INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITIES OF *ORTHOSIPHON STAMINEUS* TOWARDS COLORECTAL CANCER

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by

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DEDICATION

This thesis is dedicated to

My beloved mother and my late father

To

Brothers, sisters

To

My beloved wife, sons and daughters

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{نَرْفَعُ دَرَجَاتٍ مَّن نَّشَآءُ وَفَوْقَ كُلّ ذِي عِلْمٍ عليم } (76- سورة يوسف)

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TABLE OF CONTENTS

Page

CHAPTER TWO - MATERIALS AND METHODS

CHAPTER THREE - ANTI-ANGIOGENIC SCREENING AND PHYTOCHEMICAL STUDY OF *ORTHOSIPHON STAMINEUS*

CHAPTER SEVEN - CONCLUSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ACS	American Cancer Society
Ala	Alanine
AlCl3	Aluminium chloride
Ang-2	Angiopoietin 2
APC	Adenomatous Polypsis Coli
Are	Arginine
Asp	Asparagine
BA	Beutilinic acid
BFGF	Basic fibroblast growth factor
BM	Basement membrane
Cap	Capecitabine
CCD	charge-coupled device
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CoMFA	Comparative molecular field analysis
COX	Cyclooxygenases
CRCs	Colorectal cancers
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
Del-1	Developmental endothelial locus-1
DNA	Deoxyribose nucleic acid
DEPC	dissolved in diethyl pyrocarbonate
DQSAR	Dimension quantitative structure activity relationship
DMSO	Dimethyl sulfoxide
EC	Endothelial cells
ECGS	Endothelial cell growth supplements
ECM	Endothelial cell medium
ELISA	Enzyme-linked immunosorbant assay
FDA	Food and drug administration

FGF	Fibroblast growth factor
FTIR	Fourier transform infrared spectrometry
G-CSF	Granulocyte colony-stimulating factor
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
H	Hour
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factors
HIV	Human immunodeficiency virus
HIV-1	HIV-1 Human immunodeficiency virus type 1
His	Histidine
HMWK	High molecular weight kininigen
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
IL1R1	Interleukin-1 receptor type 1
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-7	Interleukin-7
Ile	Isoleucine
IP	Intraperitoneal injection
IP-10	Interferon-inducible protein-10
JEV	Japanese encephalitis virus
Leu	Leucine
LPS	Lipopolysaccharide
Lys	Lysine
MAPK	Mitogen-activated protein kinases
MCTS	Multicellular tumor spheroids
Met	Methionine
MMPs	Matrix metalloproteinase
MSI	Microsatellite instability
MTT	3-(4, 5-dimethylthiazol-2-yl)- 2,5 diphenyltetrazolim bromide

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OX	Oxaliplatin
OA	Orthosiphol A
OLA	Oleanolic acid
PAs	Plasminogen activators
PBS	Phosphate-buffered saline
PBS-T	PBS with 0.1% tween 20
PC	Pericytes
PDB	Protein Data Bank
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptors
PEDF	Pigment epithelium-derived factor
Pg	Picogram
PGE2	Prostaglandin E2
PLGF	Placental growth factor
Phe	Phenylalanine
PMA	Phorbol myristate acetate.
Pro	Proline
P/S	Penicillin/streptomycin
RA	Rosmarinic acid
Ras-GAP	Gguanosine triphosphatase-activating protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT_PCR	Real -Time Polymerase Chain Reaction
Ser	Serine
SPARC	Secreted protein acidic and rich
sVEGFR1	soluble VEGF receptor-1

TAMs	Tumor-associated macrophages
TGF	Transforming growth factor
TGF- β	Transforming growth factor beta
Thr	Threonine
TMF	3’-hydroxy-5,6,7,4’-tetramethoxyflavone
TNF-α	Tumor necrosis factor alpha
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
TXA2	Thromboxane A2
Tyr	Tyrosine
UV-vis	Ultra-violet visible
Val	Valine
VEGF	Vascular endothelial growth factor
VEGFR-1,2	Vascular endothelial cell receptors -1,2
VEGI	Vascular endothelial growth inhibitor
WHO	World Health Organization
WNT	Wingless-type MMTV integration site family

LIST OF SYMBOLS

Symbol	Meaning
Å	Angstrom
γ	Gamma
β	Beta
α	Alpha
<	Less than
>	More than
μ	Micro
C	Celsius
%	Percent

PENYIASATAN MEKANISME MOLEKUL YANG MENDASARI AKTIVITI ANTI-TUMOR DAN ANTI-ANGIOGENIK *ORTHOSIPHON STAMINEUS* TERHADAP KANSER USUS

ABSTRAK

Teh *Orthosiphon stamineus* Benth. (Lamiaceae) digunakan secara meluas dalam perubatan tradisional. Kajian terbaru menunjukkan bahawa 50% ekstrak ethanolik daripada *Orthosiphon stamineus* (EOS) dan sebatian aktif, asid rosmarinik (RA), memaparkan kesan-kesan anti-angiogenik, anti-radang dan anti-tumor yang ketara dalam pelbagai model eksperimen. Walau bagaimanapun, mekanisme yang mendasari sifat-sifat ini tidak dinilai dengan sepenuhnya. Kajian yang dijalankan ini bertujuan untuk menilaikan lagi mekanisme molekul yang mendasari anti-tumor dan anti-angiogenik. Dalam model eksperimen penghijrahan, perkembangan dan pembentukan tiub, cerakin kedua-dua EOS dan RA aktif menyebabkan perencatan ketara terhadap fungsi sel endothelial manusia (HUVECs) yang penting bagi merangsang proses angiogenesis. Dalam kedua-dua kajian *in vitro* dan *in vivo*, penindasan besar neovaskularisasi dalam model aorta tikus, CAM dan plug matrigel juga diperhatikan. Kajian cerakin multipleks menunjukkan pengurangan faktor pertumbuhan utama bagi lata pro-angiogenik dan perkembangan tumor iaitu faktor pertumbuhan endothelial vaskular (VEGF), faktor pertumbuhan fibroblast asas (b-FGF), transformasi faktor pertumbuhan transformasi (TGF-α), faktor nekrosis tumor (TNF-β) dan interleukin-1, 2, 7. Induksi terhadap agen anti-tumor iaitu interferon (IFN-α, β) dan faktor perangsang koloni makrofaj granulosit (GM-CSF) secara *in vitro* dan *in vivo* juga diperhatikan. EOS dan RA juga menyebabkan penurunan yang ketara perantara-perantara radang pro-angiogenik, enzim cyclooxygenase (COX), TNF-α, IL-1 dan tahap nitrik oksida (NO) yang penting untuk tumorigenesis. Lebih-

lebih lagi, EOS dan RA telah menghalang expresi gen secara signifikan dalam tisu tumor usus termasuk *HIF-α, WNT, KDR* dan *COX2*. Tambahan pula, EOS menghalang mercu tanda metastasis secara meluas iaitu pencerobohan dan pengagregatan tumor yang dibuktikan secara tomografi pendarfluor molekul (FMT) melalui pengimejan *in vivo* dan analisis histopatologi. Penemuan ini bertepatan dengan kesan rencatan pada tumor penggalak faktor angiogenesis dalam model mencit xenograft. Simulasi interaksi molekular dalam silico terhadap penanda biologi aktif EOS mengesahkan pertalian pengikat baik dan kesan modulatori kukuh terhadap faktor angiogenik dan tumorigenik. Ia mungkin disebabkan oleh kandungan fenolik dan flavonoid yang tinggi dalam EOS turut mengenakan kesan anti-tumor yang signifikan melalui modulasi pro-radang dan pengantara-pengantara angiogenesis melalui kesan hapus-sisa radikal bebas yang ketara. Kesimpulannya, hasil keseluruhan menyokong dan mengesahkan bahawa sifat-sifat anti-angiogenik dan anti-tumor EOS dan RA dibuktikan melalui kesan pemodulasian signifikan terhadap faktor-faktor utama pertumbuhan dan perantara.

INVESTIGATION OF MOLECULAR MECHANISMS UNDERLYING THE ANTI-TUMOR AND ANTI-ANGIOGENIC ACTIVITIES OF *ORTHOSIPHON STAMINEUS* TOWARDS COLORECTAL CANCER

ABSTRACT

Orthosiphon stamineus Benth. (Lamiaceae) tea is widely consumed traditionally for its vast medicinal value. Recent studies revealed that 50% ethanolic extract of *Orthosiphon stamineus* (EOS) and its active compound, rosmarinic acid (RA), displayed significant anti-angiogenic, anti-inflammatory and anti-tumor effects in various experimental models. However, the mechanisms underlying these properties have not been fully evaluated. The present work aims to further evaluate the molecular mechanisms underlying its anti-tumour and anti-angiogenic properties. In migration, proliferation and tube formation assay, both EOS and its active RA caused significant inhibition of human endothelial cell (HUVECs) functions crucial for promotion of angiogenesis. Both *in vitro* and *in vivo* studies revealed significant suppression of neovascularisation in rat aortic ring, CAM and matrigel plug. Multiplex array studies showed reduction of key growth factors for pro-angiogenic cascade and tumor development i.e. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), transforming growth factor alpha (TGF-α), tumor necrosis factor (TNF-β) and interleukin-1, 2, 7. Induction of anti-tumor agents i.e. interferon (IFN-α, β) and granulocyte macrophage colony stimulating factor (GM-CSF) both *in vitro* and *in vivo* was also noted. In addition, EOS and RA also caused a marked reduction of pro-angiogenic inflammatory mediators, cyclooxygenase (COX) enzyme, TNF-α, IL-1 and nitric oxide (NO) level vital for tumorigenesis. Moreover, EOS and RA significantly inhibited the genes expression

in colorectal tumor tissue including *HIF-α, WNT, KDR* and *COX2*. Furthermore, EOS extensively inhibited invasion and tumor aggregation evidenced by fluorescent molecular tomography (FMT) *in vivo* imaging and histopathological analysis. These findings coincide with its inhibitory effects on tumor promoting angiogenesis factors in nude mice xenograft. *In silico* molecular interaction simulations on EOS active biomarkers confirms good binding affinity and strong modulatory effect towards the angiogenic and tumorigenesis factors. It is likely the high phenolic and flavonoids content in EOS also exert a significant anti-tumor effect via modulating pro-inflammatory and angiogenesis mediators through their significant free radicals scavenging effect. In conclusion, overall results strongly substantiates EOS and RA anti-angiogenic and anti-tumor properties evidenced by their significant modulatory effect on key associated growth-factors and mediators.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer

Cancer is a malignant disease, which affects different parts of the body resulting in pathologic changes, genetic and epigenetic disorders factors which may act together or in sequence to cause cancer. Cancer occurs when groups of normal cells grow abnormally fast, losing control of cell division or with slow cell death (apoptosis) consequently, transformed from normal cells into malignant cells (Vanhoecke et al., 2005; Giansanti et al., 2011). Cancer is not a single disease but syndrome which comprises of group of multiple diseases. There are more than 100 various types of cancer that are named according to the sites where a cancerous growth originates.

The cancer cells are characterized by their invasion of the nearby tissue and spreading through the blood stream and lymphatic system to other organs or tissue (metastasis) (Zhong and Bowen, 2006). Cancer that initiates in the organs such as breast is called breast cancer and cancer that starts in the lung is called lung cancer and so on.

Metastasis is the final step of cancer and the major cause of death resulting from cancer. Bone metastases are the most common cause of cancer pain. Usually, under normal conditions, cells grow and divided automatically in order to replace the damaged cells or produce new cells. At times this orderly process goes wrong probably due to problem with the genetic material (DNA). Mutations are generally

caused by internal or external cellular damage and thereby the normal cells are converted into malignant cells. To date, the resistance of cancer cells towards cancer therapy has recognized one of the major problems in treating the disease hence much research been made towards the understanding of cancer biology and treatment using advanced protocols like radiotherapy and chemotherapy. There are two types of tumors, classified based on their growth and spread. Tumors that do not spread to other parts of the body and are incapable of recurrence are referred to as benign tumors. However, tumors are called malignant when a tumor cell invades the surrounding tissues and spreads to other parts of the body (Hanahan and Weinberg, 2011).

1.1.1 Cancer epidemiology

Cancer is a major public health problem and the second killer disease after cardiovascular diseases which cause of illness and mortality worldwide. In 2002, an estimated 10.9 million new cases of cancer incidence and mortality were reported globally with 6.7 million deaths (Parkin et al., 2005). In 2013, Bray reported that about 29 million people were living with cancer (Bray et al., 2013) and there were an estimated 7.6 million deaths (13% of all deaths) in 2008 (Gutschner and Diederichs, 2012).

World Health Organization (WHO) reported that cancer incidence and cancer-related mortality has increased remarkably, with 14 million new cases and 8.2 million deaths in 2012. Among all the cancers, the five most commonly diagnosed cancers in men were lung cancer, followed by prostate, colorectal, stomach, and liver cancers. While for women, the five most commonly diagnosed cancers were breast, followed by colorectal, lung, cervix, and stomach cancers. In general, the most

frequent source of cancer death was lung cancer with an estimated mortality rate of 1.59 million cases followed by liver cancer with 745,000 cases, stomach cancer with 723,000 cases, colorectal cancer with 694,000 cases, breast cancer with 521,000 cases and oesophageal cancer with 400,000 cases. Incidence, morbidity and mortality of cancer is expected to rise by more than 70% in the next two decades, which means that the incidence of cancer cases will increased from 14.1 million in 2012 to 22 million within the next two decades (Organization, 2014).

Incident rate of cancer in more developed areas was highest compared with the least developed areas. On the other hand, the mortality cases were much higher in less developed region, because of the economic costs, lack of diagnosis, late detection and treatment (Torre et al., 2015). Incidences of cancer have been on rise in both developed and developing countries.

Cancer is the main cause of death among adults aged 40 to 79 years and is the first or second leading cause of death in every age group among women (Boffetta and Parkin, 1994; Siegel et al., 2015). In 2015, it is estimated that 1,658,370 new cancer cases will be diagnosed and 589,430 cancer deaths in the USA (Siegel et al., 2015). However, overall cancer death rates decreased in 2011 with 168.7 per 100,000 populations from 215.1 (per 100,000 populations) in 1991. The 22% decrease in cancer deaths from 1991 to 2011 was a result of early detection, decrease in smoking, new drugs and treatment. Advances in cancer prevention approaches have also been introduced (Siegel et al., 2015).

According to the third edition of International Classification of Diseases for Oncology (ICD-O), cancer can be divided into five categories based on the primary and initial tumor, as bellow;

a) Carcinoma starts in tissue that covers the internal organs or epithelial cell; it can be grouped into different subtypes such as, adenocarcinoma, squamous cell carcinoma, transitional carcinoma and basal cell carcinoma.

b) Lymphoma and myeloma that start in the cells of the immune system

c) Leukemia progresses in blood formation tissue like bone marrow.

d) Central nervous system cancers are cancers that originate in the tissues of the spinal cord and brain.

e) Sarcoma is initiated in cartilage, bone, blood vessels, fat, connective tissue and muscle (Fritz et al., 2000)

1.1.2 Cancer in Malaysia

In 2008, the WHO's Globocan reported that cancer is one of the leading causes of death in Malaysia with an estimate of 30,000 annual cases. Based on the latest health facts 2013 reported by the Ministry of Health (MoH) of Malaysia, the incidence of cancer in Malaysia increased from 32,000 new cases in 2008 to 37,400 in 2012. This number may be expected to increase to 56,932 by 2025, if no proper prevention strategy or good lifestyle.

Breast cancer is the most common cancer among Malaysian followed by colorectal and lung cancer, with one in 19 Malaysians developing breast cancer, one in 33 developing colorectal cancer and one in 40 developing lung cancers. For men, lung cancer is the most frequent cancer followed by cancer of nasopharynx, colon, leukaemia, rectum and prostate. In women, the most frequent cancers are that of the breast followed by cervix, colon, ovary, leukaemia and lung (Lim et al., 2002).

1.1.3 Development and progression of cancer

To date, the causes of cancer are not completely understood. Cancer originates from single a mutated cell which starts to divides in uncontrolled manner exceeding normal cells, these aggressively proliferating cells can invade and destroy neighboring tissues and may spread to other parts of the body (metastasis), unlike normal cells which are self-regulated, restricted growth potential and on ability of metastasis.

The mutation may occur due to random genetic damage by endogenous factors, such as intrinsic chemicals of DNA bases, the abnormality or error in DNA replication which can be attributed to carcinogens such as infectious agent, chemicals, radiation, or free redials during metabolism (Ames, 1989; Hall and Angele, 1999; Bertram, 2000). The mutated cells grow fast until it form colony, these transformed cells divide more and more via altering the environment in a manner that favors the growth mutated cells over normal cells.

The first stage of transformed cells is a group of highly divided cell with normal appearance (hyperplasia). More transformation to hyperplastic leads to abnormal looking cells (dysplasia). The next stage of the transformation of mutated cells cancerous may take between 5-20 years for the transition of benign carcinogenic phase to the fully developed malignant stage where the neoplasia can be detected clinically.

The last stage is termed as ‘progression’, where further genetically changes take place resulting in the increase of proliferation and metastasis (Marshall, 1991; Weinberg, 1996; Compagni and Christofori, 2000; Kintzios and Barberaki, 2004) . Genetic change (mutations) and external factors react together in sequence and target

two groups of normal regulatory genes (proto-oncogenes and tumor suppressor gene), which transfer to the cancer causing gene. Proto-oncogenes are genes encode proteins that are found in every cell, which stimulate cell proliferation, differentiation and development (Sherr, 2004). This normally helps in cells homeostasis. The genes that activated by mutation are called oncogenes, which can be produced by six major factors: growth factors, transcription factor, growth factors receptors, chromatin remodelers, apoptosis regulation, and signal transducers factors (Croce, 2008) (Table 1.1). In contrast, the gene of which the inhibition is by mutation is called the tumor-suppressor gene (Table 1.2). Oncogenes accelerate the tumor cells when activated by mutation. The normal cell process is a balance between tumor-suppressor genes and oncogenes. Tumour-suppressor genes are normal genes which inhibit tumor formation by controlling the cell division, apoptosis and repair DNA mistakes that occur during DNA replication. They act as the "brakes" for the cell cycle. Tumor-suppressor genes mutations lead to a growth of cancer by inactivating that inhibitory function of these genes. In addition, environment and lifestyle, including tobacco, obesity, infectious agents, alcohol, hyperglycemia, food carcinogens, sunlight, stress, and environmental pollutants are major causes of cancer which includes about 90-95% of cases and the remaining 5-10% are due to genetic defects (Anand et al., 2008).

Table 1.1: List of oncogenes.

Oncogenes	Activation/function	Cancer
Abl	Promote cell growth through tyrosine kinase activity	Chronic myelogenous leukemia (Croce, 2008)
Myb	Transcription factor	Colon carcinoma and leukemia
Trk	Receptor tyrosine kinase	Colon and thyroid carcinomas
C-myc	A transcription factor that promotes cell proliferation and DNA synthesis	Leukemia; breast,stomach, lung, cervical, and colon carcinomas; neuroblastomas and glioblastomas (Weber,1987)
HER2/neu	Over-expression of signalling kinase due to gene amplification	Breast and cervical carcinomas (Weber,1987)
Af4 / hrx	Fusion affects the hrx transcription factor / methyltransferase	Acute leukemias
Akt-2	Encodes a protein-serine / threonine kinase	Ovarian cancer
KRAS	promoting cell survival and apoptosis suppression	colorectal carcinomas and lung cancer (Croce, 2008)
Alk/npm	Translocation creates a fusion protein with nuclear phosmin (npm)	Large cell lymphomas
Aml1	Encodes a transcription factor	Acute myeloid leukemia
Aml1/mtg8	A new fusion protein created by the translocation	Acute leukemias
Axl	Encodes a receptor tyrosine kinase	Hematopoietic cancers
Bcl 2, 3, 6	Block apoptosis (programmed cell death)	B-cell lymphomas and leukemias
Dbl	Guanine nucleotide exchange facto	Diffuse B-cell lymphoma

Table 1.2: Some of tumor suppressor genes.

Tumor suppressor genes	**Activation/function**	**Cancer**
APC(denomatous polyposis coli)	Signaling through adhesion molecules to the nucleus	Colorectal carcinomas (Santos, 2009)
BRCA1, BRCA2	DNA Damage Repair	breast cancers; ovarian cancers (Yoshida, 2004)
DCC	Netrin-1 receptor. Regulation of cell proliferation and apoptosis of intestinal epithelium.	Colorectal carcinomas
DPC4 (SMAD4)	Transcriptional factor involved in development; Implicated in metastasis and tumor invasiveness.	Colorectal tumors, pancreatic neoplasia
MADR2/JV18 (SMAD2)	Mediates signaling from growth factor receptors. Assists in transport of SMAD4 into nucleus.	Colorectal cancer
MLH1&MSH2	DNA single-nucleotide mismatch-repair defect permitting the accumulation of oncogenic mutations and tumor-suppressor loss	Colorectal cancer (Sarrió, 2003)
NF1	RAS GTPase activating protein (RAS-GAP)	Neurofibromatosis type 1
p53	Cell cycle regulation, apoptosis	Bladder, breast, colorectal, esophageal, liver, lung, prostate, and ovarian carcinomas; brain tumors, sarcomas, lymphomas, and leukemias (Santos, 2009)
RB	Binds to, and inhibits, the E2F transcription factor. Halts cell cycle progression	Retinoblastoma, sarcomas; bladder, breast, esophageal, prostate, and lung carcinomas
TGFBR2	Receptor responsible for signaling pathways mediating growth arrest and apoptosis	Colorectal and ovarian cancer

1.1.4 Cancer pathology and genetic events of tumorigenesis

In 2000, Hanahan and Weinberg proposed 10 main cellular procedures based on transformation and development of normal cells to establish malignant neoplastic tissue (Hanahan and Weinberg; 2000; Negrini et al., 2010; Hanahan and Weinberg, 2011).These basic hallmarks capabilities acquired during tumor development are:

1- Sustaining proliferatives signalling. This is one of the characterise of cancer cells, which can be acquired by various pathways and is defined by the cells ability to grow constantly without external signals and produce their own growth factors,and the corresponding receptor molecules by the autocrine proliferative stimulation. In addition, they stimulate normal cells and tumor-associated cells by producing paracrine signals, in order to support the cancer cells by forming different growth factors(Gutschner and Diederichs, 2012).
2- Inducing angiogenesis, which activate quiescent endothelial cells in order to grow new blood cells. The growth and metastatic of tumors require the formation of new blood vessels. Therefore, during tumor development and progression the "angiogenic switch" is activated and maintained to support the neoplastic growth by suppling nutirient and oxygn throw the new blood vessels. Tumor cells activate the "angiogenic switch" by countervailing inhibitors, thus inducing and sustaining the angiogenesis substances will occur (Hanahan and Folkman, 1996).
3- Evading growth suppressors (antigrowth).
4- Resisting of programmed cell death (apoptosis).
5- Limitless replication potential. Cancer cells know how to renew themselves continuously.

6- Enhancement tissue metastasis and invasion. Benign tumors are not harmful if they do not spread to other parts of the body. While the malignant tumor which invade surrounding tissues and spread to the other parts of the body (Hanahan and Weinberg, 2011).

7- Genomic instability and mutation are characteristics of most of tumors which generate random mutations during DNA repair and drive tumor development (Negrini et al., 2010; Hanahan and Weinberg, 2011).

8- Evading immune surveillance, in particular by T and B lymphocytes, macrophages, and natural killer cells.

9- Tumor-promoting inflammation.

10- Deregulating cellular energetic (Figure 1.1).

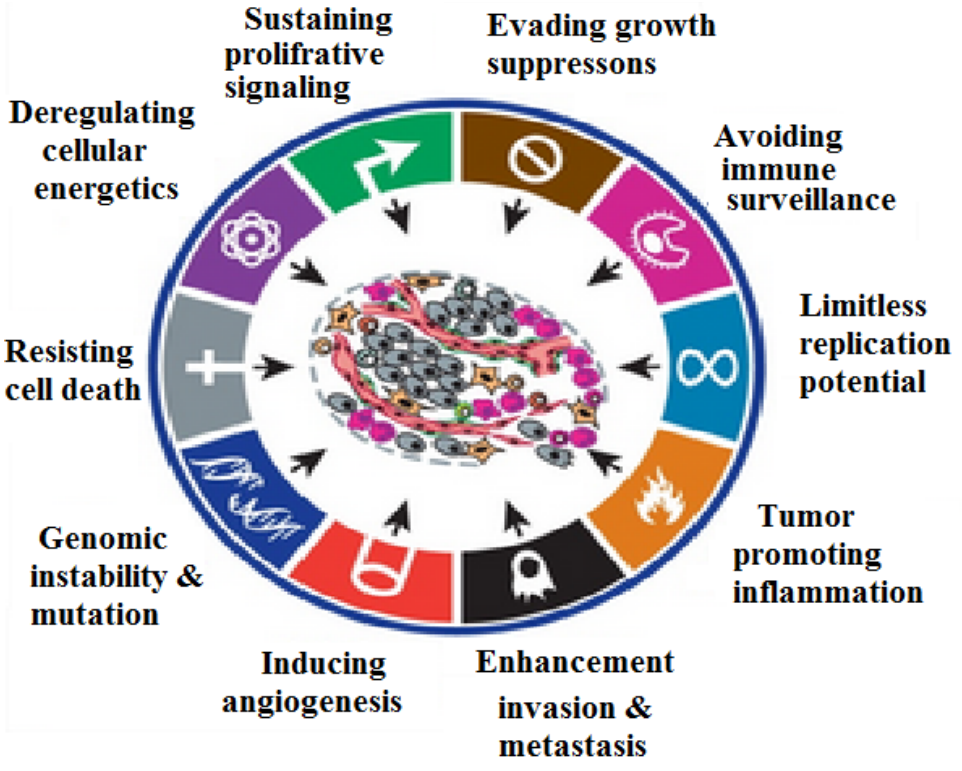

Figure 1.1: Ten hallmarks of cancer acquired during cancer progression. Adapted from (Hanahan and Weinberg, 2011).

1.2 Colorectal cancer

Colorectal cancer (CRC) is a multistep process of epithelial cells transformation into malignant cells, which is caused by the sequential order of genetic, growth factors and epigenetic mutations (Pancione et al., 2012). Several researchers have been reported that development and rise of colorectal tumors associated with specific mutation including microsatellite instability (MSI), adenomatous polypsis coli (APC) gene, stabilization and translocation of β-catenin, chromosomal instability (CIN), Kirsten-rat sarcoma virus (KRAS), TP53, loss of the 18q21 gene cyclooxygenase-2 (COX-2), and mutations in transforming growth factor β II receptor (TGFβR2) (Markowitz and Bertagnolli, 2009; Kanthan et al., 2012) (Figure 1.2).

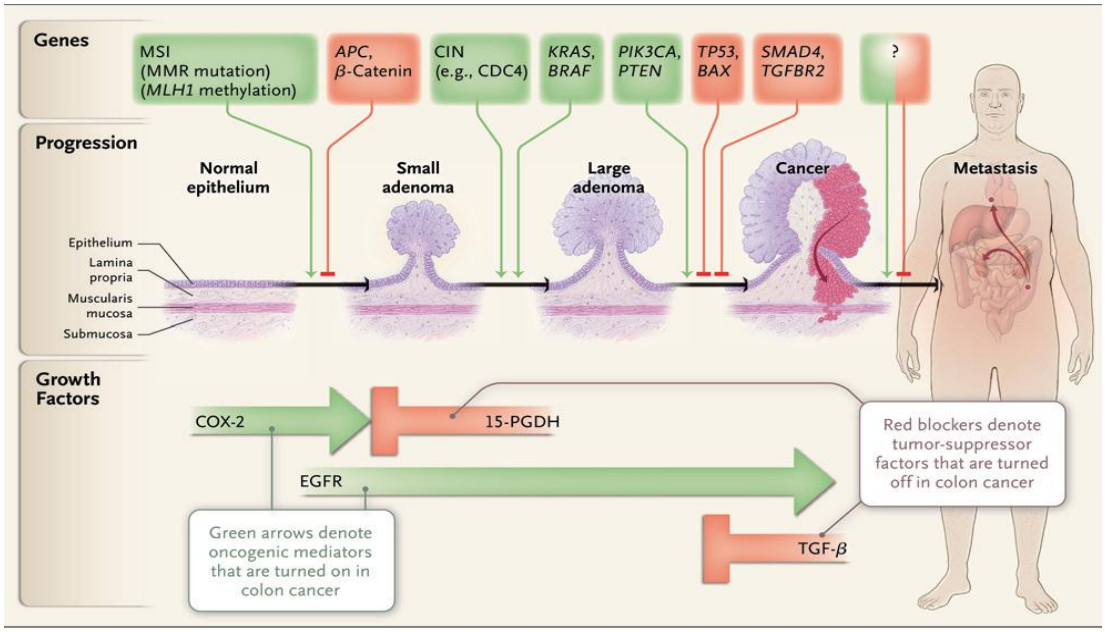

Figure 1.2: Genetic alterations frequently associated with CRC progression
Adapted from (Markowitz and Bertagnolli, 2009)

Previous studies were illustrated the role of the APC suppressor gene in the early stages of colorectal carcinoma. Inactivation of APC gene is associated with accumulation of intracellular β-catenin which plays a central role in cell adhesion

and acts as a transcription factor of the Wnt signaling pathway (Yang et al., 2006). Stimulation of Wnt/β-catenin signaling pathway leads to activation of T-cell factor/lymphoid enhancing factor-1 (TCF/LEF1) transcription factors and subsequently to the expression of several target genes including COX-2, cyclin D1 and c-Myc that are concerned in tumorigenesis of colorectal carcinoma and several other cancers (MacDonald et al., 2009). In addition, oncogenic mutations in K-ras were found to play essential role in tumorigenesis of colorectal carcinoma and their presence indicates poor prognosis (Conlin et al., 2005). Besides the hereditary APC alteration and other acquired genetic changes there are other associated genetics, enzymes and antigenic that have been found to play a central role in the adenoma-carcinoma sequence. Various carcinogenic factors have been described which also contribute in the adenoma and carcinoma formation such as familial history of colonic neoplasia, acromegaly, ulcerative colitis, drinking, smoking and consumption of red meat.

1.2.1 Epidemiology of colorectal cancer

Colorectal cancer incidence in man is higher than women with an overall sex ratio of the age standard rate, of 1.4:1 (Ferlay et al., 2010). In man, it is the third most commonly diagnosed malignant neoplasm worldwide (663,000 cases, 10.0% of the total) (Siegel et al., 2015; Scholefield and Eng, 2014), and the second most common cancer in women (570,000 cases, 9.4% of the total), beside it is the third leading cause of cancer deaths, accounting for 600,000 deaths each year (Roper and Hung, 2013). In the United States, colorectal cancer is the third leading cause of cancer deaths (9% of estimated cancer deaths in both men and women in 2012) (Scholefield and Eng, 2014).

Globally, CRC is a burden; the incidence rate is ten times higher in regions with the highest rate, such as Australia and Canada than in regions with the lowest rates, such as India, while the mortality rate is five times higher in regions with the highest rates than it is in regions with the lowest rates (Scholefield and Eng, 2014). The American Cancer Society (ACS) estimates 132,700 new cases of colorectal cancer in women and men and that 49,700 will die as a result of it in the United States in 2015 (Smith et al., 2015), compared to the incidence 148,300 new cases and 56,600 deaths in 2002. CRC is the second most common malignancy in Malaysia. Life styles, heredity, diet and micronutrient malnutrition are few putative etiology in CRC. The incidence of CRC is higher in Malaysia compared to incidence in Indian subcontinent possibly due to low intake of dietary insoluble fibre, higher animal diet and red meat content of food. The epidemiological data on CRC in Malaysia are fragmentary and insufficient. Moreover there is no specific control or preventive measure taken by ministry of health to detect CRC early in Malaysia. Colonoscopy and Fecal occult blood test are rarely advocate for early detection. Therefore it is very rare to find CRC patients in early stages. The stage distribution of CRC patients in Malaysia is shifted to right with majority being presented in late stages (III & IV). In few earlier studies stage per stage survival of CRC are lower compared to western counterparts (Biswal et al., 2002).

1.2.2 Chemotherapeutics of colorectal cancer

Chemotherapy is a type of cancer treatment that uses one or more of medicinal drug to destroy the cancer cells. Up to know, no curative therapy is available for most types of cancer including colon cancer. The available treatments are used to prolong the life span of cancer patients. Previously, cytotoxic agents such as 5-

fluorouracil (5-FU), oxaliplatin (OX) and capecitabine (Cap) were used to treat colorectal cancer. The various combinations of these agents were extensively studied in phase II and phase III clinical trials such as IFL (irinotecan, 5-FU and LV), FOLFOX (5-FU, OX and LV), FOLFIRI (5-FU, LV and irinotecan) and CapOx (capecitabine/oxaliplatin). All of these combinations showed improvement in the therapeutic outcome than with mono-therapy (Cercek and Saltz, 2008). After the development of the monoclonal antibodies bevacizumab (anti-VEGF), panitumumab (human anti-EGFR) and Cetuximab (chimeric human-mouse anti-EGFR), several combinations of these agents, with the cytotoxic drugs have been studied in phase II and phase III clinical trials. In general, the results show that the combination of anti-angiogenic factors either anti-VEGF or anti-EGFR antibodies with cytotoxic agents resulted in increased therapeutic outcomes than each individual therapy (Cercek and Saltz, 2008).

1.3 Tumor angiogenesis

1.3.1 Physiologic and pathologic angiogenesis

Angiogenesis or neovascularization is the multistep physiological process of generating new blood vessels from pre-existing vasculature. It is an essential requirement for normal physiological conditions such as the process during the development of the organs in new-borns, during wound healing, vascular system in embryonic development of the placenta during pregnancy and for the reproductive function of adults (Tonnesen et al., 2000; Auerbach et al., 2003; Sagar et al., 2006). Nevertheless, angiogenesis play fundamental role in numerous pathologic disorders of many diseases, such as rheumatoid arthritis, psoriasis, cardiovascular, blindness, obesity, ischemia, cornel neovascularisation, diabetic retinopathy, tumor growth,

tumor propagation, metastasis formation and inflammatory diseases (Folkman, 1971; Folkman, 1995; Auerbach et al., 2003; Hanyu et al., 2009; Prager et al., 2011;). In 2004, Hoeben and others reported angiogenesis in adults as being tightly controlled by a physiological balance between the stimulatory (pro-angiogenic) and inhibitory (anti-angiogenic) signals.

Several studies reported that angiogenesis may be an excellent therapeutic target for the treatment of tumor and other angiogenesis dependent disease (Ferrara, 2002; Carmeliet, 2005). Therfore, insufficiency of angiogenesis may occur in reduction the tumor growth, invasion and metastasis (Folkman, 1974; Chia et al., 2010).

Therapeutically, targeting angiogenesis has been widely regarded as an attractive approach for cancer therapy (Hoeben et al., 2004). For that reason, determining the pro-angiogenic or anti-angiogenic effects of the molecules currently used in cancer treatment is crucial (Folkman, 1971; Carmeliet, 2005).

1.3.2 Angiogenesis cascade events

Angiogenesis depends on the interactions of the endothelial cell with the extracellular matrix compounds. There are multistep processes to develop effective angiogenesis. Regulated steps, which involve the formation of blood vessels, are initiation by biological signals, which lead to the activation of the receptors on the endothelial cell by the angiogenic growth factors (Gupta and Qin, 2003). The activated cells, which cover the blood vessel walls, start to release proteases enzymes (Matrix Metalloproteinase such as MMP9) that cause pericytes to detach and degrade the extracellular matrix and basement membrane, which allows the underlying

endothelial cells to escape from the blood vessel walls. Following this, the front lines of endothelial cells migrate toward the angiogenic stimulus (Fischer et al., 2006). The migrating cells start proliferation to form solid sprouts that link to adjacent vessels using adhesion molecules called integrins.

The sprouts fuse with other sprouts to form loops. The new blood vessels formed are lined by vascular basal lamina. Finally, blood starts to flow through the new vessels (Eliceiri and Cheresh, 2001; Hoeben et al., 2004). Targeting any of these steps can inhibit the formation of new blood vessels, thus it could be striking approach for treating angiogenesis-related diseases, most notably cancer (Cardenas et al., 2011) (Figure1.3).

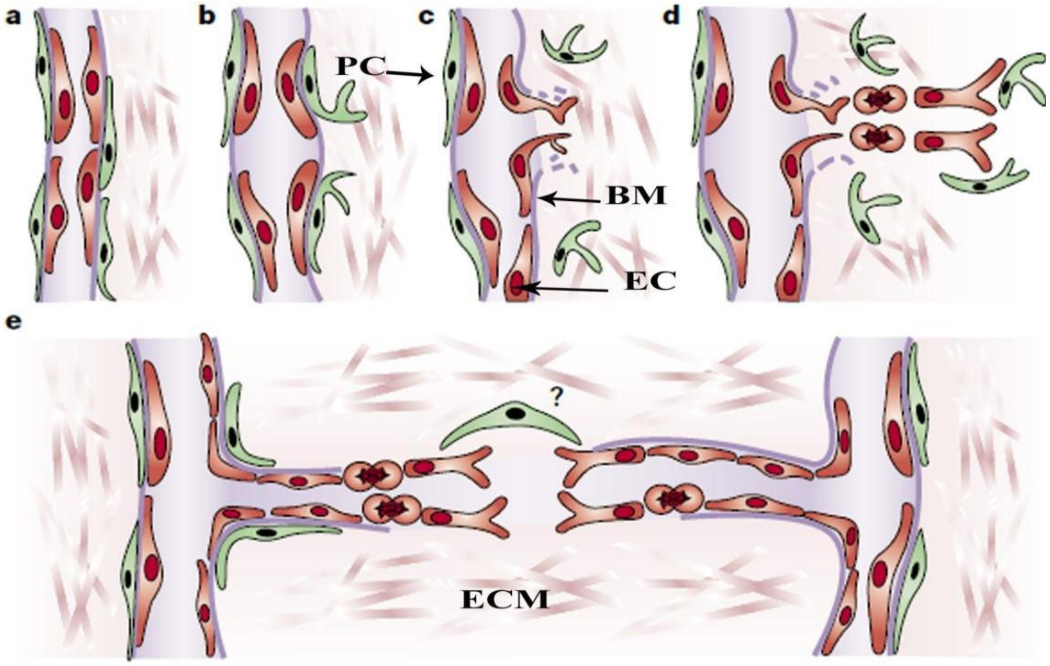

Figure 1.3: Angiogenesis Cascade. (a): Blood vessels (b): Pericytes (PC) detach, blood vessels dilate before basement membrane (BM) and extracellular matrix (ESM) gets degraded (c) The underlying endothelial cells escape from the blood vessels wallto allows endothelial cells (EC) to migrate into perivascular space towards angiogenic stimuli, (d) after that, the endothelial cells proliferate, following each other, guided by pericytes, (e) endothelial cells adhere to each other and formed a lumen which is accompanied by basement membrane formation and attachment by pericytes. Finally, the blood vessels sproutingfuse with other sprouts to form loops which formation of new circulatory systems. Adapted from (Bergers & Benjamin 2003).

1.3.3 Regulation of angiogenesis

Angiogenesis is tightly regulated process that controlled by balance between pro-angiogenic (stimulators) and anti-angiogenic (inhibitors) molecules. In their review article, Liekens and his co-workers narrate the angiogenesis process in three steps: the first step is the degradation of the extracellular matrix, the second is the regulation of angiogenic modulators, including the growth factors and the cytokines and enzymes, and the third level is the cell-cell and the cell-matrix interactions (Liekens et al., 2001).

The first step in the formation of new vessels is the proteolytic breakdown of the basement membrane underlying endothelial cells, in order for them to migrate and invade the stroma of surrounding tissues. This process requires the activity of the plasminogen activators (PAs) and the matrix metalloproteinase MMPs (Mignatti and Rifkin, 1996). The activity of both PAs and MMPs is controlled either at their expression level, at the activation level by the proteolytic enzymes, or at the level of their inhibitors, such as the tissue inhibitor of metalloproteinase and the plasminogen activator inhibitors (Liekens et al., 2001).

Subsequent to the proteolytic degradation of the extracellular matrix and under the influence of a variety of growth factors, the frontline endothelial cells start to proliferate and migrate through the degraded matrix towards angiogenesis stimuli Several modulators of angiogenesis, including inducers and inhibitors, have been described so far: the vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), the angiopoietins 1 and 2 (Ang-1 and 2), angiostatin, endostatin, interferons α and γ (IFN-α and γ) and several other growth factors (Liekens et al., 2001). The regulation of angiogenesis depends on the balance between the

stimulators and inhibitors of the process; when the pro-angiogenic growth factors predominate, then proliferation and migration of endothelial cells is increased and this consequently leads to the formation of new blood vessels and angiogenesis can be halt when anti-angiogenic modulators dominate pro-angiogenic mediators (Table 1.3). The cell adhesion molecules, besides the proteases enzymes and growth factors, play a critical role in the regulation of the angiogenesis cascade of events.

Cell adhesion molecules are classified into four families such as immunoglobulin supergene family, cadherins, and the integrins. The integrins, for example, mediate the interaction of endothelial cells with the extracellular matrix during invasion and migration. Also, the cell adhesion molecules are required for cell–cell and cell– extracellular matrix interactions, which are required for lumen formation and the construction of functional capillary loops (Bischoff, 1997).

Table 1.3: Example of pro and anti-angiogenic factors.

Pro-angiogenic (stimulators)	Anti-angiogenic (Inhibitors)
VEGF	Interferons a/b
bFGF/aFGF	Canstatin
PDGF	VEGI
PIGF	Tumstatin
TGFα/β	Angiostatin
Del-1	IL-12
TNF-a	Vasostatin
IL-8	Platelet factor-4
HGF	Thrombospondin
PD-ECGF	Endostatin
Angiogenin	16-kd prolactin fragment
IL-3	PEDF
Midkine	2 methoxyestradiol
Leptin	53-kd antithrombin III
Follistatin	Prothrombin fragments 1 and 2
G-CSF	Domain 5 of HMWK
Proliferin	Restin
Pleiotrophin	Maspin
HIV Tat	SPARC
Plasminogen activators, MMPs	IP-10
----	IL-18

Abbreviations: FGF, fibroblast growth factor; PlGF, placental growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; Del-1, developmental endothelial locus-1; TNF-α, tumor necrosis factor alfa; VEGI, vascular endothelial growth inhibitor; IL, interleukin; HGF, hepatocyte growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; PEDF, pigment epithelium-derived factor; HMWK, high molecular weight kininigen; G-CSF, granulocyte colony-stimulating factor; HIV Tat, human immunodeficiency virus TAT; IP-10, interferon-inducible protein-10; SPARC, secreted protein acidic and rich in cysteine.

1.3.4 Anti-angiogenic targets

The formation of new blood vessels is a complicated multistep process. Agents that suppress or stop neovascularization often do so by interfering with an essential step in this process, such as: (a) Reduction of endothelial cell activation, which may be achieved via the inhibition of growth factor signal production, inhibition of receptors production or inhibition of the binding between signals and receptors, (b) Targeting of endothelial cell proliferation, (c) inhibition of endothelial cell migration, (d) inhibition of endothelial cell differentiation to form a three dimensional tube-like structure and (e) stimulation of apoptosis in endothelial cells (Zhang and Bicknell, 2001).

1.3.5 Anti-angiogenic therapies

Angiogenesis is the hallmark of cancer, which plays an important role in tumor growth, invasion, and metastasis, so blockade of angiogenesis has been viewed as an effective strategy for the therapy of tumor growth and progression. Therefore, targeting angiogenesis became of great therapeutic value to cancer and other angiogenesis related diseases, it work by starving the tumor and suppress its growth rather than targeting neoplasic cells (Folkman, 1971; Quesada et al., 2006).

There are several current strategies for the inhibition of angiogenesis, which include antisense mRNA, monoclonal antibodies, receptor antagonists, and soluble receptors. More than forty anti-angiogenic drugs are being tested in human cancer patients in clinical trials all over the world. These can be divided into three groups according to the target point. The first group includes drugs that inhibit the growth of endothelial cells, such as endostatin and combretastatin A4, which induce the

apoptosis of endothelial cells (Kerbel and Folkman, 2002), whereas curcumin is an inhibitor of proliferation and cell cycle progression of endothelial cell (Singh et al., 1996). The second group includes drugs that block angiogenesis signaling, such as Avastin® and Interferon-alpha, which inhibits the production of basic fibroblast growth factor (b-FGF) and VEGF (Zhang and Bicknell, 2003). The third group consists of drugs that block extracellular matrix breakdown, such as inhibitors of matrix metalloproteinase (MMPs) and Pericytes (PC), which work by inhibiting the breakdown of extracellular matrix and thus interfere with the invasion and migration of endothelial cells.

Other new drugs, such as the tyrosine kinase inhibitors (Erlotinib, Sorafenib and Sunitinib) block the activity of multiple growth factor receptors, such as VEGF and platelet-derived growth factor receptors (PDGFRs) (Tabernero, 2007; Gotink and Verheul, 2010). Currently, seventeen anti-angiogenic agents have been approved as anti-cancer therapies by the American Food and Drug Administration (FDA) (Bodnar, 2014) (Table 1.4). These include small molecule tyrosine kinase inhibitors directed against pro-angiogenic growth factor receptors and monoclonal antibodies directed against specific pro-angiogenic growth factors or their receptors (Samant and Shevde, 2011; Bodnar, 2014).

Table 1.4: FDA-approved angiogenesis inhibitors (Adapted from Bodnar, 2014)

Inhibitor	Trade name (manufacturer)	Type of drug	Target	Clinical usage
Bevacizumab	Avastin (Genentech	Monoclonal antibody	VEGFR1–2 tyrosine kinase	Metastatic CRC, NSCLC,glioblastoma, metastatic RCC
Cetuximab	Erbitux (Bristol-Myers Squibb)	Monoclonal antibody	EGFR tyrosine kinase	Metastatic CRC, RCC
Panitumumab	Vecitbix (Amgen)	Monoclonal antibody	EGFR	Metastatic CRC
RanibizumabLucentis	(Genentech)	Monoclonal antibody	VEGF-A	Wet age-related macular degeneration
Trastuzumab	Herceptin (Genentech)	Monoclonal antibody	HER2	Advanced RCC
(Axitinib	Inlytan (Pfizer)	Small-molecule inhibitor	VEGFR1–3	Advanced RCC
Cabozantinib	Cometriq (Exelixis)	Small-molecule inhibitor	VEGFR2	c-Met Metastatic medullary thyroid cancer
Erlotinib	Tarceva (Genentech)	Small-molecule inhibitor	EGFR tyrosine kinase	Advanced or metastatic NSCLC
Everolimus	Afinitor (Novartis)	Small-molecule inhibitor	mTOR, PI3/AKT pathway	Advanced RCC,pancreatic neuroendrocrintumor, SEGA
Imiquimod	Aldara (Medicis)	Small-molecule inhibitor	TLR-7	Actinic keratosis, basal cell carcinoma
Pazopanib	Votrient (GlaxoSmithKline)	Small-molecule inhibitor	VEGFR, PDGFR, c-Kit	Advanced RCC
Regorafenib	Stivarga (Bayer)	Small-molecule inhibitor	VEGFR1–3, PDGFR, FGFR, Kit, Raf, RET	Metastatic CRC
Sunitinib	Sutent (Pfizer)	Small-molecule inhibitor	VEGFR1–3, PDGFRb, RET	Advanced RCC, GIST, pancreatic neuroendocrine tumor
Sorafenib	Nexavar (Bayer/Onyx)	Small-molecule inhibitor	VEGFR1–3,PDGFRb, Raf-1	Advanced RCC, advanced HCC
Temsirolimus	Torisel (Wyeth)	Small-molecule inhibitor	mTOR	Advanced RCC
Vandetanib	Caprelsa (AstraZeneca)	Small-molecule inhibitor	VEGFR, FGFR	Medullary thyroid cancer
Pegabtanib	Macugen (OSI Pharmaceuticals)	Pegylatedaptamer	VEGF	Wet age-related macular degeneration

1.4 Correlation between cancer and angiogenesis

1.4.1 Pro and anti-angiogenic mediators

Pro-angiogenic factors are one of the most critical tumor markers that play an important role in neoplastic transformation and the progression of microvessel growth in cancer. It is initiated by the secretion of growth factors with angiogenic properties, such as vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), basic fibroblast growth factor (b-FGF) and epidermal growth factor (EGF), nerve growth factor (NGF) platelet-derived growth factor (PDGF), Interleukin 1, 2 &7, Interferon (IFNs) and Granulocyte macrophage colony stimulating factor (GM CSF), (Prager et al., 2011). These growth factors stimulate angiogenesis via the binding to their relevant receptors which are mainly expressed in endothelial cells (Figure 1.4).

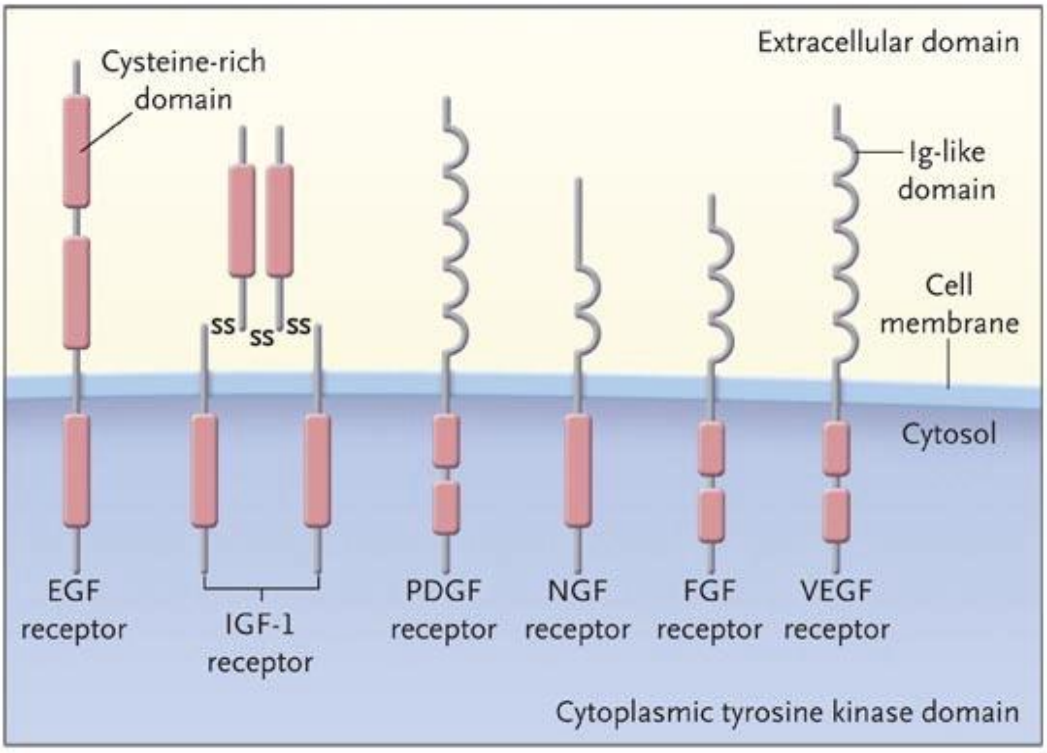

Figure 1.4: Growth factors receptors.

1.4.1.(a) Vascular endothelial growth factor

VEGFA is the prototype member of a gene family that also includes VEGFB, VEGFC, VEGFD and placenta growth factor (PLGF) (Figg and Folkman, 2008). It is a dimeric glycoprotein that binds strongly with vascular endothelial cell receptors called VEGF receptor-2 (VEGFR-2), which is a member of a receptor tyrosine kinase family (Shibuya, 2011).This is an important protein involved in developing a new blood supply, like the formation of new blood vessels from pre-existing ones (angiogenesis) and the formation of new blood vessels from non-pre-existing ones (vasculogenesis). In addition to a secreted endothelial-specific growth factor that is strongly VEGFR-2-implicated in all aspects of pathological vascular-endothelial-cell biology, dimerization of the receptor is followed by autophosphorylation, which leads to the activation of the angiogenic cascade (Olsson et al., 2006; Koch and Claesson-Welsh, 2012). Since, a close relationship between several pathologies and angiogenesis has been clarified; various angiogenic inhibitors have been studied. Many of these inhibitors are directed against VEGF or its receptors, which are considered to play a key role in angiogenesis (Niu and Chen, 2010). Thus targeting angiogenesis could be a strategy to combat angiogenesis-dependent diseases. In the case of cancer, most tumors require a more extensive blood supply to provide nutrition in order to support rapid growth (Veeravagu et al., 2007; Wang et al., 2015).

1.4.1.(b) Hypoxia inducible factor-1

Hypoxia inducible factor-1 (HIF-1) is a transcription factor which plays critical role in the regulation of multiple aspects of tumorigenesis such as nutritional stress,