ANTI-CANCER STUDIES OF β-CARYOPHYLLENE, A NATURAL COMPONENT ISOLATED FROM *AQUILARIA CRASSNA* ON COLORECTAL CANCER

by

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This thesis is dedicated to
my people in Iraq who have
long been suffering from the war

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LIST OF ABBREVIATIONS

˚C	Degree Celsius
5-FU	5-Fluorouracil
β-C	β-caryophyllene
ALT	Alternative Lengthening of Telomeres
APC	Adenomatous Polypsis Coli
ATCC	American Type Culture Collection
BA	Betulinic Acid
Bad	Bcl-xL/Bcl2-associated death promoter
Bax	Bcl2 associated X protein
Bcl2	B-cell lymphoma 2
Bcl-Xl	B-cell lymphoma extra large
bFGF	Basic Fibroblast Growth Factor
bp	Base Pair
BER	Base-excision repair
BSA	Bovine Serum Albumin
CAM	Cellular adhesion molecules
CCD-18Co	Normal human fibroblast
CO_2	Carbon dioxide
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor

EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme linked immunosorbant assay
EOs	Essential oils
Fas	Tumor necrosis factor superfamily receptor 6
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF-2	Fibroblast Growth Factor-2
FMT	Fluorescence Molecular Tomography
FTIR	Fourier transforms infrared spectroscopy
GCMS	Gas Chromatography Mass Spectrometry
HCT 116	Human colorectal carcinoma cell line
HER-2	Human Epidermal growth factor Receptor 2
HIF-1α	Hypoxia Inducible factor -1- alpha
HIFs	Hypoxia Inducible Factors
HSPs	Heat shock proteins
HTRA	High-temperature requirement factor
HUVECs	Human umbilical vein endothelial cells
IAP	Inhibitor Apoptotic Proteins
IC_{50}	Median inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IFN-α and γ	Interferons alpha and gamma
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
KBr	Potassium Bromide

K-ras	Kirsten-rat sarcoma virus
K-ras/PI3-K	K-ras/Phosphatidylinositol 3-kinases
K-ras/RAF	K-ras/Murine leukemia viral oncogene homolog 1
K-ras/RAL	K-ras/ K-ras related protein
LC_{50}	Median lethal concentration
LD50	Lethal Dose 50
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAPK	Mitogen-Activated Protein Kinase
MAPK/ERK	MAPK-Extra cellular signal regulated enzyme kinase
MAPK/JNK	Mitogen-Activated Protein Kinase/Jun N-terminal Kinase
MCF-7	Human hormone sensitive and invasive breast cancer cell
MEM	Minimum Essential Medium
MeOH	Methyl alcohol
MMPs	Matrix Metalloproteinase
MMR	Mismatch epair
mTOR	Mammalian Target of Rapamycin
MTT	Thiazolyl blue tetrazolium bromide
NER	Nucleotide-excision repair
NF-kB	Nuclear factor kappa B
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
OD	Optical density
PBS	Phosphate buffered saline

PDGFR	Platelets Derived Growth Factor Receptors
PE	Plating Efficiency
PPM	Part Per Million
PS	Penicillin/Streptomycin
SD	Standard Deviation
SF	Survival Fraction
SPF	Specific Pathogen Free
STAT	Signal transducer and activator of transcription
TGF	Tumor growth factor
TGF-α	Transforming Growth Factor-alpha
TGF-β	Transforming Growth Factor-beta
TGFβR2	Transforming Growth Factor-β II Receptor
TKI	Tyrosine Kinase Inhibitor
TLC	Thin-layer chromatography
TLRs	Toll like receptors
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular Endothelial Growth Factor
VEGFR 1 and 2	Vascular Endothelial Growth Factor Receptors 1 and 2
XIAP	X-linked inhibitor of apoptosis protein

KAJAN ANTI-KANSER β-CARYOPHYLLENE, KOMPAUN SEMULAJADI DARIPADA *AQUILARIA CRASSNA* TERHADAP KANSER USUS

ABSTRAK

Aquilaria crassna (Thymelaeceae) telah digunakan dalam perubatan tradisional penduduk Asia untuk merawat jangkitan, penyakit kronik dan keradangan. Tumbuhan ini telah dilaporkan sebagai sumber yang kaya dengan komponen bioaktif. Dalam kajian ini, dua ekstrak disediakan daripada kulit batang *A. crassna* menggunakan etanol dan kaedah hidrolisasi. Minyak pati (EOs) yang diperoleh daripada kedua-dua ekstraksi diuji sitotoksisiti menggunakan empat jujukan sel kanser dan satu jujukan sel normal, termasuk HCT 116, PANC-1, MCF-7, PC 3 dan HUVECs. EOs daripada penyulingan hidro menunjukkan kesan sitotoksik yang sederhana tetapi terpilih terhadap sel HCT 116 kanser usus dengan nilai IC_{50} 28.0±1.5μg/mL. Seterusnya kesan EOs penyulingan hidro terhadap angiogenesis dikaji secara *in vitro* dan *ex vivo* dan EOs ditemui merencat secara langsung pembentukkan tiub endothelial dan percambahan salur mikro aorta tikus. Analisis fitokimia bagi EOs dijalankan mengguna spektometri TEM, FTIR, dan GC-MS telah menunjukkan kandungan yang tinggi β-caryophyllene, 1-phenanthrenecarboxylic acid, dan α-caryophyllene, benzenedicarboxylic acid, azulene dan cyclodecene. Kajian toksikologi menunjukkan EOs mempunyai profil keselamatan yang baik dengan LD_{50} melebihi 2000 mg/mL. Kajian tumor xenograf bagi EOs menunjukkan aktiviti antikanser komponen ini terhadap kanser usus manusia adalah pada 200 mg/kg. Fraksinasi berpandukan bioaktiviti EOs terhadap jujukan – jujukan sel kanser menunjukkan β-caryophyllene (β-C) sebagai komponen bioaktif utama seperti yang dikenal pasti menggunakan FT-IR, NMS dan MS. Kajian sitotoksisiti

menunjukkan β-C mempunyai kesan anti proliferatif selektif terhadap sel kanser usus (IC_{50} 19 μM). Komponen ini menyebabkan apoptosis, kondensasi nuklear, dan fragmentasi DNA yang mendedahkan badan apoptotik. Keputusan daripada ujian jajaran apoptotik antibodi manusia menunjukkan bahawa β-C menurunkan regulasi protin sel survival Survivin, HSPs, dan XIAP dan pada masa yang sama menaikkan regulasi penunjuk pro-apoptosis p21. β-C juga mempunyai kesan antiangiogenik dengan merencat migrasi sel endotelial, percambahan salur mikro aorta tikus, dan penyekatan faktor pertumbuhan endothelial vaskular (VEGF). β-C juga mempamerkan aktiviti anti tumor yang poten pada model xenograf ektopik dan ortotopik kanser usus, menyebabkan penurunan yang jelas dalam ketumpatan salur mikro dan peningkatan dalam apoptosis/ nekrosis tisu tumor. Keseluruhannya, dapat disimpulkan bahawa β-C menunjukkan aktiviti perencatan yang kuat terhadap pertumbuhan tumor usus melalui satu mekanisma yang melibatkan induksi apoptosis dan penyekatan angiogenesis.

ANTI-CANCER STUDIES OF β-CARYOPHYLLENE, A NATURAL COMPONENT ISOLATED FROM *AQUILARIA CRASSNA* ON COLORECTAL CANCER

ABSTRACT

Aquilaria crassna (Thymelaeceae) has been used in traditional Asian medicine to treat infections, chronic pain and inflammation. This plant has been reported as a rich source of bioactive constituents. In this study, two extracts were prepared from the stem bark of *A. crassna* using ethanol and hydrodistillation methods. The resulting essential oils (EOs) from both extraction approaches were subjected for cytotoxicity studies using four cancer cell lines and one normal cell line, namely HCT-116, PANC-1, MCF-7, PC 3 and HUVECs. Hydrodistilled EOs displayed moderate cytotoxic effect but exhibited selectivity against colorectal cancer HCT-116 cells with the IC_{50} value (28.0 ±1.5μg/mL). Subsequently, the effect of hydrodistilled EOs on angiogenesis was investigated *in vitro* and *ex vivo* and it was found that EOs directly inhibits endothelial tube formation and sprouting of rat aorta microvessels. Phytochemical analysis of the EOs was carried out using TEM, FTIR, and GC-MS spectrometry revealing high amount of β-caryophyllene, 1-phenanthrenecarboxylic acid, and α-caryophyllene, benzenedicarboxylic acid, azulene and cyclodecene. Toxicology studies showed that the EOs had a good safety profile with LD_{50} being more than 2000 mg/ml. Xenograft tumor studies of EOs exhibits anticancer activity in dose-dependent manner towards human colorectal cancer at 50, 100 and 200 mg/kg. Bioactivity-guided fractionation of EOs towards cancer cell lines reveals β-caryophyllene (β-C) as a key bioactive compound as verified using FT-IR, NMR and MS. Cytotoxicity studies show that β-C has selective anti-proliferative effect towards colorectal cancer cells (IC_{50} = 19 μM). The compound

causes apoptosis, nuclear condensation and DNA fragmentation revealing apoptotic bodies. Results of human antibody apoptotic array study demonstrated that β-C downregulates the cell survival proteins Survivin, HSPs and XIAP and at the same time upregulates the pro-apoptosis marker p21. β-C was also found to be strongly antiangiogenic by inhibiting endothelial cell migration, tube formation, sprouting of rat aorta microvessels and suppression of Vascular Endothelial Growth Factor (VEGF).

β-C also exhibits potent anti-tumor activity in ectopic and orthotopic xenograft model of colorectal cancer causing clear decrease in microvessel density and increase in apoptosis/necrosis in tumor tissue. Overall, it can be concluded that β-C exhibits strong inhibitory activity against colorectal tumor growth through a mechanism that appears to involve apoptosis induction and angiogenesis suppression.

CHAPTER ONE : INTRODUCTION

1.1 Cancer biology

Cancer is the general name of a heterogeneous group of more than 100 diseases. It arises from dysregulation in normal cellular mechanisms; and it is characterized by alterations in the expression of multiple genes, leading to local tissue invasion, which may eventually turn into metastasis (Pecorino, 2012). It is widely accepted that tumorgenesis is a multistage-process. This suggestion has been supported by a vast array of experimental models, involving cell culture systems, animal models, and human subjects exposed to chemical or physical carcinogens (Pedraza-Fariña, 2006). Three distinct processes have been implicated in cancer development: The first, initiation, is highly associated with external carcinogenic agents causing DNA mutations and genetic damage to normal cells. Such damage may be irreversible if DNA does not undergo proper repair, leading to increased susceptibility of cancer formation (Sandoval and Esteller, 2012). The second, promotion, makes the major part of the latent period of carcinogenesis. It involves initiated cells undergoing gradual cell proliferation, leading to the emergence of uncontrolled growth and a malignant tumor (Vincent and Gatenby, 2008). The third, progression, represents the terminal stage and the end product of carcinogenesis. It is characterized by cell hyperproliferation and metastasis. In this stage, tumor cells undergo dissemination, and break away from their primary sites to invade nearby tissues via the circulatory system or a transport mechanism (Valastyan and Weinberg, 2011).

Genes can be classified into three groups: gatekeepers, caretakers and landscapers (Michor et al., 2004): Gatekeepers are oncogenes and tumor suppressor genes which regulate proliferation and differentiation related pathways in the cell. Misregulation of

these genes may promote abnormal cell growth. In fact, gatekeeper gene P53 is the most commonly mutated gene in human cancers. About 50% of all human cancers are associated with structural alterations in TP53 alleles (Van Heems et al., 2007).

Caretaker genes on the other hand prevent genomic instability in the cell. Genomic integrity is directly dependent on DNA repair mechanisms, such as mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER). Therefore, mutations in caretaker genes lead to accelerated transformation of a normal cell to a neoplastic one (Croc, 2008).

Landscaper genes refer to those genes which organize cells' interactions with their surrounding microenvironment. Alterations in these genes do not directly affect cellular growth, but rather generate an abnormal environment which might allow carcinogenesis and cell transformation to ensue (Ashworth et al., 2011).

1.2 Hallmarks of cancer

During carcinogenesis, cells undergo a multistep process to acquire specific characteristics that can promote cancer development, including these six essential hallmarks: sustaining proliferative signaling, evading growth suppressors, evading programmed cell death (apoptosis), enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. These six key features of cancer have been recently revised to add two more hallmarks: the ability to reprogram cell metabolism to generate adequate energy, and the ability to avoid immune destruction. Malignant growths are also believed to acquire additional characteristics which enable them to promote inflammation, and genome instability (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Hanahan and Awada, 2012) (Fig 1.1).

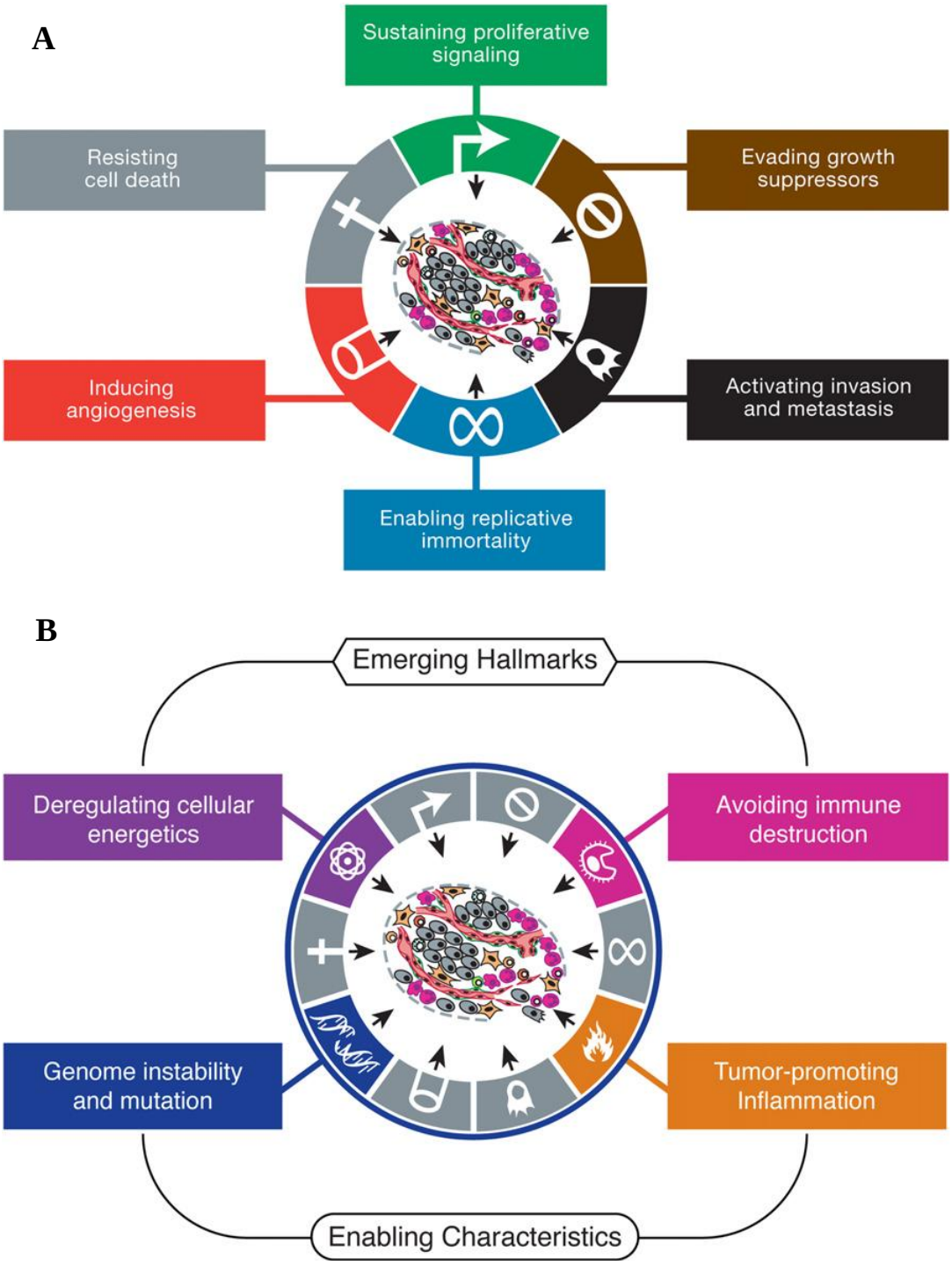

Figure 1.1: The Hallmarks of Cancer. A: The original six hallmark capabilities as described by Hanahan and Weinberg (2000). B: Emerging 'hallmarks' and enabling characteristics. Adapted from Hanahan and Weinberg (2011). Reprinted with permission (License number 3446980210269), Elsevier Ltd.

1.2.1 Sustaining proliferative signalling

During normal cell growth, differentiation and proliferation is regulated by growth signals. These stimulatory growth factors are carefully transmitted into the cell by components of the extracellular matrix; trans-membrane receptors; and intracellular signaling domains. Cancer cells, on the other hand, are able to sustain continuous proliferation by multiple ways. Some cancer cells produce their own growth factors, like tumor growth factor-α, and corresponding receptors via an autocrine stimulation process. This enables them to respond to their own growth factors in a positive feedback mechanism. Signaling cascades are deregulated in tumor cells, leading to overexpression of growth factors, which then bind to cell surface receptors making cancer cells hyper-responsive (Abbott et al., 2006; Hanahan and Weinberg, 2011; Gutschner and Diederichs, 2012). Moreover, tumor cells can switch on extracellular matrix receptors, which would initiate the downstream pathways of these receptors, causing inappropriate cell behavior. Ligand-activated growth signaling via integrins linked to components of the extracellular matrix can trigger SOS-Ras-Raf-MAP kinase pathways. These major cascades are vital to cancer cells growth. About 25% of all human tumors are associated with an altered structure of Ras proteins; and 40% of human melanomas are linked to mutations which affect the structure of Raf proteins (Hanahan and Weinberg, 2011).

1.2.2 Evading growth suppressors

In normal tissues, maintenance of cell proliferation, a state of quiescence, and tissue homeostasis is mediated by several antiproliferative growth signals. Such signals include soluble growth inhibitors, and immobilized inhibitors located in the extracellular matrix and on the surfaces of nearby cells (Gutschner and Diederichs,

2012). Cancer cells, however, escape growth suppression mechanisms. Many of the coding genes of tumor suppressing proteins, which operate in various ways to inhibit cell growth and proliferation, have been discovered by virtue of their physiological functions. Most of the antiproliferative signals are funneled through the retinoblastoma protein (pRb). In a hypophosphorylated state, pRb blocks proliferation by altering the function of a key transcription factor called E2F. This factor controls the expression of the genes necessary for cell cycle progression (Hanahan and Weinberg, 2011).

Transforming growth factor-beta (TGF-β) is a distinctive anti-proliferative factor which prevents pRb from undergoing alteration or phosphorylation. TGF-β induces the expression of p21, a factor which blocks the phosphorylation of pRb by inhibiting the cyclin-dependent kinase (CDK) complexes that are responsible for pRb phosphorylation. Thus, disorders affecting the responsiveness of TGF-β may result in tumor development (Massague, 2008).

1.2.3 Resisting cell death

Normal cells have to maintain equilibrium between the net rates of proliferation, and cell death. Tumors can only develop once cells have managed to evade programmed cell death (Fulda et al., 2010). The apoptotic machinery comprises two main components: sensors and effectors. Sensors are cell surface receptors that bind to survival factors, such as IGF-1, IGF-2 and IL-3; and to the death factors conveyed by Fas ligands and TNF-α. Sensors are responsible for monitoring the extracellular and the intracellular environments. They respond to abnormalities, like DNA damage (Hanahan and Weinberg, 2000). On the other hand, apoptosis effectors' pathways involve the caspase family, which can be activated by Fas death receptors, or cytochrome C. However, tumor cells have developed strategies to avoid apoptosis. For instance, hypoxia, DNA

damage and oncogene over-expression can induce p53-dependent apoptosis (Harris, 1996); however, many human tumors present an ability to inactivate p53 tumor suppressor gene. It has been estimated that about 2.4 million annual cancer cases worldwide are linked to p53 mutations (Richardson, 2013).

1.2.4 Enabling replicative immortality

In addition to avoiding anti-proliferative cell signaling pathways and generating self-sufficient growth signals, tumor cells are not bound by the normal limits of cell replication. In contrast to normal cells, which have fixed lifespans, cancer cells undergo unlimited replication (Gutschner and Diederichs, 2012). As observed in normal cell culture systems, the replication potential of normal cells is normally limited by replicative senescence. A normal cell cannot have more than 50 to 70 divisions. Moreover, most normal human cells in the body suffer a systematic losts of telomeric DNA (repetitive DNA sequences in the form of TTAGGG repeats). It is estimated that with the duplication of each chromosome, about 50–100 base pairs are lost from the end of each telomere. Telomeres ultimately shorten to a critical length, which results in the loss of telomere protection, leading to chromosomal instability and loss of cell viability (Abbott et al, 2006; Meena et al, 2015). These limitations in cell replication act as barriers preventing normal cells from undergoing malignant transformation. Tumor cells have two mechanisms to circumvent the loss of telomeres: (I) They trigger telomerase expression to add telomeric repeats at the ends of chromosomes. (II) They undergo processes of Alternative Lengthening of Telomeres (ALT). Telomerase reactivation was observed in 90 % of all malignant tumors (Gutschner and Diederichs, 2012); and the remaining 10 % of human malignant tumors have been found to utilize

ALT mechanisms for unlimited proliferation, and to achieve immortality (Lau et al, 2013).

1.2.5 Inducing angiogenesis

The growth of new capillary blood vessels, referred to as angiogenesis, is an essential process in tumor progression that offers more nutrients and oxygen supplies. Tumor mass formation would be limited to 1-2 mm^2 if tumor cells could not undergo this fifth hallmark of cancer (Dimova at el, 2014). The formation of new blood vessels induced by tumor cells not only secures the supply of nutrients and oxygen but also allows tumors to secrete their toxic wastes and migrate to other sites (Gutschner and Diederichs, 2012). Angiogenesis is tightly regulated at the molecular level by way of a balance between inducing signals (bFGF and VEGF) and endogenous inhibitors (endostatin and angiostatin). However, an angiogenic switch is activated during tumor development, which leads to continuous formation of blood vessels to help nourish the tumor. To achieve sustained angiogenesis, tumor cells may overexpress inducing signals or downregulate endogenous inhibitors. An imbalance between inducers and inhibitors is a prominent feature of angiogenesis (Folkman, 2002).

1.2.6 Tissue invasion and metastasis

The final offstage in cancer development is known as metastasis; the diffusion of tumor cells from their primary sites to distant tissues. About 90 % of cancer-related deaths are attributable to metastasis, which occurs due to the systemic nature of cancer and the resistance of circulated cancer cells to current therapeutic approaches (Valastyan and Weinberg, 2011). Cancer invasion and metastasis are complicated processes: The initial steps of tissue invasion involve the activation of signal cascades that regulate

cytoskeletal dynamics, the turnover of the extracellular matrix, and cell-cell adhesion molecules (e.g., cadherin family) in tumor cells. Tissue invasion is followed by malignant cells migration to nearby tissue. Eventually, metastasis occurs as invading cancer cells connect to blood vessels; breakdown cellar membranes and endothelial walls; and disseminate through the circulatory system or other transport systems to colonize distant organs (Friedl and Alexander, 2011).

1.2.7 Genomic instability and mutations

Unlike normal tissues, the majority of human tumors are highly heterogeneous in nature because they arise from multiple clones of cancer cells as a consequence of genetic mutation(s), altered environmental conditions, and reversible changes in cell properties (Meacham and Morrison, 2013). The genetic integrity of non-cancerous cells is generally maintained by a highly coordinated process in which DNA replication is well systematized, with the overall probability of an error not exceeding a single base misalignment for every billion nucleotides. This high accuracy is owed to DNA polymerase, the enzyme that builds identical strands of DNA and ensures that the sequence remains immaculate (Loeb et al, 2003). Cytotoxic carcinogens can cause genetic instability by altering the cellular microenvironment, leading to the breakdown of essential components of the cell's genome maintenance machinery (Abbott et al, 2006). Carcinogens may also cause epigenetic modifications i.e., changes affecting the functions of genes rather than their genetic structures (Kanwal and Gupta, 2012). Dysfunctional 'caretaker' genes contribute to accelerated accumulation of mutations. The products of these genes are responsible for detecting DNA damage and activating the repair machinery. They also work to inactivate mutagenic agents before any DNA damage occurs (Hanahan and Weinberg, 2011). Experimental studies estimate the

number of acquired genetic mutations in cancerous cells to be more than 10,000 (Loeb et al, 2003; Sieber et al, 2003). In fact, genomic instability is at the heart of all the stages of tumorgenesis, from pre-cancerous lesions to tissue invasion and metastasis (Negrini et al, 2010).

1.2.8 Tumor-promoting inflammation

Inflammation is the cornerstone of multiple cancer hallmarks. Inflammatory mechanisms can contribute to a stable tumor's microenvironment by providing bioactive molecules that maintain proliferative signaling (growth factors), limit apoptosis (survival factors), and promote angiogenesis (proangiogenic factors). Inflammation can also cause the recruitment of extracellular matrix constituents that facilitate invasion and metastasis (Hanahan and Weinberg, 2011). Tumor microenvironments contain many different inflammatory cells and mediators which increase the production of reactive oxygen species and lead to oxidative DNA damage, and a weakened DNA repair machinery. Actually, a normal inflammation is self-limiting: It demonstrates active resolution via cell death and the clearance of metabolic wastes and immune cells. A chronic inflammation, on the other hand, is strongly associated with an increased risk of tumorgenesis. Approximately, 20 % of human tumors are associated with chronic inflammations caused by microbial infections, irritants or autoimmune diseases (Crusz and Balkwill, 2015). In reality, inflammation and tumor growth are two closely interrelated processes which enhance and facilitate one another. In certain type of cancers, inflammation can trigger the initiation process in cancer cells; and cancer cells can in return promote the inflammation further by enhancing the expression of proinflammatory transcription factors (NF-κB) and

inflammatory enzymes (COX-2). This gives rise to a rich and complex platform of inflammatory responses within the tumor's microenvironment (Mantovani et al. 2008).

1.3 Angiogenesis

The term of angiogenesis originates from two Greek words, "angêion", which means vessel, and "genesis" (birth or emergence). It has been used to refer to the formation of micro-vascular networks from preexisting vascular vessels (Ishak at el, 2014). Angiogenesis is a highly regulated physiological process. It is normally restricted to embryonic development, and is self-limiting in adults. During adulthood, it may be observed during wound healing or the female reproductive cycle. Pathological angiogenesis, however, is often associated with dysregulated growth factors and unstable blood vessels. It is destructive for the tissues and underlies the pathogenesis of many medical conditions, such as cancer, rheumatoid arthritis, obesity, asthma and diabetes (Carmeliet, 2005; Ellenberg et al, 2010). The role of angiogenesis in cancer development was controversial until 1971 when Judah Folkman, the "father of angiogenesis," proved that tumor growth was angiogenesis-dependent, as mentioned by Stephenson et al. (2013). Now, angiogenesis is regarded as one of the essential hallmarks of cancer and metastasis. It highlights the fact that tumors cannot grow beyond a few millimeters without sufficient oxygen and nutrients (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2011). Since most solid tumors are reliant on neovascularization, angiogenesis related research has recently been invaluable to cancer therapy, because targeting tumor angiogenesis using antiangiogenic agents that inhibit or starve the growth of new blood vessels opens new avenues for the treatment of cancer. It has been estimated that more than 500 million people worldwide can benefit from antiangiogenic or proangiogenic treatments (Carmeliet, 2005; Nassar et al, 2011).

1.3.1 Pathological angiogenesis

Pathological and physiological angiogenesis processes utilize the same signaling pathways, featuring a cascade of highly coordinated cellular functions which drive the establishment of new blood vessels in response to an increasing demand for oxygen and nutrients. However, the main deference between the two is that pathological angiogenesis is persistent and irresolvable. Moreover, it is characterized by disorganized and tortuous vessel structures (Chung and Ferrara, 2011). Tumor vessels are often distinguishable from a physiological vasculature as their growth is driven by a tumor's microenvironment, which is rich in tumor secretory factors and predominated by hypoxia. In fact, hypoxia is responsible for triggering the 'angiogenic switch' to allow a tumor to grow further and metastasize (Weis and Cheresh, 2011). Furthermore, tumor angiogenesis relies on four molecular mechanisms which contribute to tumor development: sprouting, intussusception, mimicry and co-option.

1.3.1 (a) Sprouting angiogenesis

The prominent strategy for tumor progression is sprouting angiogenesis, which allows tumors to grow beyond 2–3 mm^3 in size (Fig 1.2). However, the initiation of the sprouting process requires a cascade of events which drive blood vessels to sprout. These events include the degradation of the endothelial basement membrane; the migration of endothelial cells into connective tissues; the activation of tip cells; and the proliferation of the stalk cells induced by certain growth factors, like vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor (FGF-2), and delta-like 4 (Dll4). This is followed by the formation of solid sprouts of endothelial cells connected by complicated vascular loops (Li et al, 2014; Ribatti and Crivellato, 2012).

Furthermore, sprouting angiogenesis is an invasive process associated with extracellular proteolytic activities mediated by matrix metalloproteinases (MMPs). MMPs can degrade the constituents of the extra cellular matrix (ECM) and facilitate sustained sprouting (Ebrahem et al, 2010; Van Hinsbergh and Koolwijk, 2008).

1.3.1 (b) Intussusceptive angiogenesis

The term "intussusception" is generally used to refer to the maturation of vascular networks through the formation of new blood vessels resulting from the insertion and extension of lumenal tissue pillars within the capillaries (Styp-Rekowska et al, 2011). In contrast to sprouting, intussusception is a simple mechanism with a higher morphogenic potential, and fewer energetic and metabolic activities as it does not require cell proliferation, degradation of the basement membrane, migration or tube formation (Makanya et al, 2009). Additionally, intussusceptive angiogenesis not only relies on angiogenic growth factors but it is also stimulated by higher intra-vascular blood flow rates and/or higher levels of intra-vascular shear stress (Gianni-Barrera et al, 2011; Styp-Rekowska et al, 2011).

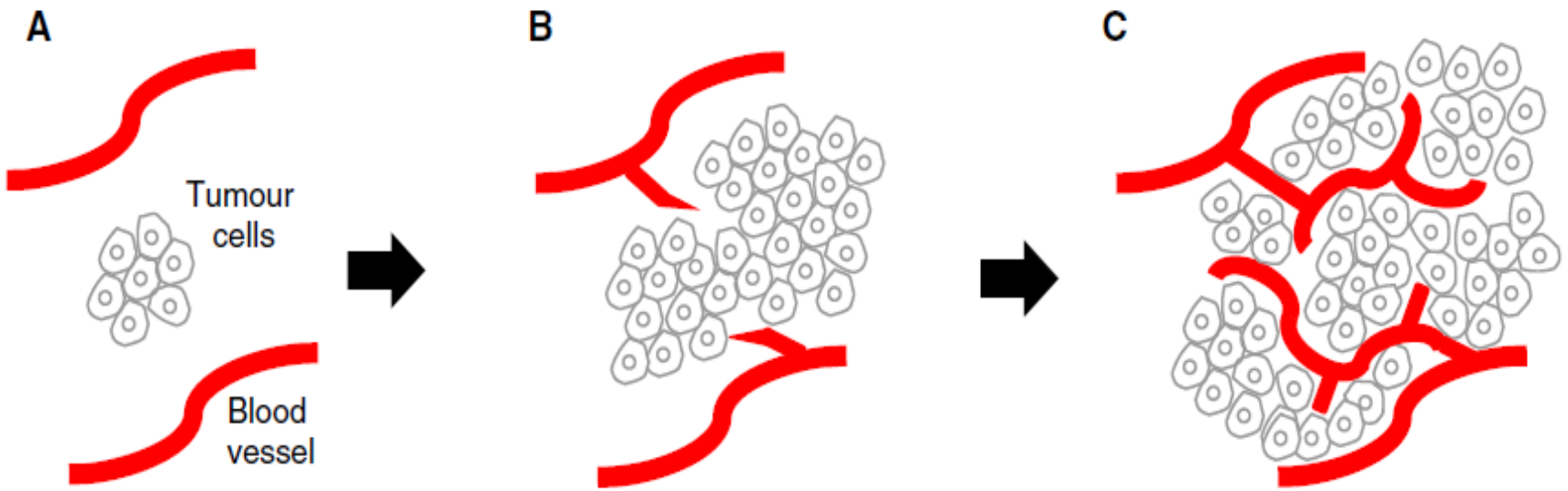

Figure 1.2: The role of sprouting angiogenesis in tumor growth. (A) when a tumor volume is small, it can obtain oxygen and nutrients from existing local blood vessels (B) As the tumor grows beyond the capacity of local blood vessels, soluble pro-angiogenic factors are released to trigger the sprouting of new vessels from local existing ones (C) sprouting vessels provide a blood supply to the tumor; and this is required in order for the tumor to grow beyond 2–3 mm^3 in size (Vasudev et al, 2014).

1.3.3 (c) Vasculogenic mimicry

Vascular mimicry describes processes related to cell plasticity which occurs mainly in aggressive tumors, and in which tumor cells organize and mimic endothelial cells to make tube-like structures containing plasma and red blood cells without the participation of endothelial cells (Chung and Mahalingam, 2014). This phenomenon has been observed in several tumor types, and was described for the first time in malignant melanoma when tissue sections of uveal and cutaneous melanomas displayed patterns and networks of Schiff-acid-positive channels in the microcirculation of melanoma cells, which coincided with lower survival rates and poor prognosis (Hillen and Griffioen, 2007).

1.3.3 (d) Vascular co-option

Vessel co-option is an alternative mechanism that enables tumors to secure a supply of blood by hijacking existing vasculatures and facilitating tumor cells migration towards host blood vessels in well-vascularized organs, such as the brain, lungs, and liver (Donnem et al, 2013). Vascular co-option has been implicated in 30 % of all colorectal cancer metastasis to the liver (Frentzas et al, 2014). In addition to being important in tumor development, vessel co-option has been associated with tumor aggressiveness and it is believed to be a factor in antiangiogenic drug resistance (Fidler and Kripke, 2015).

1.3.4 Vascular endothelial growth factor (VEGF)

Seven members of the VEGF family have been identified so far, including the mammalian VEGF ligands, which comprise five glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the placenta growth factor (PlGF). The other two members are

an analogous ligand found in the *Orf* viruses and known as VEGF-E; and an analogue in the snake venom of *Trimeresurus flavoviridis* known as VEGF-F. The activities of the VEGF family are mediated by three tyrosine kinase receptors: VEGFR1, VEGFR2, and VEGFR3 (Yamazaki et al, 2009; Zhuang and Ferrara, 2015). Each of the VEGF ligands demonstrates different binding specificity and affinity towards those VEGEF receptors. For example, VEGF-A, VEGF-B and PlGF tend to bind to VEGFR1; VEGF-A favors binding to VEGFR2; and VEGF-C and VEGF-D bind to VEGFR3. The proteolytic activities of VEGFC and VEGFD contribute to its affinity for VEGFR2 (Zhuang and Ferrara, 2015). Undoubtedly, the role of VEGFs in angiogenesis has been predominately the most researched in the field since the initial discovery of VEGFs. Findings in that capacity have provided significant insight into the mechanisms that underlie angiogenesis (Goel and Mercurio, 2013).

Standing out in VEGF family, VEGF-A is considered to be the main stimulator of angiogenesis. It exerts it effect by activating VEGFR-1 and VEGFR-2, and is responsible for triggering many steps in the angiogenesis cascade of endothelial cells, such as proliferation, migration and survival,(Waldner et al, 2010). VEGF-A can be secreted by tumor cells and spread in the surrounding area, leading to substantial increase in vascular permeability due to activated VEGFR-2 (Shibuya, 2009). In addition to VEGF-A pro-angiogenic effect, it contributes to tumor-related immunosuppression by inhibiting the migration of T-lymphocytes to the tumor site and effecting the accumulation of immature dendritic cells (Voron et al, 2014). Unlike VEGF-A, VEGF-B does not have a pro-angiogenic activity. Its role in the molecular mechanisms of angiogenesis is poorly understood (Li et al, 2012). Nevertheless, recent studies have shown VEGF-B to be highly expressed in tumor tissue, contributing to tumor progression and poor prognosis (Lautenschlaeger et al, 2013; Angelescu et al,

2013). Other members of the VEGF family, like VEGF-C and VEGF-D, are known to be lymphangiogenic stimulators: They are over-expressed in various tumor types and act to promote lymph node invasion and metastasis (Kopfstein et al, 2007; Matsuura et al, 2009). Predominantly expressed in the placenta, but also detectable in the heart, lungs and skeletal muscles, placenta growth factor (PlGF) is known to be a factor in tumorigenesis. However, its role in tumor angiogenesis and progression remains poorly understood and controversial. On one hand, overexpression of PlGF in mouse melanoma has been linked to hypervascularization of the skin, a phenotype that is associated with tumor invasion and pulmonary metastases due to increased MMPs activation (Marcellini et al, 2006). On the other hand, PIGF was shown to suppress tumor growth in fibrosarcoma and Lewis lung carcinoma cell lines by remodeling and normalizing their respective vascular phenotypes, leading to significant reduction of tumor microvascular density and branch formation (Hedlund et al, 2009). Overall, VEGF-mediated signaling is key to various angiogenic processes in tumor cells, such as cell proliferation, migration and survival. It plays a vital role in tumor angiogenesis and progression. Hence, targeting VEGFs, their receptors and/or their downstream signals should precipitate tumor vessel regression and hinder tumor progression (Figg and Folkman, 2008; Goel and Mercurio, 2013).

1.3.5 Antiangiogenic therapy

In 1971, Judah Folkman hypothesized that angiogenesis did not initiate malignancy but rather promoted tumor growth and metastasis; and ever since, many attempts have been made to treat cancer by using therapeutic agents that disrupt tumor angiogenesis, acting in the developmental stage in particular (Kubota, 2012). Angiogenic inhibitors have been approved for use against many cancer types, such as hepatocellular carcinoma,

renal cell carcinoma, breast cancer and colorectal cancer (Sennino and McDonald, 2012). The first proof of concept for an antiangiogenic approach was presented in the 1990s as the effect of interferon alpha was demonstrated against angioblastoma. Later, thalidomide was shown to treat myeloma successfully in 1999. In February 2004, the Food and Drug Administration (FDA) approved the use of an antiangiogenic drug, Avastin® (Bevacizumab), for the treatment of metastatic colorectal cancer (Folkman, 2004). With Avastin® reaping $2.5 billion in revenue in 2008, many pharmaceutical industries were encouraged to invest in the discovery of new angiogenic inhibitors (Moran, 2008). Until the year 2015, the (FDA) had approved up to 12 new antiangiogenic drugs: bevacizumab, sunitinib, sorafenib, regorafenib, pazopanib, vandetanib, cabozantinib, zivafl, ibercept, pegaptanib, ranibizumab and aflibercept (Zhuang and Ferrara, 2015).

1.3.6 Mechanisms of antiangiogenic therapy

In the last three decades, antiangiogenic therapy for cancer has gone from an interesting hypothesis to an accepted treatment approach for many cancer types. Mechanistically, antiangiogenic agents work by interfering with the key steps of angiogenesis. VEGF family plays key roles in the development and progression of many solid tumors. Understanding the molecular pathway of angiogenesis has been an essential step towards cancer treatment. Recently, several VEGFA-VEGFR2 blockers have been approved for clinical use in cancer (Duda, 2012). According to their mechanism of action, The National Cancer Institute classified antiangiogenic agents into three categories including, agents that directly inhibit endothelial cells (including integrin antagonists), agents capable of interfering with signaling cascades, and agents that inhibit the ability of endothelial cells to break down extracellular matrix (Al-Husein et

al, 2012). Moreover, approved antiangiogenic agents can be classified into three major classes that target VEGF.

1.3.6 (a) Anti-VEGF monoclonal antibodies

Bevacizumab (Avastin, Genentech/Roche) is a recombinant humanized monoclonal antibody, emerged as the first effective antiangiogenic agents that binds to all VEGFA isomers and neutralizes their activities by blocking the binding of VEGFA to VEGFR1 and VEGFR2 (Zhuang et al, 2015). Despite the strong antitumor activity of bevacizumab, it does not provide robust benefit as a monotherapy in many advanced cancer cases, except glioblastoma and renal cell carcinoma (Arrillaga-Romany et al, 2014). With the advantage of exquisite selectivity for their target and longer half life, bevacizumab approved in combination with chemotherapy or cytokine therapy for several advanced metastatic cancers, including metastatic colorectal cancer, lung cancer, pancreatic cancer and metastatic breast cancer (Potente et al, 2011). However, the mechanistic basis of the interaction between Anti-VEGF antibody and chemotherapy is still elusive, and many proposed models need to be clinically validated (Ellis and Hicklin, 2008).

1.3.6 (b) Small molecule inhibitors

Sunitinib (Sutent, Pfizer) is an orally administered multi-TKI that targets VEGFR-1,-2,-3, PDGFR, Flt-3 and RET. It was the first FDA approved agent for the management of gastrointestinal stromal tumor, and it is also approved for advanced renal cell carcinoma, metastatic pancreatic neuroendocrine tumors (Limaverde-Sousa et al, 2014). Although, it has been reported that sunitinib significantly targeted myeloid derived suppressor cells (MDSCs) and decrease their accumulation in the peripheral blood and

suppression metastatic renal cell carcomas. This reduction in immune suppression would be a rationale approach for sunitinib and immunotherapy combination for the treatment of certain tumor types (Finke et al, 2011). Recently, an extended- access trial in metastatic renal cell carcinoma has significantly provided considerable knowledge of the efficacy and safety of sunitinib and enabled 4543 patients with vast-ranging disease states to receive sunitinib treatment. The safety profile of sunitinib was consistent with the earlier reports, no unexpected long-term adverse events were seen, and clinical benefit was reported in all ages (Gore et al, 2015).

Sorafenib (Nexavar, Bayer and Onyx) is a novel bi-aryal urea; it is also an orally administered multikinase inhibitor, with wide range of activity against RAFkinases and several receptor tyrosine kinases. Sorafenib targets VEGFR-2 and VEGFR-3, PDGFRb, Flt-3, and c-Kit (Ranieri et al, 2012). It has pronounce anti-tumor activity in *in vitro* studies, preclinical xenograft models of different potent cancer type including, colon, pancreatic, lung, renal and ovarian cancer. In addition, it is the only standard clinical treatment against advanced hepatocellular carcinoma (Chen et al, 2015).

Axitinib (Inlyta, Pfizer) is a strong molecule inhibitor with selective specificity for VEGF-1, VEGF-2, and VEGF-3. In January 2012, axitinib received FDA approval for the treatment of renal cell carcinoma RCC (Al-Husein et al, 2012). Besides the potent antiangiogenic activity, axitinib have been reported to exhibit cytotoxic and immunomodulatory effects via induces DNA damage and increase the expression of P21 in RCC cells, which can be responsible for axitinib anti-tumor efficacy (Morelli et al, 2015).

1.3.6 (c) VEGF-Trap

Aflibercept (Eylea, Regeneron and Bayer) is a VEGF decoy receptor, also known as VEGF-Trap, consisting of binding domains of VEGFR-1 and VEGFR-2 fused to the Fc portion of human immunoglobulin(Ig) gamma chain, which makes it a disulfide dimer (Zhuang et al, 2015). In November 2011, aflibercept was approved by the (FDA) for the treatment of neovascular age-related macular degeneration (AMD), which is the leading cause of blindness among the old people (Stewart et al, 2012). In contrast to bevacizumab, which only binds to VEGFA isomers, aflibercept have a wider binding spectrum to VEGF-A, VEGF-B and PlGF, and it has a higher affinity for VEGFA than bevacizumab (Gaya and Tse, 2012). However, several clinical studies supported further evaluation of aflibercept in combination with chemotherapy in various malignancies. Consequently, aflibercept showed significant improve the response rate of progression-free survival (PFS) and overall survival (OS) in advanced colorectal cancer, whereas in advanced non-small-cell lung cancer it was found to improve the PFS but not OS (Limaverde-Sousa et al, 2014).

1.4 Cell Death

Cell death is an essential biological process of the normal embryological development, preservation of tissue homeostasis, morphogenesis and metamorphosis of multicellular organisms. In general, the number of cells in any tissue is controlled by an equilibrium process between cell proliferation and cell death. Imbalance of this control is an essential hallmark of cancer cells. However, mammalian cell death subdivided into three modes of death including apoptosis, autophagy and necrosis (Fig 1.3) which plays key roles in cell death decisions, based on morphological and biochemical differences. Apoptosis and autophagy are the two fundamental types of programmed cell death