ANTICANCER STUDIES OF KOETJAPIC ACID PURIFIED FROM *SANDORICUM KOETJAPE* MERR.

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ANTICANCER STUDIES OF KOETJAPIC ACID PURIFIED FROM *SANDORICUM KOETJAPE* MERR.

BY

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This thesis is dedicated to...

My parents

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Praise to Allah, the most merciful who gave me determination and strength to complete this work.

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TABLE OF CONTENTS

CHAPTER THREE- RESULTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATION

ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
ATCC	American type culture collections
A431	Epidermal carcinoma cell line
BA	Betulinic acid
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2 associated X protein
BBL probe	Broadband invers probe
BCAP31	B-cell receptor associated protein 31
BCL-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
BC1	Human Breast cancer cell line
BID	BH3 interacting domain death agonist
BSA	Bovine serum albumin
CAM	Chick chorioallantoic membrane
Cdc2	Cell division control protein 2
Cdc42	Cell division control protein 42
$CDCl_3$	Deuterated chloroform
CMV	Cytomegalovirus
Col2	Human colon cancer cell line
CO_2	Carbon dioxide
ddH_2O	Deionised distilled water

DEVD	A tetrapeptide sequence substrate for 3/7 caspases
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-RC	DNA replication complex
DR	Death receptor
DSC	Differential scanning calormetry
E1F4E	Eukaryotic translation initiation factor 4
ECGS	Endothelial cell growth supplement
EDRF	Endothelium derived relaxing factor
ELK 1/SRF	ETS Like gene 1/spectral repeat finder
eNOS	Endothelial nitric oxide synthase
ESI	Electrospray Ion
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FDA	Food and Drug Administrations
FGF	Fibroblast growth factor
g	Gram
GTP	Guanosine triphosphate
h	Hour
HCT 116	Colon cancer cell line
Hep G2	Liver cancer cell line
HIF-1	Hypoxia inducible factor one
HIFCS	Heat inactivated fetal calf serum
HPLC	High performance liquid chromatography

HRP	Horseradish Peroxidase
HT-1080	Human sarcoma cell line
HUVECs	Human Umbilical Vein Endothelial cells
HV	High Voltage
IC_{50}	Inhibitory concentration of 50%
ICAD/DFF-45	Inhibitor of caspase activated DNAse / DNA fragmentation factor 45
IFN	Interferon
IL	Interleukin
KA	Koetjapic acid
KB	Human nasopharyngeal carcinoma cell line
KBr	Potassium bromide
KB-V1	Drug resistance nasopharyngeal carcinoma cell line
kV	Kilovolts
LC-MS	Liquid chromatography mass spectrometry
LEHD	A tetrapeptide sequence substrate for caspase 8
LETD	A tetrapeptide sequence substrate for caspase 9
LNCaP	Human prostate cancer cell line
Lu 1	Human lung cancer cell line
MAPK	Mitogen activated protein kinase
MAPK/ERK	MAPK-Extra cellular signal regulated enzyme kinase
MAPK/JNC	MAPK-C-Jun amino terminal kinase
MCF 7	Breast cancer cell line
Me 12	Human melanoma cell line
mg/ml	Milligram/millilitre

MHz	Megahertz
Min	Minute
Ml	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger Ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolum bromide
MYC/MAX	A signal transduction pathway
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa B
NIH	National institutes of health
Notch	A signal transduction pathway
NO	Nitric oxide
N2	Nitrogen gas
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PE	Plating efficiency
pg/ml	Picogram per millilitre
PRb	Retinoblastoma tumour suppressor protein
pRb- E2F	A signal transduction pathway
Psi	Pound per square inch
P1GF	Placental growth factor

p53	Tumor protein 53
P-338	Murine lymphocytic leukemia
PS	Penicillin/Streptomycin
RB	Retinoblastoma
RF	Resonance Frequency
ROCK-1	Rho-associated protein kinase 1
SF	Survival fraction
SMAD	Mother against decapentaplegic
STM	signal transduction modulators
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGF-β	Tumour growth factor beta
TMS	Tetramethylsilane
TNF 1	Tumour necrosis factor 1
TPA	12-O-tetradecanoyl phorbol-13-acetate
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
VPP	Volts, Peak-to-Peak
WHO	World health organization
Wnt	wingless-int , a signal transduction pathway
µg/ml	Microgram/ millilitre
ZR-75-1	Human breast carcinoma cell line
^{1} H-NMR	Hydrogen Nuclear Magnetic Resonance

LIST OF SYMBOLS

%	Percent
Å	Angstrom
D_x	Calculated crystal density
K	Kelvin
ºC	Degree Celsius
U_{eq}	Anisotropic temperature factor
U^{ij}	Temperature factor
U*iso*	Isotropic temperature factor
α	Alpha
ß	Beta
Δ	Stoichiometric variable
έ	Epsilon
Θ	Theta
K	Kappa
Φ	Phi
Ψ	Psi

APPENDICES

ABSTRAK

Kajian Anti Kanser Asid Koetjape yang Ditulenkan dari *Sandoricum koetjape* Merr.

Barah kolorektum yang melibatkan kolon, rektum dan kanal dubur merupakan keadaan kemaglinan ketiga paling berbahaya di dunia. Penyakit itu disifatkan oleh angiogenesis yang meluas dan usikan pelbagai jenis onkogen dan gen-gen penghalang pertumbuhan tumor termasuk p53, Wnt, hipoksia, NFκB, Notch dan kinase MAP. Mensasarkan satu atau lebih laluan-laluan ini meningkatkan pemilihan ejen-ejen kemoterapeutik kerana laluan-laluan ini adalah penting untuk pertumbuhan sel-sel kanser dan percambahan. Asid koetjapik ialah seco-A-ring (empat gelang-A) oleanene triterpena yang wujud secara semulajadi di dalam spesis *Sandoricum koetjape* yang tumbuh di Malaysia dan di rantau Asia Tenggara. Dalam kajian ini, satu cara baru penulenan asid koetjapik telah dibangunkan untuk menghasilkan kristal-kristal tulen asid koetjapic pada kadar hasil yang tinggi. Struktur sebatian ini telah direlaikan dan disahkan menggunakan pelbagai kaedah fizikal dan kimia yang termasuk: kristalografi sinar-x, ^{1}H NMR, IR dan spektra jisim, dan penentuan takat lebur. Kegiatan sitotoksik asid koetjapik telah ditaksir menggunakan ujian MTT ke atas empat jenis sel kanser manusia iaitu; karsinoma kolorektal (HCT 116), kanser payudara hormon sensitif (MCF 7), kanser payudara rintangan hormon (MDA-MB-231) dan kanser hati (Hep G2), sebagai tambahan, dua jenis sel normal manusia telah diuji; kolon (CCD-18Co) dan sel-sel endotelium pembuluh tali pusat (HUVECs). Keputusan telah menunjukkan asid koetjapik ialah satu agen sitotoksik sederhana tetapi mempamerkan pemilihan menentang sel HCT

116 dengan IC_{50} (kepekatan separuh perencat) 18.88 μg/ml. Mekanisme-mekanisme sel dan molekul aktiviti antikanser asid koetjapik ke atas barah kolorektum manusia telah dikaji. Asid koetjapik didapati telah menyebabkan 'apoptosis' dalam sel-sel barah kolorektum HCT 116. Perubahan-perubahan awal yang mengiringi proses apoptosis telah dikaji melalui penilaian pengaktifan caspase. Asid koetjapik didapati telah menyebabkan pengaktifan 'caspase' hilir dan 'caspase' huluan. Kegiatan apoptotik seterusnya disahkan dengan permerhatian perubahan akhir morfologi di dalam sel-sel yang dirawat; asid koetjapik telah menyebabkan penyerpihan nuklear dan pemeluwapan serta gangguan dalam fungsi mitokondria. Kesan asid koetjapik pada aktiviti faktor-faktor transkripsi 10 laluan utama yang terlibat dalam proses karsinogenesis kanser kolon dan kanser yang lain juga dikaji. Keputusan telah menunjukkan bahawa sebatian itu telah menyebabkan pengawalaturan menurun isyarat laluan-laluan Wnt, HIF-1, MAP / ERK / JNK dan MYC/Max dan pengawalaturan menaik isyarat laluan NFκB. Di samping itu, kajian juga telah dijalankan untuk menyiasat ciri-ciri 'antiangiogenic' asid koetjapik sebagai kanser kolon adalah sangat angiogenic.

Hasil ujikaji telah menunjukkan yang asid koetjapik mempunyai kegiatan 'antiangiogenic' yang berkesan dengan menghalang pembentukan tiub endotelium, penghijrahan, dan penindasan ekspresi Faktor Pertumbuhan Pembuluh Endotelial (VEGF). Keputusan kajian ini secara jelasnya menekankan potensi antikanser asid koetjapik terhadap barah kolorektum.

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ABSTRACT

Colorectal cancer which involves the colon, rectum and the anal canal is the third most common malignancy worldwide. The disease is characterized by extensive angiogenesis and perturbation of a variety of oncogenes and tumor suppressor genes including, p53, Wnt, hypoxia, NF-κB, Notch, MAP kinase. Targeting one or more of these pathways increases the selectivity of the chemotherapeutic agents as these pathways are important for cancer cells growth and proliferation. Koetjapic acid is a naturally occurring seco-A-ring oleanene triterpene present in the *Sandoricum koetjape* species that grows in Malaysia and the Southeast Asian region. In this study, a new method of koetjapic acid purification was developed to produce pure koetjapic acid crystals at high yield. The structure of the compound was resolved and confirmed using various physical and chemical methods which include: X-ray crystallography, ^{1}H NMR, IR, and mass spectra, and melting point determination. The cytotoxic activity of koetjapic acid was assessed using MTT test against four human cancer cell lines namely; colorectal carcinoma (HCT 116), hormone sensitive breast cancer (MCF 7), hormone resistance breast cancer (MDA-MB-231) and liver cancer (Hep G2) cell lines, in addition, two normal human cell line were tested; colon (CCD-18Co) and umbilical vascular endothelial cells (HUVECs) . The results showed that KA is a modest cytotoxic agent but exhibited selectivity against HCT 116 cell line with IC_{50} 18.88 μg/ml. The cellular and molecular mechanisms of

anticancer activity of koetjapic acid towards human colorectal cancer were investigated. Here we find that koetjapic acid induces apoptosis in HCT 116 colorectal cancer cells. The early changes accompanying the apoptosis process were investigated by assessment of caspase activation. Koetjapic acid was found to cause activation of downstream and upstream caspases. The apoptotic activity was further confirmed by observing the late morphological changes in the treated cells; Koetjapic acid caused nuclear fragmentation and condensation as well as disruption in the mitochondrial functioning. We also investigated the effect of KA on the activity of the transcription factors of the major ten pathways involved in carcinogenesis of colon cancer and other cancers as well. The results showed that the compound causes down-regulation of Wnt, HIF-1, MAP/ERK/JNK and Myc/Max signaling pathways and up-regulation of the NF-κB signaling pathway.

In addition, we also investigate the antiangiogenic properties of koetjapic acid as colorectal cancer is highly angiogenic. The results showed that koetjapic acid has significant antiangiogenic activity by inhibiting endothelial tube formation, migration, and suppression of Vascular Endothelial Growth Factor (VEGF) expression. The results of this study clearly highlight the anticancer potential of KA towards colorectal cancer.

CHAPTER 1: INTRODUCTION

1.1. Cancer:

Cancer is a group of more than 100 different diseases which characterized by uncontrolled cellular growth, local tissue invasion and distant metastasis (Chabner, 2006). It has been difficult to develop an accurate definition for cancer. The reputed British oncologist Willis has defined cancer as "an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change" (Cotran et al., 1999).

The mechanisms by which cancer arises are incompletely understood. The cancer is assumed to develop from cells in which the typical managing mechanisms of the cells proliferation and growth have been altered. Recent proofs strengthen the notion of carcinogenesis as a genetically regulated multistage process (Mediana and Fausel, 2008). The first step in this process is 'initiation' which starts by exposure of cells to carcinogenic substances which lead to genetic damage that, if not repaired, results in irreversible mutations. The mutated cells grow till formation a colony. The second stage is 'promotion', in which carcinogens or other factors modify the environment in a way supports growth of mutated cells over normal cells (Mediana and Fausel, 2008). The next stage is 'transformation' of mutated cells to cancerous cells, about 5-20 years may require for the transition of benign carcinogenic phase to the fully developed malignant stage where the cancer can be detected clinically. The last stage called 'progression', where further genetically changes take place leading to increase the proliferation and metastasis (Weinberg, 1996, Compagni and Christofori, 2000).

1.2. Cancer Epidemiology:

Cancer is the major public health problem in many parts of the world. Over ten million new cases of cancer, with over six million deaths were estimated in the year 2000 (Parkin, 2001). The estimated numbers of cancer incidence and mortality in 2002 were markedly increased with 10.9 million new cases and 6.7 million deaths (Parkin et al., 2005). Even developed countries suffering from cancer, in USA 2677860 new cases of cancer have been diagnosed in 2009. In that same year, more than 562,340 cancer related deaths occurred which represents 25% of all deaths making cancer as the second leading cause of death after heart diseases (Jemal et al., 2009).

The most common cancer in men is prostate cancer which accounts for 25% of newly diagnosed cancer, followed by cancers of lung and bronchus, and colon and rectum. While the most common cancer in women is breast cancer which represents 27% of all new cancers in women, followed by cancers of lung and bronchus, and colon and rectum (Jemal et al., 2009).

Cancers of the lung and bronchus, prostate, and colorectal in men, and cancers of the lung and bronchus, breast, and colorectal in women are the most common fatal cancers. These four cancers form 50% of cancer deaths among men and women (Jemal et al., 2009).

The 5-year survival rates have been increased as new drugs and treatment approaches have been introduced; in 1930s, only 20% of cancer patients survived five years or more after treatment. In 1940s the survival rates increased to 25%. During the 1960s, the 5-years survival was 33%, and in the 1970s it was 38%. Between year 2000-2005, 51% of cancer patients survived for five years or more (Rodriguez and Case, 2005).

However, while the survival rate increases, the incidence and mortality rates also keep on growing (Parkin, 2001). According to WHO deaths from cancer are projected to continue rising. The estimated deaths in 2030 is around 12 million (Mutanen and Pajari, 2011). This contradiction between increasing the 5-years survival rate and increasing the worldwide death rates due to cancer can be explained by increased life anticipation, since cancer incidence increases with the age (Ries et al., 2000, Jemal et al., 2009).

1.3. Genetic and Molecular Basis of Cancer:

Developing cancer cells select mutations having two basic functions: mutations which increase the activity of the proteins they code for or mutations which inactivate gene function. The gene whose activity increases by mutation is called oncogene, while gene inactivated by mutation is called tumor suppressor gene.

Oncogenes are involved in signaling pathways which stimulate proliferation, while human suppressor genes code for proteins which normally act as checkpoints to cell proliferation. These alterations occur by carcinogenic agents like radiation, chemicals or viruses (somatic mutations), or they may be inherited (germ-line mutation) (Croce, 2008, Bertram, 2000, Mediana and Fausel, 2008, Yarbro et al., 2005).

Six major pathways must be activated or inactivated in the genes of normal cells to convert to cancerous cell (Hanahan and Weinberg, 2000), these are: development of independence in growth stimulatory signals (e.g., activation of a family of oncogenes called human epidermal growth factor receptor (Cohen and Carpenter, 1975)), development of a refractory state to growth inhibitory signals (e.g., mutations in suppressor genes p53 (Feng et al., 2008)), development of resistance to programmed cell death (e.g., over expression of *Bcl-2* genes (Kroemer, 1997)), development of an infinite

proliferative capacity (e.g., over expression of telomeras enzyme (Holt and Shay, 1999)), development of angiogenic potential i.e., the capacity to form new blood vessels and capillaries (e.g., over expression of VEGF (Folkman, 1995)), and tissue invasion and metastasis (e.g., over expression of Myc oncogenes (Kawashima et al., 1988)). Table 1.1 depicts some genes related with cancer incidence.

1.4. Cell Death and Apoptosis:

Cell death is a key event in biology. Cells die via two main processes; either necrosis or apoptosis (programmed cell death) (Kerr et al., 1972). Apoptosis is firmly regulated by complex molecular signaling systems. Apoptosis plays a key role in development, morphogenesis, tissue remodelling and disposing of aged or damaged cells. The initial definition of apoptosis was morphological: "Dying cells exhibit a characteristic pattern of changes, including cytoplasmic shrinkage, active membrane blebbing, chromatin condensation, and, typically, fragmentation into membrane-enclosed vesicles (apoptotic bodies)" (Wyllie et al., 1980). In this process the cells activate an intracellular death programme and kill themselves in a controlled way. Because apoptotic cells shrink during this process, they are rapidly digested, thus, there are no leakages of their contents (Raff, 1998). Figure 1.1 demonstrates the morphological changes occurring during the apoptosis process.

Table 1.1 Examples of some oncogenes and tumor suppressor genes (Kintzios, 2003).

Type of gene	*Gene*	*Cancer type*
Oncogene	*PDGF*	Glioma
Oncogene	*Erb-B*	Glioblastoma, breast
Oncogene	*RET*	Thyroid
Oncogene	*CDKN2*	Melanoma
Oncogene	*Ki-ras*	Lung, ovarian, colon, pancreatic
Oncogene	*HPC1*	Prostate
Oncogene	*N-ras*	Leukemia
Oncogene	*N-myc*	Neuroblastoma, glioblastoma
Oncogene	*Bcl-1*	Breast, head, neck
Oncogene	*MDM2*	Sarcomas
Oncogene	*c-myc*	Leukemia, breast, stomach,lung
Oncogene	*BCR-ABL*	Leukemia
Tumor suppressor gene	*p53*	Various
Tumor suppressor gene	*RB*	Retinoblastoma, bone, bladder, small cell lung, breast
Tumor suppressor gene	*BRCA1*	Breast, ovarian
Tumor suppressor gene	*BRCA2*	Breast
Tumor suppressor gene	*APC*	Colon, stomach
Tumor suppressor gene	*MSH2, MSH6, MLH1*	Colon
Tumor suppressor gene	*DPC4*	Pancreas
Tumor suppressor gene	*CDK4*	Skin
Tumor suppressor gene	*VHL*	Kidney

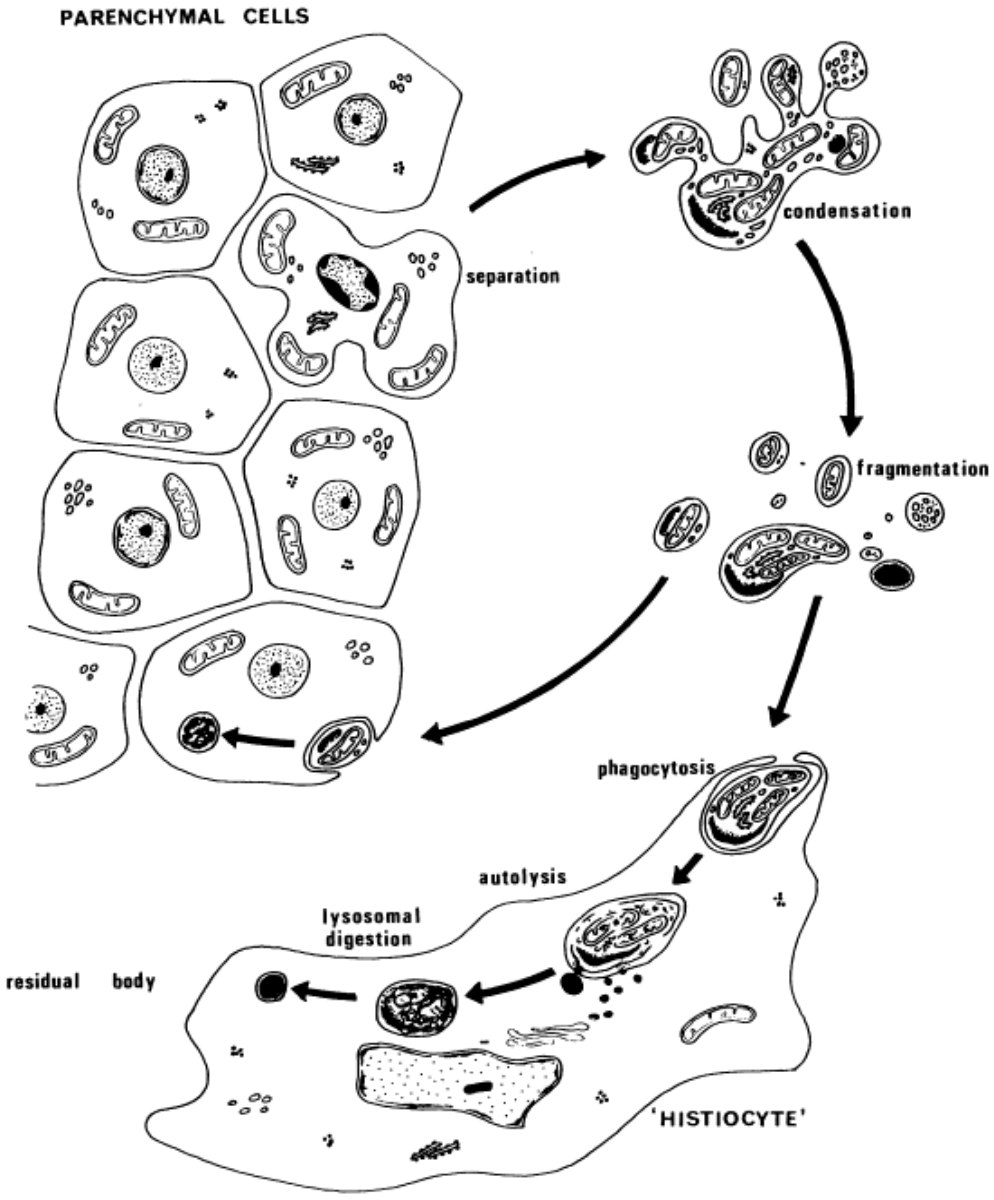

Figure 1.1 Cell morphological changes occurring during apoptosis process (Kerr et al., 1972).

In contrast, necrosis is a term that describes uncontrolled process in which death takes place after exposure to an acute injury. The cells swell and burst, spilling their content over the surrounding tissue and cause inflammation (Raff, 1998). Table 1.2 depicts comparison between some of the major features of apoptosis and necrosis. Any change in apoptosis rate in the body, either increasing or decreasing may cause many diseases. Apoptosis hyper-activation associated with neurodegenerative diseases (e.g., Parkinson's and Alzheimer's syndromes), hematologic diseases (e.g., Aplastic anaemia and lymphocytopenia) and disease characterized with tissue damage (e.g., myocardial infarction). On the other hand, increasing cell survival via apoptosis inhibition is related to autoimmunity diseases (e.g., Systemic lupus erythematosus) and tumor growths (Chamond et al., 1999).

The notion of a strong link between apoptosis and cancer developed after studying the tumor growth kinetics. These studies showed that changes in 'cell loss factor' have influence on tumor growth or regression. Further studies revealed a high rate of apoptosis occurrence in tumors treated with cytotoxic agents that regressed after treatment (Kerr et al., 1972).

Apoptosis can be induced through a number of pathways by proteins that control the cell cycle machinery including p53, Wnt, hypoxia, NF-κB, Notch and MAPKs pathways (Ghobrial et al., 2005). Any defect in these regulatory pathways have been related to many malignancies (Kaufmann and Hengartner, 2001).

Table 1.2 Comparison between some of the major features for apoptosis and necrosis (Devarajan, 2009).

Feature	Apoptosis	Necrosis
Cell volume	Decreased	Increased
Plasma membrane integrity	Preserved	Lost
Plasma membrane structure	Characteristic blebbing	Lost
Cell-cell adhesion	Lost early	Typically preserved
Cell matrix adhesion	Lost early	Lost late
Exfoliation of cells	Early, as single cells	Late, as sheets of cells
Chromatin	Condensed	Preserved
Nuclear fragmentation	Characteristic	Absent
Cytosolic contents	Preserved	Released
Apoptotic bodies	Characteristic	Absent
Phagocytosis	Characteristic	Absent
Inflammatory response	Absent	Characteristic

1.4.1. Apoptosis Pathways:

Apoptotic signaling events can be divided into two major pathways based on the mechanism of initiation: the intrinsic pathway which mainly depends on mitochondrial changes, and the extrinsic pathway which is activated via death receptors. Although different molecules take part in the core machinery of both apoptosis signaling pathways, a crosstalk exists at multiple levels (Ghobrial et al., 2005).

Apoptotic caspases could be classified into two classes, effector (downstream) caspases, which are responsible for the cleavages that disassemble the cell, and initiator (upstream) caspases, which initiate the proteolytic cascade and activate the effector caspases. Effector caspases include caspase 3, 6 and 7; their function is cleaving the polypeptides that go through proteolysis in apoptosis process. Table 1.3 depicts examples of some cellular caspase substrates classified according to their function in apoptosis (Lamkanfi et al., 2002).

Upstream caspases include caspase 8 and caspase 9. The upstream caspases can be initiated through the extrinsic pathway or the intrinsic pathway. The extrinsic pathway starts with ligation of the death receptors (e.g., CD95 (Fas) and the tumor necrosis factor-α receptor 1 [TNFR1], DR3, DR4, DR5, and DR6). The ligation results in receptor trimerization followed by binding of the adaptor molecule FADD to the cytoplasmic domain of the receptor. FADD in turn activates caspase 8 zymogen. The caspase 8 enzyme will then cleave procaspases 3 and 7 (Medema et al., 1997, Cohen, 1997, Ghobrial et al., 2005, Boatright and Salvesen, 2003, Earnshaw et al., 1999). On the other hand, changes in the conformation or activity of Bcl-2 protein initiate the intrinsic apoptotic pathway. Bcl-2 is a protein located in the outer mitochondrial membrane.

Upon activation of pro-apoptotic members of Bcl-2 such as Bak and Bax the mitochondrial membrane potential decreases, as a result the mitochondrial permeability increases which allows the releasing of cytochrome c.

Table 1.3 Examples of some cellular caspase substrates classified according to their function in apoptosis (Lamkanfi et al., 2002).

Effect on the cell	Substrate	Caspase
Disassembly of the cytoskeleton, loss of cell to cell contact, disintegration and fragmentation of the cell.	Actin	8
	Plectin	8
	α-Adducin	3
	β-Catenin	3,7,8
	E-cadherin	3,7
	Desmoglein-3	3,7
	Vimentin	3,6,7,9
	Cytokeratin-18	3,6,7
	Fak	3,7
Blebbing of the membrane	ROCK-I	3
Nuclear breakdown	Lamin A	6
	Lamin B	3
Chromatin condensation	Acinus	3
DNA degradation	ICAD/DFF-45	3
Loss of DNA repair	PARP	3,7,9
Inhibition of DNA replication	DNA-RC	3
	Topoisomerase	3
Disintegration of the Golgi complex	Golgin-160	2
Inhibition of the transport from the endothelium reticulum to the Golgi	BCAP31	3,8
Disruption of the mitochondria and amplification of the apoptotic signal	Bid	3,8
	BAX	3
	Bcl-2	3
	Bcl-X_L	1,3

In turn, cytochrome c activates caspase 9 zymogen which activates caspases 3 and 7. Figure 1.2 demonstrates the extrinsic and intrinsic pathways of apoptosis process (Boatright and Salvesen, 2003, Inoue et al., 2009).

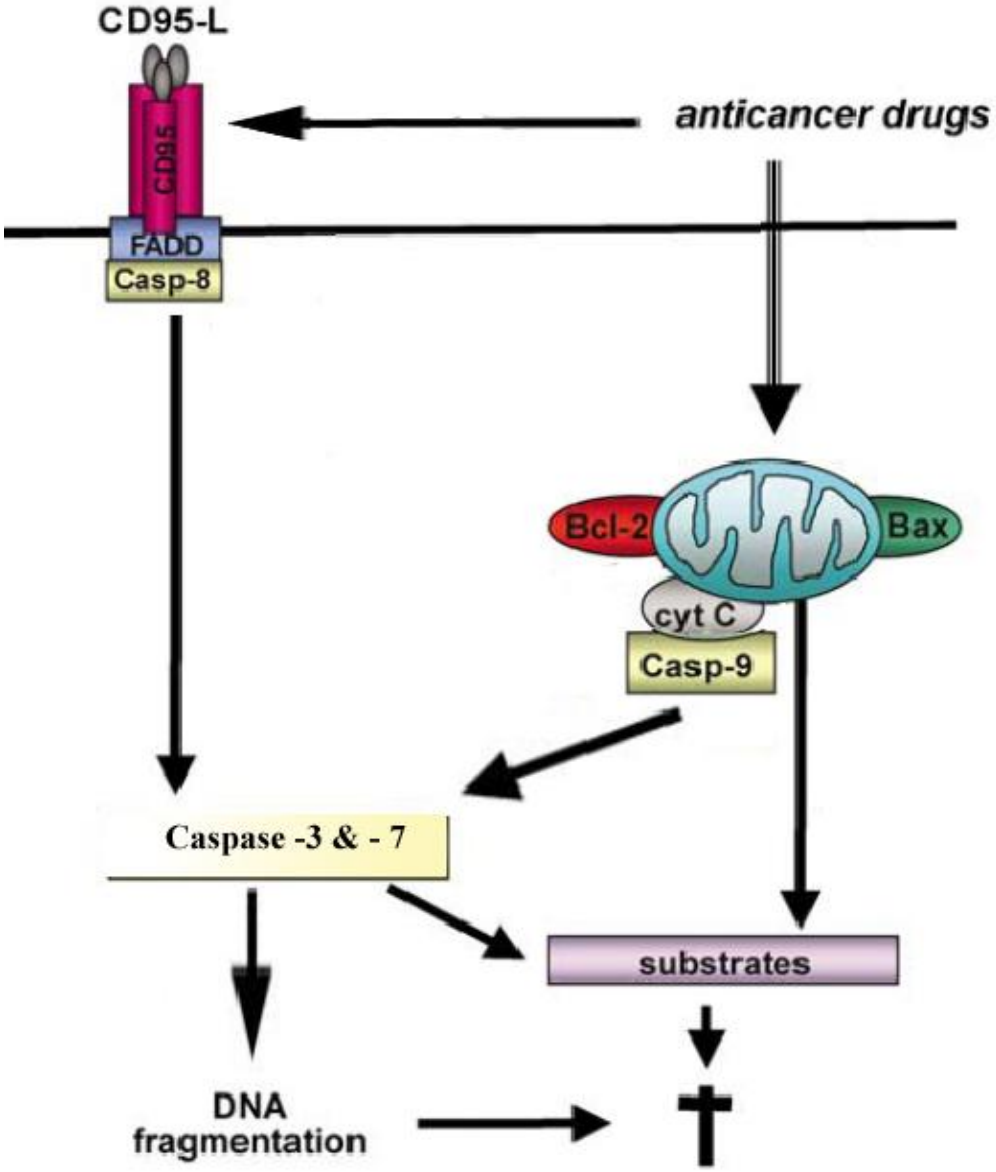

Figure 1.2 The extrinsic and the intrinsic pathways of apoptosis, adopted from (Fulda and Debatin, 2006).

1.4.2. Signal Transduction Pathways in Cancer:

Cancer cells have the ability to change the surrounding environment in a way that will assist them to grow and proliferate. They respond to any internal or external circumstances by increasing or decreasing the expression of proteins which can adjust the situations in favour of increasing the proliferative, invasive and metastatic properties (Hanahan and Weinberg, 2000). The reciprocal communications between the external or internal circumstances and protein expression level take place via activation of a cascade of intracellular biochemical reactions which is also called signal transduction pathways (Lobbezoo et al., 2003). Each pathway starts with ligation of extracellular receptors. The receptor activation is translated into biological response by activation of proteins (transcriptional factors) which then translocate into the nucleus and bind with the DNA in specific binding sites (promoters) and trigger the transcription of mRNAs which later translated to proteins (Eccleston and Dhand, 2006).

Oncogenic gene mutations results in a constitutive activation of signal transduction elements, simulating a condition of permanent activation of the receptor, even in the absence of the relevant growth factor (Hanahan and Folkman, 1996).

Wnt, Notch, TGF-β, Myc/Max, Hypoxia, MAPK pathways were reported to be hyper-activated in cancerous cells (Clevers, 2004, Miyazawa et al., 2002, Fang and Richardson, 2005, Soucek et al., 2008, van Es and Clevers, 2005a).

On the other hand, mutations in tumor suppressor genes lead to deactivation of some pathways which may serve as checkpoints of cells proliferation such as p53 (Feng et al., 2008). These pathways can be targeted with signal transduction modulators (STMs) in order to treat cancer. The STMs can modulate the pathway activity at many levels such as blocking cell surface receptors, blocking the mediators between

extracellular signals and the transcriptional factor, deactivate the binding between the transcriptional factors with the promoters or inhibiting the effects of further downstream genes (Lobbezoo et al., 2003).

STMs have attracted attention of many researchers. Many STMs compounds are being investigated in preclinical studies or in clinical trials. Additionally, there are two approved STMs drugs which have been commercially marketed; trastuzumab and imatinib (Lobbezoo et al., 2003).

1.4.2 (a) Wnt /β-catenin Signaling Pathway:

Wnt signaling pathway plays a crucial role in development process as well as cancer by controlling gene expression, cell behaviour, cell polarity and cell adhesion (Cadigan and Nusse, 1997). Wnt signals work through three pathways: Wnt /β-catenin pathway (referred to as canonical Wnt pathway) and the non-canonical Wnt/Ca^{+2} and Wnt/JNK pathways (Moon et al., 2002).

The mutations of many components of Wnt /β-catenin pathway were detected in many types of human cancers such as: colon cancer, melanoma, prostate and breast cancer (Morin et al., 1997, Verras and Sun, 2006, Lin et al., 2000, Chien et al., 2009). Moreover, it was found that 80% of sporadic colon cancer patients have mutation in a tumor suppressor gene called APC, which function was identified as a down-regulator of Wnt pathway (Calvert and Frucht, 2002). It is widely accepted now that mutations either in APC or Wnt /β-catenin pathway are the earliest events in colon oncogenesis (Kinzler and Vogelstein, 1996).

The Wnt /β-catenin pathway controls the expression of a number of important oncogenes such as: c-Myc, cyclin D1 and matrix metalloproteinase genes which are vital

in carcinogenesis as well as angiogenesis (Dihlmann and Magnus, 2005). Down-regulation of Wnt pathway with the aim of decreasing these genes expression could regress the tumor proliferation as verified in one study which targeted expression of cyclin D1 (Tetsu and McCormick, 1999).

1.4.2 (b) Notch Signaling Pathway:

Notch cell signaling pathway is involved in a variety of cellular functions such as cell fate specification, differentiation, proliferation, apoptosis, adhesion, migration, and angiogenesis (Bolos et al., 2007). The signaling cascade starts with the ligation of the extracellular four isoforms of Notch receptors (Kojika and Griffin, 2001). In the 1990s, the relation between Notch pathway and cancer was identified after a study which showed that 10% of T-cell lymphoblastic leukemia patients have constitutive activation of Notch 1 receptor (Callahan and Raafat, 2001). Further *in vivo* and *in vitro* studies supported the idea that activation of any of Notch isoforms is well-correlated with tumor growth and aggressiveness properties (Callahan and Raafat, 2001). Hyper-activation of Notch pathway signaling has been noticed in many types of cancer, including pancreas, breast, colon, renal, melanoma and lung cancers (Wang et al., 2006, Farnie and Clarke, 2007, Sun et al., 2009, Radtke and Clevers, 2005, Strizzi et al., 2009, Collins et al., 2004).

Many studies reported the strong relation between Notch and Wnt pathways in colon cancer (van Es and Clevers, 2005b, De Strooper and Annaert, 2001, Fre et al., 2009). In mutant APC mice (the tumour suppressor gene of Wnt pathway), it was found that Wnt pathway signaling as well as Notch pathway were hyper-activated, the results strengthen the hypothesis that Notch signaling might be in a downstream of Wnt

pathway. Moreover, the two pathways may work synergistically, hence both Notch and Wnt inhibitors may be combined in colon cancer treatment (van Es and Clevers, 2005b). Several approaches to block Notch pathway have been under investigations, among them: antisense, RNA interference and monoclonal antibodies (Nickoloff et al., 2003).

1.4.2 (c) p53 Signaling Pathway:

The p53 gene mutation is extremely common on all cancers; p53 is suppressed in more than 50% of all human cancer cases. p53 mutations causes activation of other oncogenic pathways, making tumor more aggressive and resistant to chemotherapy as well as radiation (Kumar et al., 2004). The relation of p53 and cancer was presented in 1980s and p53 has been called as a "Guardian of the Genome'' referring to its ability in induction of apoptosis and cell cycle arrest. p53 protein encodes many type of genes which are involved in cell cycle, apoptosis and angiogenesis. p53 controls cell death by regulating the two apoptotic pathways genes, the death receptor Fas and DR-5 genes which are involved in extrinsic pathway as well as Bax, Bak and Bid proteins which are involved in the mitochondrial pathway (Frank et al., 2004). The impact of p53 in apoptosis process was demonstrated in a study which showed that the apoptosis process has been slowed down significantly in p53 knockout-mice and as a result the tumor became more drug resistance (Lowe et al., 1993).

Restoring the p53 protein and correction its defects may perhaps be useful in treating cancer. Different approaches have been used which have showed remarkable success in many cancers such as cervical, head and neck, lung, ovarian and prostate (Clayman et al., 1995).

1.4.2 (d) TGF-β Signaling Pathway:

TGF-β signaling pathway is described as a double-edged sword, the tumor suppressor and oncogenic properties of this pathway were reported in many studies (Akhurst and Derynck, 2001, Sánchez-Capelo, 2005, Akhurst, 2002). In term of tumor suppression properties of TGF-β, one study have shown that TGF-β defect- mice were more susceptible to tumor incidence than normal mice (Tang et al., 1998). Besides, transgenic mice in which the TGF-β is hyper-activated were found to be more resistant for mammary tumor formation (Pierce et al., 1995). On the other hand, it was confirmed that tumor cells secret TGF-β proteins *in vitro* more than normal cells (Roberts et al., 1983). TGF-β plasma concentrations as well as TGF-β urinary excretion rate of cancer patients were higher than normal values (Nishimura et al., 1986, Tsushima et al., 1996). Additionally, a strong correlation between TGF-β concentrations and tumor metastasis, invasive and angiogenesis has been confirmed (Bierie and Moses, 2006). All these studies indicate that TGF-β has a negative impact on tumor prognosis (Tsushima et al., 1996). Many studies conclude that the over expression of TGF-β pathway can work as tumor suppressor gene at the early stages of cancer, however, after that this pathway serve as an oncogenic pathway and supports angiogenesis, metastasis and invasive properties of tumor cells (Bierie and Moses, 2006, Massagué, 2008). Nevertheless, the obvious mechanistic explanation of the dual effects is still ambiguous.

Targeting this pathway has shown promising results in cancer treatment, using techniques such as antisense and ligand-receptor binding inhibition by using antibodies targeting the TGF-β protein or the receptors (Massagué, 2008). However, the pharmaceutical companies still fear to produce any target of this pathway because of the

non-selectivity and potential side effects which may arise from the dual activity (Akhurst, 2002).

1.4.2 (e) Cell Cycle (pRB/ E2F) Signaling Pathway:

The retinoblastoma tumor suppressor (pRB) is an essential contributor in apoptosis and cell cycle processes. The pRB gene which encodes pRB proteins has been found to be mutated in approximately 50% of all human tumors. Additionally, genes encoding upstream regulators of pRB have been reported to be mutated in the remaining 50% of all human tumors (Frank and Yamasaki, 2004).

Studies on retinoblastoma cases showed that more than 40 % of clinical cases are hereditary due to the inactivation of pRB tumor suppressor gene (Draper et al., 1992). Using DNA cloning techniques, the influence of pRB protein was confirmed in other type of cancers such as bladder, breast, lung, leukemia and prostate (Weinberg, 1991). *In vitro* experiments which involved introducing pRB protein in cancer cells causes inhibition of cell proliferation at stage S of the cell cycle (Bandara and La Thangue, 1991). The role of pRB has been noticed in other types of cancer such as pituitary adenocarcinomas, pheochromacytomas and thyroid C-cell adenomas as have been shown in pRB knockout mice tumor model (Harrison et al., 1995, Nikitin et al., 1999).

The pRB signaling pathway is activated by binding the pRB protein with many transcriptional factors, among them E2F seems to be the most important (Bandara and La Thangue, 1991). The active dimer then binds with its promoters which control the expression of many vital genes involved in cell death process such as c-Myc, thymidylate synthase, N-Myc, cdc2, thymidine kinase, cyclin A, dihydrofolate reductase and DNA polymerase (Helin and Ed, 1993).

1.4.2 (f) NF-κB Signaling Pathway:

NF-κB suppresses cell death and supports cell growth, metastasis and angiogenesis. More than 200 target NF-κB genes have been identified, among them: Myc, Rel, and Cyclin D1-4 which are involved in cell cycle regulation, Bcl-2, Bcl-Xl, A1/Bf-1 which play important roles in the apoptosis process, VEGF gene which is essential in angiogenesis process, and urokinase plasminogen activator which plays important role in cell metastasis (Pahl, 1999). Previous studies have shown that NF-κB safe guards tumor cells from apoptosis (Barkett and Gilmore, 1999). NF-κB knocked-out mice experiments proof the oncogenic roles of this pathway (Beg et al., 1995). In other studies, activation of this pathway inhibited tumor regression and cell apoptosis (Li et al., 1999, Chaisson et al., 2002, Schmidt-Supprian et al., 2000).

The oncogonic activity of NF-κB inspired researchers to synthesize compounds that target this pathway; for instance, cinnamaldehyde which was reported as an apoptosis inducer agent acting via mitochondrial pathway, has been reported recently as a potent NF-κB pathway inhibitor (Hyeon et al., 2003, Reddy et al., 2004).

1.4.2 (g) Myc/Max Signaling Pathway:

Myc/Max pathway has been found to be hyper-activated in 70% of all human cancer cases which strongly suggesting the oncogenic nature of this pathway (Nilsson and Cleveland, 2003). The Myc/Max pathway was also found to play important roles in the cell cycle process, and the lack of this protein prevents the cell cycle from proceeding beyond the S phase (Heikkila et al., 1987). Besides its role in cell proliferation, Myc/Max also plays a role in triggering the angiogenic switch in favour of

angiogenesis initiation (Pelengaris et al., 1999). Dimerization with Max and then binding to DNA are necessary to exhibit all Myc biological effects (Evan et al., 1992).

A significant tumor regression in transgenic mice was attained by knocking-down the Myc/Max which paving the way to a more directed efforts in aim of targeting this pathway (Pelengaris and Khan, 2003).

1.4.2 (h) MAPK Signaling Pathways:

There are three sub-families of MAPK proteins: extracellular signal regulated enzyme kinases (MAPK/ERK), p38 MAPKs and the c-Jun amino terminal kinase (JNKs). While the main function of MAPK/JNKs and MAPK/ERKs are in cell cycle, regulation of mitosis, migration and apoptosis, the MAPK/p38 function is involved in inflammation (Johnson and Lapadat, 2002).

The activation of these pathways is attained by ligation the extracellular receptor Ras. Upon activation, the MAPK signaling requires the activation of three MAPK chains; starting with MAPKKK which then activates MAPKK which consequently activates MAPK (Makin and Dive, 2001).

Upon treatment with MAPK signaling inhibitors, the cell cycle process is arrested and cell proliferation is inhibited in many cell lines such as: smooth muscle, epithelial, T lymphocytes, fibroblasts and hepatocytes cell lines (Meloche and Pouyssegur, 2007).

The mechanisms by which these pathways exhibit their functions were studied thoroughly. The ability of MAPKs to regulate the cell growth was explained by it is ability to control global protein syntheses by activating the translation of the initiation factor eIF4E as well as by direct regulation of ribosomal gene transcription (Stefanovsky et al., 2001, Morley and McKendrick, 1997). Also, it has been found that the production

of pyrimidine - which is important in DNA and RNA synthesis- is under the control of this pathway (Evans and Guy, 2004). This pathway is essential for G1- to S-phase progression. MAPKs also serve in stabilization of c-myc protein (Sears et al., 2000). Additionally, MAPKs down regulate more than 170 tumor suppressor genes such as: *Tob1*, *JunD* and *Ddit3* which inhibit cell growth and proliferation (Yamamoto et al., 2006).

Based on the crucial oncogenic activities, huge efforts were attempted in order to produce inhibitors of the ERK and JNK pathways, some of the them are in clinical trials (Kohno and Pouyssegur, 2006).

1.5. Angiogenesis:

Angiogenesis research is the cutting edge technology that is currently being heavily exploited in the cancer field (DeWitt, 2005). Angiogenesis research will probably change the face of medicine in the next decades, more than 500 million people worldwide are expected to benefit from pro- or anti-angiogenesis treatments (Carmeliet, 2005). Angiogenesis is a process of new blood vessel development orchestrated by a range of angiogenic factors and inhibitors. This process is tightly regulated and self limiting in some cases such as wound healing, normal growth process and reproductive function (Folkman and Klagsbrun, 1987). In contrast, when this process is deregulated, diseases such as cancer, rheumatoid arthritis, obesity and diabetic blindness can be formed (Carmeliet, 2005, Folkman, 1995). Angiogenesis plays an important role in cancer growth without which, tumors will be unable to expand beyond 1 to 2 mm^3 (Folkman and Cotran, 1976). Cancer cells within the tumor will then use the newly formed blood vessels as a port to metastasize to other localities (Weidner et al., 1991).

Since the interdependency and a close relationship between angiogenesis, cancer growth and metastasis has been well-established, much effort have been invested into development or discovery of antiangiogenic compounds activity to target cancer and variety of other angiogenic related ailments.

1.5.1. The Vascular Endothelial Growth Factor (VEGF):

As the size of the tumor increases, oxygen demand increases causing a state of hypoxia (Fu et al., 1976). The hypoxic state in the tumor spring forth oxygen free radicals which in turn activates vascular endothelial growth factor (VEGF) triggering the angiogenesis event (Mukhopadhyay et al., 1995). VEGF (referred to also as VEGF-A) had been regarded as a heparin binding angiogenic growth factor exhibiting high specificity for endothelial cells (Gospodarowicz et al., 1989). VEGF is responsible for triggering various steps in the angiogenesis cascade such as proliferation, migration and cell survival (Ferrara, 2002). The tumor regression and inhibition can be achieved by deactivating VEGF activity via neutralizing antibodies or by introduction of dominant negative VEGF receptors (Kim et al., 1993). The VEGF was found significantly upregulated at the levels of RNA and protein in most types of cancer. The high concentration of VEGF in cancer patients is associated with poor prognosis as well as with low survival (Paley et al., 1997).

The activities of VEGF family are mediated by three tyrosine kinase receptors, VEGFR-1 (Flt l), VEGFR-2 (Flkl/KDR), and VEGFR-3 (Flt4) (Ferrara et al., 2003). VEGF –A belongs to a gene family that includes placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. The main difference between these proteins is the type of receptors they bind and activate; PlGF, VEGF-B can bind with VEGFR-1,

VEGF-C and VEGF-D bind with VEGF-3, and VEGF-A can bind with two receptors: VEGFR-1 and VEGFR-2 (Shibuya, 2001).

Alternative exon splicing results in five different isoforms of VEGF-A: VEGF121, VEGF143, VEGF165, VEGF189, and VEGF206. VEGF165 is the predominant isoform as well as it have the strongest signal transduction because its ability to bind with an extracellular molecule called neuropilin-1, the binding with neuropilin-1 results in increasing the affinity with VEGFR-2 to about ten folds (Park et al., 1993, Shibuya, 2001).

1.5.2. Angiogenesis Process Cascade:

Angiogenesis is a sophisticated multistep process. Figure 1.3 illustrates the angiogenesis process steps. Increasing of VEGF-165 expression is crucial in initiating the angiogenesis process (Nagy et al., 2003). Angiogenesis starts with dilatation of blood vessels to increase the permeability to the angiogenesis signals. Then, the pericytes which covers the blood vessels, detach and the vascular basement membrane and extracellular matrix gets degraded (Fig.1.3b), which allow for the underlying endothelial cells to migrate into the perivascular space towards chemotactic angiogenic stimuli (Fig.1.3c) (Ko et al., 2007). The migrated endothelial cells proliferate, loosely following each other into the perivascular space and form migration columns (Fig.1.3d). Then, the endothelial cells differentiate; cells' shape change in a way that facilitates the cell-cell adherence which then forms a lumen (tube-like structure). Perivascular cells are attracted, and a vascular basal lamina is produced around the newly formed vessels. The details are still vastly obscure for the last stages when vascular sprouts fuse with other sprouts to form loops (Fig. 1.3e) (Bergers and Benjamin, 2003, Carmeliet, 2005).

1.5.3. Cancer is Angiogenesis Dependent:

Many studies have confirmed the hypothesis that tumor growth is reliant on neovascularisation process. Any significant increment in tumor size must be in synchrony with increment in the blood supply and blood vessels size (Folkman, 1990). The hypothesis was confirmed by many experimental evidences. In one study, tumors implanted in places where there is no probability of new blood vessels to grow such as the aqueous fluid of the anterior chamber of the eye remained viable, avascular, and limited in size (<1 mm^3). When the cells were implanted on iris vessels, they induced new blood vessels formation which grew hastily reaching 16,000 times their original size within two weeks (Gimbrone et al., 1972). Another evidence that strengthen this hypothesis was the detection of exponential and rapid growth of tumors that were implanted on the chorioallantoic membrane of the chick embryo after blood vessels formation (Knighton et al., 1977). Studies on subcutaneously implanted tumors showed

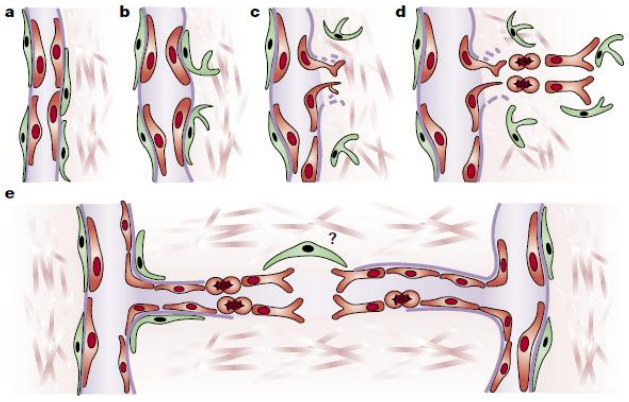

Figure 1.3 The angiogenesis process cascade. Blood vessels grow from pre-existing capillaries (a). (b) First, pericytes (green) separate, blood vessels dilate and the basement membrane and ECM is degraded. (c) The endothelial cells (red) migrate into the perivascular space. (d) Endothelial cells proliferate, and are presumably guided by pericytes. (e) Endothelial cells adhere to each other and create a lumen (Bergers and Benjamin, 2003).

that the blood vessels form approximately 1.5% of the tumor volume; this is number represent 400% increase over normal subcutaneous tissue (Thompson et al., 1987).

1.5.4. The Angiogenic Switch:

About five to twenty years may be needed for transition from benign carcinogenic phase to the fully developed malignant stage where the cancer can be perceived clinically (Mediana and Fausel, 2008). Dormancy stage occurs when tumor cells proliferate but the rate of tumor cell death (apoptosis) counterbalances this proliferation and maintains the tumor mass in a steady state (Ribatti et al., 1997). At this stage, there are a balance between two contrary signals; angiogenesis signals like VEGF (Ferrara et al., 2003) and antiangiogenesis signals (e.g., Endostatin, Angiostatin) (Kim Lee Sim et al., 2000). Therefore, the angiogenesis process starts only when the net balance between these contrary signals is tipped in favour of angiogenesis initiation (Hanahan and Weinberg, 2000, Hanahan and Folkman, 1996). Accordingly, identification and interrupting of the factors and the circumstances which increase the probability of angiogenesis initiation may keep the cancerous cells in the stage of dormancy (Gullino, 1978). The studies showed that angiogenesis process can be triggered by a variety of signals include metabolic stress (e.g., hypoxia or hypoglycaemia), mechanical stress (e.g., pressure generated by proliferating cells), immune/inflammatory response (immune/inflammatory cells that have infiltrated the tissue), and genetic mutations (Hanahan and Weinberg, 2000). These circumstances cause synthesis or release of angiogenic factors such as VEGF (Ribatti, 2009).

1.5.5. Hypoxia:

Hypoxia is defined as a decrease in the oxygen supply to a level insufficient to maintain cellular function. The cells become hypoxic if it is located too far away from blood vessels. Due to cell proliferation and tumor growth, the cells in the core of tumor gets hypoxic (Carmeliet, 2005). Hypoxic cells are more invasive and metastatic, and more resistant to be killed by chemotherapy or radiation (Melillo, 2007).

Recent evidence demonstrated the impact of activation of hypoxia inducible transcription factors (HIFs) in hypoxic cells in angiogenesis process (Zhong et al., 1999). Binding the HIFs with the DNA induces expression of several angiogenic factors including VEGF, nitric oxide synthase, platelet-derived growth factor (PDGF), and others (Ahmed and Bicknell, 2009, Carmeliet, 2005). The critical step in induction of this pathway is the stabilization of the HIFs. The most important two members of HIFs are HIF-1 and HIF-2. HIF-1 is ubiquitously expressed, while HIF-2 is expressed only in endothelial cells and in the kidney, heart, lungs and small intestine (Wang et al., 1995, Semenza, 2001). HIF-1 complex is a heterodimer consisting of two DNA binding proteins, HIF-1α and HIF-1β. The expression of HIF-1α is tightly regulated by oxygen, while the HIF-1β is expressed constitutively (Bracken et al., 2003, Wang et al., 1995). Under normoxic conditions, HIF-1α is rapidly degraded due to enzymatic prolyl-hydroxylation. However, under hypoxic conditions the stability and half life of HIF-1α increased remarkably. Accordingly, HIF-1α dimerizes with HIF-1β. The heterodimer is then translocated to the nucleus and activates the promoter region of target genes (Wang et al., 1995). As the expression of the chief factor in the angiogenesis, VEGF, and many angiogenic pathways is related directly with the activation of HIF-1, the search for drugs targeting HIF is currently receiving a lot of attention (Semenza, 2003). The notion of