

***IN VITRO AND IN VIVO LIPOSOMES
FORMULATION STUDIES OF ORTHOSIPHON
STAMINEUS EXTRACT FOR LUNG CANCER***

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by

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This thesis is dedicated to ...
My beloved mother and my late father,
My beloved wife,
My brothers and sisters

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LIST OF ABBREVIATIONS

A	Absorbance
A549	Human lung adenocarcinoma cell line
AIF	Apoptotic inducing factor
ALT	Alanine aminotransferase
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ARNT	Aryl hydrocarbon nuclear translocator
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the curve
Bcl	B-cell lymphoma protein
CAM	Chorioallantoic membrane
Caspase	Cysteine aspartic acid-specific protease
CCD-18Co	Human normal colonic fibroblast
CCEE	Counter current exchange extraction
CgA	Chromogranin A
cIAP-2	Cellular inhibitor of apoptosis protein 2
C_{\max}	Maximum observed concentration
λ_{\max}	Abundance absorbance
ΔH	Enthalpy
c-myc	Myelocytomatosis oncogene cellular homolog
CNE	Human nasopharyngeal carcinoma cells
CO ₂	Carbon dioxide

CTGF	Connective tissue growth factor
2D	Two-dimensional
3D	Three-dimensional
DAD	Diodearray UV-detector
DDW	Deionized distilled water
DISC	Death-inducing signalling complex
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
E	Standardized 50% ethanol extract of <i>O. stamineus</i> leaves
EA.hy926	Human normal endothelial cell line
EDTA	Ethylenedinitrilotetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EUP	Eupatorin
FGFR	Fibroblast growth factor receptor
FTIR	Fourier transform infrared spectrometry
FR	<i>Firefly/Renilla</i>
G	Gavage
GGT	Gamma-glutamyl transferase
GH	Growth hormone
Hb	Haemoglobin
HCT 116	Human colorectal carcinoma cell line
HEPA	High efficiency particulate air

HIFBS	Heat-inactivated fetal bovine serum
HIF	Hypoxia inducible factor
HPLC	High performance liquid chromatography
HREs	Hypoxia response elements
HSP	Heat shock proteins
HUVEC	Human umbilical vein endothelial cell line
IAP	Inhibitor of apoptosis protein
IC ₅₀	Half-maximal inhibitory concentration
ICH	International Conference on Harmonization
i.e.	That means
IGF	Insulin-like growth factor
IGF-1SR	IGF type I somatomedin receptor
IGFBP	IGF binding protein
J	Joule
JNK	c-Jun amino terminal kinase
K562	Human blood cancer cells
LD ₅₀	Lethal dose of 50% of the tested animals
LOD	Limit of detection
LOQ	Limit of quantification
M199	Growth medium (Earle's salt)
MCF 7	Human breast cancer cell line
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MEM	Minimum essential medium
MIF	Migratory inhibitory factor
MMP	Matrix metalloproteinase

MTD	Maximum tolerated dose
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
mRNA	Messenger ribonucleic acid
NC	Negative control
NEAA	Non-essential amino acids
NIF	Necrotic inducing factor
NF- κ B	Nuclear factor-kappaB
NSCLC	Non-small-cell lung carcinoma
OD	Optical density
OECD	Organization for Economic Cooperation and Development
ORP-150	Oxygen-regulated protein-150
O.S	<i>Orthosiphon stamineus</i> Benth.
O.S extract	Standardized 50% ethanol extract of <i>Orthosiphon stamineus</i> leaves
<i>O. stamineus</i>	<i>Orthosiphon stamineus</i> Benth.
p53	Tumour suppressor protein 53
PBS	Phosphate buffer saline
PC	L- α -phosphatidylcholine
PC-3	Human prostate cancer cell line
PCV	Packed cell volume
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDI	Polydispersity index
PE	Plating efficiency
PG	L- α -phosphatidyl-DL-glycerol sodium salt
PIGF	Placenta growth factor
pRB	Retinoblastoma tumour suppressor

PS	Penicillin/streptomycin solution
pVHL	Von Hippel-Lindau tumour suppressor protein
r	Radius
R ²	Correlation coefficient
RA	Rosmarinic acid
RBC	Red blood cells
RDW	Red blood cell distribution width
RH	Relative humidity
RI	Refractive index
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase HPLC
RSD	Relative standard deviation
R _t	Retention time
SCLC	Small cell lung cancer
SD	Standard deviation
SD rats	Sprague Dawley rats
SEM	Scanning electron microscopy
SF	Surviving fraction
SIN	Sinensetin
SMAC	Second mitochondrial-derived activator of caspase
SPF	Specific pathogen free
SPSS	Statistical Package for the Social Sciences
STMs	Signal transduction modulators
sTNFR1	Soluble tumour necrosis factor receptor-1
TEM	Transmission electron microscope
TGF-β	Transforming growth factor-β
T _{max}	Time of maximum observed concentration

TMB	Tetramethylbenzidine
TMF	3'-hydroxy-5,6,7,4'-tetramethoxyflavone
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
TRAILR	TRAIL receptor
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
UV-Vis	Ultraviolet visible
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor
v/v	Volume/volume
WBC	White blood cells
Wnt	Wingless-Int.
w/v	Weight/volume
w/w	Weight/weight
xIAP	x-linked mammalian inhibitor of apoptosis protein

LIST OF UNITS

Å	Angstrom
cm	Centimetre
cP	Centipoise
dl	Decilitre
g	Gram
h	Hour
hPa	Hectopascal
kcps	Kilo counts per second
kg	Kilogram
L	Litre
m	Metre
M	Molar
mbar	Millibar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mm ³	Cubic millimetre
mM	Millimolar
mmol	Millimole
mV	Millivolt
mW	Milliwatt
nm	Nanometre
pg	Picogram (=10 ⁻¹² of a gram)
rpm	Rounds per minute

U	Unit
μg	Microgram
μl	Microlitre
μm	Micron
μmol	Micromole

LIST OF SYMBOLS

α	Alpha
β	Beta
Δ, δ	Delta
ε	Epsilon
κ	Kappa
λ	Lambda
π	Pi
$^{\circ}\text{C}$	Degree Celsius
%	Percent

**KAJIAN *IN VITRO* DAN *IN VIVO* FORMULASI LIPOSOM EKSTRAK
ORTHOSIPHON STAMINEUS UNTUK KANSER PARU-PARU**

ABSTRAK

Dalam kajian ini, potensi anti-angiogenik dan anti-tumor ekstrak terpiawai 50% etanol daun *Orthosiphon stamineus* yang telah diseragamkan dinilai secara *in vitro* dan *in vivo* atas kanker paru-paru manusia. Sistem penyampaian obat liposomal dihasilkan dari ekstrak ini bagi memperbaiki bioketersediaan, efikasi, kestabilan dan profil keselamatan ekstrak. Tiga formulasi telah dihasil berdasarkan ekstrak ini: ekstrak nisbah “phospholipid”, “phospholipid” dan kandungan kolesterol. NP2, iaitu formulasi yang mengandungi nisbah 1:1 ekstrak kepada “phosphatidylcholine” dengan 20% kolesterol, telah menunjukkan kecekapannya dalam pemerangkapan, integriti, kestabilan dan efikasi liposom yang optimum. Liposom dianalisis secara kualitatif dan kuantitatif dengan menggunakan mikroskop cahaya, TEM dan SEM, penyebaran cahaya dinamik (yang telah menunjukkan formasi vesikel nano berbentuk sfera yang utuh dengan permukaan yang licin dan membran dwilapisan yang tertutup sepenuhnya), “zetasizer” dan potensi zeta (yang melaporkan vesikel anion nano dengan diameter dinamik 125 – 132 nm). NP2 telah menunjukkan sifat penghantaran sebatian bioaktif menonjol dan profil kestabilan yang terbaik apabila dibandingkan dengan ekstrak-ekstrak lain yang diuji selama enam bulan ($P \leq 0.05$), pada suhu 30°C atau lebih rendah. Ekstrak ini dan liposom menghalang proliferasi sel kanker adenokarsinoma manusia (A549) dan sel endotelium normal manusia (EA.hy926), serta memaparkan potensi anti-angiogenik yang tinggi seperti yang ditunjukkan di dalam ujian aorta tikus *ex vivo*. Analisis

TEM ke atas sel A549 telah menunjukkan perubahan-perubahan morfologi “apoptotic” yang ketara, menunjukkan pengambilan liposom oleh sel-sel. Ekstrak ini juga menghalang pengkolonian “in vitro”, pencerobohan sel-sel dan penghijrahan sel melalui kebergantungan dos-masa. Kajian “protein array” dan “dual-luciferase” menunjukkan lebih ekspresi pelbagai protein yang mengawal apoptosis, termasuk TGF- β dan MAPK / ERK, TRAILR-1, Bid, Bax, caspase 3, IGFBP-1 dan IGFBP-5; serta memperturunkan regulasi beberapa laluan-laluan lain seperti WNT, Bcl-2, cIAP-2, livin, IGFBP-3, IGFBP-6 dan IGF-1SR. Ekstrak ini dan NP2 merencatkan kolonisasi, penghijrahan dan pembentukan tiub sel-sel EA.hy926, serta menurunkan ekspresi VEGF dan HIF-1 α yang mengakibatkan aktiviti angiogenesis. Data kajian *in vivo* keberkesanan ekstrak ini dan formulasi NP2 didedahkan sebagai ejen anti-tumor yang kuat ke atas kanser paru-paru manusia menerusi model tumor xenograft serta pengaktifan ekspresi VEGF. Kajian farmakokinetik menunjukkan liposom meningkatkan bioavailabiliti oral dan peningkatan profil farmakokinetik. Kajian toksik “acute” dan “sub-acute” menunjukkan margin keselamatan yang baik untuk formulasi NP2. Liposom (NP2) menunjukkan efisiensi yang lebih baik secara signifikan dari segi peningkatan “bioavailability” dan kestabilan berbanding dengan ekstrak yang tidak diformulasikan (E) ($P \leq 0.05$). Kesimpulannya, hasil kajian ini menunjukkan ekstrak O.S mempunyai aktiviti anti-tumor yang poten terhadap karsinoma paru-paru dan formulasi liposom NP2 dapat meningkatkan efikasi anti-tumornya.

**IN VITRO AND IN VIVO LIPOSOMES FORMULATION STUDIES OF
ORTHOSIPHON STAMINEUS EXTRACT FOR LUNG CANCER**

ABSTRACT

In this study, anti-angiogenic and anti-tumour potencies of the standardized 50% ethanol extract of *Orthosiphon stamineus* leaves (O.S extract) were assessed towards human lung cancer *in vitro* and *in vivo*. Liposomal drug delivery system was developed from the O.S extract to improve bioavailability, efficacy, stability and safety profiles of the O.S extract. Three formulation was developed based on the extract : phospholipid ratio, phospholipid and cholesterol content. NP2, which is a formulation consisting of 1:1 extract to phosphatidylcholine ratio with 20% cholesterol, showed optimum entrapment efficiency, integrity, stability and efficacy of the liposomes. Liposomes were characterized qualitatively and quantitatively using light, TEM and SEM microscopy (which shows intact formation of nano-spherical shaped vesicles with mostly smooth surface and completely closed bilayer membrane), zetasizer and zeta potential (which reports anionic nano-vesicles with dynamic diameter 125-132 nm). NP2 shown significantly better release properties of the bioactive compounds and good stability profile than the O.S extract for six months stability studies ($P \leq 0.05$) at 30°C or below. The O.S extract and the liposomes partially inhibited proliferation of human lung adenocarcinoma (A549) and human normal endothelial cells (EA.hy926), and revealed potent anti-angiogenic potency as in *ex vivo* assay of rat aortic ring. TEM analysis of A549 cells shows obvious apoptotic morphological alterations, with indication of the cellular uptake of the liposomes. It also inhibited *in vitro* colonization, cells invasion and cell migration

in a dose–time dependent manner. Protein array and dual-luciferase studies show overexpression of various apoptotic-regulating proteins, including TGF- β and MAPK/ERK, TRAILR-1, Bid, Bax, caspase 3, IGFBP-1 and IGFBP-5; and down-regulation of some other pathways, such as WNT, Bcl-2, cIAP-2, livin, IGFBP-3, IGFBP-6 and IGF-1SR. The O.S extract and NP2 inhibited EA.hy926 cells colonization, migration and tube formation, and also down-regulated the expression of VEGF and HIF-1 α indicating anti-angiogenic activity. *In vivo* studies reveal potent anti-tumour activity of the O.S extract and the NP2 liposome formulation towards human lung cancer in xenograft tumour model and decreased VEGF expression. Pharmacokinetic studies show that the liposomes increased oral bioavailability and improved pharmacokinetic profile. Acute and sub-acute toxicity studies reveal good margin of safety for the NP2 formulation. The liposomes (NP2) show significantly better efficiencies with enhanced bioavailability and stability compared to the non-formulated O.S extract (E) ($P \leq 0.05$). All-in-all, the results of this study indicates that the O.S extract has potent anti-tumour activity towards lung carcinoma and the NP2 liposome formulation improves its anti-tumour efficacy.

CHAPTER ONE

INTRODUCTION

1.1 Cancer:

1.1.1 Overview of Cancer:

Cancer is an ambiguous disease, characterized by immortality of the cells and excessive proliferation frequencies, which might be related to an uncontrolled genetic mutation. There are two types of immortal cells; hyperplasia (normal cells) which is benign; and dysplasia (abnormal cells) which is malignant and can cause tumour development (Yarbro et al. 2011). Tumours might still at the tissues of origin or undergo metastases processes. Metastases is the invasion of the cancer cells from the original tissues into the neighbouring tissues where they form new tumours (Shevde and Welch 2003).

1.1.2 Cancer Epidemiology:

Cancer is one of the most public health problems worldwide. It is the main cause of many deaths, where one in every four deaths is related to cancer in the United States of America (Siegel et al. 2014). Globally, there is an estimation of 14.1 million new cancer cases with 8.2 million deaths annually of 27 different cancer types (Torre et al. 2015).

Cancer incidences increase with the increase of the aging population which presence and the prevalence of risk factors. Less developed countries account more than 55% of cancer incidences with about 65% of deaths from cancers worldwide.

This might be due to prevalence variations of risk factors, life styles, lower levels of health expenditure, detection tests, availability of treatment and education. Cancer incidences and mortalities attack males at a higher rate more than females (Igene 2008; Ferlay et al. 2010; Jemal et al. 2011; Torre et al. 2015). Annually, more than 3.5 million cancer cases have been recorded in Eastern Asia (Ferlay et al. 2010).

Lung cancer is the leading cause of cancer mortality among males and a second leading cause of cancer death among women worldwide; breast cancer is the leading cause of cancer mortality among females worldwide. The other most common cancers recorded in males are prostate, colon, pancreas and liver respectively; while in females the most commonly reported cancers are lung, colon, pancreas and ovary (Ferlay et al. 2010; Siegel et al. 2014; Torre et al. 2015; Torre et al. 2016).

Lung cancer represents 12.7% of the total cancer incidents. It is also the most serious and fatal cancer, with a fatality percentage of about 18% among all deaths from cancers globally. It is estimated that around 1.6 million lung cancer deaths and about 1.8 million new lung cancer cases occur annually worldwide, (Igene 2008; Ferlay et al. 2010; Torre et al. 2016).

Cancer can be prevented by applications of various effective methods, including controlling of tobacco smoking, vaccination and early detection (Jemal et al. 2011; Torre et al. 2015).

1.1.3 Causes and Risk Factors of Cancer:

Environmental carcinogens play pivotal role in cancer incidences. They can cause genetic mutation that may enhance abnormal cell proliferations. The risk of the disease proportionally increases with exposure durations and the type of the environmental factors. Environmental carcinogens include many factors such as tobacco smoking, obesity, diet, sun rays, radiation, pathogens (*Helicobacter pylori*, papilloma viruses and hepatitis B or C virus among others) and some chemical agents (such as asbestos, industrial waste and pesticides) (Wang and Chen 2001).

Some hormones significantly induce the development of some cancers such as those in prostate and breast cancers which are influenced by testosterone and oestrogen (Cuzick 2008). Lifestyle, race, aging, sex, sexual activity, heredity, pregnancy and physiological stress also participate in cancer occurrence (Kintzios and Barberaki 2004). Genetics play a crucial role in cancer control. There are two types of genes: proto-oncogenes (which stimulate tumour development) and tumour suppressor genes (which block the mechanism of cell division and prevent tumour development) (Kintzios and Barberaki 2004; Weeraratna 2005).

1.1.4 Classification of Cancer Types:

Cancer types can be classified according to various aspects as the following:

1.1.4(a) Origin Tissue of Tumour:

Cancer can be classified into three types, including carcinoma (if the tumour originates in epithelial cells); sarcoma (if the tumour emerges in the connective tissue) and glioma (if the tumour grows in non-neuronal brain cells). Carcinoma is the most common type (Yarbro et al. 2011).

1.1.4(b) Target Organs of Tumours:

Cancer attacks various organs of the body with different prevalence percentages. The most common type of cancers nowadays is lung cancer. Other cancer types include breast, colorectal, bladder, kidney, lymphoma, leukaemia, ovarian, prostate, stomach, skin pancreatic, and uterine cancers (Kintzios and Barberaki 2004).

1.1.5 Genetic and Molecular Control of Cancer:

Genetics control of cancer is developed via two basic types of mutations of some selected proteins; the first mutation leads to increment of activity of the proteins (the hyper-activated gene is known as oncogene), the other type of mutation resulted in inhibition of the activity of the proteins (the in-activated gene is known as tumour suppressor gene) (Kintzios and Barberaki 2004; Weeraratna 2005).

Oncogenes participate in signalling pathways that induce cell proliferation, whereas suppressor genes involved in proteins that act normally as checkpoints to cell proliferation. Carcinogenic agents (such as radiation, chemicals or viruses) can induce genetic mutations (somatic mutations). Mutation might be also inherited (germ-line mutation) (Bertram 2000; Croce 2008; Yarbrow et al. 2011).

Hyper-activation or inactivation of many genetic pathways plays a crucial role in converting normal human cells into cancerous cells (Hanahan and Weinberg 2000). Activation of oncogenes family of transmembrane tyrosine kinase receptors, named human epidermal growth factor receptor (EGFR), enhances cancer initiation. It includes four transmembrane glycoproteins (HER1/Erb-B1, HER2/neu/Erb-B2, HER3/Erb-B3 and HER4/Erb-B4) (Cohen and Carpenter 1975; Scaltriti and Baselga

2006). Over expression of telomerase enzyme enhances cell proliferation (Holt and Shay 1999). Many genes related to cancer development are depicted in table 1.1.

Table 1.1 Genes involved in carcinogenesis (Kintzios and Barberaki 2004; Weeraratna 2005).

Oncogenes	Sites of cancer	Tumour suppressor genes	Sites of cancer
Bcl-1	Breast, head and neck	APC	Colon and stomach
BCR-ABL	Leukaemia	BRCA1	Breast and ovarian
c-myc	Lung, leukaemia, stomach and breast	BRCA2	Breast
CDKN2	Melanoma	CDK4	Skin
Erb-B	Glioblastoma and breast	DPC4	Pancreas
HPC1	Prostate	MSH2, MSH6 and MLH1	Colon
Ki-ras	Lung, ovarian, colon and pancreatic	p53	Various
MDM2	Sarcomas	RB	Retinoblastoma, bone, bladder, lung and breast
N-myc	Neuroblastoma and glioblastoma	VHL	Kidney
N-ras	Leukaemia		
PDGF	Glioma		
RET	Thyroid		

1.1.6 Cancer Angiogenesis:

Since 1970s, many studies had been carried out to improve cancer treatment strategies, by targeting tumour vasculature by disrupting various steps of neovascularization development. Growth process of new blood vessels from the existing vascular bed is commonly known as angiogenesis. This is a complex process that controls normal physiological development of new blood vessels, placental development during pregnancy, wound healing processes and for menstruation cycle

through series of processes, including endothelial cell proliferation, migration as well as tube formation cascades (Folkman 1971; Charnley et al. 2008; Ahmed and Bicknell 2009). Unregulated angiogenesis contributes in development of some pathological conditions, such as cancer (tumour growth), psoriasis, obesity and diabetic retinopathy. For tumour growth up to larger than one mm³, it requires more supplementation with oxygen and nutrients. Therefore, new blood vessels are developed to connect these supplies into the tumour. Diminishing development of new blood vessels leads to starvation of the tumour cells and less chance for tumour to survive. The angiogenesis process also plays a pivotal role in metastasis of cancer cells to other localities (Folkman 1985; Tonini et al. 2003; Eskens 2004).

1.1.6(a) Regulation of Angiogenesis via VEGF:

Vascular endothelial growth factor (VEGF), pro-angiogenic mitogens, has been described in the past as vascular permeability factor (VPF), which regulates microvascular leakage (Senger et al. 1990). VEGF family includes various pivotal factors that control blood, nervous and lymphatic systems during embryonic development and processes of wound healing. Even in adults, VEGFs still participate partially in conserving vessels homeostasis (Ahmed and Bicknell 2009).

VEGFs regulate neovascularization related to some pathological changes and illnesses such as cancer (Olsson et al. 2006). Hypoxia-inducible factor-1 (HIF-1) strongly stimulates VEGF expression. In addition, some oncogenes that activate HIF-1 expression stimulate VEGF transcription; this induces tumour angiogenesis pathways (Shweiki et al. 1992; Ahmed and Bicknell 2009).

There are many identified members of VEGF family, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF). On the

other hand, there are selected tyrosine kinase receptors (VEGFRs), such as VEGFR-1, VEGFR-2 and VEGFR-3. Various studies have revealed that VEGF-A controls development of blood vessel and angiogenesis by binding abilities with VEGFR-1 and VEGFR-2 receptors. Activation of VEGF-C and VEGF-D is regulated by their binding with VEGFR-2 and VEGFR-3 for initiation of vascularization and controlling of the lymphatic system. VEGF-E binds only to VEGFR-2 in viruses selectively (Fong et al. 1995; Ferrara 2001; Ahmed and Bicknell 2009). There are different isoforms of VEGF-A have been identified, including VEGF₁₂₁, VEGF₁₄₅, VEGF₁₄₈, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉ and VEGF₂₀₆ (Niu and Chen 2010).

A binding of VEGF to VEGFR-2 receptor through kinase domains signalling pathway mediates various processes, including endothelial cell proliferation, differentiation, migration and survival activities. VEGFs stimulate phosphorylation of tyrosine residues, which has resulted in controlling of the signals of VEGFR-2. There are four autophosphorylation sites, recognized by VEGFs, including tyrosine's 951, 996, 1054 and 1059 (Dougher and Terman 1999; Takahashi et al. 2001; Zeng et al. 2001; Jussila and Alitalo 2002).

Transphosphatidylation reaction of VEGF activates a mediatory enzyme (phospholipase D) that controls angiogenesis phenotype. This activation depends on tyrosine phosphorylation and protein kinase C. It regulates many processes that initiate angiogenesis pathways (such as endothelial cell migration) (Seymour et al. 1996). VEGF also activates nitric oxide (NO) synthase and stimulates nitric oxide production by a mediation of VEGFR; this augments controlling of sprouting of new blood vessels and potentiates endothelial cell proliferation and migration (Ziche et al. 1997; Garcia-Cardena et al. 1998).

1.1.6(b) Role of Hypoxia in Angiogenesis Pathway:

Invasion and metastasis are two interrelated processes, required for tumour growth. Tumour development is significantly controlled by an “angiogenic switch” through balance between the proangiogenic and anti-angiogenic factors. Hypoxia is a common proangiogenic factor (Folkman 2002). Various environmental and epigenetic influences also control neovascularization of tumour and metastases (such as acidosis, hypoxia and hypoglycaemia (Ahmed and Bicknell 2009)).

Hypoxia is the situation of diminution of oxygen supply which stimulates tumour growth. Hypoxia is mediated by transcription factors known as hypoxia-inducible factors (HIFs). Hypoxia-inducible factor 1 (HIF-1) is the major one of HIFs. Structure of HIF-1 includes two DNA-binding proteins (heterodimer), HIF-1 α and HIF-1 β (aryl hydrocarbon nuclear translocator (ARNT)) (Charnley et al. 2008; Ahmed and Bicknell 2009).

There are another two HIFs: HIF-2 α and HIF-3 α , which control angiogenesis in addition to HIF-1 α . HIF-2 α has lesser participations in adult and tumour angiogenesis than the other two (i.e. HIF-1 α and HIF-3 α); but it plays a vital role in embryonic development (Leek et al. 2002).

Hypoxia activates genetic expression of VEGF (Shweiki et al. 1992). There are many other extra-cellular pathways that participate in regulating hypoxia induced angiogenesis, such as notch/delta, ephrin/Eph receptor, netrins/UNCs, roundabouts/slits, endothelins-1 and 2, adrenomedullin, semaphorins, angiogenin, neuropillins, plexins and various intracellular protein families (hedgehog and sprout) (Pilch et al. 2001; Bicknell and Harris 2004; Ahmed and Bicknell 2009).

In addition, hypoxia controls many other angiogenic pathways, including inducible endoplasmic reticulum oxygen-regulated protein-150 (ORP-150), which is induced as a VEGF chaperone (Ozawa et al. 2001); leptin gene up-regulated by HIF-1 (Ambrosini et al. 2002); and connective tissue growth factor (CTGF), which is a potent angiogenic factor in human breast cancer cells (Shimo et al. 2001). Furthermore, HIF-1 α regulates stromal cell-derived factor-1 (CXCL12), which leads to induction of VEGF expression and up-regulation of migratory inhibitory factor (MIF) (Hitchon et al. 2002; Bacher et al. 2003). Likewise, hypoxia hyper-activates the expression of placenta growth factor (PlGF) (Green et al. 2001), and many other polypeptide angiogenic factors, including some metabolites of glycolysis (such as pyruvate and lactate) (Murray and Wilson 2001). Angiopoietins are a family of cell-specific molecules (Ang-1, 2, 3 and 4) which activate angiogenesis process via binding to Tie receptors (Makrilia et al. 2009).

At normal conditions of oxygen supply, HIF-1 α binds to E3 ubiquitin ligase which contains the Von Hippel-Lindau tumour suppressor protein (pVHL); this enhances an enzymatic proline-hydroxylation, co-activated in the presence of iron, which resulted in proteasomal degradation of HIF-1 α (Ivan et al. 2001). Once the oxygen tension decreases up to less than 2% (due tumour growth), then, the cancer cells protect the degradation of HIF-1 α to maintain tumour survival even under hypoxic situation. HIF-1 α resists the degradation and accumulates in the nucleus, followed by formation of a complex by ligation with HIF-1 β and transcriptional co-activators (CBP/p300). This results in induction of selected genes that have hypoxia response elements (HREs). These genes include VEGF, proteases enzymes (matrix metalloproteinase (such as MMP9)), proteins involved in invasion (urokinase

receptor and plasminogen activator-1) and proteins involved in glycolytic pathway (Harris 2002; Wenger 2002).

Reactive oxygen species (ROS) also control signalling pathways of various cellular functions (such as angiogenesis and tumour growth) (Ushio-Fukai and Alexander 2005; Xia et al. 2007). Various studies had reported that the endogenous H₂O₂ (converted to OH⁻ via Fenton reaction) stimulated expression of VEGF (Ruef et al. 1997). H₂O₂ can also induce many steps of angiogenesis cascades, including endothelial cell proliferation and migration (Yasuda et al. 1999). It also stimulates microtubules morphogenesis (Shono et al. 1996). Hypoxia produces ROS, which has resulted in induction of various angiogenesis processes, including tube formation (Lelkes et al. 1998). Anti-oxidant activity results in a potent scavenging of ROS, this might support anti-angiogenic mechanism (Xia et al. 2007).

Nowadays, there are many anti-angiogenic agents, which have been approved for treating cancers. These drugs are used as adjuvants to chemotherapy regimens such as the combination of the anti-angiogenic agent bevacizumab with the chemotherapeutic agent paclitaxel and/or carboplatin, which augmented their efficiency in lung cancer therapy. Other recently developed anti-angiogenic agents include axitinib, aflibercept, brivanib, nintedanib, cediranib, pazopanib, sorafenib, ramucirumab, sunitinib, imatinib and vatalanib (Sandler et al. 2005; George and Moore 2006; Vlahovic et al. 2006; Medinger and Mross 2010; Aggarwal et al. 2012).

1.2 Apoptosis:

The ultimate goal of all strategies of cancer treatment is to enhance the death of cancer cells via induction of apoptotic cascades. Apoptosis, programmed cell death, is a strictly controlled and energy-dependent cell death process, which is not involved in necrosis or inflammation. It is a slow process but it can be induced by various internal or external causes that participate in regulation of normal or abnormal cell death. Induction of apoptosis is accompanied with activation of caspases; this has resulted in cell membrane becoming blebbing and condensation of nuclear chromatin, which consequently lead to a shrinkage of cells, losses of mitochondrial membrane permeability, formation of apoptotic bodies and finally phagocytosis of the cell debris (O'Driscoll et al. 2006; Elmore 2007; He et al. 2014).

Necrosis, uncontrolled cell deaths, is different from apoptotic cells. It is characterised by some alterations of cellular morphology, including cytoplasmic swelling, rupture of plasma membrane and cytoplasmic organelles dilatation. This brings to excessive cell death and inflammation of the surrounded area. The necrotic cells are partially eliminated by phagocytosis (internalization mechanisms). Necrotic cell death plays a vital role in development of embryonic and adult tissues (Kroemer et al. 2005; Krysko et al. 2006; Golstein and Kroemer 2007).

Necroptosis, another mechanism of controlled cell death, refers to the process of co-regulation of apoptosis and necrosis. Some studies revealed that necrosis could be controlled by a complex mechanism (Galluzzi and Kroemer 2008; Gunther et al. 2012).

Some studies have highlighted some events in this respect. Necrotic processes can be controlled by some genetic factors and specific receptors (Golstein and

Kroemer 2007). In addition, inactivation of caspases may be resulted in mixed processes of apoptosis and necrosis (Galluzzi and Kroemer 2008; Rosenbaum et al. 2010).

Necroptosis also can be regulated by many other factors, including necrostatins (Degterev et al. 2005), serine/threonine kinase activity (Kroemer et al. 2009), necrotic inducing factor (NIF), apoptosis-inducing factor (AIF), RIP1, cyclophilin-D, and poly(ADP-ribose) polymerase-1 (PARP-1) (Galluzzi and Kroemer 2008).

1.2.1 Apoptosis in Health and Diseases:

Normally, apoptosis equilibrium plays a pivotal role in the development of the body, such as in the development of placenta during pregnancy (Sharp et al. 2010). Balance between apoptosis and cell proliferation processes augments the efficiency of the autoimmune system to control various diseases (such as rheumatoid arthritis) (Eguchi 2001).

Cancer develops when the balance of the apoptotic processes is uncontrolled, leading to excessive immortal cells and induced cell proliferation. On the contrary, excessive apoptosis causes neurodegenerative diseases, such as Parkinson and Alzheimer disorders (Schiffer 2006; Srivastava 2007).

1.2.2 Apoptosis Pathways:

There are various characteristic alterations occurring in cells during apoptotic cell death, due to the presence of specific signals. Apoptosis can be enhanced through various pathways by proteins that play important roles in controlling cell cycle mechanism, including Wnt, Notch, hypoxia, NF- κ B, p53 and MAPKs

pathways. Any deregulation in these apoptotic pathways can result in many malignancies (Kaufmann and Hengartner 2001; Ghobrial et al. 2005).

Apoptotic signalling pathways are divided into two major types depending on the mechanism of initiation, extrinsic and intrinsic pathways. The extrinsic pathway is activated via cell death receptors, while the intrinsic pathway depends on mitochondrial changes. There are some other molecules that also incorporate with the mechanism of both extrinsic and intrinsic signalling pathways of apoptosis (Ghobrial et al. 2005).

Caspases (cysteine aspartic acid-specific proteases) are a family of enzymes that play an essential role in cell apoptosis process. Caspases are activated at the early apoptotic steps (Eguchi 2001), leading to hyper-activation of some other factors of apoptosis (Kass et al. 1996). Caspases are produced in the cells as proenzymes (procaspases) as inactive forms of two subunits in which they are activated by apoptotic inducing factors (Substrates) (Guy S 2003). There are at least 14 known caspases in human cells. They are sub-grouped depending on their activities into three classes namely apoptosis activators, apoptosis executioners and inflammatory mediators. Apoptosis activators (initiator or upstream caspases) initiate the proteolytic processes; they include caspases 2, 8, 9 and 10. Apoptosis executioners (effector or downstream caspases) control cleaving of polypeptides (proteolysis) and disassembling the cells, including caspases 3, 6 and 7. Inflammatory mediators activate pro-inflammatory factors (such as cytokine), including caspases 1, 4, 5, 11, 12, 13 and 14 (Lamkanfi et al. 2002; Guy S 2003; Fan et al. 2005).

1.2.2(a) Extrinsic Apoptotic Pathway:

Extrinsic apoptotic pathway is also known as death receptor-mediated apoptotic pathway. Recently, studies had shown that the extrinsic pathway was initiated through binding of some inducing ligands with the death receptors (the most prominent ligands include CD95 (Fas), DR3, DR4, DR5 and DR6), produced ceramides, clustering (trimerization) of death receptors, followed by ligation of the adaptor molecule (FADD) with the cytoplasmic domain of the receptor. Then, FADD activates caspase 8, which subsequently cleaved procaspases 3 and 7. This had resulted in induction of apoptotic signalling pathway. Stimulation of tumour necrosis factor (TNF) receptors also conducted with the induction of apoptosis. In which, the binding of tumour necrosis factor-related apoptosis inducing ligand (TRAIL) to the corresponding receptors (TRAILR-1 and TRAILR-2) led to formation of a death-inducing signalling complex (DISC) which subsequently activated caspases 8 and 10 respectively (Medema et al. 1997; Schulze-Osthoff et al. 1998; Rath and Aggarwal 1999; Boatright and Salvesen 2003; Ghobrial et al. 2005; Johnstone et al. 2008).

1.2.2(b) Intrinsic Apoptotic Pathway:

Intrinsic apoptotic pathway is also called as mitochondrial-mediated apoptotic pathway. It is initiated by various intracellular death signals, including DNA damage, exposure to reactive oxygen species (ROS), toxins, hypoxia, radiations and hyperthermia. Mitochondria and a family of B-cell lymphoma-2 (Bcl-2) proteins significantly control intrinsic apoptosis pathway; mitochondria contain apoptotic inducing factor (AIF) with Bcl-2 pro-apoptotic proteins. This decreases the permeability of the mitochondrial membrane by co-activity of Bcl-2 family which dysregulates some caspases leading to release of cytochrome *c*, follows by formation

of apoptosome complex that activates caspases (9, 3 and 7) and amplifies the apoptotic signals (Desagher and Martinou 2000; Boatright and Salvesen 2003; Elmore 2007; Inoue et al. 2009). The extrinsic apoptotic pathways also initiate the intrinsic apoptotic signalling pathways; that caspase 8 can activate the pro-apoptotic protein Bid (Li et al. 1998; Portt et al. 2011).

Studies had identified 25 different types of Bcl-2 proteins; all had been allocated outside the mitochondrial membrane. The Bcl-2 proteins include some pro-apoptotic proteins (such as Bax, Bak, Bid, Bcl-10, Bad, Bim, Bik, and Blk) and other anti-apoptotic proteins (such as Bcl-2, Bcl-W, BclX, Bcl-XL, Bcl-XS, BAG) (Elmore 2007).

The Bcl-2 proteins are sub-grouped into three main subsets depending on their functions and Bcl-2 homology (BH) domains. The first group is characterized by the presence of only a single Bcl-2 homology-3 domain (BH-3 only). It includes Bad, Bim, Bid, Bmf, Noxa and Puma. The second group has multiple Bcl-2 homology (BH) domains; it includes Bax, Bak and Bok proteins. The third group characterized by presence of all four BH domains (multiple BH domains), it includes Bcl-2, Bcl-W, Bfl-1, Bcl-XL and Mcl-1 proteins (Petros et al. 2004; Ashkenazi 2008; Kang and Reynolds 2009).

Down-regulation of the pro-apoptotic Bcl-2 family proteins or overexpression of anti-apoptotic Bcl-2 family members might be resulted with chemotherapy resistance in many human cancers, so that it requires the use of combination of anti-cancer agents (Kang and Reynolds 2009).

There are some proteins inactivating the mitochondrial pathway comprising of inhibitors of apoptosis proteins (IAPs), which include x-linked mammalian inhibitor

of apoptosis protein (xIAP), cellular inhibitor of apoptosis (cIAP), livin and survivin (Elmore 2007; LaCasse et al. 2008; He et al. 2014).

Epithelial cell proliferation and differentiation are regulated by various factors, including growth hormone (GH) and insulin-like growth factors (IGF-I and IGF-II). IGFs are polypeptides that have potent mitogenic effects on breast cancer cells. The availability and actions of IGFs are controlled by six high-affinity binding proteins (IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and IGFBP-6) and their proteases in various cells including breast cancer and lung cancer (Coutts et al. 1994; Mol et al. 1997; Dziadziuszko et al. 2008).

Derailment of excretion and expression of GH and IGF may guide to an uncontrolled proliferation of the mammary gland; this might cause growth of benign or malignant tumours (Arteaga and Osborne 1989; Mol et al. 1997). IGF-I is expressed by breast fibroblast; it is overexpressed in breast cancer cells via stimulation of IGF type I somatomedin receptor (IGF-1SR). Blockade of this receptor is an important strategy for counteracting human cancer (Arteaga and Osborne 1989; Mizukami et al. 1990).

IGF-II has a greater binding potential to various receptors than IGF-I, which is related to a wide range of biologic functions of IGF-II. Although some research studies had reported that IGF-II possesses potent anti-apoptotic efficiency towards many neoplastic cell types, the function of IGF-II was not completely understood. The overexpression of IGF-II is related to various but not all cancers, where it has shown a regulation of about 50% only of lung cancer cases. IGF-II has no relation with increased risk of some other lung cancer cases (Takanami et al. 1996; Rozen et al. 1997; Moorehead et al. 2003; Dziadziuszko et al. 2008).

The expression of IGFBPs modulates the effects of IGF on cancer cells, in which they may stimulate or inhibit the effect of IGFs (Ritvos et al. 1988; Blum et al. 1989; Owens et al. 1993). IGFBP-3 is considered as the dominant IGFBP in serum for circulation of IGF-I; it maintains and prolongs the blood circulation of IGF-I. So that, deregulation of IGFBP-3 renders the carcinogenic effect of IGF-I (Figueroa and Yee 1992; Perks and Holly 2000). It had been reported that IGFBP-1 may act as a negative modulator of IGF-I by blocking of IGF binding to its receptors, leading to reduction of the proliferation of cancer cells (Figueroa and Yee 1992; Perks and Holly 2000). Several studies had reported inhibitory effects of the identical proteins IGFBP-2 and IGFBP-4 on cell growth (Clemmons 1992; Figueroa and Yee 1992). Despite structural similarity of IGFBP-3 and IGFBP-5, some studies revealed that overexpression of IGFBP-5 inhibited cell proliferation and antagonized the anti-apoptotic potential of IGF-I (Clemmons 1992; Rozen et al. 1997).

IGFBP-6 has a special binding affinity to IGF-II. It inhibits prognosis of various IGF-II-dependent cancers (Bach 2005). Some studies reported overexpression of IGFBP-6 in various cancer cells, including lung cancer (Wegmann et al. 1993; Van Doorn et al. 1999).

The underlying mechanism of IGFs and IGFBPs is complicated and still not completely understood. There are some other factors, suggested to influence the production and activity of IGFs. Various research studies had shown that some of these proteins revealed bifunctional effects (Clemmons 1992; Perks and Holly 2000).

Reactive oxygen species (ROS) (such as OH⁻ and NO) participate in controlling apoptotic mechanism and regulating angiogenesis cascades (Watson et al. 1997; Wedi et al. 1999; Simon et al. 2000). Higher cell content of ROS inhibits the

mitochondrial apoptotic processes (Simon et al. 2000). NO induces heat shock proteins (HSP) which increase the intracellular glutathione content and inhibit apoptosis (Arrigo 1998). Extrinsic and intrinsic pathways are also controlled by the heat shock proteins (HSP) which including HSP10, 27, 60, 70 and 90. HSP27 reduces a release of cytochrome *c*; HSP70 and HSP90 inhibit formation of apoptosome complex. Whereas, HSP10 and HSP60 induce activation of executioner caspases (Zhivotovsky and Orrenius 2003).

Anti-angiogenic agents inhibit endothelial cell proliferation and migration. They also induce endothelial and tumour cells apoptosis, that the efficiency of anti-cancer treatment is augmented by the combination of chemotherapy and angiogenesis inhibitors (Folkman 2003).

1.2.3 Common Signal Transduction Cancer Pathways:

Cancer cells can modify the surrounding environment to facilitate their growth and proliferation, metastasis and invasion; through various mechanisms, including increasing or decreasing the expression of some proteins (Hanahan and Weinberg 2000). Signal transduction pathways is related to activation of cascades of intracellular biochemical reactions that lead to the changing expression level of some responsible proteins for communications with the internal or external circumstances of cancer cells (Lobbezoo et al. 2003).

Each pathway begins with ligation of extracellular receptors. The activation of receptors is translated into biological action that activates proteins (transcriptional factors), followed by its translocation into the nucleus, and binding with specific binding sites in the DNA (promoters). This triggers the transcription of mRNAs which is translated to proteins (Martin 2003; Eccleston and Dhand 2006).

Mutation of oncogenic gene establishes the activation of signal transduction elements which simulates a condition of receptor activation permanently even if there is no relevant growth factor (Hanahan and Folkman 1996). Many pathways were found to be hyper-activated in cancer cells. These pathways include Wnt, Notch, Myc/Max and hypoxia pathways (Van Es and Clevers 2005; Soucek et al. 2008; Galluzzo and Bocchetta 2011; Jiang et al. 2015). On the other hand, tumour suppressor genes mutations cause deactivation of some other pathways which are in relation with cells proliferation, such as p53 (Ahrendt et al. 2003; Feng et al. 2008).

Signal transduction modulators (STMs) can target cancer pathways selectively for cancer treatments. The STMs play important roles in modulation of pathways activities, such as blocking receptors on cell surface, blocking the mediators between extracellular signals and the transcriptional factor, deactivating binding of the transcriptional factors with some promoters, or reducing the effects of some other downstream genes (Lobbezoo et al. 2003).

In addition, a large number of research studies had been conducted to investigate the use of many STM compounds for treatments of cancer, like imatinib and trastuzumab which have been approved to be used commercially (Lobbezoo et al. 2003; Nassar 2010).

1.2.3(a) Wnt/ β -catenin Signalling Pathway:

Wingless-Int. (Wnt) signalling pathway is a fundamental pathway that participates in cell-cell signalling to control many processes, including gene expression, cell behaviour, adhesion and polarity process in normal cells as well as cancer cells (Cadigan and Nusse 1997). Wnt signals are controlled by three

pathways; Wnt/ β -catenin pathway (canonical Wnt pathway), Wnt/ Ca^{+2} (non-canonical Wnt pathway) and Wnt/JNK pathways (Moon et al. 2002).

Studies revealed mutations of many components of Wnt/ β -catenin pathway are critical for development of many types of human cancer, such as lung adenocarcinoma, colon cancer and breast cancer (Morin et al. 1997; Lin et al. 2000; Jiang et al. 2015). Moreover, the protein level of β -catenin was significantly upregulated leading to induce expression of stem cell protein Oct-4, accompanied with high expression of Wnt pathway and the oncogene cyclin D. This enhanced cell proliferation, invasion and colony formation efficiencies. Wnt/ β -catenin pathway makes complex with TCF/LEF transcriptional factors which controls expression of many other oncogenes, including c-Myc, cyclin D1 and matrix metalloproteinase genes; which induce angiogenesis as well as carcinogenesis cascades (Dihlmann and von Knebel Doeberitz 2005; Gehrke et al. 2009; Jiang et al. 2015).

Tumour regression can be counteracted by down-regulation of Wnt pathway, which plays an important role for cancer treatment (Tetsu and McCormick 1999; Jiang et al. 2015).

1.2.3(b) Notch Signalling Pathway:

Notch signalling pathway plays a crucial role of various cellular activities, such as cell proliferation, differentiation, apoptosis, fate specification, adhesion and migration, as well as angiogenesis (Bolós et al. 2007). Signalling cascade is initiated by binding of the four extracellular isoforms of Notch receptors (Kojika and Griffin 2001).

In vitro and *in vivo* research studies revealed that hyper-activation of any Notch isoform receptor promoted tumour growth and aggressiveness enhanced development of many types of cancer, including lung, pancreas, colon, breast and renal cancer (Callahan and Raafat 2001; Collins et al. 2004; Van Es and Clevers 2005; Wang et al. 2006; Farnie and Clarke 2007; Strizzi et al. 2009; Sun et al. 2009; Galluzzo and Bocchetta 2011).

Blocking of Notch pathway will be useful for cancer treatment; such inhibitors of Notch pathway include RNA interference, antisense and monoclonal antibodies. Wnt and Notch pathways may work synergistically to increase signalling cascades of cancer, so that combining of both Wnt and Notch inhibitors will potentiate their anti-cancer efficiency (Nickoloff et al. 2003; Van Es and Clevers 2005).

1.2.3(c) p53 Signalling Pathway:

Mutation of p53 gene is commonly occurred in most cancers, in which p53 is suppressed in more than 50% cases of human cancer. There are some other mechanisms leading to inactivation of p53 gene but they are not related to intragenic mutation. The p53 mutations resulted in activation of some other oncogenes, which lead to more aggressive and resistant tumour (Ahrendt et al. 2003; Wang and El-Deiry 2004).

The p53 gene induces apoptosis and cell cycle arrest; so that it is called guardian of the genome. It controls cell death by regulating two apoptotic pathway genes; extrinsic pathway (via regulation of death receptor Fas and DR-5 genes), and mitochondrial pathway (via regulation of Bax, Bak and Bid proteins) (Lowe et al. 1993; Burns and El-Deiry 2003; Nassar 2010). Repairing the defects of the p53

protein may play an important role in treating cancer (Campling and el-Deiry 2003; Toyooka et al. 2003).

1.2.3(d) TGF- β Signalling Pathway:

Transforming growth factor-beta (TGF- β) signalling pathway plays a pivotal role in many biological processes, among other in cell growth, differentiation, apoptosis and angiogenesis. Over expression of TGF- β inhibits tumour growth and development in early stage of cancer, such as lung cancer tumour. It inhibits a group of proteins called mothers against decapentaplegic proteins (SMAD). Many other studies indicated the negative impacts of TGF- β on tumour growth (Thiery 2002; Jeon and Jen 2010; Nassar 2010).

TGF- β is also known as a double-edged sword. Various studies reported the tumour suppressor and oncogenic properties of TGF- β pathway. TGF- β promotes tumour metastases and invasiveness by inducing EMT and angiogenesis. The mechanism of the dual effects of TGF- β , however is still ambiguous (Akhurst and Derynck 2001; Akhurst 2002; Sánchez-Capelo 2005; Jeon and Jen 2010; Toonkel et al. 2010).

There are many approaches for developing effective therapies for lung cancer via inducing of hyper-expression TGF- β signalling pathway (Thiery 2002; Jeon and Jen 2010).

1.2.3(e) Cell Cycle (pRB/ E2F) Signalling Pathway:

Retinoblastoma tumour suppressor (pRB) has a critical contribution in cell cycle and apoptosis processes. Mutation of the pRB gene has been detected in about 50% of all human tumours (Yamasaki 2003; Baldi et al. 2011). Heredity is

responsible for more than 40% of clinical detected cancer cases due to mutation of pRB gene. The effect of pRB gene mutation is detected using DNA cloning techniques in many types of cancers, including breast, lung, prostate and leukaemia (Weinberg 1991; Draper et al. 1992; Baldi et al. 2011).

In vitro studies found that introducing pRB protein into cancer cells had resulted in inhibition of cell proliferation and cell cycle (Bandara and La Thangue 1991). Activation of the pRB signalling pathway through a binding of the pRB protein with a various transcriptional factors (E2F is the most important one) and a binding of active dimers with corresponding promoters that activate the expression of many other vital genes, and responsible for cell death process including c-Myc, N-Myc, thymidylate synthase, cdc2, kinase, cyclin A, thymidine, dihydrofolate reductase and DNA polymerase (Bandara and La Thangue 1991; Helin and Ed 1993).

1.2.3(f) NF- κ B Signalling Pathway:

Nuclear factor-kappaB (NF- κ B) signalling pathway suppresses cell death and enhances multiple steps in carcinogenesis such as cell growth, proliferation, invasion, metastasis and angiogenesis processes. It also regulates immune system and inflammatory responses. Studies have identified more than 200 protein targets (transcriptional factors) of NF- κ B genes, including Rel, Myc, and Cyclin D1-4 (which play important roles in cell cycle regulation); Bcl-2, Bcl-XL, A1/Bf-1 (which involve in apoptosis cascades); VEGF gene (which plays a key role in angiogenesis process); and urokinase plasminogen activator (which participates in regulation of cell metastasis processes (Pahl 1999; Chen et al. 2011).

Activation of NF- κ B protects tumour cells from apoptosis (Barkett and Gilmore 1999; Tang et al. 2006; Chen et al. 2011). Many research studies have shown that targeting of NF- κ B pathway using inhibitory agents can be used as a promising anti-cancer agents for many cancers, including lung cancer and leukaemia (Ka et al. 2003; Tang et al. 2006; Chen et al. 2011).

1.2.3(g) Myc/Max Signalling Pathway:

Myc/Max pathway is an oncogene; research data have reported that Myc/Max pathway is overexpressed in more than 70% of all human cancers. Myc oncogene family includes c-Myc, N-Myc and L-Myc encode a group of nuclear phosphoproteins which regulate cell growth (Zajac-Kaye 2001; Nilsson and Cleveland 2003). The Myc/Max pathway plays a very important role in various cell cycle process and angiogenesis. Myc is dimerized with its partner protein (Max), then, bounded to DNA to exhibit the biological effects. Diminution of this protein reduces cell proliferation and duplication (Heikkila et al. 1987; Evan et al. 1992; Pelengaris et al. 1999; Rudin and Poirier 2014).

Targeting of Myc/Max pathway by down-streaming its expression is an effective and tumour-specific cancer therapy (Soucek et al. 2008; Romero et al. 2014).

1.2.3(h) MAPK Signalling Pathways:

There are three sub-groups of mitogen activated protein kinases (MAPKs), including the extracellular signal regulated enzyme kinases (MAPK/ERK), c-Jun amino terminal kinase (MAPK/JNK) and p38 MAPKs. MAPK/JNKs and MAPK/ERKs participate in regulation of cell cycle, mitosis, apoptosis and migration.