3p24, and 3p26; and an unexpected 0.7 Mbp segmental increase at 3p21.

A study by Scully et. al., (2000) showed that the most common regions for amplification were identified at chromosomes 11 and 17, whilst the most commonly deleted regions were at chromosome 3 (3p14) and 9 (9p21). Pathare et. al., (2009) reported that the most common gains were on chromosome regions 8q, 9q, 11q13, 7p, 3q, 20q, 20p and 5p and the commonly deleted regions were observed on regions 3p, 8p, 18q and 11q14-qter.

3.11.2 Studies on arrayCGH

In OC, an arrayCGH study conducted by Ivy et. al., (2008), who focussed on chromosome 3p region, found that the genomic alteration at the 3p region increase with tumour grade. Another study on oral cancer using arrayCGH in patients aged 40 and 50 years and with smoking habit, showed the specific different genomic profiles as compared to the classically described studies on FISH and CGH (O'Regan et. al., 2006).

An earlier study by Garnis et. al., (2004) found that multiple regions of chromosome 8q (8q22 to q24) were amplified in head and neck SCCs. Garnis et. al., (2009) found that the most commonly observed alteration in high grade dysplasia (HGD)/carcinoma in situ (CIS) lesions was gain at chromosome 20p, 1p, 2q, 3q, 5q, 7q, and 8p. They also discovered deletion of chromosome 3p and gain of chromosome 8q. Besides that, alterations were commonly observed on chromosome 5p, 9q, 11q, and 19p in invasive tumours. Another study by Baldwin et. al., (2005) has suggested novel alterations in OC which includes copy number gains at 3q23, 5p15.2, 7p12.3-13, 7q21.2, and 7q35 and copy number losses at 2p15, 4q34.3, and 16q23.2.

In another study by O'Regan et. al., (2006) in young OC patients with smoking habit, tumours from the older cohort manifested deletions involving 3p and 9p21 and gains involving 1p, 5p, 7p, 11p (subtelomeric regions), 3q, 5q33, 7p, 8q, 11q13, and 20q13. In young patients the alterations involved 1p, 3p (subtelomeric), 3q, 11p, 11q13, 11q13-q14, 14q32.1, and 22q11.2. They also discovered that from the tumour size (T) classification of OC, they found that the number of aberrations did increase with increasing T classification but this trend was not statistically significant. Sparano et. al.,

(2006) in their study on 0.9 Mb arrayCGH distinct resolution found that the most frequently gained regions were located at chromosome 3q, 5p, 8q, 9q and 20q. Conversely, they found that regions 3p, 8p, 13q and 18q were the most frequently deleted in head and neck cancer.

Chen et. al., (2004) reported that most gained genes were at chromosome 17q21-tel, 20q, 11q13, 3q27-29 and the X chromosome where selected genes located on chromosome 11q13 and 20q showed significant association with lymph node metastasis. Most of the arrayCGH studies were done in the Western countries, Japan and some parts of the Asian region; therefore the study on arrayCGH technology was applied in this research. **Table 3.2** demonstrated the summary of chromosomal alterations in OC reported by previous studies worldwide.

Table 3.2: Summary of chromosomal alterations in OC reported by previous studies worldwide.

Alteration	Chromosome	References	
		CGH	**arrayCGH**
Amplification	3q region	Rosin et. al., (2002), Garnis et. al., (2003), Panthare et. al., (2009)	Chen et. al., (2004), Baldwin et. al., (2005), O' Regan et. al., (2006), Sparano et. al., (2006), Ivy et. al., (2008), Cathie et. al., (2009)
	8q region	Veltman et. al., (2003), Miriam et. al., (2002), Panthare et. al., (2009)	Garnis et. al., (2004), Baldwin et. al., (2005), O' Regan et. al., (2006), Sparano et. al., (2006), Cathie et. al., (2009), Takeno et.al., (2009)
	11q region	Rosin et. al., (2002), Scully et. al., (2000)	Chen et. al., (2004), Baldwin et. al., (2005), O' Regan et. al., (2006)
	7p region	Rosin et. al., (2002)	Baldwin et. al., (2005), O' Regan et. al., (2006)
	20q region	Rosin et. al., (2002), Panthare et. al., (2009)	Chen et. al., (2004), O' Regan et. al., (2006), Sparano et. al., (2006)
Deletion	3p region	Garnis et. al., (2003), Scully et. al., (2000), Panthare et. al., (2009)	O'Regan et. al., (2006), Sparano et. al., (2006), Ivy et. al., (2008), Cathie et. al., (2009)
	9p region	Scully et. al., (2000)	Baldwin et. al., (2005), O' Regan et. al., (2006)
	18q region	Scully et. al., (2000)	Baldwin et. al., (2005), Sparano et. al., (2006)

3.12 Validation Technique - Real Time quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR technology offers fast and reliable quantification of any target sequence in a sample (Burgos et. al., 2002). SYBR Green I dye was used in qPCR analysis to determine copy number and gene expression as it provided a reliable alternative to traditional blotting methods and is much more cheaper compared to TaqMan protocols (Vilalta et. al., 2002). Both detection formats have about the same detection limit, reproducibility, and dynamic range (Deprez et al., 2002, Wilhelm et al., 2003).

Other application usage for qPCR are as follow: firstly, to measure the mRNA levels in various gene expression studies based on mRNA to cDNA transcription and quantification of cDNA, secondly, to analyze genomic DNA and measure gene copy numbers for the detection of gene deletions or amplifications in tumours or as a molecular assessment of bacterial and viral pathogen loads (Kato et. al., 2004). Absolute and relative quantification methods (Klein, 2002) were used to quantify the copy number. Absolute quantification determines the exact copy concentration of target gene by relating the *C*T value to a standard curve (Yu et. al., 2005) while relative quantification presents the amount of target gene in a sample relative to reference genes.

Recently, this method has become a popular method for gene copy number measurements. In contrast, traditional methods for measuring DNA copy numbers, such as FISH, are difficult to perform in high throughput and fail to detect small deletions/duplications (Ginzinger, 2002, Rooms et. al., 2005) besides being expensive.

CHAPTER 4

MATERIALS AND METHODOLOGY

4.1 Study Design

This is a descriptive study looking into chromosomal alterations in OSCC using fresh frozen oral cancer tissues from the Malaysian Oral Cancer Database and Tumour Bank System (MOCDTBS) coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC) from the University of Malaya (UM).

4.2 Sample Selection

4.2.1 Test Samples

A total of 20 fresh frozen tumour tissues diagnosed as OSCC were included in this study. The patients's socio-demographic data which included risk habits (smoking, betel quid chewing and drinking), age, gender and ethnicity together with patient's clinicopathological parameters; tumour grade, tumour size (T), lymph node status (N) and TNM group staging were obtained from the MOCDTBS.

4.2.2 Reference Samples

Genomic DNA (gDNA) extracted from buffy coat from healthy man and woman without any cancer were obtained from MOCDTBS and used as reference samples in this study. Only one specific patient from each gender male and female was used in this study to avoid cross contamination as well as to better characterize the copy number polymorphisms (Jaillard et. al., 2010).

As this is part of a major study from a project – "Oral Cancer & Precancer in Malaysia – Risk Factors, Prognostic Markers, Gene Expression and Impact on Quality of Life", the same ethics approval was used for this study. The approval was given by the Medical Ethics Committee, Faculty of Dentistry, and University of Malaya [Medical Ethics Committee approval no: DF 0306/001/ (L)].

4.3 Criteria

4.3.1 Inclusion criteria

- OSCC tissues with more than 70% tumour were included in this study
- The fresh frozen tumour tissues selected were from indigenous patients of Sarawak and Malaysian Indian patients

4.3.2 Exclusion criteria

- OSCC tissues with less than 70% tumour were excluded from this study
- OSCC tissues from other than Indigenous and Indian patients were excluded from this study

4.4 Processing of Frozen Tissue Sample

A series of 750 µm thick fresh frozen tumour tissue sections that was mounted in Optimal Cutting Temperature Compound (OCT) was sectioned using a cryostat and stained with Haematoxylin and Eosin (H & E). Reference slides of 5 µm were cut at the beginning and the end of 750 µm tissue cut to microscopically confirm the existence of tumour cells. The tumour tissue sections were collected in 1.5 ml sterile micro centrifuge tubes. The frozen tumour tissues were macro-dissected to ensure that the sample contained 70% of epithelial tumour tissue (the diagnosis was confirmed by oral pathologists).

4.5 Genomic DNA (gDNA) Extraction Procedure

For test and reference samples, DNA from frozen tissue (test) and blood (reference) was isolated by using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc, Chartworth, CA) according to the manufacturer's instructions with minor modifications. During the initial process, after the incubation at 55°C and shaking at 450 rpm in thermomixer (Qiagen, Inc, Chartworth, CA), 4 µl of RNase A (100 mg/mL) (Qiagen, Inc, Chartworth, CA) was added and incubated for 2 minutes at room temperature to avoid protein and RNA cross contamination.

At the end of the process, final volume of Tris EDTA (TE) buffer used was 100 µl instead of 200 µl suggested by the manufacturer, due to the small size of the sectioned samples, and to increase the DNA yield. Detailed protocol is as in **Appendix A.** A260/280 ratio and concentration (ng/ul) of gDNA was measured by NanoDrop

Spectrophotometer (Thermo Fischer Scientific Inc) with minimum ratio at 1.8 for each sample used.

4.6 Oligonucleotide arrayCGH Protocol

60-mer Oligonucleotide arrayCGH was performed using the Human Genome CGH microarray 44K (Agilent Technologies, Santa Clara, CA, USA). ArrayCGH application uses two fluorescent colour process which consist of Cyanine 3-dUTP and cyanine 5-dUTP to measure DNA copy number alterations (CNAs) changes in test samples versus reference samples (Jon et. al., 2009). It consist of six main steps: 1) Amplification of DNA (gDNA), 2) WGA DNA Labelling, 3) Microarray Hybridization 4) Washing of microarray chips, 5) Scanning of microarray by Agilent Scanner Control version 7 and Feature Extraction 9.0.14 and 6) Microarray Analysis by Workbench Standard Edition 5.0.14 software.

For Step 1, 100 ng of genomic gDNA from the test sample and 100 ng from the reference sample were used to amplify representative DNA. GenomePlex Whole Genome Amplification (WGA) Kit (Sigma Aldrich Co. Ltd) was used to generate representative amplification of gDNA. The detailed protocol is as in **Appendix B.** After the amplification of gDNA, the DNA was then purified by using Sigma GenElute PCR Clean-Up Kit (Sigma Aldrich Co. Ltd) according to manufacturer's instruction **(Appendix C).**

The WGA DNA was labelled by using Agilent Genomic DNA Labelling Kit PLUS (Agilent, California, USA) to differently label DNA with fluorescent-labelled nucleotide which included Cyanine 5-dUTP (Cy 5) dye for test sample while the reference sample is labelled by Cyanine 3-dUTP (Cy 3) dye. Detailed description of steps 2 to 4 is attached in **Appendix D** and performed according to manufacturer's instructions.

Microarray was scanned using the Agilent scanner and the image was analyzed using Agilent Feature Extraction (FE) Software version 9.5 (CGH_QCM_Feb 2007 protocol). QC report was generated for each patient and Derivative Log Ratio Spread (DLRS) was used as quality criteria to determine aberrations. Details are attached in **Appendix E.**

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4.7 Validation of *DUSP22* gene using RT-qPCR

Arising from the genes list generated from the arrayCGH above, *DUSP22* which was amongst the most commonly amplified genes, was selected for the validation process. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to detect the presence of the selected gene and to determine copy number and gene expression changes.

To detect copy number changes, twenty (20) gDNA from OSCC samples that overlapped with the previous arrayCGH cases and twelve (12) independent samples obtained from MOCDTBS were used. The additional of independent samples were important to determine the efficiency of arrayCGH technique as well as to relate any significant correlation with a larger size. A gDNA from a healthy patient was also

included as a control. Besides copy number study, the expression of the *DUSP22* gene was also examined at gene expression (GE) level by using thirty (30) cDNA samples from OSCC patients and three (3) healthy individuals obtained from MOCDTBS using RT-qPCR method. RT-qPCR was applied on an ABI Fast Applied Biosystems 75000HT sequence detection system (Applied Biosystem, Foster City, CA, USA) with the usage of fluorescent SYBR Green Dye Kit.

Primers for *DUSP22* gene and internal control primers namely 1q24.2 were designed by using The National Centre for Biotechnology Information (NCBI) databases and the sequences for both were tabulated in **Table 4.1**. According to Diskin et. al., (2009), this chromosome region of 1q24.2 gave the most stable condition for copy number study. The qPCR were performed in a 25µl reaction volume containing 12.5µl SYBR® Green I Dye, 2µl of combined forward and reverse primers (*DUSP22* gene or internal control), 50 ng of gDNA and double distilled water (ddH^2O). Sample was then amplified on a Fast Applied Biosystems 75000HT Sequence Detection System.

The quantitative analyses was then measured by the detection of relative threshold for fluorescence (Comparative CT - ΔΔCt method) with the standard conditions as followed: denatured at 50ºC for 2 minutes, followed at 95ºC for 10 minutes, 40 cycles of 95ºC for 15s and lastly at 60ºC for 1 minute. Each run was performed in triplicates. During each annealing cycle, the emission of SYBR Green fluorescent was acquired. The same method was applied for gene expression study with minor modifications on annealing temperature.

RQ value for each sample was multiplied by 2 to calculate copy number (CN) changes (ABI Foster City, CA, USA). Cut off point for most likely copy number for each unknown samples were determined by the RQ results between 1.414 and 2.449 that most likely represent a normal copy number of 2. Anything below or above these thresholds is considered a deletion (CN=1) or a duplication (CN=3 or more) respectively (D'Haene et. al., 2010). For gene expression (GE) study, the RQ value used was generated directly from qPCR analysis results.

Table 4.1: Primer sequences for internal control and *DUSP22* gene

Gene	Sequences
DUSP22	F : CGGTCCCTGCCGCTGACTTG R : GGTGGCTGAAGGCGAACGCT
Internal Control (1q24.2)	F : CAAGTGCCAACAGAGTTGC R : AATGAAGGAAGAGAATCAGTTCAG

4.8 Statistical Analysis

The statistical analysis was done using Statistical Package for Social Sciences (SPSS version 19) software. The relationship of chromosomal alteration regions with socio-demographic data (gender, ethnicity and habits) and selected clinicopathological parameter (TNM stage, tumour size (T), lymph node status (N), tumour grade and tumour site) was analyzed using Chi Square or *Fischer's* exact test whenever appropriate.

For data analysis, clinicopathological parameters were grouped together as follows: For TNM Staging group, it was divided to two categories where Stage I & II was combined in group 1 while Stage III & Stage IV in group 2. For tumour site, it was divided to two categories: tongue and others [buccal mucosa (BM), gum, palate, lip, floor of mouth (FOM)]. Primary tumour size (T) parameter was categorized to two groups; T1 & T2 and T3 & T4 combination. With regards lymph node status (N), cases are divided into positive and negative status. For habits, analysis was categorized into two groups i.e., 1) all chewing (either single habit of chewing only or any combination of two or three habits) and 2) no habit.

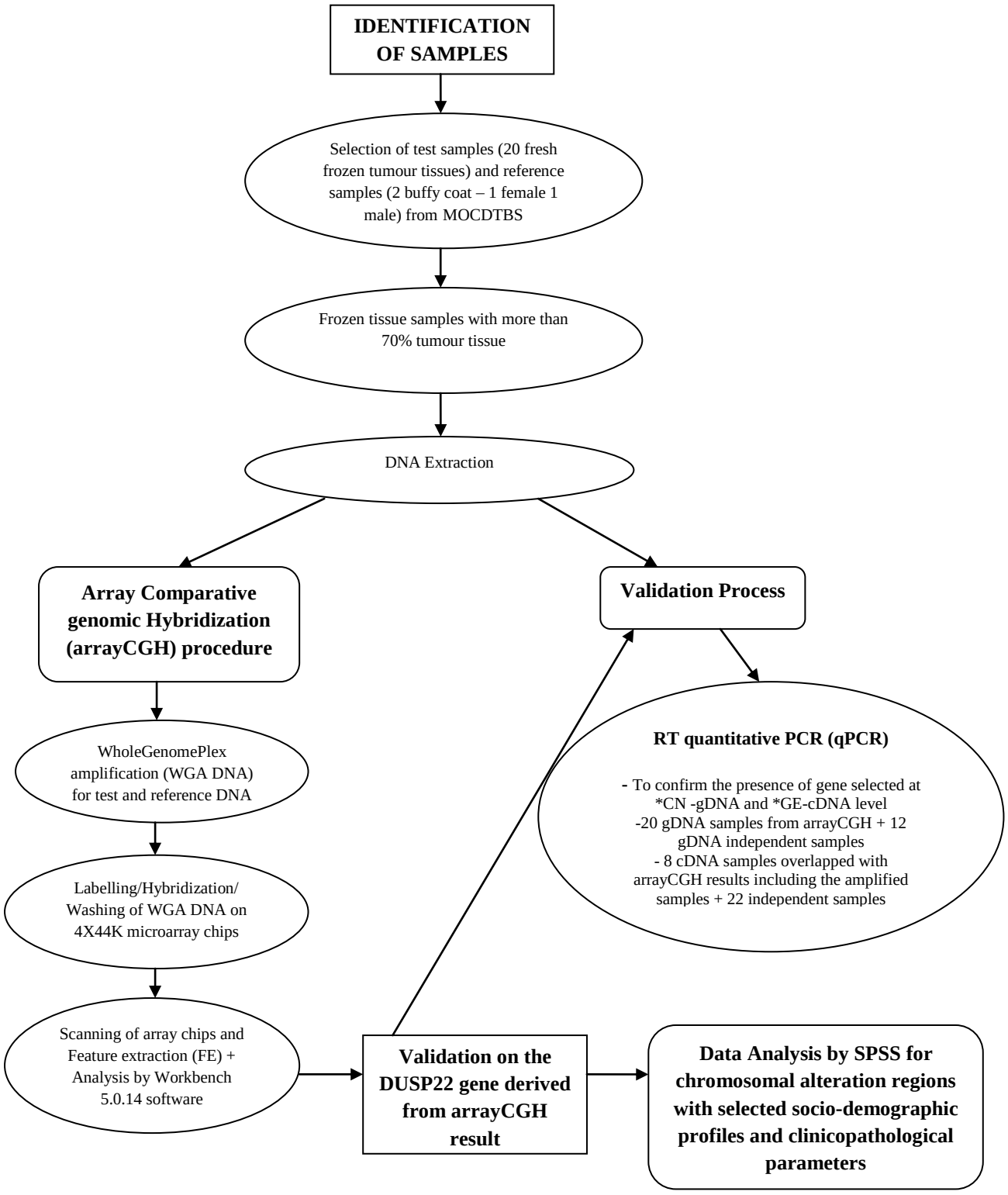

Figure 4.1: Flow Chart of the Summary for the Research Methodology

CHAPTER 5

RESULTS

5.1 Socio-demographic Characteristics of the Study

Out of the 20 OC patients in this study, 60% were Indians and 40% were indigenous of Sarawak. The overall mean age was 60.85±SD 10.85 years old with the age ranging from 26 to 78 years. There were 80% female and 20% male patients. Half of these cases (50%) practiced chewing habit, 15% a combination of smoking, drinking and chewing while remaining 35% patients had no habits as stated in **Table 5.1**. The details of demographic data are as shown in **Appendix F.**

Parameter		n (%)
Gender (n=20)	Female	16 (80)
	Male	4 (20)
Habit (n=20)	Chewing only	10 (50)
	Drinking + Smoking + Chewing	3 (15)
	No habit	7 (35)
Ethnic (n=20)	Indian	12 (60)
	Indigenous of Sarawak	8 (40)

Table 5.1: Sociodemographic profile of 20 OC cases

5.2 Characterizing Chromosomal Alterations and Gene Identification in OC

5.2.1 Chromosomal Alteration

Analysis of the arrays for the 20 OC cases revealed consistent regions of copy number changes. The mean number of alterations detected in this study which included amplification and deletion was 51.75± SD 42.68 per tumour with a range ratio of 10 to 197 per tumour. For amplification, the mean was 25±SD 27.11 per tumour, with a range of 0 to 117. For cases showing deletion, the mean number of alteration was 26.75±SD 18.36 with a range of 4 to 90.

The most common alteration detected in this study was deletion (51.8%) compared to amplification (48.2%). Fourty-four (44) regions of interest were found deleted on the chromosomes. The deleted regions were as presented in **Table 5.2** with the most frequently deleted regions detected at 19p13.3 (75%), followed by 19p13-p13.11 (65%) and 19q13.3 (65%) regions. Forty-one (41) regions were found amplified on the chromosomes. Amplification was mostly detected at 8q24.3 (60%), followed by 8q11.1-8q11.2 (55%) and 3q26 (55%) regions. Details involving regions are tabulated in **Table 5.2**. A graphical overview on analysis data using Agilent Workbench Standard Edition 5.0.14 software is shown in **Figure 5.1.**

Figure 5.2 demonstrated the image overview of chromosomal alterations distribution for frequently deleted and amplified regions as mentioned earlier, namely chromosome 3, 8 and 19. The images were extracted from Genomic Workbench software and results generated were based on 20 cases of OC patients.

Table 5.2: 44 deleted regions and 41 amplified regions detected in OSCC cases (n=20)

Alteration (%)	Amplification	Deletion
≥ 50	8q24.3 (60%), 8q11.1-8q11.2 (55%), 3q26 (55%), 3q36.2-q29 (50%), 9q34.1-34.3 (50%), 11q12.2-q12.3 (50%) and 17q21.3 (50%)	19p13.3 (75%), 19p13-p13.11 (65%), 19q13.3 (65%), 8p21.3 (60%), 16p13.3 (60%), 19q13.4 (55%), Xq28 (55%), 9q34.1-34.3 (50%), 16q24.3 (50%), 17q25.3 (50%), 21q22.3 (50%), 21q22.1 (50%), and 22q13.3 (50%)
≤50	1q21.3 (45%), 1p36.33-p33 (45%), 6p25.3 (45%), 7p22.1 (45%), 16p11.2 (40%), 14q11.2 (40%), 14q12 (40%), 17q25.3 (40%), 17q21.3-22 (40%), 19p13.1 (40%), Xq28 (40%), 11p11.2 (35%), 16p13.3 (35%), 22q13 (35%), 1p13 (30%), 5p15.3-p12 (30%), 6p21.33 (30%), 7q11.2 (30%), 8q24.22 (30%), 9p21 (30%), 12q13 (30%), 19q13 (30%), 20q13 (30%), 22q11 (30%), 1q44 (25%), 2q37.3 (25%), 3p21.31 (25%), 6p21.1 (25%), 6p22 (25%), 9q33.3 (25%), 12p13.31 (25%), 14q32.33 (25%) and 20q11 (25%)	10q26.3 (45%), 8q24.3 (45%), 11p15.5 (45%), 17q21.31 (45%), 17q21.33 (45%), 17p13.3 (45%), 22q13.1 (45%), Xp22.33-p11.3 (40%), 1p36.3 (35%), 3p21 (35%), 5q31 (35%), 6p21 (35%), 6p22.1 (35%), 18q23 (35%), 12q24.3 (35%), 2q37.3 (30%), 4p16.3 (30%), 5q35.3 (30%), 8p11 (30%), 9p21 (30%), 11q13 (30%), 11q23.3 (30%), 20q13.3 (30%), 1q21 (25%), 2q35 (25%), 3p26.3 (25%), 4p15.2-p14 (25%), 7q21 (25%), 18q11.2 (25%), and 14q12 (25%)

**Highlighted regions AMP and DEL were selected to correlate the chromosomal alterations with selected clinicopathological parameters and socio-demographic data.

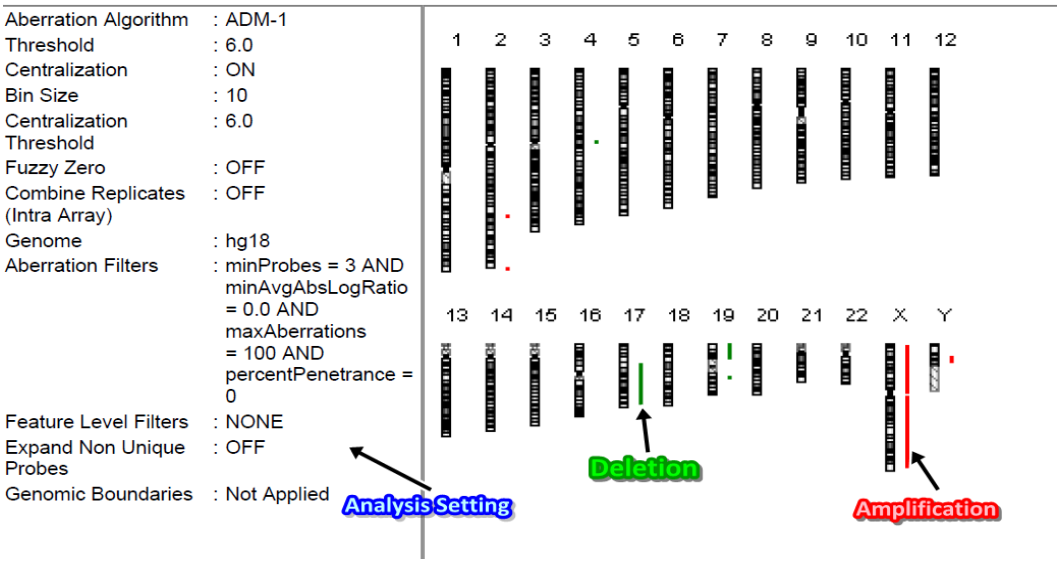

Figure 5.1 : This is an arrayCGH genome view for Sample A01. Chromosome was determined as amplification when the signal of Cy5 was in red colour whilst detected as deletion when the signal of Cy3 appeared in green colour. The analysis of arrayCGH profile was carried out according to the parameter settings suggested by manufacturer.

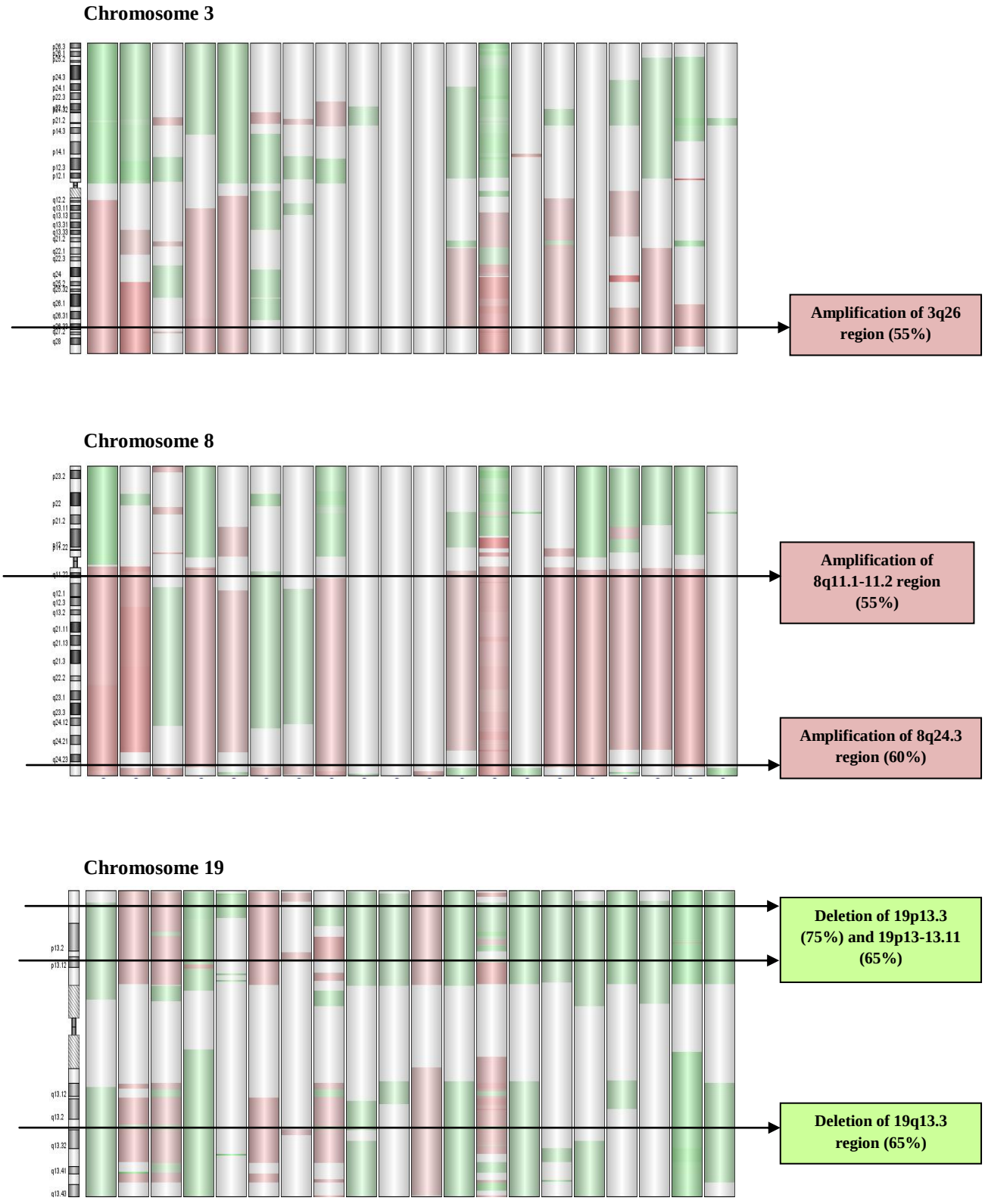

Figure 5.2: Patterns and distribution of chromosomal alterations in arrayCGH result (n=20) for selected altered chromosome 3, 8, and 19.

5.2.2 Genes identified

From **Table 5.3**, the most common amplified gene identified was *DUSP22* located at chromosome 6p25.3 in 35% cases, followed by *KIAA0146* (8q11.21) with 30% cases. Conversely, for deleted regions, the most common genes detected were *FBXO25* (8p23.3), *INPP5A, NKX6-2, C10orf92* (10q26.3), *CDH4, TAF4, LSM14B* (20q13.3) with 30% cases recorded for each gene. **Table 5.3** showed details of genes identified (cases with more than 30%) and its function as reported in previous studies. Other genes less than 30% cases identified in arrayCGH study are tabulated in **Table 5.4.** A list of other genes identified in this study is attached in **Appendix G.**

Table 5.3: Frequency of amplified and deleted genes in OSCC on arrayCGH analysis (n=20)

Gene	Symbol	Locus	%	Gene Function (http://www.genecards.org/)
Amplification				
Dual Specificity Protein Phosphatase 22	*DUSP22*	6p25.3	35	Activates the JNK signalling pathway. May play role as a signalling factor
Uncharacterized protein KIAA0146	*KIAA0146*	8q11.21	30	Unknown function but up-regulated in vascular endhotelial cells treated with interleukin-4 (IL4)
Deletion				
F-box only protein 25	*FBXO25*	8p23.3	30	May play a role in accumulation of expanded polyglutamine (polyQ) protein huntingtin (HTT)
Inositol-1,4,5-trisphosphate 5 phosphatase precursor	*INPP5A*	10q26.3	30	May play role in a signal-terminating reaction
Homeobox protein Nkx-6.2	*NKX6-2*	10q26.3	30	Unknown function but together with DNA binding assays, this gene might be important for differentiated oligodendrocyte function and in the regulation of myelin gene expression
Chromosome 10 open reading frame 92	*C10orf92*	10q26.3	30	Unknown function
Cadherin-4 precursor	*CDH4*	20q13.3	30	As a gene promoter element
Transcription initiation factor TFIID subunit 4	*TAF4*	20q13.3	30	Multimeric protein complex that plays a central role in mediating promoter responses to various activators and repressors
Protein LSM14 homolog B	*LSM14B*	20q13.3	30	May play a role in control of mRNA translation

5.3 Relationship between Chromosomal Alterations and Selected Clinicopathological Parameters and Socio-demographic Data

All patients were staged according to the UICC TNM Classification of Malignant Tumours (2005). There was one missing data for TNM stage grouping, one missing data for grading and tumour size (T) each. Clinicopathological parameters for each case are detailed in **Appendix H.** Details on statistical analysis are in **Appendix I.**

The three most frequent chromosomal alterations for amplified regions (8q24.3, 8q11.1-11.2 and 3q26) and deleted regions (19p13.3, 19q13.3 and 19p13-p13.11) were selected to determine their relationship with selected socio-demographic characteristics (gender, ethnicity and habits) and clinicopathological parameters (TNM stage, tumour size (T), lymph node status (N), tumour grade and tumour site) to satisfy objective 3. **Appendix K** shows the details of clinicopathological measurement for each parameter studied.

None of the socio-demographic characteristics and clinicopathological parameters showed significant differences for the most altered region **19p13.3 (Table 5.4)**. With respect to tumour site, even though the region was deleted for all seven cases (100%) in tongue, the relationship shown was not significant (p=0.114). For TNM Stage group parameter, there was no significant difference (p=0.245) between region 19p13.3 and staging group although all seven cases (100%) for Stage I & II was deleted. On the other hand, the tumour size (T) parameter indicated no difference (p=0.087) even eight cases of T1 and T2 (100%) were deleted in this region demonstrated the effect of small sample size. Meanwhile, tumour grade parameter showed no difference (p= 0.582) with eight (88.9%) from nine cases were well differentiated and found deleted on this region.

For N status, positive group and negative group were commonly found deleted in this region, nevertheless, the difference demonstrated no significant at p=1.00.

As for ethnic groups, region 19p13.3 was deleted in ten (83.3%) out of twelve cases in Indian as compared to five indigenous and showed no difference at p= 0.347. This region was found deleted in three (75%) out of four male distribution with no significant difference (p=1.00). Conversely, the proportion of all chewing habit group indicated that 76.9% were deleted as compared to no habit group with no significant difference p=1.00.

Table 5.4: Relationship between deleted region 19p13.3 and clinicopathological parameters and socio-demographic characteristics in OSCC cases (n=20)

Parameter	**Cases**	**19p13.3 status**		***p* value**	**Parameter**	**Cases**	**19p13.3 status**		***p* value**
	N=20	Deleted	Not deleted			**N=20**	Deleted	Not deleted	
Gender					**TNM Stage**				
Male	4	3 (75%)	1 (25%)	1.00*	Stage I & II	7	7 (100%)	0 (0%)	0.245*
Female	16	12 (75%)	4 (25%)		Stage III & IV	12	8 (66.7%)	4 (33.3%)	
Ethnicity					**Tumour grade**				
Indian	12	10 (83.3%)	2 (16.7%)	0.347*	Well	9	8 (88.9%)	1 (11.1%)	0.582*
Indigenous	8	2 (25%)	6 (75%)		Moderate	10	7 (70%)	3 (30%)	
Habit					**Lymph Node (N)**				
All chewing	13	10 (76.9%)	3 (23.1%)	1.00*	Positive	10	8 (80%)	2 (20%)	1.00*
No habit	7	5 (71.4%)	2 (28.6%)		Negative	10	7 (70%)	3 (30%)	
Tumour Site					**Tumour Size (T)**				
Tongue	7	7 (100%)	0 (0%)	0.114*	T1 & T2	9	9 (100%)	0 (0%)	0.087*
Others	13	8 (61.5%)	5 (38.5%)		T3 & T4	10	6 (60%)	4 (40%)	

**Fischer's exact test*

There was no significant difference for deleted region at **19q13.3** with all clinicopathological and demographic parameters **(Table 5.5).** TNM deletion at region 19q13.3 also showed no significant difference (p=0.173) with staging. Six (85.7%) of seven Stage I and II cases were found deleted in this region. Deletion on this region also did not show any significant difference between the tumour groups (p=0.350). Seven (77.8%) of nine cases were found deleted at 19q13.3. On the other hand, seven of 10 (70%) lymph node positive cases showed deletion at 19q13.3, however, no significant difference was noted (p= 0.650). Assessing to tumour grade, seven of 10 (70%) moderately differentiated tumours showed deletion at 19q13.3, compared to five of nine (55.6%) well differentiated tumours. However, no significant difference was observed between the two groups (p=0.650).

This region was found deleted in ten female patients as compared to two male patients, however, no significant difference was found (p=1.00). With regards to ethnicity, eight of 12 Indian patients showed deletion at 19q13.3 (66.7%) compared to four of eight cases (50%) in the indigenous group. The result was also not significant (p=0.648). With regards tongue site, region 19q13.3 was found deleted in five (71.4%) cases and this also did not show any significance difference (p=0.642) with other sites.

For the chromosome region of **19p13-p13.11**, all of the tongue carcinomas showed deletion while only 46.2% from other sites (buccal mucosa, gum, palate) showed deletion. This relationship was found to be statistically significant (p=0.044). There was no significant difference (p=0.333) between this deleted region and TNM staging even though 85.7% of Stage I & II tumours showed deletion on this region compared to 58.3% for Stage III & IV. Similarly, there was also no significance in the occurrence of deletion for this

region (p=0.141) between tumour size even though there was a higher number T1 and T2 tumours with deletion at this site was seen compared to the T3 & T4 tumours. Tumour grade also showed no significant difference (p= 0.628) even though seven (77.8%) of nine were well differentiated tumours showed deletion in this region.

Meanwhile, this region was found deleted in eleven (11) female cases compared to in two the males and this result also showed no significant difference (p=0.587). With regards to ethnicity, nine (75%) out of 12 cases of Indian ethnicity showed deletion in this region, but this is also not significant (p= 0.356). Details of statistical analysis are shown in **Table 5.5**.

Table 5.5: Summary of relationship between selected chromosomal alterations and clinicopathological parameters and socio-demographic characteristics in OSCC (n=20) (Deleted regions)

Parameter	n=20	19q13 Status		*p* value	19p13-p13.11 status		*p* value
		DEL	Not DEL		DEL	Not DEL	
Gender							
Male	4	2 (50%)	2 (50%)	1.00	2 (50%)	2 (50%)	0.587
Female	16	10 (62.5%)	6 (37.5%)		11 (68.8%)	5 (31.2%)	
Ethnicity							
Indian	12	8 (66.7%)	4 (33.3%)	0.648	9 (75%)	3 (25%)	0.356
Indigenous	8	4 (50%)	4 (50%)		4 (50%)	4 (50%)	
Habit							
All Chewing	13	8 (61.5%)	5 (38.5%)	1.00	8 (61.5%)	5 (38.5%)	1.00
No habit	7	4(57.1%)	3 (42.9%)		5 (71.4%)	2 (28.6%)	
Tumour site							
Tongue	7	5 (71.4%)	2 (28.6%)	0.642	7 (100%)	0 (0%)	0.044
Others	13	7 (53.8%)	6(46.2%)		6 (46.2%)	7 (53.8%)	
TNM stage							
Stage I & II	7	6 (85.7%)	1 (14.3%)	0.173	6 (85.7%)	1 (14.3%)	0.333
Stage III & IV	12	6(50%)	6 (50%)		7 (58.3%)	5 (41.7%)	
Tumour grade							
Well	9	5 (55.6%)	4 (44.4%)	0.650	7 (77.8%)	2 (22.2%)	0.628
Moderate	10	7 (70%)	3 (30%)		6 (60%)	4 (40%)	
Lymph Node (N)							
Positive	10	7 (70%)	3 (30%)	0.650	6 (60%)	4 (40%)	1.00
Negative	10	5 (50%)	5 (50%)		7 (70%)	3 (30%)	
Tumour Size (T)							
T1 & T2	9	7 (77.8%)	2 (22.2%)	0.350	8 (88.9%)	1 (11.1%)	0.141
T3 & T4	10	5 (50%)	5 (50%)		5 (50%)	5 (50%)	

***DEL** = *Deleted region*

Three regions showing amplification at **8q24.3, 8q11.1-11.2** and **3q26** were selected for analysis. Amplification at 8q24.3 region indicated that indigenous ethnic accounted 75% of all cases, however no significant difference was seen (*p*=0.373). Cases with no habit practiced constitute 71.4% of all cases reported and the difference was also not significant (*p*=0.642). of 12 cases reported amplified in region 8q24.3, other sites which included buccal, gum and palate represented nine cases and this also showed no significant difference (*p*=0.356). With regards to TNM staging, Stage III & IV demonstrated 66.7% of cases amplified but no significant difference was noted (*p*=0.377). Meanwhile, a total of 70% moderately differentiated tumours were showed amplification in this region and the result is also not statistically significant (*p*=0.370). Group T3 and T4 tumours showed amplification in 70% of cases. No significant difference was observed *(p*=0.370) **(Table 5.6).**

For region **8q11.1-11.2**, assessing across the ethnic groups, six of eight patients (75%) of indigenous ethnicity was found amplified with no significant difference (*p*=0.197). Similarly, there was no significant difference (*p*=0.374) seen between tumour site groups, between tongue and others even though amplification was detected in 71.4% of tongue cases. Habitual quid chewers showed that 61.5% of cases showed amplification in this region and the difference was discovered not significant (*p*=0.642). With regards to TNM staging, lymph node status, tumour grade and tumour size parameters, it was demonstrated that the difference was small among individual, and the results were also not statistically significant (*p*=1.00).

The proportion of amplification on region **3q26** for tongue site was recorded at 85.7% but there was no significant difference (p=0.070) seen. With respect to ethnicity, the amplification in this region for indigenous patients was reported in 75% of cases, and the difference was also not significant (p=0.197). In the habits group, 'no habit' practiced group showed amplification in 71.4% of cases, and the results also showed no significant difference (p=0.374). Likewise, there was also no significance in the occurrence of amplification for this region (p=0.633) between tumour stage even though there was a higher number of cases detected with amplification for Stage I & II tumours as compared to Stage III & IV tumours. For gender, lymph node status and tumour size, marginal differences were seen between groups but no significant difference was reported (p=1.00). Results correlating the selected altered regions 8q24.3, 8q11.1-11.2 and 3q26 and clinicopathological parameters are as shown in **Table 5.6.**

Table 5.6: Summary of relationship between selected chromosomal alterations and clinicopathological parameters and socio-demographic characteristics in OSCC (n=20) (Amplification regions)

Parameter	n=20	8q24.3 Status		*p* value	8q11.1-11.2	status	*p* value
		AMP	Not AMP		AMP	Not AMP	
Gender							
Male	4	2 (50%)	2 (50%)	1.00	1 (25%)	3 (75%)	0.285
Female	16	10 (62.5%)	6 (37.5%)		10 (62.5%)	6 (37.5%)	
Ethnicity							
Indian	12	6 (50%)	6 (50%)	0.373	5 (41.7%)	7 (58.3%)	0.197
Indigenous	8	6 (75%)	2 (25%)		6 (75%)	2 (25%)	
Habit							
All Chewing	13	7 (53.8%)	6 (46.2%)	0.642	8 (61.5%)	5 (38.5%)	0.642
No habit	7	5 (71.4%)	2 (28.6%)		3 (42.9%)	4 (57.1%)	
Tumour site							
Tongue	7	3 (42.9%)	4 (57.1%)	0.356	5 (71.4%)	2 (28.6%)	0.374
Others	13	9 (69.2%)	4 (30.8%)		6 (46.2%)	7 (53.8%)	
TNM stage							
Stage I & II	7	3 (42.9%)	4 (57.1%)	0.377	4 (57.1%)	3 (42.9%)	1.00
Stage III & IV	12	8 (66.7%)	4 (33.3%)		7 (58.3%)	5 (41.7%)	
Tumour grade							
Well	9	4 (44.4%)	5 (55.6%)	0.370	5 (55.6%)	4 (44.4%)	1.00
Moderate	10	7 (70%)	3 (30%)		6 (60%)	4 (40%)	
Lymph node (N)							
Positive	10	6 (60%)	4 (40%)	1.00	5 (50%)	5 (50%)	1.00
Negative	10	6 (60%)	4 (40%)		6 (60%)	4 (40%)	
Tumour size (T)							
T1 & T2	9	4 (44.4%)	5 (55.6%)	0.370	5 (55.6%)	4 (44.4%)	1.00
T3 & T4	10	7 (70%)	3 (30%)		6 (60%)	4 (40%)	

Continue…..

Parameter	**Cases**	**3q26 status**		***p* value**	**Parameter**	**Cases**	**3q26 status**		***p* value**
	N=20	Amplified	Not Amplified			**N=20**	Amplified	Not Amplified	
Gender					**TNM stage**				
Male	4	2 (50%)	2 (50%)	1.00	Stage I & II	7	5 (71.4%)	2 (28.6%)	0.633
Female	16	9 (56.3%)	7 (43.8%)		Stage III & IV	12	6 (50%)	6 (50%)	
Ethnicity					**Tumour grade**				
Indian	12	5 (41.7%)	7 (58.3%)	0.197	Well	9	3 (33.3%)	6 (66.7%)	0.650
Indigenous	8	6 (75%)	2 (25%)		Moderate	10	5 (50%)	5 (50%)	
Habit					**Lymph node (N)**				
All chewing	13	6 (46.2%)	7 (53.8%)	0.374	Positive	10	5 (50%)	5 (50%)	1.00
No habit	7	5 (71.4%)	2 (28.6%)		Negative	10	6 (60%)	4 (40%)	
Tumour site					**Tumour size (T)**				
Tongue	7	6 (85.7%)	1 (14.3%)	0.070	T1 & T2	9	5 (55.6%)	4 (44.4%)	1.00
Others	13	5 (38.5%)	8 (61.5%)		T3 & T4	10	6 (60%)	4 (40%)	

***AMP** = *Amplified region*

5.4 Validation of results using RT-qPCR of *DUSP22* gene

5.4.1 Melt curve for RT-qPCR analysis

Figure 5.3 shows the melt curve for gene expression (GE) and copy number (CN) study for both *DUSP22* gene and internal control primers (chromosome 1q24.2). The temperature for the *DUSP22* gene was stabilized at 88ºC whilst the internal control primers were at 77ºC.

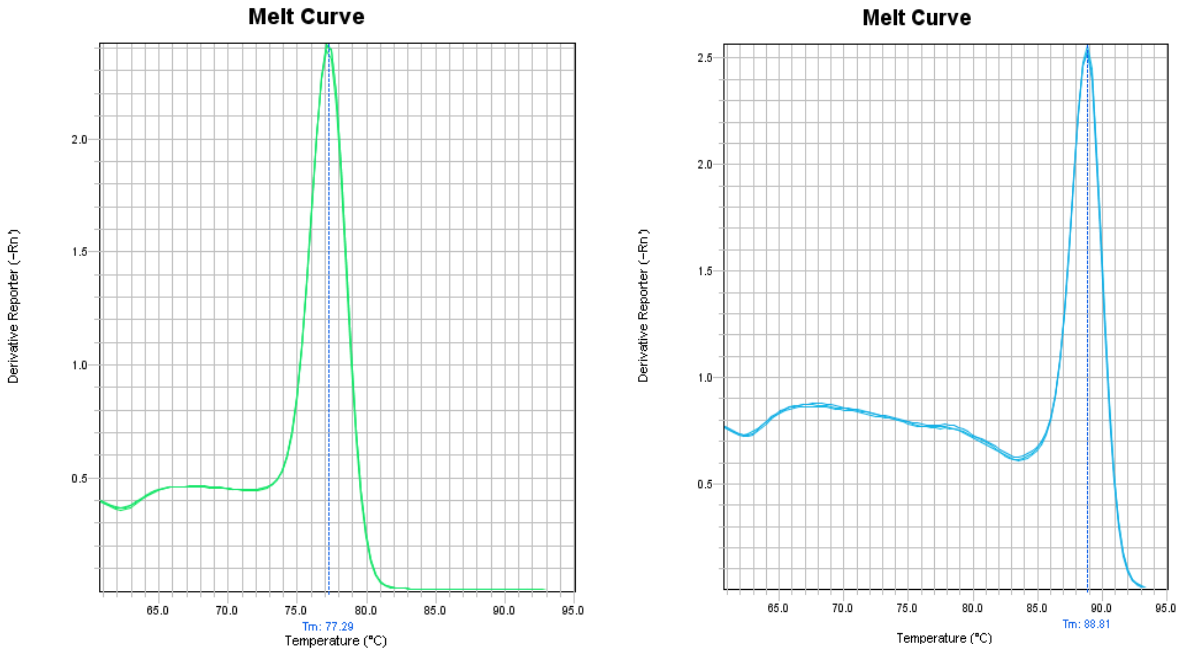

Figure 5.3: Melt curve for the *DUSP22* gene and internal control primers

5.4.2 The distribution of copy number (gDNA) for *DUSP22* gene

Out of thirty two (32) gDNA cases validated, amplified copy number changes was observed in 53.1% of the cases, five samples were down-regulated (15.6%) and 31.3% showed normal changes. The mean fold change was 2.96±SD2.48 with a minimum value of 0.0232 to maximum 12.9192 copy number changes. The distribution of all 32 cases for copy number changes in this study is shown in **Figure 5.4**.

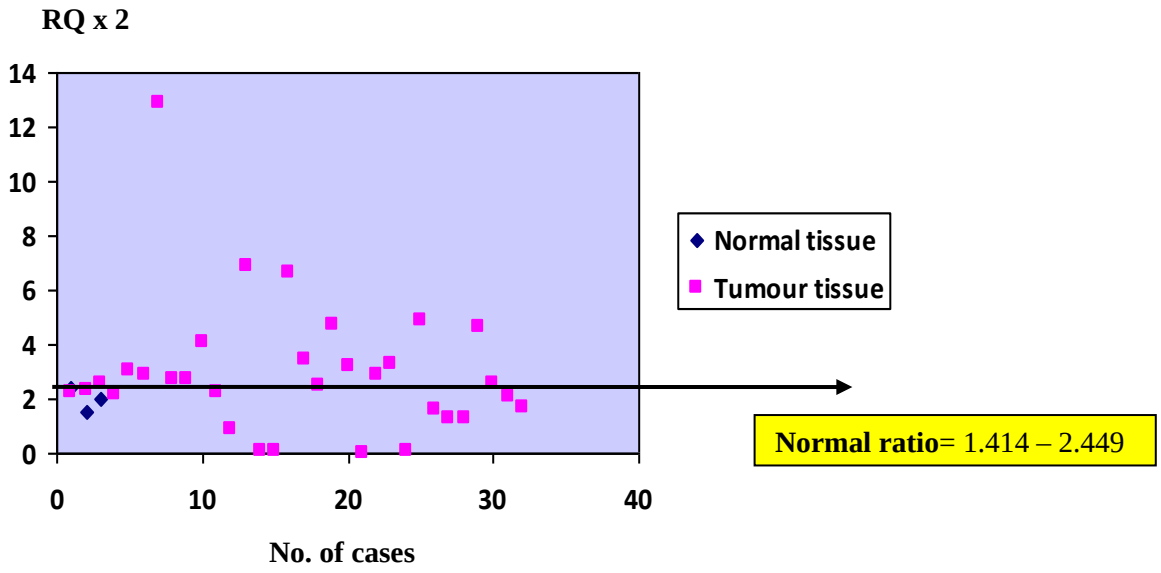

Figure 5.4: Distribution of copy number fold changes in 32 samples for *DUSP22* gene (gDNA samples) as compared to normal tissue

5.4.3 The distribution of gene expression (cDNA) for *DUSP22* gene

Results of GE study on thirty (30) cDNA samples showed over-expression of *DUSP22* gene in ten (33.3%) samples while 67.7% of other samples were distributed at normal rate. The mean of the gene expression changes was 2.95±SD6.73 with a range value of 0.1585 to 33.796. **Figure 5.5** shows the distribution of gene expression in 30 cDNA samples.

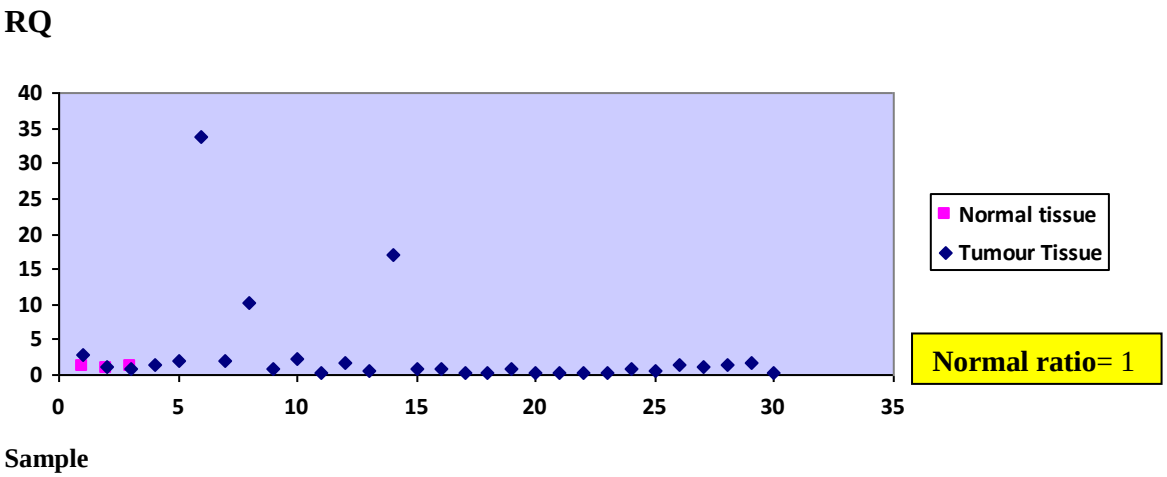

Figure 5.5: Distribution of gene expression (GE) in 30 tumour and normal tissue for *DUSP22* gene (cDNA) samples

5.4.4 Similarity between arrayCGH, copy number (CN) and gene expression (GE) Study

From arrayCGH results (**Table 5.3**), seven (35%) of 20 patients showed amplification of the *DUSP22* gene. Following this result, *DUSP22* gene was further analyzed and validated using RT-qPCR. To determine the accuracy of the arrayCGH technique, validation was carried out at CN level first and later at GE level to verify gene expression changes and copy number variation. CN was found in five out of seven (71.4%) cases analyzed whilst two cases was detected to have normal copy numbers **(Table 5.7** and **Figure 5.6).** Analysis at GE level demonstrated consistent over-expression results for all seven samples that showed amplification in arrayCGH **(Table 5.7** and **Figure 5.6).**

Case No.	arrayCGH	CN (DNA)	GE (cDNA)
OC01	✓	✓	✓
OC02	✓	✓	✓
OC03	✓	✓	✓
OC04	✓	✓	✓
OC05	✓	✓	✓
OC06	✓	x	✓
OC07	✓	x	✓

✓ : Over-expression
x : Normal change/No over-expression

Table 5.7: Validation of arrayCGH results through CN and GE

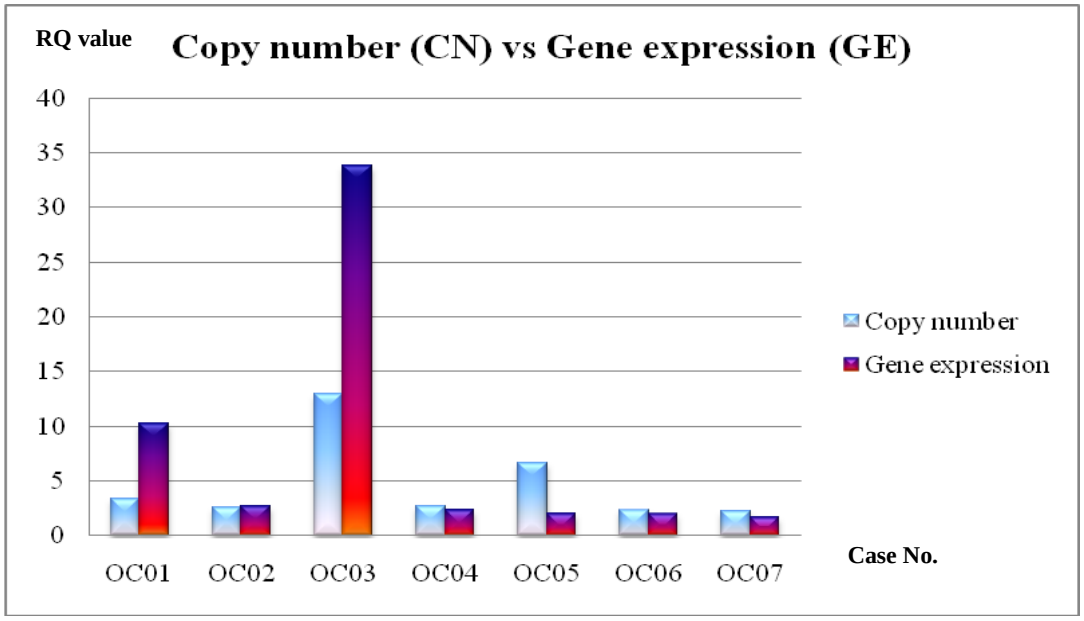

Figure 5.6: The similarity between copy number and gene expression study for 7 samples that were amplified in arrayCGH

Besides the case samples from arrayCGH, the independent samples results for both CN and GE were tabulated in **Table 5.8**. It showed the fold changes on 32 samples of CN study and 30 samples of GE studies for *DUSP22*. The raw data on CN and GE are tabulated in **Appendix J.**

Table 5.8: Details of RQ values of *DUSP22* gene for copy number (gDNA) and gene expression (cDNA)

Case No.		**Fold - Changes (RQ value)**	
	arrayCGH	Copy Number (CN)	Gene Expression (GE)
OC01	Amplified	3.3142*	10.2125**
OC02	Amplified	2.5982*	2.7342**
OC03	Amplified	12.9192*	33.796**
OC04	Amplified	2.7304*	2.3264**
OC05	Amplified	6.6462*	2.0043**
OC06	Amplified	2.293	1.9463**
OC07	Amplified	2.28	1.6855**
OC08	Not amplified	2.137	-
OC09	Not amplified	3.0303*	-
OC10	Not amplified	2.9066*	-
OC11	Not amplified	2.6976*	0.287
OC12	Not amplified	4.0978*	-
OC13	Not amplified	2.2536	-
OC14	Not amplified	0.897	-
OC15	Not amplified	0.0232	-
OC16	Not amplified	0.0842	-
OC17	Not amplified	0.06	-
OC18	Not amplified	2.8406*	-
OC19	Not amplified	4.6594*	-
OC20	Not amplified	0.0602	-
OC21	-	3.4048*	0.5782
OC22	-	2.5542*	0.927
OC23	-	4.7016*	1.2114
OC24	-	3.1872*	-
OC25	-	4.9126*	16.9786**

OC26	-	1.6384	0.6054
OC27	-	1.2516	-
OC28	-	1.2844	0.7901
OC29	-	2.0023	-
OC30	-	6.907*	1.6489**
OC31	-	-	0.7881
OC32	-	-	0.8785
OC33	-	-	0.299
OC34	-	-	0.3847
OC35	-	-	0.8939
OC36	-	-	0.3633
OC37	-	-	0.1585
OC38	-	-	0.3358
OC39	-	-	0.4128
OC40	-	-	0.7459
OC41	-	-	0.2924
OC42	-	-	1.421
OC43	-	-	1.0736
OC44	-	-	1.3614
OC45	-	-	1.6454**

***** Copy number gain
** Over-expression

CHAPTER 6

DISCUSSION

6.1 Limitation of Study

This is a descriptive study, thus the sample size for this study was determined based on previous journals that have been reported worldwide on chromosomal alterations where the size was within the range of 10 to 35 cases. Besides, due to budget constraint, the sample size for this study was small (n=20). Even though arrayCGH is an advanced platform to detect chromosomal alterations, however, this technique requires the use of expensive materials and equipments (Bradley et. al., 2007). Therefore, the results obtained from this study would not reflect the whole Malaysian OC patients scenario due to the small sample size which will reduce the power of the study (Macfarlane, 2003). Nevertheless, this study can be a platform to achieve more large scale study on chromosomal alterations in the future in order to get the whole view of affected patients.

Worldwide, 90% of well known risk factors that are proven to be associated with OC include alcohol abuse and quid chewing habit (Patmore et. al., 2005). Hence, this study focused on patients with chewing habits and no habit to determine the association between habit with chromosomal alterations or genes detected. From MOCDTBS-UM database, there were nine hospitals involved in collecting tissues for OC. Only cases from selected hospitals namely University of Malaya (UM), Hospital Tengku Ampuan Rahimah (HTAR), Hospital Kuala Lumpur (HKL) and Hospital

Umum Sarawak (HUS) were involved in this study. According to the National Cancer Registry (NCR) of Malaysia from 2003-2006 in peninsular Malaysia, for tongue and mouth cancer, the highest rate of cancer cases was detected among Indian females and males (Zainal et. al., 2006, Lim et. al., 2008). Hence, the subjects among Indian ethnicity were involved. However, the selection of the cases in this study may cause bias as only selected hospitals that showed high distribution of chosen ethnicities were selected.

Another limitation was the very limited information regarding chromosomal alterations and genes associated especially for OC in Malaysia. Therefore, insufficient references for data comparison were obtained. This study relied on the reports of chromosomal alterations from Western countries chromosomal alterations research and some parts of Asia especially India, Japan and Taiwan for comparison (Scully et. al., 2000, Rosin et. al., 2002, Baldwin et. al., 2005, O'regan et. al., 2006, Garnis et. al., 2009).

6.2 Chromosomal Alteration

Chromosomal alterations such as inactivation of TSGs by chromosomal deletion and creation of oncogenic fusion genes by chromosomal amplification may act as a fundamental pathophysiological event in human carcinogenesis (Le Beau, 1997). The biological behaviour of a tumour is affected by genetic alterations of tumour cells (Ueno et. al., 2002). In this study, the occurrence of chromosomal deletion was slightly higher as compared to the amplifications. This result is consistent with several other studies on breast cancer, ovarian cancer, lung cancer, prostate cancer and head and neck cancer (Scully et. al., 2000, Squire et. al., 2002, Baldwin et. al., 2005). Besides, this study was

supported by Bockmul et. al., (1997), who reported that metastatic HNSCCs carry more DNA losses and cause the inactivation of TSGs that were critically needed for the development of metastatic ability (Patmore et. al., 2005). Further studies have been conducted to determine the importance of chromosomal deletions in the development of metastasis in, for instance solid renal tumours. However, how these alterations lead to metastasis remains unclear (Qin, 2002) as a lot of factors contribute to chromosomal alterations.

Other studies suggested that deletion always occurs during early tumorigenesis and causes loss of function of TSGs and oncogenes (Beckmann et. al., 1997). A study by Platzer et. al., (2002) on colon cancer demonstrated that genes that showed significant increase expression in association with chromosome amplification are few in number and are not geographically clustered together; therefore chromosomal amplifications do not result in global induction of gene expression. However, according to Beckmann et. al., (1997), chromosomal amplification of oncogenes always occurs during the late stage of tumour development. Gene amplification is also a major mechanism of oncogene activation and has been associated with poor prognosis in human cancer (Myllykangas et. al., 2007).

It is generally believed, though, that the number of alterations increases steadily during cancer progression: oral leukoplakia has fewer chromosomal aberrations than OC (Weber et. al., 1998) and lower tumour stage (T1) is associated with fewer alterations as compared to higher tumour stage (T2) (Okafuji et. al., 2000). Thus, the number of alterations seems to change depending on the evolution of the tumour from normal, to hyperplasia, to severe dysplasia and invasive squamous cell carcinoma (Patmore et. al., 2005, Tsantoulis et. al., 2007).

6.2.1 Most Amplified regions detected (8q24.3, 8q11.1-11.2 and 3q26)

Through arrayCGH, high-throughput and quantitative analyses of copy number changes of gains and losses at high resolution genome can be detected (Baldwin et. al., 2005). In this study, the most common amplified region detected was located at chromosome 8q. This finding is consistent with other studies in head and neck cancer (Squire et. al., 2002, Garnis et al., 2004, Baldwin et. al., 2005, O'Regan et. al., 2006, Sparano et. al., 2006, Garnis et. al., 2009, Pathare et. al., 2009). Other than head and neck cancer, alteration in 8q region is also common in cancers from the breast (Cingoz et. al., 2003, Ghaffari et. al., 2007), prostate (Steiner et. al., 2002), colon (Platzer et. al., 2002), pancreatic cancer (Schleicher et. al., 2007) and esophagus (Ueno et. al., 2002). The frequency of amplification for 8q region slightly differs from one study with another. The differences may be due to geographic variation and lifestyle influences of population studied (Baldwin et. al., 2005).

Interestingly, gain on chromosome 8q was already described as a marker of development of aggressive prostate cancer (Van Den Berg et. al., 1995, Steiner et. al., 2002) with the common amplified region revealed at 8q24. Other studies have shown that the amplification of 8q in OSCC is due to the formation of isochromosome and unbalanced structural rearrangements (Rosin et. al., 2002, Garnis et. al., 2009). 8q24 is commonly detected in invasive head and neck squamous carcinomas that are amongst the cytogenetically most complex tumours (Hermsen et. al., 2001 and 2005). Nevertheless, the target gene for this region is not yet known, but a number of potential oncogenes are located on the long arm of chromosome 8. A variety of oncogenes that have not yet been implicated in cancer development but are functionally related to

processes involved in tumorigenesis is believed to be located on 8q (Van Dekken, 2003, Garnis et. al., 2004b).

Several genes of interest, such as *MYC* oncogene and *PTK2,* are located at chromosome 8q23-24-ter. Activation of the oncogene *c-MYC* on chromosome region 8q24 has frequently been implicated in oral carcinogenesis, but some studies have suggested other genes on 8q (Garnis et. al., 2004b). Other potential oncogenes located on 8q are as follow: *EIF3S3, PSCA, RCAS1, FAK, MMP16,* and *ANGPT1*. In this study, several frequent genes detected at chromosome 8q included *PRKDC* and *UBE2V2*.

The next most commonly amplified region is 3q. Gains in 3q are also a common finding in advanced OC (Shang et. al., 2006). The amplification of 3q26 is a common event in head and neck SCC (Singh et. al., 2002). Squire et. al., (2002) showed that this region was the most amplified region (64%) in their study on OSCC whilst Baldwin et al., (2005), showed that gain on chromosome 3q was also frequently detected. Overall, the amplification of region 3q26 in this study was consistent with previous studies reported in OC (Scully et. al., 2000, Rosin et. al., 2002, Singh et. al., 2002, Chen et. al., 2004, O'Regan et. al., 2006). Besides head and neck cancer, amplification in this region is also frequently seen in other cancers such as lung, larynx and pharynx carcinomas, and esophagus cancer (Hermsen et. al., 2001 and 2005, Peralta et. al., 2007, Gen et. al., 2010). The amplification of the 3q26.3 locus has been shown to be associated with progression to invasive cancer and is a negative prognostic factor in head and neck SCC (Singh et. al., 2002).

A study by Massion et. al., (2003) demonstrated that in high-grade pre-invasive lesions which include severe dysplasia and carcinoma in situ, region 3q is amplified in 75% of invasive SCC, suggesting that the amplification of 3q may be a marker for transition to an invasive phenotype. Results of this study are supported by Singh et. al., (2002) and Foster et. al., (2005) who described that the amplification on chromosome 3q25–26 is associated with a pre-invasive lesion that progressed to a subsequent carcinoma.

Previous cytogenetics and CGH studies suggested a few number of oncogenes, such as *BCL6, PIK3CA, SCCRO,* telomerase RNA and *AIS* gene were mapped to region 3q26-28, a frequently gained chromosomal segment. Among them, *SCCRO* and *PIK3CA* may play a role in the pathogenesis of oral SCC through the amplification at 3q26. *SCCRO* appears to be a significant predictor of regional metastasis and may be a marker for tumour aggressiveness and clinical outcome in HNSCC (Singh et. al., 2002, Tabot et. al., 2004). In this study, several genes detected include *SERPIN12, WDR49, ZBBX*, and *COL8A1*. To the best of the writer's knowledge, no reports from previous studies have described the exact oncogenes related to this amplified region yet in OC.

6.2.2 Most Deleted regions detected (19p13.3, 19p13-13.11 and 19q13.3)

In this study, the most frequently deleted region occurred at chromosome 19p13.3, followed by 19p13-13.11 and 19q13.3. Previously, Garnis et. al., (2009) has reported deletion on the whole arm of region 19p in invasive tumours of the head and neck. The occurrence of deletion on chromosome 19 was rare in OC. Only few recent journals have reported it. According to Yu et. al., (2008), 19p13 region was demonstrated to have LOH in both the stroma and the epithelial compartments of head

and neck SCC. A study conducted by Gunduz et. al., (2006) has detected high allelic LOH on chromosome 19p. 19p was also found in other cancers namely breast (Oesterreich et. al., 2001), brain (Sobottka et. al., 2000), cervical (Wingo et. al., 2009) and lung (Rodriguez-Nieto and Sanchez-Cespedes, 2009). Deletion on chromosome 19 would lead to the loss of gene function, thus inhibiting apoptosis leading to cell proliferation, progression and metastasis.

In another study on Peutz–Jeghers syndrome (PJS), a rare autosomal-dominant disease associated with a predisposition to benign and malignant tumours, results showed large genomic deletions in the region of 19p13.3 with the detection of *STK11/LKB1* tumour suppressor gene (Le Meur et. al., 2004). Besides, Hemminki et. al., (1998) hypothesized that the deletions of 19p13.3 locus were exclusively inherited from the unaffected parent supporting the presence of a TSG at the telomeric end of chromosome 19. A study by Zhang et. al., (2003) indicated that they have identified the high levels of deletion on chromosome 19p13.1 in hepatocellular carcinoma (HCC) and also found that these high levels might be related to metastasis.

Chromosome 19p13 regions contain *EXT3* and *CDKN2D* genes that play a role as TSG in most cancers (Merrer et. al., 1994). Other genes on chromosome 19p13.3 include the death-associated protein kinase 3 (*DAPK3*) that induces morphological changes in apoptosis when over-expressed in mammalian cells, suggesting a role in the induction of apoptosis, while the *BAX* gene acts as an apoptotic activator (Jung et. al., 2006). The *LKB1* gene, also known as *STK11,* is somatically inactivated through point mutations and large deletions in lung tumours, demonstrating that *LKB1* is a target of LOH (Rodriguez-Nieto and Sanchez-Cespedes, 2009). Similarly, the *BRG1/ SMARCA4* gene (19p13) was also suggested as a TSG and may play distinct roles in nucleosome

remodelling or regulate the expression of different downstream genes (Gunduz et. al., 2006). From this study, frequent genes deleted were *PPAP2C* and *MIER2.*

Deletion on chromosome 19q13 is an uncommon finding in human cancers and has been mostly reported to associated with either OC (Tsantoulis et. al., 2007), neuroblastoma, gliomas (Chou et. al., 1996, Alaminos et. al., 2005) or ovarian cancer (Bicher et. al., 1997). Interestingly, report by multiple journals on Tsantoulis et. al., (2007) study, showed that 19q region was found amplified **(Table 6.1).** The presence of genomic deletions in the 19q13 chromosomal region is commonly reported in neuroblastomas and gliomas. This strongly suggests the presence of a potential putative TSG on this region (Alaminos et. al., 2005). Numerous studies failed to relate genes of interests that are linked to the chromosomal alterations of 19q13.

In 1995, Apte and his colleagues suggested that genes such as *BAX* that is located at chromosome 19q13.3–q13.4 might play a role in apoptosis. Other genes that were reported to link with regions 19q13.3 include the myelin-related gene *EMP3* that is thought to be involved in cell proliferation and cell-cell interactions (Ben Porath and Benvenisty, 1996, Taylor and Suter, 1996). A study by Alaminos et. al., (2005) suggested that *EMP3* hypermethylation might act as a marker of poor outcome in neuroblastoma patients.

6.2.3 Other Alterations detected in OC and comparison with previous studies

To date, a variety of chromosomal alterations found in OC has been reported by numerous studies worldwide. A summary of common chromosomal alterations reported in many cancers such as head and neck (Weber et. al., 1998, Okafuji et. al., 2000, Scully et. al., 2000, Rosin et. al., 2002, Squire et. al., 2002, Chen et. al., 2004, Garnis et. al., 2004a and 2004b, Baldwin et. al., 2005, Patmore et. al., 2005, O'Regan et. al., 2006, Garnis et. al., 2009) are tabulated in **Table 6.1**. The pattern of alterations was based on a survey conducted by Tsantoulis et. al., (2007) in more than 50 HNSCC studies reported. The important regions involved in other studies (**Table 6.1)** were concurrent with results of this study i.e., the amplification of chromosome 11q (50%) and chromosome 17q (50%). For deleted regions, results of this study has similar findings for chromosome 8p (60%), 21q (50%), 3p21 (35%) and 18q (35%).

Chrom.	Amplification	LOH or Deletion
1	-	1p36.3
2	-	2q32–35, 2q35, 2q36
3	3q25-ter	**3p13–14, 3p21, 3p25**
4	-	**4p14–4p15, 4q25, 4q31–32**
5	5p	**5q21–22**
6	-	6q13, 6q25
7	7p11	7q31
8	**8q22, 8q23-ter**	**8p21, 8p22, 8p23**
9	-	9p21
10	-	10q23, 10q26
11	**11q13**	11q22.2–q22.3
12	12p12.2–p13	-
13	-	13q14.3
14	14q31–q32.2	-
15	15q15	-
16	16q23–q24	-
17	**17q24–25**	17p13.1
18	18p	**18q**
19	19q	-
20	20q	20p11.2, 20q12–13.1
21	-	**21q11.1, 21q21, 21q22.1**
22	-	22q13

* Most frequently altered regions in OC (highlighted)

Table 6.1: Summary of common chromosomal alterations in OC (Tsantoulis et. al., 2007)

6.3 Chromosomal Alterations and its association with clinicopathological parameters

Invasive head and neck SCC are amongst the cytogenetically most complex tumours, and at present there is not much consensus on the prognostic value (Peralta et. al., 2009). Several prognostic factors such as socio-demographic data and clinicopathological parameters may influence the survival of oral cancer patients (Chen et. al., 2007). Therefore, arrayCGH findings are needed to establish correlations with clinicopathological parameters to further define genomic regions involved and to identify new chromosomal regions that involved in the pathogenesis of OC. In this chapter, we will discuss several clinicopathological parameters that can be utilised to predict patient's outcome, recurrence and overall survival.

6.3.1 Socio-demographic Profile

Worldwide, OC is known to affect more males than females with an approximate ratio of 1.5:1, respectively (Warnakulasuriya, 2009). The male: female ratio in the writer study was 1:3 as in Malaysia; oral cancer is higher in females compared to males (Zainal et. al., 2006, Lim et. al., 2008). Loss of 19p13.3 region was detected in > 75% of cases in both male and female patients. Similarly, amplification of chromosome 8q24.3, 8q11, and losses of region 19q13.3 and 19p13-13.11 were observed in more than 60% of patients respectively. The gender factor was not statistically significant with chromosomal deletion on 19q13 and 19p13, as more samples need to be added in to get more meaningful correlation.

High incidence rates for OSCC were widely detected in South and Southeast Asia (India, Pakistan and Taiwan), Latin America and the Caribbean, Pacific regions, Eastern Europe, and some parts of the Western (France) [Parkin et. al., 2001, Thames Cancer Registry (TCR), 2007]. Another studies reported that, the prevalence of OC is also generally higher in ethnic minorities in other developed countries (Scully and Bagan, 2009a and 2009b, Tomar et. al., 2004). In peninsular Malaysia, OC is most commonly found in Indian patients (Zainal et. al., 2006, Lim et. al., 2008). Deletion of region 19p13.3, 19q13.3 and 19p13-13.1 among Indian patients were observed in more than 83%, 66.7% and 75% respectively. It may suggest that losses on these regions play roles in OC progression in Indian ethnicity. Conversely, amplified regions of 8q24.3, 8q11.1-11.2, and 3q26 were found higher in indigenous patients with 75% distribution. This difference may be due to different geographic variation and lifestyles habits of the populations (Mehrotra et. al., 2006).

A study by Van Wyk et. al., (1993), found that Asian women who chewed betel nut had a 44-fold increase risk of developing OC compared to non-users. BQ chewing is a common ancient practice in many Asian countries including India, Taiwan and Malaysia (Wang et. al., 2003, Gupta and Ray, 2004). In this study, losses of 19p13.3, 19q13.3 and gain of 8q11.1-11.2 were detected in >60% of patients with the chewing habit. On the other hand, losses of 19p13-13.1, and gains of 8q24.3 and 3q26 were observed in >70% of patients with no habit practice. Again, the association of these regions and habit factor seem to be undefined as different patients may practice their betel chewing habits differently.

6.3.2 Clinicopathological Parameters

6.3.2.1 Primary tumour site

The most commonly reported OC sites include the floor of the mouth (FOM) and lateral borders of the tongue. Overall, the tongue, is the most common (40- 50%) site for OSCC in European and American population. In Asian population, patients usually suffer from cancer of the buccal mucosa (BM) due to BQ or tobacco chewing habits. SCC of the BM was found in 40% of OSCC in Sri Lankan population (Moles et. al., 2007). Losses of 19p13.3 and 19q13.3 were detected in 100% of tongue cancer in this study, whilst >70% in 19p13-13.1, 8q11.1-11.2 and 3q26. This association was found significant for the 19q13.3 region. Interestingly, to the best of our knowledge, this finding on relationship between tongue and deletion of 19q13.3 has never been report yet in OC.

In this study, amplification at 8q24.3 was frequently reported in BM, gum, palate, FOM and was observed in >69% cases. These results may indicate that, the anatomic site of primary tumour can predict tumour prognosis and this may link to the lymphatic drainage of these locations through the deep cervical chain (Jerjes et. al., 2010). However, a study by Kademani et. al., (2005) suggested that the tumour site has no effect on patient's survival. On the other hand, study by Carinci et al., (1998) and Woolgar and Scott, (1995) indicated that the site of origin of OC is an important prognostic factor. According to Montoro et. al., (2008), tongue cancer has poor prognosis as it is more aggressive in terms of local invasion and spread, difficult to control and has complex biological behaviour.

6.3.2.2 Tumour size & thickness (T)

Tumour size (T) has been linked to cervical involvement and poor prognosis (Woolgar, 2006). Tumour thickness is an additional prognostic factor that has been correlated with survival (Wenzel et. al., 2004). A study by Huang et. al., (2009), has found significant difference between the 4 mm and 5 mm tumour thickness in OSCC. It is now accepted that thickness is a more accurate predictor of nodal metastasis, and survival than tumour size (Woolgar, 2006). Jerjes et. al., (2010) suggested that tumour depth of invasion is a good prognostic indicator as compared to tumour size. In this study, we could not find any significant relationship between tumour size (T) and chromosomal alterations namely gains of 8q24.3, 8q11.1-11.2. 3q26 and losses of 19p13.3, 19p13-13.1 and 19q13.3.

In chromosome 19p13.3, 100% of patients with T1 and T2 size showed loss of this region compared to 60% in the T3 and T4 group. Losses of chromosome 19p13-13.1, 19q13.3 and gain of 3q26 were also detected in >70% of T1 and T2 tumours. This may suggest a possible relationship with chromosomal alterations and small tumour size. Further investigations on genes involved on these regions should be done in order to get a better idea on the biology progression of OC. Conversely, gains of 8q24.3 and 8q11.1-11.2 were frequently observed in >70% of T3 and T4 tumours. Results of this study concur with that done by Ueno et. al., (2002) in esophageal cancer.

6.3.2.3 Lymph node Sstatus (N)

A worse prognosis is expected in patients with nodal disease especially with the presence of extracapsular spread (Greenberg, 2003). Distant metastasis was reported to occur in 5-25% of OSCC patients (Calhoun et. al., 1994), commonly in uncontrolled locoregional and N-stage diseases, especially N2 and N3. Many studies confirmed that tumour size (T) and tumour depth are independent prognostic factors in tongue cancer with a consistently adverse effect on lymph node metastasis (N) and survival rate (Teixeira et. al., 1998, Korpi et. al., 2008). More than 70% of patients with losses on region 19p13.3 and 19q13.3 showed positive lymph node status. Losses of 19p13-13.1, gains of 8q11.1-11.2, 3q26, 8q24.3 were observed in >60% of patients with negative lymph node status. No reports on these chromosomal alterations on lymph node status (N) were found in OC.

However, a study by Ueno et. al., (2002), has suggested that gain of chromosome 8q24 were significantly linked to nodal metastasis in esophageal cancer. On the other hand, a study conducted by Singh et. al., (2002) stated that gain of 3q was not related with nodes status. On the contrary, study by Nakao et. al., (2009), found that loss on region 3q was associated with lymph node status in colorectal cancer and greatly affects patient prognosis. The detection of copy number alterations linked to node metastasis is useful for elucidating the genetic mechanism of node metastasis and for estimating node metastasis before treatment (Nakao et. al., 2009). This may suggest that there are multiple pathways of node metastasis involve in cancer carcinogenesis (Ogino and Goel, 2008). Genetic alterations for those patients with lymph nodes metastasis were more complex than without metastasis (Al-Mulla et. al., 2006).

6.3.2.4 TNM staging system

Tumour stage, size and anatomical differences probably contributed to the difference in prognosis (Garzino-Demo et. al., 2006). TNM system is known to be one of the good indicators of tumour prognosis. The TNM classification adapts from UICC relates well to the overall patient survival (Scully and Bagan, 2009a & 2009b). According to Schroeff et. al., (2009), the earlier the tumour stage, the better the prognosis and the less complicated is the treatment.

Though not statistically significant, loss on region 19p13.3 was seen in 100% cases in Stage I & II patients. Similarly, it was revealed that, losses of region 19q13.3, 19p13-13.11 together with gain on 3q26 were observed in more than 86% and 71% of Stage I & II cases respectively. Though not significant, the writer hypothesized that these alterations may play an important role in the development of OSCC. Gain of 3q26 region in stage I and II in this study was similar to results of studies done by Okafuji et. al., (1999) and Ueno et. al., (2002). On the other hand, the loss of 19q13.3 was observed to be significantly involved in late stages of the disease i.e. Stage III and IV with bone marrow metastasis (Mora et. al., 2001).

For region 8q24.3, gain was found in 67% of patients in Stage III and IV, therefore suggesting that this region may play role in tumour progression. This finding is similar to that by Ueno et. al., (2002) who studied esophageal cancer and found that region 8q24.3 was highly expressed in advanced stage (Stage III and IV) compared to early stages. A study on OC by Singh et. al., (2002) and Montoro et. al., (2008) showed that there is no association between TNM stage and 3q alteration. A study by Saramaki et. al., (2001), Steiner et. al., (2002) and Schleicher et. al., (2007) also showed that

amplification of 8q occur as a late event in prostate carcinogenesis. The inconsistent in the result is slightly different maybe due to the different ethnic composition or geographical location (Takeno et. al., 2009).

Early-to-moderate-stage of OSCC defined by the American Joint Committee on Cancer which involved stages I-III (Greene et al., 2002) is most often treated surgically, whilst in advanced stage IV disease, multidisciplinary non-surgical approaches are being used to improve disease control, prolong survival, and maintain an acceptable quality of life for patients (Haraf et. al., 2003, Bernier et. al., 2004, Cooper et. al., 2004).

6.3.2.5 Tumour grading

Tumour grade is still widely used clinically as a prognostic variable in OC, but most studies often confirmed it to be of little value in prognostication (Al-Rajhi et. al., 2000, Okamoto et. al., 2002, O-charoenrat et. al., 2003, Woolgar, 2006). Well differentiated tumor cells resemble normal cells and tend to grow and spread at a slower rate than undifferentiated or poorly differentiated tumor cells, which lack the structure and function of normal cells and grow uncontrollably (Oral Cancer Foundation, 2011). It is widely accepted that prognosis is better in the early stage cancers, especially those that are well-differentiated (Scully and Bagan, 2009b). Most studies consider this grading system as a poor indicator of outcome for cancer patients (Po et. al., 2002, O-charoenrat et. al., 2003, Woolgar, 2006).

In our study, there were no significant differences between well differentiated and moderately differentiated tumours with chromosomal gains (8q24.3, 8q11.1-11.2, 3q26) and losses (19p13.3, 19p13-13.1, 19q13.3). However, losses of region 19p13.3 and 19p13-13.1 were observed in well differentiated tumours in more than 89% and 78% cases respectively.

On the other hand, loss of 19q13.3 and gains of 8q24.3, 8q11.1-11.2 and 3q26 were detected in > 50% of moderately differentiated tumours. A study by Oga et. al., (2001) found that 3q gain was significantly linked with poorly differentiated (advanced cancer) tumours. This might explain the higher frequency of moderately differentiated tumours with gain on 3q26.

Nevertheless, due to the variability of the results and their dependence on stage, site and other factors, large studies are required to resolve potential conflicts regarding better cancer prognosis. Overall, based on the results generated by arrayCGH in this study, the tumour site of OSCC seems to be one of the most predictive factors in patient prognosis. Further studies should be focus on potential genes and pathways that may play role in the progression of OC on tongue cancer.

Based on chromosomal alteration results in this study that are consistent with previous studies in various cancers, we can conclude that arrayCGH is a promising tool to detect gains and losses in cancer at a high resolution. This study has proved that even though an amplification method of WGA DNA samples were used during arrayCGH processing, the results generated still offered consistent findings with those studies that use the direct method. Hence, for future research on arrayCGH, as DNA material of cancer patients is valuable in research, 100 ng DNA samples is sufficient for arrayCGH

study as compared to 1.5 µg DNA required in direct method processing. Several studies (Suzanne et. al., 2005, Rusakova et. al., 2007) have applied the same method in arrayCGH processing, and yet the output was still consistent with the direct method.

6.4 Genes identified in arrayCGH

A number of genes were identified in this study **(Table 5.3** and **Appendix H)**. The gene, dual specificity phosphatase 22 (*DUSP22)* was selected for validation with RT-qPCR to confirm its presence as it appeared to be the most frequent in this study. The writer hypothesized that the up-regulation of *DUSP22* gene could interfere with the tumorigenic or metastasic potential of OSCC. *DUSP22* gene is located at chromosome 6p25.3 **(Table 5.2).** This region showed that 45% of cases are amplified. *DUSP22* gene is also known as following name *MKP-x, MKPX, JSP1, JKAP,* Low molecular weight dual specificity phosphatase 2, Mitogen-activated protein kinase phosphatase x, *JSP-1*, *LMW-DSP2,* JNK-stimulating phosphatise-1, MAP kinase phosphatise x and *LMWDSP2* (NCBI, 2010).

In this study, the copy number gain of the *DUSP22* gene was found in 53.1% of cases. So far there has been no report on *DUSP22* gene status and clinicopathological data related to OSCC, therefore the explanation regarding the relationship of this gene and clinicopathological parameters will have to wait for scientific data from other studies beside OSCC.

Besides copy number variation study, study on *DUSP22* at GE level was also performed. Out of 30 cases studied, *DUSP22* gene was observed in 33.3% of the cases. Further analysis on a larger population of over 100 patients in these two ethnicities may

help explain the role of the *DUSP22* gene in OC. Overall, the writer suggested and demonstrated that this gene may have no participation in the mechanism of OSCC growth and metastasis as reports regarding this gene functions still unclear. Further analysis at the protein level should be done in order to fully confirm it. Additionally, the *DUSP22* gene cannot be considered as a useful prognostic marker for OSCC yet as many further studies should be conducted.

The *DUSP22* gene was found in the cell nucleus and cytoplasm (Patterson et. al., 2009) and was first discovered in 2001. *DUSP22* gene is expressed in various types of tissues and cells, suggesting that it may participate in essential biological processes (Li et. al., 2010). Study by Sekine et. al., (2009) showed that over-expression of *DUSP22* reduces ERa activity and strongly indicated that *DUSP22* gene plays an important role in the ERa mediated transcription through a negative feedback loop in breast cancer cells. Another study showed that *DUSP22* gene has been shown to regulate T cell antigen receptor signalling through *ERK2* gene (Alonso et al., 2002). Study by Chen et. al., (2002), suggested that *DUSP22* gene acted as a specific positive regulator for the JNK signalling pathway and is required for the cytokine induced activation of the JNK pathway.

Sekine et. al., (2006) also demonstrated that *DUSP22* gene regulates interleukin 6 (*IL-6*)-/leukaemia inhibitory factor (*LIF*)-mediated signalling by dephosphorylating the *STAT3* gene. Sekine also demonstrated that *LMW-DSP2/DUSP22* gene is an important regulator of *STAT3* functions in the downstream of *IL-6/LIF* signalling, and may thus play critical roles in the progression of *IL-6-related* diseases. *DUSP22* gene has been originally shown to regulate JNK activation (Aoyama et. al., 2001, Shen et. al., 2001, Chen et al., 2002) in early studies. The *DUSP22* gene may also regulate JNK

signalling pathway in breast cancer cells (Sekine et. al., 2009). The JNK pathway plays broad roles in cellular response to various forms of stresses, growth stimulation, and apoptosis (Zhang et. al., 2007). *DUSP22* gene acts preferentially on the c-JUN n-terminal kinase (JNK) and *p38 MAPKs*, playing a role in apoptosis and cell proliferation (Fachin et. al., 2009). Li et. al., (2010) demonstrated that *JKAP or DUSP22*, an atypical DUSP, suppressed Src-induced *FAK* phosphorylation and reduced cell migration.

However, for OC, there is no research published yet on this gene. The physiological role of individual *MAPK* phosphatases such as *DUSP22* gene has been difficult to assess due to cross-pathway specificity and some functional redundancy. The discovery of DUSPs occurred recently about 6 years ago, which has initiated a large amount of interest in their role and regulation. A study had showed that the DUSP group has been implicated as major modulators of critical signalling pathways that are dysregulated in various diseases (Patterson et. al., 2009). The current literature hints at the potential of the atypical DUSPs as important signalling regulators, but the conflict remained unsolved.

CHAPTER 7

CONCLUSION

The conclusions for this study were:

1. The first objective was to detect the chromosomal alterations in OSCC using arrayCGH and this study has summarized that the chromosomal alterations detected on 3q and 8q were the most common amplifications observed in oral cancer while chromosome 19p and 19q were the most deleted regions observed.

2. The second objective was to determine the relationship between selected regions with socio-demographic characteristics and selected clinicopathological parameters, and this study showed that only region 19p13-p13.11 showed significant relationship (p=0.044) with tumour site while other regions either amplified or deleted demonstrated no significant differences between socio-demographic data or clinicopathological parameters.

3. The third objective was to identify genes involved in OSCC by arrayCGH and this study showed 62 of genes were identified including oncogenes and tumour suppressor genes reported to be linked with OC progression.

4. The fourth objective was to validate one of the identified genes using quantitative RT-qPCR technique. A gene namely *DUSP22* generated from the arrayCGH genes list was validated at both CN and GE level. The *DUSP22* gene was

found over-expressed and showed copy number gains, therefore the study was consistent with arrayCGH results.

RECOMMENDATION

Based on the results of this study, it is recommended that for future study, chromosomal alterations should be conducted on a larger scale of sample size in order to get more meaningful and significant association between the results obtained and socio-demographic profile or clinicopathological parameters. Future studies should also be narrowed down to only Indian ethnic or indigenous population only in order to get more specific information regardless of ethnic distribution and lifestyle. Perhaps, it may help determine unique prognostic indicators for every ethnic group more thoroughly.

It is a hoped that this study can be a platform to Malaysian researches to get more understanding regarding chromosomal alterations in OC and its benefits to improve patient prognosis through early detection. Further studies on potential genes that may play a role in OC (tabulated in this study) should be conducted at various levels, namely functional genomics, proteomics or metabolomics level. The pattern of chromosomal alterations and expression can determine the mystery behind the development of OC in cells. Analysis on patient survival should be also included in order to get the prognostic factor.

Overall, it is strongly recommended that this study should be continued in the future with downstream analysis to ensure the knowledge is expanded. Absolute quantitative qPCR, next gene sequencing, FISH, Chromogenic in Situ Hybridization (CISH), or Spectral Karyotyping (SKY) studies should be implemented in selected

potential genes to detect its presence in OC. Besides, huge studies on chromosomal alteration regions such as 8q, 3q, 19 p and 19q should be investigated further by FISH or SKY to get a deeper understanding of the roles of these regions in OC progression.

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APPENDIX A

Detailed protocol on DNA Extraction from Fresh Frozen Tissue

Firstly, The heat block or water bath was equilibrated to 70°C. 750 µM of fresh frozen tissue was cut into small pieces and placed in a steriled 1.5 ml tube. 20 µl of proteinase K and 180 µl of Qiagen buffer ATL were added into the tube and was incubated from 3 hours up to overnight at 55°C shaking in a thermomixer at 450 rpm. After that, the sample was centrifuged for 30 seconds at 6,000 x g to collect the sample at the bottom of the tube. The sample was cool to room temperature for a minute and was centrifuged again for 30 seconds at 6,000 x g to collect the sample at the bottom of the tube. The sample was adjusted to 240 µl with nuclease-free water.

4 µl of RNase A was added to sample, mixed by vortexing and incubated for 2 minutes at room temperature. Then, the sample was centrifuged for 30 seconds at 6,000 x g to collect the sample at the bottom of the tube. 200 µl Buffer AL was added to each sample, mixed thoroughly by vortexing, and incubated at 70°C for 10 minutes in a heat block or water bath. Next, the sample was centrifuged for 30 seconds at 6,000 x g. 200 µl of PCR grade absolute ethanol (99-100%) was added to each sample, was mixed and centrifuged for 30 seconds at 6,000 x g to collect the sample.

The mixture was then distributed into DNeasy Mini spin column placed in 2 ml collection tubes and was centrifuged at 6000 x g for 1 minute. Flow-through and collection tubes were discarded. The spin column was placed in a new 2 ml collection tubes. 500 µl Buffer AW1 was added into the column, and was centrifuged for 1 minute at 6000 x g. 500 µl of AW2 was put into the column, and was centrifuged for 3 minutes at 20,000 x g to dry the DNeasy membrane. Flow-through and collection tube were discarded. The mixture was centrifuged again at full speed for 3 minutes.

After that, the spin column was placed in a clean 1.5 ml microcentrifuge tube, and 50 to 100 µl of AE buffer was added directly into the DNeasy column membrane. The column was incubated at room temperature for 1 minute, and then was centrifuged for 1 minute at 6,000 x g to elute. The gDNA concentration and purity was measured on nanodrop to determine the absorbance at 230 nm, 260 nm, and 280 nm, and calculate the DNA concentration, the OD260/OD280 ratios

APPENDIX B

GenomePlex Complete Whole Genome Amplification (WGA) Kit procedure

Fragmentation

DNA sample was isolated and concentration was quantified by UV absorption (260 nm). 100 ng of gDNA was measured. 1 µl of 10x Fragmentation Buffer and nuclease free water PCR grade were added to make a total volume of 10 µl reaction in a 0.2 ml PCR tube. The tube was then placed in a thermal block at 95 °C for **EXACTLY** 4 minutes. The incubation is very time sensitive. Any deviation may alter results. Immediately the sample was cool on ice, and then was briefly centrifuged to consolidate the contents.

Library Preparation

2 µl of 1x Library Preparation Buffer was added to each sample. 1 µl of Library Stabilization Solution was added.Vortexed thoroughly, consolidate by centrifugation, the mixture was placed in thermal cycler at 95 °C for 2 minutes. The sample was cool on ice, centrifuged, and returned to ice. 1 µl of Library Preparation Enzyme was added, vortexed thoroughly, and centrifuged briefly. The sample was placed in a thermal cycler and incubated as follows:

16 °C for 20 minutes

24 °C for 20 minutes

37 °C for 20 minutes

75 °C for 5 minutes

4 °C hold

The samples was then removed from thermal cycler and centrifuged briefly. Samples may be amplified immediately or stored at –20 °C for three days.

Amplification

A master mix was prepared prepared by adding the following reagents to the 15 µl reaction from above step.

7.5 µl of 10X Amplification Master Mix

47.5 µl of Nuclease-Free Water

5 µl of WGA DNA Polymerase

The sample was vortexed thoroughly, centrifuged briefly, and the thermocycling procedure was performed. The following profile was used using thermocycler to amplify sample.

Initial Denaturation: 95 °C for 3 minutes

Denature: 94 °C for 15 seconds (14 cylces)

Anneal/Extend: 65 °C for 5 minutes

After cycling was completed, the reactions was maintained at 4 °C or stored at –20 °C until ready for analysis or purification.

APPENDIX C

Sigma GenElute PCR Clean-Up Kit Protocol

All centrifugations (spins) were at 12,000 – 16,000 x g. A Gen Elute Miniprep Binding Column was inserted (with a blue o-ring) into a provided collection tube, if not already assembled. 0.5 ml of the Column Preparation Solution was added to each miniprep column and was centrifuged at 12,000 x g for 30 seconds to 1 minute. The eluate was discarded.

5 volumes of Binding Solution were added to 1 volume of the PCR reaction and mix. The solution was transferred into the binding column. The column was centrifuged at maximum speed 16,000 Xg for 1 minute. The eluate was discarded, but the collection tube was retained.The binding column was placed into the collection tube. 0.5 ml of diluted Wash Solution was added to the column and centrifuged at maximum speed for 1 minute. The eluate was discarded, but the collection tube retained. The column was replaced into the collection tube.

The column was centrifuged at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Any residual eluate was discarded as well as the collection tube. The column was transferred to a fresh 2 ml collection tube. 50 µl of Elution Solution or water was applied to the center of each column. The sample was incubated at room temperature for 1 minute. To elute the DNA, the column was centrifuged at maximum speed for 1 minute.

APPENDIX D

Details procedures for array CGH from labelling to washing chip

3 µg of amplified WGA DNA was used for Fluorescent Labelling. Nuclease-free water was added to the gDNA to make a total volume of 26 µl. 5 µl of Random primers was added to each reaction tube to make a total volume of 31 µl. The mixture was mixed well gently. The sample tube was then transferred to a thermomixer and was incubated at 95° C for three minutes, and then moved to ice and was incubated once again in ice for five minutes. Labelling Master Mix was prepared by mixing 10 µl of 5X labelling buffer, 5 µl of 10X dNTPs, 3 µg of WGA DNA, 3 µl Cyanine *5* or *3* -dUTP (1.0mM), 1 µl of Exo-Klenow Fragment enzyme and nuclease free water to make a total volume of 50 µl. The procedure was performed on ice to maintain the environment of the enzyme.

After that, the mixture was incubated in a water bath at 37° C for two hours and additional 10 minutes incubated was performed at 65° C to inactive the enzyme, and then moved to ice. The mixture was purified by using Microcon YM-30 Filter as instructed by manufacturer (Milipore, Massachusetts, USA) to increase the hybridization concentration. The yield and specific activity of the labelled WGA DNA was then measured and the QC report for labelled gDNA must be within the range of measurement as in **Table 1**:

Table 1: Expected yield and specific activity

Input gDNA (µg)	Yield (µg)	Specific activity of Cy 3 (pmol/µg)	Specific activity of Cy 5 (pmol/µg)
3.0	**7 to 10**	**35 to 55**	**25 to 40**

- Cy 5 = for DNA tumour sample
- Cy 3 = for DNA normal sample

After the labelled WGA DNA measurement, the appropriate labelled cy 5 sample and cy 3 sample was combined to make a total mixture volume of 39 µl for Microarray Hybridization process.

39 µl mixture of labelled test WGA DNA and reference WGA DNA, 5 µl of Cot-1 DNA (1.0 mg/ml), 11 µl of 10X blocking agent, 55 µl of 2X Hybridization Buffer was mixed into 1.5 ml microcentrifuge tube and transferred to a thermomixer at 95° C for incubation for three minutes. Immediately, the sample was transferred to water bath at 37° C and was incubated for 30 minutes and was centrifuged for one minute at 17,900 x g to collect sample at the bottom of the tube.

100 µl of hybridization sample mixture was dispensed onto the Hybridization Chamber gasket microarray 4 x 44K slide (Agilent, California, USA) in a drag and disperse manner and was fully loaded before placing the Unrestricted HD 4x44K microarray slide (Agilent, California, USA). The microarray slide was then placed facing down the gasket slide to make sandwich pair. Slowly, SureHyb chamber cover (Agilent, Calfornia, USA) was placed onto the sandwiched slide and was clamped tighten. Vertically, the assembled chamber was rotated to wet the slides and asses the mobility of the bubbles. The slide chamber was placed in the rotator rack in a hybridization oven set to 65° C with rotation at 20 rpm for 24 hours incubation.

At temperature room, after the slide was taken out from the oven, the sandwiched pair slide was disassembled in Oligo aCGH Wash buffer 1. A slide rack was placed into slide staining dish Oligo aCGH Wash Buffer 1 and washed for 5 minutes. Then, the pre-warmed glass dish was filled with Oligo aCGH buffer 2 heating element and maintained at 37°C for 1 minute. Lastly, the glass dish that contained

sample slides was transferred to Acetonitrile in fume hood and was stirred for 1 minute. The slide was scanned immediately to minimize impact of environmental oxidants on signal intensities.

The slide containing the hybridized sample was assembled into Agilent microarray slide holder in the scanner carousel. The following was the default scan setting: a) Scan density and resolution was set at 5µm. b) Scan region was set to scan area (61 x 21.6 mm). c) Dye channel was set to Red and Green. Green & Red PMT was set to 100%. d) The automatic file naming was selected with Prefix 1 and was set to Instrument Serial Number and Prefix 2 was set to Array Barcode. e) The Extended Dynamic Range check box was cleared.

CGH_QCM_Feb 2007 program was selected to extract microarray CGH data. Finally, FE QC Report was generated and QC metric was determined. QC Metric was set to monitor the experiment consistency and determine the successful of microarray experiments.

Table 2: Microarray QC Metrics for high DNA quality samples

Metric	Excellent	Good	Poor
BGNoise	<5	5 to 10	>10
Signal Intensity	>150	50 to 150	<50
Signal to Noise	>100	30 to 100	<30
Reproducibility	<0.05	0.05 to 0.2	>0.2
DLRSpread	<0.2	0.2 to 0.3	>0.3

The image (.tif) file that was generated from scanning step was added to the FE project.

Analysis of arrayCGH data was obtained from Agilent Genomic Workbench 5.0 software with the guidance from manufacturer's guidelines with minor modification. Copy Number Alteration (CNA) and genes associated with OSCC were observed from this step.

APPENDIX E: Details on arrayCGH analysis procedure

Microarray QC Metrics for high DNA quality samples

These metrics are only appropriate for high-quality DNA samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are exported to a table in the Feature Extraction QC report (select **CGH_QCM_Feb07** in Project Properties before extraction) and in CGH Analytics. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics including the biological sample source, quality of starting gDNA, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the normal ranges that Agilent has observed when analyzing well established and characterized cell lines using version 5.0 protocol, Oligonucleotide Array-Based CGH for Genomic DNA Analysis.

Table 15

Metric	Excellent	Good	Poor
BGNoise	< 5	5 to 10	> 10
Signal Intensity	> 150	50 to 150	< 50
Signal to Noise	> 100	30 to 100	< 30
Reproducibility	< 0.05	0.05 to 0.2	> 0.2
DLRSpread	< 0.2	0.2 to 0.3	> 0.3

QC Chart Tool

At times the Feature Extraction program is used in a production environment, where the biological samples are similar, microarray processing protocols are identical and monitoring run-to-run consistency is an important goal. The Feature Extraction program can help monitor this consistency with the optional QC Chart Tool. The QC Chart Tool extracts summary statistics from a set of Feature Extraction output text files and can be used to generate metric sets that can be imported into the Feature Extraction program for analysis of each batch of microarrays processed. Only one metric set can be assigned to a Feature Extraction project. When that project is run, the Feature Extraction program summarizes the metric statistics on each microarray's QC report and

shows if the thresholds (if any) were exceeded. In addition, at the end of the project, a summary chart can be opened to display graphically what the results are for each metric for each microarray. The QC Chart Tool can be downloaded at www.agilent.com/chem/FEQCMetrics.

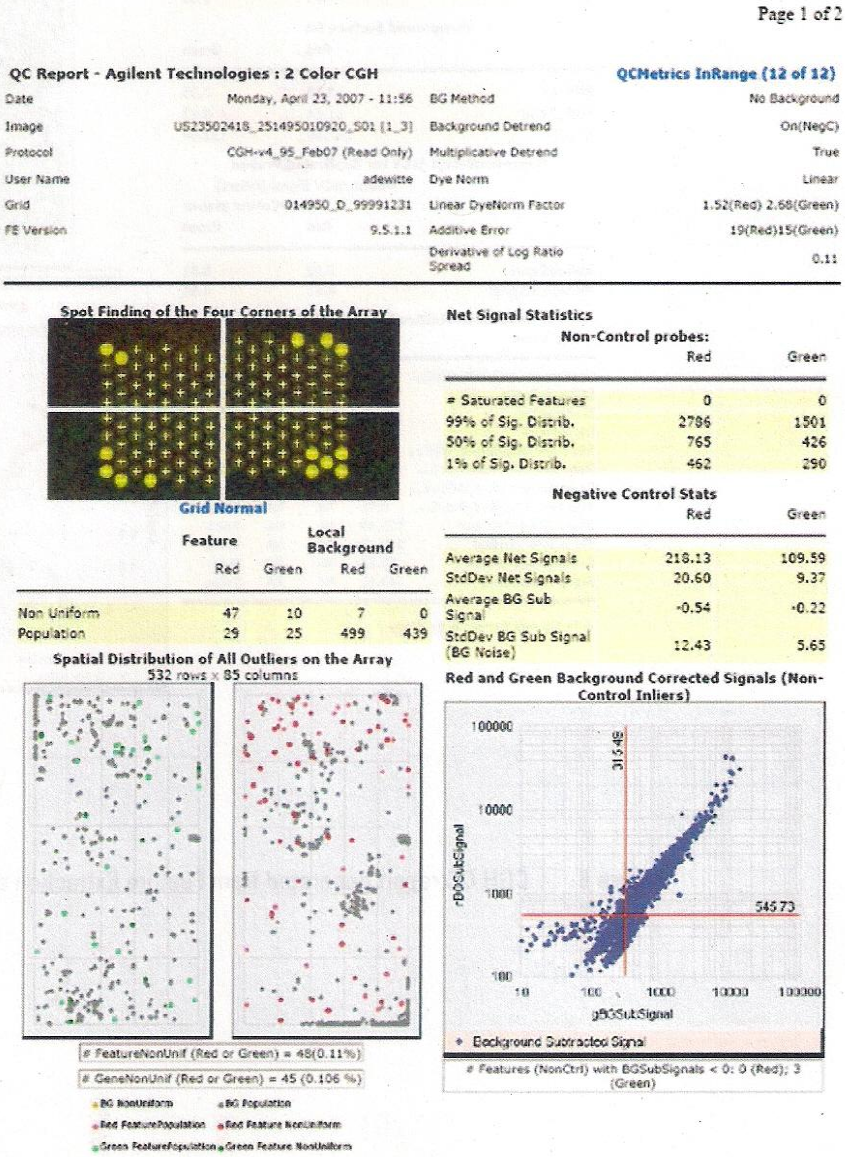
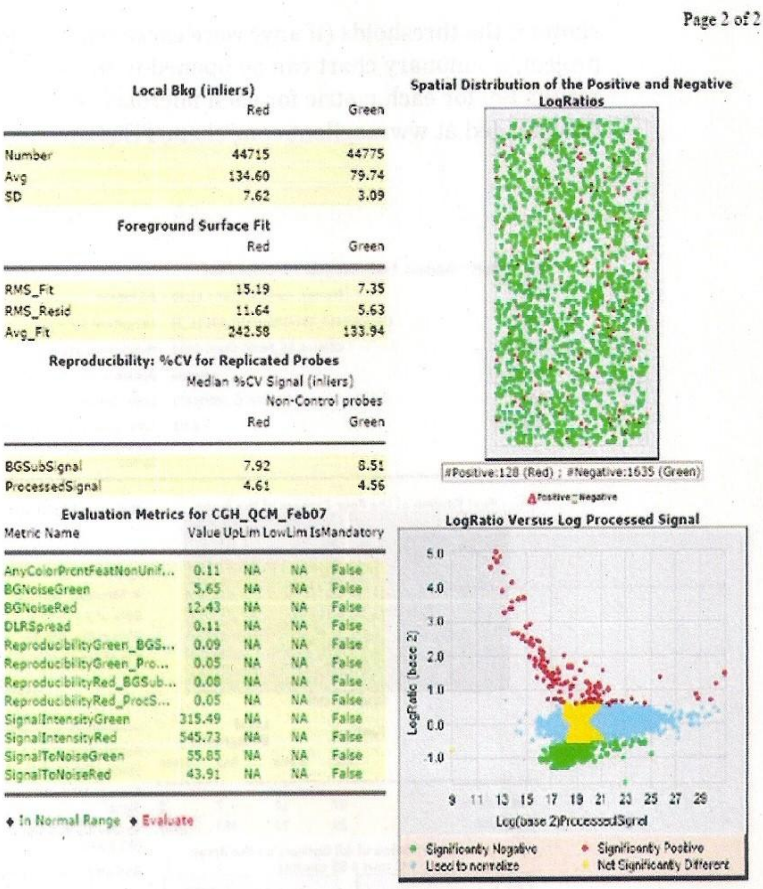


Figure 5 CGH QC report generated from Feature Extraction software v9.5, page 1



Page 2 of 2

Local Bkg (inliers)

	Red	Green
Number	44715	44775
Avg	134.60	79.74
SD	7.62	3.09

Foreground Surface Fit

	Red	Green
RMS_Fit	15.19	7.35
RMS_Resid	11.64	5.63
Avg_Fit	242.58	133.94

Reproducibility: %CV for Replicated Probes

Median %CV Signal (inliers)
Non-Control probes

	Red	Green
BGSubSignal	7.92	8.51
ProcessedSignal	4.61	4.56

Evaluation Metrics for CGH_QCM_Feb07

Metric Name	Value	UpLim	LowLim	IsMandatory
AnyColorPrcntFeatNonUnif...	0.11	NA	NA	False
BGNoiseGreen	5.65	NA	NA	False
BGNoiseRed	12.43	NA	NA	False
DLRSpread	0.11	NA	NA	False
ReproducibilityGreen_BGS...	0.09	NA	NA	False
ReproducibilityGreen_Pro...	0.05	NA	NA	False
ReproducibilityRed_BGSub...	0.08	NA	NA	False
ReproducibilityRed_ProcS...	0.05	NA	NA	False
SignalIntensityGreen	315.49	NA	NA	False
SignalIntensityRed	545.73	NA	NA	False
SignalToNoiseGreen	55.85	NA	NA	False
SignalToNoiseRed	43.91	NA	NA	False

♦ In Normal Range ♦ Evaluate

Spatial Distribution of the Positive and Negative LogRatios

#Positive:128 (Red) ; #Negative:1635 (Green)

LogRatio Versus Log Processed Signal

Figure 6 CGH QC report generated from Feature Extraction software v9.5, page 2

APPENDIX F: Socio-Demographic profiles of 20 patients of OSCC

No	IRPA	Age	Smoking	Quid Chewing	Drinking	Gender	Ethnic
1	01-0016-07	74	No	Yes	No	F	India
2	01-0027-07	78	No	Yes	No	F	India
3	01-0035-08	26	No	No	No	F	India
4	01-0052-05	69	Yes	Yes	Yes	F	India
5	01-0059-07	55	No	Yes	No	F	India
6	11-0008-05	57	No	Yes	No	M	indigenous
7	04-0006-09	54	No	No	No	M	India
8	04-0009-08	65	No	Yes	No	F	India
9	04-0013-08	62	No	Yes	No	F	India
10	04-0018-08	59	No	No	No	M	India
11	06-0014-07	56	No	No	No	F	India
12	06-0033-08	70	No	Yes	No	F	India
13	06-0036-08	64	No	Yes	No	F	India
14	11-0004-07	58	No	Yes	No	F	indigenous
15	11-0008-04	56	No	Yes	No	F	indigenous
16	11-0009-05	71	No	No	No	M	indigenous
17	11-0009-08	68	No	No	No	F	indigenous
18	11-0011-07	53	Yes	Yes	Yes	F	indigenous
19	11-0014-06	59	No	No	No	F	indigenous
20	11-0040-05	63	Yes	Yes	Yes	F	indigenous

APPENDIX G

Other genes identified in all 20 cases with ratio less than 30%

Amplified gene	Deleted gene
OR4F3, OR4F16 & OR4F29 (1p36.33), PRKDC (8q11), PLEKHG6 (12p13.31), SNRNP25, POLR3K & RHBDF1 (16p13.3) = 20% each	*VPS53, FAM57A (17p13.3), CHL1 (3p26.1), CNTN6, CNTN4 (3p26.3), and ZNF596 (8p23.3)* = 25% each
EGFR (7p12), TMEM167B (1p13.3), PDE4DIP (1q12), ZNF12 (7p22.1), PRKAR1B (7p22.3), UBE2V2 (8q11.21), ODF3 (11p15.5), and FAM101B (17p13) = 15% each	*TTLLIO, TNFRSF18 (1p36.33), CDH18 (5p14.3), LGSN (6pter-q22.33), NLRP6 (11p15), ATHL1, RIC8A, BET1L (11p15.5), HBA2, HBA1, HBQ1 (16p13.3), PPAP2C, MIER2 (19p13.3), USP25, POTED (21q11.2), C21orf34 (21q21.1)* = 20% each
	PRKRA (2q31.2), PDE6B (4p16.3), PDE4D (5q12), IFITM5 (11p15.5), NOC4L, GALNT9 (12q24.33), NOVA1, STXBP6 (14q12), RAB11FIP3, C16orf10 (16p13.3), SS18, PSMA8 (18q11.2), CDH7 (18q22.1), BID (22q11.21) = 15% each

APPENDIX H

Clinicopathological parameters for all OSCC cases (n=20) (arrayCGH)

No	IRPA	Tumour Grade	Tumour Size	Lymph Nodes (N) Status	Stage	Site
1	01-0016-07	Well	T2	N1	III	Buccal Mucosa
2	01-0027-07	Moderate	T4	Nx	IVA	Buccal Mucosa
3	01-0035-08	Well	T1	NO	I	Tongue
4	01-0052-05	Moderate	T3	NO	III	Tongue
5	01-0059-07	Moderate	T1	N2a	IV	Buccal Mucosa
6	11-0008-05	Moderate	T4	NO	IV	Buccal Mucosa
7	04-0006-09	Well	T2	NO	II	Tongue
8	04-0009-08	Well	T2	NO	II	Buccal Mucosa
9	04-0013-08	Well	T2	N2B	IVA	Buccal Mucosa
10	04-0018-08	Well	T1	NO	I	Tongue
11	06-0014-07	Moderate	T1	NO	I	Tongue
12	06-0033-08	Moderate	T3	N1	III	Buccal Mucosa
13	06-0036-08		Tis	NO	O	Buccal Mucosa
14	11-0004-07	Moderate	T3	N2c	IVA	tongue & palate
15	11-0008-04	Well	T2	N1	III	gum
16	11-0009-05	Well	T4	N2c	IVA	Lip, gum, floor of mouth
17	11-0009-08	Moderate	T3	NO	III	left hard & Soft palate
18	11-0011-07	Moderate	T4	N3a	IVB	tongue
19	11-0014-06	Moderate	T4	N2b	IVA	Gum & Palate
20	11-0040-05	Well	T4	N2c	IVA	Tongue

* NO and Nx were classified as negative
* N1, N2 and N3 were classified as positive

APPENDIX I: Chromosomal Alteration Statistical Analysis

Broder's Classifications * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Broder's Classifications	Well Differentiated	4	5	9
	Moderately Differentiated	7	3	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.269(b)	1	.260		
Continuity Correction(a)	.437	1	.508		
Likelihood Ratio	1.281	1	.258		
Fisher's Exact Test				.370	.255
Linear-by-Linear Association	1.202	1	.273		
N of Valid Cases	19				

a Computed only for a 2x2 table

b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Broder's Classifications * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Broder's Classifications	Well Differentiated	5	4	9
	Moderately Differentiated	6	4	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.038(b)	1	.845		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.038	1	.845		
Fisher's Exact Test				1.000	.605
Linear-by-Linear Association	.036	1	.849		
N of Valid Cases	19				

a Computed only for a 2x2 table

b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Broder's Classifications * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Broder's Classifications	Well Differentiated	6	3	9
	Moderately Differentiated	5	5	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.540(b)	1	.463		
Continuity Correction(a)	.073	1	.788		
Likelihood Ratio	.544	1	.461		
Fisher's Exact Test				.650	.395
Linear-by-Linear Association	.511	1	.475		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Broder's Classifications * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Broder's Classifications	Well Differentiated	8	1	9
	Moderately Differentiated	7	3	10
Total		15	4	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.017(b)	1	.313		
Continuity Correction(a)	.198	1	.656		
Likelihood Ratio	1.061	1	.303		
Fisher's Exact Test				.582	.333
Linear-by-Linear Association	.963	1	.326		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.89.

Broder's Classifications * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Broder's Classifications	Well Differentiated	7	2	9
	Moderately Differentiated	6	4	10
Total		13	6	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.693(b)	1	.405		
Continuity Correction(a)	.114	1	.735		
Likelihood Ratio	.704	1	.401		
Fisher's Exact Test				.628	.370
Linear-by-Linear Association	.656	1	.418		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.84.

Broder's Classifications * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Broder's Classifications	Well Differentiated	5	4	9
	Moderately Differentiated	7	3	10
Total		12	7	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.425(b)	1	.515		
Continuity Correction(a)	.031	1	.861		
Likelihood Ratio	.426	1	.514		
Fisher's Exact Test				.650	.430
Linear-by-Linear Association	.402	1	.526		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.32.

Tumour Size * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Tumour Size	T1 and T2	4	5	9
	T3 and T4	7	3	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.269(b)	1	.260		
Continuity Correction(a)	.437	1	.508		
Likelihood Ratio	1.281	1	.258		
Fisher's Exact Test				.370	.255
Linear-by-Linear Association	1.202	1	.273		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Tumour Size * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Tumour Size	T1 and T2	5	4	9
	T3 and T4	6	4	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.038(b)	1	.845		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.038	1	.845		
Fisher's Exact Test				1.000	.605
Linear-by-Linear Association	.036	1	.849		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Tumour Size * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Tumour Size	T1 and T2	5	4	9
	T3 and T4	6	4	10
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.038(b)	1	.845		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.038	1	.845		
Fisher's Exact Test				1.000	.605
Linear-by-Linear Association	.036	1	.849		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.79.

Tumour Size * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Tumour Size	T1 and T2	9	0	9
	T3 and T4	6	4	10
Total		15	4	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.560(b)	1	.033		
Continuity Correction(a)	2.471	1	.116		
Likelihood Ratio	6.097	1	.014		
Fisher's Exact Test				.087	.054
Linear-by-Linear Association	4.320	1	.038		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.89.

Tumour Size * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Tumour Size	T1 and T2	8	1	9
	T3 and T4	5	5	10
Total		13	6	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.316(b)	1	.069		
Continuity Correction(a)	1.760	1	.185		
Likelihood Ratio	3.557	1	.059		
Fisher's Exact Test				.141	.091
Linear-by-Linear Association	3.141	1	.076		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.84.

Tumour Size * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Tumour Size	T1 and T2	7	2	9
	T3 and T4	5	5	10
Total		12	7	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.571(b)	1	.210		
Continuity Correction(a)	.604	1	.437		
Likelihood Ratio	1.611	1	.204		
Fisher's Exact Test				.350	.220
Linear-by-Linear Association	1.488	1	.223		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.32.

Nodes Status * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Nodus Status	Negative	6	4	10
	Positive	6	4	10
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000(b)	1	1.000		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.675
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.00.

Nodes Status * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Nodus Status	Negative	5	5	10
	Positive	6	4	10
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.202(b)	1	.653		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.202	1	.653		
Fisher's Exact Test				1.000	.500
Linear-by-Linear Association	.192	1	.661		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.50.

Nodes Status * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Nodus Status	Negative	5	5	10
	Positive	6	4	10
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.202(b)	1	.653		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.202	1	.653		
Fisher's Exact Test				1.000	.500
Linear-by-Linear Association	.192	1	.661		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.50.

Nodes Status * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Nodus Status	Negative	8	2	10
	Positive	7	3	10
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.267(b)	1	.606		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.268	1	.605		
Fisher's Exact Test				1.000	.500
Linear-by-Linear Association	.253	1	.615		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.50.

Nodes Status * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Nodus Status	Negative	6	4	10
	Positive	7	3	10
Total		13	7	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.220(b)	1	.639		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.220	1	.639		
Fisher's Exact Test				1.000	.500
Linear-by-Linear Association	.209	1	.648		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.50.

Nodes Status * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Nodus Status	Negative	7	3	10
	Positive	5	5	10
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.833(b)	1	.361		
Continuity Correction(a)	.208	1	.648		
Likelihood Ratio	.840	1	.359		
Fisher's Exact Test				.650	.325
Linear-by-Linear Association	.792	1	.374		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.00.

TnM Staging * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
TnM Staging	Stage I and II	3	4	7
	Stage III and IV	8	4	12
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.028(b)	1	.311		
Continuity Correction(a)	.283	1	.594		
Likelihood Ratio	1.027	1	.311		
Fisher's Exact Test				.377	.297
Linear-by-Linear Association	.974	1	.324		
N of Valid Cases	19				

a Computed only for a 2x2 table

b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.95.

TnM Staging * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
TnM Staging	Stage I and II	4	3	7
	Stage III and IV	7	5	12
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.003(b)	1	.960		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.003	1	.960		
Fisher's Exact Test				1.000	.663
Linear-by-Linear Association	.002	1	.961		
N of Valid Cases	19				

a Computed only for a 2x2 table

b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.95.

TnM Staging * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
TnM Staging	Stage I and II	5	2	7
	Stage III and IV	6	6	12
Total		11	8	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.833(b)	1	.361		
Continuity Correction(a)	.186	1	.667		
Likelihood Ratio	.853	1	.356		
Fisher's Exact Test				.633	.337
Linear-by-Linear Association	.789	1	.374		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.95.

TnM Staging * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
TnM Staging	Stage I and II	7	0	7
	Stage III and IV	8	4	12
Total		15	4	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.956(b)	1	.086		
Continuity Correction(a)	1.290	1	.256		
Likelihood Ratio	4.280	1	.039		
Fisher's Exact Test				.245	.128
Linear-by-Linear Association	2.800	1	.094		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.47.

TnM Staging * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
TnM Staging	Stage I and II	6	1	7
	Stage III and IV	7	5	12
Total		13	6	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.534(b)	1	.216		
Continuity Correction(a)	.529	1	.467		
Likelihood Ratio	1.657	1	.198		
Fisher's Exact Test				.333	.238
Linear-by-Linear Association	1.453	1	.228		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 2.21.

TnM Staging * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
TnM Staging	Stage I and II	6	1	7
	Stage III and IV	6	6	12
Total		12	7	19

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.423(b)	1	.120		
Continuity Correction(a)	1.132	1	.287		
Likelihood Ratio	2.631	1	.105		
Fisher's Exact Test				.173	.144
Linear-by-Linear Association	2.296	1	.130		
N of Valid Cases	19				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 2.58.

Tumour Site * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Tumour Site	Tongue	3	4	7
	Others	9	4	13
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.319(b)	1	.251		
Continuity Correction(a)	.449	1	.503		
Likelihood Ratio	1.311	1	.252		
Fisher's Exact Test				.356	.251
Linear-by-Linear Association	1.253	1	.263		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

Tumour Site * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Tumour Site	Tongue	5	2	7
	Others	6	7	13
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.174(b)	1	.279		
Continuity Correction(a)	.375	1	.540		
Likelihood Ratio	1.205	1	.272		
Fisher's Exact Test				.374	.272
Linear-by-Linear Association	1.116	1	.291		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.15.

Tumour Site * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Tumour Site	Tongue	6	1	7
	Others	5	8	13
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.105(b)	1	.043		
Continuity Correction(a)	2.418	1	.120		
Likelihood Ratio	4.461	1	.035		
Fisher's Exact Test				.070	.058
Linear-by-Linear Association	3.900	1	.048		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.15.

Tumour Site * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Tumour Site	Tongue	7	0	7
	Others	8	5	13
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.590(b)	1	.058		
Continuity Correction(a)	1.832	1	.176		
Likelihood Ratio	5.170	1	.023		
Fisher's Exact Test				.114	.083
Linear-by-Linear Association	3.410	1	.065		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.75.

Tumour Site * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Tumour Site	Tongue	7	0	7
	Others	6	7	13
Total		13	7	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.799(b)	1	.016		
Continuity Correction(a)	3.673	1	.055		
Likelihood Ratio	7.953	1	.005		
Fisher's Exact Test				.044	.022
Linear-by-Linear Association	5.509	1	.019		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 2.45.

Tumour Site * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Tumour Site	Tongue	5	2	7
	Others	7	6	13
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.586(b)	1	.444		
Continuity Correction(a)	.082	1	.774		
Likelihood Ratio	.600	1	.439		
Fisher's Exact Test				.642	.392
Linear-by-Linear Association	.557	1	.456		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

Gender Distribution * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Gender Distribution	Male	2	2	4
	Female	10	6	16
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.208(b)	1	.648		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.205	1	.651		
Fisher's Exact Test				1.000	.535
Linear-by-Linear Association	.198	1	.656		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.60.

Gender Distribution * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Gender Distribution	Male	1	3	4
	Female	10	6	16
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.818(b)	1	.178		
Continuity Correction(a)	.619	1	.432		
Likelihood Ratio	1.857	1	.173		
Fisher's Exact Test				.285	.217
Linear-by-Linear Association	1.727	1	.189		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.80.

Gender Distribution * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Gender Distribution	Male	2	2	4
	Female	9	7	16
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.051(b)	1	.822		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.050	1	.823		
Fisher's Exact Test				1.000	.625
Linear-by-Linear Association	.048	1	.827		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.80.

Gender Distribution * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Gender Distribution	Male	3	1	4
	Female	12	4	16
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000(b)	1	1.000		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.718
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 1.00.

Gender Distribution * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Gender Distribution	Male	2	2	4
	Female	11	5	16
Total		13	7	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.495(b)	1	.482		
Continuity Correction(a)	.014	1	.907		
Likelihood Ratio	.478	1	.489		
Fisher's Exact Test				.587	.439
Linear-by-Linear Association	.470	1	.493		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.40.

Gender Distribution * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Gender Distribution	Male	2	2	4
	Female	10	6	16
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.208(b)	1	.648		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.205	1	.651		
Fisher's Exact Test				1.000	.535
Linear-by-Linear Association	.198	1	.656		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.60.

Ethnic Distribution * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Ethnic Distribution	Indian	6	6	12
	Indigenous	6	2	8
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.250(b)	1	.264		
Continuity Correction(a)	.425	1	.514		
Likelihood Ratio	1.288	1	.256		
Fisher's Exact Test				.373	.260
Linear-by-Linear Association	1.188	1	.276		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 3.20.

Ethnic Distribution * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Ethnic Distribution	Indian	5	7	12
	Indigenous	6	2	8
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.155(b)	1	.142		
Continuity Correction(a)	1.019	1	.313		
Likelihood Ratio	2.228	1	.136		
Fisher's Exact Test				.197	.157
Linear-by-Linear Association	2.047	1	.152		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.60.

Ethnic Distribution * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Ethnic Distribution	Indian	5	7	12
	Indigenous	6	2	8
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.155(b)	1	.142		
Continuity Correction(a)	1.019	1	.313		
Likelihood Ratio	2.228	1	.136		
Fisher's Exact Test				.197	.157
Linear-by-Linear Association	2.047	1	.152		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.60.

Ethnic Distribution * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Ethnic Distribution	Indian	10	2	12
	Indigenous	5	3	8
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.111(b)	1	.292		
Continuity Correction(a)	.278	1	.598		
Likelihood Ratio	1.095	1	.295		
Fisher's Exact Test				.347	.296
Linear-by-Linear Association	1.056	1	.304		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

Ethnic Distribution * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Ethnic Distribution	Indian	9	3	12
	Indigenous	4	4	8
Total		13	7	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.319(b)	1	.251		
Continuity Correction(a)	.449	1	.503		
Likelihood Ratio	1.311	1	.252		
Fisher's Exact Test				.356	.251
Linear-by-Linear Association	1.253	1	.263		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

Ethnic Distribution * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Ethnic Distribution	Indian	8	4	12
	Indigenous	4	4	8
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.556(b)	1	.456		
Continuity Correction(a)	.078	1	.780		
Likelihood Ratio	.554	1	.457		
Fisher's Exact Test				.648	.388
Linear-by-Linear Association	.528	1	.468		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 3.20.

All Habit * 8q24.3

Crosstab

Count

		8q24.3		
		Yes	No	Total
Combine Habit	With habit	7	6	13
	No habit	5	2	7
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.586(b)	1	.444		
Continuity Correction(a)	.082	1	.774		
Likelihood Ratio	.600	1	.439		
Fisher's Exact Test				.642	.392
Linear-by-Linear Association	.557	1	.456		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

All Habit * 8q11.1-8q11.2

Crosstab

Count

		8q11.1-8q11.2		
		Yes	No	Total
Combine Habit	With habit	8	5	13
	No habit	3	4	7
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.642(b)	1	.423		
Continuity Correction(a)	.109	1	.742		
Likelihood Ratio	.642	1	.423		
Fisher's Exact Test				.642	.370
Linear-by-Linear Association	.610	1	.435		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.15.

All Habit * 3q26

Crosstab

Count

		3q26		
		Yes	No	Total
Combine Habit	With habit	6	7	13
	No habit	5	2	7
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.174(b)	1	.279		
Continuity Correction(a)	.375	1	.540		
Likelihood Ratio	1.205	1	.272		
Fisher's Exact Test				.374	.272
Linear-by-Linear Association	1.116	1	.291		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.15.

All Habit * 19p13.3

Crosstab

Count

		19p13.3		
		Yes	No	Total
Combine Habit	With habit	10	3	13
	No habit	5	2	7
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.073(b)	1	.787		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.072	1	.788		
Fisher's Exact Test				1.000	.594
Linear-by-Linear Association	.070	1	.792		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.75.

All Habit * 19p13-p13.11

Crosstab

Count

		19p13-p13.11		
		Yes	No	Total
Combine Habit	With habit	8	5	13
	No habit	5	2	7
Total		13	7	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.196(b)	1	.658		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.199	1	.656		
Fisher's Exact Test				1.000	.526
Linear-by-Linear Association	.186	1	.666		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 3 cells (75.0%) have expected count less than 5. The minimum expected count is 2.45.

All Habit * 19q13.3

Crosstab

Count

		19q13.3		
		Yes	No	Total
Combine Habit	With habit	8	5	13
	No habit	4	3	7
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.037(b)	1	.848		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.037	1	.848		
Fisher's Exact Test				1.000	.608
Linear-by-Linear Association	.035	1	.852		
N of Valid Cases	20				

a Computed only for a 2x2 table
b 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

APPENDIX J

Results Summary for RT-PCR (cDNA Level) 30 samples (independent samples)

Target : DUSP22 gene

No	Sample	CT Mean	CT (SD)	C (Mean)	C (SE)	C	RQ	Fold	RQ (min)	RQ (Max)	CT Mean	CT (SD)
1	04-0009-08	29.5393	0.3911	-1.5457	0.4085	-1.4511	2.7342	3	1.2457	6.001	31.0849	0.5896
2	01-0011-10	30.0073	0.2173	-0.3713	0.5849	-0.2767	1.2114	1	0.393	3.734	30.3786	0.9895
3	01-0004-09	29.9289	0.0333	0.0147	0.6644	0.1093	0.927	1	0.2581	3.3294	29.9141	1.1502
4	11-0039-05	30.5016	0.1685	-0.6296	1.1297	-0.535	1.6489	1	0.1647	12.7432	31.1311	1.9495
5	01-0059-07	29.6134	0.4254	-1.0553	0.4158	-0.9607	1.9463	2	0.8743	4.3324	29.9141	1.1502
6	06-0033-08	29.0133	1.3297	-5.1734	2.4057	-5.0788	33.796	34	0.3298	3463.635	34.1867	3.9489
7	06-0036-08	30.2115	0.2178	-1.0962		-1.0017	2.0023	2			31.3077	
8	11-0040-05	27.9219	0.329	-3.4468	0.3076	-3.3523	10.2125	10	5.6496	18.4604	31.3687	0.4191
9	06-0034-09	30.3629	0.9085	0.2454	0.7616	0.34	0.7901	1	0.1825	3.4212	30.1175	0.7808
10	11-0004-07	30.3313	0.158	-1.3127	0.508	-1.2181	2.3264	2	0.8753	6.1836	31.644	0.8655
11	11-0009-05	30.7094	0.1526	1.7064	0.417	1.801	0.287	1	0.1286	0.6403	29.0031	0.706
12	01-0035-08	29.6093	0.437	-0.8477	0.5382	-0.7532	1.6855	2	0.5983	4.7481	30.457	0.8234
13	04-0019-05	29.0456	0.0289	0.5642	0.2674	0.7239	0.6054	1	0.3619	1.0129	28.4814	0.4623
14	06-0005-10	29.3841	0.1029	-4.2454	1.7553	-4.0856	16.9786	17	0.5792	497.75	33.6295	3.0386
15	01-0058-07	31.0397	0.1094	0.1838	0.353	0.3436	0.7881	1	0.3995	1.5545	30.8559	0.6015
16	04-0007-08	30.7906	0.19	0.0272	0.2513	0.1869	0.8785	1	0.5416	1.4249	30.7635	0.3916
17	01-0005-09	30.6168	0.1896	1.5822	0.4577	1.7419	0.299	1	0.1239	0.7214	29.0346	0.7698
18	01-0011-04	30.4546	0.0852	1.2184	0.2919	1.3781	0.3847	1	0.2194	0.6747	29.2362	0.4984
19	01-0023-09	29.4249	0.0232	0.0021	0.2333	0.1618	0.8939	1	0.5706	1.4004	29.4228	0.4033
20	04-0004-08	31.6901	0.0964	1.3011	0.5328	1.4608	0.3633	1	0.1303	1.0129	30.389	0.9178
21	06-0012-08	30.9543	0.1093	2.4978	0.0851	2.6575	0.1585	1	0.1346	0.1867	28.4565	0.0988

22	06-0029-08	30.6683	0.2813	1.4148	0.2794	1.5745	0.3358	1	0.1961	0.5748	29.2535	0.3938
23	08-0018-08	30.6599	0.1093	1.1166	0.6002	1.2763	0.4128	1	0.1301	1.3104	29.5433	1.0338
24	11-0009-07	31.2948	0.0108	0.2633	0.373	0.423	0.7459	1	0.3639	1.529	31.0315	0.6459
25	01-0002-10	29.9723	0.0697	0.5402	0.3397	0.7904	0.5782	1	0.3007	1.1118	29.432	0.5843
26	01-0048-07	30.9894	0.0549	-0.7571	0.0953	-0.5069	1.421	1	1.1828	1.7072	31.7465	0.1558
27	01-0107-08	29.9022	0.0555	-0.3526	0.0886	-0.1025	1.0736	1	0.9052	1.2733	30.2549	0.1432
28	04-0014-08	30.7037	0.1189	-0.6953	0.7087	-0.4451	1.3614	1	0.348	5.3255	31.399	1.2218
29	04-0023-09	30.5881	0.0588	-0.9686	0.4055	-0.7184	1.6454	2	0.754	3.5909	31.5567	0.6999
30	06-0031-08	30.5405	0.0276	1.524	0.1375	1.7742	0.2924	1	0.2244	0.3809	29.0165	0.2365
21	01-0007-07	31.0898	0.156	-0.7627	0.6399	-0.6682	1.2114	1	0.393	3.734	31.8526	1.0974
32	01-0018-08	31.2235	0.0755	0.3256	0.0856	0.4202	0.7473	1	0.6338	0.8811	30.8979	0.1276
33	01-0025-08	30.9497	0.0162	-0.0946	0.7063	0	1	1	0.2568	3.8937	31.0443	1.2233

APPENDIX K

TNM Staging of OC

TNM Staging of OC

Primary Tumor (T)

TX = Primary tumour cannot be assessed

T0 = No evidence of primary tumour

Tis = Carcinoma in situ

T1 = Tumour 2 cm or less in greatest dimension

T2 = Tumour more than 2 cm but not more than 4 cm in greatest dimension

T3 = Tumour more than 4 cm in greatest dimension

T4= Tumour invades adjacent structures (tongue, skin of neck and through cortical bone)

Nodal Involvement (N)

NX = Regional lymph nodes cannot be assessed

N0 = No regional lymph node metastasis

N1 = Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension

N2A = Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension

N2B = Metastasis in multiple ipsilateral lymph nodes no more than 6cm in greatest dimension

N2c = Metastasis in bilateral or contralateral lymph nodes, no more than 6 cm in greatest dimension

N3 = Metastasis in a lymph node more than 6 cm in greatest dimension

Distant Metastasis (M)

MX = Distant metastasis cannot be assessed

M0 = No distant metastasis

M1 = Distant metastasis

Stage Grouping

Stage 0 = Tis N0 M0

Stage I = T1 N0 M0

Stage II = T2 N0 M0

Stage III = T3 N0 M0; T1 or T2 or T3 N1 M0

Stage IVA = T4 or any T lesion, or any N0, N1 or N2 lesions, and M0

Stage IVB = Any T lesion, N3 M0

Stage IVC = Any T lesion, Any N and M1