CHAPTER 1 INTRODUCTION

1.1 Background of the study

Oral cancer is the tenth most common type of cancer contributing to death worldwide (Cheng & Blumenthal, 2008; Jemal et al., 2011). Oral Squamous Cell Carcinoma (OSCC) is a type of oral cancer and subgroup of head and neck squamous cell carcinoma (HNSCC). OSCC is the sixth most common malignancy with an incidence of more than 300,000 cases yearly, of which 62% are from developing countries (Kumar et al., 2013; Parkin et al., 2005). Smoking tobacco and drinking alcohol are regarded as major risk factors for oral cancer (Petersen, 2003). Significant efforts of oral cancer researchers committed and advanced treatments in surgery, radiotherapy, and chemotherapy; however, the overall 5-year survival rate has remained less than 50% for the last decades. There is also a rising incidence of oral cancer in developed countries and among the younger population (Scully & Bagan, 2007; Warnakulasuriya, 2009). The prognosis of OSCC remains poor essentially due to late diagnosis. Therefore it is of great importance to identify specific molecular markers that could be potentially useful for early diagnosis and prevention of OSCC. Moreover, the 5-year survival rate for oral cancer is directly associated to pathological stage at the time of diagnosis, thus early diagnosis and treatment remain the main keys in improving the overall survival rates of patients diagnosed with this disease.

An epigenetic phenomenon is a heritable alteration in the gene function without sequence changes, which involves the enzymatic addition of methyl groups to deoxycytidine residues to CpG dinucleotides. Alterations in the established epigenetic patterns usually lead to changes in gene expression, which can cause transcriptional repression. The classic epigenetic changes include DNA methylation, histone modification (acetylation, methylation and phosphorylation), chromatin remodelling and microRNA interference, which have been

shown to be involved in post-transcriptional silencing. DNA methylation is one of the epigenetic phenomena that occur at the molecular level involving cell regulation with regards to development and differentiation, aging, and diseases such as multiple sclerosis, diabetes, schizophrenia and cancers (Shaw, 2006; Supic et al., 2009). In terms of DNA methylation, cancer cells show genome-wide hypomethylation occurring at many genomic sequences, such as repetitive elements, retrotransposons, and introns resulting in genomic instability and site-specific Cytosine-Guanine (CpG) islands (Esteller, 2008). A recent study that compared colorectal cancer tissue with normal tissue suggests an important alteration at the CpG island shores (Irizarry et al., 2009). Notably, one of the main gene inactivation mechanisms involved in cancer progression is the promoter hypermethylation of CpG islands. Silencing of certain tumour suppressor genes (TSGs) leads to the development of many solid tumours, which may occur via hypermethylation of CpG islands located in the promoter region without the presence of genetic alterations (Herman & Baylin, 2003; Jones, 2012). The hypermethylation of CpG dinucleotides, especially at the CpG sites of TSGs, shows the dramatic effects on gene expression and this process is now widely recognized as either a causative or correlative event in carcinogenesis.

Recent advanced technology for high throughput genome-wide DNA methylation analyses of microarray is a potential diagnostic tool for methylation profiling (Kim & Kim, 2010). The advanced microarray technology has enabled researchers to generate large amounts of data which include approximately the entire known human genome, and has shown that many genetic alterations can be involved in carcinogenesis (Bibikova et al., 2004). Alterations in over one hundred oncogenes and TSGs have now been implicated in some cancers (Hayslip & Montero, 2006; Stransky et al., 2011; Vogelstein & Kinzler, 2004). Thus, a microarray technology was applied to analyse the methylation status of genes simultaneously and

discover the epigenetic signatures that distinguish normal from tumour tissues of OSCC in this study.

The promoter hypermethylation can also be detected by methylation-specific polymerase chain reaction (MSPCR) (Herman, Graff, Myöhänen, Nelkin, & Baylin, 1996) or by imunohistochemical (IHC) staining of the proteins coded for by the genes (Turbin et al., 2008, Rexhepaj et al., 2008). The advancement of this MSPCR technique has demonstrated a simple, rapid and cost-effective assessment for promoter methylation status. The methylation technique provides information about the methylation status, which improves the sensitivity of detection of 1 methylated allele in a background of 1000 unmethylated alleles, when detecting the tumour-specific signals in oral cancer (Cottrell & Laird, 2003). Thus, MSPCR was applied to confirm the microarray data in the study. In addition, the IHC staining was used to identify changes in protein expressions associated with the promoter hypermethylation (Rexhepaj et al., 2008, Turbin et al., 2008) in the OSCC cases.

1.2 Rationale of the study

The precise molecular mechanisms involved in the development and progression of OSCCs remain unclear. Furthermore, the discovery of TSGs often fails to be expressed in the absence of a detectable genetic mutation. This has recently led to greater research emphasis on cancer epigenetic studies, especially in the DNA methylation (Feinberg et al., 2006). Thus, the detailed study of methylation profiling pattern in normal and pathological conditions is very crucial for a better understanding of OSCC aetiology and its early detection (Moskalyov et al., 2007). Moreover, identification of new genes that are hypermethylated in OSCC can be used as biomarkers for better detection, as well as for an individual's cancer risk assessment of

recurrence and/or progression after diagnosis, and personalized medicine (Phé et al., 2010; Schmezer & Plass, 2008).

1.3 Significance of the study

Direct benefits of OSCC methylation profiling include probable advances in hypermethylation patterns which have been well characterized in many cancers. The specific patterns of hypermethylation are indicative of specific cancer types, have prognostic values and can help to guide the best course of treatment (Laird, 2003; Ludwig & Weinstein, 2005) for OSCC. An in-depth understanding of epigenetic alterations underlying oral cancer for identifying possible molecular therapeutic targets can enhance usage of molecular targeted therapy in clinics. The findings of this research could reveal that molecular biomarkers and gene detection will become a reality for the care of patients with OSCC.

The analyses of advanced methylation technologies have enabled us to validate the signature candidate gene methylation levels and protein expressions; and also to correlate these epigenetic alterations with carcinogenic pathways. The findings can ultimately lead to better cancer diagnostic, prognostication and new therapeutic strategies (including molecular-targeted therapy) that will benefit patients afflicted with oral cancer in the near future. Therefore, the observations made from this research are important in providing a stepping stone for further research into exploring the biological functions of the signature candidate genes, or roles involving methylation mechanisms and their interactive pathways leading to oral carcinogenesis.

1.4 Objectives of this study:

1. To investigate the methylation profiling expressions of OSCC.
2. To explore the hypermethylated genes for the epigenetic alterations associated with OSCC.
3. To determine the protein expression of hypermethylated genes.
4. To correlate the protein expressions with specific genes methylation levels in OSCC.
5. To correlate the characteristic of DNA hypermethylation patterns with demographic and clinicopathological data, and survival rate of the OSCC patients.

The hypotheses of the study are:

1. There is a difference in methylation profiling expressions between normal subjects and OSCC patients.
2. There is a difference in gene hypermethylation levels and protein expressions between normal subjects and OSCC patients.

CHAPTER 2 LITERATURE REVIEW

2.1 Oral cancer

Oral cancer is an epithelial neoplasia commonly initiated as a focal clonal overgrowth of altered stem cells near the basement membrane, and then expanding upward to replace the normal epithelium in the oral cavity (Werning, 2007). The basal cell of the oral epithelium has a higher than normal mitotic activity, any disturbance in quality and quantity of cell-regulating proteins can lead to carcinogenesis (Sapp at el., 1997). This malignancy commonly involves the tongue, gum, cheek, palate, lip and floor of the mouth (Garzino-Demo et al., 2006). Its oncogenic transformation is initially from normal epithelium through hyperplasia to dysplasia to carcinoma in situ and lastly, invasive carcinoma (Saranath, 2000). Recurrence of OSCC at the local tumour site or regional metastases through the lymph nodes of the neck to form secondary cancer can occur, despite a very advanced form of cancer treatment by surgery and/or radiotherapy. This suggests that the cancer treatment fails to target the small amounts of cancerous cells that still remain in the body. As such, in order to cure cancer with a higher success rate, the current conventional surgical and/or radiotherapeutic treatments must be critically examined. Modification of the treatment protocols using newer technology such as molecular-targeted therapy need to be further investigated.

2.1.1 The world scene

Oral cancer is one of the common human cancers worldwide for which it is ranked 10th most common for men and 13th most common for women. In 2008, it was reported in the global burden of cancer (GLOBOCAN) that 263,900 new oral cancer cases were diagnosed and 128,000 deaths occurred annually with 65.4% of oral cancers occurring in the less developed

countries such as in Asia (Jemal et al., 2011). The incidence of oral cancer is higher in developing countries as compared to developed countries. Although OSCC accounts for less than 5% of malignant tumours in developed countries, it is the most common malignancy in parts of South Asia and South East Asia, accounting for up to two third of malignant tumours in these regions (Warnakulasuriya, 2009). In the United States of America (USA), 30,000 people are diagnosed with oral or pharyngeal cancer annually for which the incidence rate is similar between African-American and Caucasian females, but is 30% higher amongst males of African-American descent if compared with Caucasian males. Squamous cell carcinomas (SCCs) account for the majority of the cases in the USA and are mainly related to tobacco and alcohol exposures (Sturgis et al., 2004). Approximately 90% of oral cancers are presented as squamous cells in origin, thus they are usually referred as OSCC (Walker et al., 2003). Despite significant efforts and commitment of oral cancer researchers in recent years, the overall survival rate has remained at less than 50%, which is lower than cervical and breast cancers.

2.1.2 The Malaysian scene

Oral cancer is one of the emerging health problems within Malaysia due to the increase in risky habits such as smoking, betel quid chewing and excessive alcohol drinking being practised amongst the local population (Zain, 2001; Zain & Ghazali, 2001). The most common form of oral cancer diagnosed in Malaysia is OSCC (Ng & Siar, 1997). According to National Cancer Registry (NCR), oral cancer is the sixteenth most common cancer for females and is ranked number twenty one for males for overall cancer incidence per 1000,000 populations in 2002 (Lim et al., 2008). It is ranked seventh and third most common cancer for Indian males and females with 4.5% and 6.5% respectively of all cancers (Lim et al., 2008). Based on the third report of the NCR on clinical data from government hospitals in

Peninsular Malaysia, it was reported that the highest incidence of oral cancer (excluding tongue cancer) occurred for those of Indian ethnicity, followed by the Malays and then the Chinese (Lim et al., 2008). This can be seen quite prominently amongst Indian females for whom an age standard ratio (ASR) of 14.4 per 100,000 population was observed, in comparison with the Malays and the Chinese both of whom only reached ASR values of 0.8 and 0.6 per 100,000 respectively (Lim et al., 2008).

2.2 Carcinogenesis

Carcinogenesis is a progression of a normal healthy cell to a potentially malignant cell, which undergoes an autonomous proliferation. It is a multistep and multifactorial complex mechanism containing a variety of genetic and epigenetic alterations. These alterations include silencing of gene expression and cell functions involve in regulating cell signalling, growth, survival, angiogenesis, and cell cycle control which are fundamental to cell homeostasis (Gordana et al., 2009). For decades, gene mutations seemed to be the most important alterations in human cancers. However, in recent years, it has been shown that epigenetic alterations, especially alterations in DNA methylation patterns play a vital role in carcinogenesis. Both hypermethylation of CpG rich areas, namely CpG islands which are mostly located at the 5' end of the transcription start site (TSS) of the activated transcribed genes, and hypomethylation of transcriptional inactive genomic regions, frequently happen in various cancers (Enokida & Nakagawa, 2008; Esteller, 2011). Silencing of tumour suppressor genes (TSGs) by the promoter hypermethylation, or induction of oncogenes by the promoter hypomethylation are frequent mechanisms in different types of cancer. This may potentially be of increasing diagnostic and therapeutic importance since the DNA methylation alterations are reversible (Iglesias-Linares et al., 2010). Therefore, methylation analysis may provide

promising clinical applications that includes the development of new biomarkers and prediction of the therapeutic response or prognosis.

2.3 Cancer staging and grading

The most widely used cancer staging system is the TNM (Tumour/nodes/metastases) system (Table 2.1). The TNM system has been well accepted by the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) (Edge & Compton, 2010). The cancer staging system describes with roman numerals of I to IV, and indicates the extent that the cancer has spread. A higher number indicates a more advanced cancer and a worse outcome is more likely. This system includes the identification of the size and/or extent of the primary tumour (T) with degree of invasion (scale from 1-4), the quantity of spread to nearby lymph nodes (N) with the degree of involvement (range 0-3), and the presence of metastasis to other parts of the body (0 or 1). A number is added to each letter to indicate the size and/or extent of the primary tumour and the severity of cancer spread. This staging system is used for most types of cancer, except for brain tumours and haematological malignancies. Clinicians can plan appropriate treatment and estimate patient's prognosis by knowing the stage of disease. This system has evolved over time and continues to change as scientists gain more knowledge about cancer. In addition, physical examinations, imaging procedures, laboratory tests, pathology reports, and surgical reports can provide more information to determine the stage of a cancer.

At the same time, histological grade of the tumour is usually assessed as well. The grade score increases with the lack of cellular differentiation. Grade I is referred as well differentiated if they appear similar to normal cells, and grade III if they appear to be poorly differentiated (Pindborg et al., 1997).

Bryne et al. (1992) proposed the multifactorial grading system using deep invasive margins when tumour's morphological features are to be evaluated in the grading system. The pattern of invasion is described as individual, non-cohesive and cohesive tumour cells, well defined tumour islands and cords (Bryne et al., 1992).

Table 2.1: TNM clinical staging categories with 5-year survival rate for cancer (Edge & Compton, 2010)

Stage	TNM Classification	5-year survival rate
I	T1 N0 M0	85%
II	T2 N0 M0	66%
III	T3 N0 M0 or T1,T2,T3, N1 M0	41%
IV	Any T4 lesion or Any N2 or N3 or Any M1 lesion	9%

2.4 Cancer prognosis

Patients' survival depends on the cancer staging after diagnosis. Patients' survival rates for early stage detection are about 5 times higher than that of late stage cancers. If the cancer is symptomatic, it is typically quite advanced and overall survival is poor, but it would depend on the type and if metastases to other parts of the body or organs had already occurred. Usually, a stage I cancer patient has an average 5-year survival of approximately 85%-90%. This drops to about 50-60% for a more invasive tumour at stage II, and without node involvement it can reach 70%. Cancers diagnosed at stage III with positive regional lymph nodes have an average 5-year survival of 30%-40%, whilst cancers at stage IV that is accomplished distant metastases the 5-year survival is less than 9% (Table 2.1).

2.5 Risk factors for oral cancer

Risk factors that predispose to OSCC are lifestyle factors such as tobacco consumption, excessive alcohol drinking, and betel-quid chewing. Human papillomavirus (HPV), other

infections, or host defences may also be relevant. In addition, genetic factors such as single nucleotide polymorphisms (SNPs) or mutation may influence the risks (Scully & Bagan, 2009). Other predisposing factors to oral cancer development include diet, occupational risk, poor oral health, and radiation or sunlight exposure. The main aetiological factors of causing OSCC in western countries are excessive alcohol consumption and tobacco use, which act on genetically susceptible individuals, whereas in the Asian countries, betel quid chewing habit is seen as another important risk factor (Chen et al., 1999; Zain & Ghazali, 2001; Warnakulasuriya,2009).

2.5.1 Lifestyle factors - tobacco, alcohol and betel-quid chewing

2.5.1.1 Tobacco and betel-quid chewing

Smoked tobacco or smokeless tobacco consumption is one of the major risk factors for OSCC (Warnakulasuriya & Ralhan, 2007). Cigarette smokes contain more than 7,000 chemicals whereby hundreds of them are toxic. Nitrosamines, formed by nitrosation of nicotine during the curing of tobacco leaves and other tobacco alkaloids present in tobacco smoke can alkylate DNA bases. This form of DNA damage is repairable if recognised by the various repair enzymes. However, excessive smoking leads to the impairment of multiple DNA repair pathways such as adduct reversal as well as base and nucleotide excision repair pathways (Cogliano et al., 2004). The free radicals contained in the cigarette smoke will lead to the oxidation of the thiol group of the antioxidant enzymes glutathione-S-transferase, glutathione reductase, superoxide dismutase, catalase and glutathione peroxidase which renders the enzymes ineffective and leaves the cell exposed to oxidative stress (Patel et al., 2005). Another mechanism involved in smoking induced carcinogenicity is cytochrome P450-mediated α-hydroxylation of nucleotides causing the formation of DNA adducts (Cogliano et al., 2004). Arecoline, an aldehyde found in cigarette smoke can induce the transition of

cytosine (C) to thymine (T) by binding to 5-Methylcytosines (5-MCs) present in gene promoter regions which is associated with the development of lung cancer (Feng et al., 2006). Promoter methylation of p16 has been observed in normal oral mucosa of 9.7% of cigarette smokers, supporting the fact that p16 hypermethylation is an early event in HNSCC (Ventorin von Zeidler et al., 2004). Elevation of the OSCC risk for males and females were found by observing the effects of betel quid chewing with or without tobacco. There is also a relationship between OSCC and betel-quid (areca nut) usage, a habit found in about 20% of the world's population, especially in Asian communities (Cogliano et al., 2004). Areca nut is carcinogenic to humans as confirmed by many studies (Scully & Bagan, 2009). Chewing betel quid induces local irritation and trauma to the oral mucosa, causing chronic inflammation, oxidative stress and cytokine production (Lai & Lee, 2006; Shillitoe, 2009). Gene expression may be altered by a main component in the areca nut, arecoline, by hypermethylation mechanism in blocking TSGs of p14, p15 and p16, inhibiting the p53 by repressing DNA repair and triggering DNA damage responses (Tsai et al., 2008).

2.5.1.2 Excessive alcohol consumption and mouthwash

A suspected carcinogen, acetaldehyde is oxidized by alcohol dehydrogenases (ADHs) from alcohol and acetaldehyde is then degraded to a non-carcinogenic substrate, acetate by aldehyde dehydrogenases (ALDH) in the liver. Acetaldehyde has been found to hinder DNA nucleotide methyltransferases (DNMTs) in a defective metabolic pathway of alcohol metabolism. Aberrant methylation can result in the increased oncogene expression (hypomethylation) and TSG suppression (hypermethylation). The worldwide prevalence of 25% oral cancers is attributed to tobacco usage (smoking and or chewing), 7–19% to alcohol drinking, and more than 50% attributed to betel quid chewing in areas of high chewing prevalence (Petti, 2009). Smoking increases the acetaldehyde burden following excessive

alcohol consumption, which enhances the activation of pro-carcinogens present in tobacco due to increased metabolic activation (Purohit et al., 2012).

Synergistic effects of cigarette smoking, heavy alcoholic consumption, and betel-quid chewing were found in the studies (Wu et al., 2006; Zygogianni et al., 2011). Extracellular nicotine worked synergistically on the arecoline-induced cytotoxicity, which plays a vital role in getting higher risk of developing oral cancer (Chang et al., 2001).

It has been reported that excessive use of alcoholic mouthwash increased the risk of acquiring oral cancer for smokers up to nine times (OR 9.15) compared with the non-alcoholic mouthwash which had an OR of nearly five times (OR 4.96) (McCullough & Farah, 2008). However, Warnakulasuriya (2009) summarized that alcoholic mouthwash is still a debatable risk factor for oral cancer. The molecular link between mouthwash use and oral cancer detected in some of the epidemiological studies may be explained by the local carcinogenic effects of acetaldehyde with cumulative exposure in the oral cavity (Lachenmeier, 2010).

2.5.2 Infectious factors

Infections induced by bacteria, fungus and virus may inhibit cell apoptosis, trigger cell proliferation, interfere with cellular signalling mechanisms and up-regulate tumour promoters that can lead to carcinogenesis (Meurman, 2010). Chronic infections or oral ecological-periodontal disease or loss of teeth are characterized by an inflammatory response of oxidative stress leading to carcinogenesis. In addition, the proliferation of ketone-producing and nitrate-reducing microorganisms may contribute to the increase of carcinogens present (Abnet et al., 2008). A study conducted by Kolenbrander et al. (2005) on biofilm formation

has observed that 16S rRNA gene amplification was dominated by Streptococcus spp., *Actinomyces* spp., *Veillonella* spp., *Prevotella* spp., *Neisseria* spp., *Gemella* spp., *Abiotrophia* spp., *Rothia* spp. and *Clostridia* class. The mechanism of microbial interactions with dental bacterial plaque causing periodontal disease is by synthesizing acetaldehyde from alcohol by both oral Streptococci and Neisseria. Thus, oral infections may trigger malignant transformation in tissues of the oral cavity (Meurman & Bascones-Martinez, 2011).

Viral infections of Herpes Simplex virus (HSV), Epstein–Barr virus (EBV) and Human Papilloma Virus (HPV) have been found to be related to OSCC development (Termine et al., 2008; Jalouli et al., 2010; Demokan & Dalay, 2011). The occurrence of OSCC increases in females who have cervical carcinoma related to HSV, and this also increases the risks in their partners. These tumours are likely to represent transmission between the couple (Haddad et al, 2008). The HSV, types-1 and -2 have been investigated for the possible associations with human cancers. Levels of antibody to HSV-1 together with smoking have been reported to be higher in patients with oral cancer than those in the control group (Shillitoe, 2009). However, the HSV's involvement in oral cancer is also a debatable risk (Warnakulasuriya, 2009).

EBV is widespread in all populations, its DNA can be occasionally found in normal oral mucosa and lesions that include oral cancer. However, controversy between many studies was found, where an EBV gene expression in oral cancer have or have not shown the viral protein expressions that are or are not associated with malignancy. So, it is difficult to predict the role of EBV in oral cancer pathogenesis (Wu et al., 2006; Griffith et al., 2007; Shillitoe, 2009).

A recent study has shown that HPV E7-protein increases the *de novo* methyltransferases enzymatic activity by directly interacting with DNMT1, which may be used to control cellular proliferation pathways (Burgers et al., 2006). Moreover, some tumour formation may be associated with HPV, HSV and EBV infections, even though the viruses' roles in causing the tumours need to be evaluated carefully.

2.5.3 Genetic instability

Genetic instability may be inherited or acquired thereby making a person more susceptible to oral cancer. Single Nucleotide polymorphisms (SNPs) in cytochrome P450 and glutathione S-transferase genes have been implicated in oral cancer (Hua et al., 2012). Polymorphisms of glutathione S-transferase genes (GSTM1, GSTT1 and CSTP1) are also found to be related to cancers by several studies (Dialyna et al., 2003; Hashibe et al., 2003; Srivastava et al., 2005; Hua et al., 2012). The function of this gene family involves metabolizing carcinogens, repairing DNA damage and controlling cell growth. Therefore, SNPs leads to changes in amino acid sequence, will cause malfunction of enzymes encoded by this gene family and consequently increase the risk for cancer.

2.5.4 Diet and nutrition

A diet with a low fruit and vegetable intake but rich in animal origin and fat is associated with increased risk for developing oral cancer (Edefonti et al., 2010). A study conducted by Petridou et al. (2002) observed that vegetables, fruits, micronutrients, dairy products and olive oil can play an important role in protecting cells against oral cancers. A meta-analysis demonstrated a 50% significant reduction of oral cancer risk after an additional daily serving of fruits and vegetables (Pavia et al., 2006).

Dietary factors that are involved in one-carbon metabolism, provides the interaction of nutrients and DNA methylation because they influence the supply of methyl groups, and therefore the biochemical pathways of methylation. These nutrients include folate, vitamin B 12, vitamin B 6, methionine, and choline (Davis & Uthus, 2004; Gilbert & Liu, 2010).

Folate, a water soluble vitamin, is a major component of the one-carbon metabolism. It cannot be synthesized *de novo* by mammals; therefore their cellular level depends on dietary intake. The folate has a capacity in modulating DNA methylation levels, which has a protective effect against colorectal carcinogenesis (Kim, 2004). However, it has become increasingly evident that folate possesses dual modulatory effects on colorectal carcinogenesis, which depend on the timing and dose of folate intervention. Furthermore, a study has shown that folate deficiency has an inhibitory effect, whilst folate supplementation promotes the progression of established colorectal neoplasms (Kim, 2007).

Methionine, an essential amino acid, is the main source for S-adenosylmethionine (SAM), the primary methyl group donor for most of the methylation reactions in the body (Selhub & Miller, 1992). It has been reported methionine supplement induced binding of methyl CpG binding protein 2 to reelin promoter as well as CpG hypermethylation in frontal cortex of a mouse, causes schizophrenia in methionine-induced mouse model (Dong et al., 2005)

2.5.5 Host defences

Certain viruses manage to find alternate ways to adapt to the host defence system by regulating their gene expression through modulation of DNA methylation; which thus allows a virus to silence its gene activation to favour its establishment of persistent infection and evades the host immune defence (Tao & Robertson, 2003). Tsai et al. (2002) also

demonstrated that the EBV oncogene product, latent membrane protein 1, inactivates E-cadherin gene expression through activation of DNA methyltransferases.

There is evidence of host defences against carcinogens or repair or defence mechanisms that include genetic, immune and dietary defects. The evidence associated to host defences with an increased risk of OSCC include organ transplantation, Fanconi anaemia and dyskeratosis congenita (Scully & Bagan, 2009).

2.5.6 Chemical carcinogenic substances

Chemical carcinogenic substances such as asbestos and coal tar can lead to genetic damage in normal cells which normally would undergo apoptosis. This carcinogenic exposure can cause genotoxic alterations in normal cells leading to irreversible cellular mutations if passed on during the next cell cycle. The mutated cells have a selective growth advantage leading to the development of a clonal population of neoplastic cells. A study revealed effects of oral carcinogenesis from carcinogens of coal tar, 20-methylcholanthrene, DMBA, and 4-NQO levels of polyamine synthesis as well as nucleolar organizing regions (NORs) were increased in animal models, along with the progression of oral carcinogenesis (Tanaka., 2011). In addition, a variety of compounds are considered to be epigenetic carcinogens as they result in an increased incidence of tumours; that may be caused by epigenetic changes in homeobox genes, DNA repair genes, and epigenetic regulatory enzymes (Zhou et al., 2012).

A recent study conducted by Tabish et al. (2012) on DNA damaging chemicals such as benzene, hydroquinone, styrene, carbon tetrachloride and trichloroethylene, found that these chemicals can cause DNA hypomethylation via oxidative stress pathways activation. Increased DNA methylation of p16 promoter was observed in exposures to the environmental

chemical pollutant, arsenite, where arseniasis patients were found to have higher p16 alteration when compared with people who did not have any history of arsenite exposure (Majumdar et al., 2010; Zhang et al., 2007).

2.6 Molecular alterations in cancers

The fundamental mechanisms involved in carcinogenesis are the overexpression of oncogenes and TSGs silencing. Promoter CpG hypermethylation is one of the epigenetic alterations that represents an alternative mechanism apart from genetic factors for silencing TSGs. It is also noticeable that epigenetic and genetic factors are often involved in multiple cellular pathways such as cell cycle regulation, DNA repair, apoptosis, angiogenesis, and cell-to-cell adhesion during the process of tumour growth and progression. Cell cycle control is disturbed particularly by oncogene over-expression or amplification which drives cell proliferation. TSGs on the other hand, are negative growth regulators involved in cell protection by regulating cellular trafficking, DNA damage response and apoptosis (Weinberg, 1991). A plethora of sequential accumulation of genetic and epigenetic defects in oncogenes and TSGs respectively, are involved in OSCC pathogenesis (Scully, 2011; Argiris et al., 2008; Saranath, 2000). Moreover, alterations in more than a hundred oncogenes and TSGs have now been implicated in various cancers (Hayslip & Montero, 2006; Stransky et al., 2011; Vogelstein & Kinzler, 2004).

2.6.1 Oncogenes

Generally, oncogenes are derived from function gains in cellular proto-oncogenes. Proto-oncogenes are normal genes that are involved in cell growth and division. Alterations or mutations, chromosomal rearrangement, and gene amplification in these genes lead to the development of oncogenes, which can promote or allow excessive and continuous cell

growth and division. Genes whose protein products have been found to be very important for normal cell growth signalling and whose over-expression or mutation leads to unchecked cell growth and carcinogenesis, are defined as “oncogenes”. Oncogenes are broadly divided into five categories. They are growth factors or growth factor receptors such as hst-1, int-2, EGFR/erbB, c-erbB-2/Her-2 and sis; intracellular signal transducers such as ras, raf and stat-3; transcription factors such as myc, fos, jun and c-myb; cell cycle regulators such as Cyclin D1, and those involved in apoptosis such as bcl-2 and Bax (Croce, 2008).

However, it has also been described that the proto-oncogenes transcription depression by hypomethylation that leads to increased mutation rates and causes chromosome instability, is an early hallmark of tumour cells (Eden et al., 2003; Mascolo et al., 2012).

2.6.2 Tumour suppressor genes

TSGs are genes that normally function in the growth control mechanism by regulating cell cycle arrest, apoptosis, cell adhesion, and DNA repair. TSG functions can be disturbed by several mechanisms that include point mutations, deletion, binding with cellular and viral proteins, or by TSG silencing from hypermethylation - all of which can lead to carcinogenesis (Ha & Califano, 2006). p53 is a TSG, which plays an important role in maintaining genome stability involving cell repair or apoptosis, and is implicated in a majority of malignancies, either spontaneous or inherited.

The loss functions of TSGs which often occurs in tumours, has been ascribed to be more frequently associated with epigenetic silencing through methylation rather than genetic defects in the OSCC (Jithesh et al., 2013). Additionally, gene hypermethylation correlates with transcriptional inactivation that can serve as an alternative repression to the promoter

region other than mutations for TSGs. Studies have shown that the silencing of TSGs is recognized as a vital role in cancer development (Jones & Baylin, 2002). An example of p16 methylation is the epigenetic inactivation of a TSG, which abrogates cell cycle control, escaping from senescence and then inducing cell proliferation that leads to the abnormal cell growth in SCC (Gonzalez et al., 1997; Rosas et al., 2001). Nearly 50% of the genes that cause familial forms of cancer when mutated in the germ line are known to undergo methylation-associated silencing in various sporadic forms of cancer. Similarly, there has been an increase in candidate of TSGs being identified to be associated with promoter hypermethylation silencing in certain cancers (Herman & Baylin, 2000). Several genes including p16/CDKN2A, DAPK1, MGMT, TIMP3, TCF21, RASSF1 and C/EBPalpha have been found to harbour hypermethylated regulatory sequences that could lead to the repression of gene expression or silencing in some cancers (Schmezer & Plass, 2008; Josena et al., 2011; Fukushige & Horii, 2013).

2.7 Epigenetic biomarkers for cancers

DNA methylation biomarker is defined as a molecular target that undergoes DNA methylation changes in carcinogenesis. Many genes show great specificity as DNA methylation biomarkers for early cancer diagnosis, prognosis, therapy response, and cancer recurrence detection (Fukushige & Horii, 2013). Usage of DNA methylation as a biomarker has been especially focused on early cancer detection. Furthermore, methylation markers are a stable target that allows flexibility for assay development. Therefore, gene promoter hypermethylation can be used not only as biomarkers for the early detection of HNSCC but also to improve prevention strategies and therapy outcomes (Schmezer & Plass, 2008).

A recent finding of one of the methylated genes, glutathione S-transferase gene (GSTP1), was detected in 80-90% of prostate cancer patients, but was not in benign hyperplastic tissues. Its detection specificity may be applied as a diagnostic marker for prostate cancer detection. Since specific CpG islands hypermethylation can be a cancer biomarker by applying various types of biological fluids and biopsy specimens, urine samples via a non-invasive approach may become a possible clinical application for the detection of gene methylation (Nakayama et al., 2004; Payne et al., 2009; Fukushige & Horii, 2013).

In addition, p16 or CDKN2A methylation has been used as a biomarker by using the sputum of smokers for early detection of lung cancer. The p16 hypermethylation has been associated with poorer outcome of colorectal cancer, and a similar finding of death associated Protein kinase (DAPK) was also evident in lung cancer (Brock et al., 2008; Model et al., 2007). Recently, ABHD9, AOX1 and RERG, novel genes with tumour specific DNA methylation in colorectal cancer were found using whole-genome methylation arrays (Øster et al., 2011).

There are currently very few studies that have been evaluated for DNA methylation as a biomarker for treatment response with DNMT and HDAC inhibitors. If these inhibitors manage to reactivate functions of TSGs, then DNA methylation may be used as a biomarker to predict treatment response with these epigenetic drugs. Application of detection of methylation level in plasma and serum can monitor for the established disease after cancer therapy (Sozzi et al., 2003). Thus, particular gene hypermethylation can be a predictor for cancer treatment response. Promoter hypermethylation of O 6-methylguanine-DNA methyltransferase (MGMT) is an independent predictor biomarker with a favorable outcome in glioblastoma patients treated with alkylating agents, carmustine (Weller et al., 2009).

Although many promising DNA methylation biomarkers have been identified for diagnostic purposes, their use in clinical practices is still presently very limited. This is due to lack of diagnostic specificity and sensitivity that is required in running a diagnostic test (Cottrell & Laird, 2003). Thus, panels of biomarkers may be needed in order to ensure sufficient test specificity and sensitivity. As such, the application of DNA-hypermethylation as tumour markers in routine clinical practice requires rapid, quantitative, accurate, and cost-effective techniques. Moreover, the objective criteria for gene selection in tissue-specific methylation suited to different tumour types are extensively required for methylation analysis (Tokumaru et al., 2004).

2.8 Cancer therapy

Surgery, radiotherapy, and chemotherapy are conventional treatment modalities of oral cancer, which are designed to stop the spread of cancer by killing cancerous cells. Unfortunately, due to unselective nature and toxicity effects of radiotherapy and chemotherapy, many of the body's healthy cells are also damaged. As a result, the most recent research of cancer treatment is now focused on the molecular biology of oral cancer in an attempt to target selected pathways involved in carcinogenesis.

With the increased understanding of molecular mechanisms and basic pathways in the pathogenesis of SCC, these pathways may be modified, and rational approaches in cancer therapy at the molecular level may be invented as molecular targeted therapy. Some of the new approaches depend on tumour biology and aim to specifically inhibit tumour growth and metastasis by targeting the tumour microenvironment or vasculature (Jain, 2005), or tend to focus on specific protein or signal transduction pathways (Adjei & Hidalgo, 2005). By blocking the signals that inhibit the cancerous cells to divide uncontrollably, targeted cancer

therapies aim to stop the growth and division of cancerous cells. One example is the usage of "signal-transduction inhibitors" to block receptors of specific enzymes on the cancer cell surface. In addition to that, "apoptosis-inducing drugs" may cause cancerous cells to undergo apoptosis by interfering with synthesis process and allowing the cancerous cell to die (Ghobrial et al., 2005). Another method includes the usage of "angiogenesis inhibitors" that can prevent the growth of new blood vessels in the surrounding tissue of solid tumour by not creating a new blood circulatory system to other parts of the body (Brannon-Peppas & Blanchette, 2012; Ferrara & Kerbel, 2005).

Current research related to molecular targeted therapies has shown that epigenetic pharmaceuticals could be a putative replacement or adjuvant therapy, and possibly enhance the effects of these current cancer treatments (Wang & Chiao, 2010). The epigenetic mechanism shows reversible effects on proto-oncogene and tumour suppressor sequences by conformational changes in histones, directly affecting cancer progression (Iglesias-Linares et al., 2010). The aim of epigenetic therapy is to reverse the causal epigenetic aberrations in cancer, leading to the restoration of normal epigenome. In particular, the most current molecular candidates for new drug targets that are capable of reversing aberrant DNA methylation and histone acetylation patterns by inhibiting histone acetyltransferasehistone, lysine methyltransferases, protein arginine methyltransferases and HDACs have been extensively explored (Dowden et al., 2010; Iglesias-Linares et al., 2010).

These types of cancer therapies may have a profound impact on personalized medicine that is based on molecular target selection of each individual patient's tumour biology (Jain, 2002). Eventually, these cancer therapies should be able to provide more selective treatment in

comparison to conventional cancer treatments, resulting in fewer normal cells being harmed, the reduction in treatment side effects, and overall quality of life improvement.

2.9 Epigenetics

Literally "epi- (Greek: over, above) genetics" incorporates mechanisms that regulate gene expression but do not alter the DNA sequence itself, leading to inheritable changes in the phenotype. Epigenetic processes include genomic imprinting (Hore, Rapkins, & Graves, 2007), gene silencing (Jones & Baylin, 2007; Lande-Diner et al., 2007), X-chromosome inactivation, nuclear reprogramming (Yang et al., 2007), and some elements of carcinogenesis (Jones & Baylin, 2007; O'Sullivan & Goggins, 2013). Epigenetic events play a vital role in multiple genes and cell signal processes, including DNA repair, cell cycle, carcinogen metabolism, intercellular responses, apoptosis and angiogenesis. The epigenetic alterations refer to any heritable modifications in gene expression without alterations or coded of the DNA sequence. They do not affect the underlying base-pair sequence, whereas genetic aberrations change the expression by altering the sequence of adenine–thymine and cytosine–guanine base pairs (Shaw et al., 2007). They occur more frequently than gene mutations and may persist for the entire cell life and even for multiple generations (Kyrgidis et al., 2010). DNA methylation is an epigenetic phenomenon that has dramatic effects on gene expression and this process is now widely recognized as either a causative or correlative event in carcinogenesis (Baylin, 2005). It involves all or even only a few of the CpG islands may result in a closed chromatin structure and consequently in silencing the transcription of the gene (Ahuja & Issa, 2000).

Epigenetic changes are dynamic and reversible processes. Epigenetic takes part in facilitating the wide diversity of cell types. Besides its physiologic function, epigenetic changes can appear aberrantly during aging and development causing expression disequilibrium which may lead to pathologies. The classification of epigenetic modification which occurs in human cells is known as DNA methylation, histone deacetylation, and microRNAs.

2.9.1 Significance of epigenetic studies

Epigenetic alteration represents an alternative mechanism in carcinogenesis, as opposed to genetic factors, such as gene mutations and deletion, by inactivating TSGs. Both epigenetic and genetic factors often work together in affecting multiple cellular pathways in cell-cycle regulation (p14, p15, p16), DNA repair (Hmlh1, BRCA1, MGMT)), apoptosis (DAPK, APAF-1), cell-to-cell adhesion (CDH1, CDH3) and carcinogen metabolism (GSTP1) which leads to tumour progression (Momparler, 2003; Fan, 2004; Rodríguez-Paredes & Esteller, 2011). Furthermore, a tumour-type specific profile of promoter hypermethylation exists in particular cancers that allow these hypermethylation genes to be used as biomarkers for malignancy.

Epigenetic changes may occur due to environmental factors, aging, and genomic imprinting and disease. An attractive aspect to the study of epigenomic dysregulation in disease is that the epigenetic changes should be reversible, whereas genetic mutations are difficult to complement with gene therapy approaches. Nowadays, the available drugs which are globally applied include DNMTs and histone deacetylase inhibitors (Hellebrekers et al., 2006); but the disease changes might be limited to a subset of the genome. Research areas recognized for future work on epigenetics include: (a) basic epigenetic mechanisms in cancer need further investigation; (b) technology development in the area of epigenetics, such as high-throughput

quantitative assays and increased sensitivity or specificity is essential for the early detection and risk assessment of cancer; (c) clinical application of epigenetic changes to cancer prevention and risk assessment needs further investigation.

2.9.2 Epigenetic interaction

2.9.2.1 Epigenetics and aging

Epigenetic aberrations such as global hypomethylation potentially decreases and promoter hypermethylation will increase as people age (Issa, 2012). Animal studies showed loss of genomic imprinting (Bennett-Baker et al., 2003) and age-related reactivation X-linked chromosome gene (Wareham et al., 1987). During the aging process of monozygotic twins, differential patterns of DNA methylation and histone modifications were observed in the study (Fraga et al., 2005).

2.9.2.2 Epigenetics and gender

Susceptibility of epigenetic alterations may be influenced by gender, even though the involved mechanisms are not fully understood. The female gender has been reported to be highly related with CDKN2A gene in colorectal cancer (Lind et al., 2004) and CDG1 in lung cancer (Vaissière et al., 2009). Controversially, gender has a protective effect with RASSF1A and ESR1 in lung cancer (Lai et al., 2005). In addition, the female gender has also been associated with a low level of global hypomethylation in HNSCC (Hsiung et al., 2007).

2.9.2.3 Epigenetics and diets

Diet has been implicated in many pathways involved in carcinogenesis including DNA methylation. Epigenetic events constitute an important mechanism by which dietary bioactive components can selectively activate or inactivate gene expression that leads to cancer

susceptibility. The most compelling is nutrients involved in one-carbon metabolism due to the facts that methyl (CH_3-) groups for DNA methylation are derived from one-carbon methyl donors. This suggests an intrinsic link between one-carbon nutrients and epigenetic alterations. Dietary compounds important for the one-carbon metabolism include folate, methionine, and s vitamin B6 and vitamin 12 (Davis & Uthus, 2004; Gilbert & Liu, 2010). Low folate intake has been related to an increased incidence of colorectal cancer (Choi & Mason, 2002). Molecular changes in tumour cells in relation to folate intake may provide insights into the role of one-carbon metabolism in carcinogenesis (Choi & Mason, 2002; Giovannucci, 2002). In addition, one study observed that soy phytoestrogens may have cancer-protective effect by preservation of normal methylation pattern (Davis & Uthus, 2004). Other investigations conducted on green tea (epigallocatechin-3-gallate (EGCG)) showed that polyphenols affect DNA methylation by binding and inhibiting DNMTs activity, resulting in the reactivation of methylation-silenced TSG in human skin cancer cells. Thus, epigenetic mechanism of action of EGCG may contribute to the chemoprevention of skin cancer (Nandakumar et al., 2011).

2.9.3 DNA methylation

The epigenetic alteration most studied in the human cancer cell is DNA methylation. The number of genes with aberrant methylation in the cancer cell is still unknown, but it is estimated that approximately 5% (approximately 1,500–2,000) of the human genome can be aberrantly methylated in a cancer cell (Schuebel et al., 2007).

DNA methylation is a post-replicative modification of the DNA molecule itself, in which methyl groups are added to cytosine nucleotides in specific areas of the gene by the enzyme DNA methyltransferase (Herman & Baylin, 2003). DNA methylation directly switches off

gene expression by preventing the binding of transcription factors in the genes. DNA methylation regulates gene expression either directly or by influencing the histone modification that influences gene expression.

2.9.3.1 DNA methylation mechanism

DNA methylation is a heritable, tissue- and species-specific modification of mammalian DNA (Cross & Bird, 1995). Two different DNA methylation mechanisms are known as the *de novo* methylation (Fraga & Esteller, 2007) and maintenance methylation (Chao & D'Amore, 2008; Church & Pruitt, 2002) (Figure 2.1). Firstly, the *de novo* methylation is an additive process of methyl group to an unmethylated cytosine as cells divide and age (Fraga & Esteller, 2007). Such epigenetic modifications may be due to a mutation at the DNA level (for example, a point mutation that changes a CG pair to GG), or they may develop in response to nutrients such as folate and vitamin B12, and environmental factors such as toxins, infections and hypoxia (Bayarsaihan et al., 2010).

The phenomenon of epigenetic change over time was recently demonstrated in a study of 40 pairs of monozygotic twins by quantifying genome-wide methylation (Fraga et al., 2005). Fraga et al. (2005) found that epigenetic differences between older twin pairs were significantly greater than those between younger twins. Secondly the maintenance methylation is inheritance of DNA methylation patterns during cell division, involving a copying process of pre-existing methylation patterns from mother strand to a newly synthesized DNA strand. The maintenance methylation is to ensure conservation of methylation pattern during replication process. Aberrant patterns of the *de novo* methylation can lead to disease and various cancers by altering the gene expression in a given cell type at

a given time. For example, a silenced proto-oncogene losing its methylation status or an unmethylated TSG being silenced can both lead to carcinogenesis.

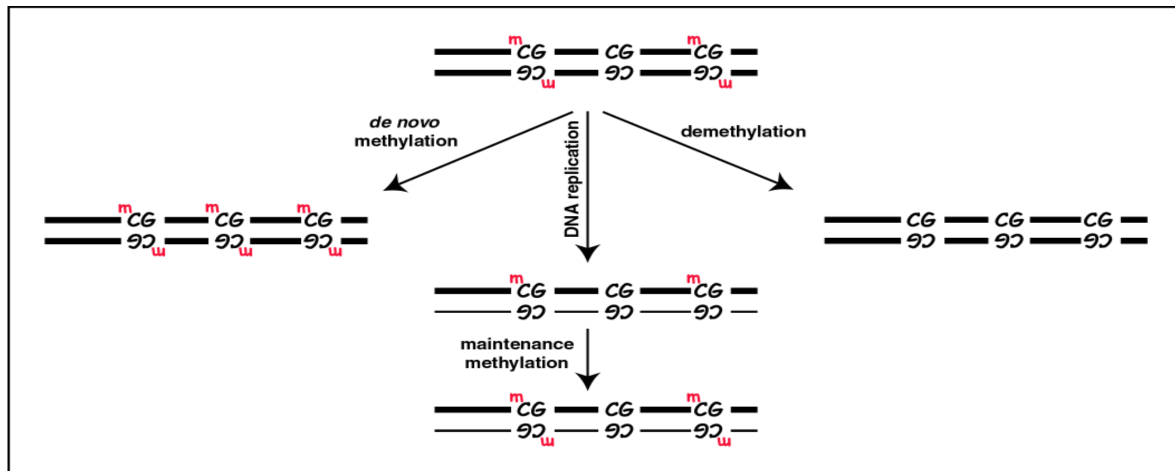


Figure 2.1 Illustration of de novo methylation and maintenance methylation processes. In vertebrates DNA methylation occurs mainly in CpG dinucleotides depicted here as CG. Methyl residues are depicted as ‘m’. De novo methylation is the introduction of methyl groups at previously unmethylated cytosines. Existing methylation pattern can be erased by demethylation. Maintenance methylation ensures conservation of the methylation pattern during replication by copying the methylation pattern from the mother strand to the daughter stand (Easwaran, 2003).

2.9.4 CpG islands

Few decades have passed since Riggs, Holliday and Pugh proposed that cytosine DNA methylation in eukaryotes could act as a stably inherited modification affecting gene regulation and cellular differentiation and transmitting the silenced state to daughter cells (Holliday & Pugh, 1975; Riggs, 2008). Cytosine methylation is one of the most extensively studied epigenetic processes. In the mammalian genome, DNA methylation plays a vital role in ensuring the accurate epigenetic inheritance; its process takes place only at 5′ -cytosine preceding to a guanosine base in a CpG dinucleotide (Bird, 2002). In view of this dinucleotide is actually underrepresented in most of the genomic regions, but short regions of

0.5–4 kb in length, known as CpG islands, are rich in CpG content (Bird, 2002; Takai & Jones, 2002). The CpG islands are mostly found in the transcriptionally inactive proximal promoter regions of most of the genes and normally are unmethylated in normal cells (Figure 2.2) or even heterochromatin sections of the genome, suggesting that CpG island methylation is correlated with DNA compaction. Methylation of CpG islands is often associated with delayed replication, condensed chromatin and transcription initiation inhibition. Initial sequencing and analysis of the human genome conducted by the International Human Genome Sequencing Consortium for CpG islands predicted that a total of 28,890 CpG islands is present in a mammalian genome, with 70-80% of CpG dinucleotides are methylated (Lander et al., 2001). However, in 5′ promoter regions of approximately 60% of CpG islands can be found. Therefore, it is speculated that CpG islands can overlap with the promoter region and even extend into exonic regions, with the exception of X-chromosomally inactivated genes, imprinted genes and tissue-specifically expressed genes that are generally unmethylated (Gardiner-Garden & Frommer, 1987).

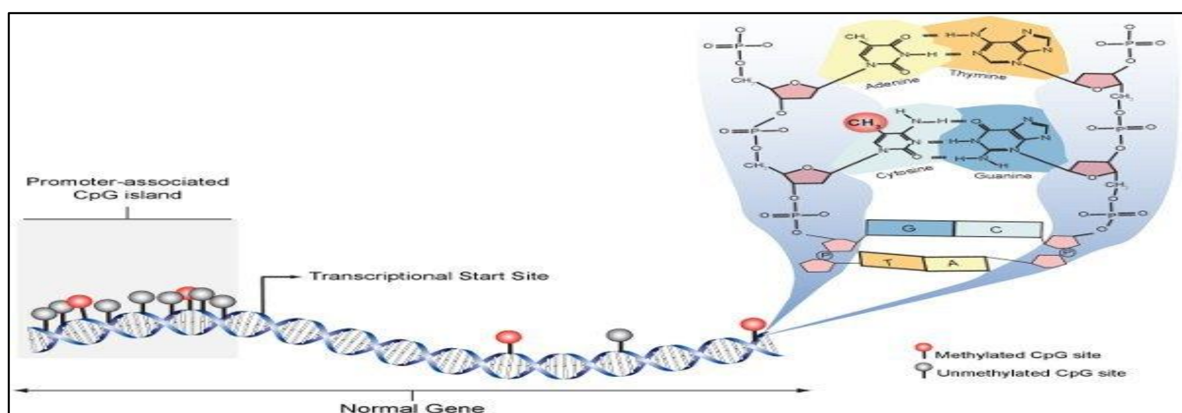


Figure 2.2 Methylated CpG schematic. In the normal cell, promoter-associated CpG islands are predominantly unmethylated (grey) whereas CpG sites within gene bodies are sparse and generally methylated (red). The panel on the right expands the molecular structure of DNA at an individual CpG site and shows methylation with a CH3 molecule at carbon 5 of cytosine (Patterson et al., 2011).

2.9.4.1 CpG methylation

DNA methylation is a transfer process of a methyl group (-CH3) from a universal methyl group donor called S-Adenosyl-L-Methionin (SAM) to a 5' carbon of cytosine bases (Lieber & Packer, 2002) (Figure 2.3) by methyltransferase I and II. SAM, an important methyl group donor in various biochemical reactions, is derived from the amino acid L-methionine (Williams et al., 2005).

CpG islands of growth-regulatory gene promoter regions have often found hypermethylated in tumours, an event causing the transcriptional "silencing" of TSGs (Bonazzi et al., 2009), thus contributing to carcinogenesis. Schuebel et al. (2007) reported that 5% of known gene promoters have aberrant methylation in a typical solid tumour. In addition, aberrant CpG hypermethylation due to DNMTs overexpression are attributed by down regulation of TSGs. A recent research reported some genes hypermethylation correlates with increased DNTM3B levels in colorectal tumours (Ibrahim et al., 2011). These genes are predicted to be essential for carcinogenesis based on their presumed functions which otherwise seemed not to be frequently mutated in such cancers (Jones & Baylin, 2002).

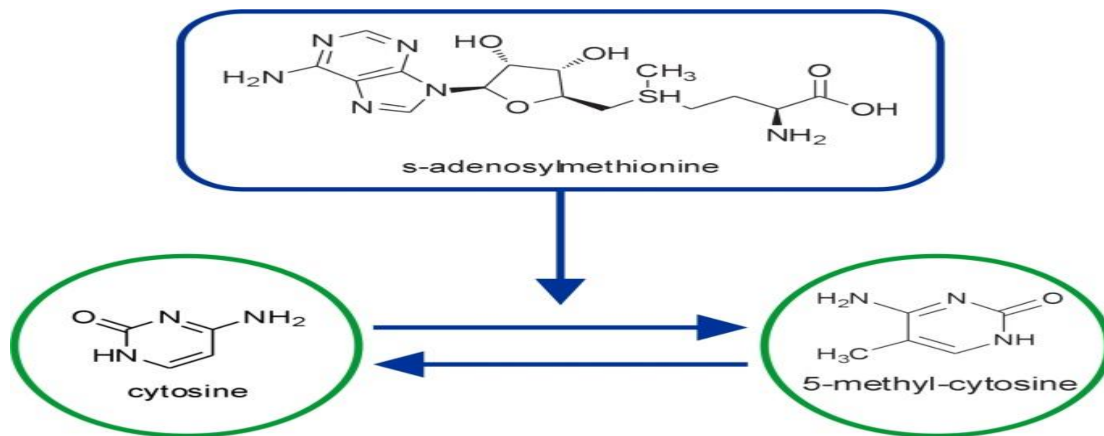


Figure 2.3 Methylation of cytosine. The donor of the methyl group is the active form of methionine, S-adenosyl-L-methionine (SAM) and its addition to cytosine is realised at the carbon-5 position (Lieber & Packer, 2002).

2.9.4.2 DNA nucleotide methyltransferases

DNA methylation is a key enzymatic process mediated by DNMTs. In mammals, three major DNMTs, namely DNMT1, DNMT3a, DNMT3b (Figure 2.4) are involved in covalently transferring a methyl group to the C5 position of cytosine residues, by changing the DNA conformation (Cheng & Blumenthal, 2008; Rottach et al., 2009). All mammalian DNMTs are encoded by their own single gene, and consist of catalytic and regulatory regions except DNMT2 (Xu et al., 2010).

DNMT1 interact with methylated or unmethylated CpG dinucleotides during chromosome replication (Chen & Li, 2006) and DNA repair (Mortusewicz et al., 2005) in maintaining established methylation pattern or *de novo* methylation. DNMT1 has a 30- to 40-fold preference for hemimethylated daughter strands during DNA replication to maintain the established methylation patterns across successive cell divisions.

DNMT2 appears to provide an example of divergent evolution: it was named based on its high sequence and structural similarity to known DNA DNMT. However, it does not methylate DNA but methylates cytosine 38 in the anticodon loop of tRNA aspartic acid methyltranferase (Goll et al., 2006; Rai et al., 2007). DNMT2 (TRDmt1) is the first RNA methyltransferase to be identified in humans. It is the most widely conserved DNMT protein with homologues in plants, fungi, and animals (Rai et al., 2007).

Two of active *de novo* DNMTs, DNMT3a and DNMT3b, and one regulatory factor, DNMT3-Like protein (DNMT3L) belong to the DNMT3 family. The DNMT3a and DNMT3b have similar domain arrangements: both contain a variable region at the N terminus, a Cys-rich 3-Zn-binding domain, and a C-terminal catalytic domain. DNMT3a and DNMT3b are essential for the *de novo* methylation during cell development by methylating previously unmodified CpG residues to methylated CpG residues, but can lead to aberrant methylation in cancer cells (Fabbri et al., 2007). They also interact with miRNA in maintaining global gene expression patterns (Veeck & Esteller, 2010). DNMT3L is a protein which is homologous to the other two DNMTs but has no intrinsic DNA DNMTs catalytic activity (Lukasik et al., 2006). It is physically associated with DNMT3a and DNMT3b by assisting them in DNA binding ability and modulating their catalytic activity.

Interestingly, a recent study has proposed that DNMTs are not only involved in CpG methylation, but also activates demethylation of 5-methyl CpGs through deamination and DNA glycosylase, and base excision repair proteins (Wu & Zhang, 2010).

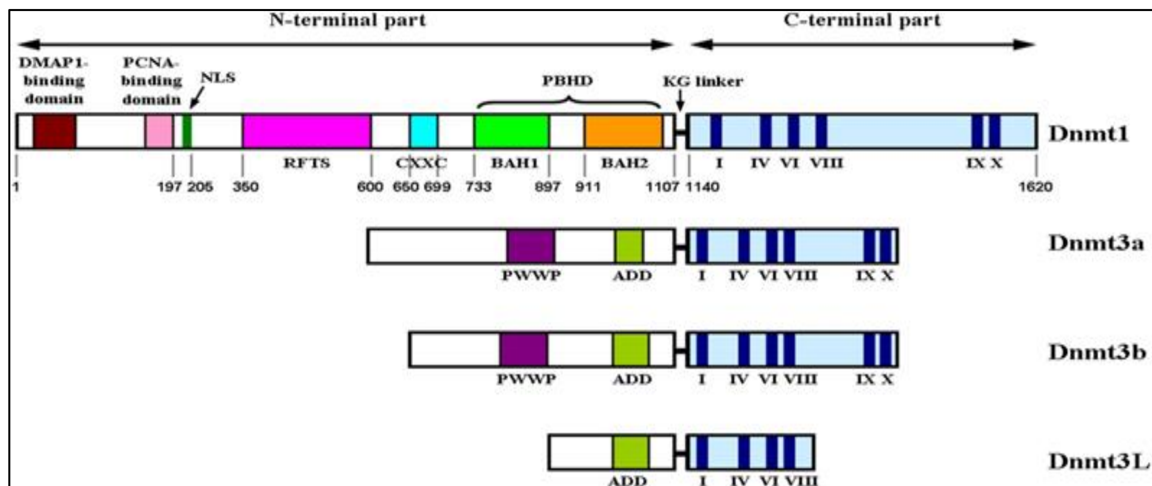


Figure 2.4 Schematic drawing_of known members of the DNMT super-family are summarized. DNMT 1 is essential for maintenance methylation. Function of DNMT 2 is so far unknown. DNMT 3A and DNMT3B are essential for de novo methylation. DNMT 3L is a co-factor protein for DNMT 3A and DNMT 3B (Cheng & Blumenthal, 2008).

2.9.5 Effects of DNA methylation on gene transcription

The overall methylation state in a cell might also be a precipitating factor in carcinogenesis as evidence suggests that genome-wide hypomethylation can lead to chromosome instability and increased mutation rates (Baylin et al., 2001). An epigenetic pathway of transcriptional inactivation for many TSGs has also been identified in many different cancers especially on promoter hypermethylation in OSCC studies (Balmain et al., 2003; Ha & Califano, 2006; Herman & Baylin, 2003; Manel Esteller, 2011).

Recently, hypermethylation of CpG dinucleotides has been identified as being involved in the TSGs inactivation. Its involvement in all or even a few of the CpG islands may result in a closed chromatin structure and consequently in silencing the transcription of the gene (Ahuja & Issa, 2000; Baylin et al., 2001). A number of studies have shown that TSGs silencing is recognized as a vital role for developing cancer. This hypermethylation correlates with

transcriptional inactivation that can serve as an alternative repression in the promoter region other than mutations in TSGs (Ahuja & Issa, 2000; Baylin et al., 2001; Esteller & Herman, 2002; Herman & Baylin, 2003). It is observed in most of neoplasm and is associated with the inappropriate transcriptional silencing of genes (Baylin et al., 2001; Jones & Baylin, 2002). Razin (1998) has demonstrated that the gene silencing effect on methylated regions is associated with the interaction of 5-MCs binding proteins with other components of chromatin. The interaction makes the DNA inaccessible to transcription factors through the changes of histone deacetylation and chromatin structure (Bestor, 1998). Surprisingly, such promoter hypermethylation is at least and possibly more common as the disruption of classic TSGs in human cancer by mutation.

2.10 Histone modifications

Histones are basic proteins consisting of a global domain and histone tail, which protrude out of the nucleosome. DNA is wrapped around an octamer of histones consisting of a histone 3 and histone 4 tetramer, and two histone 2A and histone 2B dimers. This structure is called a nucleosome, the basic unit of building block of chromatin (Luger et al., 1997). These histone tails are subject to post-translational modifications including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, as well as lysine ubiquitination and sumoylation (Yang & Seto, 2008). These modification patterns on histone tails are involved in gene regulation through the histone amino-terminal tails protruding from the surface of the nucleosome and on the globular core region (Margueron et al., 2005).

2.11 High throughput methylation analysis

The need for genome-wide techniques has apparently increased due to the great demand of possible targets for molecular-targeted therapies. Techniques such as the methylated DNA

immunoprecipitation combined with microarray analyses (MeDIP-chip), or approaches with epigenetically active drugs combined with gene expression microarrays which allow the analysis of ~29.000 genes at a time, have become available over the last few decades (Babinsky, 2011). Nowadays, multiple advancements in sequencing and microarray technologies of high throughput methylation methods have enabled many tools to be developed for large-scale methylation screening (O'Sullivan & Goggins, 2013). The following points list out some of the advanced tools available nowadays in the market:

1. Utility of fragments cloned from CpG island libraries (Estécio et al., 2007).
2. Bead based array-like (Bibikova et al., 2006).
3. DNA precipitation with methyl-binding proteins or antibodies that recognize methyl cystosine (ChIP-chip) (Weber et al., 2005; Zhang et al., 2006).
4. Methylation sensitive restriction endocleases with or without fractionation of the genome (Adrien et al., 2006; Balog et al., 2002).
5. Generation sequencers such as the Roche Genome Sequencer FLX (Taylor et al., 2007).
6. Hpall tiny fragment Enrichment by Ligation-mediated PCR (HELP) assay (Khulan et al., 2006).
7. MASS utilizes enzyme McrBC (Ibrahim et al., 2006)
8. Modification method of HELP and MASS (Kamalakaran et al., 2009).
9. Representational oligonucleotide microarray analysis (ROMA) (Lucito et al., 2003).

The above mentioned tools have their own implications and advantages in technological usages. DNA microarray and next generation sequencing are the most reliable and informative methods applied today (Lippman et al., 2005).

2.11.1 Microarray

The availability of microarray technology has encouraged researchers to carry out extensive studies using data available on the entire known human genome, and these studies have reported more than one hundred gene alterations are implicated in carcinogenesis. During the past few years, three major types of microarrays using tissue, protein and DNA platforms have been developed (Angenendt, 2005; Nazmul-Hossain et al., 2008). Tissue microarray technology has been developed in an effort to overcome the limitations of standard histologic methods. It immobilizes small amounts of biopsy tissues from multiple subjects on glass slides for IHC processing (Shergill et al., 2004). Protein microarrays, peptides or intact proteins are immobilized for detection by antibodies. Applications of protein microarrays include assessment of enzyme–substrate, protein–protein and DNA-protein interactions (Angenendt et al., 2003). DNA microarrays are the most widely utilized applications of microarray technology, where thousands to tens of thousands of data points may be generated in each experiment.

In epigenetic application, hundreds of thousands of SNPs can be genotyped, or genomic applications carried out for which mRNA transcripts are interrogated as a measure of gene expression. Nowadays, the continuous utility of the novel genomics tools, DNA microarrays are increasing due to their ability to incorporate a large number of the genes into a single assay, turning into promising tools for the genome-wide analysis of transcripts.

2.11.2 DNA methylation microarrays

Profiling DNA methylation across the genome is vital to understand the influence of epigenetics especially DNA methylation for normal biology and disease. There has been a revolution in DNA methylation analysis technology over the past decade where analyses that

were previously restricted to specific loci can now be performed on a genome-scale and entire methylomes can be characterized at single-base-pair resolution. Genome-wide methylation assays were developed mostly based on two platforms. The first platform is based on pharmacological reactivation of epigenetically regulated genes combined with gene expression microarray analysis whilst the second platform is comprised of microarray analyses of DNA fragments after immunoprecipitation of methylated DNA fragments.

DNA microarrays have been developed and include cDNA microarray, high-density oligonucleotide microarrays, bead-based microarray, and microelectronic arrays (Roy et al., 2002). A bead-based microarray platform depending on genotyping of bisulfite-converted genomic DNA shows a high level of assay multiplexing, and scalable automation for sample handling and data processing.

A quality control study has been conducted for inter-laboratory reproducibility, inter-platform and technical reliability of DNA microarray using breast cancer samples. These reliability tests have made it possible for molecular tests to become an important tool in tailoring cancer treatment and anticancer drug responses for personalized cancer medicine management (van't Veer & Bernards, 2008).

2.11.3 Cytosine microarray

One of the DNA methylation microarrays is a 450K cytosine microarray, which is an alternative option to determine a genome-wide DNA methylation profile. The latest HumanMethylation 450 beadchip assay, Illumina, Infinium Methylation 450K covers 99% of all reference sequences (RefSeq) genes and approximately 450,000 CpGs overall, is for the high-resolution, genome-wide DNA methylation profiling of human samples to be carried out.

This microarray analysis includes CpG and CNG sites, CpG islands, shores (the 2 kb flanking the CpG islands), shelves (the 2kb flanking the shores), open sea, non-coding RNA (microRNAs and long non-coding RNAs) and sites surrounding the transcription start sites (-200 bp to -1,500 bp, 5'-UTRs and exons 1) for coding genes, but also for the corresponding gene bodies and 3'-UTRs (Sandoval et al., 2011). If compared with sequencing approaches, DNA methylation arrays are a low-cost alternative, which allows the profiling of a large number of samples, although this occurs at a reduced resolution.

2.11.4 Microarray application in oral cancers

A variety of applications of microarrays in oral cancer identification include the early diagnosis of oral potentially malignant disorders with high transformation potential, the identification of malignancy in tissue biopsies and comparison of genetic alterations at different pathological stages. Applications are also in the sub-classification of histologically identified tumours, the identification of biomarkers and prognosticators, and the area drug discovery (Bibikova et al., 2009; Ramaswamy & Golub, 2002; van't Veer et al., 2002). The usage of microarrays is particularly important as additional information to complete clinical information for accurate cancer diagnosis. This is to ensure that cancer patients receive appropriate treatment.

In oral potentially malignant disorders such as leukoplakias and erythroplakias, microarrays have been used to identify genes that could serve as biomarkers for dysplastic lesions which have the potential to progress to cancer (Carinci et al., 2005). By comparison of normal tissues with histopathologically classified potentially malignant or malignant lesions, these studies reveal that aberrant methylation can begin very early in tumour progression (Baylin et al., 2001). Most of the important pathway abnormalities in cancer include the loss of cell

cycle regulation, altered function of transcription factors, altered receptor function, disruption of cell adhesion or motility, inactivation of signal transduction pathways, loss of apoptotic signals and lastly genetic instability (Baylin, 2005). Characterization of molecular pathways that are dysregulated leading to malignant transformations can be targeted for early diagnosis and therapy.

2.12 Methylation analysis

DNA methylation patterns are established early in development, modulated during tissue specific differentiation, and disrupted in diseases including cancer. To understand the biological role of DNA methylation and its role in human disease, precise, efficient and reproducible methods are required to detect and quantify individual 5-MCs in CpG dinucleotides. The bisulphite conversion protocol is a gold standard for DNA methylation analysis, which facilitates DNA methylation identification and quantification at single nucleotide resolution.

2.12.1 Bisulfite-modification based method

Chemistry of cytosine deamination by sodium bisulphite is involved in steps of sulphonation, hydrolic deamination and alkali desulphonation. Sulphonation involves the addition of bisulphite to the 5-6 double bond of cytosine. Hydrolic deamination is a hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative. Lastly, alkali desulphonation is a removal of the sulphonate group by an alkali treatment to give a uracil in bisulfite-modification process (Figure 2.5) (Patterson et al., 2011).

In a brief for the bisulfite-modification process, sodium bisulfite deaminated unmethylated cytosine that is situated in single-stranded DNA and sodium 5, 6-dihydrocytosine-6 sulphonate forms at a low pH. When pH rises to basic conditions, sodium bisulfite will degrade and unmethylated cytosine will transform into uracil (Piperi & Papavassiliou, 2011). Bisulfite preferentially deaminates cytosine to uracil in a single stranded DNA - 5-MCs are refractory to bisulphite-mediated deamination. Upon PCR amplification, uracil is amplified as thymine while 5-MCs residues remain as cytosines, allowing methylated CpGs to be distinguished from unmethylated CpGs by the presence of a cytosine (C) versus thymine (T) residue during sequencing. These reaction conditions result in the complete conversion of approximately 99.5-99.7% of every target DNA sequence (Frommer et al., 1992).

The resultant products with converted single strand DNA will replace the uracil to thymine and can be analysed by various techniques such as MSPCR, bisulfite sequencing, real-time PCR, combined bisulfite restriction analysis (COBRA), methylation-sensitive single nucleotide primer extension (MS-SNuPE), microarrays, pyrosequencing and the most recent developed technique of quantitative real-time PCR, methylation-sensitive high-resolution melting (MS-HRM) (O'Sullivan & Goggins, 2013). All methods share the same procedure of modifying DNA with sodium bisulfite as the first step and subsequently PCR amplification with primers specific for modified DNA. This technique has a higher sensitivity and specificity if compared with non bisulfite-modification techniques (Shapiro et al., 1974; Susan et al., 1994).

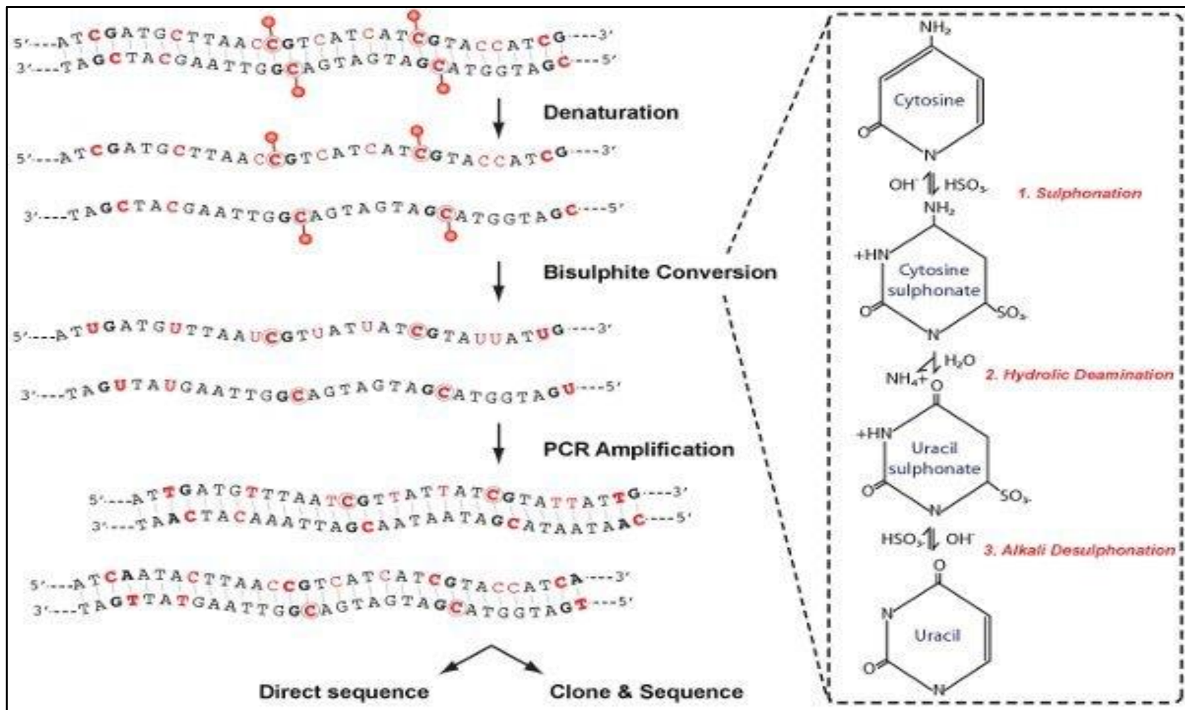


Figure 2.5 Schematic chemical conversion of DNA methylation analysis. Analysis of DNA methylation includes four main stages as shown; denaturation, bisulphite conversion, PCR amplification and analysis. In the right panel, modifications to the cytosine molecule that occur during bisulphite conversion of sulphonation, hydrolic deamination and alkali desulphonation are depicted (Patterson et al., 2011).

2.12.2 Gene-specific methylation analysis

Recently, two major techniques involved in research of gene-specific in methylation status are the bisulfite-modification based method, and the methylation-sensitive restriction enzymes. Most of the techniques used to detect DNA methylation are based on PCR methods, and are therefore extremely sensitive. DNA methylation can be analysed easily by qualitative or quantitative polymerase chain reaction (PCR)-based methods. In the bisulphite modification-PCR amplification approaches, quantitative MSPCR and Methyl-Light mainly rely on a methylation change at the PCR primer binding site (and or Taqman probe site for quantitative PCR). As a result, these tests are prone to false-negative results where the gene

promoter might have been methylated (but not at the primer binding site). Bisulfite sequencing is comparatively time-consuming if compared with others.

Methylation-sensitive restriction enzyme PCR technique combines methylation-sensitive restriction enzyme digestion and PCR. After enzyme digestion, PCR products are obtained if the enzyme does not digest the methylated CpG sites within the specified DNA region (Galm & Herman, 2005; Wong, 2006)**.** Examples of PCR products are COBRA, Ms-SNuPE, and quantitative real-time MS-HRM that allows the quantitative analyses of DNA methylation.

2.12.3 Methylation-specific polymerase chain reaction (MSPCR)

MSPCR is a rapid, cost-effective, highly sensitive method that can specifically assess the methylation status of virtually any group of CpG sites within a CpG island. It is independent of methylation-sensitive restriction enzymes or radioactive reagent usage. This assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated cytosines to uracils whilst leaving the methylated cytosines remains unmodified, and is lastly followed by subsequent amplification with primers specific for methylated versus unmethylated DNA (Wong, 2006). This method requires specific PCR primers that are designed to distinguish between methylated and unmethylated DNA sequences.

The great sensitivity of this technique allows qualitative methylation analysis from DNA obtained not only from fresh frozen tissues, peripheral blood, bone marrow, or body fluids but also from paraffin-embedded samples. Although MSPCR is a simple, sensitive and specific method for determining the methylation status of virtually any CpG-rich region, one of its limitations is that it exclusively detects the methylation status within two short sequences targeted by the MSPCR primer. If the incomplete converted sequence occurs in

bisulfite-converted DNA, a false possible result may be generated. MSPCR primers are designed to flank one or more of the CpG sites, or is near the 3’ end. This condition makes the primers more selective for methylated template. The specificity and sensitivity of primers are significantly related to primer design. The advantages of this technique include shorter duration and that only small amounts of DNA are required for methylation status detection (Galm & Herman, 2005; Wong, 2006).

2.12.4 Methylation-sensitive restriction enzymes methods

Restriction-sensitive enzymes are used to digest cytosine in unmethylated DNA sequences whilst the methylated sequences are left untouched. This method does not change the DNA sequences. Disadvantages of this method include the very limited amount of potential unmethylated site that can be probed, and false positive results could occur if there is incomplete digestion in enzymatic treatment occurs.

2.12.5 Methylation-sensitive high-resolution melting (MS-HRM)

PCR-based methylation detection applications led to the development of the MS-HRM technique. MS-HRM technology is being increasingly applied in research laboratories and has the potential for future application in diagnostic settings. The MS-HRM analysis is a recently developed technique that has an enormous potential for detecting DNA sequence changes. This temperature gradient analysis was to identify the methylation level of the DNA sequence over a period of 10 years; where methylated and unmethylated sequences generate different sequences after bisulfite treatment, resulting in a significant change in the melting curve between methylated and unmethylated PCR products. The methylation status can be estimated by comparing the melting profiles of unknown PCR products to melting profiles of PCR products from a serial standard DNA mixture of methylated and unmethylated templates

(Wojdacz & Dobrovic, 2007). It provides a sensitive detection of methylation status in a labour- and cost-efficient manner (Wojdacz & Dobrovic, 2007). Moreover, it investigates the methylation status of imprinted loci as well as the identification of heterogeneous methylation (Galm & Herman, 2005). Recent new instruments combined with DNA intercalating dyes, for example, EvanGreen that can be used at saturating concentrations allowing the discrimination of sequence changes in PCR amplicons without manual handling separately for PCR products. The recent application of MS-HRM to mutation scanning and SNP genotyping as well as DNA methylation studies have been demonstrated (Wojdacz & Dobrovic, 2007).

2.13 Immunohistochemistry

IHC was established by Coons and Jones for detecting bacteria using an immunofluoresent technique since 1941 (Coons et al., 1941). IHC became the standard tool in diagnostic pathology and research for the detection of protein expression in the late 1970s. The problem of low reproducibility and standardization of the IHC technique, especially in the fixative used for pretreatment of specimens, detection methods, and result interpretation still remain unsolved (Wester et al., 2000; Cregger et al., 2006). Furthermore, it may be impossible to standardize all potential variables in IHC. Nevertheless, the interpretation of IHC results may be standardized through the usage of new quantitative methods where the inherent subjectivity of the assessment of in-situ protein concentration is overcome by quantifying the protein concentration. Thus, more objective quantitative scoring methods using automated systems in the analysis of IHC have been implemented recently (Levsky & Singer, 2003; Turbin et al., 2008; Rexhepaj et al., 2008).

Chromogenic or fluorescence techniques are widely used in quantitative histochemistry. The end-product of immunostaining will be deposited at the site of the antigen. The antigen may be located in a specific cellular location, such as nuclear, organellular, cytoplasmic, membranous, or extracellular locations. After photographic capture, the reaction product may be quantified by image-analysis software (Latham et al., 1996; Levsky & Singer, 2003).

2.13.1 Technical aspect of immunohistochemistry

IHC techniques involve a serial of processes, beginning with antigen retrieval to unmask antigens hidden by formalin cross-link or other fixations using pressure cooking, protease treatment, microwaving or heating techniques (Shi et al.,1997; Shi et al. 2001). The first definite step of IHC is the primary antibody incubation step following antigen retrieval treatment. Then, a specific secondary antibody that is tagged with biotin and horseradish peroxidase is added. Lastly, a detection reagent, a chromogen or fluorescent tagged molecule is applied for visualizing of the primary antibody (Polak & Van Noorden, 1997).

CHAPTER 3 MATERIALS AND METHODS

3.1 Study design

A cross sectional study was conducted using archived samples to analyse the methylation profiling of OSCC. A workflow of this study is depicted in Figure 3.1

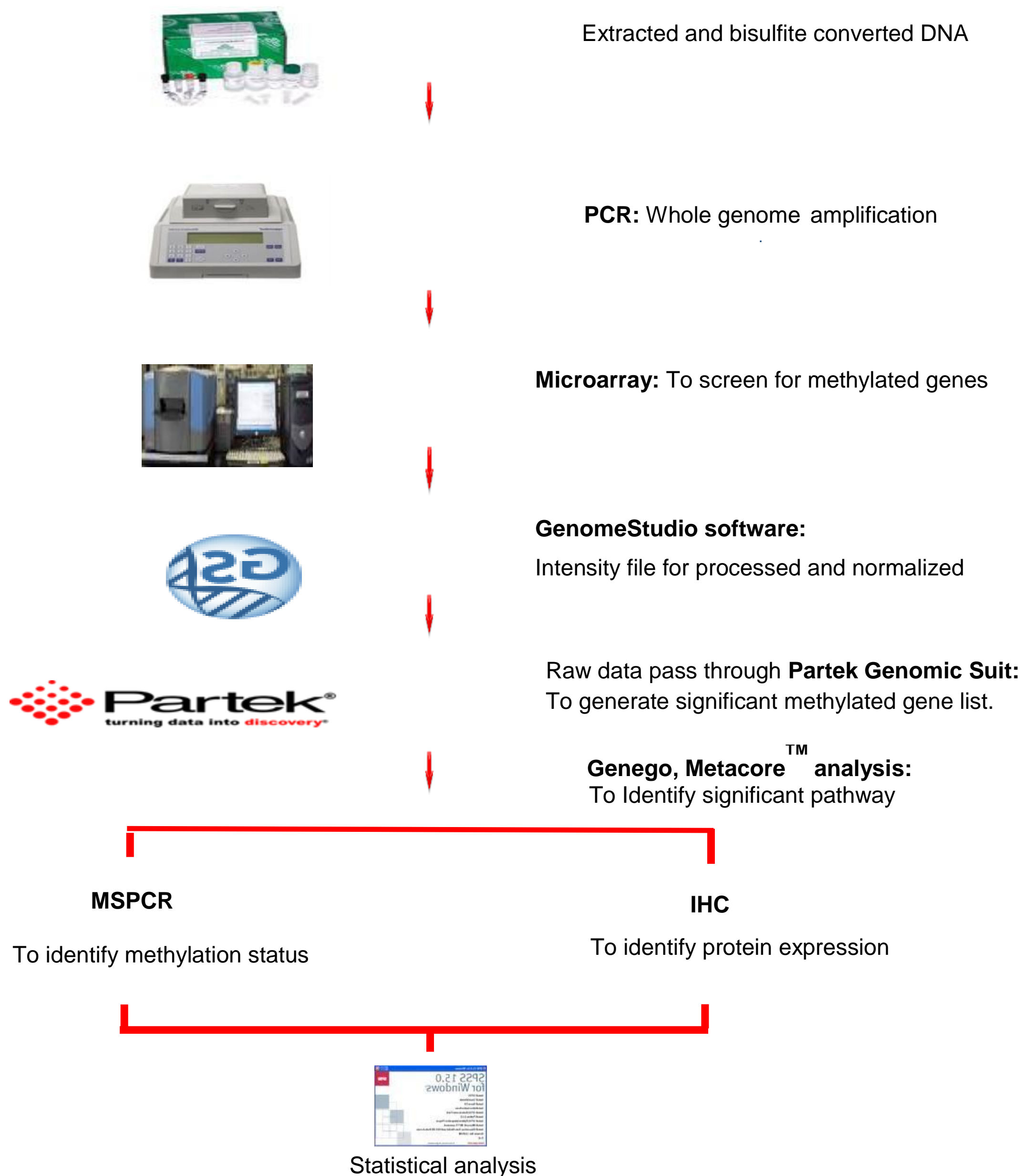


Figure 3.1 Project workflow

3.2 Study population

Archived specimens were used for this study. Human genomic DNA was extracted from four snap frozen healthy tissues surrounding impacted teeth, twenty independent snap frozen OSCC tissues for microarray analysis, and 40 independent formalin fixed and paraffin embedded (FFPE) OSCC tissues for validating significant hypermethylated genes using the MSPCR and IHC assays. The selected normal tissues were from healthy subjects who had no clinical lesions in their oral mucosa and non-smokers, non-alcoholics and non-betel quid chewers. All of these selected were new cases and had not undergone any anticancer treatment prior to this. An oral pathologist confirmed the histopathological findings and all samples used had more than 70% of the tumour samples for OSCC or more than 70% epithelium for the healthy tissues.

The samples and relevant clinical data were obtained from the Malaysia Oral Cancer Database and Tissue Bank System (MOCDTBS) coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC). This study was approved by the Medical Ethics Committee of the Faculty of Dentistry, University of Malaya (Ethic reference no: DF OP1101/0049 (L)).

3.3 DNA extraction

3.3.1 Snap frozen tissues

A total of 0.1 mg snap frozen specimen was sectioned using a cryostat (Leica Microsystem, Wetzlar, Germany) at a temperature of -20°C. A reference slide was prepared from representative tumour tissues and stained using Haematoxylin and Eosin (H&E) staining (Appendix A) to confirm the diagnosis and to gauge the percentage of tumour cells in the tissue. The sections from macrodissected tissues that showed more than 70% tumour cells

were then selected for DNA extraction. QIAamp® DNA Extraction Mini Kit (Qiagen, Germany) was used for DNA extraction following the manufacturer's procedure. The purity of the extracted genomic DNA was analysed by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) with an extinction coefficient for double stranded DNA (50ng-cm/μl). The quality of DNA was checked using 1% agarose gel electrophoresis. DNA samples with $OD_{260/280}$ ratio from 1.80 - 2.00 were selected for further downstream application. Overall, the DNA purity of the samples had an average absorbance ratio of 1.89 ± 0.10. The extracted genomic DNA were frozen and stored at –20°C until required for the experiment.

3.3.2 Formalin fixed paraffin embedded (FFPE) tissues

Tumour tissues were surgically removed from the oral cancer patients during treatment by an oncology team, immediately fixed in 10% neutral buffered formalin, and then embedded in paraffin and prepared as FFPE blocks. Selected blocks in excess of diagnosis were retrieved from the MOCDTBS for use in this study. Four μm tissues were sectioned from the FFPE block using a microtome and 20 of these sections from each block were transferred into a centrifuge tube containing deparafinization reagent (Qiagen, Germany), vortexed, and centrifuged. The supernatant was discarded and the procedure repeated sequentially with 90% and 70% ethanol respectively. After centrifugation at 16 000 *g* for 20 minutes at 4°C, cell pellets were treated for Proteinase K digestion at 50°C for 4-16 hours. DNA extraction using QIAamp® DNA Extraction Mini Kit (Qiagen, Germany) was carried out following the manufacturer's procedure. The quality and purity of the genomic DNA were analysed by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) and the quality of DNA was checked by 1% agarose gel electrophoresis. The genomic DNA was frozen and stored at -20°C.

3.3.3 Bisulfite-converted DNA

The extracted genomic DNA was bisulfite-converted using the EpiTect Bisulfite Kit (Cat. No. 59104, Qiagen, CA) according the manufacturer's protocol. One ng to 2 µg of extracted genomic DNA was chemically modified by sodium bisulfite which changed the unmethylated cytosine into uracil. Conversely, this does not happen with methylated cytosine. The product contained unconverted cytosine where they had been previously methylated, and had cytosine converted to uracil if they were previously unmethylated.

A total volume of 20 µl solution of the bisulfite mix reagent and genomic DNA was added into microfuge tubes for PCR amplification using Eppendorf Mastercycler Gradient PCR (Germany). The following cycling conditions were performed:- first denaturation step: 5 minutes for 95°C, first incubation step: 25 minutes for 60°C, second denaturation step: 5 minutes for 95°C, second incubation step: 85 minutes for 60°C, third denaturation step: 5 minutes for 95°C, third incubation step: 175 minutes for 60°C, and lastly holding at 20°C. The bisulfite-converted DNA was then purified with lysis buffer containing 10ug/ml carrier RNA and centrifuged in spin columns for 1 minute. After that, washing with wash buffer was carried out and centrifuged at a maximum speed for 1 minute. Desulfonation buffer was then added in the spin column and incubated for 15 minutes at room temperature and centrifuged. Wash buffer was added to the spin column and centrifuged again before the spin column was placed in a heating block at 56°C for 5 minutes. This step enables the evaporation of any remaining liquid. Finally, 20 µl of elution buffer was added to the spin column and inserted into 1.5 ml of microcentrifuge tubes. Final PCR product can be eluted by centrifugation for 1 minute at approximately 15,000g (12,000 rpm). The DNA products were qualified by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies) with an extinction coefficient for single stranded DNA (33ng-cm/µl). The purified bisulfite-converted DNA was

ready for immediate analysis or it could be stored at or below -20°C for later use. For long term storage, the samples would ideally be stored at or below -70°C.

3.4 Microarray assay

Microarray experiment was conducted on the Infinium Illumina methylation bead array (Illumine, San Diego, USA). A total of 200 ng of total bisulphite converted DNA was applied to a bead-based microarray following the manufacturer's protocol (Illumine, San Diego, USA). In brief, the bisulfite converted DNA was subjected to whole genome amplification by DNA polymerase. Amplified products were then enzymatically fragmented and purified from dNTPs, primers and enzymes, and applied to chip via allele specific annealing to either the methylation specific probe or the non-methylation probe. There are two bead types for each CpG site per locus on the chip. Each locus tested is differentiated by different bead types since there are over 200,000 bead types available. Each bead type is attached to single stranded 50-mer DNA oligonucleotides that differ in sequence only at the free end; and this type of probe is known as an allele specific oligonucleotide. One of the bead types will correspond to the methylated cytosine locus and the other will correspond to the unmethylated cytosine locus which has been converted into uracil during bisulfite treatment, and later amplified as thymine during whole genome amplification. Hybridization was followed by single base extension with hapten labelled dideoxynucleotides. ddCTP was labelled with biotin while ddATP, ddUTP and ddGTP were labelled with 2,4-dinitrophenol (DNP). After incorporation of these hapten labelled ddNTPs, multi-layered immunohistochemical assays were performed by repeated rounds of staining with a combination of antibodies to differentiate the two types. After staining, the chip was scanned by BeadArray Reader to show the intensities of the unmethylated and methylated bead types (Bibikova et al., 2011).

3.4.1 Microarray data analysis

3.4.1.1 Genome Studio Data Analysis

The raw data were analysed by GenomeStudio Data Analysis Software (Illumine, San Diego, USA), and fluorescence intensity ratios between the two bead types were calculated. In the diploid human genome, a ratio value of 0 equals a non-methylation of the locus, a ratio of 1 equals to total methylation, and a value of 0.5 means that one copy is methylated and the other is not.

After the detection of the methylation status, the microarray files were further analysed and this included normalization of the raw data to reduce experimental variations, background and average normalization, and preforming standard statistical tests on the results.

The single-site resolution data could then be compiled into several types of figures for visualization and analysis, such as line plots, bar graphs, scatter plots, histograms, dendrograms, box plots, or heat maps. Here, scatter plots are used to correlate the methylation data, bar plots to visualize relative levels of methylation at each site tested, and heat maps are used to cluster the data to compare the methylation profile at the sites tested.

The array signals were uploaded to the Illumina's Genome Studio software for background normalization and filtered by β values for methylation levels using the Illumina's Genome Studio software (Bibikova et al., 2011). Samples which showed fluorescence intensity with $p<0.001$ were included in the study. The methylation level was defined as follows: Hypermethylated and hypomethylated alleles as those having an average β value of more than 0.6, and below 0.4 of the overall mean for all samples respectively. Wilcoxon rank sum test with a p value of 0.001 was corrected with 5% of false discovery rate corrections (FDR)

for multiple testing corrections (Susan et al., 1994). Multiple testing corrections allow a justification of p value based on test numbers being performed. Five percent of the FDR is allowed; having a 5% chance of 1 false positive in every 500 genes. The FDR adjusts the p value of 0.05 to reflect the frequency of false positive occurrences in the gene list. Differences in average β values between the two groups are presented along with the details of methylation probes where the differentially methylated probes between normal subjects and patients were identified. Only selected differentially hypermethylated probes in OSCC patients that passed the filtration criteria were further analysed by Partek Genomic Suite 6.5.

3.4.1.2 Partek Genomic Suite and Genego, Metacore™ analysis

The selected data of differentially methylated genes was then exported to the Partek Genomics Suite 6.5 (Partek Inc., USA). Unsupervised analysis of hierarchical clustering was obtained for distribution of subjects with healthy and OSCC tissue samples. List with significant methylated genes was generated using one-way ANOVA with $p < 0.05$ and fold change > 2.0 (Kron et al., 2009) and then subjected to Genego, Metacore™ to determine their biological pathway associated with carcinogenesis.

3.5 Methylation-specific polymerase chain reaction

The protocol was performed according to the manufacturer's instructions (EpiTect MSP Kit, Qiagen, CA). Unmethylated and methylated DNA controls (Catalog NO. D5014, ZYMO, Orange, CA, USA) were used as negative and positive controls, respectively; and double distilled water (ddH_2O) was used as a blank control in all the experiments.

Primers were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems, USA) based on primer design criteria (Appendix B). Details of the primers are shown in Table 3.1. The bisulfite-converted DNA served as a template using primers specific for the methylated or the modified unmethylated sequences. The primers were designed to anneal the methylated bisulfite-converted sequences within a gene. Thus, the bisulfite-converted DNA served as a template using primers specific for the methylated or the modified unmethylated sequences.

The bisulfite-converted DNA was subjected to PCR amplification using the Eppendorf Mastercycler Gradient PCR (Germany) with the following cycling conditions: - Initial activation step: 95°C for 10 minutes; 35 cycles in the denaturation step: 94°C for 15 seconds; annealing step: Tm of primer; extension step: 72°C for 30 seconds; and final extension: 72°C for 10 minutes. Five microliters of the PCR products were loaded into 1% agarose gels, electrophoresed and visualized under image analyser (Typhoon 9410 variable mode imager, Amersham Biosciences; Baie d'Urfe, Quebec, Canada) after staining with the SYBR Safe DNA gel stain (Invitrogen, USA).

3.5.1 Statistical analysis for comparisons between patients' demographic profiles and clinicopathological characteristics

Statistical analysis for comparisons between patients' demographic and clinicopathological data and the selected genes were analysed using Pearson Chi-square or Fisher's Exact for categorical variables, and independent sample T-tests for continuous normally distributed variables. The patients' demographic data included in the data analysis were age, gender, alcohol consumption, tobacco smoking, betel quid chewing habits, tumour sites, pathological stages, invasive front and tumour grading. The data were analysed by the Statistical Package for Social Sciences (SPSS software, version 17, Chicago, USA). When a p value was found to be less than 0.05, statistical difference was regarded as significant.

Table 3.1 Details of primers used in methylation-specific polymerase chain reaction

Genes	UCSC reference location	Methylated		Unmethylated		Annealing temperature (°C)	Product length (bp)
		Forward primer	Reverse primer	Forward primer	Reverse primer		
P16	chr9:21967751-21994490	5′TTATTAGAGGGTGGGGCGGATCGC3’	5′GACCCCGAACCGCGCCGTAA3′	5′TTATTAGAGGGTGGGGTGGATGT3	5′CAACCCCAAACCACAACCATA A3′	61	156
DDAH2	chr6:31695894-31698245	5'TTGGATTACGGTCGTGTC 3'	5'ACGAAAACTAACCTTCCCG3'	5'TTTTTGGATTATGGTTGTGTT3'	5'ATACAAAAACTAACCTTCCCAC3'	55	167
DUSP1	chr5:172197482-172199606	5'AGTTTGGAAAATTAAAGGAGC3'	5'ATACCCACGTTACCTCCATA3'	5'GGAGTTTGGAAAATTAAAGGAGT3'	5'CAATACCCACATTACCTCCATA3	53	155
CELSR3	chr3:48698335-48701667	5'TAGATTAGGCGTTCGGTTTC3'	5'AAAAAATAACCTCGACGAACC3'	5'GAGTAGATTAGGTGTTTGGTTTT3'	5'CCAAAAAAATAACCTCAACAAACC3'	50	136
PIKCR5	chr17:8868469-8869372	5'GTAGTTGGGATTATAGGCGC3'	5'ACGAATCACGAAATCAAAAA3'	5'GGGTAGTTGGGATTATAGGTGT3'	5'AAACAAATCACAAAATCAAAAA3'	52	157
TP73	chr1:3566445-3569636	5'TGAAGATGTGCGAGTTAGTC3'	5'TCTTAAAAAATCGCGTCAAT3'	5'TTATGAAGATGTGTGAGTTAGTT3'	5'ACATCTTAAAAAATCACATCAAT3'	55	125
MEF2D	chr12:5364720-53646071	5'AAGGAGATTTTTTCGGTTTC3'	5'TTTCTTCCGTACAATACTCGA3'	5'ATTAAGGAGATTTTTTTGGTTTT3'	5'CTTTTCTTCCATACAATACTCAAA3'	53	105
RRM2	chr2:10262173-10263481	5'TCGTTTTGTTTTGGTTGTTC3'	5'GCGAACTCACCGTATTCTC3'	5'TAGTTGTTTTGTTTTGGTTGTTT3'	5'CCCACAAACTCACCATATTCTC 3'	54	115
BCL2	chr18:60986621-60988286	5'GTTGTGTTATCGGCGTTC3'	5'CTCGAAACGTCCCTAAACA3'	5'GTTGTGTTATTGGTGTTT3'	5'CTCAAAACATCCCTAAACA3'	54	134

3.5.2 Survival analysis

Survival rates and curves were estimated using the Kaplan-Meier method and comparisons of prognostic subgroups of demographic, clinicopathological data and genes hypermethylation with survival rate were conducted using the log-rank test, respectively. The association was considered statistically significant if $p < 0.05$.

3.6 Immunohistochemical staining

Archival FFPE tissue blocks of normal oral mucosa (n=4) and OSCC (n= 40) were retrieved from OCRCC. To construct tissue microarray block, small core biopsies were taken from non-necrotic, morphologically representative areas of FFPE tumour tissues and assembled on a recipient paraffin block. This was performed using a semi-automatic tissue arrayer minicore (Alphelys, SAS, France). The biopsied core was 3.0 mm in diameter which was sufficient for assessing the morphological features in the tissues, and 40 cores were assembled on a recipient paraffin block. After construction, 4 µm sections were cut and H&E staining was performed on the initial slide to verify the histology.

All these selected cases were histologically verified and diagnosed according to the classification of the World Health Organization (WHO) by an oral pathologist. The confirmed OSCC cases were used as positive control on each IHC run. The protocol was performed according to the manufacturer's instructions (Dako, USA, Capinteria, CA). The primary antibodies applied for immunohistochemical analysis were rabbit, mouse or goat monoclonal or polyclonal antibodies to specific hypermethylated genes of DDAH2, DUSP1, RRM2 and MEF2D respectively with 30 minutes of incubation time (Table 3.2). The immunohistochemical staining was performed with DAKO REAL EnVision Detection

System, Peroxidase/DAB$^+$, Rabbit/Mouse (K500711, Dako, USA, Capinteria, CA) (Kämmerer et al., 2001).

Table 3.2 Details of antibodies used in immunohistochemical assay

Name of proteins	Catalogue number	Positive control tissues	Primary antibodies	Nuclear or cytoplasmic staining	Dilution factor
DDAH2	Ab87064	Human colon tissues	Rabbit polyclonal antibody against human DDAH2	Cytoplasmic	1:100
DUSP1	ab1351	Human colon tissues	Goal polyclonal antibody against human DUSP1	Cytoplasmic	1:200
RRM2	ab57653	Human appendix tissues	Mouse monoclonal antibody to human RRM2	Cytoplasmic	1:450
MEF2D	ab32845	Human appendix tissues	Rabbit polyclonal antibody to human MED2F	Nuclear	1:450

3.6.1 Protocol for detection of protein expression of selected genes using IHC

The protocols are as listed in Appendix C

3.6.1.1 Dewaxing, deparafinization and rehydration

Slides were labelled and placed in a slide holder. The slides were put in an oven at 60 ^{o}C for 30-60 minutes for dewaxing. The slides were then submerged for 3 minutes each in three different chambers of xylene to deparaffinize the FFPE tissues. The tissues were then rehydrated in a graded alcohol series of 95% and 70% for 3 minutes each and this was followed by rinsing in running water. After that, the slides were incubated in 100 mM Tris phosphate buffer (TBS) (pH 9.0) for 10-35 minutes prior to an antigen retrieval process.

3.6.1.2 Antigen retrieval

For antigen retrieval, the slides were placed in a plastic container containing 250 ml of 10 mM citrate buffer (pH 6.0). The container was placed in an 8-quarter programmable pressure cooker filled with 2-3 L of ddH_2O and the slides were pressurized on a low setting for 5 minutes. Once finished, pressure was released and the container was removed from the pressure cooker and cooled to room temperature for 15 minutes.

3.6.1.3 Blocking

A hydrophobic glue marker (Pap Pen, Research Products International Corp, Mt. Prospect, IL) was used to draw a hydrophobic barrier around each tissue specimen. To quench endogenous peroxidase activity of the tissues, 3% of hydrogen peroxide (H_2O_2) in methanol was applied to each slide and incubated for 5 minutes in a humidity chamber. The slides were then washed 3 times in with 0.05% Tween-20 for 3 minutes each, and then in ddH_2O for 6 minutes.

3.6.1.4 Staining

Primary monoclonal and polyclonal antibodies were pre-diluted with 100 mM TBS (pH 9.0) according to dilution factors as shown in Table 3.2. The slides were placed in the humidity chamber for 30 minutes. A negative control slide without antibody incubation was stained along with the other slides. The slides were then rinsed twice in the TBS with 0.05% Tween-20 for 3 minutes each and placed back into the humidity chamber. Subsequently, 10% of horseradish peroxidase (HRP) from the Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) was applied on each slide before incubating for 30 minutes. The slides were then washed twice, each for 3 minutes in the TBS containing 0.05% Tween-20. Two drops of diaminobenzidine (DAB) substrate (1 ml DAB and 20ul DAB chromogen) were applied to each slide and incubated for 10 minutes. Finally, the slides were washed three times (2 minutes each) in the TBS with 0.05% Tween-20.

3.6.1.5 Counterstaining

For counterstaining, the slides were briefly dipped once into the Hematoxylin solution, followed immediately with two separate rinses in ddH2O.

3.6.1.6 Tissue preservation

The slides were serially dehydrated in graded alcohol of 70%, 95% and 100% for 2 minutes each. Two drops of Vectomount (Vector Laboratories, Burlingame, CA) were added onto each slide and a coverslip was carefully placed on each slide without trapping air bubbles. The slides were left to dry overnight before being analysed under the light microscope.

3.6.2 Image scoring analysis of protein expressions of selected genes in IHC stained specimens

Photomicrographs with power magnification of 40 times were taken by a CCD camera DP50 (Olympus, Tokyo, Japan) attached to an Olympus BX61 Fluorescence microscope (Olympus America, Inc., Center Valley, PA, USA). Cell count and tumour areas were measured in each photomicrograph to detect brownish positive IHC staining using digital image analysis software (AnalySIS LS Professional version 5.0; Olympus America, Inc., Center Valley, PA, USA). Colour threshold detection of the AnalySIS Professional software was used to determine positive (brown pixels) and negative (blue pixels) stained cells per tumour area. The tumour tissues present in the images were marked and included for image analysis. Data are expressed as the presence of positive cells in percentages (positively brown stained divided by tumour area). The evaluation was done qualitatively by taking the definite positive brown staining at the specific cellular location of each protein (Durlej et al., 2010). In the study, MEF2D expression was associated with distinct nuclear staining of the cells, whereas DDAH2, DUSP1 and RRM2 revealed cytoplasmic staining of the cells. IHC staining scoring were examined by an evaluator without prior knowledge of the methylation status of MSPCR.

The cut off threshold for positive and negative immunostaining was dependent on the median percentage of each gene's reactivity as described previously (Sis et al., 2005). Percentage of the median value for each gene was used to differentiate between positive and negative immunostainng. The percentage of median value of immunostaining demonstrated in tumour tissues for DDAH2 was 10.0%, 5.13% for DUSP1, 17.8% for MEFD2, and 20.0% for RRM2. Protein expression with a value of more than the median percentage was considered to be overexpressed.

3.6.3 Statistical analysis of protein expressions of selected genes

Quantitative data obtained as continuous abnormally distributed variables were presented as median (interquartile) for percentage of protein expressions. The statistical analysis of association between protein expressions of selected genes in OSCC was analysed using the Mann-Whitney U test for continuous abnormally distributed variables. Association of gene hypermethylation and protein expression was conducted by Chi-square and Fisher's exact tests. Correlations between protein expressions of different genes and a patient's age were performed using Spearman's rho test for continuous abnormally distributed variables. The correlation of protein expression strength between genes was considered to be weak if correlation (r) was close to 0 and considered strong if it was close to 1 with a significant value of $P < 0.01$ (two-tailed). The data were analysed by SPSS software, version 17 (SPSS, Chicago, USA). Statistical significance was accepted at $p < 0.05$.

CHAPTER 4 RESULTS

4.1 Methylation microarray analysis

4.1.1 Study population

Twenty frozen tissue samples of OSCC and 4 normal tissues were collected over a time period of five years from 2005 to 2010 for microarray assay. These samples were obtained from 10 male patients with a mean age of 57 ± 18.90 years and 10 female patients with a mean age of 64.5 ± 13.62 years. In this study, a total of four cases of tumour pathological stage I, five cases of stage II, five cases of stage III and six cases of stage IV were obtained for the microarray assay.

4.1.2 Illumina's Genome Studio software analysis

The methylation levels of 45,000 CpG sites were measured by Infinium methylation assay and are presented as a value from 0 (completely unmethylated) to 1.0 (completely methylated). In the Illumina's Genome Studio software analysis, one of the 4 normal tissue samples was categorized and filtered as an outlier during the data handling and excluded from the study.

In this study, mean methylation value differences between normal tissues with different stages of OSCC cases were obtained. Line plot (Figure 4.1) and box plot (Figure 4.2) represent the difference in methylation values (average β value) between normal tissues (average β value < 0.4) and OSCC tissues (average β value > 0.6) in CpG sites and tumour pathological stages. These values were averaged from all patients within each CpG site. The difference in values is averaged for all patients within each CpG site. All CpG sites within a PCR product are grouped together. The results demonstrated that normal samples segregated differently from clinical samples as shown in Figure 4.1 and Figure 4.2.

Histogram of representative genes' group methylation profiles of average β value for p16, DDAH2 and DUSP1 alleles were distinctly differentiated between normal and 4 pathological stages (Figure 4.3.1-4.3.3). A distinct profile shows lower average β value in normal subjects if compared with pathological stage I, II, III and IV for p16, DDAH2 and DUSP1 alleles. The analysis of the methylation profile resulted in a gene list with 34 promoter-associated hypermethylated genes with an average β value of 0.4 in methylation ($p < 0.001$) (Appendix D).

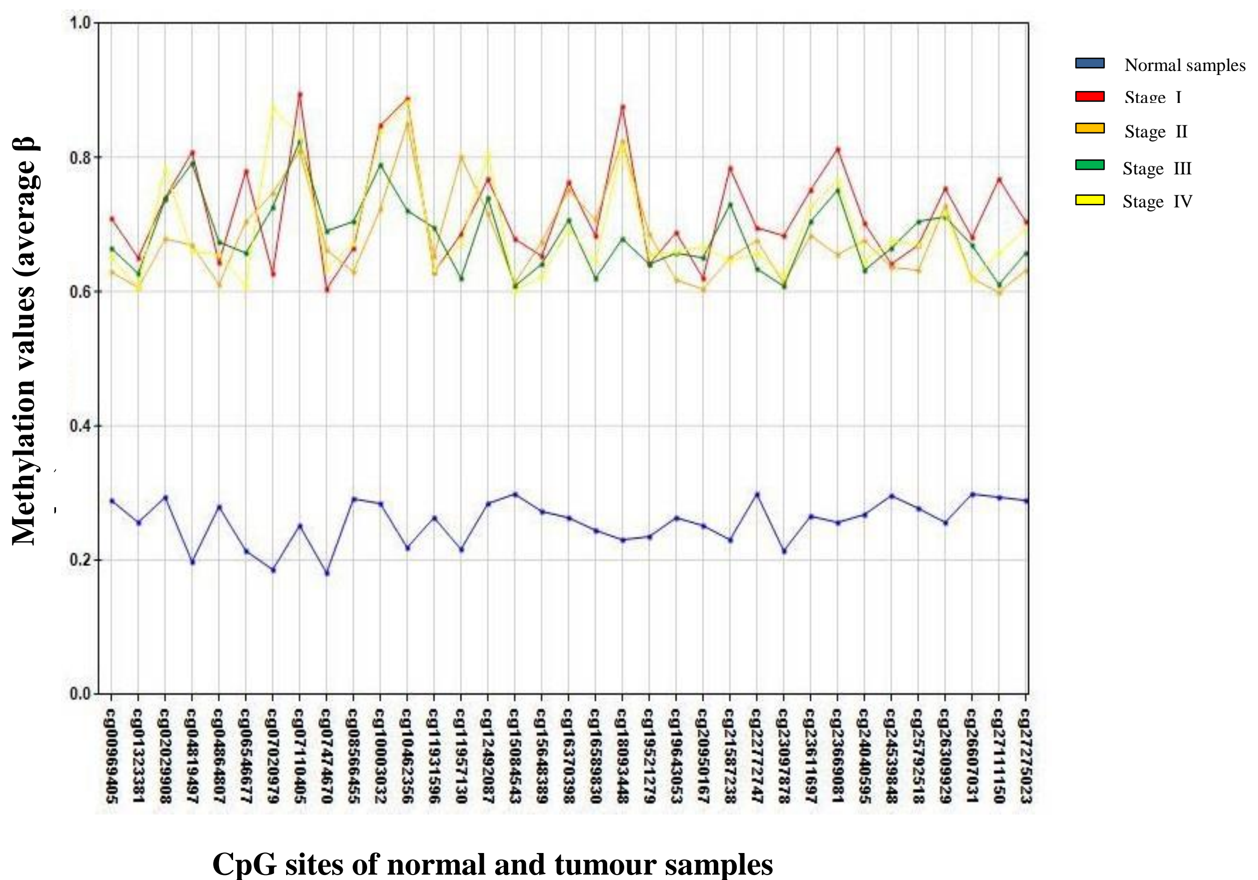
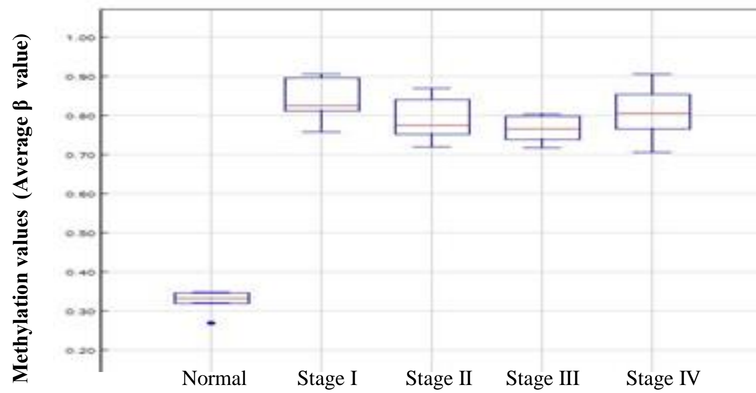
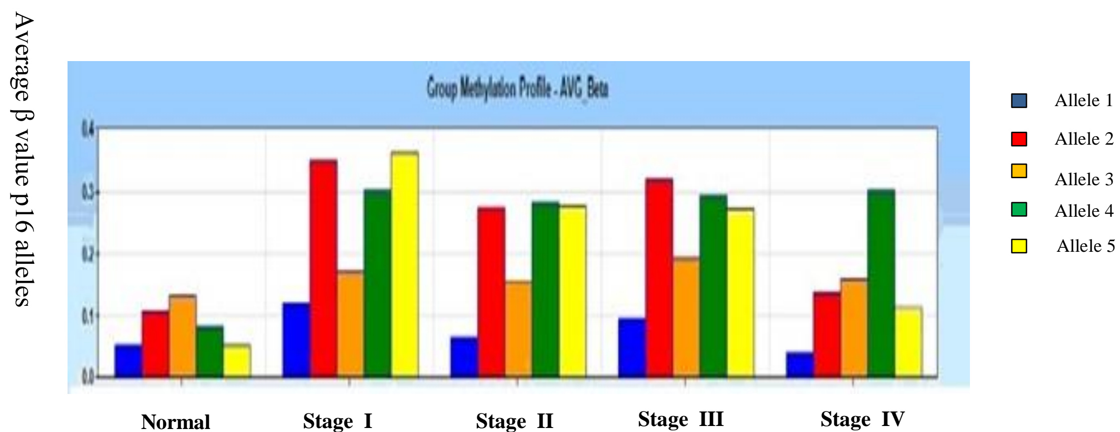


Figure 4.1. The line plot shows unsupervised hierarchical clustering of CpG sites of normal tissues and tumour samples (Stage I-IV) with methylation values (average β value). Normal samples (blue line) segregated differently from tumour samples (stage I in red line, stage II in orange-coloured line, stage III in green-coloured line, stage IV in yellow-coloured line) in OSCC.



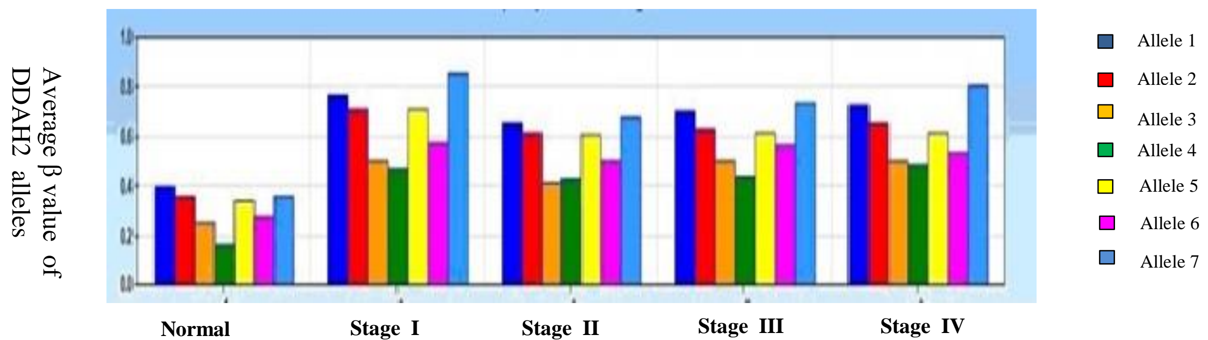
Normal and tumour pathological stages (Stage I, II, III and IV)

Figure 4.2. Mean methylation value difference between normal tissues with different stages of OSCC cases. The box plot represents methylation difference values between samples of normal (average β value < 0.4) and OSCC (Stage I, II, III and IV) (average β value > 0.6).



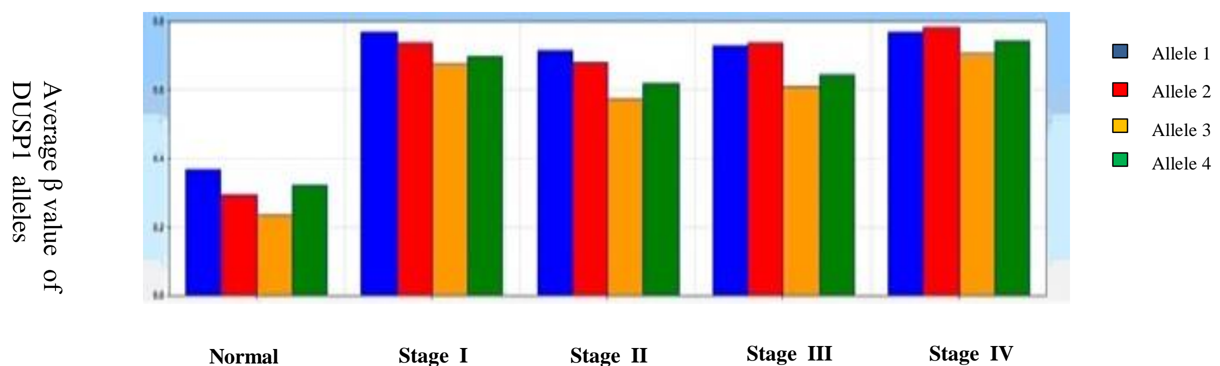
Normal and tumour pathological stages (Stage I, II, III and IV)

Figure 4.3.1. Histogram of group methylation profiles of five different p16 alleles average β value between normal and 4 pathological stages (Stage I, II, III and IV).



Normal and tumour pathological stages (Stage I, II, III and IV)

Figure 4.3.2. Histogram of group methylation profiles of seven different DDAH2 alleles average β value between normal and 4 pathological stages (Stage I, II, III and IV). DDAH2 alleles are representative in colour of dark blue, red, orange, green, yellow, pink and light blue.



Normal and tumour pathological stages (Stage I, II, III and IV)

Figure 4.3.3. Histogram of group methylation profiles of DUSP1 alleles average β value between normal and 4 pathological stages (Stage I, II, III and IV). Four different DUSP1 alleles are representative in colour of blue, red, orange and green.

4.1.3 Partek Genomic Suite 6.5 assay

Data obtained from the Illumina's Genome Studio software were analysed by the Partek Genomic Suite software. A separate hierarchical clustering analysis was performed on normal (n=3) and tumour tissues (n=20). The data set was clustered using a standard hierarchical method with the Pearson's correlation to determine the distance function. The cluster set of normal and tumour samples (stage I, II, III and IV) clearly segregated the normal from tumour samples (Figure 4.4, Figure 4.5).

The unsupervised hierarchical clustering (Figure 4.4) and principle component analysis (Figure 4.5) of methylation status of normal and tumour samples (stage I, II, III and IV) showed that normal samples were clustered differently from clinical samples. However, there was no clear separation between the clustering patterns among the four different pathological stages.

A total number of 1318 loci were differentially methylated between tumour and normal samples by at least a 2 fold change and FDR value <0.05 (Figure 4.6) - 1080 loci (fold change 2.0001 - 30.1393) were hypermethylated, and 238 loci (fold change -2.00025 - -7.99067) were hypomethylated with p < 0.0001 (Figure 4.7). A gene list of 89 promoter hypermethylated loci was generated (Appendix E), where 69 loci are located in the islands of promoter, 1 in the north shelf, 10 in the north shore, 5 at the south shore of promoter regions and 4 were unspecific regions. Representative hypermethylated genes of p16 (FC=+2.85385 p=0.002357), DUSP1 (FC=+2.56631 p=5.49E-08), DDAH2 (FC=+2.82636 p=0.000934), PIK3R5(FC=+3.18124 p=0.001524), CELSR3 (FC=+15.0254 p=0.000736), TP73 (FC=+2.2665 p=0.001507), RRM2 (FC=+2.54062 p=0.001067), MEF2D (FC=+2.54282 p=0.002182), and one hypomethylated gene of BCL2 (FC= -2.15965 p=0.000201556) were

selected for further study. Gene selection criteria included their biological functions, $p < 0.001$ and a methylation fold change for microarray data validation using MSPCR and IHC assays. These selected genes do not necessary reflect the greatest statistical significance or greatest methylation fold change.

The Partek Genomic Suite visualization also demonstrated the selected significant enrichment differences in p16, DDAH2, DUSP1, PIKC3R5, CELSR3 and BCL2 genes in their selected probe regions respectively ($p < 0.001$) as shown in Figure 4.8.1-4.8.6. The entire gene's significant enrichment differences corresponded to the designated primer regions for MSPCR analys

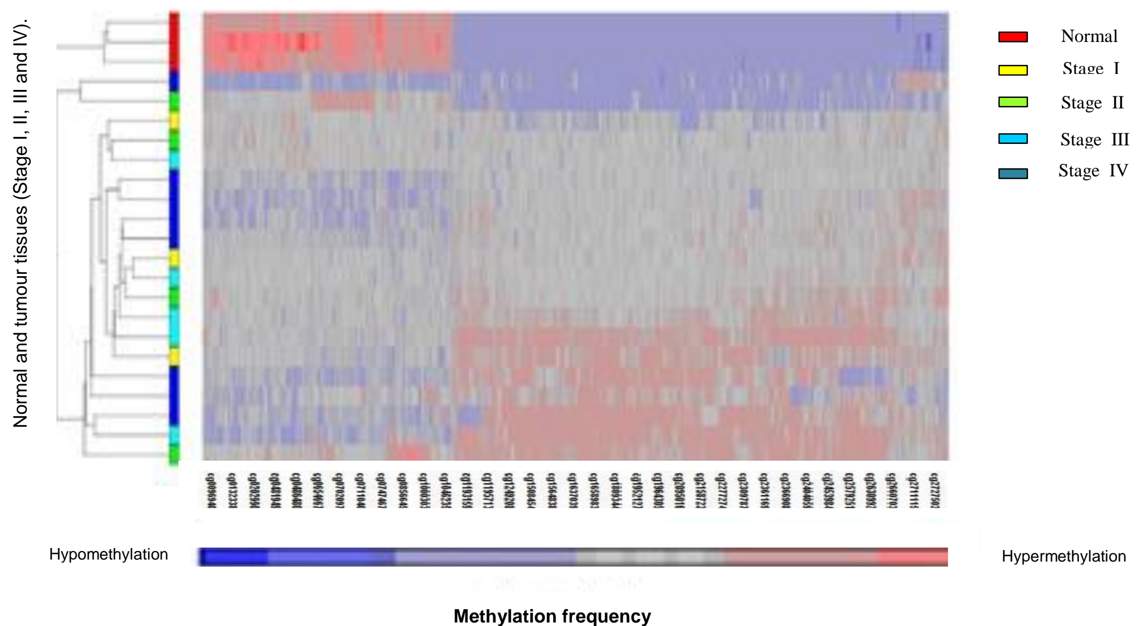


Figure 4.4. Heatmap of methylation frequency of differentially methylated genes in OSCC. Unsupervised hierarchical clustering was performed on gene methylation profiles for the normal tissues (n=3) and tumour tissues (n=20, stage I, II, III and IV). The heat map of differentially methylated genes based on clustering is shown in the figure. Each row represents a sample and each column represents a CpG loci. Red colour indicates hypermethylated CpG sites and blue colour indicates hypomethylated CpG sites.

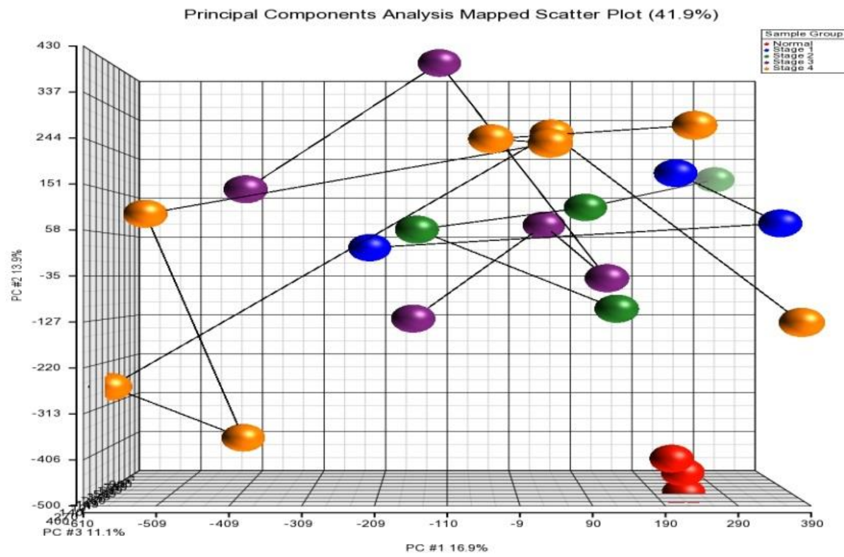


Figure 4.5. Principle Component Analyses segregated the normal samples (red colour) from tumour samples (stage I: blue; stage II: green; stage III: purple; and stage IV: orange)

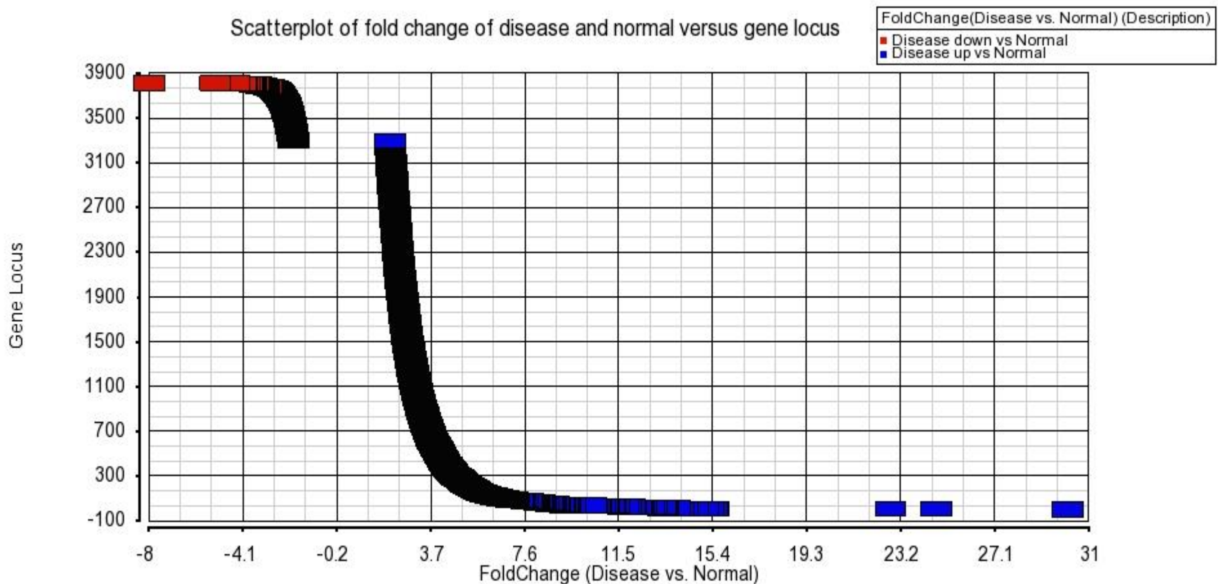


Figure 4.6. Scatter plot of fold change of normal and tumour tissues with gene locus. A number of loci are differentially hypermethylated between tumour and normal samples with fold change of 2.0001 to 30.13 and hypomethylated with fold change of -2.00025 to -7.99067.

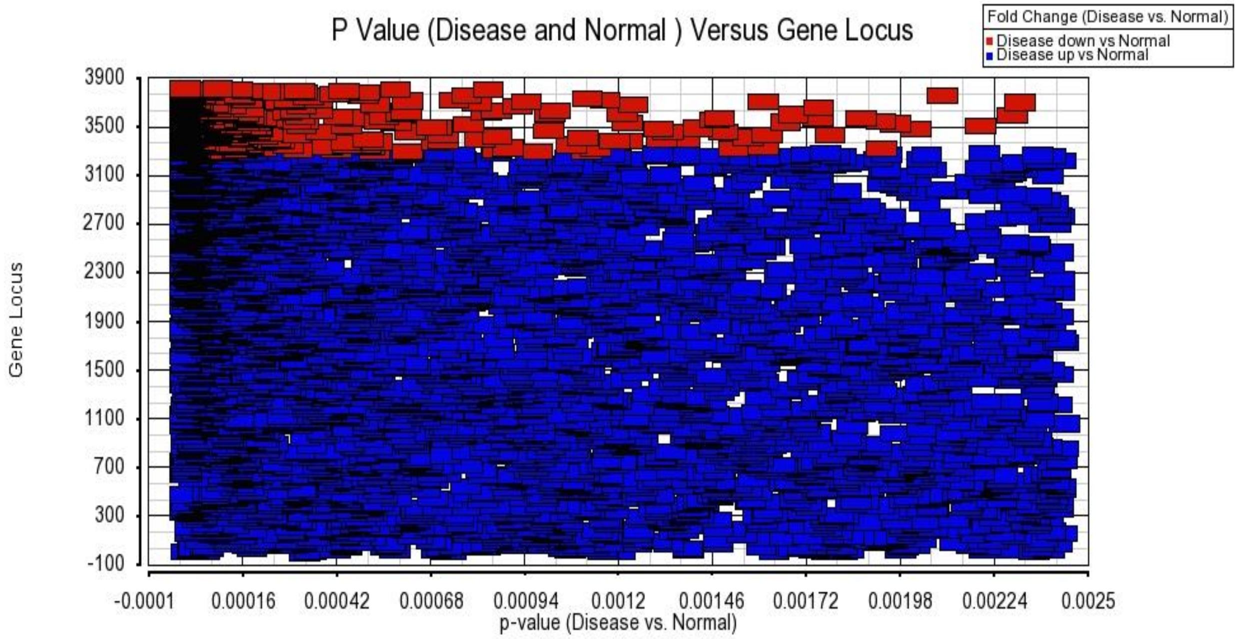


Figure 4.7. Distribution chart of p-value with locus for hypomethylated genes and hypermethylated genes. Figure shows hypermethylated loci are represented in blue boxes and hypomethylated loci are represented in red boxes. All loci that selected in the study was based on $p < 0.0001$.

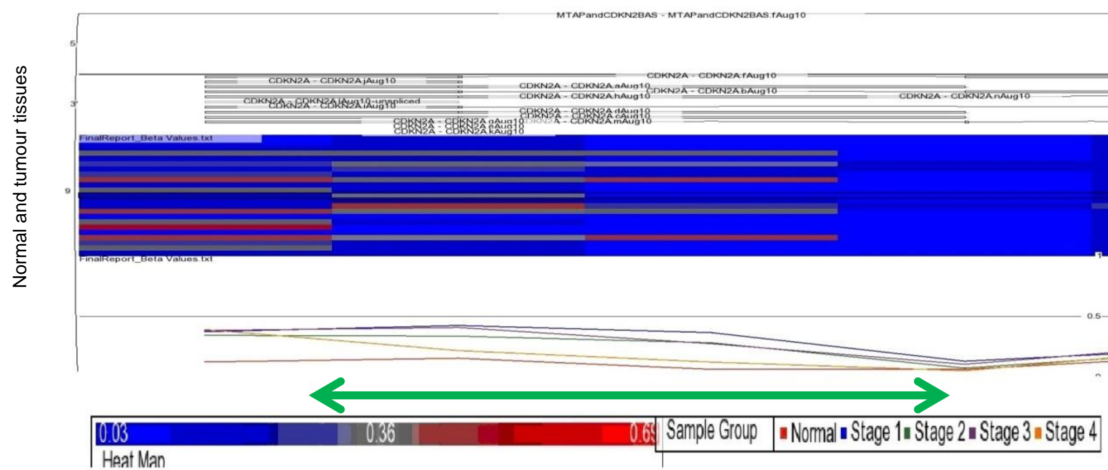


Figure 4.8.1. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe of p16 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

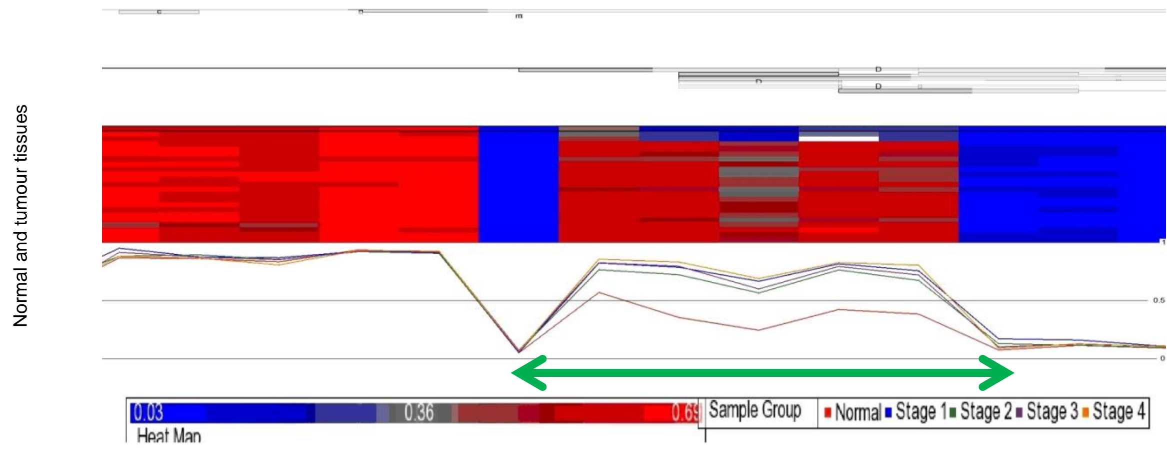


Figure 4.8.2. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe of DUSP1 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

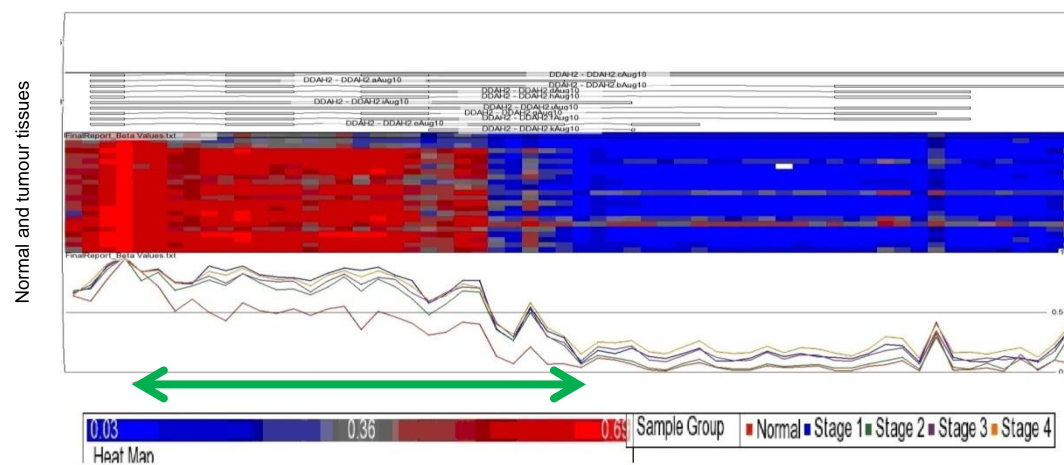


Figure 4.8.3. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe DDAH2 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

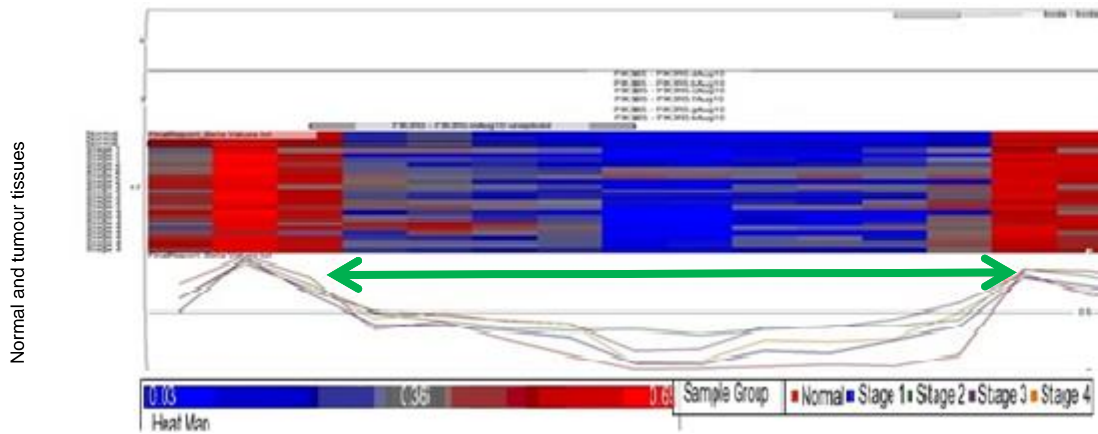


Figure 4.8.4. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe of PIKC3R5 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

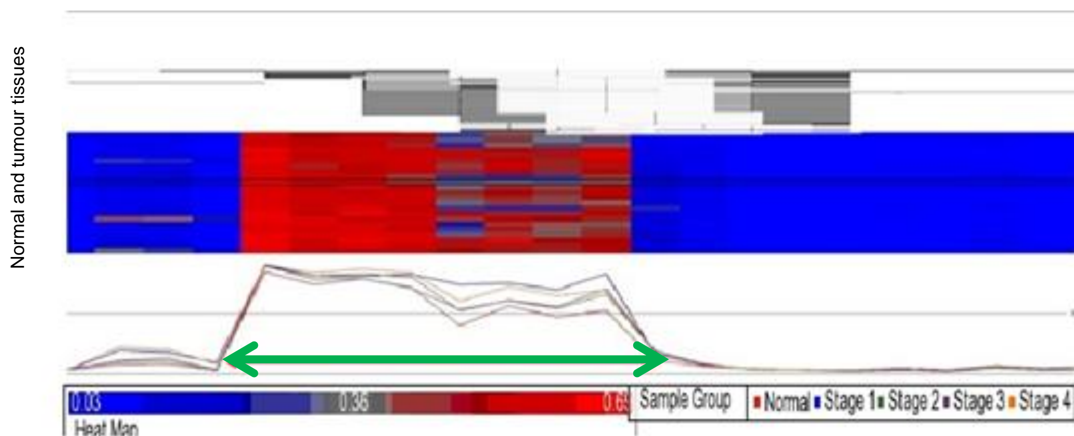


Figure 4.8.5. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe of CELSR3 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

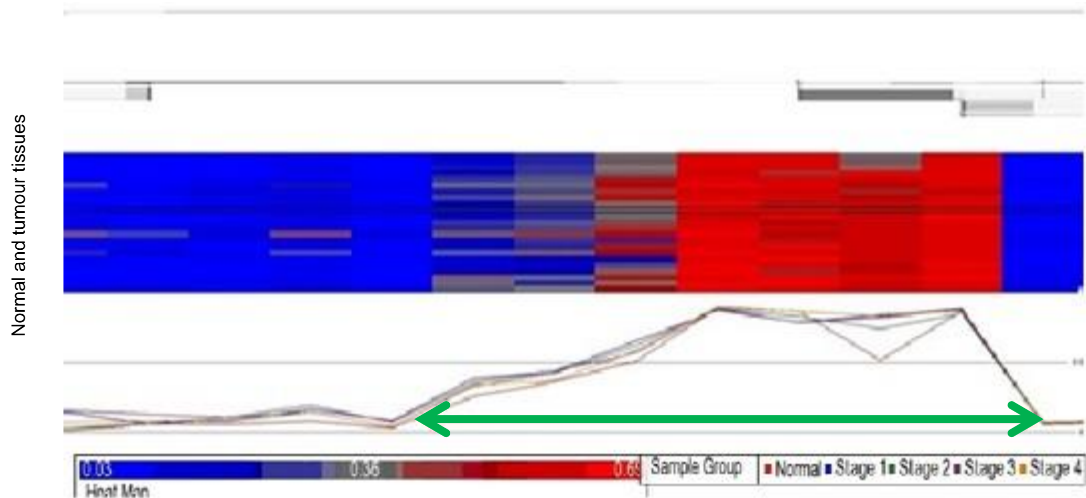


Figure 4.8.6. Representative of Partek Genomic Suite Visualization. The upper panel of each shows the heat map for each probe of BCL2 gene in normal and four pathological stages. The line graphs in the lower panel of each show log2 ratio of β values of each probe between normal and four pathological stages with clear separation (indicated with green arrow).

4.1.4 Signalling pathway analysis of hypermethylated genes from patients with OSCC

To elucidate which biological pathways are involved in OSCC progression, the hypermethylated gene list was further mapped using the MetaCore^{TM} analytical suite 4.5 for significant pathway analysis. A summary of the significant pathways ($p<0.001$) identified from the hypermethylated genes of OSCC is illustrated in Table 4.1. The top ten pathways were selected in the study. In depth biological analysis by the MetaCore^{TM} analytical suite 4.5 revealed that the most significant pathway involved in hypermethylation was immune response of function of MEF2 in T lymphocytes pathway (p=6.696E-03). The hypermethylated genes involved in this pathway include MEF2D and MEF2. These two genes, MEF2D and MEF2 genes, were also involved in the development role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis pathway (p=7.776E-03), cardiac hypertrophy of Ca (2+)-dependent NF-AT signalling in cardiac hypertrophy (p= 8.634E-03), and immune response of gastrin in the inflammatory response

(p=1.247E-02). The MEF2 gene was also hypermethylated in the transcription role of heterochromatin protein 1 (HP1) family in transcriptional silencing (p=5.329E-02). The second top most pathway was immune response of DAP12 receptors role in NK cells (p=7.776E-03) which revealed genes of HLA-C and HLA-B. The other two hypermethylated genes, RRM2, small RR subunit were predominantly in pathways of dCTP/dUTP metabolism (p=1.461E-02), dATP/dITP metabolism (p=2.283E-02 and ATP/ITP metabolism (p=3.734E-02). PI3K regulation class IB (p101) gene was hypermethylated in the last top ten pathway of apoptosis and survival of beta-2 adrenergic receptor anti-apoptotic (p= 5.565E-02). Thus, the two most predominant genes, MED2F and RRM2 were further analysed by MSPCR and IHC.

Table 4.1. Significant biological pathway associated with hypermethylated genes of OSCC (Enrichment analysis pathway maps by GeneGo software)

No	Enrichment by Pathway Maps	p value	Genes
1	Immune response_Function of MEF2 in T lymphocytes	6.696E-03	MEF2D, MEF2
2	Immune response_Role of DAP12 receptors in NK cells	7.776E-03	HLA-C, HLA-B
3	Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis	7.776E-03	MEF2D, MEF2
4	Cardiac Hypertrophy_Ca(2+)-dependent NF-AT signalling in Cardiac Hypertrophy	8.634E-03	MEF2D, MEF2
5	Immune response_Gastrin in inflammatory response	1.247E-02	MEF2D, MEF2
6	dCTP/dUTP metabolism	1.461E-02	RRM2, Small RR subunit
7	dATP/dITP metabolism	2.283E-02	RRM2, Small RR subunit
8	ATP/ITP metabolism	3.734E-02	RRM2, Small RR subunit
9	Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	5.329E-02	MEF2
10	Apoptosis and survival_Beta-2 adrenergic receptor anti-apoptotic action	5.565E-02	PI3K reg class IB (p101)

4.2 Methylation-specific polymerase chain reaction analysis

The selected genes of p16, DDAH2, DUSP1, CELSR3, PIK3R5, TP73, MEF2D, RRM2 and BCL2 were assessed using a quantitative technique, MSPCR in independent FFPE samples. Table 4.2 shows the methylation status and frequencies of promoter hypermethylation. Promoter hypermethylation of positivity of p16 (n=31, 77.5%), DDAH2 (n=32, 80%), DUSP1 (n=35, 87.5%), CELSR3 (n=35, 87.5%), PIK3R5 (n=32, 80%), TP73 (n=37, 92.5%), MEF2D (n=28, 70%), RRM2 (n=32, 80%) and BCL2 (n=18, 45%) correlated with the microarray data. For unmethylation status, p16, DDAH2, DUSP1, CELSR3, PIK3R5, TP73, MEF2D, RRM2 and BCL2 genes had 22.5% (n=9), 20% (n=8), 12.5% (n=5), 12.5% (n=5), 20% (n=8) , 7.5% (n=3), 30% (n=12), 20% (n=8) and 55% (n=22) respectively. Representative agarose gel images are shown in Figure 4.9.1-4.9.9. Details of hypermethylated and unmethylated gene status and percentage of gene methylations are listed in Table 4.2.

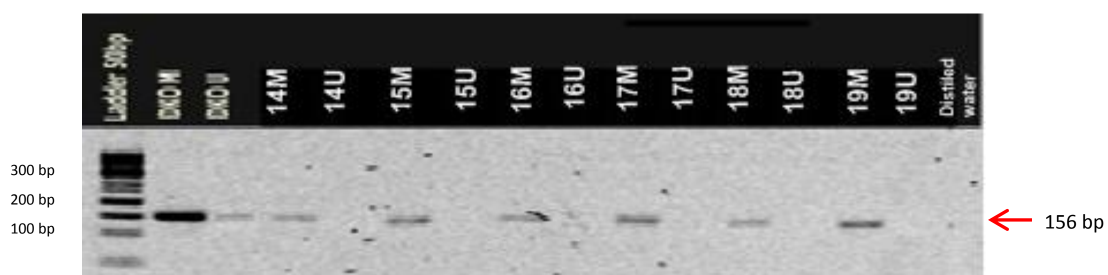


Figure 4.9.1. Representative agarose gel electrophoretic images of methylation status for gene of p16 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Lanes 16: Distilled water used as negative control. Sample no. 14,15,16,17,18 and 19 show methylated status

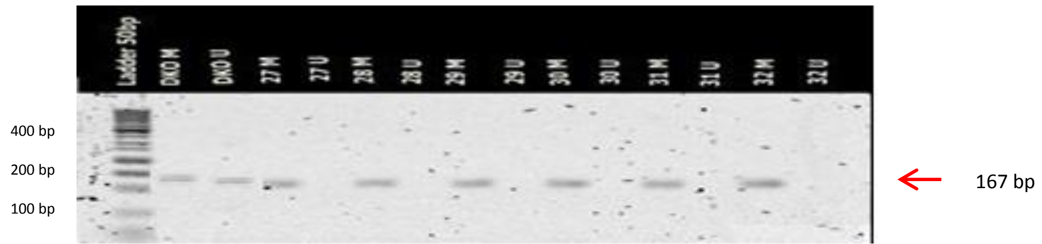


Figure 4.9.2. Representative agarose gel electrophoretic images of methylation status for gene of DDAH2 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Sample no. 27, 28, 29, 30, 31 and 32 show methylated status.

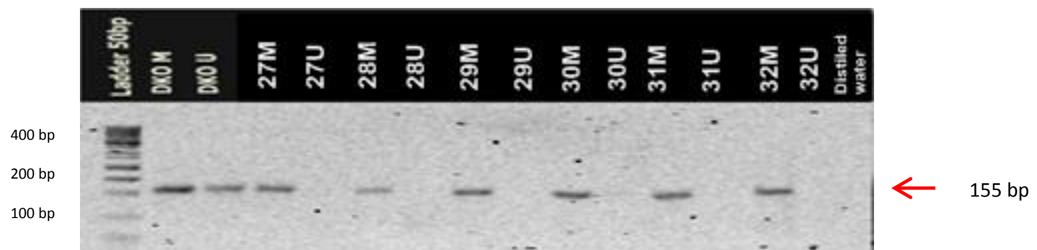


Figure 4.9.3. Representative agarose gel electrophoretic images of methylation status for gene of DUSP1 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Lanes 16: Distilled water used as negative control. Sample no. 27, 28, 29, 30, 31 and 32 show methylated status.

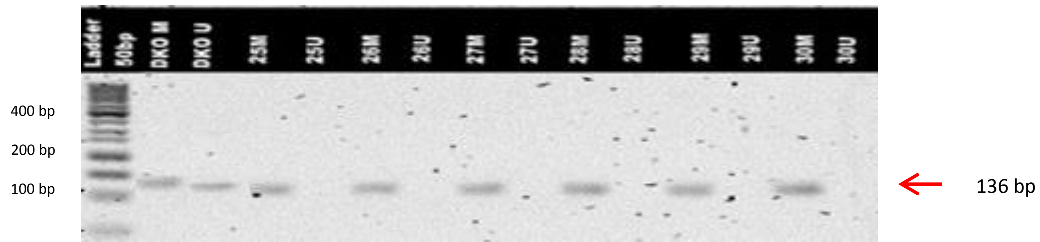


Figure 4.9.4. Representative agarose gel electrophoretic images of methylation status for gene of CELSR3 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Sample no. 25, 26, 27, 28, 29 and 30 show methylated status.

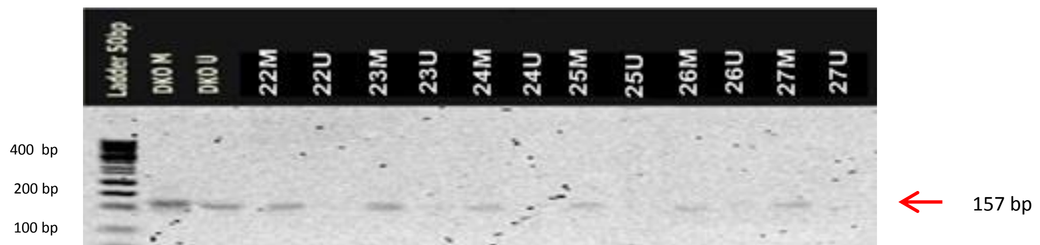


Figure 4.9.5. Representative agarose gel electrophoretic images of methylation status for gene of PIK3R5 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Sample no. 22, 23, 24, 25, 26 and 27 show methylated status.

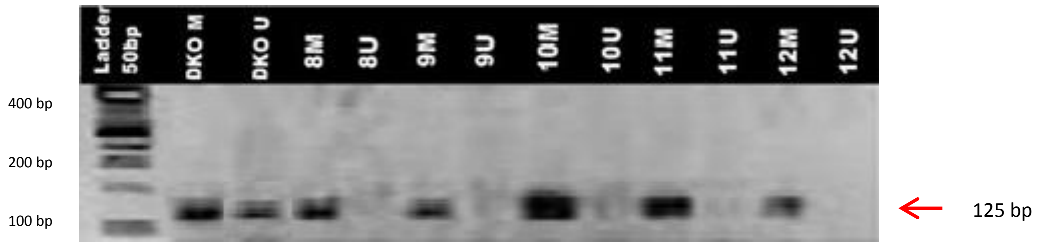


Figure 4.9.6. Representative agarose gel electrophoretic images of methylation status for gene of TP73 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-13 represent methylation status of tumour samples. Sample no. 8, 9, 10, 11 and 12 show methylated status.

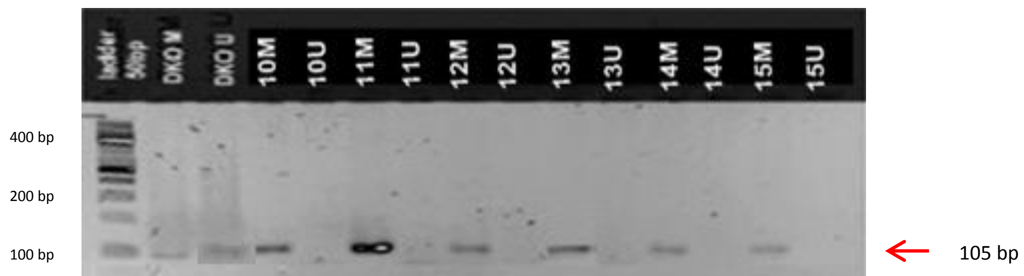


Figure 4.9.7. Representative agarose gel electrophoretic images of methylation status for gene of MEF2D in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Sample no.10, 11, 12, 13, 14 and 15 show methylated status.

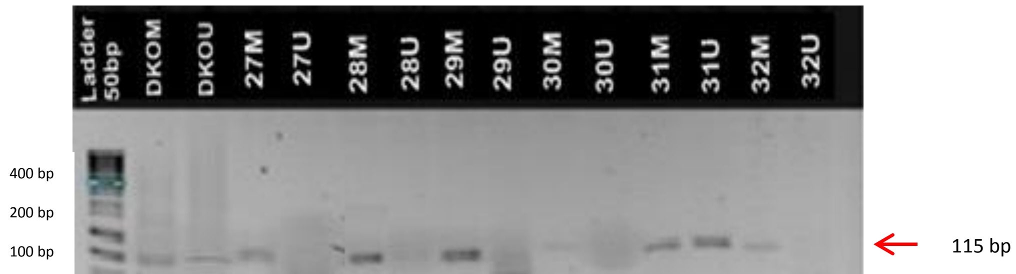


Figure 4.9.8. Representative agarose gel electrophoretic images of methylation status for gene RRM2 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-15 represent methylation status of tumour samples. Sample no. 27, 28, 29, 30, 31 and 32 show methylated status.

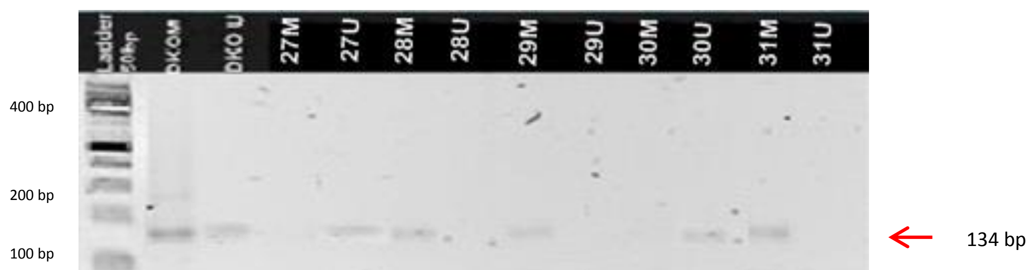


Figure 4.9.9. Representative agarose gel electrophoretic images of methylation status for gene of BCL2 in methylation control and tumour samples. DKO represents methylation control; M represents methylated alleles and U represents unmethylated alleles. Lane 1: ladder marker of 50 bp. Lanes 2 and 3: universal methylated (DKO M) and unmethylated control (DKO U). Lanes 4-13 represent methylation status of tumour samples. Sample no. 28, 29, and 31 show methylated status.

Table 4.2. Methylation status and percentage for methylated genes of p16, DDAH2, CELSR3, DUSP1, PIKCR3, TP73, MEF2D, RRM2 and BCL2

No.	Hypermethylated genes								
	p16	DDAH2	DUSP1	CELSR3	PIK3R5	TP73	MEF2D	RRM2	BCL2
1	M	M	M	M	M	M	M	M	UM
2	M	UM	UM	M	M	M	M	M	UM
3	M	M	M	M	M	M	UM	UM	UM
4	M	UM	M	M	UM	M	UM	UM	UM
5	M	M	M	M	M	M	M	M	UM
6	M	M	M	M	M	M	M	M	UM
7	M	M	M	M	M	M	M	M	UM
8	M	UM	UM	M	M	M	UM	M	M
9	M	M	M	M	M	M	UM	M	M
10	M	UM	M	M	M	M	M	M	M
11	M	M	M	M	M	M	M	UM	M
12	M	M	M	M	M	M	M	M	UM
13	UM	M	M	M	UM	M	M	M	UM
14	M	M	M	M	M	M	M	M	UM
15	M	M	M	M	UM	M	M	M	UM
16	M	M	UM	UM	UM	UM	M	M	UM
17	M	M	M	UM	M	M	UM	M	UM
18	M	M	M	UM	M	M	M	UM	UM
19	M	M	UM	UM	M	M	M	M	UM
20	M	M	M	UM	UM	M	M	M	UM
21	M	M	M	M	UM	M	M	M	M
22	M	M	M	M	M	M	UM	UM	M
23	M	UM	M	M	M	UM	M	UM	M
24	M	UM	UM	M	M	M	M	M1	M
25	M	UM	M	M	M	M	M	UM	M
26	UM	M	M	M	M	M	UM	M	M
27	UM	M	M	M	M	M	M	M	UM
28	M	M	M	M	M	M	M	M	M
29	M	M	M	M	M	M	UM	M	M
30	UM	M	M	M	M	M	UM	M	UM
31	M	M	M	M	UM	M	M	M	M
32	UM	M	M	M	M	M	M	M	M
33	M	M	M	M	M	M	M	M	UM
34	UM	M	M	M	M	M	M	M	UM
35	M	M	M	M	M	M	M	UM	M
36	M	M	M	M	M	M	M	M	M
37	UM	M	M	M	M	UM	M	M	M
38	M	M	M	M	M	M	UM	M	UM
39	UM	M	M	M	UM	M	UM	M	UM
40	UM	M	M	M	M	M	UM	M	M
Percentage, % (No of cases) of methylation	77.5 (n=31)	80 (n=32)	87.5 (n=45)	87.5 (n=35)	80 (n=32)	92.5 (n=37)	70 (n=28)	80 (n=32)	45 (n=18)

Indication:

M = Methylated status

UM = Unmethylated status

4.2.1 Demographic and clinicopathological parameters of OSCC patients

Table 4.3 shows the demographic profiles and clinicopathological characteristics of the 40 OSCC patients. These characteristics of age, gender, ethnic group, habits of alcohol drinking, tobacco smoking and betel-quid chewing, tumour sites, pathological stages, invasive fronts and differentiation of tumour grading were included in the study. The ethnic groups of 40 patients consisting of 14 men and 26 women with a mean age of 59.3 years (age range of 28–80 years) consisted of Malays (n=5), Chinese (n=6) and Indians (n=29). Most of the patients (80%) were found to have alcohol drinking, tobacco smoking and/or betel-quid chewing habits, whilst only 20% of the patients abstained from them. For the tumour sites, one (2.5%) OSCC was located on the lip, 19 (47.5%) of buccal mucosa, 8 (20%) gum and 12 (30%) tongue. For the pathological TNM (Tumour staging), four (10%) cases each were TNM stage I and III, six (15%) stage II, and 26 (65%) were at stage IV. For the invasive front (POI staging), the total amount of no graded cases was recorded as 3 (7.5%), 31 (77.5%) as non-cohesive, and 6 (15%) as cohesive staging. The number of differentiation (tumour grading) of OSCC for a well differentiated grade was reported at 13 (32.5%), moderate at 26 (65%), and one each (2.5%) for poorly differentiated and no graded case.

4.2.2 Association between patients' demographic profiles, clinicopathological characteristics and methylation status of p16, DDAH2, DUSP1, CELSR3, PIKC3R5, TP73, MEF2D, RRM2 and BCL2 genes

The Chi Square and Fisher's Exact tests, and independent sample T-tests of SPSS version 17.0 were applied to determine the association between patients' demographic profiles, clinicopathological characteristics with p16, DDAH2, DUSP1, CELSR3, PIKC3R5, TP73, MEF2D, RRM2 and BCL2 genes. A $p < 0.05$ was considered statistically significant. There was a significant association found between p16 and TP73 hypermethylation with patients' tumour site (p=0.001 and 0.006), and CELSR3 and TP73 hypermethylation with patients'

invasive stages (p=0.008 and 0.025). In addition, DDAH2 and CELSR3 hypermethylation was found significantly associated with patients' age (p=0.042 and 0.044). However, there was no statistical significance of the association between p16, DDAH2, DUSP1, CELSR3, PIKC3R5, TP73, MEF2D, RRM2 and BCL2 genes with other demographic and clinicopathological data except the aforementioned characteristics as shown in Table 4.3.

Table 4.3. Demographic profiles, clinicopathological characteristics and gene methylations of OSCC patients

Character-istics	Percent-age, %	P values (Chi-square or Fisher Exact tests)								
		p16	**DDAH2**	**DUSP1**	**CELSR3**	**PIKC3R5**	**TP73**	**MEF2D**	**RRM2**	**BCL2**
Gender										
Male	14 (35%)									
Female	26 (66%)	0.546	0.168	0.631	1.000	1.000	0.279	0.477	0.686	0.747
Ethnic										
Malay	5 (12.5%)									
Chinese	6 (15.0%)									
Indian	29 (72.5%)	0.214	0.473	0.560	0.728	0.770	0.669	0.563	0.514	0.379
Age Range (Years)										
20-39	4 (10%)									
40-59	15 (37.5%)									
60-79	19 (47.5%)									
> 80	2 (5%)	0.196	**0.05***	0.574	0.468	0.902	0.240	0.704	0.693	0.968
Habit										
No habit	8 (20%)									
Quit habit	1 (2.5%)									
Alcohol/Tobacco /Betel quid	31 (77.5%)	0.855	0.541	0.575	0.113	0.402	0.365	0.764	0.563	0.390
Tumour Sites										
Buccal mucosa	19 (47.5%)									
Gum	8(20%)									
Tongue	12 (30%)									
Lip	1 (2.5%)	**0.001***	0.700	0.601	0.797	0.922	**0.006***	0.247	0.701	0.708

Table 4.3, continued

Character-istics	Percent-age, %	P values (Chi-square or Fisher Exact tests)								
		p16	DDAH2	DUSP1	CELSR3	PIKC3R5	TP73	MEF2D	RRM2	BCL2
<u>Pathological TNM stage</u>										
I	4 (10%)									
II	6 (15%)									
III	4 (10%)									
IV	26 (65%)	0.327	0.587	0.325	0.300	0.282	0.619	0.194	0.608	0.646
<u>Invasive front (POI staging)</u>										
Cohesive	6 (15%)									
Non-cohesive	31 (77.5%)									
No stated	3 (7.5%)	0.247	0.542	0.278	**0.008**	0.819	**0.025**	0.980	0.137	0.448
<u>Differentiation (Tumour grading)</u>										
Well	13 (32.5%)									
Moderate	25 (62.5%)									
Poorly	1 (2.5%)									
No stated	1 (2.5%)	0.862	0.858	0.735	0.876	0.463	0.959	0.610	0.845	0.280

*Significant difference for promoter hypermethylation in p16 and TP73 hypermethylation with patients' tumour site (p=0.001 and 0.006), CELSR3 and TP73 hypermethylation with patients' invasive stages (p=0.008 and 0.025) by Chi-square or Fisher's Exact test.

4.3 Survival analysis

The OSCC patient survival data were calculated using Kaplan-Meier and log-rank tests. In survival rate data, there were two missing pieces of information due to lost contact during the follow-up time of 38 weeks. The patients' survival time were found to range from 2 to 87 weeks with a median follow-up time of 28.5 weeks.

In this study, the overall 5-year survival rate was 38.1% of OSCC patients (95% CI=27.1, 49.1) (Figure 4.10). The relative survival rate for patients' demographic profiles, clinicopathological characteristics and gene hypermethylations are shown in Table 4.4 and Figure 4.11.1-4.11.17.

There were no significance observed between the patients' relative survival rate and ethnicity (X^2=0.188, p=0.911), age (X^2=0.466, p=0.926), (X^2=2.715, p=0.438), tumour sites (X^2=3.390, p=0.335), pathological stages (X^2=1.411, p=0.842), invasive front (X^2=1.604, p=0.448) and tumour grading, (X^2=2.977, p=0.226). In addition, there were also no significance observed between the patients' relative survival rate and hypermethylation of p16 (X^2=1.338, p=0.226), DDAH2 (X^2=0.365, p=0.546), DUSP1 (X^2=0.093, p=0.760), PIKCR5 (X^2=0.094, p=0.759), TP73 (X^2=0.006, p=0.940), MEF2D (X^2=0.235, p=0.628), RRM2 (X^2=0.084, p=0.772), CELSR3 (X^2=0.090, p=0.764) and BCL2 (X^2=0.001, p=0. 980). All of the patients' demographic and clinicopathological parameters, and gene hypermethylations did not influence the overall survival rate (p>0.05), except for the gender difference (X^2=3.636, p=0.050) (Figure 4.11.3). The gender of patients had a significant difference in the survival rate with 24.2% for males and 46.5% for females.

Table 4.4. Survival analysis of patients' demographic profiles, clinicopathological characteristics, and genes' hypermethylation status

Characteristics		Mean of overall survival			Log rank (Mantel Cox) test	
		Estimate	95% confidence interval (CI)		X^2	p value
			Lower bound	Upper bound		
Ethnic	Malay Chinese Indian Overall	33.8 38.2 36.9 38.1	8.7 15.6 24.4 27.1	58.8 60.7 49.4 49.1	0.188	0.911
Age	20-39 40-59 60-79 > 80 Overall	43.7 35.4 32.7 34.0 38.1	6.6 21.0 17.8 0.0 27.1	80.7 49.8 47.6 68.6 49.1	0.466	0.926
Sex	Male Female Overall	24.2 46.5 38.1	6.4 7.4 5.6	36.8 61.2 49.1	3.636	***0.050**
Habits	No habit Betel quid Smoke Alcohol Overall	32.1 30.9 76.0 36.6 38.1	16.0 16.8 60.8 19.2 27.1	48.3 44.8. 91.2 54.0 49.1	2.715	0.438
Tumour sites	Buccal mucosa Gum Tongue Lip Overall	29.2 61.5 38.1 24.0 38.1	14.7 32.6 23.6 24.0 27.1	43.7 90.4 52.6 23.0 49.1	3.390	0.335
Pathological stages	No Stage Stage 1 Stage 2 Stage 3 Stage 4 Overall	14.0 46.3 28.0 52.2 36.9 38.1	14.0 22.6 5.8 16.9 22.5 27.1	14.0 69.9 50.2 87.4 51.2 49.1	1.411	0.842
Invasive stages	Nil Non cohesive Cohesive Overall	23.3 38.2 45.8 38.1	6.21 24.9 21.9 27.1	40.5 51.4 69.7 49.1	1.604	0.448

Table 4.4; continued

Characteristics		Mean of overall survival			Log rank (Mantel Cox) test	
		Estimate	95% confidence interval (CI)		X^2	p value
			Lower bound	Upper bound		
Tumour grading	Well differentiated	33.4	17.7	49.0	2.977	0.226
	Moderate differentiated	41.2	26.9	55.4		
	Poor differentiated	8.00	8.0	8.0		
	Overall	38.1	27.1	49.1		
p16	Unmethylated	53.7	25.21	82.2	1.338	0.247
	Methylated	33.8	22.8	44.7		
	Overall	38.1	27.	49.1		
DDAH2	Unmethylated	34.1	19.6	48.5	0.365	0.546
	Methylated	36.8	24.6	49.0		
	Overall	38.1	27.1	49.1		
DUSP1	Unmethylated	29.0	5.3	52.7	0.093	0.760
	Methylated	38.8	27.2	50.6		
	Overall	38.1	27.1	49.1		
PIK3R5	Unmethylated	33.5	20.6	56.4	0.094	0.759
	Methylated	38.4	26.3	50.4		
	Overall	38.2	27.1	49.1		
TP 73	Unmethylated	23.0	0.0	52.1	0.006	0.940
	Methylated	37.8	26.8	48.9		
	Overall	38.1	27.1	49.1		
MEF2D	Unmethylated	35.7	15.1	56.4	0.235	0.628
	Methylated	38.7	26.4	51.2		
	Overall	38.1	27.1	49.1		
RRM2	Unmethylated	27.4	18.3	36.5.	0.084	0.772
	Methylated	39.5	26.6	52.4.		
	Overall	38.1	27.1	49.1		
CELSR3	Unmethylated	36.4	10.7	62.1	0.090	0.764
	Methylated	37.4	25.7	49.0		
	Overall	38.1	27.1	49.1		
BCL2	Unmethylated	37.7	22.9	52.5	0.001	0.980
	Methylated	37.7	21.5	54.1		
	Overall	38.1	27.1	49.1		

*Significant difference was found between sex difference with survival rate (p=0.050) by log rank test.

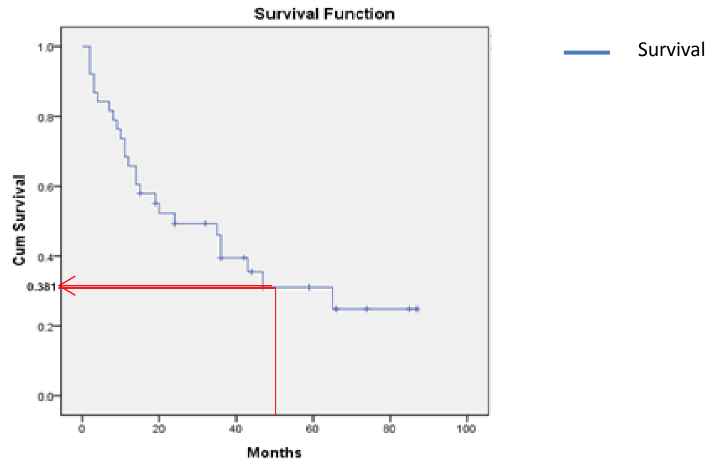


Figure 4.10. 38.1% of overall survival rate in OSCC patients (95% CI=27.1, 49.1) showed in Kaplan-Meier curve.

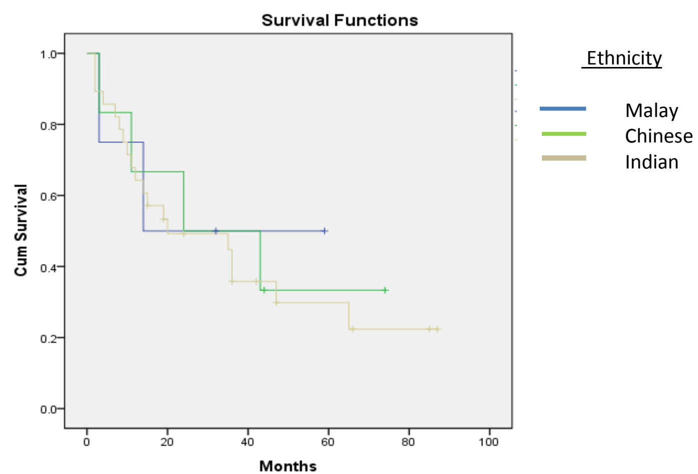


Figure 4.11.1. Relative survival of ethnicity demonstrated in Kaplan-Meier survival curve. The curve shows ethnicity have no influence on the patients' overall survival rate (X^2=0.188, P=0.911).

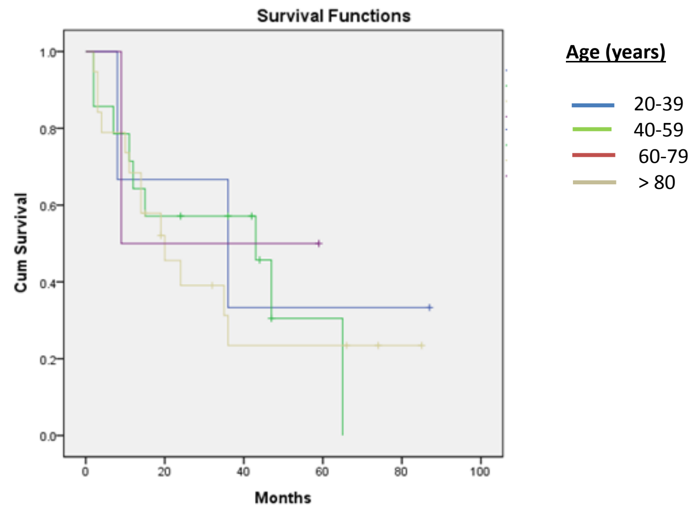


Figure 4.11.2. Relative survival of age demonstrated in Kaplan-Meier survival curve. The curve shows age has no influence on the patients' overall survival rate (X^2=0.466, P=0.926).

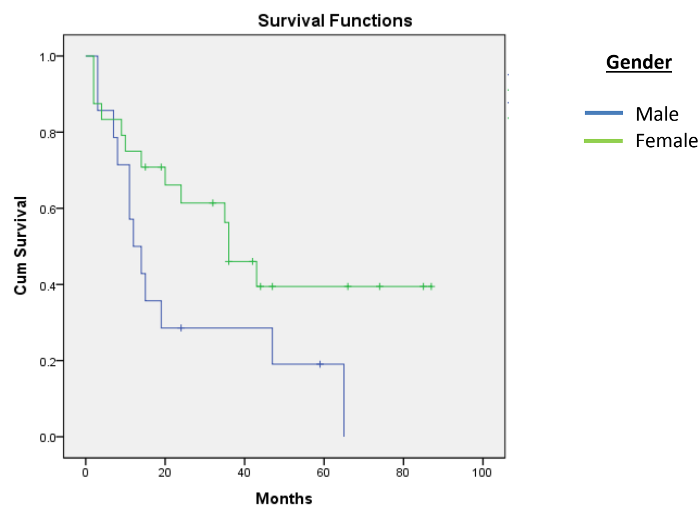


Figure 4.11.3. Relative survival of gender demonstrated in Kaplan-Meier survival curve. The curve shows gender has influence on patients' overall survival with 24.2% for male and 46.5% for female (X^2=3.636, p=0.050).

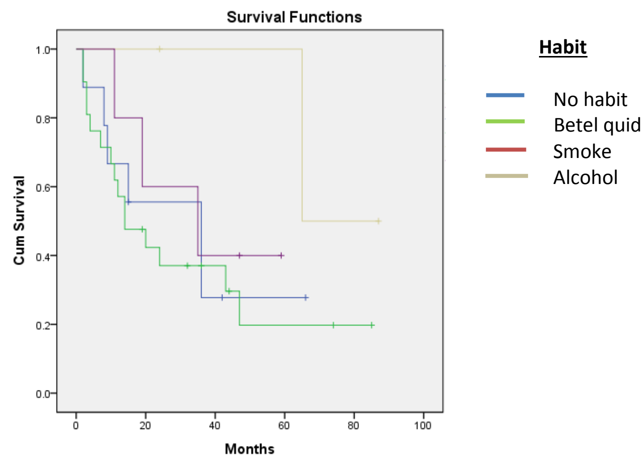


Figure 4.11.4. Relative survival of habits demonstrated in Kaplan-Meier survival curve. The curve shows habits have no influence on the patients' overall survival rate (X^2=2.715, p=0.438).

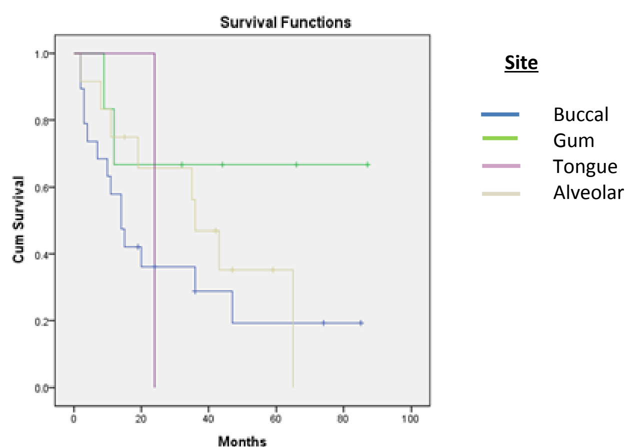


Figure 4.11.5. Relative survival of tumour sites demonstrated in Kaplan-Meier survival curve. The curve shows tumour sites have no influence on the patients' overall survival rate (X^2=3.390, p=0.335).

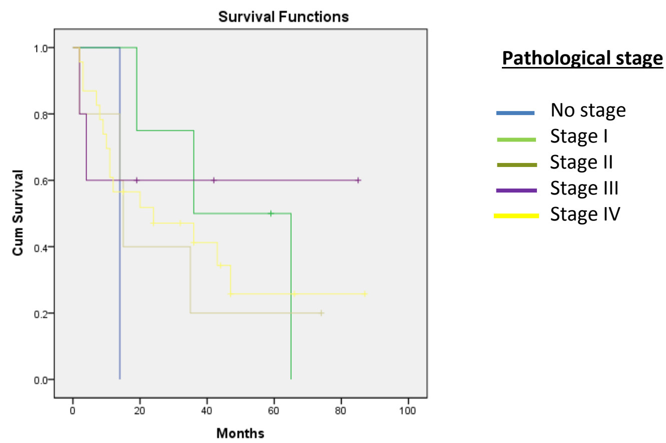


Figure 4.11.6. Relative survival of pathological stages demonstrated in Kaplan-Meier survival curve. The curve shows pathological stages have no influence on the patients' overall survival rate (X^2=1.411, p=0.842).

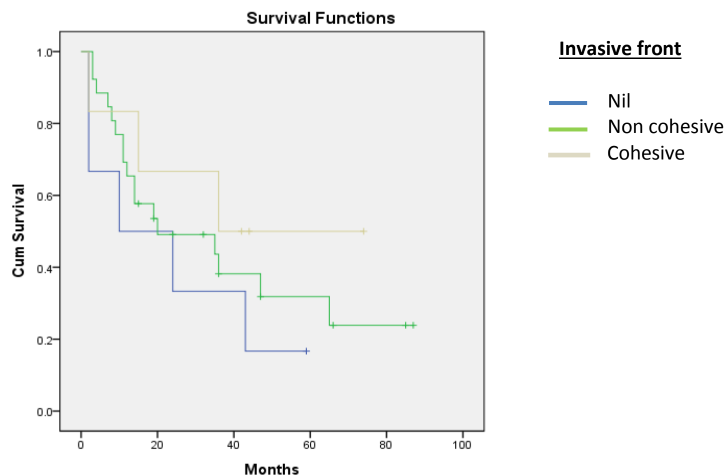


Figure 4.11.7. Relative survival of invasive front demonstrated in Kaplan-Meier survival curve. The curve shows invasive stages have no influence on the patients' overall survival rate (X^2=1.604, p=0.448).

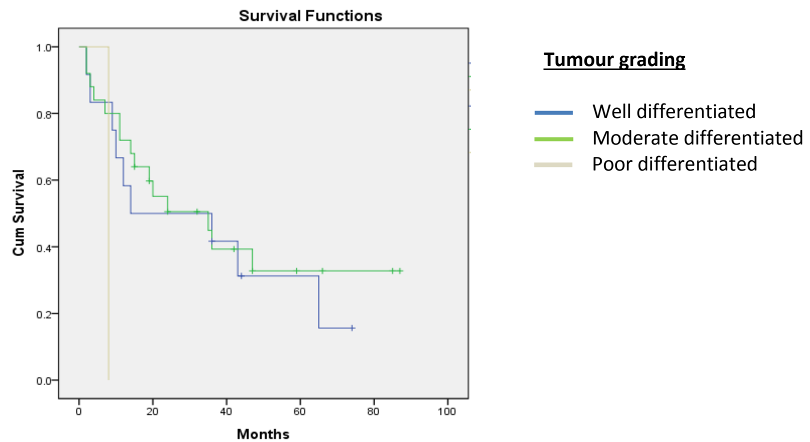


Figure 4.11.8. Relative survival of tumour grading demonstrated in Kaplan-Meier survival curve. The curve shows tumour grading has no influence on the patients' overall survival rate (X^2=2.977, p=0.226).

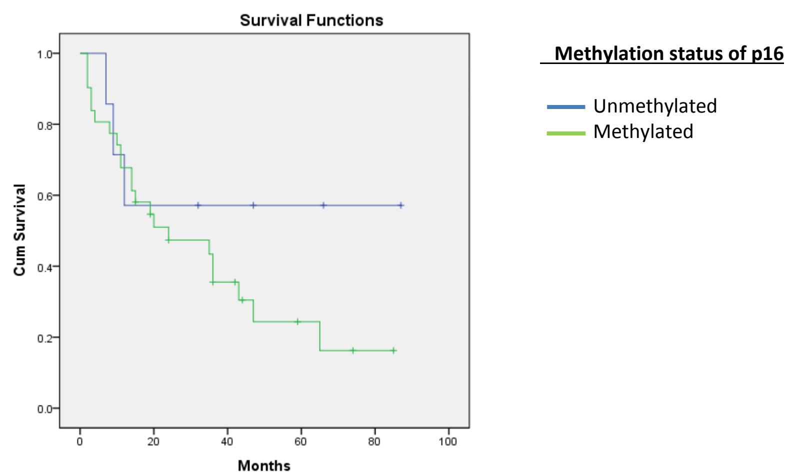


Figure 4.11.9. Relative survival of p16 methylation demonstrated in Kaplan-Meier survival curve. The curve shows p16 methylation has no influence on the patients' overall survival rate (X^2=1.338, p=0.247).

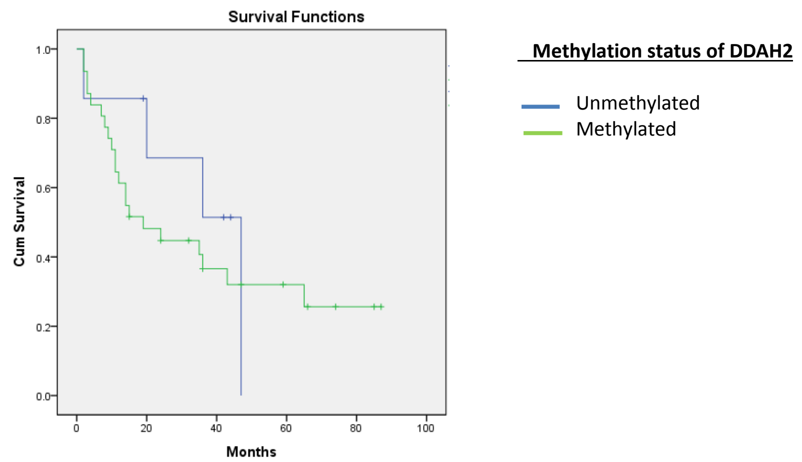


Figure 4.11.10. Relative survival of DDAH2 methylation demonstrated in Kaplan-Meier survival curve. The curve DDAH2methylation has no influence on the patients' overall survival rate (X^2=0.365, p=0.546).

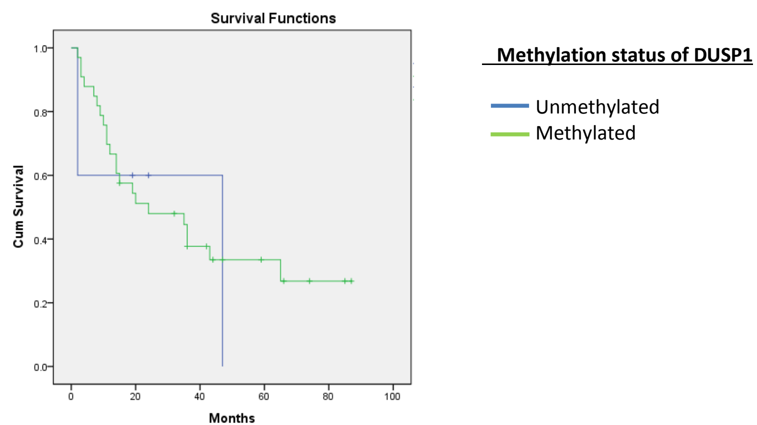


Figure 4.11.11. Relative survival of DUSP1 methylation demonstrated in Kaplan-Meier survival curve. The curve shows DUSP1methylation has no influence on the patients' overall survival rate (X^2=0.093, p=0.760).

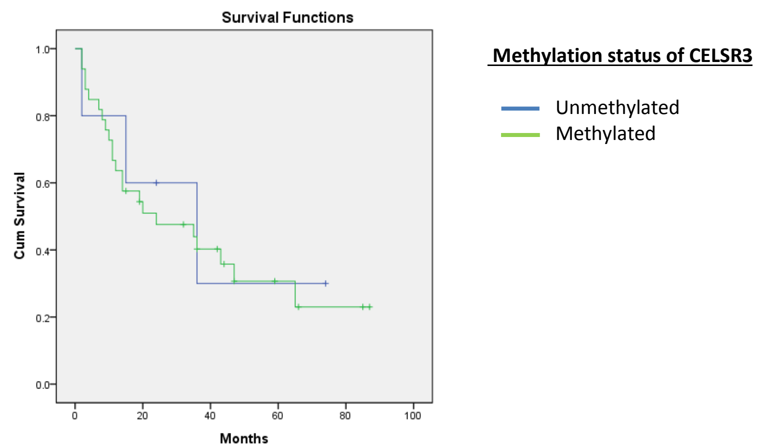


Figure 4.11.12. Relative survival of CELSR3 methylation demonstrated in Kaplan-Meier survival curve. The curve shows CELSR3 methylation has no influence on the patients' overall survival rate (X^2=0.090, p=0.764).

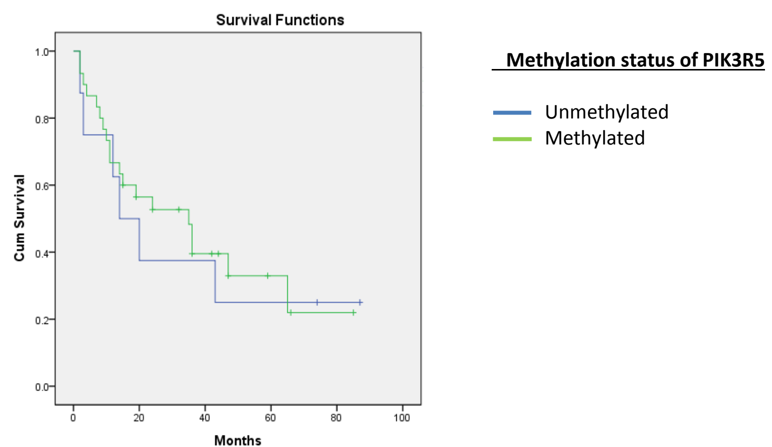


Figure 4.11.13. Relative survival of PIK3R5 methylation demonstrated in Kaplan-Meier survival curve. The curve shows PIK3R5 methylation has no influence on the patients' overall survival rate (X^2=0.094, p=0.759).

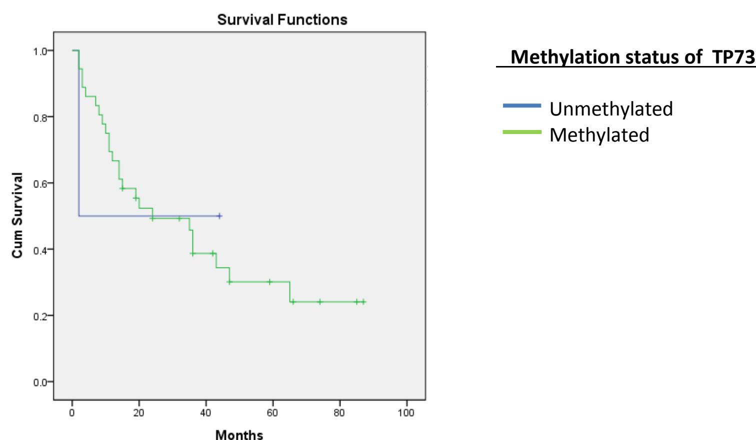


Figure 4.11.14. Relative survival of TP73 methylation demonstrated in Kaplan-Meier survival curve. The curve shows TP73 methylation has no influence on the patients' overall survival rate (X^2=0.006, p=0.940).

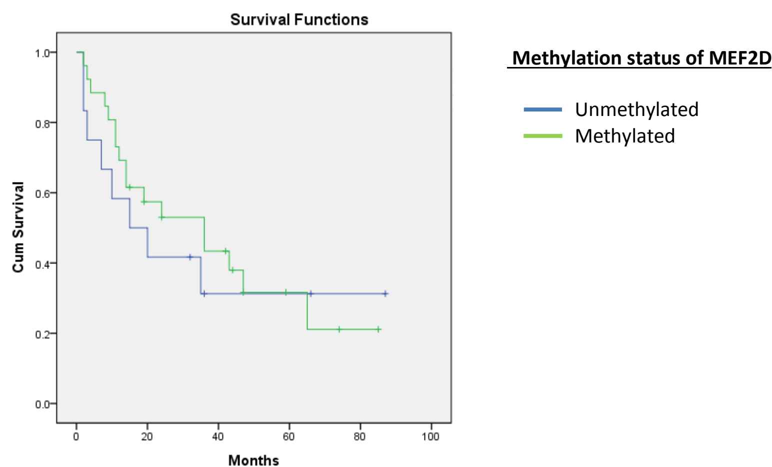


Figure 4.11.15. Relative survival of MEF2D methylation demonstrated in Kaplan-Meier survival curve. The curve shows MEF2D methylation has no influence on the patients' overall survival rate (X^2=0.235, p=0.628).

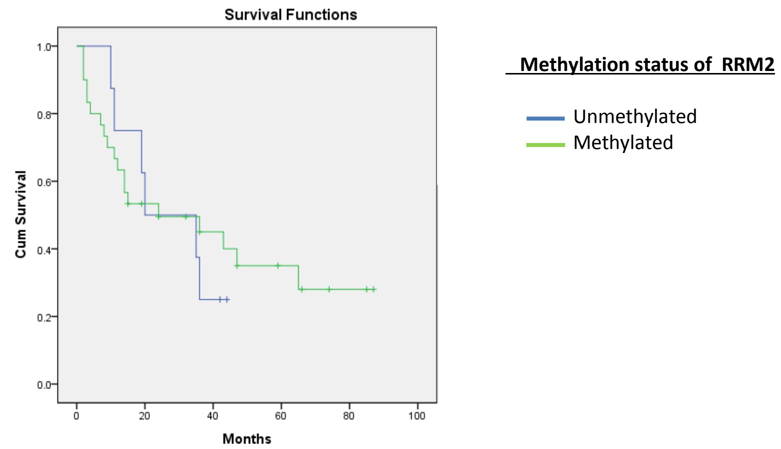


Figure 4.11.16. Relative survival of RRM2 methylation demonstrated in Kaplan-Meier survival curve. The curve shows RRM2 methylation has no influence on the patients' overall survival rate (X^2=0.084, p=0.772)

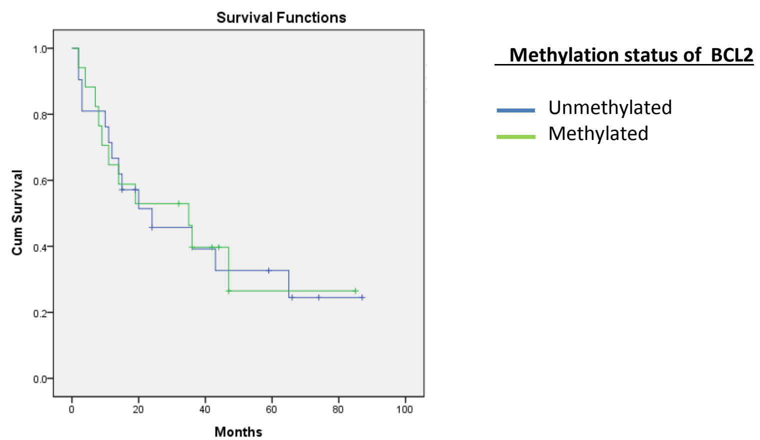


Figure 4.11.17. Relative survival of BCL2 methylation demonstrated in Kaplan-Meier survival curve. The curve shows BCL2 methylation has no influence on the patients' overall survival rate (X^2=0.001, p=0.980).

4.4 Immunohistochemical analysis

To detect the protein expression levels of DDAH2, DUSP1, MEF2D and RRM2, IHC analysis was performed on 4 normal oral mucosa and 40 OSCC tissues. Negative immunostaining of OSCC tissue was observed in negative control sections of DDAH2 (Figure 4.12.1), DUSP1 (Figure 4.12.4), MEF2D (Figure 4.12.7) and RRM2 (Figure 4.12.10). All normal mucosa demonstrated positive immunostaining in DDAH2 (Figure 4.12.2), DUSP1 (Figure 4.12.5), MEF2D (Figure 4.12.8) and RRM2 (Figure 4.12.11). MEF2D demonstrated nuclear immunostaining (Figure 4.12.9), while DDAH2, DUSP1 and RRM2 showed cytoplasmic immunostaining (Figure 4.12.3, 4.12.6 and 4.12.12) in OSCC tissue.

A low percentage of cases of positive immunostaining was demonstrated in tumour tissues for DDAH2 at 30% (12/40), DUSP1 with 27.5% (11/40), MEF2D with 25% (10/40) and RRM2 with 22.5% (9/40) (Table 4.5).

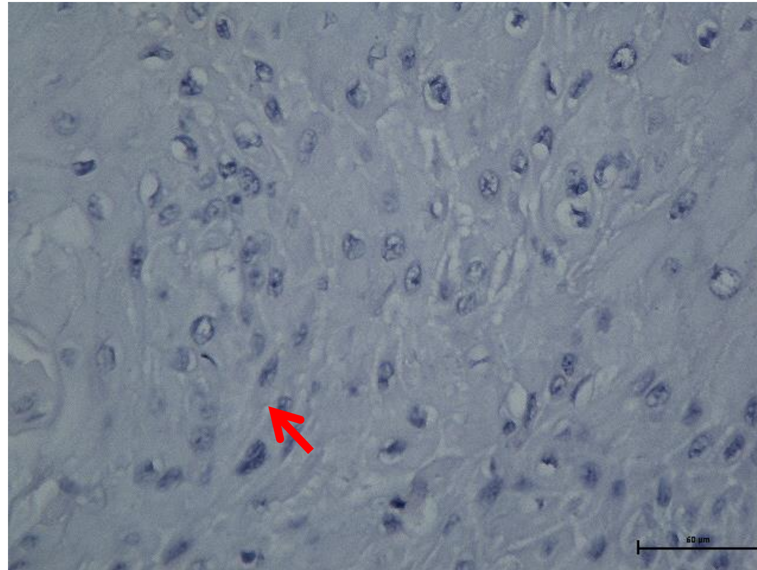


Figure 4.12.1. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against DDAH2. No staining of was observed in the cytoplasm (with red arrow) of the tumour cells of the negative control section (40X magnification).

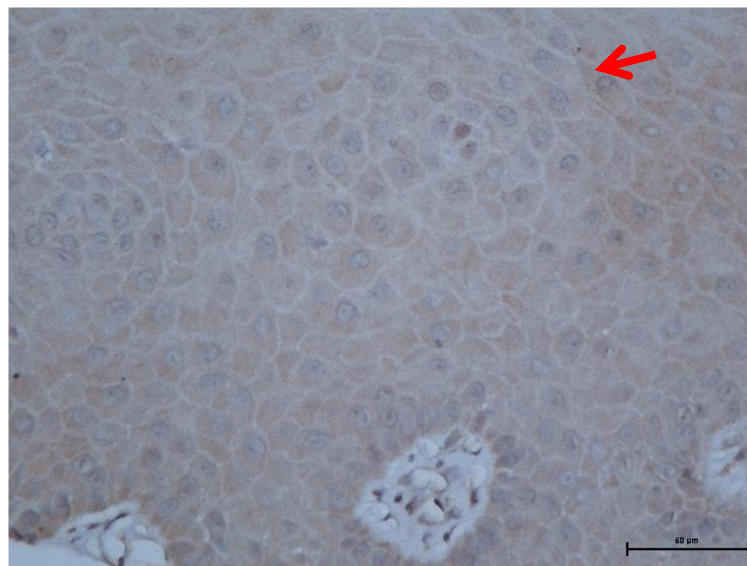


Figure 4.12.2. Formalin-fixed paraffin-embedded section of representative normal squamous cells was stained with the antibodies against DDAH2. Positive immunostaining of DDAH2 was observed in most of the cytoplasm (with red arrow) of the normal epithelium (40X magnification).

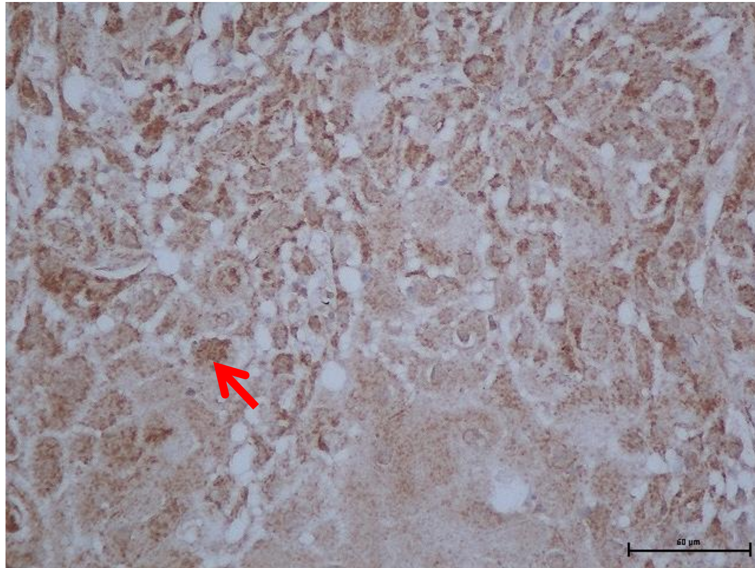


Figure 4.12.3. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against DDAH2. Positive staining of DDAH2 was detected in most of the cytoplasm (with red arrow) of the tumour cells (40X magnification).

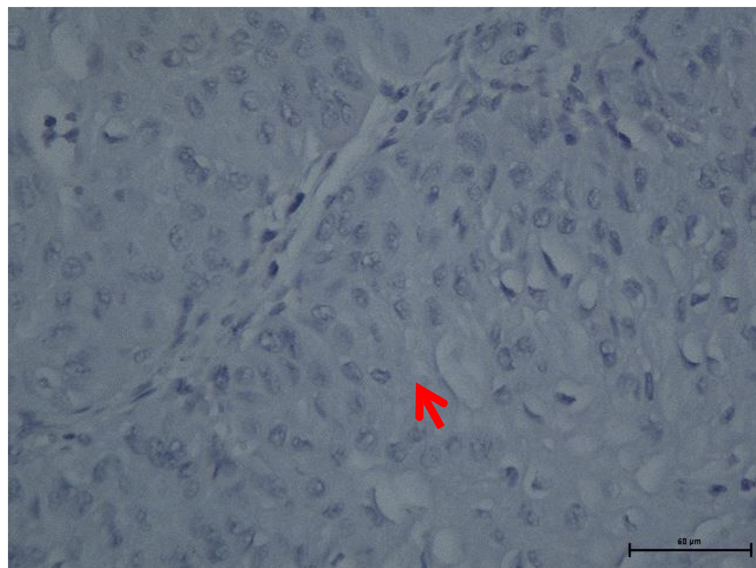


Figure 4.12.4. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against DUSP1. No cytoplasmic immunostaining (with red arrow) shows in the tumour cells of the negative control section (40X magnification).

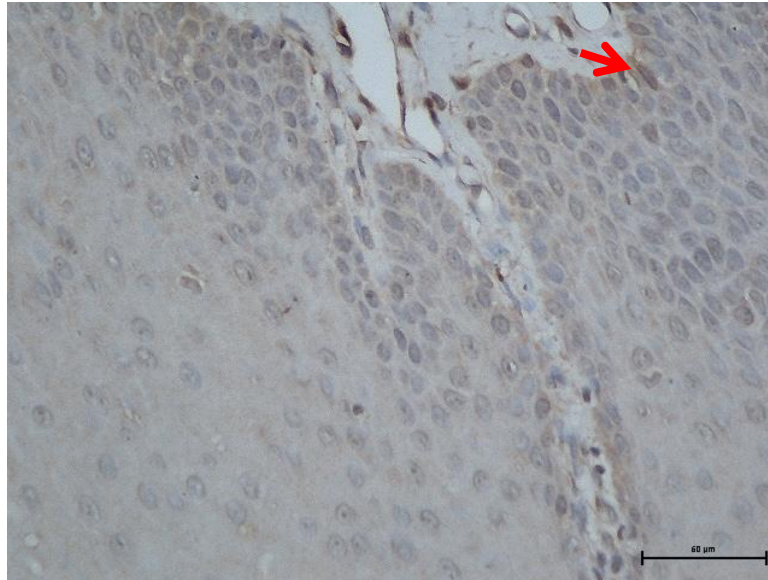


Figure 4.12.5. Formalin-fixed paraffin-embedded section of representative normal squamous cells was stained with the antibodies against DUSP1. Weak positive cytoplasmic immunostaining (with red arrow) of DUSP1 shows in the normal epithelium (40X magnification).

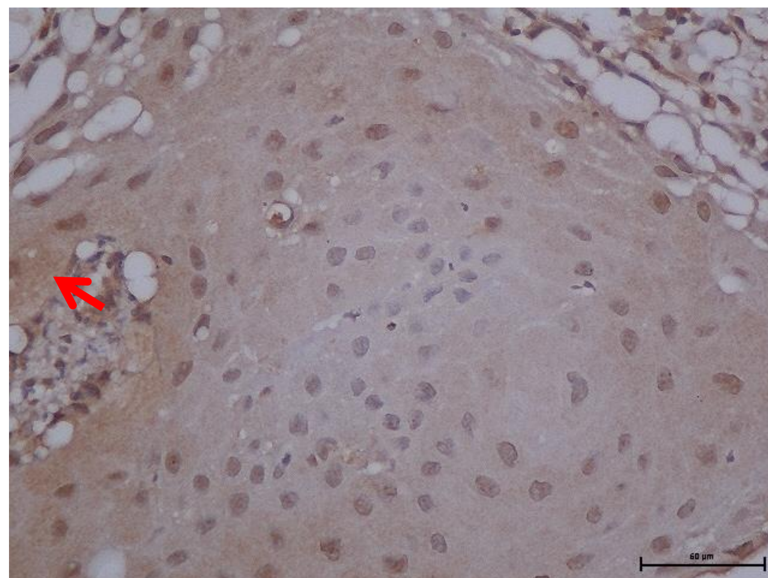


Figure 4.12.6. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against DUSP1. Positive DUSP1 cytoplasmic staining (with red arrow) was detected in the tumour cells (40X magnification)

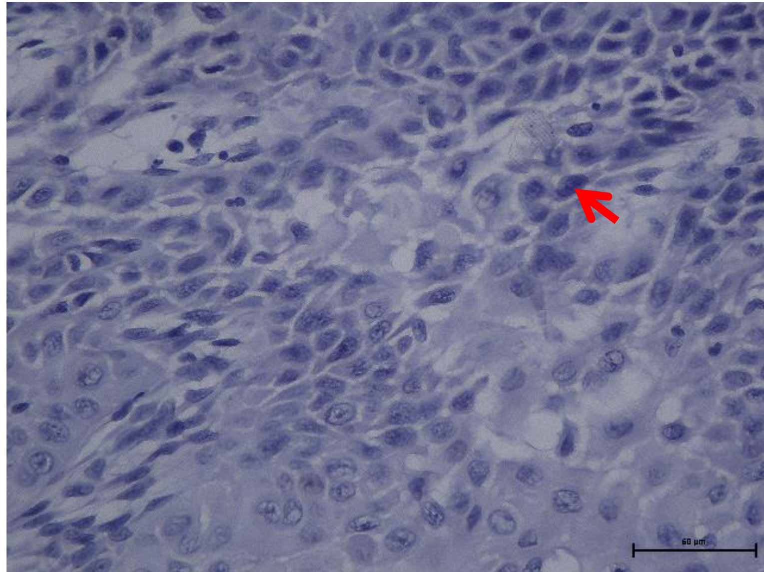


Figure 4.12.7. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against MEF2D. Negative MEF2D nuclear staining (with red arrow) shows in the tumour cells of the negative control section (40X magnification).

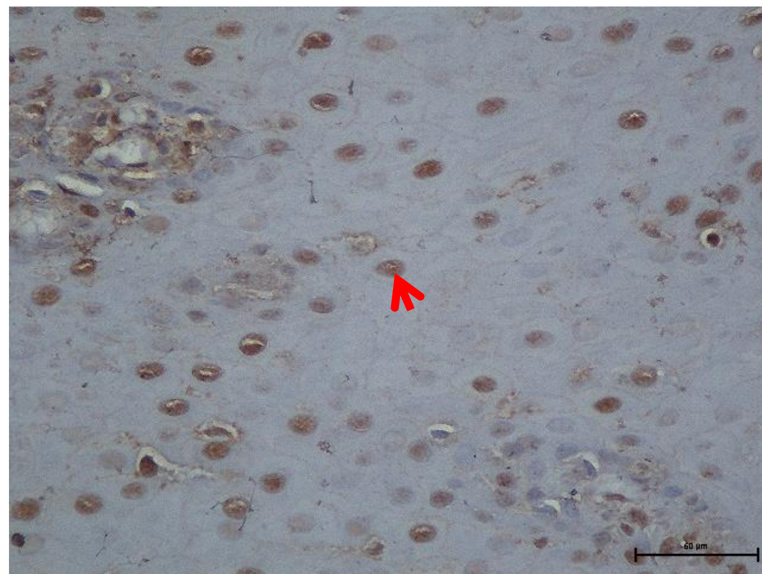


Figure 4.12.8. Formalin-fixed paraffin-embedded section of representative normal squamous cells was stained with the antibodies against MEF2D. Positive MEF2D nuclear staining (with red arrow) shows in the normal epithelium (40X magnification).

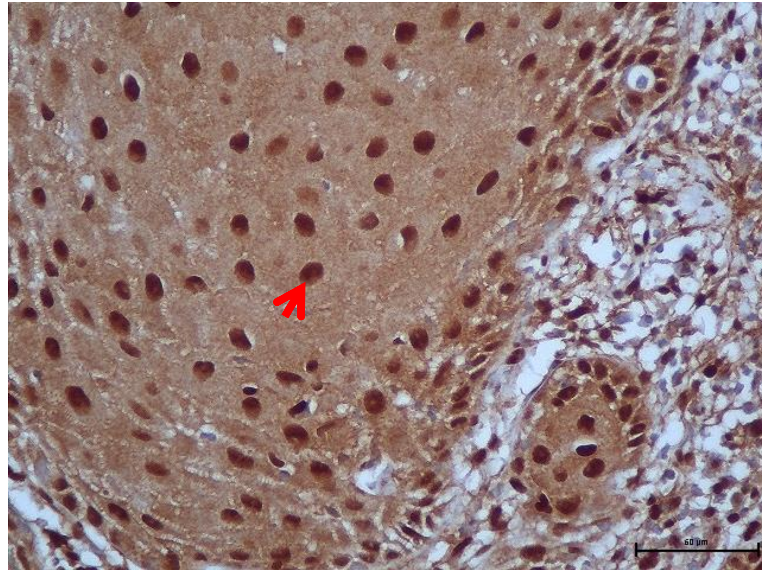


Figure 4.12.9. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against MEF2D. Positive MEF2D nuclear staining (with red arrow) was detected in the tumour cells (40X magnification).

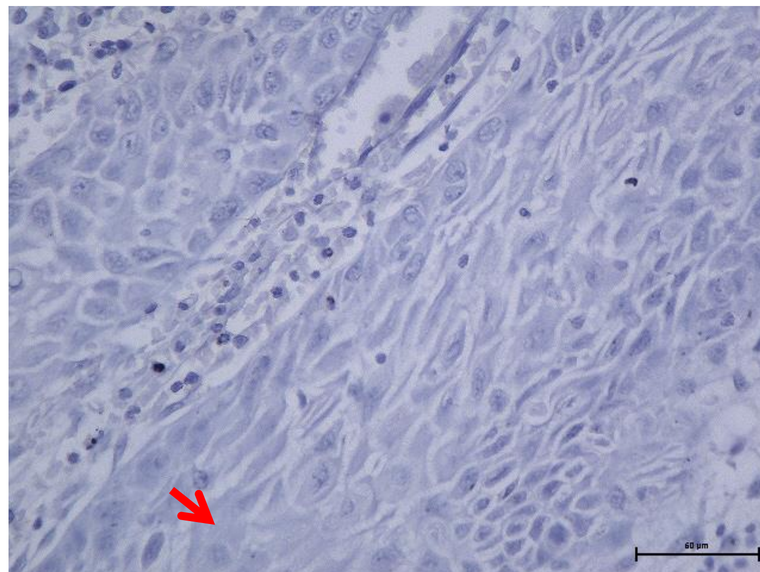


Figure 4.12.10. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against RRM2. Negative RRM2 cytoplasmic staining (with red arrow) shows in the tumour cells of the negative control section (40X magnification).

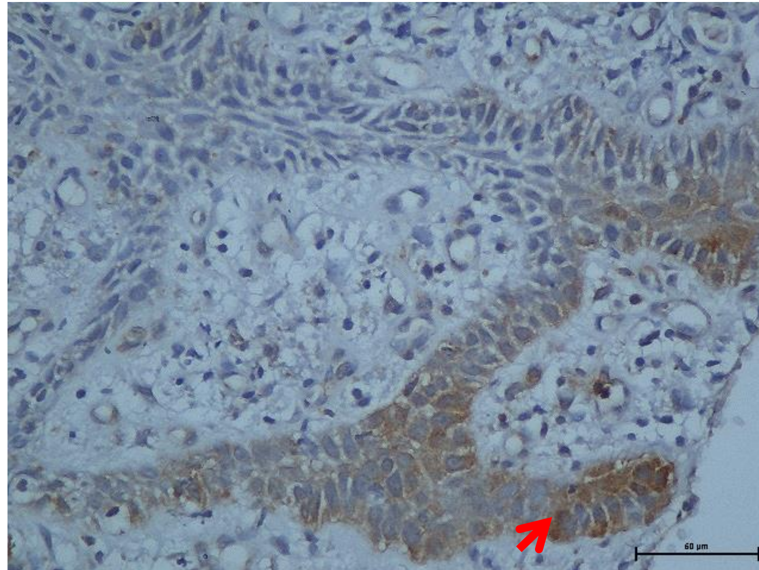


Figure 4.12.11. Formalin-fixed paraffin-embedded section of representative normal squamous cells was stained with the antibodies against RRM2. Positive RRM2 cytoplasmic staining (with red arrow) shows in the normal epithelium (40X magnification).

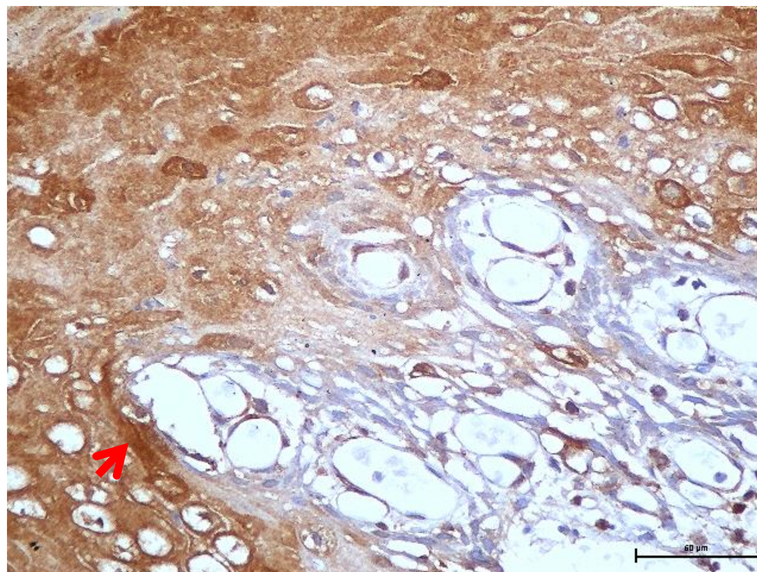


Figure 4.12.12. Formalin-fixed paraffin-embedded section of representative Oral Squamous Cell Carcinoma tissues was stained with the antibodies against RRM2. Positive RRM2 cytoplasmic staining (with red arrow) was detected in the tumour cells (40X magnification).

Table 4.5 Status and percentage of immunostaining for DDAH2, DUSP1, MEF2D and RRM2

No.	Immunostaining status of hypermethylated genes			
	DDAH2	DUSP1	MEF2D	RRM2
1	N	P	N	N
2	N	P	N	N
3	N	P	N	P
4	N	P	N	N
5	N	P	N	N
6	N	P	N	N
7	N	P	N	N
8	N	N	P	P
9	N	N	N	P
10	N	N	N	P
11	N	P	N	P
12	N	N	N	P
13	N	N	P	N
14	P	P	P	N
15	P	P	N	N
16	P	P	P	N
17	N	N	P	N
18	P	N	P	N
19	P	N	P	N
20	P	N	P	N
21	N	N	N	N
22	P	N	N	N
23	N	N	N	N
24	N	N	N	N
25	N	N	N	N
26	P	N	N	N
27	P	N	P	N
28	N	N	N	N
29	N	N	N	N
30	P	N	N	N
31	N	N	N	P
32	N	N	N	N
33	P	N	N	N
34	N	N	N	N
35	P	N	P	N
36	N	N	N	N
37	N	N	N	P
38	N	N	N	N
39	N	N	N	P
40	N	N	N	N
Percentage, % (No of cases) of immunostaining	30 (n=12)	27.5 (n=11)	25 (n=10	22.5 (n=9)

Indication:

P = Positive immunostaining

N = Negative immunostaining

4.4.1 Association between gene methylation levels and protein expressions of DDAH2, DUSP1, MEF2D and RRM2.

The Chi-square and Fisher's exact tests were conducted to investigate the association between gene hypermethylations and protein expressions. The results showed an association between gene hypermethylation level with protein expression of DDAH2 ($p=0.017$), DUSP1 ($p=0.006$), MEF2D ($p=0.002$), and RRM2 ($p=0.001$).

4.4.2 Correlation between protein expressions of DDAH2, DUSP1, MEF2D and RRM2

Spearman's rho test was conducted to correlate protein expressions of DDAH2, DUSP1, MEF2D and RRM2. In the results, no correlation was found between protein expression of DDAH2 with DUSP1 ($p = 0.584$, $r = 0.081$, CI = 95%), DDAH2 with RRM2 ($p = 0.88$, $r = 0.022$, CI = 95%), DUSP1 with MEF2D ($p = 0.071$, $r = 0.263$, CI = 95%), DUSP1 with RRM2 ($p = 0.366$, $r = 0.133$, CI = 95%) and MEF2D with RRM2 ($p = 0.823$, $r = -0.033$, CI = 95%). However, there was a significant correlation of protein expression found between DDAH2 and MEF2D ($p = 0.008$, $r = 0.378$, CI = 95%), where DDAH2 revealed a weak positive correlation with MEF2D (Appendix F).

4.4.3 Correlation between patients' age and protein expression of DDAH2, DUSP1, MEF2D and RRM2.

Spearman's rho test showed no correlation between patients' age with protein expressions of DDAH2 ($p = 0.227$, $r = -0.195$, CI = 95%), DUSP1 ($p = 0.078$, $r = 0.282$, CI = 95%), and MEF2D ($p = 0.158$, $r = -0.228$, CI = 95%) (Appendix F). However, a significant correlation was found between a patient's age with the protein expression of RRM2 ($p = 0.015$, $r = 0.381$, CI = 95%), where RRM2 revealed a weak positive correlation with the patient's age (Appendix F).

CHAPTER 5 DISCUSSIONS

The overall goal of this study was to enhance the knowledge of the epigenetic alterations in OSCC through genome-wide methylation profiling, especially to verify the established and newly found hypermethylated genes as potential clinically useful biomarkers by MSPCR and IHC analysis. Correlations between gene hypermethylation and protein expression of DDAH2, DUSP1, MEF2D and RRM2 in OSCC were also elucidated. Finally, the potential co-founding factors in oral cancer were studied for the prognostic purpose and survival rate.

5.1 Methylation microarray analysis

The result of methylation microarray analysis demonstrated the demarcation between the 20 tumour tissues and 3 normal samples using unsupervised hierarchical clustering and PCA analysis. It is concordant with those reported elsewhere (Bibikova et al., 2006; Killian et al., 2012; Nazmul-Hossain et al., 2007).

To the best of my knowledge, this is the first methylation study on Malaysian oral cancer patients using the Illumina, Infinium 450K Methylation assay which is also the latest generation of methylation microarray.

This approach provided very comprehensive and high throughput data in a genome-wide study, covering 99% of RefSeq genes. More than 450,000 methylation sites, including the promoter region of CpG islands and shores per sample at single nucleotide resolution are provided by this platform (Bibikova et al., 2011; Cheng et al., 2014; Stott-Miller et al., 2014). The recent advances in high-throughput microarray technologies have enabled investigators to study site-specific DNA methylation events on a much broader scale (O'Sullivan &

Goggins, 2013). Many hypermethylation events in CpG islands near known genes transcriptional start sites (which correlated with reduction in gene expression) have been identified. These approaches have led to the discovery of hundreds of affected genes previously not identified, which are potential prognostic DNA methylation in cancer events (Noushmehr et al., 2010; Øster et al., 2011).

To date, the Infinium 450K Methylation microarray is the most attractive, powerful and cost-effective tool available for generating quantitative DNA methylomes in healthy and diseased individuals (Dedeurwaerder et al., 2011). Furthermore, quality control of test reliability of DNA microarray conducted recently has made it possible for molecular testing to become an important tool (van't Veer & Bernards, 2008). Using the Infinium 450K methylation microarray, the present study compared the genomic DNA hypermethylation of oral cancer screened for 1080 differential sites, which showed the detailed distribution of these differential sites, establishing an oral cancer DNA methylation profile. Similar observation also reported by Stott-Miller et al. (2014). Verification of microarray results by MSPCR and IHC in the study showed that these results were reliable. This shows that methylation array analysis is very specific and useful in identifying the signature hypermethylated genes and for understanding the roles of hypermethylated genes that are involved in oral carcinogenesis (Bibikova et al., 2006; Viet & Schmidt, 2010).

Methylation microarrays have been applied on various cancers including breast, colon, stomach, pancreatic, oesophageal, lung, bladder, colorectal and ovarian cancers (Viet & Schmidt, 2010; Øster et al., 2011). Although numerous studies have been published on DNA methylation of HNSCC, very few studies have utilized microarray technology. A panel of 2

to 21 known gene-associated oral cancer sources have been employed instead in the DNA methylation studies (Viet & Schmidt, 2010).

A considerable number of differentially methylated sites had been reported in most of methylation profiling of oral cancer that conducted by other studies (Viet & Schmidt, 2008; Towle et al., 2013). Towle et al. (2013) reported methylation profile on 30 biopsies consisting of dysplastic, carcinoma in situ, OSCC and adjacent normal biopsies. This methylation profile was performed using the Infinium Human Methylation 27K platform which evaluates 27,578 CpG dinucleotides (corresponding to 14,473 unique genes). Viet & Schmidt (2008) applied the GoldenGate Methylation Array (Illumina) platform which includes 1,505 CpG loci selected from 807 cancer-related genes for methylation profile patterns in preoperative and post-operative saliva of thirteen OSCC patients. Thus, this partially explains the gene dissimilarity in OSCC studies that identified from different microarray platforms and tumour subtypes.

The methylation approach of searching for new biomarkers in oral cancer is a promising alternative since this epigenetic modification is an early, progressive and cumulative event in OSCC. Microarray technologies have been frequently applied to compare the epigenetics between normal and tumour tissues for determining biomarkers, and can contribute in diagnostic and therapeutic strategies. However, most of the clinical sample selections were from different oral tumour sites, and this explains why gene dissimilarities were identified in different experiments, as some findings suggest the possibility of gene expressions of SCC from different tumour sites which may be distinct from one another (Ginos et al., 2004; Warner et al., 2004).

In this study, further verification of candidate genes on a separate cohort of healthy and oral cancer population by MSPCR and IHC analysis have confirmed the hypermethylation in the promoter region of existing biomarkers of p16, TP73, BCL2 and DUSP1 as reported by others (Friedrich et al., 2004; Hoque et al., 2005; Kang et al., 2007; Wilcox, Baysal, Gallion, Strange, & DeLoia, 2005) and new biomarkers of DDAH2, PIK3R5, CELSR3, MEF2D and RRM2. Thus, confirmation of the gene methylation statuses using methylation specific technique, MSPCR in p16, DUSP1, DDAH2, CELSR3, PIK3R5, RRM2, MEF2D, TP73 and BCL2 genes; and IHC assay for protein expressions of DDAH2, DUSP1, MEF2D and RRM2, provide reliability of the microarray data that show 100% of concordance (Weber et al., 2005). Therefore, by using computational and gene-specific validation approaches, we identified that DUSP1, DDAH2, CELSR3, PIK3R5, RRM2 and MEF2D are the potential epigenetic biomarkers for oral cancer.

5.2 Partek Genomic Suite assay

Data obtained from Illumina's Genome Studio software were further analysed and integrated using Partek Genomic Suite software. A separate heat-map hierarchical clustering analysis was performed on normal (n=3) and tumour tissues (n=20). The data set was clustered using a standard hierarchical method with the Pearson's correlation to determine the distance function (Kron et al., 2009). The cluster set of normal and tumour samples (stage 1, 2, 3 and 4) clearly segregated the normal from tumour samples (Figure 3). However, there was no clear separation between the clustering patterns in the four different pathological stages. This may be due to the number of samples per pathological stage, as these were small in sample size in this study.

Signature candidate genes of p16, DUSP1, DDAH2, PIK3R5, CELSR3, BCL2, RRM2 and MEF2D were selected based on the $p < 0.001$, methylation fold change and their biological functions. In addition, Genomic Suite Visualization demonstrated the selected significant enrichment differences in p16, DDAH2, DUSP1, PIKC3R5, CELSR3 and BCL2 genes as their selected probe regions were further validated for microarray data validation using MSPCR and IHC assays.

Genes that were differentially hypermethylated in their promoter regions between tumour and normal samples by at least 2 fold changes and FDR value less than 0.05 (Kron et al., 2009) were further mapped to the GeneGo, Metacore^{TM} for pathway analysis. MED2F and RRM2 genes were further selected after pathway analysis for microarray data verification with MSPCR and IHC assays.

5.3 Methylation-specific polymerase chain reaction analysis

DNA methylation plays an essential role in maintaining cellular function, although methylation aberrations may lead to carcinogenesis. Thus, several methods have been developed recently to analyse gene methylation status in human cells, depending on their detection strengths and weaknesses (O'Sullivan & Goggins, 2013). One of the methods is the MSPCR which applies specific designed methylated and unmethylated primers for detecting the methylation level (Herman et al., 1996). It can be applied for distinguishing methylated from unmethylated cytosine residues. As few as 1 to 10 tumour cells among 10^4 normal cells in clinical samples can be detected (Cottrell & Laird, 2003). It is a sensitive but not quantitative technique for the methylation level (Herman et al., 1996; Towle & Garnis, 2012).

The methylation frequency of promoter regions of some important genes, such as p16 and TP73 is high in OSCC and has also been well documented in other cancer studies (Sharma et al., 2007; Towle & Garnis, 2012; Jha et al., 2012). The variation of gene methylation frequencies in individual studies may be due to the different geographical origin, which suggests population differences (Jha et al., 2012), because similar results have been observed in a study of genetic alteration in mutations. In fact, frequency variation of gene hypermethylation can also be due to cancer heterogeneity in promoter methylation, such methylation alterations were generally not correlated with gene expression (Aryee et al., 2013).

Tumour suppressor gene, p16 (chromosome 9p21.3) is a cell cycle regulator involved in the inhibition of checkpoint G1 phase progression in normal cells. The loss of p16 expression is usually connected to homozygote deletion, loss of heterozygosity, mutations and methylation. Apparently, promoter hypermethylation of p16 gene in CpG islands silences its transcription and represses gene expression act as another alternative pathway in many cancers. In this study, the p16 gene revealed that the promoter CpG island hypermethylation is associated with transcriptional silencing in OSCC, as indicated by many cancers including cervical (Jha et al., 2012), oesophagus (Kuwano et al., 2005), head and neck (Herman et al., 1995), lung (An et al., 2002), colorectal (Xu et al., 2004), breast (Sharma et al., 2007) and oral cavity (Cao et al., 2009; Dong et al., 2012; Pérez-Sayáns et al., 2011; Šupi et al., 2009; Towle & Garnis, 2012). These studies and the data here firmly demonstrate that the promoter hypermethylation is a frequent mode of gene silencing and p16 is one of the most frequently altered genes in most of the cancers.

Dimethylarginine dimethylaminohydrolase 2 (DDAH2) (chromosome 6p21.3) encodes an enzyme that is involved in nitric oxide (NO) generation by regulating cellular concentrations of methyl arginine, which in turn inhibits nitric oxide synthase (NOS) activity in normal cells (Leiper & Nandi, 2011). NO is involved in vital cell processes of vasodilation, respiration, cell migration, immune response and apoptosis. However, its dysregulation has been implicated in many pathophysiological conditions such as chronic disease and cancer (Hasegawa et al., 2006; Abhary et al., 2010; Kim et al., 2010; Korde et al., 2012), including the OSCC cases in this study. This is in contrast to the recent report by Kim et al. (2010) who showed that the DDAH2 gene expression was up-regulated in ovarian carcinoma. In this study, the DDAH2 gene was significantly hypermethylated in the promoter of CpG islands. This indicates that the novel gene, DDAH2 might play a putative role in OSCC carcinogenesis.

Dual Specificity phosphatase-like 1 (DUSP1) (chromosome 5q35.1) encodes for dual specificity protein phosphatase enzyme. It is an emerging subclass of the protein tyrosine phosphatase gene superfamily, a heterogeneous group of protein phosphatases that can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues. It acts as an activator in the MAPK signalling pathway, to dephosphorylate mitogen-activated protein kinase (MAPK) on both 'Thr-183' and 'Tyr-185' which are actively involved in protein modification, signal transduction and oxidative stress in normal cells (Keshet & Seger, 2010). In addition, DUSP1 is controlled by p53 during the cellular response to oxidative stress. It may play an important role in the human cellular response to environmental stress as well as in the negative regulation of cellular proliferation. In addition, it is also involved in angiogenesis, invasion and metastasis of non-small cell lung cancer (Keyse, 2008). High level of DUSP1 promoter hypermethylation was demonstrated in the tumour samples as reported by other studies including prostate, hepatocellular and non small cell lung

carcinomas (Rauhala et al., 2005; Calvisi et al., 2008; Keyse, 2008; Moncho-Amor et al., 2011).

Cadherin EGF LAG seven-pass G-type receptor 3 (CELSR3) (chromosome 3p21.31), belongs to a flamingo subfamily, part of the cadherin superfamily, but it does not interact with catenins. CELSR3 is a member of cell contact-mediated communication, with cadherin domains acting as homophilic binding regions and EGF-like domains involved in cell adhesion and receptor-ligand interactions. CELSR3 is crucial in controlling neural development which develops and maintains individual plastic changes throughout life by regulating planar cell polarity (PCP) through interactions with PCP proteins in WNT/PCP signalling pathway (Tissir et al., 2005; Feng et al., 2012). WNT/PCP signalling pathway is apparently implicated in embryogenesis and carcinogenesis, where aberrant activation of the WNT/PCP signalling pathway leads to malignancy through abnormal tissue polarity, invasion, and metastasis. Its' up-regulation in pancreatic cancer could be provided as a potential druggable target since its protein is located at the plasma membrane and has intriguing capabilities in WNT/PCP signalling pathway (Katoh, 2005). In addition, the dysregulation of DNA methylation has been reported recently in various cancer types including renal, bladder, hepatic and pancreatic carcinomas (Erkan et al., 2010; Morris et al., 2010; Jeong et al., 2011; Shen et al., 2012). Erkan et al. (2010) group found that in pancreatic and hepatic carcinoma CELSR3 was identified as a hypermethylated marker as reported in my finding. Moreover, Katoh et al. (2005) demonstrated that CELSR3 expression was reduced in adult brain tumours, which showed that its gene expression had undergone inactivation effect. Thus, this study predicts that hypermethylation mechanism has reduced this gene expression in OSCC cases. CELSR3 hypermethylation status in this finding suggests that it may be involved in oral carcinogenesis. Together, the data suggest an important role for CELSR3 in tumour cells

that warrants further investigation in the study. In the study, CELSR3 hypermethylation level was 87.5%, whereas 15% of CELSR3 hypermethylation activity in renal cell carcinoma (Morris et al., 2010). This controversy may be due to 1) the specific patterns of hypermethylation are indicative of specific cancer types and 2) the high specificity of primers design has targeted the gene promoter region. To the best of my knowledge, this is the first time CELSR3 is being investigated in OSCC cases. Thus, CELSR3 is a novel gene involved in epigenetic inactivation, which has not been reported elsewhere in OSCC.

Phosphoinositide-3-kinase, regulatory subunit 5 (PIK3R5), is also known as p101 (chromosome 17p13.1). Its hypermethylation level was found significant in the OSCC of this study, as reported in breast cancer (Rodenhiser et al., 2008), ovarian cancer (Dai, 2011) and non-small lung cancer (Lee et al., 2013; Lokk et al., 2012). Rodenhiser et al. (2008) reported that PIK3R5 hypermethylation is involved in the canonical pathway of ERK/MAPK signalling, B-cell receptor signalling, Integrin signalling and Huntington's disease signalling. Dai demonstrated the involvement of PIK3R5 promoter hypermethylation in AKT/mTOR pathway signalling pathway that leads to carcinogenesis in ovarian cancer (Dai, 2011). Meanwhile, Lee et al. (2013) found that PIK3R5 is one of the members of vascular endothelial growth factor (VEGF) signalling pathway that may influence an angiogenesis-dependent biological pathway, which is a critical component of carcinogenesis of non-small cell lung cancer. Lokk et al. (2012) revealed that patients with a high PIK3R5 methylation level had better survival rate than those with medium level. Based on my present knowledge during this study, this is the first report on the PIK3R5 that may act as a candidate gene that leads to OSCC carcinogenesis.

Ribonucleotide reductase small subunit M2 (RRM2) (chromosome 2p25.1) is the main component in modulating enzymatic activity of ribonucleotide reductase (RR), which is composed of RRM1 and RRM2. RRM1/RRM2 complex is the major source of deoxynucleoside triphosphates (dNTPs) for DNA replication during S phases (Lee et al., 2003). It is a rate-limiting enzyme in DNA synthesis and thus plays a pivotal role in cell growth. Increased RR activity has been shown by increasing DNA synthesis rate to serve the proliferative activity of cancer cells. Thus, aberration of the RR is dramatically associated with malignant transformation and tumour cell growth (Liu et al., 2007). These important roles have made RRM2 to be an attractive target for chemotherapeutic development (Tsimberidou et al., 2002). Methylation of CpG promoter regions of RRM2 was rare, when analysed by direct sequencing of bisulphate-modified DNA in primary renal cell carcinoma (Morris et al., 2008). However, Hsu et al. (2011) has correlated its overexpression with tumour malignancy and progression in the early stages of lung cancer. Rahman et al., also observed that RRM2 regulates BCL2 protein stability. The RRM2 gene suppression leads to increased Bcl-2 degradation in head and neck, and lung cancer, where RRM2 represses expression of the antiapoptotic protein BCL2 resulting in induction of mitochondria-mediated intrinsic apoptosis (Rahman et al., 2013). In addition, Zhang et al. (2009) found that RRM2 involved in tumour angiogenesis and growth through regulation of the expression of antiangiogenic thrombospondin-1 and proangiogenic VEGF.

Myocyte Enhancer Factor 2D (MEF2D) (chromosome 1q22) is a member of the MEF2 family of transcription factors for DNA binding proteins that activate genes transcription involved in muscle and neuronal cell differentiation. MEF2D is regulated by class II histone deacetylases (Nebbioso et al., 2009). It is a growth factor- and stress-induced gene which mediates cellular functions not only in skeletal and cardiac muscle development, but also in

neuronal differentiation and survival. It plays diverse roles in the control of cell growth, survival and apoptosis via p38 MAPKs signalling in muscle-specific and/or growth factor-related transcription. It plays a critical role in the regulation of neuronal apoptosis. It has also been identified as a candidate oncogene in murine retroviral insertional mutagenesis studies (Schwieger et al., 2009). The MEF2 signalling pathways mediate response to MAPK and calcium related signals that control survival of neurons and T-lymphocytes; which induce a known transforming oncogene, c-jun expression. Prima et al. found that MEF2D signalling pathway alteration occurs in acute lymphoblastic leukemia and suggested that MEF2D/DAZAP1 and/or DAZAP1/MEF2D contribute to leukemogenesis. They also have identified fusion proteins of MEF2D and DAZAP1 as components of novel pathways that contribute to human leukemogenesis (Prima et al., 2005). Cortese et al. (2012) revealed that MEF2D is a plasma-circulating DNA identified in an independent 38 of the prostate patient validation cohort in their epigenetic study. These findings concur with the OSCC cases of this study for MEF2D gene aberration.

TP73 gene is located on chromosome 1p36.3. It is a tumour suppressor, encodes for p73 protein which has structurally similar to p53 protein within a DNA binding domain and oligomerization and transactivation domain. It participates in the apoptotic response to DNA damage. p73 is p53-related protein that can induce apoptosis in association with cell cycle gene, p21 (Zhu et al., 1998). Its aberration is frequently deleted in meningioma (Nakane et al., 2007), and frequently hypermethylated in B cell lymphomas (Corn et al., 1999), gliomas (Watanabe et al., 2002), gastric carcinoma (Ushiku et al., 2007), colorectal carcinoma (Xu et al., 2004), cervical cancer (Jha et al., 2012) and oral cancer (Fan, 2004; Shaw, 2006; Radhakrishnan et al., 2011). The high incidence of p73 hypermethylation in various cancers has proposed that epigenetic modification of p73 via CpG island hypermethylation represents

a critical alternative mechanism for epigenetic silencing of p73, which could have important consequences for cell cycle regulation.

The B-cell lymphoma *2* (BCL2) locus is located on chromosome 18q21, encodes an integral outer mitochondrial membrane protein that inhibits caspase activity and suppresses apoptosis (Youle & Strasser, 2008). It has been reported frequently silencing in prostate tumour is due to low BCL2 promoter hypermethylation and associated with a decrease in its expression. Similar finding was also observed in this study. Although methylation levels were significantly higher for BCL2 with advanced pathological stages, an inverse correlation with mRNA expression was found in BCL2 (Carvalho et al., 2010). BCL2 hypermethylation is involved in three TNF-α-mediated apoptosis-relevant pathways, the MAPK pathway, the classical NF-kB pathway, and the TNF-α-induced caspase-dependent death signalling pathway (Kim et al., 2011). In glioma, BCL2 methylation was correlated both with global DNA methylation and another methylation of anti-apoptotic gene BclXL*,* suggesting that the common methylation mechanisms was involved in the apoptosis pathway (Shono et al., 2001). Another research conducted on 134 lung cancer by Nagatake et al. (1996) revealed that 28% cases of BCL2 promoter hypermethylation were found as the most frequent tumour-specific with low hypermethylation in lung cancer. Moreover, the results of this study demonstrated that 45% of BCL2 methylation status, which was concordant with low BCL2 hypermethylation was evident in lung cancer (Nagatake et al., 1996). However, there may be another possibility that gene transcriptional modulation was modified by traditional activators or repressors, in addition to the gene expression regulation by methylation mechanism (Tsunematsu et al., 2009). This means that BCL2 has low frequency in methylation level when compared with other studied genes in OSCC cases.

Finally, the data findings here demonstrate that the mechanism of promoter hypermethylations is the frequent mode of gene silencing and apparently they are amongst the most frequently altered genes in the OSCC cases.

5.4 Significant signalling pathway analysis of hypermethylated genes of MEF2D and RRM2

GeneGo, MetaCore^{TM} analytical suite 4.5 software was applied to identify genes in known biological pathways and/or processes that may be deregulated due to altered DNA methylation. The software identified the cellular pathways of immune response and DNA synthesis emerged as crucial players in the regulation of oral cancer progression in the study. One report about signalling pathway of gene expression in the cheek site of Malaysian OSCC patients conducted by the DASL Cancer Panel, containing 502 cancer related genes demonstrated similar cellular pathways in immune response in T lymphocytes and natural killers (Amyza, 2012). Both T lymphocytes and natural killers are important mediators in anti-tumour immunity for destructing malignant cells.

This study also highlighted gene enrichment showing aberrant DNA methylation in cancer related pathway of apoptosis. One methylation study of gastric cancer conducted on the same microarray platform showed that the dysregulation genes were involved in cancer-related pathways of regulation of the inhibitor-κB kinase/nuclear factor-κB cascade, cell differentiation, cell cycle arrest, cascade activation and cell proliferation (Cheng et al., 2014). However, since the pathway analysis was conducted on a limited set of hypermethylated genes selected, caution needs to be taken in interpreting these results.

Notably, the two predominant genes in the top significant cellular pathway found to exhibit recurring DNA methylation alteration are MEF2D and RRM2.

MEF2D

MEF2D gene predominantly involves pathways of immune response of function of MEF2 in T lymphocytes and development role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis. MEF2D transcription factor plays an important role in T cell calcium-induced apoptosis. Several factors regulate MEF2 transcription factors, including Map kinases and HDAC enzymes. Association of MEF2 with HDAC4, HDAC5, HDAC7 and HDAC9 results in deacetylation of nucleosomal histones surrounding MEF2 DNA-binding sites with subsequent suppression of MEF2-dependent genes.

In view of the development role of HDAC and CaMK in control of skeletal myogenesis pathway, MEF2D is one of the downstream transcription factors of mitogen activated protein kinase 1 (BMK1) pathway which can be induced by mitogens and or oncogenic signals that promotes cell cycle progression in tumour cells. The activated BMK1 phosphorylates and activates transcription factors of MEF2D subsequently regulate oncogenic potential of proto-oncogenes, -Jun, which participates in proliferation regulation (Kato et al., 2000).

RRM2

RRM2, the DNA damage repair-related nucleotide metabolism enzyme was predominantly activated in pathways of dCTP/dUTP metabolism, dATP/dITP metabolism and ATP/ITP metabolism. RR was a catalyst in the conversion of ribonucleotides to 2'-deoxyribonucleotides which are the precursors for DNA synthesis and repair. Endogenous ribonucleotides and deoxyribonucleotides are essential metabolites that play a critical role in

cell function, and determination of their levels is of fundamental importance in understanding key cellular processes involved in energy metabolism and molecular as well as biochemical signalling pathways. An increase of RRs activity was observed in transforming tumour cells as the DNA synthesis rate has increased to serve the proliferative activities of tumour cells. Thus, aberration of the RR is dramatically associated with malignant transformation and tumour cell growth (Liu et al., 2007). These important facts have made the RRM2 gene to become an attractive target for chemotherapeutic treatment.

5.5. Survival analysis

In the study, Kaplan-Meier survival analysis was performed to determine the differences of patients' demographic profiles, clinicopathological characteristics, and methylation of selected genes in a 5-year overall survival rate. A low 5-year overall survival rate of 38.1% was reported in this study and 18.2% in another study that conducted in Thailand (Kruaysawat et al., 2010). However, no correlation was found between survival rate and methylation status except for gender. Susceptibility of epigenetic alterations may be influenced by gender, even though the involved mechanisms are not fully understood.

In this study, gender had a significant impact on the survival rate with 24.2% for males and 46.5% for females. Therefore, in terms of gender, females appeared to have a better prognostic outcome rather than males in the OSCC survival rate of this finding. It seems controversial as one report has lower survival rates in females, but that was attributed to delay in seeking medical treatments (Leite & Koifman, 1998). In addition, Hsiung et al. (2007) also observed that the female gender has been associated with low level of global hypomethylation in HNSCC. There is a controversy in reporting no correlation between prognoses with age (Al-Rajhi et al., 2000), however, one study has shown worse prognosis in

older patients (Ribeiro et al., 2003). Survival of OSCC patients following surgical therapy has been reported to be affected by tumour size, nodal metastasis, staging, and differentiation (Lo, Kao, Chi, Wong, & Chang, 2003). In addition, Arduino et al. (2008) reported a statistically significant relationship between survival rates with histological grading, tumour size, nodal metastasis, staging, and differentiation as well as loco-regional involvement, and emphasize on the importance of tumour differentiation to predict the OSCC clinical outcome. However, the correlation between the multiple survival affecting factors of patient- and or tumour-related factors for predicting the well survival rate of cancer therapy still remains a challenge (Glare, 2005; Massano et al., 2006). The OSCC prognosis is still considered poor for the time being and it would be beneficial to rule out the molecular markers that are involved in self-sufficiency of growth signals, insensitivity to growth-inhibitor signals, evasion of apoptosis, unlimited replicative potential, ability to promote sustained angiogenesis, and capacity to invade surrounding tissue and metastasize (Hanahan & Weinberg, 2011) in order to find out the absolute and reliable prognostic markers for better cancer treatment.

5.6 Immunohistochemical analysis

IHC has become a standard assay in surgical pathology and research for protein expression, even though manual interpretation of IHC is a very subjective, time-consuming and variable process, with an inherent intra-observer and inter-observer variability (Cregger et al., 2006). Thus, automated image analysis approaches offer an opportunity for developing rapid, uniform indicators of IHC staining (Rexhepaj et al., 2008). Moreover, Turbin et al. (2008) and Rexhepaj et al. (2008) reported that automated quantitation immunostaining results do not differ from scoring of pathologist.

Protein expressions of the selected gene products were examined by IHC. The protein expression was 100% in normal mucosa tissues in this study. In the tumour tissues, the IHC results revealed a low immunostaining frequency in the signature candidates of hypermethylated genes of DDAH2, DUSP1, MEF2D and RRM2. De Bruin et al. (2007) revealed that DDAH2 is involved in apoptosis regulation showed low expression in low-apoptotic subset of rectal tumour. In addition, Zhou et al. (2009) found that the expression of DUSP1 gradually decreased in normal ovarian tissues, benign tumour, borderline tumour and primary ovarian carcinoma respectively; and its expression level in ovarian carcinoma tissues of the late pathological stage (III/IV) patients was significantly lower than the early stage (I/II) patients (Zhou, Gan, & Zhang, 2009). Moreover, Li et al. (2011) found that MEF2D expression is related to all of clinical stages with invasion and metastatic involvements of nasopharyngeal carcinoma. Furthermore, Xie et al. (2012) showed that RRM2 protein expression does not show any prognostic value even though it was associated with pancreatic adenocarcinoma.

These facts reflected that protein expressions of DDAH2, DUSP1, MEF2D and RRM2 are observed previously in other cancers rather than in OSCC, thus these four protein expressions are actually newly explored in these OSCC cases, which may be used as biomarkers for OSCC identification in the near future.

5.6.1 Association of protein expression of DDAH2, DUSP1, MEF2D and RRM2

In addition, the increase of each protein expression of the DDAH2, DUSP1, MEF2D and RRM2 genes do not significantly have coexpression to each other, except MEF2D with DDAH2 (P=0.008, r=0.378, CI = 95%) which means that the four proteins are most likely not expressed together in the OSCC cases except for MEF2D, where MEF2D interact significantly together with DDAH2. This was similar to the findings of coexpression of other genes in the breast cancer study (Van den Akker et al., 2011). Further investigation needs to be conducted for further confirmation with larger sample size.

5.7 Association of gene hypermethylation levels with protein expression of DDAH2, DUSP1, MEF2D and RRM2

In this study, there was an association observed between gene hypermethylation levels and protein expression of DDAH2 (P=0.017), DUSP1 (P=0.006), MEF2D (P=0.002), and RRM2 (P=0.001). A few other studies showed gene promoter hypermethylation was significantly associated with decreased protein expression in gastric cancer (Guan et al., 2013), choriocarcinoma (Feng et al., 2004) and ovarian cancer (Yang et al., 2012). Similar finding was reported in the present study. Furthermore, Hsu et al. (2005) also reported that an association between hMLH1 and hMLH2 hypermethylation status with protein expressions was observed in their study of 105 non-smoking female patients with lung cancer. The observed inverse correlations of the hypermethylation level of DDAH2, DUSP1, MEF2D and RRM2 with their protein expression were evident in OSCC samples of this study, suggesting that the protein expressions of DDAH2, DUSP1, MEF2D and RRM2 were silenced by promoter hypermethylation in OSCC, as promoter hypermethylation is an epigenetic change which acts as one of the pathways that leads to oral carcinogenesis (Ha & Califano, 2006).

No inverse relationship was observed between methylation status with expression level in another study (Xu et al., 2004). This means no statistically significant association between the gene hypermethylation levels with protein expression and this might be attributed to small tumour sample size and also samples selection. This may also be explained by the multiplicity of molecular mechanisms regulating gene expression by many other mechanisms including histone modifications as these modifications are known to impact the transcription regulation (Deaton & Bird, 2011).

Previous reports of similar findings about DUSP1 hypermethylation in hepatocellular carcinoma have been reported by Calvisi et al. (2008). Itoi et al. (2007) proposed poor prognostic value of high levels of RRM2 gene expression in pancreatic carcinoma. Li et al. (2012) found that the BZLF1 promoter can be transcribed by the transcription factor of MEF2D in EBV-positive tumours. In a few studies, it was reported that the gene promoter hypermethylation was significantly associated with decreased protein expression in various carcinomas (Yang et al., 2012; Guan et al., 2013) as was found this study. To the best of my knowledge, presently there are no other OSCC studies conducted on the association of DDAH2, MEF2D and RRM2 hypermethylation with their protein expressions.

5.8 Demographic profiles, clinicopathological characteristics, gene hypermethylations and protein expressions of OSCC

Patients' demographic and clinicopathological data included in this study were age, gender, alcohol drinking and tobacco smoking and betel quid chewing habits, tumour sites, pathological stages, and tumour grading.

In MSPCR for methylation analysis, there are statistically significant associations found between p16 and TP73 hypermethylation with a patient's tumour site (p=0.001 and 0.006), CELSR3 and TP73 hypermethylation with patients' invasive stages (p=0.008 and 0.025).

A study conducted by Maruya et al. (2004) found no significant correlation between methylation status of an individual gene and clinicopathological parameters including age, stage, and histological differentiation, except that methylation of P16 may be associated with the pathological stage during tumour progression. Radhakrishnan et al. (2011) reported that an absence or reduced expression of p73 in all cases clinically classified as stage IV in primary oral cancer. Lack of correlation of molecular level of hypermethylated genes with pathological stages may be due to small sample size (Woolgar, 2006) and OSCC heterogeneity (Bhargava et al., 2010). OSCC usually exhibits a heterogeneous cell population with difference in degree of differentiation. Presence or absence of metastases in cancer may be correlated with survival; therefore a pathological stage of tumour has predicted to be the outcome of tumour for many years, with varying prognostic values (Bhargava et al., 2010).

A significant correlation between of hypermethylation of TP73 promoter and both age groups (age ⩽ 45 years and age > 45 years) was observed in a study amongst passive smokers of

cervical cancer patients (Jha et al., 2012). In addition, down regulation of TP73 expression by its promoter hypermethylation leads to cervical carcinogenesis (Jha et al., 2012). These previous studies support that the TP73 hypermethylation is significant in pathogenesis of cancers including the OSCC cases in this study. CELSR3 hypermethylation has been reported recently in renal, bladder, hepatic and pancreatic carcinomas (Erkan et al., 2010; Morris et al., 2010; Jeong et al., 2011; Shen et al., 2012). This data suggests an important role for CELSR3 in tumour cells that warrants further investigation in this study as a potential prognosticator. Furthermore, CELSR3 hypermethylation is scarcely reported by others and is therefore explored in this finding. The identification of additional reliable prognosticators in OSCC has been hampered by factors such as a relatively small sample size; heterogeneity of cancer, and lastly lack of standard clinical management and laboratory protocols combined with inconsistent data reporting and records (Woolgar, 2006).

From the aforementioned studies, it can be concluded that p16, CELSR3 and TP73 promoter methylation occur frequently in various cancers including OSCC. The association between p16 and TP73 hypermethylation patterns in tumour sites, and CELSR3 and TP73 hypermethylation with patients' invasive stages may become a valuable tool for early oral cancer detection in the study.

In the IHC analysis, a significant correlation between patients' age and the hypermethylated genes of RRM2 ($p=0.015$, $r=0.381$, CI = 95%) was observed while there is no correlation with DUSP1 ($p=0.078$, $r=0.282$, CI = 95%). However, there appears to be a tendency towards such correlation for DUSP1. In view of this, researchers have proposed that epigenetic factors may also be central to controlling changes in gene expression and genomic instability during aging (Issa, 2012; Oberdoerffer et al., 2008) and age-related disease such as

cancer (Berdasco & Esteller, 2012). This means that the RRM2 may be related to the ageing process along with oral carcinogenesis. It may play an important role in aging (Oberdoerffer et al., 2008) and aging-related disease (Berdasco & Esteller, 2012; van Otterdijk et al., 2013). This also indicates that RRM2 genes will be of poor prognostic value as OSCC patient's age increase.

5.9 Limitations of the study

There were some unavoidable limitations in this study.

While microarrays are designed to give a genome-wide view of the cell on an unprecedented scale, this technology still poses some limitations outside certain research settings which inhibit its broad usage. One of the main drawbacks is costing. Each array, depending on the platform, may cost hundreds to thousands of dollars per sample (Kuo et al., 2004). On the other hand, genome-wide methylation platforms have lagged behind because of an inherent loss of DNA sequences during the bisulfite conversion. This bisulfite conversion can lead to loss of specificity during the hybridization process. Another challenge is the efficiency of handling and analysing large volumes of data generated by microarray approaches. However, there are sophisticated computational methods that have been increasingly developed to be amenable to large data sets generated from microarray experiments (Kirmizis & Farnham, 2004). As key disease pathways are identified, custom arrays containing relevant subsets of genes may eventually be integrated into clinical settings for drug therapeutic usage.

The bisulphite modification-PCR amplification approaches such as semiquantitative MSPCR mainly relies on a methylation change at the PCR primer binding. As such, these tests are prone to false-negative results, where the gene promoters might have been methylated, but not at the primer binding site. However, this problem can be solved as the selected primers in the study were designed based on the most methylated loci found in RefSeq genes.

The methylation information available in oral cancer is limited if compared with other neoplasms. Nevertheless, studies have established that aberrant hypermethylation is an important event in the carcinogenesis of OSCC. Published studies have made it possible to establish that transcriptional gene silencing is due to the methylation state of its promoter

regions. However, many newly explored methylated genes in this finding need to be validated by functional assays such as cell proliferation, migration and invasive assays. This can be done by developing in vitro models of cell cultures in the future in order to consolidate the facts of epigenetic aberrations in this study.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The primary objective of this study was to investigate methylation profiling of OSCC using DNA microarray. The microarray results have revealed that there was a difference in gene methylation levels of oral cancer tissues from normal tissues. This study has also managed to compile a list of differential hypermethylated genes in OSCC using Illumina's Genome Studio software analysis and Partek Genomic Suite analysis. Finally, significant candidate genes involved in oral cancer and pathway network that was generated by GeneGo Metacore™ software were warranted for microarray data validation. Two cellular pathways of immune response and DNA synthesis pathways were identified as major players involved in oral carcinogenesis of this study.

The second objective of this study was to explore hypermethylated genes using MSPCR assay that may be used to detect the epigenetic alterations associated with OSCC. The present study suggests that besides the established hypermethylated genes of P16, and TP73, other genes such as DDAH2, DUSP1, CELSR3, Pikc3r5, MEF2D and RRM2 hypermethylation may be important and need further study.

The third objective of this study was to determine protein expression of hypermethylated genes using immunohistochemical staining, and to correlate the specific genes methylation level with its protein expression in OSCC. DDAH2, DUSP1, MEF2D and RRM2 demonstrated low positive immunostaining in OSCC by IHC assay. These four proteins most likely do not interact amongst themselves except MEF2D, which may coexpress with DDAH2 in cancer signalling networks.

The fourth objective of this study was to correlate the protein expressions with genes methylation levels in OSCC. The inverse relations of DDAH2, DUSP1, MEF2D and RRM2 hypermethylations with their protein expressions were observed in the present study. Protein expressions of DDAH2, DUSP1, MEF2D and RRM2 actually are newly explored in this study and have not been reported before. These proteins may be used as novel biomarkers for OSCC identification in the near future.

The last objective of this study was to correlate the DNA hypermethylation patterns with the patients' demographic and clinicopathological data of tumour site, pathological stage, invasive stage and histological grading, and survival rate for predicting OSCC prognosis. The molecular prognostic study of protein expression with age was also investigated in OSCC. The results showed that the association of p16 and TP73 hypermethylation patterns in tumour site, and CELSR3 and TP73 hypermethylation with invasive stage may become valuable prognostic tools for OSCC. In addition, gender is the only demographic factor that significantly associated with an OSCC patient's overall survival rate. Furthermore, the correlation between RRM2 protein expression with patients' age was shown to be a poor prognosis of OSCC as patients' age increased.

In summary, comprehensive promoter hypermethylation biomarkers screening approach with the DNA methylation assay, and data validation by MSPCR assay for p16, DUSP1, DDAH2, CELSR3, PIK3R5, TP73, MEF2D and RRM2, and IHC analysis for DUSP1, DDAH2, MEF2D and RRM2 genes, have managed to identify potential signature candidates especially DUSP1, DDAH2, CELSR3, PIK3R5, RRM2 and MEF2D in OSCC identification and prognostication purposes.

Protein expressions of DUSP1, DDAH2, MEF2D and RRM2 genes were reduced by their promoter hypermethylation in OSCC, as promoter hypermethylation is the predominant mechanism in DUSP1, DDAH2, MEF2D and RRM2 deregulations, which seems to play an important role in oral carcinogenesis. The inverse correlation between gene hypermethylations and protein expressions in the study suggests that the hypermethylation mechanism is an important event in silencing expressions of DDAH2, DUSP1, MEF2D and RRM2 in the OSCC cases. Furthermore, protein expressions of DDAH2, DUSP1, MEF2D and RRM2 are newly explored in the OSCC cases, which may encourage deeper molecular understanding of OSCC progression. The coexpression of MEF2D with DDAH2 may demonstrate the involvement of these genes in a cellular pathway network. Hence, these biomarkers can become a helpful tool for OSCC prognostication and diagnosis, as some other genes were reported in breast cancer (Van den Akker et al., 2011).

Last but not least, it was observed that gender has a clear influence on the patients' survival rate in OSCC prognosis. Susceptibility of epigenetic alterations may be influenced by gender, even though the involved mechanisms are not fully understood. In addition, the association between hypermethylation of p16 and TP73 with tumour site, and TP73 and CELSR3 with invasive stage demonstrates OSCC prognosis in the study. Moreover, integrated available clinical data for discovering novel prognostic markers where DDAH2, CELSR3 and RRM2 are involved, the aging-related gene may indicate poor prognosis of OSCC as patient's age increase.

In conclusion, the hypothesis set in my study was true that there is a difference in methylation profiling expressions, hypermethylation levels and protein expressions between normal subjects and OSCC patients.

6.2 Recommendations

Further work will be aimed at elucidating the functional roles in cell proliferation, immigration and invasion of DUSP1, DDAH2, MEF2D and RRM2 by using in-vitro models. Treatment with a demethylation drug, 5-aza-2'-deoxycytidine in OSCC cell lines will restore gene expression. The differential expression of DUSP1, DDAH2, MEF2D and RRM2 in OSCC may have potential application as a prognostic indicator and planning for patient treatment. In addition, improvement of therapeutic immunotherapies may become a reality in the future by exploring genes involved in immune response of these OSCC cases, since immunotherapy has become one of the most promising new cancer treatment.

Direct comparison of OSCC to normal tissues by larger clinical samples in microarray assay will allow research to identify more hypermethylated-based biomarkers in OSCC in future. This will provide critical insight into the differences found in oral malignancy and may also provide a new strategy in cancer prevention and develop a molecular targeted therapy.

The rationale for epigenetic molecular-targeted prevention of oral cancer is the use of biomarkers to detect the cancer risk of pre-malignancy and malignancy. In this sense, an understanding of the biology of oral carcinogenesis will yield important advances for detecting high-risk cancer patients, monitoring preventive interventions, and assessing cancer risk and pharmacogenomics. In addition, novel chemopreventive agents based on epigenetic molecular mechanisms and targets against oral cancers will be derived from studies using appropriate animal carcinogenesis models. New approaches such as molecular-targeted agents and agent combinations in high-risk individuals are undoubtedly needed to reduce the devastating worldwide consequences of oral malignancy (Tanaka et al., 2011).

Early detection and diagnosis of oral cancer can be done by identifying reliable biomarkers which can then reduce patient morbidity and mortality. Conventional histopathological studies provide a subjective diagnosis due to human limitations, thus, new molecular diagnostic tools aid in identifying marginal surgical tissue clearance and lymph node metastases. The use of additional reliable biomarkers besides the existing/established immunohistochemical assay of p53 and p16 will act as an adjunct to routine histopathological examination in order to enhance the prognostication and cancer treatment management.

Due to the molecular and cellular heterogeneity of oral cancers, and the subsequent variability in biological behaviours, a single pathway or biomarker may have inherent limitations in terms of predicting cancer outcome. Thus, coexpression networks of several genes within linked pathways, have striking implications at a time where there is significant emphasis on developing cancer and targeted therapies (Van den Akker et al., 2011). It will make less sense if the only target is a single gene that can be functionally bypassed by deregulating other factors in the same signalling cascade. It is envisaged that clinical utilization of DNA methylation as a biomarker would be based on a panel of genes associated with oral cancer. The identification of CpG island hypermethylation in the promoter region of selected genes could provide a reliable biomarker for establishing methylation profiles in OSCC. Moreover, it is pertinent to point out the need for the use of such panels of genes as a diagnostic/screening tool on noninvasively obtained biological specimens such as serum or saliva. Thus, the development of a robust system of diagnosis of prognosis using such panels would have significant clinical implications.

Patient survival rates have remained at a disappointingly stable level despite significant development in the multimodality treatment of the disease. Therefore, there should be more

molecular diagnostic tools to explore other predictive biomarkers to overcome the existing limited conventional prognostic factors. More research and development should be done and could include a wider use of standardised computerised databases with improved methods for the retrieval and exchange of information (Woolgar, 2006). A reduced reliance on subjective interpretation along with a wider use of automated techniques and quantitative data would also be beneficial (Woolgar, 2006). Therefore, these suggestions may help clinicians to improve prognostic accuracy and conduct appropriate management for oral cancer patients.

BIBIOGRAPHY

Abhary, S., Burdon, K. P., Kuot, A., Javadiyan, S., Whiting, M.J., Kasmeridis, N., & Craig, J.E. (2010). Sequence variation in DDAH1 and DDAH2 genes is strongly and additively associated with serum ADMA concentrations in individuals with type 2 diabetes. *PLoS One, 5*(3), 1-8

Abnet, C .C., Kamangar, F., Islami, F., Nasrollahzadeh, D., Brennan, P., Aghcheli, K., & Dawsey, S. M. (2008). Tooth loss and lack of regular oral hygiene are associated with higher risk of esophageal squamous cell carcinoma. *Cancer Epidemiology Biomarkers Prevention, 17*(11), 3062-3068. doi: 10.1158/1055-9965.EPI-08-0558

Adjei, Alex A., & Hidalgo, Manuel. (2005). Intracellular signal transduction pathway proteins as targets for cancer therapy. *Journal of Clinical Oncology, 23*(23), 5386-5403.

Adrien, L. R., Schlecht, N. F., Kawachi, N., Smith, R. V., Brandwein-Gensler, M., Massimi, A., & Belbin, T. J. (2006). Classification of DNA methylation patterns in tumor cell genomes using a CpG island microarray. *Cytogenetic and Genome Research, 114*(1), 16-23.

Ahuja, N., & Issa, J. P. (2000). Aging, methylation and cancer. *Histology and Histopathology, 15*(3), 835-842.

Al-Rajhi, N., Khafaga, Y., El-Husseiny, J., Saleem, M., Mourad, W., Al-Otieschan, A., & Al-Amro, A. (2000). Early stage carcinoma of oral tongue: prognostic factors for local control and survival. *Oral Oncology, 36*(6), 508-514.

Amyza, S. (2012). *Gene expression patterns of cheek, gum and tongue squamous cell carcinoma.* (Unpublished Doctoral dissertation). University of Malaya, Kuala Lumpur, Malaysia.

An, Q., Liu, Y., Gao, Y., Huang, J., Fong, X., Li, L., & Cheng, S. (2002). Detection of p16 hypermethylation in circulating plasma DNA of non-small cell lung cancer patients. *Cancer Letters, 188*(1), 109-114.

Angenendt, P. (2005). Progress in protein and antibody microarray technology. *Drug Discovery Today, 10*(7), 503-511.

Angenendt, P., Glökler, J., Sobek, J., Lehrach, H., & Cahill, D.J. (2003). Next generation of protein microarray support materials: Evaluation for protein and antibody microarray applications. *Journal of Chromatography A, 1009*(1), 97-104.

Arduino, P. G., Carrozzo, M., Chiecchio, A., Broccoletti, R., Tirone, F., Borra, E., . . . Gandolfo, S. (2008). Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: a retrospective study of 334 cases. *Journal of Oral and Maxillofacial Surgery, 66*(8), 1570-1579.

Argiris, A., Karamouzis, M. V., Raben, D., & Ferris, R. L. (2008). Head and neck cancer. *The Lancet, 371*(9625), 1695-1709.

Aryee, M. J., Liu, W., Engelmann, J. C., Nuhn, P., Gurel, M., Haffner, M. C., Esopi D., . . . Yegnasubramanian S. (2013). DNA methylation alterations exhibit intraindividual stability and interindividual heterogeneity in prostate cancer metastases. *Science Translational Medicine, 5*(169), 169ra110-169ra110. doi: 10.1126/scitranslmed.3005211

Babinsky, V. (2011). *Identification of unknown epigenetically regulated genes in non-small cell lung cancer.* University of Vienna. Austria. Available from ProQuest Dissertations and Theses database of library of University Malaya. .

Balmain, A., Gray, J., & Ponder, B. (2003). The genetics and genomics of cancer. *Nature Genetics, 33*, 238-244.

Balog, R. P., Ponce de Souza, E. Y., Tang, H. M., DeMasellis, G. M., Gao, B., Avila, A., Luebke, K. J. & Garner H. R. (2002). Parallel assessment of CpG methylation by two-color hybridization with oligonucleotide arrays. *Analytical Biochemistry, 309*(2), 301-310.

Bayarsaihan, D. (2011). Epigenetic mechanisms in inflammation. *Journal of Dental Research, 90*(1), 9-17.

Baylin, S. B., Esteller, M., Rountree, M. R., Bachman, K. .E., Schuebel, K., & Herman, J. G. (2001). Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Human Molecular Genetics, 10*(7), 687-692.

Baylin, S. B. (2005). DNA methylation and gene silencing in cancer. *Nature Clinical Practice Oncology, 2*, S4-S11.

Bennett-Baker, P. E., Wilkowski, J., & Burke, D. T. (2003). Age-associated activation of epigenetically repressed genes in the mouse. *Genetics, 165*(4), 2055-2062.

Berdasco, M., & Esteller, M. (2012). Hot topics in epigenetic mechanisms of aging: 2011. *Aging Cell, 11*(2), 181-186.

Bestor, T. H. (1998). Gene silencing: Methylation meets acetylation. *Nature, 393*(6683), 311-312.

Bhargava, A., Saigal, S. l., & Chalishazar, M. (2010). Histopathological Grading Systems In Oral Squamous Cell Carcinoma: A Review. *Journal of Internationall Oral Health, 2*(4), 1-10.

Bibikova, M., Yeakley, J. M., Chudin, E., Chen, J., Wickham, E., Wang-Rodriguez, J., & Fan, J. B. (2004). Gene expression profiles in formalin-fixed, paraffin-embedded tissues obtained with a novel assay for microarray analysis. *Clinical Chemistry, 50*(12), 2384-2386.

Bibikova, M., Lin, Z. W., Zhou, L. X., Chudin, E., Garcia, E. W., Wu, B., & Fan, J. B. (2006). High-throughput DNA methylation profiling using universal bead arrays. *Genome Research, 16*(3), 383-393. doi: Doi 10.1101/Gr.4410706.

Bibikova, M., Le, J., Barnes, B., Saedinia-Melnyk, S., Zhou, L., Shen, R., & Gunderson, K. L. (2009). Genome-wide DNA methylation profiling using Infinium assay. *Epigenomics, 1*(1), 177-200.

Bibikova, M., Barnes, B., Tsan, C., Ho, V., Klotzle, B., Le, J. M., . . . & Shen, R. (2011). High density DNA methylation array with single CpG site resolution. *Genomics, 98*(4), 288-295.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes and Development, 16*(1), 6-21.

Bonazzi, V.F., Irwin, D., & Hayward, N. K. (2009). Identification of candidate tumor suppressor genes inactivated by promotermethylation in melanoma. *Genes, Chromosomes and Cancer, 48*(1), 10-21.

Brannon-Peppas, L., & Blanchette, J. O. (2012). Nanoparticle and targeted systems for cancer therapy. *Advanced Drug Delivery Rreviews*, 56(11):1649-59

Brock, M. V., Hooker, C. M., Ota-Machida, E., Han, Y., Guo, M., Ames, S., & Pridham, G. (2008). DNA methylation markers and early recurrence in stage I lung cancer. *New England Journal of Medicine, 358*(11), 1118-1128.

Bryne, M., Koppang, H. S., Lilleng, R., Kjaerheim, A. (1992). Malignancy grading of the deep invasive margins of oral squamous cell carcinoma has high pprognostic value. *Journal of Pathology*; 166 (4), 375-381.

Burgers, W. A., Blanchon, L., Pradhan, S., De Launoit, Y., Kouzarides, T., & Fuks, F. (2006). Viral oncoproteins target the DNA methyltransferases. *Oncogene, 26*(11), 1650-1655.

Calvisi, D. F., Pinna, F., Meloni, F., Ladu, S., Pellegrino, R., Sini, M., & Virdis, P. (2008). Dual-Specificity Phosphatase 1 Ubiquitination in Extracellular Signal-Regulated

Kinase–Mediated Control of Growth in Human Hepatocellular Carcinoma. *Cancer Research, 68*(11), 4192-4200.

Cao, J., Zhou, J., Gao, Y., Gu, L., Meng, H., Liu, H., & Deng, D. (2009). Methylation of p16 CpG Island Associated with Malignant Progression of Oral Epithelial Dysplasia: A Prospective Cohort Study. *Clinical Cancer Research, 15*(16), 5178-5183. doi: 10.1158/1078-0432.ccr-09-0580

Carinci, F., Lo Muzio, L., Piattelli, A., Rubini, C., Palmieri, A., Stabellini, G., & Scapoli, L. (2005). Genetic portrait of mild and severe lingual dysplasia. *Oral Oncology, 41*(4), 365-374.

Carvalho, J. R., Filipe, L., Costa, V. L., Ribeiro, F. R., Martins, A. T., Teixeira, M. R., & Henrique, R. (2010). Detailed analysis of expression and promoter methylation status of apoptosis-related genes in prostate cancer. *Apoptosis, 15*(8), 956-965.

Chang, M. S., Uozaki, H., Chong, J. M., Ushiku, T., Sakuma, K., Ishikawa, S.,… & Arai, K. (2006). CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. *Clinical Cancer Research, 12*(10), 2995-3002.

Chang, Y.C., Hu, C.C., Tseng, T.H., Tai, K. W., Lii, C. K., & Chou, M. Y. (2001). Synergistic effects of nicotine on arecoline-induced cytotoxicity in human buccal mucosal fibroblasts. *Journal of Oral Pathology & Medicine, 30*(8), 458-464.

Chao, W., & D'Amore, P. A. (2008). IGF2: epigenetic regulation and role in development and disease. *Cytokine & Growth Factor Reviews, 19*(2), 111-120.

Chen, T., & Li, E. (2006). Establishment and maintenance of DNA methylation patterns in mammals DNA Methylation: *Basic Mechanisms* (pp. 179-201): Springer.

Chen, Y. K., Huang, H. C., Lin, L. M., & Lin, C. C. (1999). Primary oral squamous cell carcinoma: an analysis of 703 cases in southern Taiwan. *Oral Oncology, 35*(2), 173-179.

Cheng, X., & Blumenthal, R. M. (2008). Mammalian DNA Methyltransferases: A Structural Perspective. *Structure,* 16(3), 341-350.doi: http://dx.doi.org/10.1016/j.str.2008.01.004

Cheng, Y., Yan, Z., Liu, Y., Liang, C., Xia, H., Feng, J., & Luo, H. (2014). Analysis of DNA methylation patterns associated with the gastric cancer genome. *Oncology Letters, 7*, 1021-1026.

Choi, S. W., & Mason, J. B. (2002). Folate status: effects on pathways of colorectal carcinogenesis. *The Journal of Nutrition, 132*(8), 2413S-2418S.

Coons, A. H., Creech, H. J., & Jones, R. N. (1941). Immunological properties of an antibody containing a fluorescent group. *Experimental Biology and Medicine, 47*(2), 200-202.

Church, D., & Pruitt, K. D. (2002). Accessing the Human Genome. *Current Protocols in Human Genetics*. DOI: 10.1002/0471142905.hg0609s34

Cogliano, V., Straif, K., Baan, R., Grosse, Y., Secretan, B., & Ghissassi, F. E. (2004). Smokeless tobacco and tobacco-related nitrosamines. *The Lancet Oncology, 5*(12), 708. doi: 10.1016/s1470-2045(04)01633-x

Corn, P. G., Kuerbitz, S. J., van Noesel, M. M., Esteller, M., Compitello, N., Baylin, S. B., & Herman, J. G. (1999). Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. *Cancer Research, 59*(14), 3352-3356.

Cortese, R., Kwan, A., Lalonde, E., Bryzgunova, O., Bondar, A., Wu, Y., & Petronis, A. (2012). Epigenetic markers of prostate cancer in plasma circulating DNA. *Human Moecular Genetics, 21*(16), 3619-3631. doi: 10.1093/hmg/dds192

Cottrell, Susan E., & Laird, Peter W. (2003). Sensitive detection of DNA methylation. *Annals of the New York Academy of Sciences, 983*(1), 120-130.

Cregger, M., Berger, A. J., & Rimm, D. L. (2006). Immunohistochemistry and quantitative analysis of protein expression. *Archives of Pathology and laboratory Medicine, 130*(7), 1026-1030.

Croce, C. M. (2008). Oncogenes and cancer. *New England Journal of Medicine, 358*(5), 502-511.

Cross, S. H, & Bird, A. P. (1995). CpG islands and genes. *Current Opinion in Genetics & Development, 5*(3), 309-314.

Dai, W. (2011). *Biostatistical analysis of DNA methylation profiling in ovarian cancer.* Doctoral dissertation. Imperial College London. England Available from ProQuest Dissertations and Theses database of library of University Malaya.

Davis, C. D., & Uthus, E. O. (2004). DNA methylation, cancer susceptibility, and nutrient interactions. *Experimental Biology and Medicine, 229*(10), 988-995.

de Bruin, E. C., van de Pas, S., van de Velde, C. J., van Krieken, J H., Peltenburg, L. T., Marijnen, C. A., & Medema, J. P. (2007). Gene expression profiling reveals two separate mechanisms regulating apoptosis in rectal carcinomas in vivo. *Apoptosis, 12*(9), 1671-1680.

Deaton, Aimée M., & Bird, Adrian. (2011). CpG islands and the regulation of transcription. *Genes and Development, 25*(10), 1010-1022.

Dedeurwaerder, S., Defrance, M., Calonne, E., Denis, H., Sotiriou, C., & Fuks, F. (2011). Evaluation of the Infinium Methylation 450K technology. *Epigenomics, 3*(6), 771-784.

Demokan, S., & Dalay, N. (2011). Role of DNA methylation in head and neck cancer. *Clinical Epigenetics*, 1-28.

Dialyna, I. A., Miyakis, S., Georgatou, N., & Spandidos, D. A. (2003). Genetic polymorphisms of CYP1A1, GSTM1 and GSTT1 genes and lung cancer risk. *Oncology Reports, 10*(6), 1829-1835.

Dong, Yuying, Wang, Jie, Dong, Fusheng, Wang, Xu, & Zhang, Yinghuai. (2012). The correlations between alteration of p16 gene and clinicopathological factors and prognosis in squamous cell carcinomas of the buccal mucosa. *Journal of Oral Pathology and Medicine, 41*(6), 463-469. doi: 10.1111/j.1600-0714.2012.01132.x

Dowden, James., Hong, Wei., Parry, Richard V., Pike, Richard A., & Ward, Stephen G. (2010). Toward the development of potent and selective bisubstrate inhibitors of protein arginine methyltransferases. *Bioorganic and Medicinal Chemistry Letters, 20*(7), 2103-2105.

Durlej, M., Tabarowski, Z., & Slomczynska, M. (2010). Immunohistochemical study on differential distribution of progesterone receptor A and progesterone receptor B within the porcine ovary. *Animal Reproduction Science, 121*(1), 167-173.

Duxbury, M. S., & Whang, E. E. (2007). RRM2 induces NF-κB-dependent MMP-9 activation and enhances cellular invasiveness. *Biochemical and Biophysical Research communications, 354*(1), 190-196.

Easwaran, H. P. (2003). *A complex interplay of regulatory domains controls cell cycle dependent subnuclear localization of DNMT1 and is required for the maintenance of epigenetic information.* Doctoral dissertation. Humboldt University. Available from ProQuest Dissertations and Theses database of library of University Malaya.

Edefonti, V., Bravi, F., La Vecchia, C., Randi, G., Ferraroni, M.., Garavello, W., & Decarli, A. (2010). Nutrient-based dietary patterns and the risk of oral and pharyngeal cancer. *Oral Oncology, 46*(5), 343-348.

Eden, A., Gaudet, F., Waghmare, A., & Jaenisch, R. (2003). Chromosomal instability and tumors promoted by DNA hypomethylation. *Science,* 300 (5618), 455-455.

Edge, S. B., & Compton, C. C. (2010). The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Annals of Surgical Oncology, 17*(6), 1471-1474.

Enokida, H., & Nakagawa, M. (2008). Epigenetics in bladder cancer. *International Journal of Clinical Oncology, 13*(4), 298-307.

Erkan, M., Weis, N., Pan, Z., Schwager, C., Samkharadze, T., Jiang, X., & Debus, J. (2010). Organ-, inflammation- and cancer specific transcriptional fingerprints of pancreatic and hepatic stellate cells. *Molecular Cancer, 9*(1), 88-103.

Estécio, M. R, Yan, P. S., Ibrahim, A. E., Tellez, C. S., Shen, L., Huang, T. H., & Issa, J. P. (2007). High-throughput methylation profiling by MCA coupled to CpG island microarray. *Genome Research, 17*(10), 1529-1536.

Esteller, M., & Herman, J. G. (2002). Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *Journal of Pathology, 196*(1), 1-7. doi: 10.1002/path.1024 [pii]10.1002/path.1024

Esteller, M. (2008). Epigenetics in cancer. *New England Journal of Medicine, 358*(11), 1148-1159.

Esteller, M. (2011). Epigenetic changes in cancer. *F1000 Biology Reports, 3:9*.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E.,… & Croce C.M. (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proceedings of the National Academy of Sciences, 104*(40), 15805-15810.

Fan, C. Y. (2004). Epigenetic alterations in head and neck cancer: prevalence, clinical significance, and implications. *Current Oncology Reports, 6*, 152-161.

Feinberg, A. P., Ohlsson, R., & Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nature Reviews Genetics, 7*(1), 21-33.

Feng, J., Han, Q., & Zhou, L. (2012). Planar cell polarity genes, Celsr1-3, in neural development. *Neuroscience Bulletin, 28*(3), 309-315.doi: 10.1007/s12264-012-1232-8

Feng, Z., Hu, W., Hu, Y., & Tang, MS.. (2006). Acrolein is a major cigarette-related lung cancer agent: Preferential binding at p53 mutational hotspots and inhibition of DNA repair. *Proceedings of the National Academy of Sciences, 103*(42), 15404-15409.

Feng, H., Cheung, A. N., Xue, W. C., Wang, Y., Wang, X., Fu, S.,… & Tsao, S. W. (2004). Down-regulation and promoter methylation of tissue inhibitor of metalloproteinase 3 in choriocarcinoma. *Gynecologic Oncology, 94*(2), 375-382. doi: http://dx.doi.org/10.1016/j.ygyno.2004.04.019

Ferrara, N., & Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. *Nature, 438*(7070), 967-974.

Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestar, M. L., & Benitez, J. (2005). Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences of the United States of America, 102*(30), 10604-10609.

Fraga, M. F, & Esteller, M. (2007). Epigenetics and aging: the targets and the marks. *Trends in Genetics, 23*(8), 413-418.

Friedrich, M. G., Weisenberger, D. J., Cheng, J. C., Chandrasoma, S., Siegmund, K. D., Gonzalgo, M. L., & Tsai, Y. C. (2004). Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients. *Clinical Cancer Research, 10*(22), 7457-7465.

Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., & Paul, C. L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences, 89*(5), 1827-1831.

Fukushige, S., & Horii, A. (2013). DNA methylation in cancer: a gene silencing mechanism and the clinical potential of its biomarkers. *The Tohoku Journal of Experimental Medicine, 229*(3), 173-185.

Galm, O., & Herman, J.G. (2005). Methylation-specific polymerase chain reaction. *Methods Molecular Medicine, 113*, 279-291.

Gardiner-Garden, M., & Frommer, M. (1987). CpG islands in vertebrate genomes. *Journal of Molecular Biology, 196*(2), 261-282.

Garzino-Demo, P., Dell'Acqua, A., Dalmasso, P., Fasolis, M., La Terra Maggiore, G. M., Ramieri, G., & Schena, M. (2006). Clinicopathological parameters and outcome of 245 patients operated for oral squamous cell carcinoma. *Journal of Cranio-Maxillofacial Surgery, 34*(6), 344-350.

Ghobrial, I. M., Witzig, T. E., & Adjei, A. A. (2005). Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians, 55*(3), 178-194.

Gilbert, E. R., & Liu, D. (2010). Flavonoids influence epigenetic-modifying enzyme activity: structure-function relationships and the therapeutic potential for cancer. *Current Medicinal Chemistry, 17*(17), 1756-1768.

Ginos, M. A., Page, G. P., Michalowicz, B. S., Patel, K. J., Volker, S. E., Pambuccian, S. E., & Gaffney, P. M. (2004). Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. *Cancer Research, 64*(1), 55-63.

Giovannucci, E. (2002). Epidemiologic studies of folate and colorectal neoplasia: a review. *The Journal of Nutrition, 132*(8), 2350S-2355S.

Glare, P. (2005). Clinical predictors of survival in advanced cancer. *Journal Support Oncology, 3*(5), 331-339.

Goll, M. G., Kirpekar, F., Maggert, K. A., Yoder, J. A., Hsieh, C. L., Zhang, X., . . . Bestor, T. H. (2006). Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science, 311*(5759), 395-398.

Gonzalez, MV., Pello, MF., Lopez-Larrea, C., Suarez, C., Menendez, MJ., & Coto, E. (1997). Deletion and methylation of the tumour suppressor gene p16/CDKN2 in primary head and neck squamous cell carcinoma. *Journal of Clinical Pathology, 50*(6), 509-512.

Gordana, Š., Ruzˇica K., Mirjana, B. M., Nebojša, J., Zvonko, M. (2009). Gene hypermethylation in tumor tissue of advanced oral squamous cell carcinoma patients. *Oral Oncology*, 45(12):1051-7 doi: 10.1016/j.oraloncology.2009.07.007

Griffith, C., Noonan, S., Lou, E., & Shillitoe, E. J. (2007). An oncolytic mutant of herpes simplex virus type-1 in which replication is governed by a promoter/enhancer of human papillomavirus type-16. *Cancer Gene Therapy, 14*(12), 985-993.

Guan, Z. Y., Zhang, J., Song, S. H., & Dai, D. Q. (2013). Promoter methylation and expression of TIMP3 gene in gastric cancer. *Diagnostic Pathology, 8*(1), 110-116.

Ha, P. K., & Califano, J. A. (2006). Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. *The Lancet Oncology, 7*(1), 77-82.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hasegawa, K., Wakino, S., Tanaka, T., Kimoto, M., Tatematsu, S., Kanda, T., & Kurabayashi, M. (2006). Dimethylarginine dimethylaminohydrolase 2 increases vascular endothelial growth factor expression through Sp1 transcription factor in endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology, 26*(7), 1488-1494.

Hashibe, M., Brennan, P., Strange, R. C., Bhisey, R., Cascorbi, I., Lazarus, P., & Katoh, T. (2003). Meta-and pooled analyses of GSTM1, GSTT1, GSTP1, and CYP1A1 genotypes and risk of head and neck cancer. *Cancer Epidemiology Biomarkers and Prevention, 12*(12), 1509-1517.

Hayslip, J., & Montero, A. (2006). Tumor suppressor gene methylation in follicular lymphoma: a comprehensive review. *Molecular Cancer, 5*(1), 44.

Hellebrekers, D. M. E. I., Castermans, K., Viré, E., Dings, R. P. M., Hoebers, N. T. H., Mayo, K. H., & Griffioen, A. W. (2006). Epigenetic regulation of tumor endothelial cell anergy: silencing of intercellular adhesion molecule-1 by histone modifications. *Cancer Research, 66*(22), 10770-10777.

Herman, J. G., Merlo, A. M., LI, L., Rena, G. I., Jean-Pierre, J. D., Nancy, E., & Baylin, S. B. (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Research, 55*(20), 4525-4530.

Herman, J. G., Graff, J. R., Myöhänen, S. B. D.N., Nelkin, B. D., & Baylin, S. B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proceedings of the National Academy of Sciences, 93*(18), 9821-9826.

Herman, J. G., & Baylin, S. B. (2000). Promoter-region hypermethylation and gene silencing in human cancer. *Current Topical Microbioogyl Immunology, 249*, 35-54.

Herman, J. G., & Baylin, S. B. (2003). Gene silencing in cancer in association with promoter hypermethylation. *New England Journal of Medicine, 349*(21), 2042-2054.

Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science, 187*(4173), 226-232.

Hoque, M. O., Topaloglu, O., Begum, S., Henrique, R., Rosenbaum, E., Van Criekinge, W., & Sidransky, D. (2005). Quantitative methylation-specific polymerase chain reaction gene patterns in urine sediment distinguish prostate cancer patients from control subjects. *Journal of Clinical Oncology, 23*(27), 6569-6575.

Hore, T. A., Rapkins, R. W., & Graves, J. A. (2007). Construction and evolution of imprinted loci in mammals. *Trends in Genetics, 23*(9), 440-448.

Hsiung, D. T., Marsit, C. J., Houseman, E. A., Eddy, K., Furniss, C. S., McClean, M. D., & Kelsey, K. T. (2007). Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiology Biomarkers and Prevention, 16*(1), 108-114.

Hsu, H. S., Wen, C. K., Tang, Y. A., Lin, R. K., Li, W. Y., Hsu, W. H., & Wang, Y. C. (2005). Promoter hypermethylation is the predominant mechanism in hMLH1 and hMSH2 deregulation and is a poor prognostic factor in nonsmoking lung cancer. *Clinical Cancer Research, 11*(15), 5410-5416.

Hsu, N.Y., Wu, J. Y., Liu, X., Yen, Y., Chen, C. Y., Chou, M. C., & Cheng, Y. W. (2011). Expression status of ribonucleotide reductase small subunits hRRM2/p53R2 as prognostic biomarkers in stage I and II non-small cell lung cancer. *Anticancer Research, 31*(10), 3475-3481.

Ibrahim, A. E., Arends, M. J., Silva, A. L., Wyllie, A. H., Greger, L., Ito, Y.,… & Brenton, J. D. (2011). Sequential DNA methylation changes are associated with DNMT3B overexpression in colorectal neoplastic progression. *Gut, 60*(4), 499-508.

Ibrahim, A. K., Thorne, N. P., Baird, K., Barbosa-Morais, N. L., Tavare, S., Collins, V. P., & Brenton, J. D. (2006). MMASS: an optimized array-based method for assessing CpG island methylation. *Nucleic Acids Research, 34*(20), e136-e136.

Iglesias-Linares, A., Yañez-Vico, R. M., & González-Moles, M. A. (2010). Potential role of HDAC inhibitors in cancer therapy: Insights into oral squamous cell carcinoma. *Oral Oncology, 46*(5), 323-329.

Irizarry, R. A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., & Webster, M. (2009). The human colon cancer methylome shows similar hypo-and hypermethylation at conserved tissue-specific CpG island shores. *Nature Genetics, 41*(2), 178-186.

Issa, J. P. (2012). Age-Related Variation in DNA Methylation *Epigenetic Epidemiology* (pp. 185-196): Springer.

Itoi, T., Sofuni, A., Fukushima, N., Itokawa, F., Tsuchiya, T., Kurihara, T.,…& Kasuya, K. (2007). Ribonucleotide reductase subunit M2 mRNA expression in pretreatment biopsies obtained from unresectable pancreatic carcinomas. *Journal of Gastroenterology, 42*(5), 389-394.

Jain, K. K. (2002). Personalized medicine. *Current Opinion in Molecular Therapeutics, 4*(6), 548-558.

Jain, K. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science, 307*(5706), 58-62.

Jalouli, J., Ibrahim, S. O., Mehrotra, R., Jalouli, M. M., Sapkota, D., Larsson, P. A., & Hirsch, J. M. (2010). Prevalence of viral (HPV, EBV, HSV) infections in oral submucous fibrosis and oral cancer from India. *Acta Oto-laryngologica, 130*(11), 1306-1311.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians, 61*(2), 69-90.

Jeong, P., Ha, Y. S., Cho, I.C ., Yun, S. J., Yoo, E. S., Kim, I. Y., & Kim, W. J. (2011). Three-gene signature predicts disease progression of non-muscle invasive bladder cancer. *Oncology Letters, 2*(4), 679-684.

Jha, A. K., Nikbakht, M., Jain, V., Capalash, N., & Kaur, J. (2012). p16(INK4a) and p15(INK4b) gene promoter methylation in cervical cancer patients. *Oncology Letters, 3*(6), 1331-1335. doi: 10.3892/ol.2012.655

Jha, A. K., Nikbakht, M., Jain, V., Sehgal, A., Capalash, N., & Kaur, J. (2012). Promoter hypermethylation of p73 and p53 genes in cervical cancer patients among north Indian population. *Molecular Biology Reports, 39*(9), 9145-9157.

Jithesh, P. V., Risk, J. M., Schache, A. G., Dhanda, J., Lane, B., Liloglou, T., & Shaw, R. J. (2013). The epigenetic landscape of oral squamous cell carcinoma. *British Journal of Cancer*, *108*(2):370-379.

Jones, P. A., & Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. *Nature Reviews Genetics, 3*(6), 415-428.

Jones, P. A., & Baylin, S. B. (2007). The Epigenomics of Cancer. *Cell, 128*(4), 683-692. doi: 10.1016/j.cell.2007.01.029

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics, 12*, 484-492.

Josena, K. S., Dhananjay, C., Vinod, N., Kang M, C., Raja, S., & Maria, J. W. (2011). DNA Methylation in Thyroid Tumorigenesis. *Cancers*. 3(2): 1732-1743. doi: http://dx.doi.org/10.3390/cancers3021732

Kamalakaran, S., Kendall, J., Zhao, X., Tang, C., Khan, S., Ravi, K., & Helland, A. (2009). Methylation detection oligonucleotide microarray analysis: a high-resolution method for detection of CpG island methylation. *Nucleic Acids Research, 37*(12), e89-e89.

Kämmerer, U., Kapp, M., Gassel, A. M., Richter, T., Tank, C., Dietl, J, & Ruck, P. (2001). A new rapid immunohistochemical staining technique using the EnVision antibody complex. *Journal of Histochemistry and Cytochemistry, 49*(5), 623-630.

Kang, G. H., Lee, S., Cho, N. Y., Gandamihardja, T., Long, T. I., Weisenberger, D. J., & Laird, P. W. (2007). DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. *Laboratory Investigation, 88*(2), 161-170.

Kato, Y., Zhao, M., Morikawa, A., Sugiyama, T., Chakravortty, D., Koide, N., . . . & Yokochi, T. (2000). Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. *Journal of Biological Chemistry, 275*(24), 18534-18540.

Katoh, M. (2005). WNT/PCP signalling pathway and human cancer (Review). *Oncology Reports, 14*(6), 1583-1588.

Keshet, Y., & Seger, R. (2010). The MAP kinase signalling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Molecular Biology,* 661:3-38.

Keyse, S. M. (2008). Dual-specificity MAP kinase phosphatases (MKPs) and cancer. *Cancer and Metastasis Reviews, 27*(2), 253-261.

Khulan, B., Thompson, R. F., Ye, K., Fazzari, M. J., Suzuki, M., Stasiek, E., & Montagna, C. (2006). Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Research, 16*(8), 1046-1055.

Killian, J. K., Walker, R. L., Bilke, S., Chen, Y., Davis, S., Cornelison, R., . . . & Meltzer, P. S. (2012). Genome-Wide Methylation Profiling in Archival Formalin-Fixed Paraffin-Embedded Tissue Samples. *Methods Molecular Biology, 823*, 107-118. doi: 10.1007/978-1-60327-216-2_8

Kim, J. C., & Kim, T. W. (2010). Promoter methylation of specific genes is associated with the phenotype and progression of colorectal adenocarcinomas. *Annals of Surgical Oncology, 17*(7), 1767-1776.

Kim, S. J., Kelly, W. K., Fu, A., Haines, K., Hoffman, A., Zheng, T., & Zhu, Y. (2011). Genome-wide methylation analysis identifies involvement of TNF-α mediated cancer pathways in prostate cancer. *Cancer Letters, 302*(1), 47-53. doi: 10.1016/j.canlet.2010.12.010

Kim, Y. I. (2004). Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer? *Cancer Epidemiology Biomarkers and Prevention, 13*(4), 511-519.

Kim, Y. I. (2007). Folate and colorectal cancer: An evidence-based critical review. *Molecular Nutrition and Food Research, 51*(3), 267-292.

Kim, Y. S., Do, J. H., Bae, S., Bae, D. H., & Ahn, W. S. (2010). Identification of differentially expressed genes using an annealing control primer system in stage III serous ovarian carcinoma. *BMC Cancer, 10*(1), 576-593.

Kirmizis, A., & Farnham, P. J. (2004). Genomic approaches that aid in the identification of transcription factor target genes. *Experimental Biology and Medicine, 229*(8), 705-721.

Kolenbrander, P. E., Egland, P. G., Diaz, P. I., & Palmer Jr, R J. (2005). Genome–genome interactions: bacterial communities in initial dental plaque. *Trends in Microbiology, 13*(1), 11-15.

Korde, S., Sridharan, G., Gadbail, A., & Poornima, V. (2012). Nitric oxide and oral cancer: A review. *Oral Oncology, 48*(6), 475-483.

Kron, K., Pethe, V., Briollais, L., Sadikovic, B., Ozcelik, H., Sunderji, A.,… & Bapat B. (2009). Discovery of novel hypermethylated genes in prostate cancer using genomic CpG island microarrays. *PLoS One, 4*(3), e4830.

Kruaysawat, W., Aekplakorn, W., & Chapman, R. S. (2010). Survival time and prognostic factors of oral cancer in Ubon Ratchathani Cancer Center. *Journal of the Medical Association of Thailand, 93*(3), 278-284.

Kumar, S., Dekate, K., Patel, S., & Haque, N. (2013). Oral Cancer: The Dentist's Intervention. *Journal of Contemporary Dentistry, 3*(2), 74-77.

Kuo, W. P., Kim, E. Y., Trimarchi, J., Jenssen, T. K., Vinterbo, S. A., & Ohno-Machado, L. (2004). A primer on gene expression and microarrays for machine learning researchers. *Journal of Biomedical Informatics, 37*(4), 293-303.

Kuwano, H., Kato, H., Miyazaki, T., Fukuchi, M., Masuda, N., Nakajima, M.,… & Faried, A. (2005). Genetic alterations in esophageal cancer. *Surgery Today, 35*(1), 7-18.

Kyrgidis, A., Tzellos, T., & Triaridis, S. (2010). Melanoma: Stem cells, sun exposure and hallmarks for carcinogenesis, molecular concepts and future clinical implications. *Journal of Carcinogenesis, 9*(1), 3. doi: 10.4103/1477-3163.62141.

Lachenmeier, D. W. (2010). Mouthwash controversies. *British Dental Journal, 208*(3), 95-96.

Lai, J. C., Cheng, Y. W., Chiou, H. L., Wu, M. F., Chen, C. Y., & Lee, H. (2005). Gender difference in estrogen receptor alpha promoter hypermethylation and its prognostic value in non-small cell lung cancer. *International Journal of Cancer, 117*(6), 974-980.

Lai, K. C., & Lee, T. C. (2006). Genetic damage in cultured human keratinocytes stressed by long-term exposure to areca nut extracts. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 599*(1), 66-75.

Laird, P. W. (2003). The power and the promise of DNA methylation markers. *Nature Reviews Cancer, 3*(4), 253-266.

Lande-Diner, L., Zhang, J., Ben-Porath, I., Amariglio, N., Keshet, I., Hecht, M., . . . Cedar, H. (2007). Role of DNA methylation in stable gene repression. *Journal of Biological Chemistry, 282*(16), 12194-12200.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J.,… & Chen Y. J. (2001). Initial sequencing and analysis of the human genome. *Nature, 409*(6822), 860-921.

Latham, V. H., Latham, L. E., & Oppenheimer, Steven B. (1996). Desktop computer-based image analysis of cell surface fluorescence patterning from a photographic source. *Acta Histochemica, 98*(3), 295-300.

Lee, D, Lee, G. K, Yoon, K. A, & Lee, J. S. (2013). Pathway-Based Analysis Using Genome-wide Association Data from a Korean Non-Small Cell Lung Cancer Study. *PLoS One, 8*(6). doi: 10.1371/journal.pone.0065396

Lee, Y., Vassilakos, A., Feng, N., Lam, V., Xie, H., Wang, M., & Young A. (2003). GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase, shows potent antitumor activity against a variety of tumors. *Cancer Research, 63*(11), 2802-2811.

Leiper, J., & Nandi, M. (2011). The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. *Nature Reviews Drug Discovery, 10*(4), 277-291.

Leite, I. C. G, & Koifman, S. (1998). Survival analysis in a sample of oral cancer patients at a reference hospital in Rio de Janeiro, Brazil. *Oral oncology, 34*(5), 347-352.

Levsky, J. M, & Singer, R. H. (2003). Fluorescence in situ hybridization: past, present and future. *Journal of Cell Science, 116*(14), 2833-2838.

Li, L. C., Carroll, P. R., & Dahiya, R. (2005). Epigenetic changes in prostate cancer: implication for diagnosis and treatment. *Journal of the National Cancer Institute, 97*(2), 103-115.

Li, L., Su, X., Choi, G. C. G., Cao, Y., Ambinder, R. F., & Tao, Q. (2012). Methylation profiling of Epstein-Barr virus immediate-early gene promoters, BZLF1 and BRLF1 in tumors of epithelial, NK- and B-cell origins. *BMC Cancer, 12*, 125-125. doi: 10.1186/1471-2407-12-125

Li, Y., Zhang, L., Nong, J., Bian, S., Zhao, Z., Ren, Y., & Bian, X. (2011). Expression of MEF2D on nasopharyngeal carcinoma tissues and its influence of prognostic. *Journal of Clinical Otorhinolaryngology Head and Neck Surgery, 18* (2), 840-847.

Lieber, C. S., & Packer, L. (2002). S-Adenosylmethionine: molecular, biological, and clinical aspects - an introduction. *The American Journal of Clinical Nutrition, 76*(5), 1148S-1150S.

Lim, G. C. C., Rampal, S., & Yahaya, H. (2008). *Cancer Incidence in Peninsular Malaysia, 2003-2005: The Third Report of the National Cancer Registry, Malaysia*: National Cancer Registry.

Lind, G. E., Thorstensen, L., Løvig, T., Meling, G. I., Hamelin, R., Rognum, T. O., & Lothe, R. A. (2004). A CpG island hypermethylation profile of primary colorectal carcinomas and colon cancer cell lines. *Molecular Cancer, 3*(1), 28.

Lippman, Z., Gendrel, A. V., Colot, V., & Martienssen, R. (2005). Profiling DNA methylation patterns using genomic tiling microarrays. *Nature Methods, 2*(3), 219-224.

Liu, X., Zhou, B., Xue, L., Yen, F., Chu, P., Un, Frank., & Yen, Y. (2007). Ribonucleotide reductase subunits M2 and p53R2 are potential biomarkers for metastasis of colon cancer. *Clinical Colorectal Cancer, 6*(5), 374-381.

Lo, W. L., Kao, S. Y., Chi, L. Y., Wong, Y. K., & Chang, R. C. (2003). Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *Journal of Oral and Maxillofacial Surgery, 61*(7), 751-758.

Lokk, K., Vooder, T., Kolde, R., Välk, K., Võsa, U., Roosipuu, R., & Urgard, E. (2012). Methylation markers of early-stage non-small cell lung cancer. *PLoS one, 7*(6), e39813. doi: 10.1371/journal.pone.0039813.

Lu, A. G., Feng, H., Wang, P. X., Han, D. P., Chen, X H., & Zheng, M. H. (2012). Emerging roles of the ribonucleotide reductase M2 in colorectal cancer and ultraviolet-induced DNA damage repair. *World Journal of Gastroenterology, 18*(34), 4704-4713.

Lucito, R., Healy, J., Alexander, J., Reiner, A., Esposito, D., Chi, M., . . . Troge, J. (2003). Representational oligonucleotide microarray analysis: a high-resolution method to detect genome copy number variation. *Genome Research, 13*(10), 2291-2305.

Ludwig, J. A., & Weinstein, J. N. (2005). Biomarkers in cancer staging, prognosis and treatment selection. *Nature Reviews Cancer, 5*(11), 845-856.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature, 389*(6648), 251-260.

Lukasik, S. M., Cierpicki, T., Borloz, M., Grembecka, J., Everett, A., & Bushweller, J. H. (2006). High resolution structure of the HDGF PWWP domain: a potential DNA binding domain. *Protein Science, 15*(2), 314-323.

Majumdar, S., Chanda, S., Ganguli, B., Mazumder, D. N., Lahiri, S., & Dasgupta, U. B. (2010). Arsenic exposure induces genomic hypermethylation. *Environmental Toxicology, 25*(3), 315-318.

Margueron, R., Trojer, P., & Reinberg, D. (2005). The key to development: interpreting the histone code? *Current Opinion in Genetics and Development, 15*(2), 163-176.

Maruya, S., Issa, J. P., Weber, R. S., Rosenthal, D. I., Haviland, J. C., Lotan, R., & El-Naggar, A. K. (2004). Differential Methylation Status of Tumor-Associated Genes in Head and Neck Squamous Carcinoma Incidence and Potential Implications. *Clinical Cancer Research, 10*(11), 3825-3830.

Mascolo, M., Siano, M., Ilardi, G., Russo, D., Merolla, F., Rosa, G.D., & Staibano, S. (2012). Epigenetic Disregulation in Oral Cancer. *International Journal of Molecular Sciences, 13*(2), 2331-2353.

Massano, J., Regateiro, F. S., Januário, G., & Ferreira, A. (2006). Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 102*(1), 67-76.

Mathers, J. C., Strathdee, G., & Relton, C. L. (2010). Induction of Epigenetic Alterations by Dietary and Other Environmental Factors. *Advances in genetic*, 71:3-39.

McCullough, M. J., & Farah, C. S. (2008). The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes. *Australian Dental Journal, 53*(4), 302-305.

Meurman, J. H., & Bascones-Martinez, A. (2011). Are oral and dental diseases linked to cancer? *Oral Diseases, 17*(8), 779-784.

Meurman, J. H. (2010). Infectious and dietary risk factors of oral cancer. *Oral Oncology, 46*(6), 411-413.

Model, F., Osborn, N., Ahlquist, D., Gruetzmann, R., Molnar, B., Sipos, F., & Tulassay, Z. (2007). Identification and validation of colorectal neoplasia–specific methylation markers for accurate classification of disease. *Molecular Cancer Research, 5*(2), 153-163.

Momparler, R. L. (2003). Cancer epigenetics. *Oncogene, 22*(42), 6479-6483.

Moncho-Amor, V., Ibanez de Caceres, I., Bandres, E., Martinez-Poveda, B., Orgaz, J. L., Sanchez-Perez, I., & Perona, R. (2011). DUSP1/MKP1 promotes angiogenesis, invasion and metastasis in non-small-cell lung cancer. *Oncogene, 30*(6), 668-678. doi: onc2010449 [pii] 10.1038/onc.2010.449

Morris, M. R., Gentle, D., Abdulrahman, M., Clarke, N., Brown, M., Kishida, T., & Maher, E. R. (2008). Functional epigenomics approach to identify methylated candidate tumour suppressor genes in renal cell carcinoma. *British Journal of Cancer, 98*(2), 496-501.

Morris, M. R., Ricketts, C. J., Gentle, D., McRonald, F., Carli, N., Khalili, H., & Maher, E. R. (2010). Genome-wide methylation analysis identifies epigenetically inactivated candidate tumour suppressor genes in renal cell carcinoma. *Oncogene, 30*(12), 1390-1401. doi: 10.1038/onc.2010.525

Mortusewicz, O., Schermelleh, L., Walter, J., Cardoso, M. C., & Leonhardt, H. (2005). Recruitment of DNA methyltransferase I to DNA repair sites. *Proceedings of the National Academy of Sciences of the United States of America, 102*(25), 8905-8909.

Moskalyov, E. A., Eprintsev, A. T., & Hoheisel, JD. (2007). DNA methylation profiling in cancer: From single nucleotides towards the methylome. *Molecular Biology, 41*(5), 723-736.

Nagatake, M., Osada, H., Kondo, M., Uchida, K., Nishio, M., Shimokata, K., & Takahashi, T. (1996). Aberrant hypermethylation at the bcl-2 locus at 18q21 in human lung cancers. *Cancer Research, 56*(8), 1886-1891.

Nakane, Y., Natsume, A., Wakabayashi, T., Oi, S., Ito, M., Inao, S., & Yoshida, J. (2007). Malignant transformation-related genes in meningiomas: allelic loss on 1p36 and methylation status of p73 and RASSF1A. *Journal of Neurosurgery*, 107(2):398-404.

Nakayama, M., Gonzalgo, M. L., Yegnasubramanian, S., Lin, X., De Marzo, A. M., & Nelson, W. G. (2004). GSTP1 CpG island hypermethylation as a molecular biomarker for prostate cancer. *Journal of Cellular Biochemistry, 91*(3), 540-552.

Nandakumar, V., Vaid, M., & Katiyar, S. K. (2011). Epigallocatechin-3-gallate reactivates silenced tumor suppressor genes, Cip1/p21 and p16INK4a, by reducing DNA methylation and increasing histones acetylation in human skin cancer cells. *Carcinogenesis, 32*(4), 537-544.

Nazmul-Hossain, A. N. M., Patel, K. J., Rhodus, N. L., & Moser, K. L. (2008). Microarrays: applications in dental research. *Oral Diseases, 14*(1), 25-29. doi: 10.1111/j.1601-0825.2006.01342.x

Nebbioso, A., Manzo, F., Miceli, M., Conte, M., Manente, L., Baldi, A., & Mai, A. (2009). Selective class II HDAC inhibitors impair myogenesis by modulating the stability and activity of HDAC–MEF2 complexes. *EMBO Reports, 10*(7), 776-782.

Ng, K. H., & Siar, C. H. (1997). Oral cancers in Malaysia and national oral cancer control programme. *Dentistry Journal of Malaysia, 18*, 48-51.

Noushmehr, H., Weisenberger, D. J., Diefes, K., Phillips, H. S., Pujara, K., Berman, B. P., & Bhat, K. P. (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell, 17*(5), 510-522.

O'Sullivan, E., & Goggins, M. (2013). DNA Methylation Analysis in Human Cancer. *Methods Molecular Biology,* 980,131-56

Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, ., . . . & Loerch, P. (2008). SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. *Cell, 135*(5), 907-918.

Øster, B., Thorsen, K., Lamy, P., Wojdacz, T. K., Hansen, L. L., Birkenkamp-Demtröder, K., & Andersen, C. L. (2011). Identification and validation of highly frequent CpG island hypermethylation in colorectal adenomas and carcinomas. *International Journal of Cancer, 129*(12), 2855-2866.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. (2005). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians, 55*(2), 74-108.

Patterson, K., Molloy, L., Qu, W., & Clark, S. (2011). DNA methylation: bisulphite modification and analysis. *Journal of Visualized Experiments, 21*(56), 3170. doi: 10.3791/3170.

Pavia, M., Pileggi, C., Nobile, C. G. A., & Angelillo, I. F. (2006). Association between fruit and vegetable consumption and oral cancer: a meta-analysis of observational studies. *The American Journal of Clinical Nutrition, 83*(5), 1126-1134.

Payne, S. R., Serth, J., Schostak, M., Kamradt, J., Strauss, A., Thelen, P., &. . . Morotti, A. (2009). DNA methylation biomarkers of prostate cancer: Confirmation of candidates and evidence urine is the most sensitive body fluid for non-invasive detection. *The Prostate, 69*(12), 1257-1269.

Pérez-Sayáns, M., Suárez-Peñaranda., J. M., Gayoso-Diz, P., Barros-Angueira. F., Gándara-Rey, J. M., & García-García, A. (2011). p16INK4a/CDKN2 expression and its relationship with oral squamous cell carcinoma is our current knowledge enough? *Cancer Letters, 306*(2), 134-141. doi: 10.1016/j.canlet.2011.02.039

Petersen, P. E. (2003). The World Oral Health Report 2003: continuous improvement of oral health in the 21st century - the approach of the WHO Global Oral Health Programme. *Community Dentistry and Oral Epidemiology, 31*(s1), 3-24.

Petridou, E., Zavras, A. I., Lefatzis, D., Dessypris, N., Laskaris, G., Dokianakis, G., & Trichopoulos, D. (2002). The role of diet and specific micronutrients in the etiology of oral carcinoma. *Cancer, 94*(11), 2981-2988.

Petti, S. (2009). Lifestyle risk factors for oral cancer. *Oral oncology, 45*(4), 340-350.

Phé, V., Cussenot, O., & Rouprêt, M. (2010). Methylated genes as potential biomarkers in prostate cancer. *BJU International, 105*(10), 1364-1370.

Pindborg, J. J., Reichart, P. A., Smith, C. J., & Van der Waal, I. (1997). *Histological typing of cancer and precancer of oral mucosa: Definitions and explanatory notes*: Springer.

Piperi, C., & Papavassiliou, A. G. (2011). Strategies for DNA methylation analysis in developmental studies. *Development, Growth & Differentiation, 53*(3), 287-299.

Polak, J. M, & Van Noorden, S. (1997). *Introduction to immunocytochemistry*. Bios Scientific Publishers Oxford.

Prima, V., Gore, L., Caires, A., Boomer, T., Yoshinari, M., Imaizumi, M., & Hunger, S. P. (2005). Cloning and functional characterization of MEF2D/DAZAP1 and DAZAP1/MEF2D fusion proteins created by a variant t (1; 19)(q23; p13. 3) in acute lymphoblastic leukemia. *Leukemia, 19*(5), 806-813.

Purohit, V., Rapaka, R., Kwon, O. S., & Song, B J. (2013). Roles of alcohol and tobacco exposure in the development of hepatocellular carcinoma. *Life Sciences*. 92(1):3-9.

Radhakrishnan, R., Kabekkodu, S., & Satyamoorthy, K. (2011). DNA hypermethylation as an epigenetic mark for oral cancer diagnosis. *Journal of Oral Pathology & Medicine, 40*(9), 665-676. doi: 10.1111/j.1600-0714.2011.01055.x

Rahman, M. A., Amin, A. R., Wang, D., Koenig, L., Nannapaneni, S., Chen, Z. G., & Shin, D. M. (2013). RRM2 Regulates Bcl-2 in Head and Neck and Lung Cancers: A Potential Target for Cancer Therapy. *Clinical Cancer Research,* 19(13):3416-3428.

Rai, K., Chidester, S., Zavala, C. V., Manos, E. J., James, S. R., Karpf, A. R., & Cairns, B. R. (2007). Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. *Genes and Development, 21*(3), 261-266.

Ramaswamy, S., & Golub, T. R. (2002). DNA microarrays in clinical oncology. *Journal of Clinical Oncology, 20*(7), 1932-1941.

Rauhala, H. E., Porkka, K. P., Tolonen, T. T., Martikainen, P. M., Tammela, T. L .J., & Visakorpi, T. (2005). Dual-specificity phosphatase 1 and serum/glucocorticoid-regulated kinase are downregulated in prostate cancer. *International Journal of Cancer, 117*(5), 738-745.

Razin, A. (1998). CpG methylation, chromatin structure and gene silencing - a three-way connection. *The EMBO journal, 17*(17), 4905-4908.

Rexhepaj, E., Brennan, D. J., Holloway, P., Kay, E. W., McCann, A. H., Landberg, G., & Gallagher, W. M. (2008). Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: application to measurement of oestrogen and progesterone receptor levels in breast cancer. *Breast Cancer Research, 10*(5), R89. doi:10.1186/bcr2187.

Ribeiro, K. de Cássia B., Kowalski, L P., & Latorre, Ma. do R. (2003). Perioperative complications, comorbidities, and survival in oral or oropharyngeal cancer. *Archives of Otolaryngology Head & Neck Surgery, 129*(2), 219-228.

Riggs, A. D. (2008). X inactivation, differentiation, and DNA methylation. *Cytogenetic and Genome Research, 14*(1), 9-25.

Rodenhiser, D. I., Andrews, J., Kennette, W., Sadikovic, B., Mendlowitz, A., Tuck, A. B., & Chambers, A. F. (2008). Epigenetic mapping and functional analysis in a breast cancer metastasis model using whole-genome promoter tiling microarrays. *Breast Cancer Research, 10*(4), R62. doi:10.1186/bcr2121

Rodríguez-Paredes, M., & Esteller, M. (2011). Cancer epigenetics reaches mainstream oncology. *Nature Medicine*, 330-339. doi: doi:10.1038/nm.2305

Rosas, S. L. B., Koch, W., da Costa Carvalho, M da G., Wu, L., Califano, J., Westra, W., & Sidransky, D. (2001). Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer Research, 61*(3), 939-942.

Rottach, A., Leonhardt, H., & Spada, F. (2009). DNA methylation-mediated epigenetic control. *Journal of Cellular Biochemistry,* 108(1), 43-51.

Roy, S., Khanna, S., Bentley, K., Beffrey, P., & Sen, C. K. (2002). Functional genomics: high-density oligonucleotide arrays. *Methods in Enzymology,* 353, 487-497.

Sandoval, J., Heyn, H., Moran, S., Serra-Musach, J., Pujana, M. A., Bibikova, M., & Esteller, M. (2011). Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics, 6*(6), 692-702.

Sapp, J. P., Eversole, L. R., & Wysocki, G. (1997). Contemporary oral and maxillofacial pathology. *Implant Dentistry,* 6(3), 238-239.

Saranath, D. (2000). Integrated biology and molecular pathology of oral cancer. *Contemporary Issues in Oral Cancer*, 50-51. Oxford University Press.

Schmezer, P., & Plass, C. (2008). Epigenetic aspects in carcinomas of the head and neck. *Head and Neck Oncology, 56*(6), 594-602.

Schuebel, K. E., Chen, W., Cope, L., Glöckner, S. C., Suzuki, H., Yi, J. M., & van den Bosch, S. (2007). Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. *PLoS Genetics,* 3(9), e157.

Schwieger, M., Schüler, A., Forster, M., Engelmann, A., Arnold, M. A., Delwel, R., & Olson, E. N. (2009). Homing and invasiveness of MLL/ENL leukemic cells is regulated by MEF2C. *Blood,* 114(12), 2476-2488.

Scully, C., & Bagan, J. V. (2007). Recent advances in oral oncology. *Oral Oncology, 43*(2), 107-115.

Scully, C., & Bagan, J. V. (2009). Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. *Oral Diseases, 15*(6), 388-399. doi: 10.1111/j.1601-0825.2009.01563.x

Scully, C. (2011). Oral cancer aetiopathogenesis; past, present and future aspects. *Medicina Oral Patologia Oral Cirugia Bucal,* 16(3), e306-e311.

Selhub, J. M. J. W., & Miller, J. W. (1992). The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *The Americanj Journal of Clinical Nutrition, 55*(1), 131-138.

Shapiro, R., DeFate, V., & Welcher, M. (1974). Deamination cytosine derivatives by bisulfite. Mechanism of the reaction. *Journal of the American Chemical Society, 96*(3), 906-912.

Sharma, G., Mirza, S., Prasad, C. P., Srivastava, A., Gupta, S. D., & Ralhan, R. (2007). Promoter hypermethylation of p16 INK4A,p14,ARF,CyclinD2 and Slit2 in serum and tumor DNA from breast cancer patients. *Life Sciences, 80*(20), 1873-1881.

Shaw, R. (2006). The epigenetics of oral cancer. *International Journal of Oral and Maxillofacial Surgery, 35*(2), 101-108. doi: 10.1016/j.ijom.2005.06.014

Shaw, R. J., Hall, G. L., Lowe, D., Bowers, N. L., Liloglou, T., Field, J. K., . . . Risk, J. M. (2007). CpG island methylation phenotype (CIMP) in oral cancer: Associated with a marked inflammatory response and less aggressive tumour biology. *Oral Oncology, 43*(9), 878-886.

Shen, J., Wang, S., Zhang, Y. J., Kappil, M., Wu, H. C., Kibriya, M. G., & Lee, P. H. (2012). Genome-wide DNA methylation profiles in hepatocellular carcinoma. *Hepatology*. 55(6), 1799-1808

Shergill, I. S., Shergill, N. K., Arya, M., & Patel, H. R. H. (2004). Tissue microarrays: a current medical research tool. *Current Medical Research and Opinion, 20*(5), 707-712.

Shi, S. R., Key, M. E., & Kalra, K. L. (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *Journal of Histochemistry and Cytochemistry, 39*(6), 741-748.

Shi, S. R., Cote, R. J., & Taylor, C. R. (1997). Antigen retrieval immunohistochemistry: past, present, and future. *Journal of Histochemistry and Cytochemistry, 45*(3), 327-343.

Shi, S. R., Cote, R. J., & Taylor, C. R. (2001). Antigen retrieval techniques Current perspectives. *Journal of Histochemistry and Cytochemistry, 49*(8), 931-937.

Shillitoe, E. J. (2009). The role of viruses in squamous cell carcinoma of the oropharyngeal mucosa. *Oral Oncology, 45*(4), 351-355.

Shono, T., Tofilon, P. J., Bruner, J.M., Owolabi, O., & Lang, F. F. (2001). Cyclooxygenase-2 expression in human gliomas prognostic significance and molecular correlations. *Cancer Research, 61*(11), 4375-4381.

Sis, B., Sarioglu, S., Sokmen, S., Sakar, M., Kupelioglu, A., & Fuzun, M. (2005). Desmoplasia measured by computer assisted image analysis: an independent prognostic marker in colorectal carcinoma. *Journal of Clinical Pathology, 58*(1), 32-38.

Sozzi, G., Conte, D., Leon, M. E., Cirincione, R., Roz, L., Ratcliffe, C., & Pelosi, G. (2003). Quantification of free circulating DNA as a diagnostic marker in lung cancer. *Journal of Clinical Oncology, 21*(21), 3902-3908.

Srivastava, D. S. L., Mandhani, A., Mittal, B., & Mittal, R D. (2005). Genetic polymorphism of glutathione S-transferase genes (GSTM1, GSTT1 and GSTP1) and susceptibility to prostate cancer in Northern India. *BJU International, 95*(1), 170-173.

Stransky, N., Egloff, A. M., Tward, A. D., Kostic, A. D., Cibulskis, K., Sivachenko, A., & McKenna, A. (2011). The mutational landscape of head and neck squamous cell carcinoma. *Science, 333*(6046), 1157-1160.

Stott-Miller, M., Zhao, S., Wright, J. L., Kolb, S., Bibikova, M., Klotzle, B., &. . . Stanford, J. L. (2014). Validation study of genes with hypermethylated promoter regions associated with prostate cancer recurrence. *Cancer Epidemiology Biomarkers & Prevention*, *23*(7):1331-9.

Sturgis, E. M., Wei, Q., & Spitz, M. R. (2004). Descriptive epidemiology and risk factors for head and neck Cancer. *Seminars Oncology*. *31*(6):726-33

Supic, G., Kozomara, R., Brankovic-Magic, M., Jovic, N., & Magic, Z. (2009). Gene hypermethylation in tumor tissue of advanced oral squamous cell carcinoma patients. *Oral Oncology, 45*(12), 1051-1057.

Susan, J. C., Harrison, J., Paul, C. L., & Frommer, M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Research, 22*(15), 2990-2997.

Tabish, A. M., Poels, K., Hoet, P., & Godderis, L. (2012). Epigenetic factors in cancer risk: effect of chemical carcinogens on global DNA methylation pattern in human TK6 cells. *PLoS One, 7*(4), e34674.

Takai, D., & Jones, P. A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proceedings of the National Academy of Sciences of the United States of America*. *99*(6), 3740-3745.

Tanaka, T., Tanaka, M., & Tanaka, T. (2011). Oral carcinogenesis and oral cancer chemoprevention: a review. *Pathology Research International, 2011*. doi.org/0.4061 /2011/431246

Tao, Q., & Robertson, K. D. (2003). Stealth technology: how Epstein–Barr virus utilizes DNA methylation to cloak itself from immune detection. *Clinical Immunology, 109*(1), 53-63.

Taylor, K. H., Kramer, R. S., Davis, J. W., Guo, J., Duff, D. J., Xu, D., . . . Shi, H. (2007). Ultradeep bisulfite sequencing analysis of DNA methylation patterns in multiple gene promoters by 454 sequencing. *Cancer Research, 67*(18), 8511-8518.

Termine, N., Panzarella, V., Falaschini, S., Russo, A., Matranga, D., Lo Muzio, L., & Campisi, G. (2008). HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a meta-analysis (1988–2007). *Annals of Oncology, 19*(10), 1681-1690.

Tissir, F., Bar, I., Jossin, Y., De Backer, O., & Goffinet, A. M. (2005). Protocadherin Celsr3 is crucial in axonal tract development. *Nature Neuroscience, 8*(4), 451-457.

Tokumaru, Y., Harden, S. V., Sun, D., Yamashita, K., Epstein, J. I., & Sidransky, D. (2004). Optimal use of a panel of methylation markers with GSTP1 hypermethylation in the diagnosis of prostate adenocarcinoma. *Clinical cancer research, 10*(16), 5518-5522.

Towle, R., & Garnis, C. (2012). Methylation-Mediated Molecular Dysregulation in Clinical Oral Malignancy. *Journal of Oncology, 2012*, 1-11.

Towle, R., Truong, D., Hogg, K., Robinson, W. P., Poh, C. F., & Garnis, C. (2013). Global analysis of DNA methylation changes during progression of oral cancer. *Oral Oncology, 49* (11), 1033-1042.

Tsai, C. N., Tsai, C. L., Tse, K. P., Chang, H. Y., & Chang, Y. S. (2002). The Epstein–Barr virus oncogene product, latent membrane protein 1, induces the downregulation of E-cadherin gene expression via activation of DNA methyltransferases. *Proceedings of the National Academy of Sciences, 99*(15), 10084-10089.

Tsai, Y. S., Lee, K. W., Huang, J L., Liu, Y. S., Juo, S H., Kuo, W. R., . . & Jong, Y. J. (2008). Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. *Toxicology, 249*(2), 230-237.

Tsimberidou, A. M., Alvarado, Y., & Giles, F. J. (2002). Evolving role of ribonucleoside reductase inhibitors in hematologic malignancies. *Expert Review of Anticancer Therapy, 2*(4), 437-448.

Tsunematsu, T., Kudo, Y., Iizuka, S., Ogawa, I., Fujita, T., Kurihara, H., & Takata, T. (2009). RUNX3 has an oncogenic role in head and neck cancer. *PLoS One, 4*(6), e5892. doi: 10.1371/journal.pone.0005892

Turbin, D.A., Leung, S., Cheang, M. C., Kennecke, H. A., Montgomery, K. D., McKinney, S., . . & Badve, S. (2008). Automated quantitative analysis of estrogen receptor expression in breast carcinoma does not differ from expert pathologist scoring: a tissue microarray study of 3,484 cases. *Breast cancer Research and Treatment, 110*(3), 417-426.

Ushiku, T., Chong, J M., Uozaki, H., Hino, R., Chang, M. S., Sudo, M., . . . & Fukayama, M. (2007). p73 gene promoter methylation in Epstein-Barr virus-associated gastric carcinoma. *International Journal of Cancer, 120*(1), 60-66.

Vaissière, T., Hung, R. J., Zaridze, D.., Moukeria, A., Cuenin, C., Fasolo, V., . . . Brennan, P. (2009). Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. *Cancer Research, 69*(1), 243-252.

van't Veer, L. J., Dai, H., Van De Vijver, M. J., He, Y.D., Hart, A. A.M., Mao, M., & Witteveen, A. T. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature, 415*(6871), 530-536.

van't Veer, L. J., & Bernards, R. (2008). Enabling personalized cancer medicine through analysis of gene-expression patterns. *Nature, 452*(7187), 564-570.

Van den Akker, E. B., Verbruggen, B., Heijmans, B. T., Beekman, M., Kok, J N., Slagboom, P. E., & Reinders, M. J. (2011). Integrating protein-protein interaction networks with gene-gene co-expression networks improves gene signatures for classifying breast cancer metastasis. Journal of Integrative Bioinformatics, 16; 8(2):188. doi: 10.2390/biecoll-jib-2011-188.

van Otterdijk, S. D., Mathers, J. C., & Strathdee, G. (2013). Do age-related changes in DNA methylation play a role in the development of age-related diseases? *Biochemical Society transactions, 41*(3), 803-807.

Veeck, J., & Esteller, M. (2010). Breast cancer epigenetics: from DNA methylation to microRNAs. *Journal of Mammary Gland Biology and Neoplasia, 15*(1), 5-17.

Ventorin von Zeidler, S., Miracca, E. C., Nagai, M. A., & Birman, E. G. (2004). Hypermethylation of the p16 gene in normal oral mucosa of smokers. *International Journal of Molecular Medicine, 14*(5), 807-811.

Viet, C. T., & Schmidt, B. L. (2008). Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients. *Cancer Epidemiology Biomarkers & Prevention, 17*(12), 3603-3611.

Viet, C. T., & Schmidt, B. L. (2010). Understanding oral cancer in the genome era. *Head and Neck, 32*(9), 1246-1268.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine, 10*(8), 789-799.

Walker, D. M., Boey, G., & McDonald, L. (2003). The pathology of oral cancer. *Pathology, 35*(5), 376-383.

Wang, L. G., & Chiao, J. W. (2010). Prostate cancer chemopreventive activity of phenethyl isothiocyanate through epigenetic regulation (Review). *International Journal of Oncology, 37*(3), 533-539.

Wareham, K. A., Lyon, M. F., Glenister, P. H., & Williams, E. D. (1987). Age related reactivation of an X-linked gene. *Nature,* 327(6124):725-727

Warnakulasuriya, K. A. A. S., & Ralhan, R. (2007). Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco - a review. *Journal of Oral Pathology and Medicine, 36*(2), 63-77.

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncology, 45*(4), 309-316.

Warner, G. C., Reis, P. P., Jurisica, I., Sultan, M., Arora, S., Macmillan, C., . . . & Sukhai, M. (2004). Molecular classification of oral cancer by cDNA microarrays identifies overexpressed genes correlated with nodal metastasis. *InternationalJjournal of Cancer, 110*(6), 857-868.

Watanabe, T., Huang, H., Nakamura, M., Wischhusen, J., Weller, M., Kleihues, P., & Ohgaki, H. (2002). Methylation of the p73 gene in gliomas. *Acta Neuropathologica, 104*(4), 357-362.

Weber, M., Davies, J. J., Wittig, D., Oakeley, E. J., Haase, M., Lam, W. L., & Schübeler, D. (2005). Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genetics, 37*(8), 853-862.

Weinberg, R. A. (1991). Tumor suppressor genes. *Science, 254*(5035), 1138-1146.

Weller, M., Stupp, R., Reifenberger, G., Brandes, A. A., van den Bent, M. J., Wick, W., & Hegi, M. E. (2009). MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nature Reviews Neurology, 6*(1), 39-51.

Werning, J. W. (2007). *Oral cancer: diagnosis, management, and rehabilitation*. Thieme Medical Publishers, Inc.

Wester, K., Wahlund, E., Sundström, C., Ranefall, P., Bengtsson, E., Russell, P. J., & Busch, C. (2000). Paraffin section storage and immunohistochemistry: effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. *Applied Immunohistochemistry and Molecular Morphology, 8*(1), 61-70.

Wilcox, C. B., Baysal, B. E., Gallion, H. H., Strange, M. A., & DeLoia, J. A. (2005). High-resolution methylation analysis of the BRCA1 promoter in ovarian tumors. *Cancer Genetics and Cytogenetics, 159*(2), 114-122.

Williams, A. L., Girard, C., Jui D., Sabina, A., & Katz, D. L. (2005). S-adenosylmethionine (SAMe) as treatment for depression: a systematic review. *Clinical Investigative medicine,* 28(3), 132-139.

Wojdacz, T. K., & Dobrovic, A. (2007). Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Research, 35*(6), e41-e41. doi: 10.1093/nar/gkm013

Wong, I. H. N. (2006). Qualitative and quantitative polymerase chain reaction-based methods for DNA methylation analyses. *Methods in Molecular Biology, 336*, 33-43.

Woolgar, J. A. (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncology, 42*(3), 229-239.

Wu, I. C., Lu, C. Y., Kuo, F. C., Tsai, S. M., Lee, K. W., Kuo, W. R., & Ko, Y. C. (2006). Interaction between cigarette, alcohol and betel nut use on esophageal cancer risk in Taiwan. *European Journal of Clinical Investigation, 36*(4), 236-241.

Wu, S. C., & Zhang, Y. (2010). Active DNA demethylation: many roads lead to Rome. *Nature Reviews Molecular Cell Biology, 11*(9), 607-620.

Xie, H., Lin, J., Thomas, D. G., Jiang, W., & Liu, X. (2012). Ribonucleotide reductase M2 does not predict survival in patients with resectable pancreatic adenocarcinoma. *International Journal of Clinical and Experimental Pathology, 5*(4), 347-355.

Xu, F., Mao, C., Ding, Y., Rui, C., Wu, L., Shi, A., & Xu, Z. (2010). Molecular and enzymatic profiles of mammalian DNA methyltransferases: structures and targets for drugs. *Current Medicinal Chemistry, 17*(33), 4052-4071.

Xu, X. L., Yu, J., Zhang, H. Y., Sun, M. H., Gu, J., Du, X., & Zhu, J. D. (2004). Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World Journal of Gastroenterology, 10*(23), 3441-3454.

Yang, G., Zhang, H., Liu, Y., Zhou, J., He, W., Quick, C. M., & Fan, C. Y. (2012). Epigenetic and immunohistochemical characterization of the Clusterin gene in ovarian tumors. *Archives of Gynecology and Obstetrics*, 1-7.

Yang, X. J., & Seto, E. (2008). Lysine acetylation: codified crosstalk with other posttranslational modifications. *Molecular Cell, 31*(4), 449-461.

Yang, X., Smith, S. L., Tian, X. C., Lewin, H. A., Renard, J. P., & Wakayama, T. (2007). Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nature Genetics, 39*(3), 295-302.

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews Molecular Cell Biology, 9*(1), 47-59.

Zain, R. B. (2001). Cultural and dietary risk factors of oral cancer and precancer - a brief overview. *Oral Oncology, 37*(3), 205-210.

Zain, R. B., & Ghazali, N. (2001). A review of epidemiological studies of oral cancer and precancer in Malaysia. *Annals of Dentistry University of Malaya, 8*, 50-56.

Zhang, A. H., Bin, H. H., Pan, X. L., & Xi, X. G. (2007). Analysis of p16 gene mutation, deletion and methylation in patients with arseniasis produced by indoor unventilated-stove coal usage in Guizhou, China. *Journal of Toxicology and Environmental Health, 70*(11), 970-975.

Zhang, K., Hu, S., Wu, J., Chen, L., Lu, J., Wang, X., & Yen, Y. (2009). Overexpression of RRM2 decreases thrombspondin-1 and increases VEGF production in human cancer cells in vitro and in vivo: implication of RRM2 in angiogenesis. *Molecular Cancer*, 28;8:11. doi: http://dx.doi.org/10.1186/1476-4598-8-11

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, Sha., Chan, W. L., Chen, H., & Jacobsen, S. E. (2006). genome-wide high-resolution mapping and functional analysis of dna methylation in arabidopsis. *Cell, 126*(6), 1189-1201.

Zhou, J. W., Gan, N. Y., & Zhang, W. J. (2009). The expression of MKP-1 and p-ERK (1/2) in primary ovarian epithelial tumor tissues. *Journal of Molecular Cell Biology, 42*(3-4), 224-230.

Zhu, J., Jiang, J., Zhou, W., & Chen, X. (1998). The potential tumor suppressor p73 differentially regulates cellular p53 target genes. *Cancer Research, 58*(22), 5061-5065.

Zygogianni, A. G., Kyrgias, G., Karakitsos, P., Psyrri, A., Kouvaris, J., Kelekis, N., & Kouloulias, V. (2011). Oral squamous cell cancer: early detection and the role of alcohol and smoking. *Head Neck Oncology, 3*, 2. doi: 10.1186/1758-3284-3-2

Appendix A

Protocol of Haematoxylin and Eosin Staining

1. Xylene I ………………………………………………………… 5 mins.
2. Xylene II ……………………………………………………..… 4 mins.
3. Ethanol 100% ……………………………………………………. 3 mins.
4. Ethanol 95% ……………………………………………………… 3 mins.
5. Ethanol 70% ……………………………………………………… 3 mins.
6. Running water …………………………………………………… 3 mins.
7. Harris Hematoxylin ……………………………………………. 12 mins.
8. Running water …………………………………………………... 3 mins.
9. Acid Alcohol ……………………………………………………. Count to 10.
10. Running water …………………………………………………. 3 mins.
11. Potassium Acetate ……………………………………………… 4 dips.
12. Running water …………………………………………………. 3 mins.
13. Ethanol 80% …………………………………………………….. 1 min.
14. Eosin ……………………………………………………….. 6 mins.
15. Ethanol 95% …………………………………………………….. 4 dips.
16. Ethanol 95% …………………………………………………….. 4 dips.
17. Ethanol 100% (2 changes) ……………………………………… 2 mins.
18. Xylene (3 changes) ……………………………………………… 3 mins.
19. Mount in D.P.X

<u>RESULTS :</u>
Anionic tissue components…………………blue
Cationic tissue components ………………..red

Appendix B

Primer design criteria:

1. Primers should be approximately 30bp in length, to ensure specificity
2. Primer should have a similarly predicted Tm above 50°C and did not differ by more than 1-2 ^{o}C.
3. Primer should contain multiple CT bases, to ensure conversion specificity.
4. The final base at the 3' end should be a CT base to ensure amplification of converted DNA.
5. CpG dinucleotides should be avoided in the primer sequence to avoid potential bias towards methylated, unmethylated or unconverted template.
6. The length of amplicon should be no more than 450bp to ensure maximum yield.

Appendix C

Protocol of Immunohistochemical staining*:* Labelled Streptavidin Biotin (LSAB)

1. Deparaffinize and hydrate to water.
2. Antigen retrieval with microwave. - 10 mins
3. Cool down to room temperature
4. Quenching with Peroxidase Blocking Reagent (3% H_2O_2) - 5 mins
5. Rinse with TBS 1X3 mins
6. Incubation with primary antibody - 30 mins
7. Rinse with TBS 1X3 mins
8. Incubate with Link: Biotinylated secondary antibody - 30 mins
9. Rinse with TBS 2X3mins
10. Streptavidin Peroxidase complex - 15-30 mins
11. TBS 1X3
12. Incubate with DAB working solution - 10 mins
13. Washing in running water
14. Counterstain with Hematoxylin - 2-5 mins
15. Washing in running water
16. Blueing in 2% Sodium Acetate. - 5 dips
17. Washing in running water
18. Dehydrate, clear and mount

Peroxidase Blocking Reagent

DESCRIPTION: Peroxidase Blocking Reagent: Peroxidase inhibitor containing hydrogen peroxide and 15 mM sodium azid

Appendix D

Details of 34 promoter-associated hypermethylated genes of OSCC with UCSC gene accession and CpG Island name (Illumina's Genome Studio software analysis)

UCSC_REFGENE_NAME	UCSC_REFGENE_ACCESSION	UCSC_CPG_ISLANDS_NAME
DFFB	NM_014704;NM_004402	
DUSP1*	NM_004417	chr1:3775077-3775369
FEZ2	NM_001042548;NM_005102	chr5:172197482-172199606
RWDD3	NM_001128142;NM_015485	chr2:36824792-36825305
ZNF589	NM_016089	chr1:95699725-95700142
DDAH2*	NM_013974	chr3:48282425-48282817
TSC22D3	NM_004089;NM_198057;	chr6:31695894-31698245
LEPRE1	NM_022356;NM_001146289	chrX:106959378-106959914
ORMDL3	NM_139280	chr1:43232214-43233359
HYAL2	NM_003773;NM_033158	chr17:38083226-38084052
MUM1	NM_032853;NR_024247	chr3:50359839-50360042
KIF3C	NM_002254	chr19:1354420-1355350
EPHX2	NM_001979	chr2:26204554-26205649
GEMIN7	NM_001007269;NM_001007270;NM_024	chr8:27348658-27348883
MIER2	NM_017550	chr19:45594397-45595007
MAPRE2	NM_001143826	chr19:345260-345590
C14orf80	NM_001134877	chr18:32556654-32557883
VEZF1	NM_007146	chr14:105956175-105958197
ZBTB4	NM_001128833;NM_020899	chr17:56064830-56066051
		chr17:7382266-7382710

Appendix D; continued

UCSC_REFGENE_NAME	UCSC_REFGENE_ACCESSION	UCSC_CPG_ISLANDS_NAME
P16*	NM_001195132;NM_058195	chr9:21974578-21975306
HYAL2	NM_003773;NM_033158	chr3:50359839-50360042
PPT2	NM_005155;NM_030651;NM_138717	chr6:32121829-32122529
TRIM65	NM_173547	chr17:73892496-73893448
TMEM120A	NM_031925	chr12:53689227-53689570
PFDN5	NM_002624;NM_145897	chr6:31695894-31698245
CCNJ	NM_001134376;NM_001134375;	chr10:97802871-97804262
GEMIN7	NM_001007269;NM_001007270;NM_0247	chr19:45594397-45595007
NCKAP5L	NM_001037806;NR_027499;NR_027500	chr12:50221558-50222609
TAF4	NM_003185	chr20:60638612-60638982
C1orf52	NM_198077;NR_024113	chr1:85724701-85725524
TRAPPC5	NM_001042461;NM_174894;NM_0010424	chr19:7745490-7747714
TAF4	NM_003185	chr20:60639272-60639835
TRIM65	NM_173547	chr17:73892496-73893448

Genes of DUSP1, DDAH2 and p16 marked with * were selected for further study.

Appendix E

Details of 89 promoter hypermethylated genes of OSCC with island location and p value generated (Partek Genomic Suite software)

Gene name	Promoter _ CPG_Island location	Chromosome location	P value	Fold change
KCNA3	Island	chr1:111216244-111217937	0.001631	24.6852
CELSR3*	island	Chr3:48693118-48701667	0.000736	15.0254
PLXNC1	Island	chr12:94541727-94544000	0.000349	14.1601
ZNF582	Island	chr19:56904636-56905355	0.000345	13.8471
MAP1LC3A	Island	chr20:33146135-33147318	0.002268	12.7764
C17orf46	Island	chr17:43339124-43339832	0.000696	12.2603
TSPYL5	Island	chr8:98289604-98290404	0.001153	10.8674
TRIM61	Island	chr4:165878036-165878446	0.001392	10.5525
INA	Island	chr10:105036628-105038084	0.000185	10.3509
ZNF529	Island	chr19:37095680-37096589	0.000222	7.67903
ZNF844	Island	chr19:12175460-12176057	0.000696	7.53149
ZNF808	Island	chr19:53039077-53039920	0.000324	6.93332
SPDYA	Island	chr2:29033351-29034011	0.001080	6.25406
ZNF761	Island	chr19:53935089-53935291	0.000645	6.09716
ZSCAN18	Island	chr19:58609338-58609988	1.30E-05	5.68720
HLA-H	Island	chr6:29855295-29856565	0.001807	5.62426
PPP1R16B	Island	chr20:37434206-37435592	0.001031	5.61812
ITPKB	Island	chr1:226924560-226926553	0.000376	5.44636
USP44	Island	chr12:95941906-95942979	0.001043	5.34401
ZNF611	Island	chr19:53237861-53238499	0.000733	5.33032
NKAPL	Island	chr6:28226979-28227483	0.000474	4.90568
DPY19L2P2	Island	chr7:102920309-102921514	0.000976	4.71972
ELMO1	Island	chr7:37487354-37488672	0.001611	4.66954
HKR1	Island	chr19:37825101-37825756	0.000127	4.38601
ZNF845	North shore	chr19:53836795-53837495	0.000167	4.14363
FLJ45983	Island	chr10:8091374-8098329	0.000988	4.12495
BMP8B	Island	chr1:40253683-40255172	0.000547	4.12023
LOC100133991	Island	chr17:43339124-43339832	0.001262	4.10620
HLA-C	Island	chr6:31238852-31240120	0.001490	4.08982

Appendix E, continued

Gene name	Promoter _ CPG_Island location	Chromosome location	P value	Fold change
ZNF354C	Island	chr5:178487146-178487921	0.001030	4.04431
ZNF492	North shore	chr19:22817274-22817546	0.001103	4.03547
HLA-L	Island	chr6:30227320-30228255	0.001482	3.88852
WDR8	Island	chr1:3566445-3569636	0.001731	3.76379
FABP5L3	Unspecific		0.000371	2.10058
ARPC1B	Island	chr7:98990157-98990922	0.001252	3.33637
ZNF382	Island	chr19:37095680-37096589	0.001594	3.25046
PIK3R5*	Island	chr17:8868469-8869372	0.001524	3.18124
HLA-B	Island	chr6:31323946-31325211	0.000925	3.17527
AHRR	Island	chr5:343449-344535	0.002364	3.08810
UGDH	Island	chr4:39528728-39529723	0.000656	3.03321
C9orf119	Island	chr9:131037591-131038695	0.000281	2.97446
ATP6V1B2	North shore	chr8:20054546-20055027	0.001921	2.96530
TRIM59	Island	chr3:160167184-160168200	0.000339	2.95672
ZNF568	Island	chr19:37406931-37407463	0.002031	2.88949
CSDA	Island	chr12:10875137-10876180	0.000324	2.87859
PDE4C	Island	chr19:18335072-18337375	0.001892	2.87417
P16*	Island	chr9:21974578-21975306	0.002357	2.85385
DDAH2*	Island	chr6:31695894-31698245	0.000934	2.82636
GPRASP1	South shore	chrX:101906001-101907017	0.00116	2.82113
NR6A1	Island	chr9:127532040-127533691	0.000954	2.81225
ZNF578	Island	chr19:52956656-52957245	0.000254	2.77831
LTC4S	North shore	chr5:179222608-179223825	0.000138	2.69466
COX5A	Island	chr15:75229782-75230660	0.000267	2.65538
LIMD2	Island	chr17:61776890-61778733	0.001213	2.64260
ZNF321	Island	chr19:53445344-53445933	0.002135	2.59262
NUB1	North shore	chr7:151038858-151039136	0.001992	2.57423
MEF2D*	Unspecific		0.002182	2.54062
DUSP1*	Island	Chr5:172197482-172199606	5.49E-08	2.56631
RRM2*	Island	chr2:10262173-10263481	0.001067	2.54062
ZNF418	Island	chr19:58446336-58446800	8.68E-05	2.51806
C19orf57	Island	chr19:14016665-14017435	0.002252	2.49569
RBM4B	North shore	chr11:66444997-66445471	0.000141	2.42872

Appendix E, continued

Gene name	Promoter _ CPG_Island location	Chromosome location	P value	Fold change
ZNF283	Unspecific		0.006481	2.36704
CTSA	North shore	chr20:44518897-44520361	0.000188	2.36562
FAM35A	Island	chr10:88853757-88855498	0.000561	2.33510
HPDL	Island	chr1:45792419-45793301	8.57E-07	2.32230
POM121L2	Island	chr6:27279794-27280635	0.001275	2.30725
TP73*	Island	Chr1:3566445-3569636	0.00150754	2.26647
MYEF2	South shore	chr15:48470007-48470628	7.14E-05	2.26717
ARHGEF7	North shore	chr13:111805638-111806531	0.001953	2.24715
BZW2	Island	chr7:16685252-16686364	0.002295	2.24408
ZNF701	Island	chr19:53073308-53074039	0.001135	2.23376
ZNF677	South shore	chr19:53757819-53758148	0.001913	2.22286
ZNF610	North shore	chr19:52839444-52839937	0.002055	2.21386
CHD4	North shelf	chr12:6715500-6715829	0.001025	2.19070
ZPBP	Island	chr7:50132572-50132921	0.002319	2.16902
KLHL21	South shore	chr1:6661776-6663844	0.000551	2.16866
IFFO2	Unspecific		0.011708	2.15139
BCL11A	Island	chr2:60776596-60778157	0.001565	2.14449
CCDC106	North shore	chr19:56159257-56159937	0.002300	2.13559
ZNF75A	Island	chr16:3355020-3356012	0.000479	2.13414
IGF2BP1	Island	chr17:47072820-47076042	0.001266	2.10853
DPYSL2	Island	chr8:26434372-26436785	0.000146	2.08274
TBCA	Island	chr5:77071645-77072303	0.001422	2.07714
ADPRHL2	Island	chr1:36554325-36554955	0.001030	2.07696
TMEM220	Island	chr17:10632789-10633490	0.000436	2.04846
THG1L	Island	chr5:157158378-157158856	0.001672	2.03017
SENP7	Island	chr3:101231610-101232173	0.002304	2.02761
ELL2	South shore	chr5:95296368-95297438	0.002360	2.00901

Genes of CELSR3, PIK3R5, p16, DDAH2, MEF2D, DUSP1, RRM2 and TP73 marked with * were selected for further study.

Appendix F

Correlation between protein expression and patients' age

			DDAH2	DUSP1	MEF2D	RRM2	Age_Cont
Spearman's rho	DDAH2	Correlation Coefficient	1.000	.081	**.378****	.022	-.195
		Sig. (2-tailed)	.	.584	**.008**	.880	.227
		N	40	40	40	40	40
	DUSP1	Correlation Coefficient	.081	1.000	.263	.133	.282
		Sig. (2-tailed)	.584	.	.071	.366	.078
		N	40	40	40	40	40
	MED2F	Correlation Coefficient	**.378****	.263	1.000	-.033	-.228
		Sig. (2-tailed)	**.008**	.071	.	.823	.158
		N	40	40	40	40	40
	RRM2	Correlation Coefficient	.022	.133	-.033	1.000	**.381***
		Sig. (2-tailed)	.880	.366	.823	.	**.015**
		N	40	40	40	40	40
	Age_Cont	Correlation Coefficient	-.195	.282	-.228	**.381***	1.000
		Sig. (2-tailed)	.227	.078	.158	**.015**	.
		N	40	40	40	40	40

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Appendix G

List of publications and conference proceedings

<u>Publications:</u>

1. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, EFFAT O, SK TAN, V. CHONG and K.L. THONG (2013). DNA Methylation Profiling Revealed Promoter Hypermethylation-induced Silencing of p16, DDAH2 and DUSP1 in Primary Oral Squamous Cell Carcinoma. Inter J Med Sci, 2013, 10(12); 1727-1739.

2. G.H KHOR, G.A. FROEMMING, R.B. ZAIN, A.M. THOMAS, and K.L. THONG (2014). Screening of Differential Promoter Hypermethylated Genes in Primary Oral Squamous Cell Carcinoma. Asian Pac J Cancer Prev. 15 (20), 8957-8961.

3. G.H. KHOR,, K.L. THONG, G.A. FROEMMING, A.M. THOMAS, NMM NIK MOHD ROSDY and R.B. ZAIN. Human Papiloma Virus 18 detection in Oral Squamous Cell Carcinoma and potentially Malignant Lesions Using Saliva Samples. Asian Pacific J Cancer Prev, 2012, 13 (12); 6109-6113

4. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.C. TAN, J. KIM, A.M. THOMAS, and K.L. THONG. Pathways Deregulation In Oral Squamous Cell Carcinoma Using Methylation Profiling. J Dent Res 2011, 89 (Sp C), Abs 47. ISSN: 1544-0591

5. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, and K.L. THONG. Identification and Validation of Novel Aberrant Gene Promoter Hypermethylation in Oral Squamous Cell Carcinoma. Malaysian J Pathol 2012; 34 (Supplement A): P/AP/22

6. G.H. KHOR, G.A. FROEMMING, R.B. ZAIN, A.M. THOMAS, E OMAR, SK TAN and K.L. THONG. (2013). Aberrant methylation of genes in oral squamous cell carcinoma. Oral Oncology Vol 49,supplement 1, pg S6-S7, OP007.

Conference proceedings:

1. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.C. TAN, J. KIM, A.M. THOMAS, and K.L. THONG. Pathways Deregulation In Oral Squamous Cell Carcinoma Using Methylation Profiling. 25th IADR-SEA Division Annual Scientific Meeting and 22nd SEAADE Annual Meeting. 28-30/10/2011. Grand Copthorne Waterfront, Singapore. Program book. Abs 47

2. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, and K.L. THONG. DifferentiaI Hypermethylation In Oral Squamous Cell Carcinoma using microarraybased DNA methylation analysis. 1st Annual IMMB Postgraduate Colloquium 2011. 25-26/11/2012. Calton Holiday Hotel and Suites, Shah Alam. Programme & abstract book. O6 (Oral presentation).

3. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, and K.L. THONG. DifferentiaI Hypermethylation In Oral Squamous Cell Carcinoma.16th Biological Science Graduate Congress. 12-13/12/2011. National University of Singapore. Singapore. Abstract book. PP-3-08.

4. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, and K.L. THONG. DifferentiaI Hypermethylation In Oral Squamous Cell Carcinoma Using Methylation Bead Array. Human Genome Meeting 2012. 11-14/3/2012. Sydney Convention Centre, Sydney, Australia. Abstract Book. P030

5. G.H. KHOR, R.B. ZAIN, G.A. FROEMMING, A.M. THOMAS, and K.L. THONG. Identification and Validation of Novel Aberrant Gene Promoter Hypermethylation in Oral Squamous Cell Carcinoma 11th Annual Scientific Meeting. 8-10/6/2012 Crowne Plaza Mutiara Hotel, Kuala Lumpur. Proceedings and Programme Book. P/AP/22.

6. G.H. KHOR, G.A. FROEMMING, R.B. ZAIN, A.M. THOMAS, E OMAR, SK TAN and K.L. THONG. (2013). Methylation Profiling in Primary Oral Squamous Cell Carcinoma. 38th Malaysian Society for Biochemistry and Molecular Biology (MSBMB). 28- 29/8/2013. Putrajaya Marriott Hotel and Spa, Putrajaya. Won the best poster presentation.