STUDY ON THE INVOLVEMENT OF WIF-1 IN ORAL CARCINOGENESIS VIA EGFR AND WNT SIGNALING PATHWAYS

CHOW TAN WEI

UNIVERSITI SAINS MALAYSIA

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by

CHOW TAN WEI

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

(Amino acid abbreviation)No.	Amino acid that located at specific site (numerical number) of target protein sequence
$(Protein)^{P-}$	Protein with specific mutated phosphorylation site
18 S rRNA	Small component of eukaryotic cytoplasmic ribosomal ribonucleic acid
Å	Ångström, mainly used in expressing sizes of atoms, lengths of chemical bonds, and wavelengths of electromagnetic radiation
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
anti-$(Protein)^{P}$	Antibody that binds to protein with specific phophorylation site
AOM/DSS	Azoxymethane/dextran sodium sulphate
APC	Adenomatous polyposis coli
ARM	Armadillo
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAPRI	Critical Assessment of PRedicted Interactions
CCND1	Protein-coding gene for cyclin D1
CDK	Cyclin-dependent-kinase
cDNA	Complementary deoxyribose nucleic acid
ChIP	Chromatin immunoprecipitation
CKI	Casein kinase I
COX	Cyclooxygenase-2

CRD	Cysteine-rich domain
CTNNB1	Protein-coding gene for β-catenin
CYP2E1	Cytochrome P450 2E1
DKK	Dickkopf
DNA	Deoxyribonucleic acid
DVL	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent
EMT	Epithelial-to-mesenchymal transition
Epo	Erythropoietin
ERK	Extracellular signal-regulated protein kinase
Evi	*Evenness*
FBS	Fetal bovine serum
FFT	Fast Fourier Transform
FISH	Fluorescence in situ hybridization
FZD	Frizzled
G-CSF	Granulocyte-colonystimulating factor
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GPCR	G-protein-coupled receptor

GSC	Grid-based shape complementarity
GSK3β	Glycogen synthase kinase 3β
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
Hh	Hedgehog
HIF-1	Hypoxia-inducible factor-1
HIV	Human immunodeficiency virus
HNF1A	Hepatic nuclear factor 1 alpha; Protein-coding gene for TCF-1
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HRG	Heregulin
HSV	Herpes virus
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Int-1	*Integrated*
IκB	Inhibitory protein for Nuclear factor-κB
JAK	JAnus Kinase
JNK	C-Jun NH_2-terminal kinase
kDa	Kilodalton (molecular weight for protein)

LC_{50}	The concentration at which the lethality activity was 50%
LEF-1	Lymphoid enchancer factor-1
LRP	Low-density-lipoprotein-receptor-related protein
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinases
MMTV	Mouse Mammary Tumor Virus
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-κB
OD	Optical density
OSCC	Oral squamous cell carcinomas
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDK 1	Protein kinase 1
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl Fluoride
PPI	Protease and Phosphatase Inhibitor
R^2	Correlation coefficient

RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotation rate of the rotor
RTK	Receptor Tyrosine Kinase
RYK	Receptor-like tyrosine kinase
SCC	Squamous cell carcinomas
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sFRP	Secreted Frizzled-Related Protein
SH2	Src Homology 2
Shf	Shifted
SPF	Spherical polar Fourier
STAT	Signal transducers and activators of transcription
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween 20
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCF-1	T-cell factor-1
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitors
TNF	Tumor necrosis factor
Tpo	Thrombopoietin
UDP-GT	Uridine-5’-diphosphate-glucuronosyltransferase

UV	Ultraviolet
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
Wg	*Wingless*
WIF-1	WNT inhibitory factor 1
Wls	*Wntless*
WNT	Wingless and integration site growth factor
WT	Wild type
X*g*	Centrifugal force

LIST OF PUBLICATIONS

Journal article

1) Chow, T. W., Ong, M. T. and Shaharum S. (2013) WIF-1 might play additional role in carcinogenesis and cancer growth via EGFR signaling pathway. *Journal of Biochemical Technology*. 4(4). p.645-647.

Journal abstract

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Proceedings

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2) Chow, T. W., Shaharum S. and Ong, M. T. (2013) Confirmation of WIF-1/EGFR interaction via co-immunoprecipitation. *ICSCC 2013, 19-22 October, Haffkine Institute, Parel, Mumbai, India*.Mumbai: ICSCC, p.35.

3) Chow, T. W., Ong, M. T. and Shaharum S. (2012) WIF-1 might play additional role in carcinogenesis and cancer growth via EGFR signaling pathway. *3rd INFORMM Postgraduate Colloqium 2012, 26/27 March 2012, INFORMM USM*. Penang: INFORMM USM, p.8.

Conference Presentation

1) Chow, T. W., Ong, M. T. and Shaharum S. (2014) *WNT inhibitory factor-1 (WIF-1): A new role in carcinogenesis?* ASCO 2014, 30 May – 3 June, McCormick Place, Chicago.

2) Chow, T. W., Ong, M. T. and Shaharum S. (2014) *The phosphorylation of AKT and GSK3β promotes the proliferation of CAL27 cell line*. WACCE 2014, 11-12 January, EUREKA USM.

3) Chow, T. W., Shaharum S. and Ong, M. T. (2013) *Confirmation of WIF-1/EGFR interaction via co-immunoprecipitation*. ICSCC 2013, 19-22 October, Haffkine Institute, Parel, Mumbai, India.

4) Chow, T. W., Ong, M. T. and Shaharum S. (2012) *WIF-1 might play additional role in carcinogenesis and cancer growth via EGFR signaling pathway*. 3rd INFORMM Postgraduate Colloqium 2012, 26-27 March, INFORMM USM.

KAJIAN PENGLIBATAN WIF-1 DALAM ORAL KARSINOGENESIS MELALUI LALUAN ISYARAT EGFR DAN WNT

ABSTRAK

Faktor perencat WNT 1 (WIF-1) adalah perencat fisiologi dalam laluan isyarat WNT melalui domain WIF. Fungsi domain serupa EGF dalam WIF-1 masih perlu dikaji. Kajian terbaru menunjukkan peningkatan ekspresi WIF-1 dalam sel kanser yang progresif, satu fenomena yang bercanggah dengan mekanisme isyarat sel dalam perkembangan sel kanser. Kajian ini bertujuan untuk menjelaskan potensi pengikatan WIF-1 dengan EGFR, yang memulakan laluan isyarat EGFR kaskade dan/atau melalui cakap silang antara EGFR dan laluan isyarat hiliran WNT, yang akhirnya menyebabkan pembentukan kanser. Kajian dimulakan dengan analisis dok protein-protein antara WIF-1 dan molekul EGFR menggunakan pelayan talian CAPRI-tersenarai, iaitu ZDOCK, Gramm-X, HEX dan PatchDock. Pengimunomendakan bersama dan pemendapan Western telah direka untuk mengkaji pengikatan EGFR / WIF-1 dan penghasilan pengaktifan potensi bagi laluan isyarat EGFR dan WNT akibat pengikatan tersebut. Sel mutan yang stabil telah ditubuhkan untuk mengkaji cakap silang antara laluan isyarat WNT dengan EGFR. Ekspresi gen hiliran telah dilakukan menggunakan PCR masa nyata. Keputusan eksperimen mencadangkan bahawa WIF-1 boleh memainkan peranan tambahan dalam laluan isyarat EGFR melalui domain EGF-like. Pengikatan *in siliko* bagi WIF-1 kepada EGFR adalah berdaya sesuai (-616,40 kcal / mol), berbanding dengan pengikatan EGF/EGFR (-627,18 kcal / mol). Dalam pengimunomendakan bersama, WIF-1 telah terikat kepada, dan seterusnya pengimunomendakan bersama dengan, EGFR. Faktor utama dalam

hiliran bagi laluan isyarat EGFR didapati diaktifkan berikut pengikatan WIF-1 kepada EGFR. Selain itu, keputusan yang diperolehi menunjukkan pengaktifan penting bagi hiliran, tetapi bukan huluan, laluan isyarat WNT kaskade melalui cakap silang dari pengaktifan laluan isyarat EGFR oleh WIF-1. Pendek kata, data kami jelas menunjukkan bahawa WIF-1 mungkin memainkan peranan yang berbeza dalam pembentukan kanser dengan berinteraksi dengan EGFR, selain daripada bertindak sebagai ahli antagonis bagi WNT. Oleh itu, kajian semula adalah untuk mengkaji kesesuaian WIF-1 sebagai agen anti-kanser.

STUDY ON THE INVOLVEMENT OF WIF-1 IN ORAL CARCINOGENESIS VIA EGFR AND WNT SIGNALING PATHWAYS

ABSTRACT

WNT inhibitory factor 1 (WIF-1) is a physiological inhibitor in WNT signaling pathway via its WIF domain. The function of EGF-like domain in WIF-1 remains unclear. Recent studies had indicated an increase in WIF-1 expression in progressive cancer cells, a phenomenon contradicting to the known cell signaling mechanisms in cancer cell progression. The present study aimed at elucidating the potential binding of WIF-1 to EGFR, leading to initiation of EGFR downstream signaling cascades and/or via the crosstalk between EGFR and downstream of WNT signaling pathways, which ultimately resulting in cancer formation. Initial protein-protein docking between WIF-1 and EGFR molecules was performed using CAPRI-listed online servers, namely ZDOCK, GRAMM-X, HEX and PatchDock. Co-immunoprecipitation and Western blot were designed to study the binding of EGFR/WIF-1 and the potential activation of EGFR and WNT downstream signaling pathways resulting upon the binding. Stable mutant cell lines were established to study the crosstalk between WNT and EGFR signaling pathways. Downstream gene expression initiated by the crosstalk was investigated using quantitative real-time PCR. The experimental results suggested that WIF-1 could play additional role in EGFR signaling pathway via its EGF-like domain. *In silico* binding of WIF-1 to EGFR was energetically favorable (-616.40 kcal/mol), as compared to EGF/EGFR binding (-627.18 kcal/mol). In co-immunoprecipitation, WIF-1 was bound to, and hence was co-immunoprecipitated with, EGFR. Downstream key factors in EGFR

signaling pathway were found to be activated following the binding of WIF-1 to EGFR. Moreover, the results obtained showed a prominent activation of downstream, but not of upstream, cascades of WNT signaling pathway via crosstalk from WIF-1 activated EGFR signaling pathway. In short, the data obtained have clearly shown that WIF-1 might play a different role in cancer formation by interacting with EGFR, rather than acting as an antagonist of WNT members. It is therefore crucial to review the appropriateness of WIF-1 to be used as an anti-cancer agent in the field.

CHAPTER 1: INTRODUCTION

Cancer is one of the most common causes of morbidity and mortality today, with more than 14 million new cases and more than 8.2 million deaths each year globally (Stewart and Wild, 2014). In head and neck cancers, over 90% are oral squamous cell carcinomas (OSCC). OSCC is one of the top ten cancers worldwide, with broad differences in geographic distribution. It affects at least 0.3 million people annually worldwide. This disease claims the lives of almost 70,000 people each year or about eight persons every hour (Stewart and Wild, 2014). In South-East Asia especially in developing country like India, the challenges of OSCC burden may increase over 75% of cancer deaths in 2020, as compared to 2000 (Mishra and Meherotra, 2014). The major risk factors that contribute to OSCC development are tobacco use and alcohol consumption (ACS, 2014). Synergistic effects of smoking and alcohol may increase the risk for OSCC development by 30-fold (ACS, 2014). Betel quid chewing is a lifestyle behavior in as high as 40% of adult population (Petti et al., 2013).

OSCC is normally preceded from oral precancerous lesion, particularly leukoplakia and erythroplakia. Oral leukoplakia is the most common type of oral precancerous lesions detected in the early stage of OSCC development. It is a white plaque in oral mucosa which is hardly characterized clinically or pathologically. The risk of malignancy transformation of patients with oral leukoplakia is 8 to 10 times higher than those who do not. Oral leukplakia may bypass dysplasia stage and directly enter into metaplasia stage, subsequently developed into invasive carcinoma (Wang et al., 2009). Other type of oral precancerous lesion is oral erythroplakia. It is a velvety red plaque that is rarely found in the oral mucosa. As compared to

leukoplakia, erythroplakia is 17 times more prominent in developing severe dysplasia in at least 85% of OSCC cases (Scully et al., 2008).

On a related note, OSCC surgery can be very disfiguring and thus psychologically traumatic in a society that places a high value on physical appearance. Treatment of this condition often results in severe loss of oral function, and chronic discomfort including difficulty in chewing, swallowing, and speaking. With the advancement in diagnosis and treatment, the average 5-year survival rate for OSCC is about 62% (ACS, 2014). Early detection of OSCC increases the chance that a person lives 5 years after initial diagnosis; the 5-year survival rate is 81% for those diagnosed with early-stage OSCC but only 22% for persons diagnosed with advanced stage cancer. Only 35% of OSCC is detected at the earliest stage (CDC, 2000). However, the prognosis of OSCC is still unsatisfying, including delayed precancerous lesion detection, increased rate of relapse and lymph node metastases (Chang et al., 2007; Zhang et al., 2012). Hence, OSCC is a significant component of the global burden of cancer and better understanding in oral carcinogenesis is needed to improve the survival rate of patients.

The missing link between inflammation and oral carcinogenesis in molecular signaling pathway is not clear at present time. As early as 1863, Rudolph Virchow already proposed that cancers tended to occur at sites of chronic inflammation (Balkwill and Mantovani, 2001). Lately, tumor-promoting inflammation has been listed as one of the important hallmarks in carcinogenesis (Hanahan and Weinberg, 2011). This is because multiple hallmarks functions can be promoted by inflammatory-induced microenvironment. A general paradigm model for human carcinogenesis is often viewed as a multi-stage disease due to the accumulation of tumor suppressor genes and oncogenes in the cell (Luu et al. 2004). The activity of

key players involved in those cancer cell signaling pathways may serve as an alternative cancer treatment for current cancer therapy. Due to the early involvement in cell development, the interaction between epidermal growth factor receptor (EGFR) and wingless and integration site growth factor (WNT) signaling pathways are highly focused in clinical research (Musgrove, 2004; Tan et al., 2005; Shclange et al., 2007; Suzuki et al., 2007a; Taylor et al., 2007)

From the cell signaling point of view, those important factors i.e. cigarette smoking and alcohol will lead to EGFR overexpression in 90% of OSCC cases and further cascade of cell signaling may facilitate OSCC development. Given the potential of WNT inhibitory factor 1 (WIF-1) to be used as natural inhibitor of WNT signaling pathway to prevent carcinogenesis and/or the progression of cancer resulted from the activity of WNT signaling pathway, it is crucial to ensure that WIF-1 would not contribute to any unwanted negative effects in the potential cancer-inhibition function. Interestingly, WIF-1 is made up of WIF domain and 5 repeated EGF-like domains whereby EGF is one of the ligands that specifically bind to EGFR. Hence, there may be a possibility that WIF-1 might bind to the oral squamous cells at the upstream of cell signaling pathway, playing a similar role as EGF and at the same time, it may bind to the WNT molecule at the downstream of cell signaling pathway as its ordinary role. Such a combination may fool the oral cells in response to the cell signaling. Nevertheless, contradictions were found in many cases wherein the expression of WIF-1 was increased with cancer formation (Suzuki et al. 2007a; Suzuki et al. 2007b). This type of observations implies that WIF-1 might play a cancer-promoting role via other mechanism(s) in addition to its endogenous inhibitory property in WNT signaling pathway. Thus, experiment designed in this

project is to investigate the possible role for WIF-1 in oral carcinogenesis. The hypothesized mechanisms of WIF-1 in oral carcinogenesis are shown in Figure 1.1.

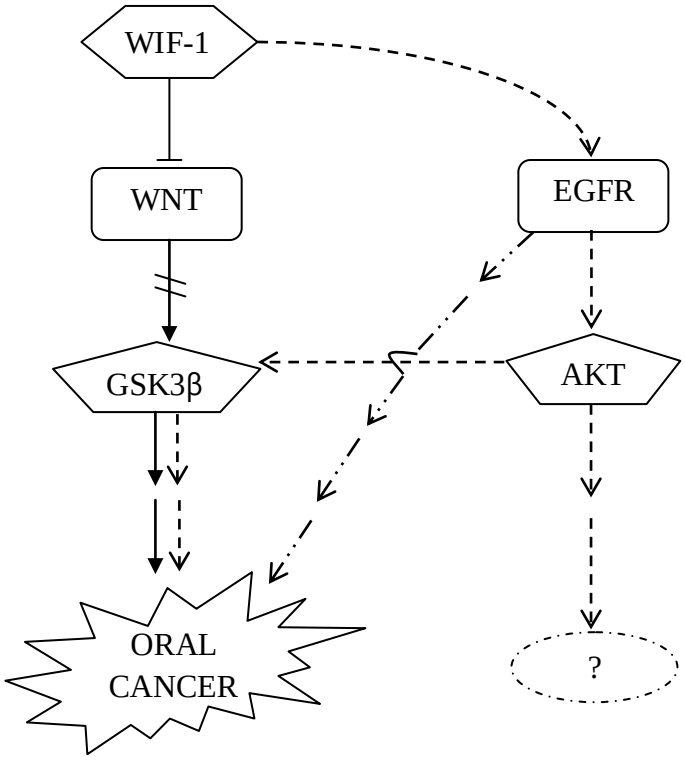

Figure 1.1: Possible role of WIF-1 in oral carcinogenesis. WIF-1 is a physiological inhibitor in WNT signaling pathway. With the presence of WIF-1, it will bind to WNT directly and prevent WNT-mediated oral carcinogenesis. However, if WIF-1 is interacting with EGFR, the downstream of EGFR signaling pathways (such as AKT) could be activated. Crosstalk between WNT and EGFR signaling pathways may be happened and result in oral carcinogenesis (Hu and Li, 2010; Bányai et al., 2012)

1.1 Objectives

1. To investigate the binding between WIF-1 and EGFR
2. To examine the potential cell signaling factors involved in oral carcinogenesis at gene expression level and at protein level.
3. To study the crosstalk between WNT and EGFR signaling pathways in oral carcinogenesis
4. To examine the downstream mechanism(s) involved in these signaling pathways to assess the newly proposed model implicated in oral carcinogenesis

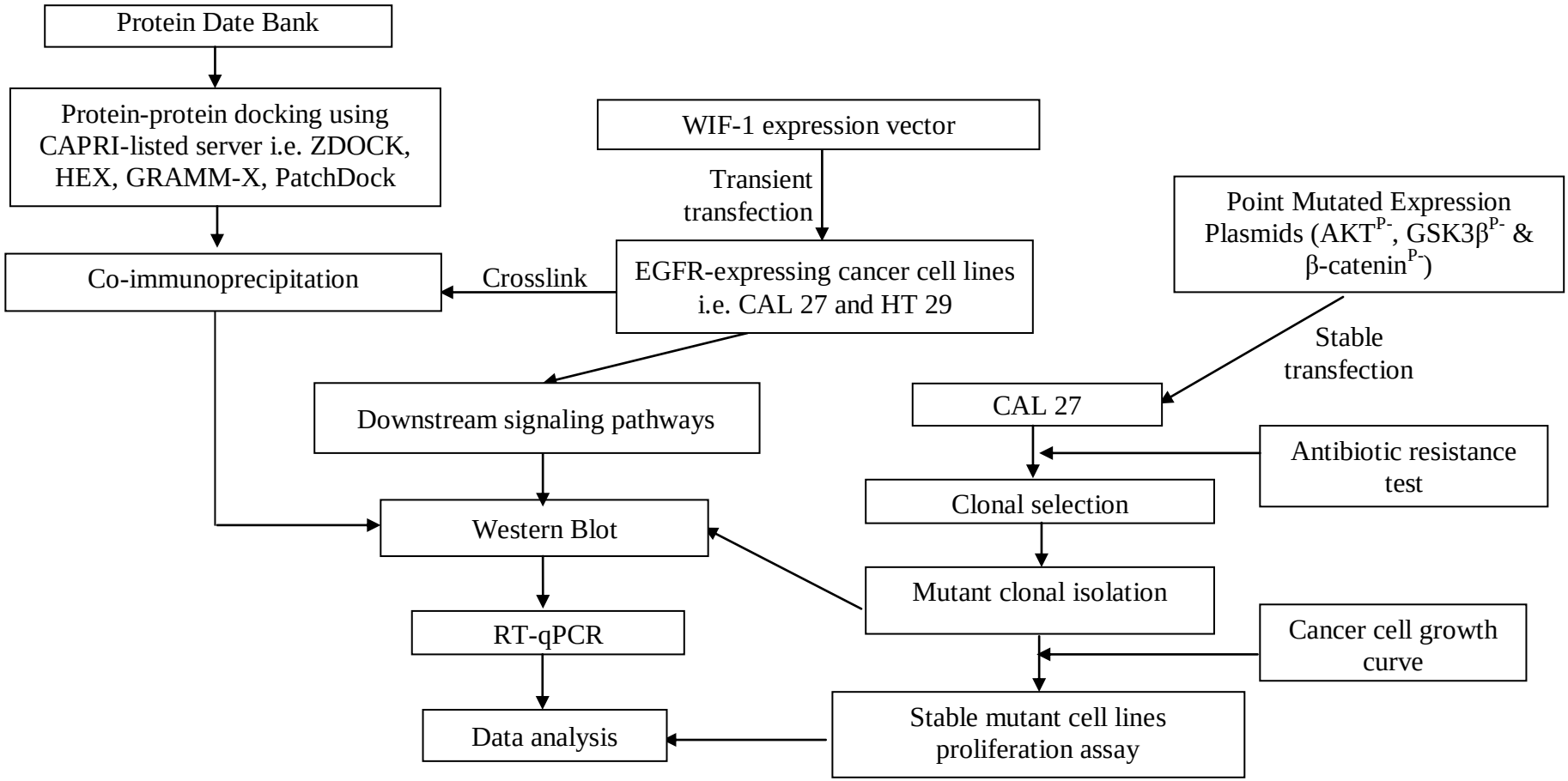

Figure 1.2: Flow chart of Research. For further explanation, please refers to Section 3.1.

CHAPTER 2: LITERATURE REVIEWS

2.1 Oral cancer

2.1.1 Background

According to World Cancer Report 2014 (Stewart and Wild, 2014), the global burden of cancer in 2012 has rose an estimated 14 million new cases per year and this figure is expected to increase to 22 million annually in next 20 years. Globally, cancer has accounted for at least 8.2 million deaths in 2012. In head and neck cancers, over 90% are squamous cell carcinomas (SCC) which are commonly found in the oral cavity. Oral squamous cell carcinomas (OSCC) consistently rank as one of the top ten cancers worldwide, with broad differences in geographic distribution.

In United States, oral cancer affects approximately 42000 people with an estimated 8390 death cases in 2014 (ACS, 2014). The rates of incidence are more than twice as high in male as in female. In Europe, OSCC accounted for 61416 new cases in 2012 with a mortality rate of 1.3% (Stewart and Wild, 2014). The incidence rate of males is double the rate of that in female. However, OSCC cases are much more serious in Asia region, with 0.1 million new cases and more than 50% mortality rate, especially in male (Stewart and Wild, 2014). Almost one-third of global cases and half of worldwide deaths from OSCC occur in this region. In Malaysia, OSCC is ranked at the fourteenth most common cancer, accounting for 2.1% of incidence rate for both genders and leads to 1.2% of mortality rate in total cancer cases (Stewart and Wild, 2014). The 5-year prevalence for adult population to develop OSCC in Malaysia is about 2.2%. In males, it is the tenth most common cancer, while it is thirteenth in females.

2.1.2 Inducing factors in oral carcinogenesis

Oral squamous cell carcinoma (OSCC) is a multifactorial development, influenced by genetic abnormalities and lifestyle behaviours. As reviewed by Stefano Petti (2009) and Jukka H. Meurman (2010), oral cancers are associated with cigarette smoking, alcohol consumption, betel quid chewing, poor oral hygiene and infection. However, the detailed mechanisms involved are yet to be explored.

2.1.2.1 Cigarette smoking

Tobacco usage in various forms has known to be a major risk factor for oral carcinogenesis (Figure 2.1). The International Agency for Research on Cancer (IARC) (2004) suggested that there is more than 60 carcinogens in each puff of cigaratte smoke. Though the direct mechanism of smoking to oral cancer is still unclear, it was believed that polycyclic aromatic hydrocarbons (PAHs) and N-nitrosamines may induce DNA damage, promote gene methylation and mutation, and cause chromosomal aberrations in target organs (Toh et al., 2010). Cigarette smoke also contains free radicals in either tar phase or gas phase which is capable of promoting oxidative damage (Pasupathi et al., 2009). However, the role of oxidative damage in causing oral cancers remains to be explored.

According to the surgeon report in U.S. Department of Health and Human Services (USDHHS) (2010), most genotoxic agents in cigarette smoke are metabolically activated by cytochrome P450 enzyme and then covalently bind to DNA to produce DNA adducts. The persistent DNA adducts can cause miscoding during DNA replication and lead to mutation in critical genes such as *KRAS* oncogenes and *TP53* tumor-suppressor genes. Some of the genotoxic agents may react with DNA directly to form DNA adducts without the process of metabolic

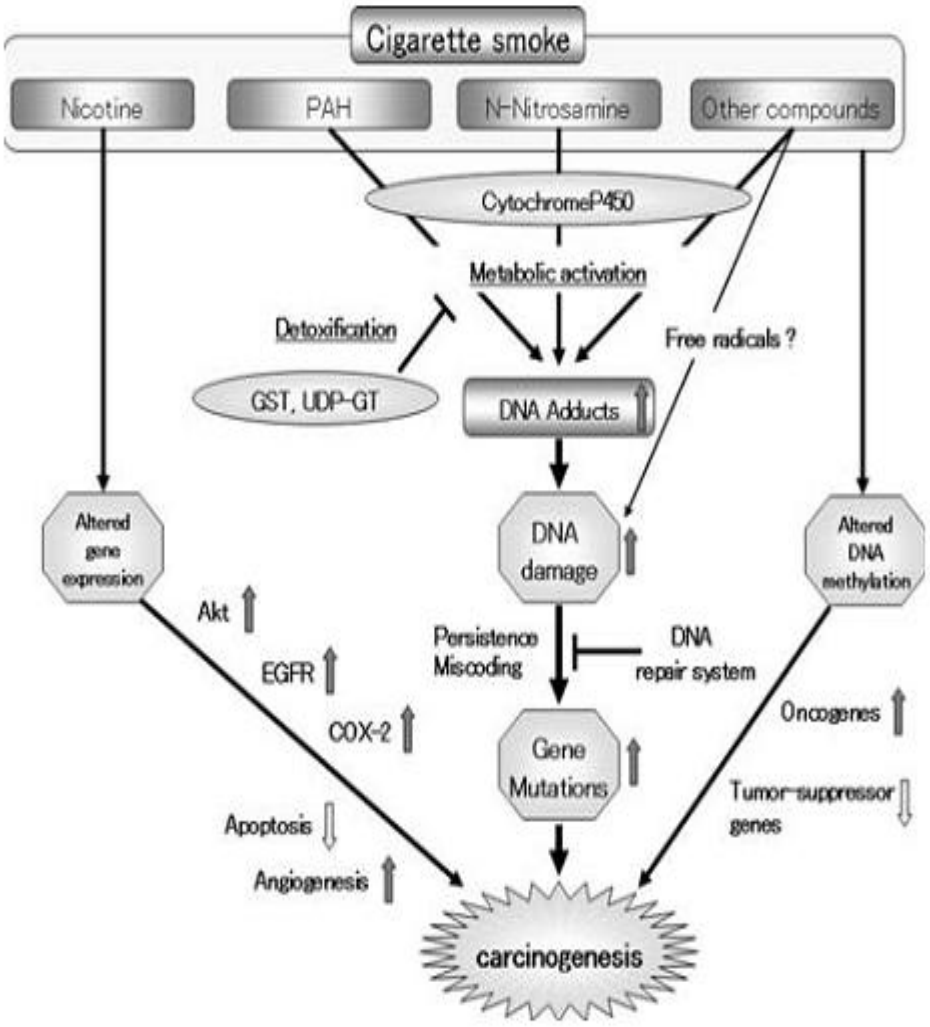

Figure 2.1: Schematic presentation of compounds in cigarette smoke and their roles in carcinogenesis. Cigarette smoke contains more than 60 carcinogens. Polycyclic aromatic hydrocarbons (PAH) and N-nitrosamine are metabolically activated by cytochrome P450 enzyme and form DNA adduct which later induce DNA damage, gene mutations and finally leads to carcinogenesis. The balance between the metabolic activation of carcinogens and detoxification were catalysed by glutathione-S-transferases (GST), uridine-5'-diphosphate-glucuronosyltransferases (UDP-GT) and etc. Nicotine may up-regulating the gene expression level of AKT, epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) and promotes angiogenesis which then lead to carcinogenesis. Free radicals in the cigarette smole may induce DNA damage while other genotoxic compounds promote DNA methylation of oncogenes and increase the expression of tumor-suppressor genes for carcinogenesis (Figure from Toh et al., 2010)

activation. The formation of DNA adduct is normally at the adenine or guanine sites and lead to the initiation of carcinogenesis (Chen et al., 2011). The balance between the metabolic activation of carcinogens and detoxification were catalyzed by numerous enzymes, including glutathione-S-transferases (GSTs), uridine-5'-diphosphate-glucuronosyltransferases (UDP-GTs), epoxide hydrolases, and sulfatases varies among individuals and is likely to affect cancer susceptibility (Stadler et al., 2008). Moreover, nicotine may be involved in the initiation and invasion of tumors via AKT pathway (Xu et al., 2007). Nicotine mimicking the mode of action of cellular growth factors and modulates their biological mechanism through the activation of nicotinic acetylcholine receptors, β-adrenoceptors or epidermal growth factor receptor (EGFR) (Chen et al., 2011). However, the roles of these receptors are cell-type dependent.

2.1.2.2 Alcohol consumption

Pure ethanol is not a direct carcinogen and can be metabolized by alcohol dehydrogenase (ADH) in the liver to form acetaldehyde (Figure 2.2). Acetaldehyde is carcinogenic and able to induce gene mutations through the formation of DNA adducts (Morse et al., 2007; Toh et al., 2010). Such actions may interfere with DNA synthesis and repair. However, the amount of acetaldehyde present in various tissues may also depend on the polymorphism of alcohol-metabolizing enzymes, i.e. ADH and aldehyde dehydrogenase (ALDH). For example, epidemiological studies demonstrated that individuals with heterozygous inactive *ALDH2*2* genotype are still able to oxidize acetaldehyde to acetate with a markedly reduced activity. Following alcohol consumption, these individuals may generate 3-fold higher concentrations of acetaldehyde in serum and saliva as compared to individuals with normal homozygosity of *ALDH2*1* genetic background (Seitz and Stickel, 2007; Yokoyama et al., 2007). Ethanol can be oxidized to acetaldehyde by normal oral microflora. However, there is limited further metabolism of acetaldehyde to acetate by oral bacteria, hence the salivary acetaldehyde concentration can be accumulated up to 10- to 100-fold higher than that in the blood (Homann et al., 2000).

In addition to that, ethanol can also be oxidized by cytochrome P450 2E1 (CYP2E1)-dependent microsomal monooxygenase system. Ethanol-mediated reactive oxygen species (ROS) are generated during alcohol metabolism by CYP2E1 (Seitz and Becker, 2007). Chronic ethanol consumption in animals and humans lead to marked induction of hepatic CYP2E1 enzyme by 10- to 20-fold as compared to non-chronic ethanol consumers. Those ethanol-mediated ROS produced by CYP2E1 may result in oxidative injury, inflammation, and lipid peroxidation (Seitz and Stickel, 2007). Therefore, the oxidative stress caused by ROS is suggested as a

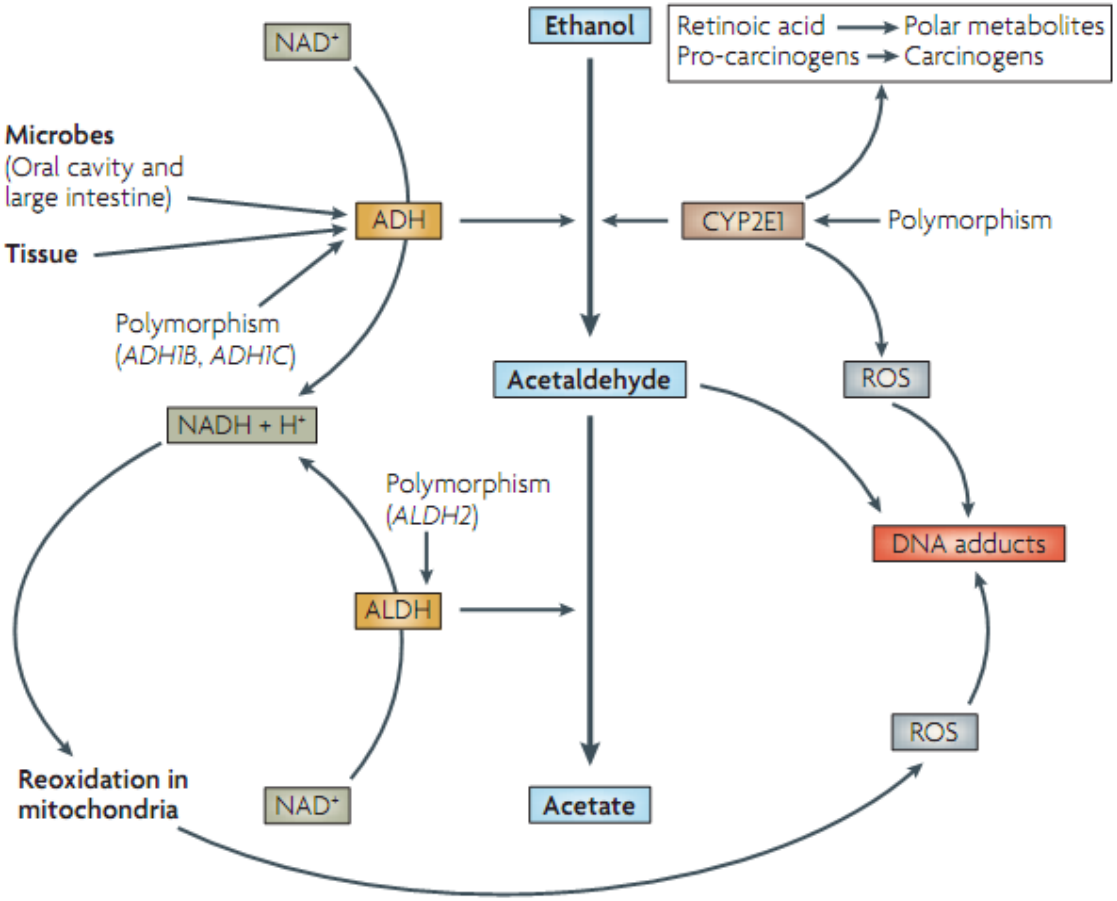

Figure 2.2: Schematic presentation of ethanol metabolism and its role in carcinogenesis. Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1), which is then further oxidized to acetate by acetaldehyde dehydrogenase (ALDH). ADH-mediated ethanol metabolism results in reduced nicotinamide adenine dinucleotide (NADH) and acetaldehyde, whereas ethanol oxidation by CYP2E1 leads to the production of acetaldehyde and reactive oxygen species (ROS). The amount of acetaldehyde present may also depends on the polymorphisms of alcohol-metabolizing enzyme, i.e. CYP2E1, ALDH and alcohol dehydrogenase (ADH). Increased CYP2E1 activity not only leads to increased generation of ROS, but also to an increased activation of various environmental pro-carcinogens present in tobacco smoke and certain diets. CYP2E1 also decreases tissue levels of retinol and retinoic acid, which have important functions in the regulation of cell growth and transdifferentiation. NADH is reoxidized to NAD^+ in the mitochondria, which may further increase the generation of ROS. The increased levels of acetaldehyde and ROS may lead to DNA adduct formation (Figure from Seitz and Stickel, 2007)

critical pathophysiological mechanism in various human diseases, including cancer. Interestingly, some literature reviews suggested an association between alcohol-containing mouthwashes and oral cancer development (McCullough and Farah, 2008; Vecchia, 2009). However, the epidemiological findings are not consistent across various studies.

2.1.2.3 Synergistic effects between cigarette smoking and alcohol consumption

Cigarette smoking and alcohol consumption is known to be associated with oral carcinogenesis either independently or synergistically in a dose-dependent fashion (Morse et al., 2007; Ganci et al., 2012). Simultaneous exposure to smoking and drinking may increase oral cancer risk by 2- to 4-fold, as compared to independent exposure to either of the risk factors (Castellsagué et al., 2004). Furthermore, alcohol may act as a natural solvent for tobacco carcinogens, enhancing and prolonging the mucosal exposure of tobacco carcinogens to target tissues (Mehrotra and Yadav, 2006; Ganci et al., 2012). In addition, cigarette smoking is found to double up the salivary acetaldehyde level by altering the oral microflora and thereby increase the bacterial ADH activity (Salaspuro and Salaspuro, 2004; Seitz and Stickel, 2007). Smoking may rapidly shift the oral microflora from Gram-negative to Gram-positive bacteria as Gram-positive bacteria able to produce high amounts of acetaldehyde as compared to Gram-negative bacteria (Salaspuro, 2003).

2.1.2.4 Betel quid and areca nut extract

Betel quid chewing is a common traditional or social practice that link to oral leukoplakia and oral submucous fibrosis in a dose-dependent manner (Sharan et al., 2012). Areca nut, also known as betel nut, can be consumed alone or combined with others additives and wrapped in a betel leaf as betel quid. The alkaloids extracted from betel quid can give stimulant sensation to betel quid chewers and increase their work capacity with higher alertness (Petti, 2009). The components of betel quid may vary among countries, regions, communities and individuals. In India, heavy betel quid chewer will add tobacco as an additive but such preparation is not being practiced in Papua New Guinea and China (Sharan et al., 2012). With the addition of tobacco in betel quid, the risk of oral precancerous development will be increased by 3-fold (Stadler et al., 2008; Petti et al., 2013).

During the chewing process of betel quid, ROS may be produced in the oral cavity due to the auto-oxidation of areca nut polyphenols under the alkaline condition enhanced by slaked lime (Adhikari and De, 2013). Such ROS formation causes detrimental effects to oral mucosa by inducing mutation and genotoxicity in relation to tumor initiation process. Besides that, ROS can also change the structure of oral mucosal to be more susceptible to the penetration of other betel quid components and environmental toxicants (Mehrotra and Yadav, 2006). Moreover, the endogenous nitrosation of areca nut may potentially generate carcinogenic nitrosamines (Petti, 2009). Areca nut-specific nitrosoamines may interact with DNA, proteins or other targets to form adducts and exerting its carcinogenic activities (Adhikari and De, 2013). In short, areca nut and/or betel quid mastication is significantly associated with oral cancer formation.

2.1.2.5 Infectious agents – bacterial, yeast and viral infection

Oral cavity has constantly exposed to a huge diversity of microorganisms. It is estimated that about 15 to 20% of oral cancer with not known risk factors are related to microbial infection and inflammation (Mager, 2006; Chocolatewala et al., 2010; Meurman, 2010). A shift of the microbial patterns in the oral cavity may be due to smoking, alcohol consumption, betel quid chewing and poor oral hygiene (Hooper et al., 2009). Interestingly, many microbial species found to be related to cancer development are highly site-specific but their roles in cancer formation are still unclear (Hooper et al., 2009). Proposed mechanism for microbial infection to initiate cancer formation can be either through chronic inflammation and disruption of eukaryotic cell cycle and signaling pathways, or by metabolism of carcinogenic products such as acetaldehyde (Chocolatewala et al., 2010). Hence, the possible relationship between microorganisms and carcinogenesis becomes an interesting matter to be concerned.

2.1.2.5.1 Bacterial infection

Alteration of the oral microbiota in oral cancer is an essential biomarker to identify specific microorganisms that prevail in tumor specimen. Interestingly, a diversity of bacteria species was isolated from the microflora population of tumourous site and control sites (Meurman, 2010). Most of the isolated bacteria are saccharolytic and acid tolerant. This characteristic enables them to survive in the hypoxic and acidic solid tumor environment. Significant salivary analysis also noted that some bacteria species are specifically detected in the saliva of oral cancer patients, but may not be detected in the tumor site (Chocolatewala et al., 2010). Bacterial agents may induce carcinogenesis through chronic infection or toxin production that interferes with

cellular signaling mechanisms. They may also act as tumor promoter, promote cellular proliferation, inhibit apoptosis and help in immune evasion (Mager, 2006). Moreover, the production of salivary acetaldehyde from alcohol metabolism by oral bacteria also explained another additional mechanism in the bacteria–oral carcinogenesis interaction (Meurman and Uittamo, 2008). In short, the carcinogenic mechanism by bacterial infection is not clear, but it was believed that the detected oral bacteria is site-specific and may play a different role in oral carcinogenic process.

2.1.2.5.2 Fungal infection

Candida albican is one of normal flora in oral cavity. Overgrowth of it is associated with poor oral hygiene and periodontitis. Chronic hyperplastic candidosis is a rare oral fungal infection (Chocolatewala et al., 2010). The invasion of candidal hyphae into oral epithelium may lead to oral leukoplakia and dysplastic changes. Candidal leukoplakias appear to be a more aggressive type in oral carcinoma transformation as compared to other types (Hooper et al., 2009). About 9 to 40% of the candidal leukoplakia cases are estimated to develop into oral carcinoma (Meurman, 2010). Animal studies have confirmed that long-term candidal infection may result in malignant transformation in oral mucosa (Hooper et al., 2009). *Candida* may induce malignant transformation by generating nitrosamine compounds as proto-oncogene activators (Hooper et al., 2009) or synergistically associated with other risk factors such as tobacco smoking and alcohol consumption (Meurman, 2010). However, there are no studies demonstrate that a control of *Candida* would reduce the incidence of oral cancer.

2.1.2.5.3 Viral infection

Without the correlation with smoking and alcohol consumption, human papillomavirus (especially HPV-16 and 18) infection alone has contributing to about 70% of oral and oropharyngeal carcinomas (Ganci et al., 2012). However, HPV infection may show a latency period in 12% of subjects with clinically healthy oral mucosa (Meurman, 2010). The repeated viral exposure may be due to certain sexual practices. The expression of viral proteins disrupted the cell cycle regulation, promote cell growth and increase the frequency of gene mutation and chromosomal instability (Chung and Gillison, 2009). In addition, herpes virus (HSV) (Shillitoe, 2009) and human immunodeficiency virus (HIV) (Epstein et al., 2005) are also reported to correlate with oral carcinogenesis with the presence of common risk factors such as smoking and alcohol consumption. Therefore, more data is needed for further conclusion.

2.1.3 Role of inflammation in oral cancer

The link between inflammation and oral carcinogenesis in molecular signaling pathway is still not clear at present time. As early as 1863, Rudolf Virchow had proposed the role of chronic inflammation in carcinogenesis (Balkwill & Mantovani, 2001). One way to describe the process linking inflammation to cancer is through the illustration of essential "hallmarks" in cancer formation (Hanahan and Weinberg, 2011). Cancer development requires the acquisition of ten fundamental properties: self-sufficient proliferation, insensitivity to anti-proliferative signals, escape from immune destruction, unlimited replicative potential, tissue invasion and metastasis, the maintenance of vascularization, genome instability and mutation, evasion of apoptosis, deregulation of cellular energetic and most importantly, tumor-promoting

inflammation. Inflammation can foster multiple hallmark functions by providing several bioactive molecules to the tumor microenvironment (Hanahan and Weinberg, 2011).

Acute inflammation may help in cancer regression but chronic inflammation may promote cancer progression (Philip et al., 2004). In oral carcinogenesis, epithelial inflammation is also a factor to initiate cancer formation (Hooper et al., 2009). The use of tobacco, alcohol and betel quid induce the formation of DNA adduct and free radicals which may later generate an inflammatory microenvironment for genetic alteration and cancer promotion. Besides that, chronic inflammation result from chronic microbial infection may also predispose to cancer formation (Lu et al., 2006). Inflammation-induced reactive oxygen and nitrogen species may directly or indirectly contribute to malignant cell transformation by induction of genomic stability and alterations of cellular epigenetic events (Kundu and Surh, 2008).

Cells have intrinsic mechanisms by which to prevent unregulated proliferation or the accumulation of DNA mutations. These include tumor suppressor pathways that mediate DNA repair, cell cycle arrest, apoptosis and senescence. In the effects of DNA damage or oncogenic activation, cells will either repair their DNA to prevent mutations or initiated cells will undergo cell death. If massive cell death occurred in infection or non-infectious tissue injury, loss of cell population will be replaced by the proliferation of neighbouring cells, or undifferentiated precursor cells. Many inflammatory pathways function to mediate these tissue repair mechanisms (Chen et al., 2003; Wang et al., 2005). In an extension of its physiologic role in mediating tissue repair or as a strategy in host defense to infection, the inflammatory

response may play a role in providing survival and proliferative signals to initiated cells, thereby leading to tumor promotion.

Direct evidence for a link between carcinogenesis and either host defense and tissue repair due to inflammation has yet to be developed as cells are organic microsystems with functional compartments interconnected by multiple signal pathways (Wolf, 2007). A distinct network of intracellular signaling molecules involving protein kinases, membrane receptors and transcription factors are interacting together to facilitate carcinogenesis (Kundu and Surh, 2008). To address the details of transition from inflammation to cancers and the further development of inflammation-associated cancers, it is necessary to investigate specific roles of key regulatory molecules in the oral cancer signaling pathways involved. A summary of inflammatory mechanisms that lead to cancer development is shown in Figure 2.3.

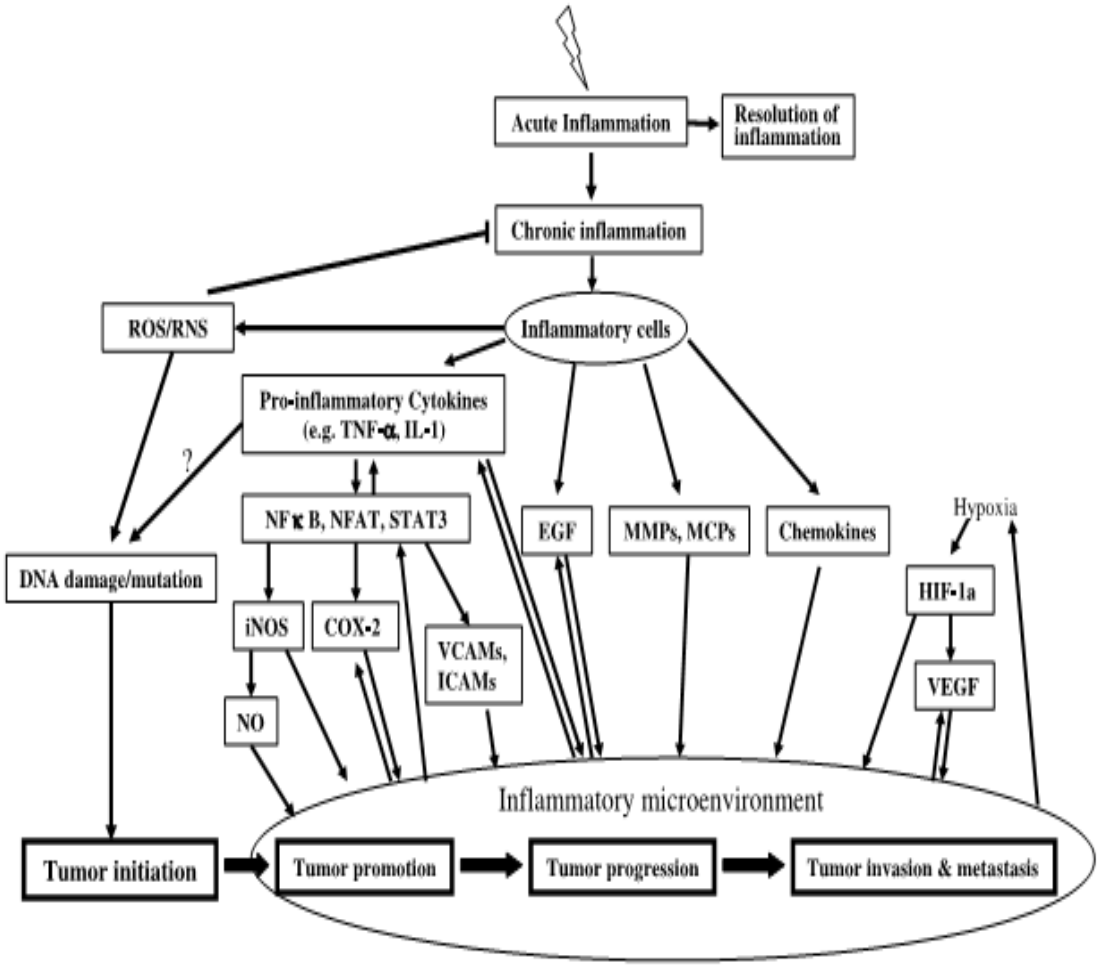

Figure 2.3: Summary of mechanisms for the involvement of inflammation in cancer development. Acute inflammation may undergo resolution of inflammation or as a factor to initiate cancer formation. Inflammatory-induced free radicals i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS) may directly or indirectly induce DNA damage or mutation, and contribute to malignant transformation. The inflammatory cells may mediate tissue repair mechanism due to the chronic inflammatory response, for cell survival and cell proliferation. The overexpression of pro-inflammatory cytokines, chemokines and etc may lead to tumor promotion. Hypoxia-induced mechanism will also take part in this inflammatory microenvironment which then leads to carcinogenesis. Tumor promotion indicates the process during which initiated cells develop into benign lesions. Tumor progression defines the process during which benign tumors progress to malignant carcinomas (Figure from Lu et al., 2006)

2.1.4 Signaling pathways involved in oral cancer

2.1.4.1 EGFR signaling pathway

Epidermal growth factor receptor (EGFR) protein was reported to be overly expressed in almost 90% of head and neck squamous cell carcinoma (HNSCC), especially in oral squamous cell carcinoma (OSCC) with poor association of gene amplification status (Bernardes et al., 2013; Maiti et al., 2013). Elevated EGFR expression happened throughout the premalignant stages of carcinogenesis, ranging from dysplastic lesions to OSCC (Mahendra et al., 2014). Besides that, overexpression of EGFR is always associated with cancer proliferation and metastasis, which may later contribute to poor prognosis and reduced survival rate (Kalyankrishna and Grandis, 2006; Ribeiro et al., 2014). Licitra et al. (2011) also reported that post-curative surgical removal of tumor cells in HNSCC patients shows an overexpression of EGFR in the normal, metaplastic and dysplastic epithelium, surrounding the resected tumor region. Such aberrant expression of EGFR may potentially trigger cancer recurrence locoregionally. Activated EGFR may promote several EGFR-related pathways such as mitogen-activated protein kinase (MAPK) for cell proliferation; AKT for cell survival and anti-apoptosis; signal transducers and activators of transcription (STAT) for gene transcriptional activity (Licitra et al., 2011). Overview of EGFR and its-related pathways will be further discussed in Section 2.2. EGFR-targeting therapy especially small molecule tyrosine kniase-specific inhibitors and monoclonal antibodies showed good anti-cancer effects in preclinical HNSCC models when administered alone or in conjugation with chemotherapy and/or radiotherapy (Choi and Meyer, 2008; Harari et al., 2009). Therefore, EGFR and its-related downstream signaling pathways are aimed to be the

hallmark for oral cancer and potential biomarkers or theraupetic candidates for oral cancer prognostic and/or survival indicator (Licitra et al., 2011).

2.1.4.2 WNT signaling pathway

Aberrant activation in WNT/β-catenin signaling pathway promotes abnormal cell proliferation, survival, and consequently results in epithelial-to-mesenchymal transition (EMT) and the development of epithelial cancers including colon, skin, liver, and ovarian cancers (Morin and Weeraratna 2003; Moon et al. 2004; Nelson and Nusse 2004; Taketo 2004; Huber et al. 2005; Reya and Clevers 2005; Clevers 2006; Blanpain et al. 2007; Polakis 2007). Additional mechanisms by which defects in the regulation of WNT signaling contribute to tumor progression probably remain undiscovered. The manifestation of cancer by aberrant WNT signaling most likely results from inappropriate gene activation mediated by stabilized β-catenin or epigenetic inactivation of WNT inhibitors. Uraguchi et al. (2004) demonstrated a panel of WNT genes expressed in cell type-specific OSCC cell lines and their immunohistochemistry results in oral carcinoma tissues showed an association between WNT3 expression and nuclear localization of β-catenin in invasive OSCC. Several target genes of β-catenin signaling have now been identified and some of their functions are consistent with the control of tumor cell growth, differentiation and survival (Major et al., 2007). No mutated β-catenin was reported in OSCC (Lo Muzio et al., 2005). Surprisingly, Prgomet et al. (2014) showed that elevated WNT5A expression in OSCC cell lines may also promote cancer cell invasion and migration via noncanonical WNT signaling pathway. Epigenetic alteration i.e. DNA methylation of WNT inhibitors contribute to early and late phases of OSCC initiation and progression (reviewed by Mascolo et al., 2012). Thus, activation of the WNT

signaling pathway can have a significant impact on tumor progression. Overview of WNT signaling pathways will be further discussed in Section 2.3. However, WNT mechanisms in oral carcinomas are still largely unexplored (Papagerakis and Pannone, 2012).

2.1.4.3 NF-κB signaling pathway

Nuclear factor-κB (NF-κB) is a dimeric nuclear transcription factor that plays a major role in immune response, cell adhesion, cell proliferation and differentiation, angiogenesis and apoptosis (Sun and Zhang, 2007). It is also a central dogma that responded to all the inflammatory stresses (Sawhney et al., 2007). Dysregulation of NF-κB activity is commonly related to inflammatory disease, autoimmune and metabolic disorders and also cancer development (Oeckinghaus et al., 2011). In cell, NF-κB is retained in the cytoplasm by its inhibitory protein IκB, in the inactivate form of NF-κB -IκB complex. In response to extracellular stimuli, IκB is subjected to phosphorylation, ubiquitination, and subsequently degraded by the proteasomal mechanism via a canonical IκB kinase (IKK) complex-dependent pathway or a noncanonical NF-κB -inducing kinase pathway. The degradation of IκB results in NF-κB activation. NF-κB then translocates to the nucleus to regulate target gene expression that involved in cell fate (Lu et al., 2006; Yan et al., 2010).

Aberrant function of NF-κB is always observed in the inflammatory-associated cancer as it can be activated by different stimuli such as inflammatory signals, hypoxia mediater, oncogenic proteins etc (Yan et al., 2010). Its activation in chronic inflammation can induce the expression of proinflammatory cytokines including tumor necrosis factor (TNF) α, interleukin (IL)-6 and -8, adhesion molecules, matrix metalloproteinases (MMP) -2 and -9, cyclooxygenase-2 (COX-2),

and inducible nitric oxide synthase (iNOS) (Lu et al., 2006; Sawhney et al., 2007; Ikebe et al., 2012). In OSCC, NF-κB was found to be highly expressed in betel quid-associated oral submucous fibrosis (Tilakaratne et al., 2006) and radiation-induced oral mucositis (Ikebe et al., 2012). The activation of NF-κB is triggered by DNA damage and ROS formation in the oral cavity. Besides that, *in vivo* study of OSCC patient's specimen concluded that NF-κB contributes to tumor invasion and OSCC hematologic and lymphatic metastases (Yan et al., 2010). Moreover, NF-κB is closely coordinated with other signaling pathways such as EGFR and WNT. Overexpression of EGFR in OSCC may upregulate its downstream signaling pathways. AKT can potentially upregulate NF-κB transactivation directly through phosphorylation of p65 domain (Magné et al., 2006), or indirectly by controlling IκB kinase (IKK) activity (Oeckinghaus et al., 2011). Aberrant function of NF-κB can also transactivate STAT3 by the release of IL-6 in the autocrine/paracrine mechanism (Squarize et al., 2006). IKKs can also transactivate MAPK in the NF-κB-independent pathway. Furthermore, IKKs can also stabilize β-catenin by competing the distinct phosphorylation sites targeted by glycogen synthase kinase 3β (GSK3β), and induce cyclin D1 expression (Oeckinghaus et al., 2011). Therefore, it is believed that NF-κB plays an important role in linking up inflammation and cancer together.

2.1.4.4 HIF-1α signaling pathway

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that is critically involved in oxygen homeostasis (Yan et al., 2014). It is made up of α and β subunits, whereby HIF-1α is the key mediator in oxygen regulation which contribute to HIF-1 activity (Li et al., 2012). In normoxia condition, HIF-1α is subject to oxygen-dependent hydroxylation and undergoes proteolytic degradation (Rodolico et