CHAPTER 1

INTRODUCTION

1.1 PROBLEM STATEMENT

Cancer is one of the main causes of death after infections and heart diseases (Johnson, 2003a). Each year, cancer incidence and mortality are generally expanding. The International Agency for Research on Cancer (IARC) reported that almost more than half of 7.6 million new diagnosed cases of cancer occur in developing nations annually. The lung is the most frequent cancer site among 11 other sites studied and after that cancer of the stomach and oral are the most common which are particularly prevalent in Asia (Nasca, 2001). Annually over 390,000 cases of oral cancer occur as a serious public health problem reported globally; two-thirds of these are found in developing countries. Approximately 200,000 deaths are being caused by this malignancy yearly. The incidence of oral cancer in Southern Asia particularly in the Indian subcontinent is high (Parkin et al., 2005).

Epidemiologic observations of the present day emphasize the complexity of cancer etiology, comprising abundant data suggesting that cancers have multiple causes and usually both unique and overlapping risk factors (Slattery & Fitzpatrick, 2009). The difference in distribution of oral cancer worldwide is influenced by the risk factors (Stewart& Kleihues, 2003). All over the world, tobacco smoking, alcohol consumption and betel-quid chewing are the three common risk factors recognized as associated with oral cancer (Johnson, 2003b; Zain & Ghazali, 2001).

Molecular mechanisms responsible for this malignancy are not well recognized although the different risk factors for oral cancer are well known. The development of oral cancer proceeds through several molecular genetic events, often after long-term exposure to environmental risk factors, particularly tobacco and /or alcohol (Rai *et al.*, 2004). It is interesting to know that the polymorphic genotypes which code for tobacco carcinogen-metabolizing enzymes (for example, glutathione s-transferase – GST) could play an important role in oral cancer susceptibility (Park *et al.*, 1999).

Although preclinical and clinical studies have shown therapeutic anti-tumor effects of *TNF-α* in several tumors (bladder, breast, prostate, neuroblastoma, non-small cell lung, colon, mesothelioma, lymphoma, ovarian, osteosarcoma & gastric cancers) (See *et al.*, 2002), there is some evidence that it may also promote the development and spread of cancer by promoting stroma formation and angiogenesis (Fràter-Schröder *et al.*, 1987; Naylor *et al.*, 1993).

TNF-α expression is mostly regulated at the transcriptional level (Raabe *et al.*, 1998) and polymorphism of the *TNF-α* gene has been intensively studied as a potential determinant of susceptibility to numerous cancers such as bladder cancer (Jeong *et al.*, 2004; Marsh *et al.*, 2003; Nonomura *et al.*, 2006),renal cell carcinoma (Nakajima *et al.*, 2001),non-small cell lung carcinoma (Shih *et al.*, 2006),cervical cancer (Govan *et al.*, 2006) and breast carcinoma (Mestiri *et al.*, 2001).

Increased serum levels of *TNF-α* have been described in patients with solid tumors, including oral carcinoma associated with adverse disease outcome (Su *et al.*, 2004). *TNF-α* expression and production *in vitro* were significantly higher in patients with GA and AA genotype therefore a G to A substitution at position −308 in the *TNF-α* promoter which is associated with increased *TNF-α* production (Kroeger *et al.*, 1997) has been subject of particular interest.

1.2 SIGNIFICANCE OF STUDY

Oral cancer is a tobacco-related disease which has an association with the poor survival rates and represents a significant problem based upon its high incidence in many parts of the world; severe functional and cosmetic defects accompany this malignancy and its treatment (Sato *et al.*, 1999). Deoxyribonucleic acid (DNA) mutations and increased production of reactive oxygen species (ROS) can be induced directly by the metabolic products from environmental exposure such as tobacco smoke, alcohol, and betel quid. These free radicals can lead to DNA damage and lipid peroxidation which could harm our body. The probability of mutations in critical oncogenes or tumor suppressor genes is increased by unrepaired damage in dividing cells (Gaudet *et al.*, 2003). Identification of inter-individual cancer susceptibility is an important factor in cancer prevention and early detection (Morita *et al.*, 1999). Some studies have also reported that specific polymorphisms in metabolism genes have a role in development of cancer in the oral cavity (Schwartz, 2000).

Many studies reported that *TNF-α* −308 G/A polymorphism was frequently linked with several cancers. It has also been significantly associated with the cancer stage and grade. While production of TNF-α is influenced by many factors (e.g. infection), genetic regulation also plays an important role (Azmy *et al.*, 2004).The –308 GA genotype of *TNF-α* gene had a statistically significant effect on TNF-α production and on the other hand, gene transcription was significantly increased. Moreover, the serum concentration of TNF-α was significantly higher in cancer patients than in the control subjects (Kakehi et al., 2010).

In Malaysia so far there is no literature on the *TNF-α* −308 G/A polymorphism and the risk of oral cancer. The only study done on relationship of *TNF-α* −308 G/A

polymorphism and oral cancer risk was the association between *TNF-α* polymorphism and the risk for OSCC in Taiwan (Liu *et al.*, 2005). This study aims to investigate the association between risk habits and TNF-α −308 G/A polymorphism with oral cancer risk in Malaysian Indian and Indigenous people.

This preliminary study should provide insight into the association between oral cancer susceptibility and genetic polymorphism of *TNF-α* −308 G/A in the two risk populations. With this study, it may be possible to advise those with risk habits to change, or to quit these habits if were able to prove a positive relation with these habits.

1.3 OBJECTIVES OF RESEARCH

This research is guided by the following objectives:

1 To determine the prevalence of *TNF-α* −308 G/A polymorphism among the Malaysian Indian and Indigenous oral cancer and non-oral cancer (normal) subjects.

2 To determine the association between *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian and Indigenous population.

3 To determine the association between different risk factors with *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian and Indigenous population.

1.4 HYPOTHESIS

The following hypotheses will be examined in this study:

i. There is an association between *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian population.

ii. There is an association between different risk factors with *TNF-α* −308 G/A genotype and oral cancer risk among the Malaysian Indian and Indigenous population.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 ORAL CANCER

2.1.1 Definition

Defining oral cancer presents some important challenges to both clinicians and researchers. Oral cancer or oral cavity canceris a subtype of head and neck cancer involving the tissue of the lips or the tongue, the floor of the mouth, cheek lining, gingival/alveolus (gum), and alveolus, palate and buccal mucosa (C00-C06). This cancerous tissue growth is located in the mouth (Blot, 1992; Zakrzewska, 1999). The term oral cancer has been used by various researchers in a different manner such as 'mouth cancer' or 'head and neck cancer' alternately with 'oral cancer'. Many attempts have been made in defining oral cancer and as yet there seems to be no uniformly accepted definition of oral cancer (Moore *et al.*, 2000).

In order to be able to compare surveys globally, the terminology for diseases should follow the International Classification of Diseases (ICD) coding system of the World Health Organization (WHO) as an international database for statistical-epidemiological study of diseases like cancer. In addition, these defined oral cancer sites were based on the WHO, tenth edition of ICD (ICD-10). The ICD provides a detailed coding system based on the first primary anatomic site of the tumor. The most common form of intra-oral malignancy is oral squamous cell carcinoma (OSCC) (Blot, 1992; Moore *et al.*, 2000; Zakrzewska, 1999).

2.1.2 Epidemiology of Oral Cancer

2.1.2.1 Global Epidemiology of Cancer

According to Parkin et al. in 2005, incidence, prevalence, mortality and survival are the primary measures of the burden of cancer worldwide which are not the same in different areas (Fig. 2.1). In regard to the study by Parkin et al. (2005), incidence is the number of new cases occurring, expressed as an absolute number of cases per year or as a rate per 100,000 persons per year. Prevalence describes the number of persons alive at a particular point in time with the disease of interest. Mortality is the number of deaths occurring, and the mortality rate is the number of deaths per 100,000 persons per year. The observed survival rate is the proportion of persons with cancer who survive for a specified period of time after diagnosis, usually 5 years (Parkin *et al.*, 2005). The Cancer Incidence in Malaysia for 2003-2005, published by the National Cancer Register (NCR) revealed that a total of 67,792 new cancer cases were diagnosed among Malaysians (Cancer Incidence in Peninsular Malaysia, 2008).

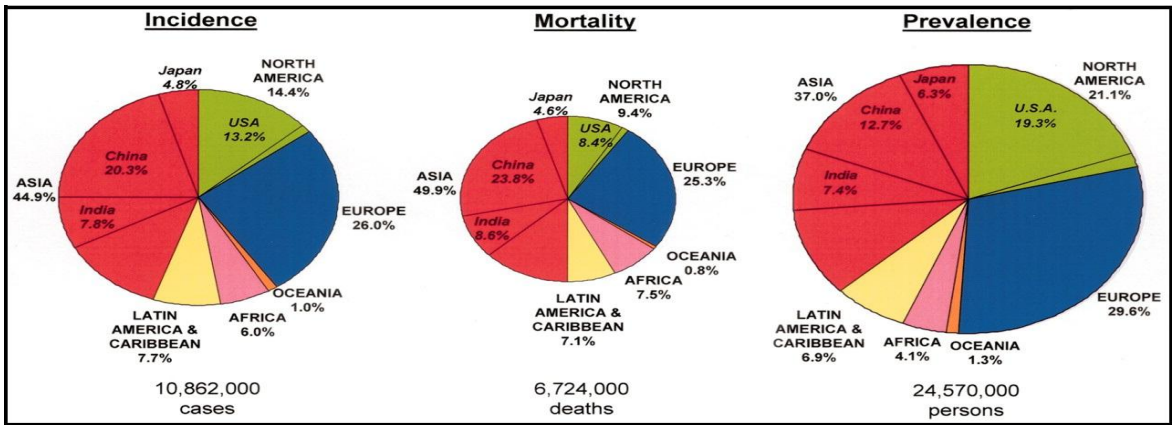

Figure 2.1: Incidence, Mortality, and Prevalence by Location (Parkin et al., 2005).

The ranking of cancers for males and females as number of new case (Incidence), together with corresponding number of deaths (Mortality) in the developed and developing countries of the world has been shown in Figure 2.2.

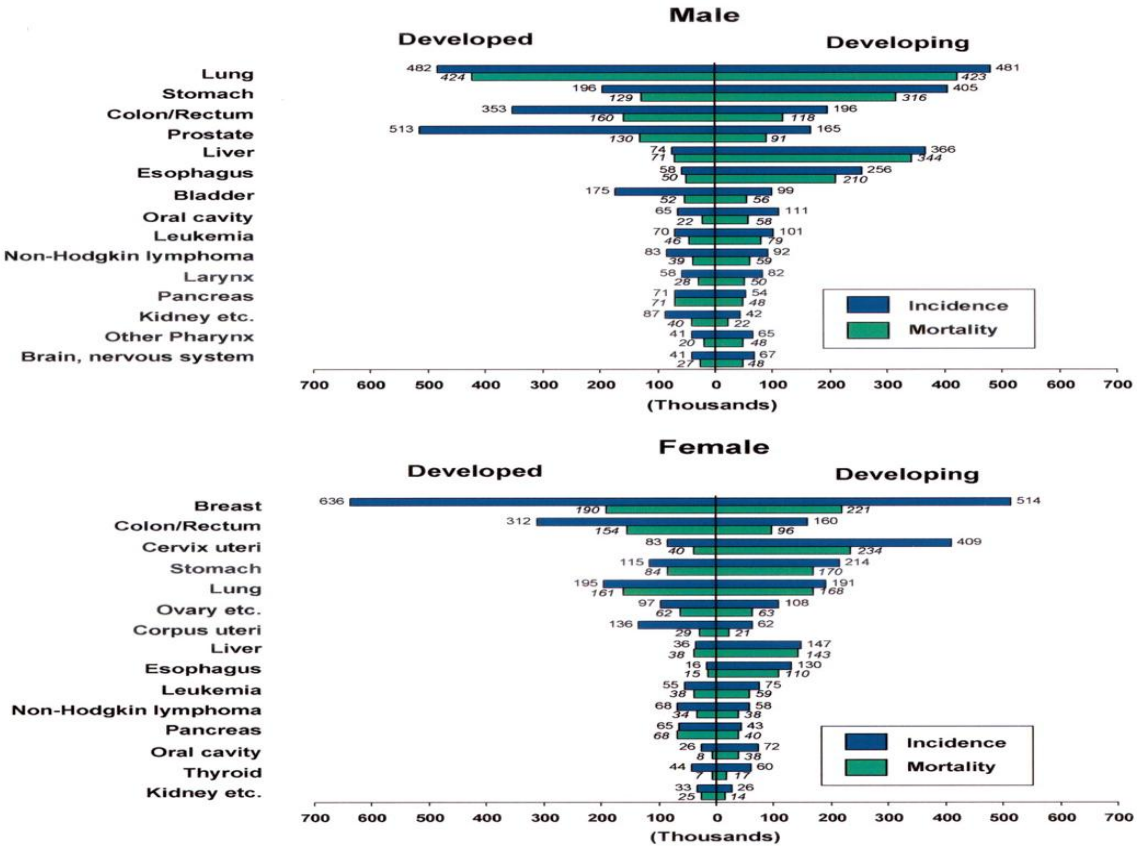

Figure 2.2: Estimated Number of New Case (Incidence) and Deaths (Mortality) in 2002. Data shown in thousands for developed and developing countries by cancer site and gender (Parkin et al., 2005).

2.1.2.2 Incidence of Oral Cancer in the World

Oral cancer was ranked 6th most prevalent malignancy throughout the world in 2007 (Fedele, 2009; Lingen *et al.*, 2008). An estimated 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) occurred in 2008 worldwide (Jemal *et al.*, 2011). The occurrence of oral cancer is especially high among men compared to women (Fig. 2.3). In some countries, outbreaks for oral cancer are different in men from 1 to 10 cases per 100,000 residents (Petersen *et al.*, 2005). Cancer of the oral cavity is more common in developing countries compared to developed countries (Fig. 2.3).In the developed countries such as the United Kingdom (UK) and United States of America (US), oral cancer accounted for 1-2% and 3% of the total cancer incidence respectively (Canto & Devesa, 2002; Stewart & Kleihues, 2003). The Age-Standardized Oral Cavity Cancer Incidence Rates are different between genders and various regions in the world (Fig 2.3). In south-central Asia, cancer of the oral cavity ranks among the three most common types of cancer. For instance, the age standardized rate (ASR) of oral cancer is 12.6 per 100,000 populations in India (Petersen *et al.*, 2005).

In Peninsular Malaysia, a total 21,464 cancer cases were diagnosed in 2003 according to the second report of the National Cancer Registry (NCR) data (Lim & Halimah, 2004). Oral cancer is the second leading cause of death due to cancers among Malaysian males in Malaysian Ministry of Health Hospitals. Malaysian Indian ethnic has the highest incidence rate which is internationally similar to that in the Indian subcontinent (CARIF, 2010).

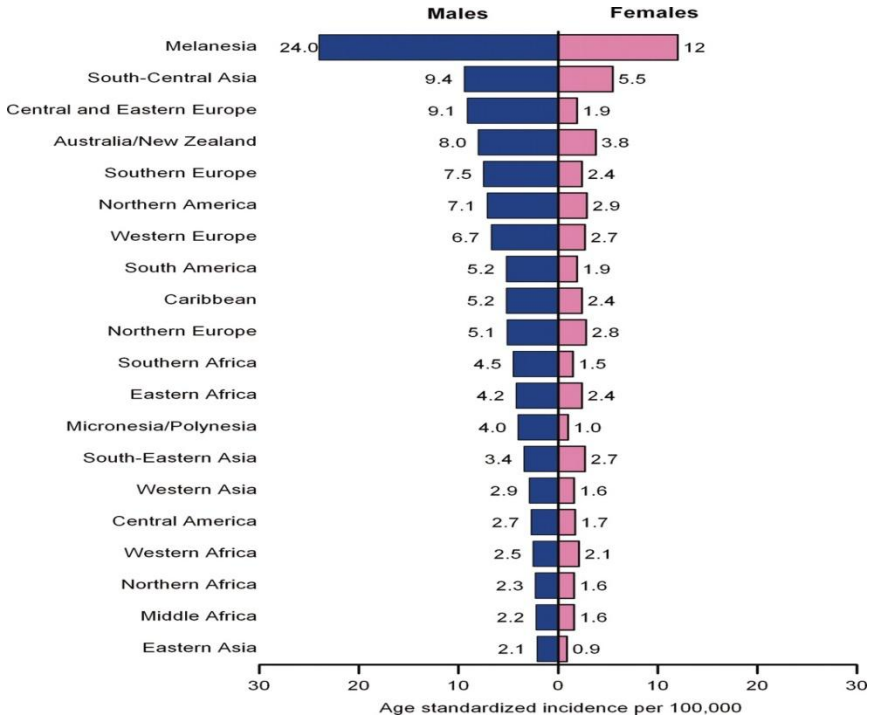

Figure 2.3: Age-Standardized Oral Cavity Cancer Incidence Rates by Gender and World Area (GLOBOCAN 2008).

The Malaysian NCR in 2003 documented oral cancer as the 6th and 3rdmost common type of malignancy among Indian males and females respectively in Peninsular Malaysia (Fig. 2.4 & 2.5) (Lim & Halimah, 2004).

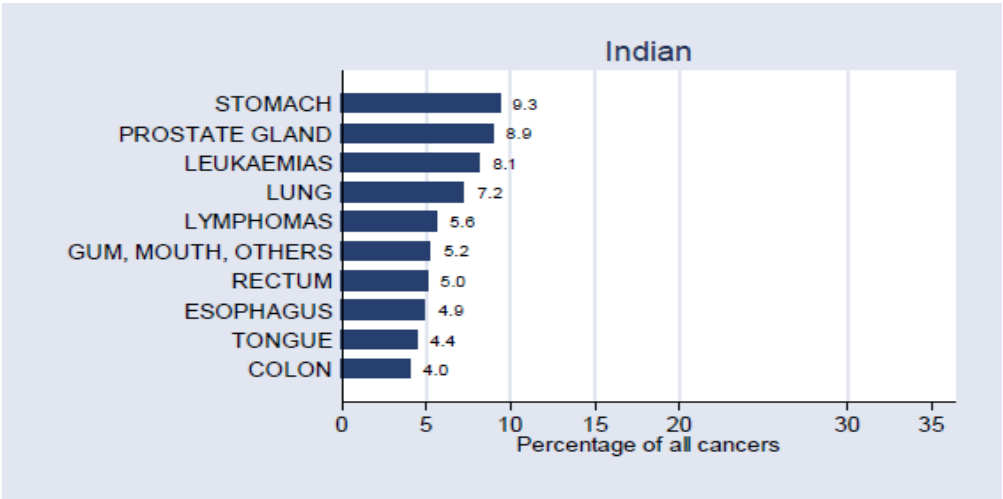

Figure 2.4: Ten most frequent cancers in Indian males (Peninsular Malaysia 2003).

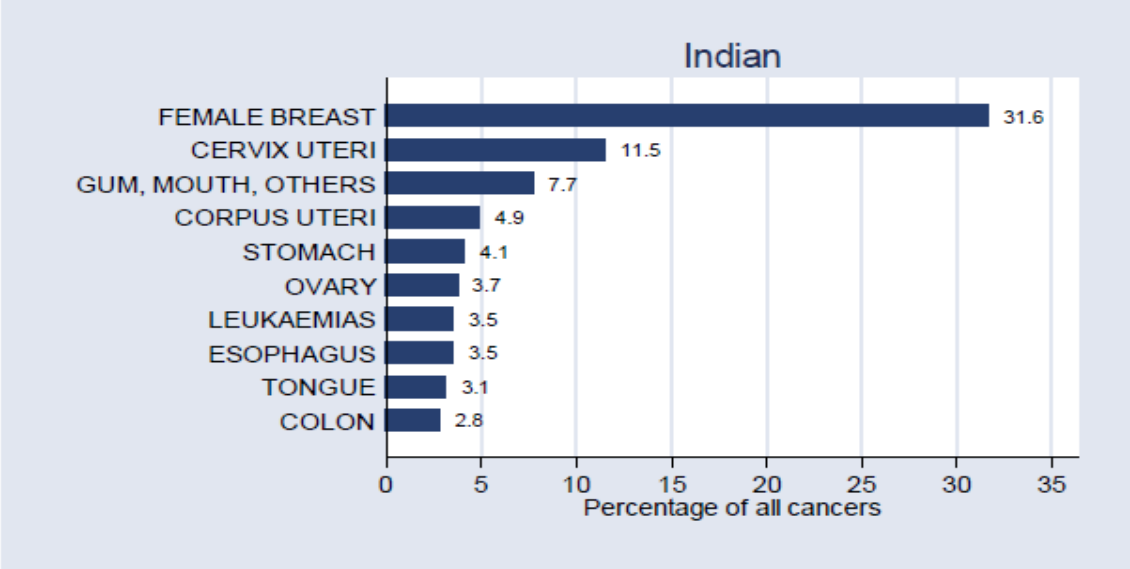

Figure 2.5: Ten most frequent cancers in Indian females (Peninsular Malaysia 2003)

2.1.2.3 Oral Cancer Mortality

The worldwide mortality of oral cavity and pharyngeal cancer was estimated to be 197,000 in 1990, in which about 100,000 deaths due to cancers of the "mouth". The mortality rate of oral cancer in 2002 increased to 127,259 cases with ASR of 2.9 and 1.5 per 100,000 for males and females respectively (Parkin *et al.*, 2005).

2.1.2.4 Gender Distribution of Oral Cancer

The highest worldwide incidence rate for oral cancer among women is reported in Bangalore, India unlike European countries (Blot *et al.*, 1996; Mathew *et al.*, 1997). Sex distribution changes considerably in different geographical regions in consequence of changing risk factors (Johnson, 2003a; La Vecchia *et al.*, 1997). In the western countries,

men are affected two or three times more often than women, largely because of their greater indulgence in alcohol and tobacco. However the occurrence of oral cancer for women can be larger than or equal to that for men in high incidence areas such as India, where chewing and sometimes smoking are also common among women, although this may differ greatly from region to region (Johnson, 2003b).

2.1.2.5 Age Distribution of Oral Cancer

Oral cancer predominantly is a disease seen in middle-aged and older persons (Neville & Day, 2002). Although the incidence of oral cancer at any age is comparatively low in western countries at 2–6% of all malignancies, on the Indian subcontinent the rate is as high as 30–40% (Llewellyn *et al.*, 2001). Cases occur prior to the age of thirty five are due to heavy abuse of different forms of tobacco (Jayant & Yeole, 1987; Johnson, 1991).

2.1.2.6 Ethnic Distribution of Oral Cancer

A significantly high number of deaths among oral cancer patients are recorded in men from the Indian subcontinent in the UK compared with the Indigenous UK population (Balarajan *et al.*, 1984). Similarly, the National Cancer Report in 2004 confirmed the overall incidence of oral cancer among Indians living in Malaysia is considerably higher than in the Malay or Chinese ethnic groups. In addition, oral cancer

appears to be most prevalent in areas with a high Asian population (Scully & Bedi, 2000; Warnakulasuriya & Johnson, 1996).

2.1.3 Sites of Oral Cancer

The prevalence and incidence of oral cancer are different among countries and depend on the site of oral cancer. Different oral cancer sites (ICD 10 C00-C06) may have had an association with different lifestyle risk habits which leading to different prognosis.

2.1.4 Molecular Epidemiology

Molecular epidemiology in molecular biology topics such as cancer susceptibility leads to increase in the chances of detecting high-risk lesions and individuals (Shetty, 2003). Molecular epidemiology has a consequential role in the growing awareness of the importance of relatively common genetic and acquired susceptibility factors in modulating risks from environmental carcinogens such as smoking of cigarette and so forth (Hong & Sporn, 1997).

2.1.5 Oral Carcinogenesis

Carcinogenesis or oncogenesis or tumorigenesis is literally the development or induction of cancer. Cancer is a multifactorial disease caused by accumulation of specific

genetic modification in the genes which code for proteins that regulate gene expression, cell division, cell differentiation and cell death (apoptosis) (Almadori et al., 2004). Oral carcinogenesis is a multistep process which leads to oncogene activation and tumor suppressor gene inactivation (Choi & Myers, 2008). It will continuously develop through a multistep process involving initiation, promotion and progression (Khan *et al.*, 2010). Stage of initiation plays an important role to cause permanent mutation which includes the DNA damage to the cells or tissue as a result of exposure to carcinogens, followed by stage of promotion which stimulates the initiated cell to divide. The development of these mutations is called progression (Oliveira *et al.*, 2007).

2.1.5.1 Oncogenes

Oncogenese are genes whose protein products have been found to be important for normal cell growth signaling and differentiation (Das & Nagpal, 2002). Oncogenes, tumor suppressor genes, and growth factors control the regulation of apoptosis and cell proliferation in oral squamous cell carcinoma. Accurate regulation of all this positive and negative signaling plays a substantial role in maintaining normal cell growth; disturbance in such a regulation can lead to neoplasia (Scully, 1992).

2.1.5.2 Tumor Suppressor Genes

Accumulation of activated genes has a basic importance, but these alone are not enough to cause oral cancer. Inactivation of negative regulatory tumor suppressor genes

is demanded. Mutations at only one of the gene copies are shown by oncogenes, while inactivation of tumor suppressor genes is done by point mutation, deletion and rearrangement in both gene copies. Because they are negative phenotype or no longer present within the cell, they are not easy to identify. We know only two genes for tumor suppressor activity in oral cancer that they are called p53 and doe-1. Deregulation of these affects cell cycle, chromosome stability, senescence, apoptosis and control of cell proliferation (Shetty, 2003).

2.2 RISK FACTORS

The three principal factors which influence most diseases are lifestyle, environmental factors and genetic susceptibility (Scully *et al.*, 2000). The causes of head and neck cancer are not completely understood. Epidemiological evidence shows there are many different factors which are widely denoted as risk factors and are not necessarily causal agents but are associated with an increased probability or risk of the occurrence of these cancers either individually or in combination (Döbróssy, 2005).

About 40% of these malignancies are known to be squamous cell carcinomas arising in the oral cavity. Oral cancer is greatly related to lifestyle, with major risk factors being tobacco smoking, smokeless tobacco products, alcohol consumption, genetic susceptibility and human papilloma virus (HPV) infections (Mignogna *et al.*, 2004; Walsh & Epstein, 2000). In addition, smoking and alcohol drinking have synergistic effects (Blot *et al.*, 1988; Hashibe *et al.*, 2009; Mignogna *et al.*, 2004).The oral cancer

risk is higher if a person is both a heavy smoker and a drinker compared with a heavy smoker, or a heavy drinker alone (Warnakulasuriya *et al.*, 2008).

The contribution of each of these risk factors is different among regions (Jemal *et al.*, 2011). Smoking and heavy alcohol drinking are estimated to have caused 42% and 16% of deaths from cancers of the oral cavity (including the pharynx) worldwide, while in high-income countries they are about 70% and 30%, respectively (Danaei *et al.*, 2005). Smokeless tobacco products and betel quid chewing with or without tobacco are also the major risk factors for oral cavity cancer in Taiwan, India, and other neighboring countries (Jemal *et al.*, 2011). The increase in the incidence rate of oral cancer in Taiwan may have been in part because of the heavy consumption of alcohol and betel quid (Ho et al., 2002).

A study done by Muttalib et al. (2002) in Malaysia showed that a total of 44.5% of 6,781 subjects declared to have one or more of the three "high-risk" habits (particularly tobacco smoking, alcohol drinking and betel quid chewing). A higher proportion of females chew betel quid while higher proportion of males smoked and used alcohol (Muttalib *et al.*, 2002).

The study by Lissowska *et al*. (2003) in Warsaw, Poland, examined smoking, drinking, diet, dental care and sexual habits as risk factors of oral cancer and pharyngeal cancer among 122 patients and 124 controls. The researchers found that some factors such as smoking and drinking cessation and increase of fresh fruit intake are possible as effective preventive measures against oral cancer. These findings show also that poor oral hygiene may be an independent risk factor. According to the assignable risk, it was defined that 57% of oral cancer cases were smokers and 31% of them were alcohol consumers in Poland (Lissowska *et al.*, 2003).

2.2.1 Tobacco

Many diseases such as oral cancer are associated with tobacco smoking (Wald & Hackshaw, 1996). It is also connected to a harmful effect on oral health, such as increasing risk of periodontal (gum) diseases (Sham *et al.*, 2003). Epidemiological studies have shown that tobacco use is a significant risk factor for the development of periodontal diseases; with the frequency of smoking, disease severity rises (Amarasena *et al.*, 2002; Bergström, 1989; Haber & Kent, 1992).

Tobacco consumption can take many forms and over 90% of patients with oral cancer use tobacco in one form or another (Warnakulasuriya *et al.*, 2005). Tobacco and alcohol can damage cells in the lining of the oral cavity and oropharynx. The cells in this layer must grow more rapidly to repair this damage. According to the American Cancer Society, investigators say that the DNA-damaging chemicals in tobacco are linked to increase in the risk of oral cancer (American Cancer Society, 2010).

Tobacco smoking is the strongest risk factor for oral cancer (Geisler & Olshan, 2001; Nair & Bartsch, 2001). Tobacco smoke contains a great number of chemical carcinogens (Boffetta, 2003). There are more than 300 carcinogens in tobacco smoke or in its water-soluble components which may leach into saliva (Johnson, 2001). Smoking of cigarette or *bidi* (a thin, cheap cigarette in India made from cut tobacco rolled in leaf) is related to increased oral cancer risk among the Indians (Rahman et al., 2003, 2005). Smokers are at dramatically increased risk for oral carcinoma, particularly squamous cell cancer (Baron & Rohan, 1996). According to a meta-analysis, on average, a three-fold increase in oral cancer risk was observed among current smokers (Gandini et al., 2008).Both intensity and duration of smoking are associated with the risk of oral cancer

while quitting smoking leads to a fall in risk (Blot et al., 1988; Castellsagué et al., 2004; Rodriguez et al., 2004).

After 10 smoke-free years, the extra risk of oral cancer from smoking almost disappears (Kuper et al., 2002). However, a recent study demonstrated that it needs 20 years or more for the risk to diminish to that of never smokers (Bosetti et al., 2008).

2.2.2 Alcohol

Alcohol use has been strongly shown as an independent risk factor in the development of oral cancer (Geisler & Olshan, 2001; Nair & Bartsch, 2001). Chronic alcohol use plays an important role in the development of cancer of the esophagus and oral cavity (Seitz *et al.*, 2004; Zakhari, 2006). All forms of alcoholic drink are dangerous if heavily used. In fact, alcohol may lead to immune suppression and nutritional deficiencies which could raise the susceptibility to carcinogens (Das & Nagpal, 2002). Beer and stout are the commonly consumed alcoholic beverages locally in Malaysia. Toddy and samsu are consumed as special home brands by the Indians and domestically manufactured rice alcohols are consumed by the Indigenous people of Sarawak (Zain, 1999).

Nowadays, the consumption of ethanol in alcoholic beverages is recognized as a carcinogenic risk by the International Agency for Research on Cancer, an extension of the World Health Organization. Alcohol abuse is linked with the mouth, pharynx, larynx and esophagus cancers (Baan *et al.*, 2007; World Cancer Research Fund and American Institute for Cancer Research, 2007; Zygogianni *et al.*, 2011). The role of ethanol in

alcoholic beverages is the same as nicotine in tobacco, when it comes to causing cancer (Ogden, 2005), although the reason for this association is not completely understood. Alcohol may affect these tissue directly (Lachenmeier, 2008); however investigators have also considered that some factors may be involved such as alcohol metabolite, acetaldehyde, and alcohol's ability to enhance mucosal penetration of other carcinogenic chemicals (International Agency for Research on Cancer, 2007).

Alcohol abuse, specifically when associated with tobacco smoking has been identified as an important risk factor for mouth cancer for almost 50 years (Ogden, 2005). Approximately 75% of upper aero-digestive tract cancers such as all oral cancers appeared in association with alcohol and tobacco consumption (La Vecchia *et al.*, 2004; Llewellyn *et al.*, 2003). However, alcohol intake still stays high in many countries. The increasing incidence of oral cancer has shown the importance of the role of alcohol alone and in partnership with other etiologic agents (Ogden, 2005).

Alcohol-containing mouthwashes can be one of the etiologic agents in the oral cancer risk family. This was proven by recent studies in Australia, Brazil and Germany. Smoking and drinking were strongly associated with an increased risk of oral cancer (Warnakulasuriya *et al.*, 2008).

2.2.3 Betel-quid

Quid is explained as substance, or mixture of substances put in the mouth or chewed and remaining in contact with the mucosa. It usually contains one or both of the two basic ingredients such as tobacco or areca nut which can be in raw, manufactured or

processed form (Zain, 1999). After caffeine, nicotine and alcohol, areca nut is the fourth psychoactive substance in the world (Gupta & Ray, 2004).

According to the WHO report in 2008, chewing tobacco quid causes cancer of the oral cavity. Chewing tobacco can be alone or with lime, betel leaf, betel nut and other compounds as a combination called paan. Corrosion of the oral mucosa, leukoplakia or submucus fibrosis, and eventually cancer are caused by both paan and paan masala (a mixture of nuts, seeds, herbs, and spices served after meals in India, Middle East and parts of Southeast Asia) especially when they contain tobacco. In Asia, betel chewing culture is a robust risk factor for developing oral cancer (Van Lerberghe & ebrary, 2008). The association has been firm beyond many countries such as in India, Philippines, Malaysia, Bangladesh, Cambodia and Thailand (Saub, 2001).

Betel quid use is highest among some of the indigenous groups in Malaysia, where the quid is mixed with tobacco. In mainstream/urban Malaysian society, tobacco and betel quid are used together by the Malaysian Indians, but it is not popular among Malays (Gupta & Ray, 2004).

In Malaysia, the single habit of chewing betel quid was most common among the Indian females (Ghani *et al.*, 2011). A study done by Muttalib et al. (2002) showed that more than 22% of the population still practiced betel quid chewing although it was more restricted to particular populations comprising Indians who working in distant plantations, the indigenous of Sabah and Sarawak and some early Malay peoples who live in rural villages (Zain & Ghazali, 2001). Most Chinese do not indulge in betel quid chewing habit (Muttalib *et al.*, 2002).

Betel quid chewing produces reactive oxygen species (ROS) that have multiple harmful effects on the oral mucosa. The ROS can play an important role directly in the tumor initiation process, by including genotoxicity and gene mutation or by attacking the

salivary proteins and oral mucosa. Finally, this will lead to structural alterations in the mucosa that may facilitate penetration by other betel quid components and environmental toxicants (Jeng *et al.*, 2001; Walker *et al.*, 2003).

2.2.4 Genetic Susceptibility

Despite the risk of tobacco smoking, alcohol drinking and quid chewing, the majority of patients who consume these substances do not get cancer. Factors that affect malignancy development in people who have been exposed to tobacco may involve a combination of exposure and genetic susceptibility which may regulate the human genes in metabolizing the risk factors mentioned above (Sreelekha *et al.*, 2001).

The role of genetics can be categorized into two groups in cancer etiology: the single (rare) genes and the more common susceptibility genes. The single (rare) genes are the normal genes which under endogenous and exogenous factors and may transform into cancer genes; the more common susceptible genes are genes which are susceptible to transformation.

High penetrance disease genes are uncommon (i.e., have a low allele frequency, typically less, or much less than 1%). If these genes are present, they cause a high risk of a particular cancer (Sinha & Caporaso, 1999).These include genes that affect DNA repair, chromosome stability, the activity of oncogene or tumor suppressor genes, cell cycle control or signal transduction, hormonal or vitamin metabolism pathways, immune function and receptor or neurotransmitter action (Caporaso, 1999).

The aim of genetic susceptibility study is to identify inherited susceptibility factors. Corroborative evidence shows that genetic factors are involved in the development of most cancer cases, involving those without a clear familial aggregation. Mutation or deletion of single gene lead to most hereditary cancer syndromes, and the inheritance patterns for some of these syndromes are often in accordance with Mendel's transmission models with family. Only a small fraction of cancer cases in humans are interpreted by hereditary cancer syndromes, because germ-line mutations of major cancer gene are scarce in the general population. On the other hand, polymorphic genes, although each conveys a proportionate small risk, may lead to the incidence of many cancer cases, given their high prevalence in the general population. The risk of cancer can be increased by interaction of these genes with environmental agents (Nasca, 2001).

Genetic predisposition may also be an important factor in the development of oral squamous cell carcinoma (NEMES, 2006). It is believed that the susceptibility of inability or ability to metabolize carcinogens or procarcinogens is inherited by definite individuals (Kumar & Zain, 2004; Scully *et al.*, 2000).

Definition of the nature of these genetic factors would have huge benefit, not only to at-risk family members, who would thus take particular care to avoid other risks, but in understanding of molecular mechanisms of oral carcinogenesis, opening the way to better prevention and treatment (Johnson, 2003a).

2.2.5 Viral, Candida Infection, Diet and Other Risk Factors

Oral or head and neck squamous cell carcinoma development is also affected by Candida infection, immune suppression, the use of mouthwash, syphilis, dental factors,

poor oral hygiene, oral sex practice and occupational risks (Johnson, 2003a; Talamini *et al.*, 2000).

The role of viruses such as Human Papilloma Virus (HPV) and Human herpes Virus (mainly Epstein-Barr Virus) and Herpes Simplex Virus (HSV) have been implicated in oral cancer (Scully, 2005). Also, *Candida albicans*has been implicated in the pathogenesis of oral premalignant lesions (Kumar & Zain, 2004).

Dietary factors and nutrition seem to be important in preventing oral precancer and cancer as has been shown in a number of recent studies. Antioxidants which are contained in fruits and vegetables seem to have a preventive effect (Reichart, 2001). According to a study by Talamini *et al*. (2000) occasional (Odds Ratio (OR) = 0.7) or frequent (OR = 1.3) practice of oral sex, and homosexual intercourse (OR = 1.0, men only) did not seem to affect oral cancer risk (Talamini *et al.*, 2000). Several studies had shown that poor oral hygiene has been assumed as a risk factor for oral cancer (Franco *et al.*, 1989; Schildt *et al.*, 1998).

2.3 TUMOR NECROSIS FACTOR-ALPHA (*TNF-α*)

One of the most important groups of proteins is cytokines which regulate and mediate inflammation and angiogenesis. Cytokines include interleukins (ILs), tumor necrosis factors (TNFs) and certain growth factors (GFs) (Serefoglou *et al.*, 2008).

TNF-α and-β are key molecules in immune responses to infection, and both play important roles in the pathogenesis and clinical manifestations of parasitic diseases. There are many reports about several polymorphic variants with the potential to affect

cytokine levels in patients with autoimmune diseases and parasitic and bacterial infection (Kaelan *et al.*, 2002).

Tumor necrosis factor (TNF, cachexin or cachectin and formally known as tumor necrosis factor alpha) is a member of a group of cytokines that arouse severe phase reaction in systemic inflammation (Deng *et al.*, 2008). The primary role of TNF is to regulate immune cells.Also apoptotic cell death and inflammation are induced, and tumorigenesis and viral replication are inhibited by TNF (Raychaudhuri & Raychaudhuri, 2009). Dysregulation and, in particular, overproduction of TNF can be effective in increase of susceptibility to a variety of human diseases, as well as cancer (Ruuls & Sedgwick, 1999; van den Berk *et al.*, 2010).

TNF-α was initially described as a cause of tumor necrosis and was associated with cachexia-inducing states such as cancer and infection. Phagocytic cells normally produce and secrete this cytokine, where it has anti-tumor and pro-inflammatory functions. There are significant amount of *TNF-α* in other tissues, especially in adipocytes, and small amounts are shown in skeletal and cardiac muscle of both mice and humans (Ni *et al.*, 2009).

2.4 *TNF-α* −308 G/A POLYMORPHISM AND CANCER RISK

There are many polymorphisms in the *TNF-α* gene such as −376, −308, −238 and −163 which are located at positions in the promoter region of the *TNF- α* gene. These four polymorphisms are found to be G to A transition polymorphisms (Xia *et al.*, 1998).

The G−308A TNF-α polymorphism is a transition mutation in which guanine (G) is changed to adenine (A) in the −308 location at the *TNF-α* promoter region. Previous studies show that a general increase in transcriptional activity of TNF-α may affect the level of TNF-α through the G−308A *TNF-α* polymorphism (Brand *et al.*, 2001).

Many studies have been done on the *TNF-α* −308 G/A polymorphism and the risk of cancer such as lung, cervical, breast and prostate cancer. Some of these studies demonstrated that *TNF-α* −308 G/A polymorphism could play an important role as genetic factor in cancer development.

A significant association between the −308 G/A polymorphism in the promoter region of *TNF-α* and the susceptibility to lung cancer was shown in China (Shih *et al.*, 2006). On the contrary, a study has indicated that there was no association between *TNF-α* −308 polymorphism and risk of lung cancer among non-Hispanic Caucasians (Engels *et al.*, 2007).

Also the association between the G−308A *TNF-α* promoter polymorphism and the risk for invasive cervical cancer (ICC) was studied by Duarte *et al.* (2005). In summary, they showed that the presence of the high producer allele −308A in the *TNF-α* gene seems to be associated with an increased risk for the ICC development (Duarte *et al.*, 2005). According to Fang *et al.* (2010), the *TNF-α* −308 G allele is a risk factor for developing breast cancer, especially for Caucasians (Fang *et al.*, 2010). Another study demonstrated that the polymorphism in −308 region of *TNF-α* is associated with prostate cancer (OH *et al.*, 2000).

2.5 *TNF-α* −308 G/A POLYMORPHISM AND ORAL CANCER RISK

Extensive research has shown that functional polymorphisms affecting gene expression of *TNF-α* are strongly associated with increased risk of oral cancer (Serefoglou *et al.*, 2008).

A study showed a strong association of *TNF-α* high expression alleles with an increased risk of oral cancer among German and Greek population (Yapijakis *et al.*, 2009). Serefoglou *et al.*,(2008) has also indicated functional polymorphisms affecting gene expression of interleukins IL-4, -6, -8, and -10 as well as TNF-α are strongly associated with an increased risk for OSCC in Greece (Serefoglou *et al.*, 2008).

Another study evaluated the association of *TNF-α* promoter polymorphism and subsequent risk for OSCC among 192 patients and 146 healthy case controls for the first time in Taiwan. The findings showed that the -308 TNF G (tumor necrosis factor G allele) was higher in patients with OSCC compared to the controls (91.2% vs. 82.2%; $p = 0.02$) (Liu *et al.*, 2005). Another study among Asian Indians was done by Gupta *et al.*, 2008 suggesting that *TNF-α* −308 G/A may be related to susceptibility of OSCC. These SNPs may be useful as a marker for high-risk groups among Asian Indians. On the contrary, only the study done by Chiu *et al.* 2001 which observed no association between *TNF-α* −308 G/A polymorphism and risk of oral cancer (Chiu *et al.*, 2001) in Taiwan.

CHAPTER 3

METHODOLOGY

3.1 STUDY DESIGN

This research is based on case-control study on Indian and Indigenous groups in Malaysia. Samples were recruited during the period of 2005 to 2009.

3.2 STUDY SUBJECTS

A total of 143 oral cancer patients (98 Indian & 45 Indigenous) who were diagnosed, histologically confirmed and untreated and 79 controls (57 Indian & 22 Indigenous) were taken among normal volunteers who neither themselves nor their family have any history of cancer formed the sample of the study.

They were recruited by the oral cancer research and coordinating centre (OCRCC). The OCRCC is an oral cancer data bank which develops and maintains the system of data and sample (tissue, blood, DNA, etc.) collection, processing and storage through the Malaysian Oral Cancer Database & Tissue Bank System (MOCDTBS).

This data bank comprises information of related parameters that are extracted from patients referred to nine chosen centers such as: the Dental Faculty University of Malaya (UM), Universiti Sains Malaysia (USM), Universiti Kebangsaan Malaysia (UKM), and the Ministry of Health Malaysia specialist clinics at the General Hospitals of Kuala

Lumpur, Selangor, Perak, Kelantan, Sabah and Sarawak. These OCRCC data were stored in a standardized manner referring to socio demographic data; risk habits (smoking, alcohol drinking and betel-quid chewing), diagnosis, clinical staging, histological grading and follow-up information for future checking against estimation of disease outcome and behavior.

The OCRCC arose from a partnership between two universities, the University of Malaya (UM) and the Universiti Sains Malaysia (USM); the Cancer Research Initiatives Foundation (CARIF); and the Ministry of Health Malaysia. The OCRCC is the major coordinating partner developing the MOCDTBS. The OCRCC coordinates the collection of data and samples from many hospital-based centers that see patients with oral cancer and precancerous lesions based across the country. The database also contains control samples obtained from volunteers.

All relevant clinical and socio demographic data on diagnosis, location, risk habits, age, gender and ethnic group for this study was obtained from the MOCDTBS.

All patients fulfilled both the inclusion and exclusion criteria have formed the sampling frame for the study.

Inclusion Criteria

Cases are included:

- Patients who were newly diagnosed pathologically as oral squamous cell carcinoma (OSCC) at the nine selected centers.
- Patients who have not been treated previously by radiotherapy or chemotherapy.
- Patients who have either genomic DNA in the nuclei acid bank (at OCRCC-UM and CARIF-Cancer Research Initiatives Foundation) or blood samples.

Controls are included:

- Persons who do not have oral cancer, potentially malignant lesions or other cancers.
- Persons who neither themselves nor their family have any history of cancer
- Persons who have either genomic DNA in the nuclei acid bank (OCRCC-UM and CARIF) or blood samples.

Exclusion Criteria

Cases are excluded:

- Patients who were non-Malaysian citizen.
- Patients who already had cancer and are currently treated (patients with recurrence of cancer).

Controls are excluded:

- Persons who had some kinds of diseases which have an association with desired risk factors in the study (smoking, alcohol drinking, betel-quid chewing).
- Persons who either themselves or their family have history of cancer.

We also did not use any sampling methods in this study because it was expected that all patients were not available in the sampling frame. Informed consent was obtained from the participants for the umbrella project Oral Cancer and Precancer in Malaysia – Risk Factors, Prognostic Markers, Genetic Expression & Impact on Quality of Life, IRPA RMK 8 Project No: 06-02-03-0174 PR 0054/05-05 where this current project is a part of the umbrella project. The ethical approval for the umbrella project was also obtained with the medical ethics code no. DF OP0306/0018 (L) and endorsed by Ministry of Health Malaysia.

The summary of the study is presented in below flowchart (Figure 3.1).

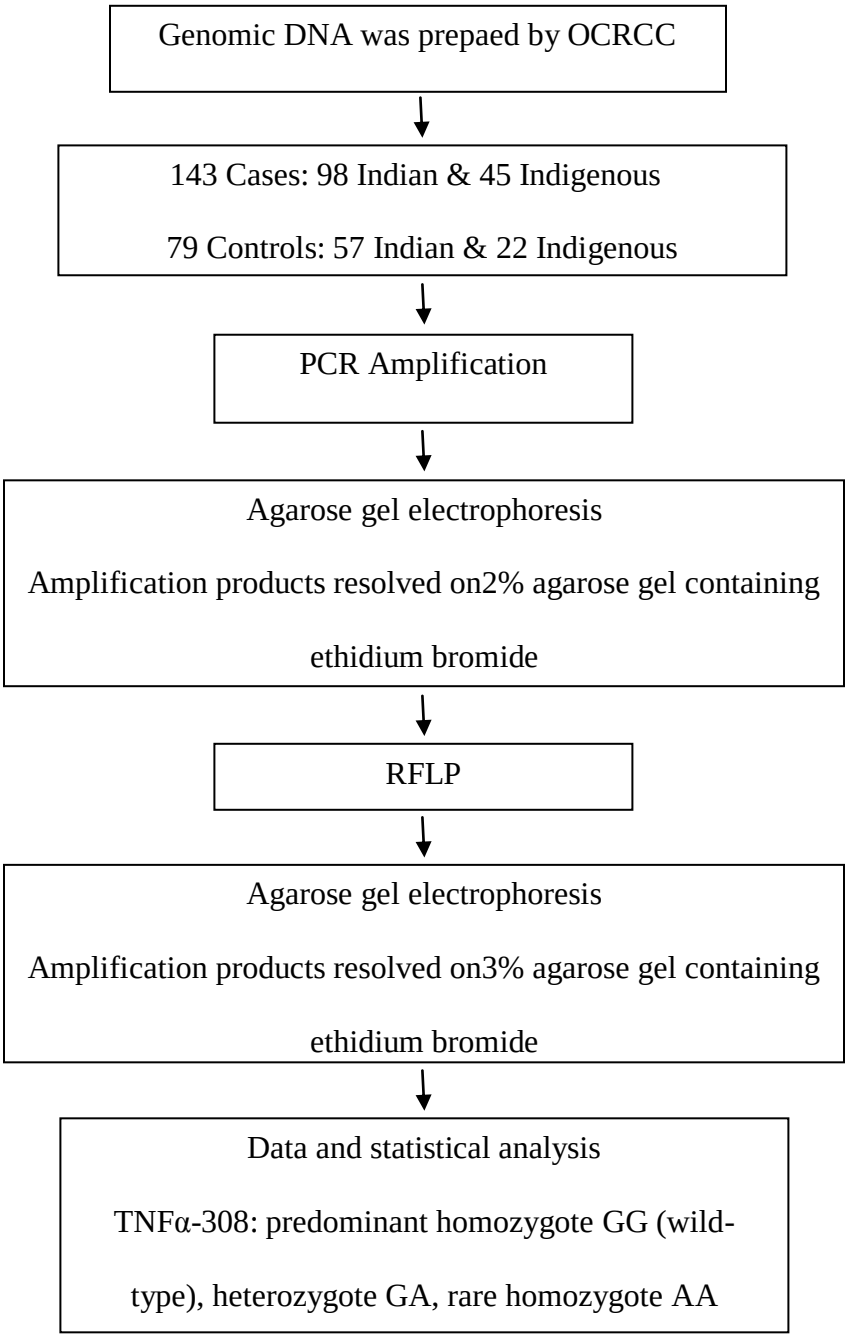

Figure 3.1: Flowchart of the methodology of the study.

3.3 SAMPLE SIZE ESTIMATION

We used the Power and Sample Size Program version 3.0.17 to determine the number of sample. We planned a study of independent cases and controls with 1 control(s) per case (M=1) according to prior data which indicated that the probability of exposure among controls (P0) was 0.183 and also the true odds ratio for disease in exposed subjects relative to unexposed subjects (OR) was 2.6 (Chiu *et al.*, 2001). In regard to this input variable, it was calculated that we had to study 90 case patients and 90 control subjects to be able to reject the null hypothesis and this odds ratio equals 1 with probability (power) 80%. The Type I error probability associated with this test of this null hypothesis (α) was 5% that it meant the Confidence Interval (CI) was 95%. In order to ensure that the power of study is at least 80% in the event that the number of control obtained is less than 90, it was decided that the number of cases be increased to 143 patients. The sample size calculation is attached in Appendix B. Some of the limitations also taken into considerations are restricted time to manage the study and higher cost incurred in obtaining DNA extraction and PCR kit, reagents, restriction enzyme (RE) and the agarose gel.

3.4 GENOMIC DNA

The entire genomic DNA used in this research was obtained from the nuclei acid bank (at OCRCC-UM and CARIF-Cancer Research Initiatives Foundation).

The Nanophotometer was used to record both the A260/280 ratio and the quantity of DNA (ng/µl) for each of the genomic DNA obtained.

3.5 GENOTYPING METHODS

3.5.1 Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

PCR-RFLP was used to detect the *TNF-α* −308 G/A SNP. We determined the GG, GA and AA genotype of TNF-α −308 by digestion of the PCR product with restriction enzymes followed by agarose gel electrophoresis. This method was proven to be reliable as shown in studies (Asghar *et al.*, 2004; Gupta *et al.*, 2008; Guzeldemir *et al.*, 2008; Liu *et al.*, 2005).

3.5.1.1 Polymerase Chain Reaction (PCR)

PCR was prepared in a 30 µl reaction volume and the amount of reagents required for each assay was shown in Table 3.1.

Table 3.1: PCR reaction mixture for genotyping *TNF-α* Codon −308 (Asghar *et al.*, 2004).

Reagent	Working Concentration	Volume (µl)
DNA template	-	0.1 µl < DNA
5× Green GoTaq Flexi Buffer	1×	6 µl
25 mM $MgCl_2$ (pH 9.0)	1.5 mM	1.8 µl
10 mM dNTPs	200 µM	0.6 µl
GoTaq DNA Polymerase (5U/µl)	1.5 U	0.3 µl
25 µM forward primer	0.5 µM	0.6 µl
25 µM reverse primer	0.5 µM	0.6 µl
dH_2O	-	Make up to 30 µl
Total		30 µl

The primer sequences were shown in Table 3.2.

Table 3.2: Primer sequences for *TNF-α* Codon −308 (Asghar *et al.*, 2004).

Polymorphic Variants	Primer sequences (5'→3')	Size	Tm
rs1800629 (G/A)	Fw: GAGGCAATAGGTTTTGAGGGCCAT Rv: GGGACACACAAGCATCAAG	24bp 19bp	70 ºC 62 ºC

Each reaction included 1xGreen GoTaq Flexi buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 1.5 U of GoTaq DNA Polymerase (5U/ µl promega, USA), 0.5µM of each of the primers and and 50-100 ng of genomic DNA. A negative control (dH_2O) was included for

each run. 5x Buffer, $MgCl_2$, dNTP and Taq Polymerase were purchased from Promega, USA.

The PCR mixture was gently mixed and spun briefly. The tubes were placed into the Gene Amp PCR system 9902 (Perkin-Elmer, Applied Biosystems) and PCR was carried out according to the cycling parameters listed in Table 3.3.

Table 3.3: PCR Conditions.

Program	Temperature	Duration	Cycle
Initial Denaturation	94ºC	5 min	1
Denaturation	94ºC	30 sec	35
Annealing	60ºC	30 sec	35
Elongation	72 ºC	30 sec	35
Final Elongation	72 ºC	7 min	1
Hold/Soak	4 ºC	∞	

3.5.1.2 Gel Electrophoresis of PCR Product

Finally, amplicons were confirmed through the expected size of the amplified fragments (147bp) by agarose gel electrophoresis (2% w/v) and visualized with ethidium bromide.

A 2% (w/v) agarose gel was prepared for electrophoresis. A DNA ladder 100bp (Promega) was used as the molecular weight marker. An aliquot 10 µl of the PCR

products was loaded into the wells. Gel electrophoresis was run at a voltage of 110 for 30 minutes. Many PCR and PCR-RFLP studies used this trustworthy method (Guptaa *et al.*, 2008; Liu *et al.*, 2005; Yapijakis *et al.*, 2009). Solution and reagents used for agarose gel electrophoresisare were shown in Appendix C.

The gel was stained with ethidium bromide (0.5 mg/ml), destained in dH_2O and then visualized using a UV transilluminator. The photo of the gel was taken using gel documentation machine, Alpha Imager (Alpha Innotech; CA, USA). Agarose, LE, Analytical Grade and 100bp DNA Ladder were purchased from Promega, USA.

3.5.1.3 DNA Sequencing

We need to double-check the interpretation of the primary data by sending some samples for sequencing. Direct sequencing of the PCR products for 40 samples was done by using the reverse primer to validate *TNF-α* −308 G/A genotypes determined previously by examining their RFLP patterns. The PCR product was purified to reduce the noise level which may interfere with interpretation of the sequence result. The PCR products were purified using Qiagene PCR product DNA purification kit according to the manufacturer's protocol (Appendix D).

After purification, agarose gel electrophoresis was done to check the purified PCR product (147bp). The concentration of purified DNA was measured by UV Spectrophotometry. The purified PCR product was sequenced by a commercial sequencing facility (First base Sequencing) to verify the amplicons.

3.5.1.4 Restriction Fragment Length Polymorphism (RFLP) Analysis

In PCR-RFLP, amplified products are digested with restriction enzyme. Restriction enzyme that cleaves the DNA specifically into different alleles is used to digest the DNA of interest, and then the alleles of each sample can be observed as a specific band pattern on the gel.

The confirmed PCR product containing *TNF-α* gene with expected band size (147bp) on agarose gel, was digested with *NcoI* restriction enzyme (New England Biolabs, USA) to determine the genotype of *TNF-α* −308 G/A.In this study, the PCR product was digested with 2 units of *NcoI* (New England Biolabs, USA).

The preparation of the RE Mix was done as in Table 3.4. Digestion was carried out at 37ºC for 4 hours and the enzyme was inactivated by heat at 60ºC for 20 minutes.

Table 3.4: Restriction enzyme reaction master mix.

Reagent	Volume (µl)
10x Buffer	2 µl
NcoI (2 units)	0.2 µl
dH_2O	7.8 µl
PCR product	10 µl
Total	20 µl

3.5.1.5 Gel Electrophoresis of digested PCR Product

Finally, three genotypes of TNF-α −308 G/A would be obtained from PCR-RFLP procedures as an output. After digestion, the restriction fragments were separated by agarose gel electrophoresis (4% w/v) and visualized with ethidium bromide (EB) to assign individuals' genotypes such as GG (predominant homozygote or wild type), GA (heterozygote) and AA (rare homozygote or polymorphism). A DNA ladder 100bp (Promega) was used as the molecular weight marker. An aliquot 5 µl of the RFLP products was loaded into the wells. Gel electrophoresis was run at a voltage of 110 for 45 minutes.

3.6 STATISTICAL ANALYSIS

3.6.1 Variables

We used the dependent (outcome) and independent (desired risk factors) variables in this study which respectively were having oral cancer and grouped into socio demographic factors, genomic polymorphism and mutation (Fig. 3.2). All of them had been obtained from the MOCDTBS.

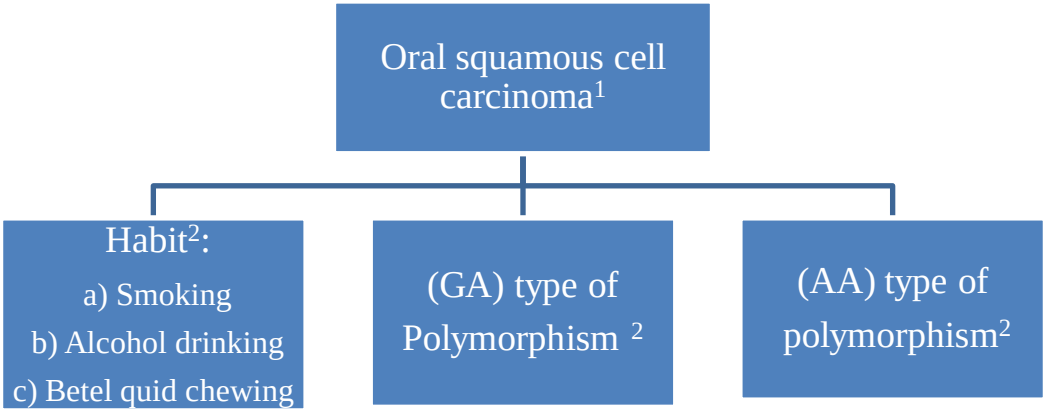

Figure 3.2: Independent and dependent variables of this study.

([1] as dependent variable & [2] as independent variables)

Entering information earned from the questionnaires and data analysis was done using the Statistical Package for the Social Sciences (SPSS) version 15.0 which is among the most widely used programs for statistical analysis in Social Science. Survey analysis was performed after the data had been checked and cleaned. The distribution and frequencies were surveyed. Classifications with small sample size and slant distribution were recorded while the differences in the frequency of various alleles and genotypes between cases and controls were evaluated by chi-square test.

The frequency and distribution of all dependent and non-dependent risk factors were calculated by descriptive statistics. Frequency and percentages were calculated for categorical variables such as gender, age, ethnic and risk factors. Independent *t*-test was used for comparison of typical distinction in sequential variables between cases and controls while chi-square test was used for characterization of the differences in continuous variables between cases and controls. Statistical significance was defined as a *p*-value of <.05 (Ghani *et al.*, 2011).

Simple logistic regression was used to achieve the objectives of this study to determine the association between *TNF-α* −308 G/A polymorphisms and oral cancer risk. Odds ratio (OR) was used to calculate the strength of association between the risk factor of interest and disease outcome. Crude OR of the association was obtained by simple logistic regression. *TNF-α* −308 G/A polymorphism genotype status (GA & AA) was compared against the wild-type (GA) which was used as the reference group (Goldin, 2007).

Data analysis was done in 4 steps to achieve 3 objectives which had been designed at first. First of all, the variables were screened and chosen with simple logistic regression analysis (Morise *et al.*, 2002). Simple logistic regression analysis would produce raw OR of the association. For analysis of genotype, the *TNF-α* polymorphism genotypes were compared against the expected genotype which has the highest activity (wild type) as in Table 3.5. All the wild type genotype was used as the reference group.

Table 3.5: *TNF-α* −308 G/A genotypes.

TNF-α genotypes	Expression
TNF-α −308 Wild type	Consist of GG genotype with highest activity
polymorphism	Consist of GA or AA genotypes with low activity

In addition, the genotype frequencies of the SNP were tested for Hardy-Weinberg equilibrium (HWE) in controls among Malaysian Indian and Indigenous groups by chi-square test. Hardy-Weinberg equilibrium (HWE) testing may be most important for data quality control (Rohlfs & Weir, 2008). We examine the implications of discrete *p*-values in HWE testing. Much evidence shows the application of departure from HWE in many usages such as inferring the existence of natural selection, challenging the statistical analysis of forensic DNA profiles, and detecting genotyping errors (Rohlfs & Weir, 2008; Zou & Donner, 2006). Hence it is necessary to understand the concept of testing genotype frequencies for fit to Hardy-Weinberg proportions (HWP) as an initial step in data analyses. A lot of information about population and patient samples (non-random mating, admixture), the accuracy of the genotyping, and selection are gained by observed deviations from HWP (Weight *et al.*, 2003).

CHAPTER 4

RESULTS

4.1 SOCIO-DEMOGRAPHIC PROFILE OF CASES AND CONTROLS

This case-control study included 143 confirmed cases of oral cancer and 79 healthy controls with no history of any kind of cancers or any family history of cancer. The age range for oral cancer patients was 28-105 years, in comparison with 22-104 years in the control group. With increasing age-group, the prevalence of oral cancer was raised and the highest prevalence recorded in the >50 years age-group (86%). All the data were analyzed based on these unmatched case-control study subjects. The selected socio demographic profiles of cases and controls are summarized in table 4.1.

Important differences between cases and controls were estimated in six variables. Ages, gender, ethnicity, smoking, alcohol drinking and betel quid chewing statuses were among the selected socio demographic profiles. With regard to age distribution analysis, cases (mean age = 63.69 ± 12.84) were significantly older than controls (mean age = 50.43 ± 16.35) (P = 0.000). Based on gender group distribution, there was a significant difference observed between cases and controls (p = .032). The distributions of male and female among controls (40.5 % & 59.5% respectively) and cases (26.6 % & 73.4% respectively) were significantly different. Female proportion was overrepresented within the cases group (73.4%) as compared to the male proportion (26.6%) and also in the group of controls, the gender distribution between females and males were represented by59.5% and 40.5% respectively.

No significant differences in ethnicity distributions were seen between the case and the control groups (p = .574). Indians and Indigenous were considered as two different ethnic groups in Malaysia. A large percentage of case group (68.5%) and control group (72.2%) were Indians compared with the proportion of Indigenous in case group (31.5%) and in control group (27.8%).

In addition, smoking, alcohol drinking and betel quid chewing are three other significant variables which follow the similar pattern of distribution. The distribution of non-smokers and non-drinkers among the cases were observed significantly lower (74.1% & 65.7% respectively) than the controls (86.1% & 78.5% respectively). In fact, the distribution of non-betel quid chewers among the cases were substantially lower (23.8%) than in the controls (79.7%). On the contrary, the proportion of betel quid chewers (76.2%) was significantly higher than non-betel quid chewers (23.8%) in the cases.

Habits were statistically significant between cases and controls (p = .038 in smoking status, p = .047 in alcohol drinking status and p = .000 in betel quid chewing status). Among cases, patients who smoked drank and chewed betel quid had a higher frequency compared to controls. No attempt was made to estimate the total amount of smoking, alcohol consumption or chewing, in terms of amount per day or duration in years, because the aim and design of the study was not to re-evaluate well established lifestyle risk factors for oral cancer.

Table 4.1: Socio-demographic profile of 143 case and 79 control subjects.

Socio demographic profile	Control Frequency(%)	Case Frequency(%)	*p*-value
Age in years	**50.43 (16.35)[a]**	**63.69 (12.84)[a]**	**.000**
Age			
≤50	**45 (57%)**	**20 (14%)**	**.000**
>50	**34 (43%)**	**123 (86%)**	
Gender			
Male	**32 (40.5%)**	**38 (26.6%)**	**.032**
Female	**47 (59.5%)**	**105 (73.4%)**	
Ethnic			
Indian	**57 (72.2%)**	**98 (68.5%)**	**.574**
Indigenous	**22 (27.8%)**	**45 (31.5%)**	
Habits			
Smoking statuse			
NO	**68 (86.1%)**	**106(74.1%)**	**.038**
Yes	**11 (13.9%)**	**37 (25.9%)**	
Drinking alcohol status			
No	**62 (78.5%)**	**94 (65.7%)**	**.047**
Yes	**17 (21.5%)**	**49 (34.3%)**	
Chewing betel quid status			
No	**63 (79.7%)**	**34 (23.8%)**	**.000**
Yes	**16 (20.3%)**	**109 (76.2%)**	
[a]Mean (*SD*)			

4.2 FACTORS ASSOCIATED WITH ORAL CANCER

4.2.1 Socio-demographic profiles and oral cancer

The results of independent *t*-test and simple logistic regression (SLR) analysis are shown in Table 4.2 for demonstration of the association between socio demographic factors and oral cancer risk. The association between age, smoking, alcohol drinking, betel quid chewing status and oral cancer risk was significantly exposed. In this study, the older patients presented to be 8.14 times higher risk of catching oral cancer than younger patients (OR 8.14, 95% CI 4.252 - 15.582).

Significant associations were observed between socio demographic profiles (gender, smoking, alcohol drinking and betel quid chewing) and oral cancer risk. The females displayed to be 1.88 times higher risk of having oral cancer as compared to the males (OR 1.88, 95% CI 1.051 - 3.369). Habitual smokers had a significantly 2.16 times higher risk of having oral cancer than non-smokers, giving an OR of 2.16 (95% CI 1.031 – 4.517). As compared to non drinkers, drinkers had a significantly 1.9 times higher risk of getting oral cancer than non-drinkers, showing an OR of 1.9 (95% CI 1.004 - 3.599) and a very significant increase in oral cancer risk was also detected among betel quid chewers compared with those who are not (OR of 12.62, 95% CI 6.457 – 24.676). The betel quid chewers tend to have 12.62 times the risk of having oral cancer than those who do not chew. On the other hand, there was no association found between ethnicity and oral cancer risk. Hence, among all the socio demographic profiles which we studied, five factors were discovered to be significantly associated with oral cancer risk at univariate level.

Table 4.2: Association between socio demographic profiles and oral cancer by simple logistic regression analysis

Factors	Oral Cancer		Crude OR	95% CI	*p*-value
	Control Frequency (%)	Case Frequency (%)			
Age in years	**50.43 (16.35)[a]**	**63.69 (12.84)[a]**	**8.14**	**4.252 - 15.582**	**.000**
Age					
≤50	**45 (57%)**	**20 (14%)**	**1**		
>50	**34 (43%)**	**123 (86%)**	**8.14**	**4.252 – 15.582**	**.000**
Gender					
Male	**32 (40.5%)**	**38 (26.6%)**	**1**		
Female	**47 (59.5%)**	**105 (73.4%)**	**1.88**	**1.051 – 3.369**	**.034**
Ethnic					
Indian	**57 (72.2%)**	**98 (68.5%)**	**1**		
Indigenous	**22 (27.8%)**	**45 (31.5%)**	**1.19**	**0.649 – 2.18**	**.574**
Smoking status					
No	**68 (86.1%)**	**106 (74.1%)**	**1**		
Yes	**11 (13.9%)**	**37 (25.9%)**	**2.16**	**1.031 – 4.517**	**.041**
Alcohol drinking status					
No	**62 (78.5%)**	**94 (65.7%)**	**1**		
Yes	**17 (21.5%)**	**49 (34.3%)**	**1.9**	**1.004 – 3.599**	**.048**
Betel quid chewing status					
No	**63 (79.7%)**	**34 (23.8%)**	**1**		
Yes	**16 (20.3%)**	**109 (76.2%)**	**12.62**	**6.457– 24.676**	**.000**

[a]Mean (*SD*)

4.3 PCR AMPLIFICATION, VERIFICATION AND RFLP OF *TNF-α* GENE

4.3.1 PCR amplification of *TNF-α* gene fragment

In total, 222 samples from both cases and control groups were amplified using the optimized conditions and the samples provided the expected amplified band size (147bp) on the agarose gel (Figure 4.1). No band was obtained with the negative control.

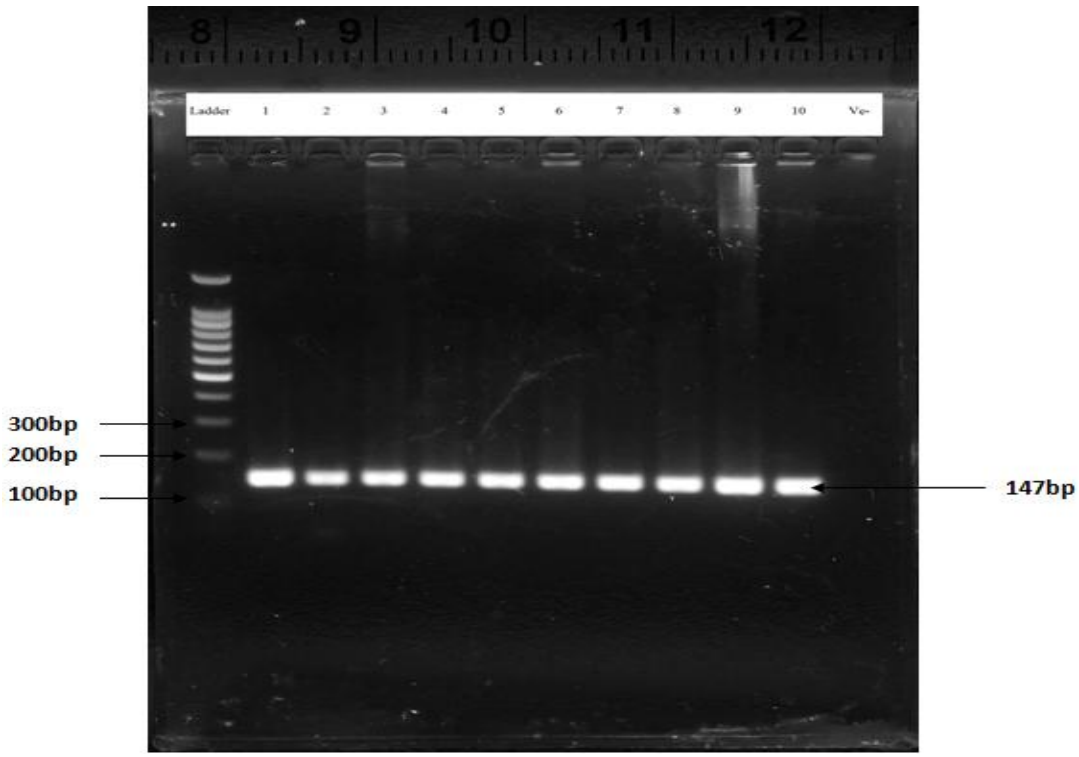

Figure 4.1: PCR products containing *TNF-α* −308 G/A SNP were checked using 2% (w/v) agarose gel run at 110 Volt for 30 minutes. First lane - 100bp DNA ladder, Lane 1-10 - PCR product of *TNF-α* −308 G/A, Last lane - PCR negative control without DNA template

4.3.2 Restriction fragment length polymorphism of *TNF-α −308* G/A fragment

After digestion of the DNA fragments (*TNF-α* −308) with restriction enzyme (*NcoI*), 3 types of TNF-α −308 G/A genotypes were seen (Table 4.3). 1: The 126 gene fragments generated from homozygous wild-type genotype of *TNF-α* −308 (GG); 2: the presence of 147bp, 126bp bands represents heterozygous genotype of *TNF-α −308* (GA); 3: 147bp represents homozygous mutant genotype of *TNF-α −308* (AA).

Figure 4.2 shows the gel profiles after RFLP analysis. In this study, 23 persons were heterozygous individuals (GA) from the 222 subjects. Three (3) persons were homozygous mutant individuals (AA) and 196 persons had homozygous wild-type genotype of *TNF- α −308* (GG).

Table 4.3: *TNF-α* codon −308 band-pattern after digestion with *NcoI*.

Genotypes		
GG Genotype Homozygote	**GA Genotype Heterozygote (Polymorphism)**	**AA Genotype Homozygote (Polymorphism)**
	147bp	**147bp**
126bp	**126bp**	

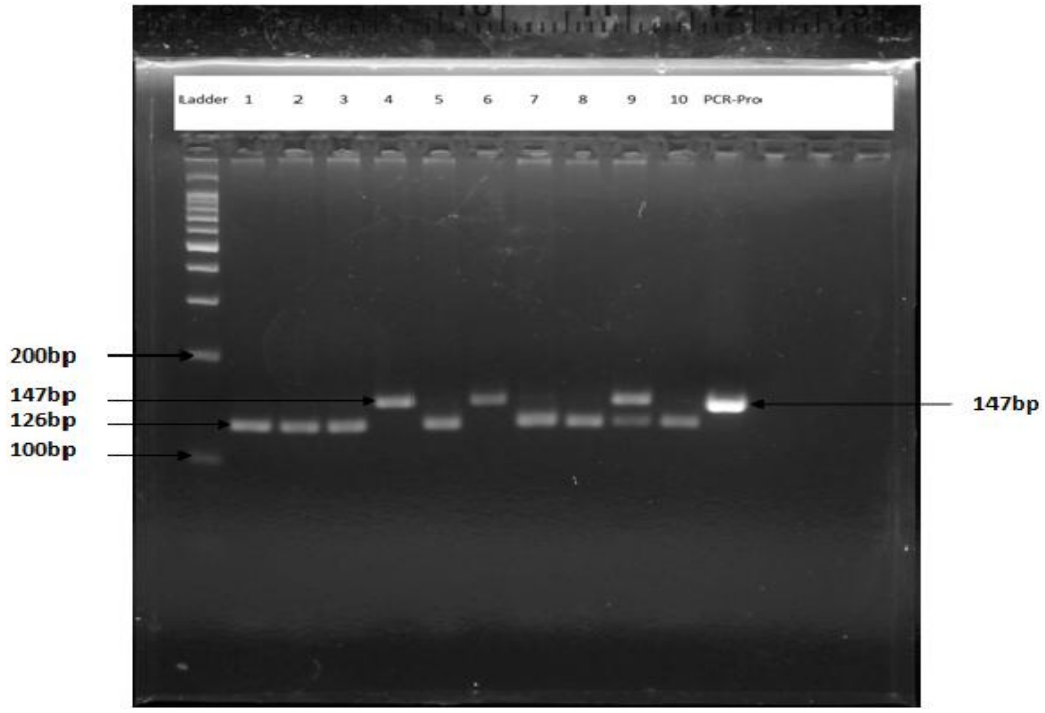

Figure 4.2: Banding patterns of the *NcoI*-digested PCR amplicons product of *TNF-α* −308: First lane - 100bp DNA ladder, Lane 1-3, 5, 7, 8 & 10 - 126bp (GG Genotype, Homozygote - Wild), Lane 4 & 6 - 147bp (AA Genotype, Homozygote - Polymorphism), Lane 9 - 147bp and 126bp (GA Genotype, Heterozygote) & Last lane - 147bp (positive control)

4.3.3 Verification of amplified *TNF-α −308* gene fragment after digestion with *NcoI* and sequencing result

The results from sequencing were compared to the sequences in National Center for Biotechnology Information (NCBI) database by Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).The nucleotide sequences of amplicons were obtained by using Biosystems Sequence Scanner software v1.0. All selective amplicons generated by the primers showed 98%-99% similarity in blast search. The DNA sequences successfully recognized the interested amplified regions of *TNF-α* gene. All the results obtained from NCBI are shown in Appendix G.

The sequencing chromatogram results of three different genotypes are shown in Figures 4.3, 4.4 and 4.5. Figure 4.3 indicates the sequencing chromatogram result of a purified PCR product with a homozygous wild-type genotype (GG) genotyping via RFLP analysis. Figure 4.4 and 4.5 show sequencing chromatogram results of purified PCR products having heterozygous and homozygous variant genotypes identified by using RFLP. All the genotyping results of selected samples obtained from RFLP were successfully verified by DNA sequencing.

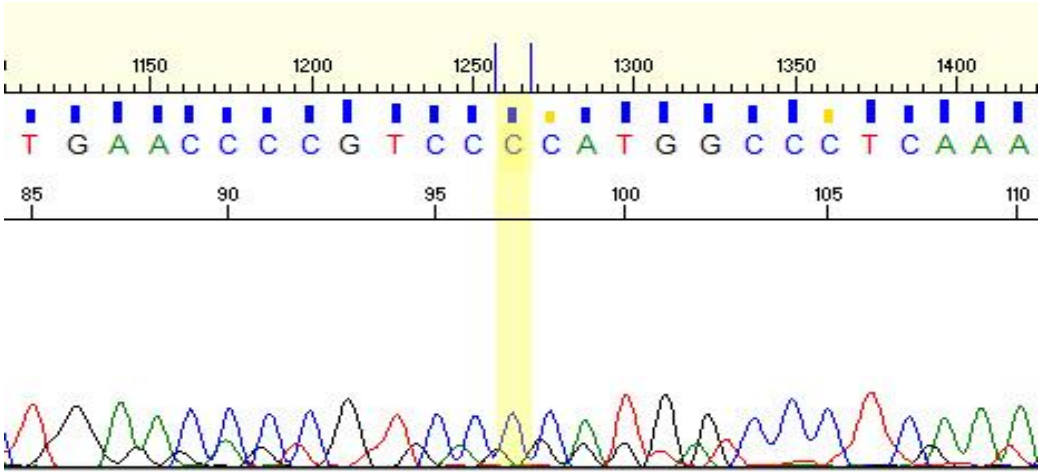

Figure 4.3: Chromatogram section showing the sequencing result for the homozygous wild-type genotype (GG) of *TNF-α* −308 G/A.
In this figure, the C on the reversed strand of DNA (bp number 97) indicates to the homozygous wild-type genotype (GG) of *TNF-α* gene codon −308.

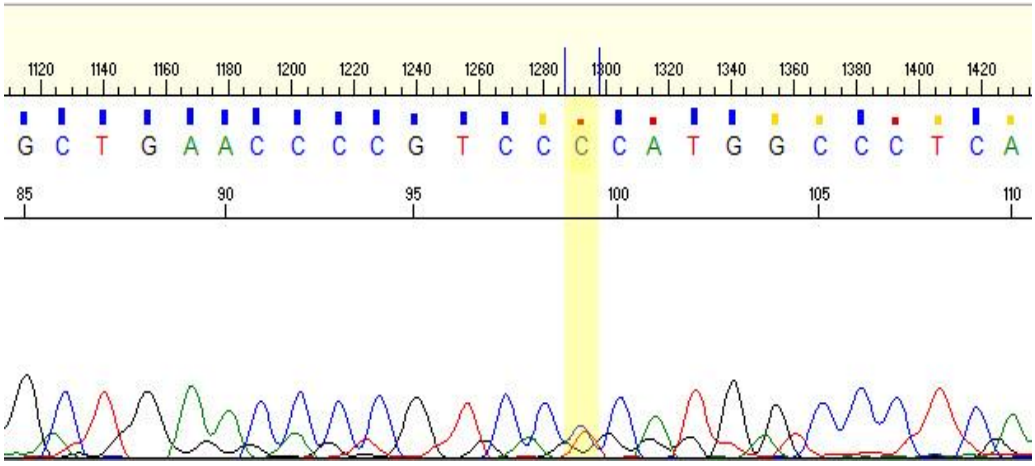

Figure 4.4: Chromatogram section showing the sequencing result for the heterozygous genotype (GA) of *TNF-α* −308 G/A.
In this figure, the C on the reversed strand of DNA (bp number 99) indicates to the heterozygous genotype (GA) of the *TNF-α* gene codon −308.

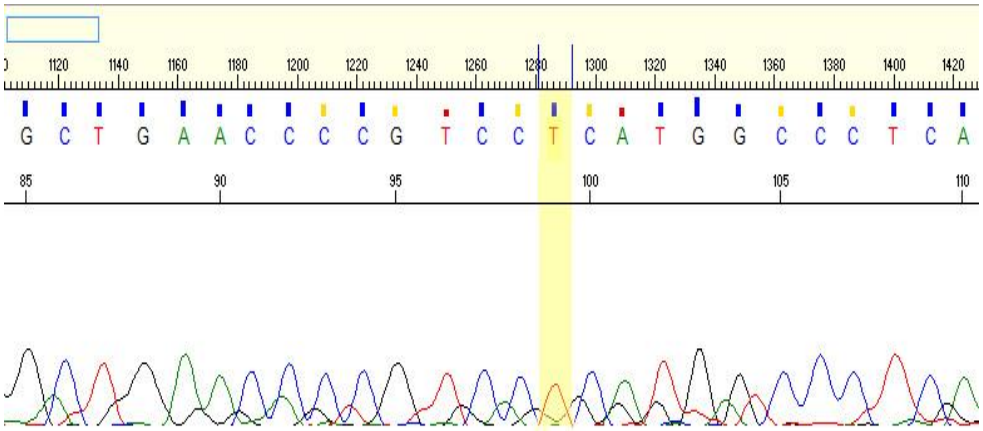

Figure 4.5: Chromatogram section showing the sequencing result for the homozygous mutant genotype (AA) of *TNF-α* −308 G/A.
In this figure, the T on the reversed strand of DNA (bp number 99) indicates to the homozygous mutant genotype (AA) of the *TNF-α* gene codon −308.

4.4 *TNF-α* −308 G/A POLYMORPHISM AND ORAL CANCER

Table 4.4, 4.5 and 4.6 show summaries of the results for the distribution of *TNF-α* −308 G/A genotypes among the Malaysian Indian and Indigenous oral cancer and non-oral cancer (normal) subjects.

Table 4.4 shows a summary of the results for the distribution of *TNF-α* −308 G/A genotypes among oral cancer and non-oral cancer (normal) subjects. The frequency distribution of GG as homozygous wild-type genotype was slightly lower for control group (87.3%) compared to cases (88.8%). The proportion of GA as heterozygous genotype was slightly higher in controls (11.4%) versus cases (9.8%).The frequency distribution of AA as homozygous mutant genotype was slightly lower for control group (1.3%) compared to cases (1.4%). There was no significant difference in wild and variant genotypes distribution between cases and controls (p = .930).

Table 4.4: Distribution of *TNF-α* −308 G/A genotypes in oral cancer patients and controls among the Malaysian Indian and Indigenous population.

Total	**Oral Cancer**		
Genotype	**Control**	**Case**	***p*-value**
	Frequency (%)	**Frequency (%)**	
GG	**69 (87.3%)**	**127 (88.8%)**	
GA	**9 (11.4%)**	**14 (9.8%)**	
AA	**1 (1.3%)**	**2 (1.4%)**	**.930**

Table 4.5 shows a summary of the results for the distribution of *TNF-α* −308 G/A genotypes between oral cancer and non-oral cancer (normal) subjects among Indians. The frequency distribution of GG as homozygous wild-type genotype was slightly lower for control group (86%) compared to cases (86.7%). The proportion of GA as heterozygous genotype was slightly higher in controls (12.3%) versus cases (11.2%). The frequency distribution of AA as homozygous mutant genotype was slightly lower for control group (1.8%) compared to cases (2%). There was no significant difference in wild and variant genotypes distribution between cases and controls among Indians (p = .974).

Table 4.5: Distribution of *TNF-α* −308 G/A genotypes in oral cancer patients and controls among the Malaysian Indian population.

Indian **Genotype**	**Oral Cancer** **Control Frequency (%)**	**Case Frequency (%)**	***p*-value**
GG	**49 (86%)**	**85 (86.7%)**	
GA	**7 (12.3%)**	**11 (11.2%)**	
AA	**1 (1.8%)**	**2 (2%)**	**.974**

Table 4.6 shows a summary of the results for the distribution of *TNF-α* −308 G/A genotypes between oral cancer and non-oral cancer (normal) subjects among Indigenous. The frequency distribution of GG as homozygous wild-type genotype was slightly lower for control group (90.9%) compared to cases (93.3%). The proportion of GA as

heterozygous genotype was slightly higher in controls (9.1%) versus cases (6.7%). There was also no significant difference in wild and variant genotypes distribution between cases and controls among Indigenous (p = .723).

Table 4.6: Distribution of *TNF-α* −308 G/A genotypes in oral cancer patients and controls among the Malaysian Indigenous population.

Indigenous **Genotype**	**Oral Cancer** **Control Frequency (%)**	**Case Frequency (%)**	***p*-value**
GG	**20 (90.9%)**	**42 (93.3%)**	
GA	**2 (9.1%)**	**3 (6.7%)**	
AA	**-**	**-**	**.723**

Table 4.7 shows a summary of the results for the association between *TNF-α* −308 G/A genotypes and oral cancer risk which was analyzed by the simple logistic regression analysis at univariate level. According to Table 4.7, the association between *TNF-α* −308 G/A genotypes (GA & AA) and oral cancer groups is not statistically significant (p = .710 and .946 respectively).

Tables 4.8 and 4.9 show no significant association between variant genotypes (GA & AA) and oral cancer risk among the Malaysian Indian and Indigenous population (GA

among the Malaysian Indian population: p = .848, AA among the Malaysian Indian population: p = .908, GA among the Malaysian Indigenous population p = .724).

Table 4.7: Association between *TNF-α* −308 polymorphism and oral cancer among the Malaysian Indian and Indigenous population by simple logistic regression analysis.

Total Genotype	**Oral Cancer**		**Crude OR**	**95% CI**	***p*-value**
	Control Frequency (%)	**Case Frequency (%)**			
GG	**69 (87.3%)**	**127 (88.8%)**	**1**		
GA	**9 (11.4%)**	**14 (9.8%)**	**1.18**	**0.487 – 2.873**	**.710**
AA	**1 (1.3%)**	**2 (1.4%)**	**0.92**	**0.082 – 10.332**	**.946**

Table 4.8: Association between *TNF-α* −308 polymorphism and oral cancer among the Malaysian Indian population by simple logistic regression analysis.

Indian Genotype	**Oral Cancer**		**Crude OR**	**95% CI**	***p*-value**
	Control Frequency (%)	**Case Frequency (%)**			
GG	**49 (86%)**	**85 (86.7%)**	**1**		
GA	**7 (12.3%)**	**11 (11.2%)**	**1.1**	**0.402 – 3.033**	**.848**
AA	**1 (1.7%)**	**2 (2.1%)**	**0.87**	**0.077 – 9.813**	**.908**

Table 4.9: Association between *TNF-α* −308 polymorphism and oral cancer among the Malaysian Indigenous population by simple logistic regression analysis.

Indigenous Genotype	**Oral Cancer**		**Crude OR**	**95% CI**	***p*-value**
	Control Frequency (%)	**Case Frequency (%)**			
GG	**20 (90.9%)**	**42 (93.3%)**	**1**		
GA	**2 (9.1%)**	**3 (6.7%)**	**1.4**	**0.216 – 9.054**	**.724**
AA	**-**	**-**	**-**	**-**	**-**

4.5 SOCIO-DEMOGRAPHIC PROFILES WITH TNF-α −308 G/A POLYMORPHISM AND ORAL CANCER

We surveyed the association between different risk factors with *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian and Indigenous population as the third objective (Table 4.10 and Table 4.11). It was found that the association between different risk factors with *TNF-α* −308 G/A polymorphism and oral cancer risk were also not significant for Malaysian Indians and Indigenous people who smoked and not smoked, drank alcohol and not drank, chewed betel quid and not chewed. All the data analyses are shown in Appendix E.

Table 4.10: Association between different risk factors with *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian population.

Indian **Genotype**	**Oral Cancer** **Control Frequency(%)**	**Case Frequency (%)**	**Crude OR**	**95% CI**	***p*-value**
Smoker GG GA & AA	**9 (100%)** **0 (0%)**	**16 (88.9%)** **2 (11.1%)**	**1** **NA**	**NS**	**NS**
Non - Smoker GG GA & AA	**40 (83.3%)** **8 (16.7%)**	**69 (86.2%)** **11 (13.8%)**	**1** **0.8**	**0.296 – 2.146**	**.654**
Alcohol drinker GG GA & AA	**12 (92.3%)** **1 (7.7%)**	**23 (85.2%)** **4 (14.8%)**	**1** **2.09**	**0.209 – 20.811**	**.531**
Non - Alcohol drinker GG GA & AA	**37 (84.1%)** **7 (15.9%)**	**62 (87.3%)** **9 (12.7%)**	**1** **0.77**	**0.264 – 2.233**	**.627**
Betel quid chewer GG GA & AA	**15 (100%)** **0 (0%)**	**72 (85.7%)** **12 (14.3%)**	**1** **NA**	**NA**	**NS**
Non - Betel quid chewer GG GA & AA	**34 (81%)** **8 (19%)**	**13 (92.9%)** **1 (7.1%)**	**1** **0.33**	**0.037– 2.877**	**.314**

NA, not available. NS, not significant

Table 4.11: Association between different risk factors with *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indigenous population.

Indigenous **Genotype**	**Oral Cancer** **Control Frequency(%)**	**Case Frequency (%)**	**Crude OR**	**95% CI**	***p*-value**
Smoker GG GA & AA	**2 (100%)** **0 (0%)**	**17 (89.5%)** **2 (10.5%)**	**1** **NA**	**NA**	**NS**
Non - Smoker GG GA & AA	**18 (83.3%)** **2 (16.7%)**	**25 (86.2%)** **1 (13.8%)**	**1** **0.36**	**0.030 – 4.281**	**.419**
Alcohol drinker GG GA & AA	**3 (75%)** **1 (25%)**	**20 (90.9%)** **2 (9.1%)**	**1** **0.3**	**0.020 – 4.418**	**.380**
Non - Alcohol drinker GG GA & AA	**17 (94.4%)** **1 (5.6%)**	**22 (95.7%)** **1 (4.3%)**	**1** **0.77**	**0.045 – 13.268**	**.859**
Betel quid chewer GG GA & AA	**25 (100%)** **0 (0%)**	**1 (100%)** **0 (0%)**	**NA**	**NA**	**NS**
Non - Betel quid chewer GG GA & AA	**19 (90.5%)** **2 (9.5%)**	**17 (85%)** **3 (15%)**	**1** **0.6**	**0.089– 4.008**	**.595**

NA, not available. NS, not significant

4.6 CHI-SQUARE (X^2) TEST OF HARDY-WEINBERG EQUILIBRIUM (HWE)

The (X^2) Test was used to determine whether observed genotype frequencies are consistent with Hardy-Weinberg equilibrium (Appendix F).

As shown in table 4.12, it was observed that the genotype frequencies of SNP were consistent with HWE in controls among Malaysian Indians and Indigenous respectively (p = .24 & p = .82).

Table 4.12: Determine Hardy-Weinberg equilibrium for controls among Indian and Indigenous population

Genotypes	**Observed**	**Expected**	***p*-value**
Indian Controls			
GG	49	48.4	
GA	7	8.3	
AA	1	0.4	.24
Indigenous Controls			
GG	20	20	
GA	2	1.9	
AA	0	0	.82

CHAPTER 5

DISCUSSION

5.1 DISTRIBUTION OF SOCIO-DEMOGRAPHIC PROFILE

In this study significant differences were verified in age, gender and risk habits namely smoking, alcohol drinking and betel quid chewing. It has been well substantiated that the incidence of oral cancer is raised with increasing age, globally (Hershkovich *et al.*, 2007; Hirota *et al.*, 2008). The highest incidence for oral cancer happens among those above forty years old and the average age at diagnosis is approximately 60 years of age (Burket *et al.*, 2003). The same distribution of oral cancer was found in this study population.

In this case-control study the difference in the mean age was highly significant between the cases and controls (p = .000). The mean age of cases was 63.69 years ± 12.84 and mean age of controls was 50.43 years ± 16.35. These patients were enlisted from the OCRCC database that included patients from different age and two ethnic groups in Malaysia who attended nine selected centers such as University Malaya's Hospital for minor sickness or cancer problems. The present findings also agree with previous findings which have been done in Malaysia by Ramanathan and Lakshimi (1976) and Ng *et al.* (1992). They said: "oral cancer in Malaysia is a disease of the older age group where majority of the patients were in the fifth to seventh decade of life" (Ng *et al.*, 1992; Ramanathan & Lakshimi, 1976).

With regard to gender distribution, there was a significant difference observed between cases and controls (p = .032) in this study. Among oral cancer studies, one study done in Hungary by Suba *et al.*(2009) showed significant difference in gender distribution between cases and controls but on the contrary, Bundgaard *et al.* (1995) had shown no significant difference in these two genders between cases and controls in Denmark (Bundgaard *et al.*, 1995; Suba *et al.*, 2009).

Therefore, it seems that several factors such as geographical area, genetic susceptibilities and lifestyles could play the role in contributing to the difference in gender distribution between cancer patients and controls. Also the gender which is more exposed to the high risk habit is another factor that could contribute to the gender difference in a special population. In our study subjects, the distribution of females (105 patients - 73.4%) was approximately three-quarters of cases group (143 patients) as compared to the distribution of males (38 patients - 26.6%). We can explain this result according to the study which has been done by Zain in 2001. There is a higher risk of getting oral cancer among females as compared with males, because most of them practice higher risk habit such as chewing betel quid (Zain, 2001).

We studied the Indian and Indigenous population in Malaysia. The ethnic distribution was not significantly different among cases and controls (p = .574). The Indians were the predominant group in cases (68.5%) and control (72.2%) and this was followed by the Indigenous (31.5% and 27.8% respectively). This prevalence of oral cancer in relation to ethnic origin in this study was almost similar to the incidence findings of oral cancer in Malaysia published by Ramanathan and Lakshimi (1976) and Ng *et al.* (1985). These two studies showed the majority of oral cancer cases were the Indians as compared to the other ethnic groups in Malaysia (Ng *et al.*, 1985; Ramanathan

& Lakshimi, 1976). Also, Zain *et al.* (1997) reported that the prevalence of oral precancer amongst Indians (4.0%) was higher than in indigenous (2.5%) (Zain *et al.*, 1997).

All the major habits, especially betel quid chewing, seem to be highly significant between case and control groups in this study. A significant difference in distribution of subjects with tobacco smoking habit was found between cases and controls (p = .038). The proportion of current and ex-smokers was higher among the cases (25.9%) than the controls (13.9%). Interestingly, most of cases have never smoked (74.1%) and there is an almost the same pattern for control subjects (86.1%). This result is not similar with other studies which showed the majority of patients with oral cancer were smokers (Cha *et al.*, 2007; Wen-Jiun *et al.*, 2011; Zygogianni *et al.*, 2011)except for the study conducted by Kietthubthew *et al.* (2001) in Southern Thailand. In addition, Ko *et al.* (1995) found that the oral cancer incidence among smoker patients was 8.4 fold higher than that among non-smoker patients (Ko *et al.*, 1995). Another study also showed the patients who smoked had a 6.41- fold increase in the risk of getting oral cancer (Castellsagué *et al.*, 2004).

Malaysian Indians who formed around 8% of Malaysia's population (Assunta & Idris, 2001) are the heaviest drinkers. A significant difference in distribution of subjects with alcohol drinking habit was found between cases and controls (p = .048). The proportion of current and ex-alcohol consumer was only one-third among the cases (34.3%) as compared to 65.9% and 56.6% of drinkers among the cases in Taiwan and Southern Thailand studies, respectively (Hung *et al.*, 1997; Kietthubthew *et al.*, 2001). It was higher than the controls (21.5%). Most of cases have never drunk (65.7%) and there is an almost the same pattern for control subjects.

According to our findings, 78.5% of the control subjects were not associated with alcohol drinking among the studied population. This might be attributed to prohibition of

alcohol consumption among Muslims; a 1996 national survey also found that 77% of non-Muslim adults were abstainers in Malaysia (WHO, 2004). In addition, the increasing importance of Islam in Malaysia as controlling factor promoted stricter attitudes towards alcohol even among the non-Muslims (Kortteinen, 2008). For instance, direct alcohol advertising is not allowed except in the state of Sabah (Assunta & Idris, 2001).Although alcohol consumption alone was not independently associated with oral cancer (Ko *et al.*, 1995) the combination of alcohol and tobacco may result in more synergized effect that would increase the risk of oral cancer (Bhurgri *et al.*, 2003).

A strong significant difference in distribution of subjects with betel quid chewing habit was found between cases and controls (p = .000). Among smoking, alcohol drinking and betel quid chewing as the major habits, betel quid chewing was the most common habit widely practiced by 76.2% of cases while 79.7% of the controls were non betel quid chewers. In this study the betel quid chewing habit was more prevalent among women (68.4%) than men (30%), with approximate ratio of 2:1. This study confirmed the previous findings that identified chewing as the strongest risk factor for oral cancer (Jayant *et al.*, 1977; Ko *et al.*, 1995; Nair *et al.*, 2004; Sankaranarayanan *et al.*, 1989), in particular for chewing products containing tobacco. Chewing products without tobacco was also an independent risk factor for cancers of the oral cavity (Znaor *et al.*, 2003).

According to previous studies such as Gupta and Ray (2004) which reported betel quid chewing as a popular old habit in the tropical areas, particularly in the Pacific Islands, South Asia, and Southeast Asian countries since the past, so the high incidence of oral cancer among those is explainable. In some cultures like Indian culture, betel quid has high symbolical value and plays an important role in traditional and religious ceremonies. It is a known fact that betel quid, with or without tobacco, is one of the major

risk factors for oral cancer among Indians (Chen *et al.*, 2008; Gupta & Ray, 2004; Jacob *et al.*, 2004).

5.2 DISTRIBUTION OF *TNF-α* −308 G/A AMONG CASES AND CONTROLS

There was no significant difference in distribution of GG, GA and AA genotypes for the *TNF-α* −308 between cases and controls (p = .930). This distribution was almost the same as shown by Franceschi and others in the south of Brazil. The distribution of the *TNF-α* −308 G/A genotypes was also not statistically significant (p = .17) among cases and controls in that population (Franceschi *et al.*, 2009).

Similar distribution of *TNF-α* −308 G/A genotypes were also reported by Liu *et al.* (2005) in Taiwan (Liu *et al.*, 2005). The GG was significantly higher (91.2% vs. 82.2%), whereas GA and AA were significantly lower (8.3% vs. 16.4% and 0.5% vs. 1.4%) in patients as compared with controls (Liu *et al.*, 2005). On the contrary, a study by Gupta *et al.* (2008) observed a significant difference in distribution of *TNF-α* −308 G/A genotypes among the cases and controls (p = .0002). The GG was significantly lower in oral cancer patients (64.89% vs. 85.7%) and GA and AA were higher (24.47% vs. 14.3% and 10.64% vs. 0.00%) as compared with controls (Gupta *et al.*, 2008).

5.3 SOCIO DEMOGRAPHIC PROFILE AND ORAL CANCER RISK

We used Simple Logistic Regression in this study which gave us Odds Ratio (OR) to assess risk estimation. Although interpretation of the OR should usually be restricted to saying the association is positive, negative or does not exist, there is an exception. It has been declared that OR almost approximate to the relative risk when the incidence of disease in a study population is less than 5-10% (when the disease is rare). The interpretation of OR in this study would be similar to the relative risk which was risk of having the disease among exposed people and non-exposed people (Campbell *et al.*, 2005; Merrill, 2010). Oral cancer is classified into this group since the occurrence of this disease is 0.04% (Zain *et al.*, 1997).

In this study, the older subjects with an average age of 63.69 years tended to get oral cancer. The older subjects (age >50 years old) had 8.14 times the risk of having oral cancer than the younger subjects (age ≤50 years old). This result was confirmed by studies done by Hirota *et al.* (2008) in Brazil and other parts of the world (Burzynski *et al.*, 1992; Cusumano & Persky, 1988; Hirota *et al.*, 2008; Llewellyn *et al.*, 2001). Other types of cancers generally follow this finding. The best reason for this statement is exposure of older people against risk factors is longer than younger people so they have higher risk of getting cancer.

In searching for the causes of oral cancer, various interesting facts become evident, which suggest an etiological relationship. Previous studies have suggested an association between gender and oral cancer. In this study, women have an 88% increased risk of getting oral cancer with an OR of 1.88 (95% CI 1.051 - 3.369). This could be attributed to the higher number of females (105 patients - 73.4%) among the cases (143 patients) and associated with betel quid (104 out of 152 patients – 68.4%). This result was

similar to second report of the NCR (2003) done by Lim and Halimah (Lim & Halimah, 2004).

In regard to the association between ethnicity and oral cancer risk there was no significant association. As a result of important factors such as small numbers of each ethnicity population as a separate group which had a great role in statistical analysis.

There was no significant value on association between different risk factors with variant genotypes of *TNF-α* −308 (GA & AA) and risk of oral cancer among Malaysian Indian and indigenous ethnicity as separate population. A large sample size is important for future design of case control studies using population-based controls.

As in other countries, smoking is one of the most important causes of cancer and other diseases in Malaysia which can be prevented (Lim, 2002). We found a significant association between smoking and oral cancer. The smoking patients had a significantly 2.16 times higher risk of having oral cancer than non-smoking patients (OR 2.16, 95% CI 1.031 – 4.517). Although some studies reported a significant association between smoking and oral cancer (Blot *et al.*, 1988; Castellsagué *et al.*, 2004; Gandini *et al.*, 2008; Warnakulasuriya *et al.*, 2010), Rahman *et al.* (2003) did not find any significant relationship between oral cancer and smoking (Rahman *et al.*, 2003).

Alcohol consumption is another high-risk activity associated with oral cancer. Some U.S. studies have demonstrated that alcohol use is a more important risk factor for oral cancer than smoking (Blot *et al.*, 1988; Mashberg *et al.*, 1981).

As compared to non drinkers, drinkers had a significantly 1.9 times higher risk of suffering oral cancer than non-drinkers, showing an OR of 1.9 (95% CI 1.004 - 3.599). Association between alcohol drinking and the risk of developing oral cancer has been reported in several studies (Baan *et al.*, 2007; Castellsagué *et al.*, 2004; Morse *et al.*, 2007; Znaor *et al.*, 2003), although Wen-Jiun *et al.* (2011) from Taiwan reported that

those “who drank only alcohol did not have an increased risk of developing oral cancer” (Wen-Jiun *et al.*, 2011). Meanwhile, Weinstein*et al.* (2002) indicated that alcohol intake tended to decrease risk of oral cancer (Weinstein et al., 2002).

Betel quid chewing has been identified as a major risk factor for oral cancer with or without the incorporation of tobacco in many countries such as Malaysia (Gupta & Ray, 2004). The strongest risk factor for oral cancer was betel quid chewing in this study. A very significant increased in oral cancer risk was also detected among betel quid chewers compared with those who are not. The betel quid chewers tend to have 12.62 times the risk of having oral cancer than those who do not chew (OR of 12.62, 95% CI 6.457 – 24.676). This finding is similar to a previous study by Ko et al. (1995) which reported a statistically significant association between oral cancer and betel quid chewing alone (Ko *et al.*, 1995). Perhaps the high numbers of betel quid chewers among the cases were because the majority of the betel quid chewers were Indian or Indigenous females. In fact, this betel quid chewing habit is still widely practiced and indulged by Indians and Indigenous people of Sabah and Sarawak (Zain *et al.*, 1997). In addition, several studies have demonstrated significant associations with betel quid chewing in relation to oral cancer (Lu *et al.*, 1996; Saub, 2001; Wen-Jiun *et al.*, 2011).

5.4 *TNF-α* −308 POLYMORPHISM AND ORAL CANCER

Genetic association studies on common DNA polymorphisms in genes of cytokines may reveal important information about the role of these factors in the susceptibility for head and neck cancer (Serefoglou *et al.*, 2008). Highly significant true

association obtained by appropriate studies may provide a useful tool for prognosis and prevention of cancers (Cooper *et al.,* 2002).Associations between single nucleotide polymorphisms (SNPs) of the *TNF-α* gene at position −308 (G/A) and the risk of developing different types of cancer have been reported in several studies (Duarte *et al.*, 2005; Engels *et al.*, 2007; Fang *et al.*, 2010; Oh *et al.*, 2000; Shih *et al.*, 2006; Wei *et al.*, 2005).

Polymorphism of *TNF-α* at position −308 (G/A) may not be a risk factor for oral cancer because we did not find a statistically significant association between the *TNF-α* −308 polymorphism and oral cancer risk. It seems that the polymorphism has no major role in increasing oral cancer risk. The observed lack of an association between the *TNF-α* −308 G/A genotype and susceptibility to oral cancer in this study is similar to that observed in a previous study (Chiu *et al.*, 2001). However, a possibility remains that we may have yielded a false negative result due to an insufficient statistical power resulting from the very low frequency of the minor allele (*TNF-α* −308A) in our sample.

5.5 SOCIO-DEMOGRAPHIC PROFILES WITH *TNF-α* −308 G/A GENOTYPES AND ORAL CANCER RISK

Generally, there were no significant associations found between smoking as a risk factor with polymorphism genotypes and oral cancer risk among the Malaysian Indian and Indigenous population separately. Association between smoking as a risk factor with variant genotypes of *TNF-α* −308 (GA & AA) and oral cancer risk has been reported by Gupta *et al.* in 2008 (Gupta *et al.*, 2008). The observed lack of an association between

smoking as a risk factor with *TNF-α* −308 polymorphism and susceptibility to oral cancer in this study is similar to that observed in a previous study (Van Dyke *et al.*, 2009).

In addition, no significant associations were observed between alcohol drinking as a risk factor with variant genotypes of *TNF-α* −308 (GA & AA) and oral cancer risk among the Malaysian Indian and Indigenous population separately. The observed lack of an association between alcohol drinking as a risk factor with*TNF-α* −308 polymorphism and oral cancer risk in this study is similar to that observed in a previous study (Trujillo-Murillo *et al.*, 2011). On contrary, a study by Lu *et al.* (2004) has indicated significant associations between alcohol drinking as a risk factor with *TNF-α* −308 G/A polymorphism and oral cancer risk (Lu *et al.*, 2004).

Also, betel quid chewing as a risk factor was not associated significantly with *TNF-α* −308 G/A variant genotype (GA & AA) and oral cancer risk among the Malaysian Indian and Indigenous population separately. Therefore, it seems there is no association between different risk factors such as smoking, alcohol drinking and betel quid chewing with *TNF-α* −308 G/A polymorphism and oral cancer risk among the Malaysian Indian and Indigenous population.

5.6 LIMITATION OF THE STUDY

There are several limitations such as: sample size, time, financial restriction, statistical power, recall bias, confined reference and so forth. The main constraint in this study is the small sample size which could not express the true population but was rather confined to this study. This is because the calculated sample size was based on the objective and oral cancer risk and as well as the feasibility of conducting the study within

the restricted resources and time limitation. The truth of these findings may only be confirmed when the study is replicated with a larger sample. A larger sample was impossible for this study because of time and financial limitations. Because of limited statistical power, precise estimation of the gene environment interaction was not feasible; odds ratios in case-control studies with small sample size would be artificially inflated. Wide ranges of the confidence intervals (95% CI) come from insufficient power of the subgroup analysis and insufficient sample size in the subgroup. Another limitation is recall bias that occurs when cases and controls recall exposures differently. Recall bias in this situation may lead to spurious associations (Rockenbauer et al., 2001).

CHAPTER 6

CONCLUSIONS

At first, it was observed that there were significant associations between socio-demographic factors such as age, gender, smoking, alcohol drinking and betel quid chewing and oral cancer risk among Malaysian Indian and Indigenous population. Also, no association was found between ethnicity and oral cancer risk among Malaysian Indian and Indigenous population.

No significant association was found between *TNF-α* −308 G/A polymorphism and oral cancer risk among Malaysian Indian and Indigenous population. In other words, this *TNF-α* −308 G/A polymorphism may not be a risk factor in oral cancer.

Lastly, no significant associations was observed between different habitual risk factors variant genotypes of *TNF-α* −308 and oral cancer risk among Malaysian Indian and Indigenous population, those were not significant.

No association was seen between *TNF-α* −308 polymorphism and oral cancer risk among the Malaysian Indian and Indigenous population. Since this is the first study in Malaysian Indian and Indigenous population and also the development of oral cancer like other cancers is based on multifactorial contribution, so additional environmental and genetic factors should be explored. A study with a larger sample size is needed to confirm our findings.

6.1 RECOMMENDATIONS FOR FUTURE STUDY

There are several possible approaches for future study that would improve the outcomes for oral cancer, especially on risk factors and their association with oral cancer. As failure to show an association between genotypes and cancer risk may partly be due to a lack of statistical power, a large sample size is important for future design of case control studies using population-based controls.

It is strongly recommended that in future studies, if frequency will be changed to higher number of patients, it may effect on results to be significant among ethnics and so forth.

Future studies should consider looking into the possible explanation on what factors make females and males differ in their risk of having oral cancer since it can help to narrow down our prevention campaign focusing only to the associated factors.

It seems that smoking alone does not contribute to getting cancer and there is a possible reason for the similar proportion of never smoker and smoker in cases and control. Therefore, it would be useful in future studies to survey the role of smoking-alcohol drinking, smoking-betel quid chewing and also smoking-alcohol drinking-betel quid chewing interaction in oral cancer development. On top of that, interpretation and classification of patients are other factors which are important to be considered in future studies. However, this was not captured in our study since there were some limitations in the secondary data used; thus this issue should be considered in future studies.