THE POTENTIAL ANTI-CANCER EFFECTS OF *PSIDIUM GUAJAVA* (GUAVA) LEAVES ON COLORETAL CANCER VIA THE INHIBITION OF ANGIOGENESIS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ang	Angiopoietin
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CXCL2	Chemokine (C-X-C motif) ligand 2
dH2O	*P. guajava* leaf distilled water extract
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	Free radical scavenging potential
EA.hy926	Human vascular endothelial cell line
EOH	*P. guajava* leaf ethanol extract
FBS	Foetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
HCT 116	Human colon carcinoma cell line
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HUVEC	Human umbilical vein endothelial cell line
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KBr	Potassium bromide
m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa-B
NH	*P. guajava* leaf n-hexane extract
NO	Nitric oxide
P. guajava	*Psidium guajava* (Guava)
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PlGF	Placental growth factor
RPMI 1640	Roswell Park Memorial Institute medium
SD	Standard deviation
TFC	Total flavonoid content
TNF	Tumour necrosis factor
TPC	Total phenolic content
UV-Vis	Ultraviolet-visible spectrophotometry
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
λmax	Maximum absorbance

LIST OF UNITS

°C	Celsius
µg	Microgram
µl	Microlitre
µm	Micrometre
cm	Centimetre
eV	Electronvolt
g	Gram
h	Hour
kcal	Kilocalorie
kg	Kilogram
m	Metre
mg	Milligram
mg GAE/g	Milligram of gallic acid equivalent per gram of tested sample
mg QE/g	Milligram of quercetin equivalent per gram of tested sample
min	Minute
ml	Millilitre
mol	Mole
nm	Nanometre
psi	Pounds per square inch
s	Seconds

POTENSI KESAN ANTIKANSER DAUN *PSIDIUM GUAJAVA* (JAMBU BATU) TERHADAP KANSER KOLOREKTUM MELALUI PERENCATAN ANGIOGENESIS

ABSTRAK

Kanser kolorektal, iaitu kanser yang terdapat dalam kolon atau rektum, adalah salah satu kanser yang mengakibatkan kematian yang paling umum. Kelaziman kanser kolorektal di Malaysia dijangka akan meningkat disebabkan oleh penuaan populasi, kemajuan sosioekonomi, serta diet dan gaya hidup yang semakin kebaratan. Pertumbuhan tumor-tumor kolorektal melebihi saiz tertentu, serta penyebaran metastatik mereka, bergantung kepada peningkatan angiogenesi di vaskulatur sekitarnya. Disebabkan daun-daun *Psidium guajava* (jambu batu) kaya dengan sebatian fenolik, flavonoid, karotenoid, dan vitamin, aktiviti-aktiviti anti-angiogenik dan antikanser ekstrak-ekstrak daun *P. guajava* diselidiki untuk menilai potensi mereka dalam rawatan kanser kolorektal yang bergantung kepada angiogenesis. Tiga ekstrak dihasilkan menggunakan air suling, etanol, dan n-heksana sebagai pelarut, dan dilabelkan sebagai dH2O, EOH, dan NH. Ekstrak-ekstrak tersebut dicirikan secara fizikal melalui kromatografi gas-spektrometer massa (GC-MS), spektroskopi ultraviolet dan sinar tampak (UV-Vis), dan spektroskopi inframerah transformasi Fourier (FTIR). Melalui kajian pemulung radikal bebas DPPH, jumlah kandungan fenolik, dan jumlah kandungan flavonoid, ekstrak EOH didapati mempunyai aktiviti antioksidan yang tertinggi. Ekstrak dH2O dan EOH menunjukkan aktiviti perencatan yang lebih banyak terhadap daya maju sel-sel EA.hy926 (endothelial vaskular manusia) dan pertumbuhan saluran darah mikro dari cincin aortik tikus berbanding dengan ekstrak NH, menunjukkan aktiviti anti-angiogenik yang lebih baik. Untuk mengkaji

kesan-kesan ekstrak dH2O dan EOH ke atas pelbagai aspek proses angiogenik, kajian-kajian penghijrahan sel, pembentukan tiub, dan pembentukan koloni telah dijalankan menggunakan sel-sel EA.hy926. Ekstrak EOH didapati jauh lebih baik daripada ekstrak dH2O dari segi menentang penghijrahan sel dan pembentukan koloni sel-sel. Kedua-dua ekstrak juga berkesan terhadap pembentukan tiub, dengan ekstrak dH2O yang mempunyai aktiviti perencatan yang lebih tinggi. Melalui kajian penetapan kadar imunosorben taut-enzim (ELISA), ekstrak EOH didapati berjaya menghalang ekspresi VEGF dari sel-sel EA.hy926. Ekstrak NH mempunyai aktiviti ingibisi yang lebih baik terhadap sel-sel karsinoma kolon HCT 116, walaupun nilai IC_{50} yang tinggi menunjukkan bahawa ketiga-tiga ekstrak tersebut tidak sitotoksik terhadap sel-sel kanser, dan ekstrak-ekstrak tersebut mungkin menghalang pertumbuhan sel-sel melalui mekanisme lain. Aktiviti perencatan kanser kolorektal oleh ekstrak dH2O dan EOH mungkin adalah melalui aktiviti anti-angiogenik mereka, kerana kedua-dua ekstrak ini menunjukkan kesan perencatan yang ketara terhadap spheroid tumor yang terdiri daripada sel-sel EA.hy926 dan HCT 116. Melalui kajian dok molekular, adalah didapati bahawa aktiviti anti-angiogenik ekstrak EOH boleh dikaitkan dengan hakitat bahawa ia mungkin mengandungi β-caryophyllene dan β-elemene, bahan-bahan kimia yang mempunyai potensi tinggi untuk menjadi perencat yang kuat terhadap HIF-1α, iaitu protein yang menggawal transkripsi VEGF. Sementara itu, aktiviti antioksidan dan antikanser ekstrak EOH mungkin disebabkan oleh kemungkinan kewujudan β-caryophyllene. Ekstrak EOH daun jambu batu telah menunjukkan kesan-kesan anti-angiogenik yang menjanjikan dalam kajian-kajian *in vitro* dan *ex vivo*. Oleh itu, kajian ini boleh disimpulkan bahawa ekstrak EOH mempunyai potensi dalam rawatan kanser kolorektal melalui perencatan angiogenesis.

THE POTENTIAL ANTICANCER EFFECTS OF *PSIDIUM GUAJAVA* (GUAVA) LEAVES ON COLORECTAL CACNER VIA THE INHIBITION OF ANGIOGENESIS

ABSTRACT

Colorectal cancer, which is the cancer that develops in the colon or rectum, is one of the most common cancers that lead to death. The prevalence of colorectal cancer in Malaysia is expected to increase due to the aging population, socioeconomic advances, and increasingly Westernised diet and lifestyle. The growth of colorectal tumours beyond a certain size, as well as their metastatic spread, is dependent on the increase in angiogenesis of their surrounding vasculature. As *Psidium guajava* (guava) leaves are rich in phenolic compounds, flavonoids, carotenoids, and vitamins, the anti-angiogenic and anticancer activities of *P. guajava* leaf extracts were investigated to assess their potential in the treatment of the angiogenesis-dependent colorectal cancer. Three extracts were produced using distilled water, ethanol, and n-hexane as solvents, and were labelled as dH2O, EOH, and NH. The extracts were physically characterised through gas chromatography–mass spectrometry (GC-MS), ultraviolet-visible spectroscopy (UV-Vis), and Fourier transform infrared spectroscopy (FTIR). Through the DPPH radical scavenging, total phenolic content, and total flavonoid content assays, the EOH extract was found to have the highest antioxidant activity. The dH2O and EOH extracts exhibited more inhibitory activity against EA.hy926 (human vascular endothelial) cell viability and microvessel growth from rat aortic rings compared to the NH extract, indicating a better anti-angiogenic activity. In order to investigate the effects of the dH2O and EOH extracts on different aspects of the angiogenic process, the cell migration, tube formation, and colony formation assays

were conducted using the EA.hy926 cells. The EOH extract performed significantly better than the dH2O extract against the cell migration and colony formation of the cells. Both extracts were also effective against tube formation, with the dH2O extract having a slightly higher inhibitory activity. Through the enzyme-linked immunosorbent assay (ELISA), the VEGF expression of the EA.hy926 cells was found to be inhibited by the EOH extract. The NH extract had a better inhibitory activity on the cell viability of the HCT 116 human colon carcinoma cell line, although the high IC_{50} values suggested that all three extracts were not cytotoxic towards the cancer cells, and the extracts may have inhibited the growth of the cells through other mechanisms. The colorectal cancer inhibitory activity of the dH2O and EOH extracts could have been through their anti-angiogenic activity, as the two extracts showed significant inhibitory effect on tumour spheroids consisting of EA.hy926 and HCT 116 cells. From the molecular docking studies, the anti-angiogenic activity of the EOH extract could be attributed to the fact that it may contain β-caryophyllene and β-elemene, which had a high potential to be a potent inhibitor to HIF-1α, a protein that mediates the transcription of VEGF. Meanwhile, the antioxidant and anticancer activities of the EOH extract could be due to the possible presence of β-caryophyllene. The EOH guava leaf extract had shown promising anti-angiogenic effect *in vitro* and *ex vivo*. Therefore, it can be concluded that the EOH extract has potential in the treatment of colorectal cancer through the inhibition of angiogenesis.

CHAPTER ONE

INTRODUCTION

1.1 Cancer

Cancer, also known as malignant tumour and neoplasm, is no single disease, but a generic term for large family of diseases defined by abnormal cell growth beyond their usual boundaries, which could also potentially metastasize, invading adjoining body parts and spread to other organs. There are over 100 types of cancers that affect humans.

According to the World Health Organisation (WHO), cancer is the second leading cause of death globally, accounting for around 8.8 million deaths, which was one out of six deaths. Around 70% of cancer deaths occur in low- and middle-income countries. The Global Health Observatory estimated that 9.6 million deaths will be by cancer in 2018, and therefore is a global epidemic that needs to be addressed. The most common cancers that led to deaths in 2015 were cancers of the lungs, liver, bowels (colorectal), stomach, and breasts (World Health Organization (WHO), 2018). The most common types of cancers that affect males are cancers of the lungs, prostate, bowels, and stomach, while cancers of the breasts, bowels, lungs, and cervix are more common for females. For children, cancers of the lymphoid blood cells (leukaemia) and brain are more common (World Health Organization (WHO), 2014) (World Health Organization (WHO), 2018).

1.1.1 Causes of Cancer

Most cases of cancer arose from the interaction between an individual's genetic factor with environmental factors, with only a small amount of cases that were caused by inherited genetics (Anand et al., 2008). The cancer-causing environmental factors, known as carcinogens, can be separated into three types – physical, chemical, and biological. Physical carcinogens include ionizing radiation and non-ionizing ultraviolet radiation, and exposure to these types of radiation are related to the cause of some invasive cancers and most non-invasive cancers such as non-melanoma skin cancers. Exposure to chemical carcinogens, such as tobacco smoke and alcohol, could lead to specific types of cancer. Tobacco smoke, in particular, is highly correlated with incidences of lung cancer (Kuper et al., 2002), while numerous cases of liver and digestive tract cancers were attributed to alcohol exposure (Schütze et al., 2011). Biological factors that cause cancer mostly come from infections of the body by pathogens, usually viruses. Examples of these cancer-causing viruses, or oncoviruses, are the human papillomavirus that causes cervical cancer, and the hepatitis B and hepatitis C viruses that cause liver cancer.

Other than environmental factors, a small minority of cancers were caused by inherited genetic mutations, consisting of less than 3–10% of all cases of cancer (Roukos, 2009).

1.1.2 Oncogenesis

The transformation from normal cells to cancer cells is caused by a series of genetic mutations in genes that regulate cell division, apoptosis, and DNA repair. According to researchers Douglas Hanahan and Robert Weinberg, tumour cells show

the “six hallmarks of cancer” – the characteristics necessary to produce malignant tumours as normal cells progress into the cancer disease, enabling tumour growth and metastasis. These characteristics include the sustained proliferation of the cells without the regulation of normal intercellular signals, continuous cell growth despite the presence of growth suppressors, resistance towards programmed cell death through apoptosis, immortality in terms of unlimited cycles of cell replication, the induction of angiogenesis for the production of new blood vessels to provide for the metabolic requirements of the cells with their unchecked growth, and the invasion of their surrounding tissues as well as metastasis to other organs (Hanahan and Weinberg, 2011).

1.1.3 Colorectal Cancer

Colorectal cancer is also known as bowel cancer or colon cancer. It was responsible for 774 000 deaths in 2015 (World Health Organization (WHO), 2018). Colorectal cancer may lead to the narrowing or blockages of the bowel, which would affect the bowel habits of the patient. Symptoms of this disease include diarrhoea or constipation, blood in stool, abdomen pain and bloating, fatigue, or unexplained weight loss.

Most cases of colorectal cancer are caused by old age and lifestyle factors such as smoking (Liang et al., 2009), a lack of physical exercise (Harriss et al., 2009), and the consumption of red meat and processed meat (Larsson and Wolk, 2006) as well as alcohol (Cho et al., 2004) (Fedirko et al., 2011) (World Health Organization (WHO), 2014). Those inflicted with inflammatory bowel diseases (Crohn’s disease and ulcerative colitis) had a greater risk of getting colorectal cancer. Less than 5% of cases

of colorectal cancer were caused by inherited genetic disorders such as hereditary non-polyposis colon cancer, Gardner syndrome, and familial adenomatous polyposis (Lynch and De la Chapelle, 2003). The risk of developing colorectal cancer is high for a person with first-degree relatives (parent, sibling or child) diagnosed with it (Kampman, 2007).

Colorectal cancer is nearly three times more common in high-income countries than in middle- to low-income countries due to the higher life expectancy, as well as the Westernised diet. It is also slightly more common in men than in women by seven to five (Ferlay et al., 2010). In Asia, while cases of colorectal cancer is higher in developed countries such as Singapore, Japan, and South Korea (International Agency for Research on Cancer, 2012) (Sung et al., 2014), the socioeconomic advancement and increasingly Westernized life style in Malaysia is expected to heighten the incidences of colorectal cancer in the near future (Veettil et al., 2017). The prevalence of colorectal cancer risk would also increase in the future due to the aging of the Malaysian population (Yusoff and Zulkifli, 2014), as about 80% of the cases of colorectal cancer in Malaysia were diagnosed in people older than 50-years-old (Lim et al., 2008). In 2014, incidences of colorectal cancer among other types of cancer were the second highest in males (2 563 cases), and third in females (1 976).

1.1.3(a) Pathogenesis of Colorectal Cancer

Tumours of colorectal cancer originate from the epithelial cells lining the colon or rectum of the gastrointestinal tract as the carcinogenic food and drinks that were ingested interact with their genetic factors. Cells that had experienced a mutation that increased the expression of their Wnt signalling pathway may develop into tumour

cells. Other mutations of signalling proteins responsible for cell apoptosis (Markowitz and Bertagnolli, 2009), as well as those for DNA repair, also contributed to the pathogenesis of colorectal cancer (Lynch and Hoops, 2002).

Colorectal cancer usually started out as a benign tumour, a polyp, which slowly became a malignant tumour over time. Around 95 % of the cases of colorectal cancer are formed from adenomatous polyps, with the rest being mucinous and adenosquamous (Kufe et al., 2010). Cancerous polyps could grow through its spread into the wall of the colon or rectum, starting from the innermost mucosa. They could also grow into blood or lymph vessels, then spread to distant parts of the body through them.

The genetic transition to malignant cells for colorectal cancer involves the sequential mutation of the *ras* gene that controls cell proliferation (Vogelstein et al., 1988), and subsequent inactivation or loss of *p53* (Baker et al., 1989) that codes for a tumour suppression protein, genetic alterations that are thought contribute to several malignant phenotypes and increased angiogenesis (Fearon and Vogelstein, 1990). These, and other genetic alterations, provide the cells with a growth advantage that allow them to outgrow other cells and form a tumour.

From adenomas, the cells gradually increase in size, undergo dysplasia, acquire villous morphology, and finally develop into carcinoma.

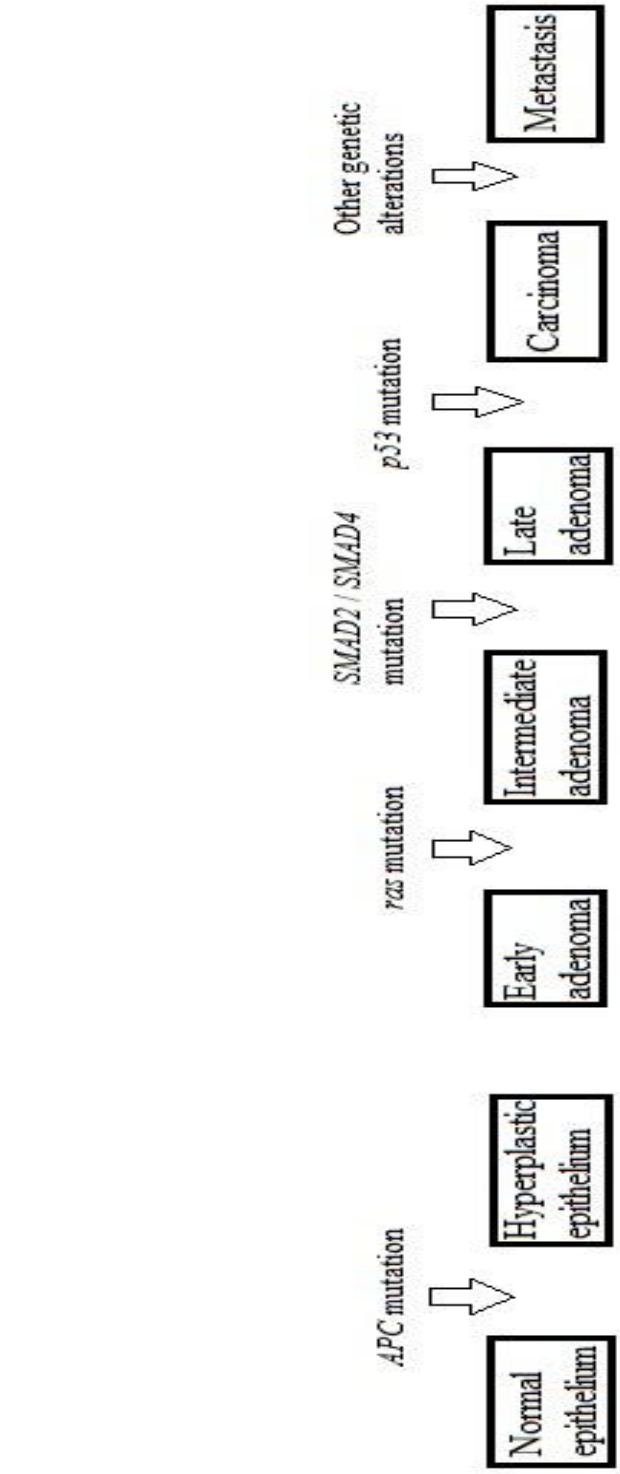

Figure 1.1 Genetic changes in colorectal cancer progression (Fearon and Vogelstein, 1990) (Joanne et al., 2005).

1.1.3(b) Treatment of Colorectal Cancer

Early stages of colorectal cancer could be treated with surgery, either through colonoscopy or the surgical removal of the infected part of the colon or rectum. Depending on the severity of a particular case, the treatment is sometimes accompanied with chemotherapy and radiation therapy to shrink the tumour before surgery, and to reduce the risks of recurrence after surgery. Targeted therapy, a type of chemotherapy with drugs such as bevacizumab and ramucirumab that specifically targets proteins contributing to cancer development, could also be used. Those with advanced colon cancer or with severe symptoms will be provided with palliative care to reduce the symptoms and complications, as well as to improve the quality of life (Amersi et al., 2004).

1.2 Angiogenesis

Angiogenesis is an important, multistep physiological process in which new blood vessels were formed from pre-existing ones. It is an essential physiological process during embryo development, wound healing, and in response to ovulation (Adair and Montani, 2010). This process includes the proliferation and migration of endothelial cells, the remodelling of the surrounding extracellular matrix, and the maturation of the new vessels formed (Kimura et al., 2000). The angiogenic process is tightly regulated, either activated or repressed through a balance between angiogenic and angiostatic stimuli as the metabolic requirements of the surrounding cells change. Changes in metabolic activity would lead to changes in vasculature through angiogenesis to provide for those requirements.

When unregulated, an overexpression of angiogenesis could result in severe tissue dysfunction and lead to the pathogenesis and development of a number of diseases, for example, rheumatoid arthritis (Paleolog, 2002), diabetic retinopathy (Crawford et al., 2009), and chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, and inflammatory bowel diseases (Koutroubakis et al., 2006). In addition, angiogenesis is a vital requirement for the metastasis of tumours. The reverse condition, insufficient vessel growth or abnormal vessel regression, would lead to or contribute to the severity of diseases as well, diseases such as cardiac and cerebral ischemia (Krupinski et al., 1994), hypertension (Struijker, 1999), and osteoporosis (Martinez et al., 2002). In contrast to physiological angiogenesis, with a vasculature that is well-ordered in structure and function, pathologically-induced angiogenesis form a vasculature that is not distributed uniformly, branch irregularly or excessively, and do not follow a clear hierarchy (Dvorak, 2005). This could have been due to an imbalance of angiogenic regulators or because they were the result of the activity of

molecular signals different from physiological angiogenesis. Also, unlike physiological angiogenesis, pathological angiogenesis is often induced by some degree of inflammation due to the recruitment of blood-borne inflammatory and angiogenic factors to sites of inflammation and wound healing (Carmeliet, 2000b).

Vasculogenesis is another physiological process which involves the formation of new blood vessels. However, while similar to angiogenesis, it is a distinct process, responsible for the formation of blood vessels *de novo* from endothelial cell precursors in the developing embryo where there were previously none. After that angiogenesis takes over the blood vessel formation process with the growth, expansion, and remodelling of these primitive vessels to form a vast, complex vascular network (Conway et al., 2001).

1.2.1 Sprouting Angiogenesis

The two types of angiogenesis that were identified, sprouting angiogenesis and intussusceptive (non-sprouting) angiogenesis (Ribatti, 2006), involve different cell types and are regulated by biological different molecules. Sprouting angiogenesis is the formation of new blood vessels through the ends of pre-existing ones, characterised by local vasodilation, increased vascular permeability, and cell proliferation (Djonov et al., 2003). Vasodilation for sprouting angiogenesis is initiated during hypoxia in response to nitric oxide (NO) in order to cater to the metabolic requirements of the cells in the region. In` most parenchymal cells, the transcription of vascular endothelial growth factor A (VEGF-A), a type of signalling protein that stimulates angiogenesis, is in part up-regulated by NO in response to a hypoxic environment. Numerous other angiogenic factors, such as angiopoietin 2 (Ang-2) (Yamakawa et al., 2003) and

inducible nitric oxide synthase (iNOS) (Melillo et al., 1995), are also up-regulated in response to hypoxia. Some of the roles of VEGF-A in angiogenesis include inducing increased vascular permeability, promoting alterations in the cell membrane structure (Kimura et al., 2000), and most importantly, as a pro-angiogenic stimulant that guides the growth of the new capillary sprout that would eventually form the new vessel. Therefore, the role of VEGF-A is irreplaceable in hypoxia-induced angiogenesis (Adair and Montani, 2010).

Through the redistribution of intercellular adhesion molecules and alterations in cell membrane structure, an increase in vascular permeability in response to NO is achieved (Eliceiri et al., 1999). The glycoprotein angiopoietin 2 (Ang-2) played a part in the reduction of inter-endothelial cell contacts, detaching the smooth muscle cells and loosening the underlying matrix for the migration of the endothelial cells (Gale and Yancopoulos, 1999). Through the degradation of the extracellular matrix, other growth factors involved in angiogenesis and cell proliferation such as the basic fibroblast growth factor (bFGF) and over 20 matrix metalloproteinases (MMPs) were liberated, along with exposing the adhesion sites hidden in the matrix (Conway et al., 2001). The MMPs are crucial in the degradation of the extracellular membranes and basement membrane structures, and are in part involved in endothelial sprouting through their interactions with other angiogenic factors (Pozzi et al., 2000).

With an increase in vessel permeability and the degradation of the surrounding matrix, the endothelial cells are free to migrate to form new vessels, a process mediated by various forms of VEGF, angiopoietins, and FGFs. FGFs are capable of stimulating endothelial cell growth and recruit inflammatory cells that produce an array of angiogenic factors (Carmeliet, 2000a). A chemotactic gradient of VEGF-A is produced, from the area with oxygen-starved cells to the pre-existing vessels. Endothelial cells

from the pre-existing vessels form sprouts that grow following the gradient, guided by endothelial tip cells with filopodia heavily endowed with VEGFR2 receptors capable of sensing differences in VEGF-A concentrations (Gerhardt et al., 2003). Endothelial stalk cells would then proliferate as they follow behind the tip cell following the VEGF-A gradient, which is how the capillary sprouts grow and elongate. The series of stalk cells would become the lumen of the new capillaries as their vacuoles develop and coalesce.

VEGF-A levels would return to normal when the local tissue had received an adequate amount of oxygen from the new capillaries. The new capillaries would then mature and stabilise through the recruitment of pericytes and the deposition of the extracellular matrix produced by mural cells (Adair and Montani, 2010). The surrounding vascular smooth muscle cells stabilise the new vessels through the inhibition of endothelial proliferation and migration, as vessels not covered by the smooth muscle cells could easily regress when angiogenic stimuli become limiting (Benjamin et al., 1998). The extracellular matrix functions as a scaffold on which the new vessels grow, and as a local store for growth factors that can be activated rapidly without *de novo* synthesis (Li et al., 2003). The pericytes that surround the endothelial cells of the vessels assist in the function specialisation of the vessels to accommodate local tissue vasculature requirements through their expression of numerous growth factors and cytokines (Hirschi and D'Amore, 1996).

1.2.2 Intussusceptive Angiogenesis

Compared to sprouting angiogenesis, the intussusceptive mode of the angiogenic process is a fairly-recent discovery. It was first observed in the developing microvasculature of the postnatal rat lung by Burri and coworkers in 1987 using scanning electron microscopy. Intussusceptive angiogenesis is a process that occurs with decreasing frequency in the vascular bed of vertebrates from birth to adulthood, as it was more frequently observed in prenatal and new-born rat myocardium than those in adults (van Groningen et al., 1991). Recently, intussusceptive microvascular growth has been observed to be involved in the capillary network expansion of numerous organs and species, including the chicken chorio-allantoic membrane (CAM) (Patan et al., 1993) (Djonov et al., 2000), retina, kidney (Djonov et al., 2002), rat myocardium (van Groningen et al., 1991), rat cerebrum (Zhang et al., 2002), and tumours (Djonov et al., 2001) (Patan et al., 2001).

The process of intussusceptive angiogenesis involves the extension of the blood vessel wall into the vessel to split it longitudinally into two, and can be divided into four distinct phases. In the first phase, an interendothelial bridge is formed through the creation of a zone of direct contact between opposing capillary walls protruding into the lumen. This is followed by the second phase, with the formation of a central perforation of the endothelial bilayer and the reorganisation of the intercellular junctions of the endothelium. The cylindrical bridge is wrapped in endothelial cells, with the presence of cytoplasmic extensions of myofibroblasts with their microfilaments within the core of the bridge. Next, an interstitial pillar core is formed through successive invasions of myofibroblasts and pericytes, and the deposition of interstitial fibres. Finally, in the last stage, the pillar increases in girth into a full-sized capillary mesh (Burri and Tarek, 1990) (Burri et al., 2004).

Intussusceptive angiogenesis is activated through friction in the vessel system from blood flow-induced shear stress and pressure induced-wall stress from blood flow, vasodilation, and blood pressure (Tomanek, 2012). Blood vessels with higher velocities of blood flow had been shown to induce intussusceptive angiogenesis in chick embryos (Thoma, 1893). The time needed for pillar formation is about 40 to 120 minutes, a rapid process that infers that the means and mechanisms required for transluminal pillar formation is constantly present and only needs to be switched on in the endothelial and supporting cells, with no alteration of gene expression required (Djonov et al., 2002).

As sprouting angiogenesis involves sprouts of endothelial cells growing towards the pro-angiogenic stimulus VEGF-A, blood vessels could be created in areas that were previously devoid of them through this type of angiogenesis. In contrast, intussusceptive angiogenesis can only occur when there are pre-existing vessels in the area, as transvascular pillar tissues were formed through the invasion of interstitial tissues into the existing vessels (Adair and Montani, 2010). However, the process of intussusceptive angiogenesis is much faster than that of sprouting angiogenesis, as it does not require endothelial proliferation and migration, but only the reorganisation of existing endothelial cells. Intussusceptive angiogenesis is therefore more metabolically efficient, and more suited for situations where fast growth is needed but resources are limited. Intussusceptive angiogenesis is not only involved in numerous organs at different levels of the systems, it may also be involved in the vascular structure remodelling to optimise vessel form and function, as the same process of pillar formation, termed intussusceptive arborisation, has been observed in the initial remodelling of primitive capillary plexuses into complex arterial and venous vascular trees (Kurz et al., 2003).

1.2.3 Biological Molecules Involved in the Angiogenic Process

Table 1.1 Angiogenesis activators and inhibitors involved in the angiogenic process.

Angiogenesis Activators	**Angiogenesis Inhibitors**
Angiopoietin-1 (Ang-1)	Angiopoietin-1 (Ang-1) (tumour angiogenesis)
Angiopoietin-2 (Ang-2) (in the presence of VEGF)	Angiopoietin-2 (Ang-2) (in the absence of VEGF)
Basic fibroblast growth factor (bFGF)	Vascular endothelial growth factor receptor (VEGFR)
Basic fibroblast growth factor (bFGF)	Endostatin
Cyclooxygenase-2 (COX-2)	Metallopeptidase inhibitor 1 (TIMP1)
Hepatocyte growth factor (HGF)	Interferon-α, -β, and -γ (IFN-α, -β, and –γ)
Hypoxia-inducible factor 1-alpha (HIF-1α)	Interleukin-4, -12, and -18 (IL-1, -12, and -18)
Interleukin-1 (IL-1)	
Matrix metalloproteinase (MMP)	
Nuclear factor kappa-B (NF-κB)	
Placental growth factor (PlGF)	
Platelet-derived growth factor (PDGF)	
Tumour necrosis factor-α (TNF-α)	
Vascular endothelial growth factor (VEGF)	

While numerous biological molecules were involved in the angiogenic process, special attention has been given to VEGF as the primary inducer of blood vessel formation in adults. Not only does it function as a key regulator of physiological and pathological angiogenesis associated with a number of diseases, it also has a direct mitogenic effect on endothelial cells (Thomas, 1996) (Shibuya, 2006). VEGF-A consists of many isoforms through alternative splicing, and they exert many of the

same functions in the angiogenic process such as increasing vascular permeability, stimulating endothelial cell proliferation and migration, and up-regulating the transcription factors of proteins associated with angiogenesis such as chemokine (C-X-C motif) ligand 2 (CXCL2), IL8, cyclooxygenase-2 (COX-2,) and the tissue factors. (Carmeliet and Collen, 1999). The predominant isoform of VEGF secreted by most normal and transformed cells is $VEGF_{165}$, which exhibits biochemical properties similar to VEGF purified from various sources (Ferrara et al., 1992). During hypoxia, an up-regulation in VEGF expression, through the increased transcription and stabilisation of its mRNA, was induced (Mukhopadhyay et al., 1995) (Ikeda et al., 1995). When VEGF-A binds with the VEGFR-2 receptors on endothelial cells, a cascade of signalling events is triggered, resulting in the phosphorylation and activation of proteins related to angiogenesis. VEGF-A triggers progenitor cells to differentiate towards the endothelial cell lineage, guides the angiogenic sprouting of endothelial tip cells (Gerhardt et al., 2003), stimulates the three-dimensional vascular tube formation, and regulates vascular permeability (Ferrara et al., 2003). The blocking of VEGF significantly decreases microvessel outgrowth (Brown et al., 1996).

The transcription factor subunit hypoxia-inducible factor 1-alpha (HIF-1α) is a major regulator of the cellular and developmental responses to hypoxia. During hypoxia, the protein degradation of HIF-1α via the ubiquitin protease pathway is prevented. The stabilised HIF-1α subunit is then free to merge with the β subunit, leading to the induction of the transcription of more than 60 genes, including angiogenic promoters VEGF (Wang et al., 1995) and angiopoietin-2 (Ang-2) (Simon et al., 2008), as well as matrix metalloproteinases-2 (MMP-2) that mediates the migration of endothelial cells in the angiogenic process (Ben-Yosef et al., 2005).

The basic fibroblast growth factor (bFGF) is another pro-angiogenic growth factor that is up-regulated during active angiogenesis. Normally stored in the vascular basement membrane and extracellular matrix, it can be activated by heparan sulfate-degrading enzymes to promote angiogenesis during wound healing and tumour development. It induces angiogenesis by increasing the proliferation of endothelial cells as well as their chemotaxic organisation into tube-like structures for the formation of new blood vessels, and its production of proteases involved in angiogenesis (Montesano et al., 1986).

The placental growth factor, or PlGF, is an angiogenic protein from the VEGF family that stimulates the growth, migration, and survival of endothelial cells (Ziche et al., 1997) (Adini et al., 2002), and also induces vasodilatation and vessel growth (Yonekura et al., 1999). While it is primarily expressed in the placenta throughout all stages of gestation, transcripts of PlGF have also been detected in the heart, lungs, thyroid glands, and skeletal muscles (Persico et al., 1999). PlGF and its corresponding receptor, VEGFR-1, are expressed minimally in the adult quiescent vasculature, but both of them are strongly up-regulated in pathological conditions (Carmeliet et al., 2001). Even though human PlGF-1 and VEGF-A are only 42% identical in terms of amino acid sequence identity, their overall three-dimensional structures are highly similar (Iyer et al., 2001), which explains the fact that they competitively bind to the same receptor and have similar biological functions. PlGF was found to function in the stimulation of the growth and migration of endothelial cell *in vitro*, and the *ex vivo* angiogenesis in rabbit avascular cornea with the same potency of those induced by VEGF and basic fibroblast growth factor (bFGF) (Ziche et al., 1997). By competing with VEGF-A for VEGFR-1 binding sites, more VEGF-A is available to bind to VEGFR-2, which amplifies the VEGF-A-driven signal for angiogenesis. In addition,

PlGF is also capable of triggering its own pro-angiogenic signalling cascade via VEGFR-1, acting on the growth, migration, and survival of endothelial cells (Ziche et al., 1997) (Autiero et al., 2003). PlGF was also found to be over-expressed in various cells during pathological conditions, such as in colorectal (Wei et al., 2005) and human breast cancer cells (Parr et al., 2005).

The activation of nuclear factor kappa-B (NF-κB), a type of protein complex that controls DNA transcription, is a significant event in the angiogenic process, as it regulates genes involved in cell survival, cell growth and migration, and the MMPs. NF-κB mediates the synthesis of cytokines tumour necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8, as well as the expression of COX-2. In the angiogenic process, NF-κB promotes the expression of MMP-2, -3, and -9 involved in the degradation of the vascular basement membrane and the remodelling of the extracellular matrix (Popov et al., 2006). The activation of NF-κB was found to be able to enhance the VEGF production in mouse lung epithelial cells through the hypoxia-induced mitogenic factor (HIMF) (Tong et al., 2006), thus promoting angiogenesis.

The angiopoietin/Tie2 system is an important regulator of angiogenesis. Angiopoietins, a family of extracellular ligands, exert their functions in vascular remodelling and angiogenesis. Together with VEGF, angiopoietin-1 (Ang-1) stimulates the production of pro-angiogenic cytokines in *ex vivo* rat aortic rings explants before the emergence of neovessels (Aplin et al., 2006), which could be due to both molecules being able to activate the Tie-2 receptor. Moreover, Ang-1 promote endothelial cell survival and is also a regulator of blood vessel remodelling and maturation.

Angiopoietin-2 (Ang-2) is an antagonist of Ang-1 in its binding with the Tie-2 receptor and a regulator of angiogenesis (Fiedler et al., 2003). It acts as a pro-angiogenic in the presence of VEGF, functioning in the destabilising of mature blood vessels in the angiogenic process before vessel sprouting could occur (Scharpfenecker et al., 2005), but acts as an anti-angiogenic signal in the absence of VEGF in inducing vessel regression (Lobov et al., 2002) . The competitive binding of Ang-2 to the Tie-2 receptor blocks the function of Ang-1 and sensitizes endothelium cells to TNF-α signals, which promotes endothelial cell survival, vessel sprouting, and angiogenesis (Maisonpierre et al., 1997). Through the destabilisation of the blood vessels, the endothelial cells are released from the pericytes and the extracellular matrix (Holash et al., 1999). In addition, Ang-2 sensitizes endothelial cells to VEGF, which leads to increased angiogenesis. The gene expression of Tie2-expressing monocytes can be regulated by Ang-2 to augment pro-angiogenic activities (Coffelt et al., 2010).

TNF-α is a pro-inflammatory cytokine secreted by monocytes in response to tissue injury, which also has the ability to induce the gene expression of VEGF and its receptors, leading to an increase of angiogenesis (Giraudo et al., 1998). TNF-α could also contribute to local angiogenesis by stimulating the release of VEGF, basic fibroblast growth factor (bFGF) (Ryuto et al., 1996), and platelet-derived growth factor (PDGF) (Rosengren et al., 2010), which promotes the proliferation and migration of endothelial cells.

Like TNF-α, IL-1 is a cytokine that is also a potent pro-angiogenic stimulus in both physiological and pathological angiogenesis. IL-1 activates the myeloid cells to produce a cascade of cytokines, which in turn activates the endothelial cells, and induces changes in their gene expression, function, and morphology towards increased angiogenesis (Voronov et al., 2007). It induces the migration of the endothelial cells

and tube formation through the activation of the p38-mitogen-activated protein kinase (MAPK) and the MAPK-activated protein kinase 2 (Jagielska et al., 2012), and also up-regulates the expression of VEGF and its receptors on endothelial cells, both of which also leads to increased angiogenesis (Berse et al., 1999).

COX-2 is an enzyme that can be induced by hypoxia and cytokines such as VEGF (Goppelt-Struebe, 1995). The down-regulation of COX-2 significantly inhibited the *in vitro* migration and tube formation of human umbilical vein endothelial (HUVEC) cells through the suppression of the angiogenesis-related molecules VEGF, Flt-1, Flk-1/KDR, ANG-1, Tie-2, and MMP-2 (Yao et al., 2011). The expression of COX-2 correlated with the levels of VEGF and PDGF expression in multiple gastric adenocarcinoma cell lines, further shown by the increased microvessel density (Tatsuguchi et al., 2004).

The hepatocyte growth factor (HGF) is a cellular growth, motility, and morphogenic factor secreted by mesenchymal cells (Sonnenberg et al., 1993). It is also a potent angiogenic factor that stimulates angiogenesis in the *in vitro* wound healing and three-dimensional collagen assays using human endothelial cells, and induces neovascularisation in the *in vivo* rabbit cornea assay at sub-nanomolar concentrations (Bussolino et al., 1992). It stimulates the cell migration, proliferation, protease production, and the invasion and organisation of vascular endothelial cells into capillary-like tubes (Rosen et al., 1997).

MMPs, also called also called matrixins, are calcium-dependent zinc-containing endopeptidases involved in the degradation of the extracellular matrix and the tissue remodelling process (Verma and Hansch, 2007). Currently, there are 23 MMPs identified in humans (Visse and Nagase, 2003). Many of them, such as MMP-

1 and MMP-7, are expressed in physiological situations involving angiogenesis. Biological molecules such as TNF-α and interleukins (ILs) stimulate MMP expression. The MMPs would then degrade the ECM and recruit inflammatory cells to mediate the adhesion, proliferation, and apoptosis of the cells in vessel walls for vascular remodelling (Parks et al., 2004).

1.2.4 Angiogenesis in the Treatment of Colorectal Cancer

Sustained angiogenesis is one of the characteristics of tumour cells, as the growth of blood vessels around the tumour was stimulated in order to supply nutrients to the tumours. It is a vital step in a tumour's development towards malignancy as it allows for tumour propagation and progression (Folkman, 1995). As the blood vessels surrounding the tumour supply the cancerous cells with the oxygen and metabolites they require, as well as serve as an effective system for cellular waste disposal, the growth of the vasculature system surrounding the tumour is the rate-limiting step for tumour progression (Bergers and Benjamin, 2003).

In order for tumours to grow beyond sizes of about 1-2 mm in diameter, the switch to the angiogenic phenotype is required. It consists of a gain-of-function event where pro-angiogenic factors are up-regulated or induced in the tumour cells, and loss-of-function events where endogenous angiogenic inhibitors are down-regulated (Hanahan and Folkman, 1996). Without switching to a sustained pro-angiogenic state, the tumour may enter a state of dormancy (Holmgren et al., 1995) or regress (Rak et al., 1995).

The increased tissue mass from the formation of the tumour causes hypoxia at the surrounding tissue microenvironment, activating the hypoxia-inducible factor-1α (HIF-1α) which in turn up-regulates the expression of pro-angiogenic factors that lead to increased angiogenesis (Dor et al., 2001). Angiogenesis is also activated through the cancer cell-driven expression of pro-angiogenic molecules such as VEGF, bFGF (Dirix et al., 1996), IL-8 (Sparmann and Bar-Sagi, 2004), placenta-like growth factor (PLGF) (Wei et al., 2005), PDGF (Saito et al., 2000), transforming growth-factor-β (TGF-β) (Tsushima et al., 1996), pleiotrophin and more (Relf et al., 1997), biomolecules that correlate with a decrease in the survival rate of colorectal cancer patients. Oncogenes such as such as K-*ras* (Okada et al., 1998), v-*Src* (Jiang et al., 1997), c-*Src* (Mukhopadhyay et al., 1995), v-*raf*, c-*fos*,(Mazure et al., 1996) and v-*yes* could also be expressed by the tumour to up-regulate angiogenic factors and induce angiogenesis (Kerbel et al., 1998). Growth factors such as VEGF, TGF-α and -β, bFGF, HGF, Ang-1 and -2, and IL-8 had been identified to have possible tumour angiogenesis-promoting activity (Bouck et al., 1996).

One of the most common genetic changes in human cancers involve the *ras* gene, results in elevated VEGF (Rak et al., 1995), bFGF (Iberg et al., 1989), TGF-α and TGF-β (Cleveland et al., 1980) (Roberts et al., 1986) in transformed epithelial cells, which leads to elevated tumour angiogenesis in addition to increased tumour cell proliferation. A reduction in VEGF activity was associated with the disruption of the mutant K-*ras* allele in human colon cancer DLD-l and HCT l16 cells, as well as a significantly more active induction of mitogenesis in HUVEC cells (Shirasawa et al., 1993) (Rak et al., 1995). It may explain the relevance of the timing of the *ras* gene mutation with the rapid disease progression of colorectal cancer (Fearon and Vogelstein, 1990) and the high frequency of K-*ras* mutation detected in polyploidy

colon tumours (Hasegawa et al., 1995). Elevated levels of TGF-α (Buick et al., 1987) and TGF-β (Jamal et al., 1994) expression were associated with *ras* oncogene mutations. Both TGF-α and –β are capable of inducing angiogenesis *in vivo*, and could also up-regulate VEGF, which leads to increased angiogenesis. Cells with the mutant oncogene v-*src* becomes tumourigenic and express high levels of VEGF mRNA and protein in a way similar to cells with H-*ras* mutations (Rak et al., 1995).

Mutations of the *p53* tumour suppressor gene, which commonly occur during the genetic transformation of colorectal carcinomas, causes an up-regulation of the mRNA of VEGF (Kieser et al., 1994). The wild-type *p53* gene regulate the expression of angiogenic inhibitor genes such as thrombospondin (Dameron et al., 1994) and the glioblastoma-derived angiogenesis inhibitory factor (Van Meir et al., 1994), as well as mediate apoptosis, cell mediation, and tumourigenicity (Donehower and Bradley, 1993) (El-Deiry et al., 1992).

VEGF is a major factor in tumour angiogenesis, as numerous types of cancers and malignant melanomas, such as those of the breast, colon, ovary, bladder, and brain, were found to produce it. The blood vessels of these tumours were also found to express high levels of VEGF receptors (Dvorak et al., 1995) (Ferrara, 1995). The degree of vascularisation of cancer tumours had been closely associated with VEGF mRNA expression (Plate et al., 1992). The VEGF produced by the tumour cells were not only localised in the tumour but also in the vasculature (Plate et al., 1992) (Brown et al., 1993), which indicates that tumour-secreted VEGF accumulates in the target cells. The mRNA of VEGF receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) was also found to be up-regulated in the endothelial cells of tumour vasculature compared to those surrounding tumour-free tissue (Plate et al., 1992). As tumour cells that express VEGF possess a growth advantage *in vivo* due to their stimulation of

angiogenesis, yet the expression of VEGF did not confer a growth advantage for tumour cells *in vitro*, it can be inferred that VEGF's role in tumour angiogenesis is due to paracrine rather than autocrine mechanisms (Ferrara, 1995). Treating nude mice bearing liver metastasis tumours of human colon carcinoma cells with anti-VEGF monoclonal antibodies resulted in a marked decrease in number and size for the tumours, with most of the tumours under 1 mm in diameter and all under 3 mm (Warren et al., 1995), which supports the hypothesis that the growth of tumours beyond a certain size requires angiogenesis for support.

The HIF-α transcriptional protein subunit was found to be overexpressed in numerous cancers and their metastases, including cancers of the colon, breast, kidney, prostate, lung, skin, ovary, and pancreas (Zhong et al., 1999). Blood vessels produced from tumour angiogenesis are highly irregular and leaky compared to regular vessels, which leads to the presence of hypoxic areas in the tumour as well as increased HIF-1α stability despite active angiogenesis. Oncogenes from *ras* and *p53* mutations, such as those in colorectal cancer, also contributes to the stabilisation of the HIF-1α protein subunit (Kikuchi et al., 2009) (Ravi et al., 2000). Since the HIF-1α is mediates the transcription of numerous genes associated with tumour angiogenesis and metastasis, it is an appealing target for anticancer strategies.

The overexpression of COX-2 contributes to the development of colorectal cancer through its promotion of VEGF activity in tumour angiogenesis (Cianchi et al., 2001). High microvessel density around the tumour, as well as increased VEGF expression, correlates to the clinical stage progression of the disease in patients (Nakasaki et al., 2002), in addition to poor relapse-free survival rate and overall survival rate of colorectal cancer (Des Guetz et al., 2006). High vessel counts in colorectal cancer also correlates with hematogenous metastasis, as an increase in

tumour neovascularization also increases the surface area for tumour cells to potentially enter the systemic circulation and lead to metastasis (Folkman, 1994).

The final stage of oncogenesis is the metastasis of the tumour. Neovascularisation of the tumour from increased angiogenesis also increases the chances of the tumour cells entering the bloodstream and travelling to other distant organs.

Suppressing the growth of blood vessels surrounding the tumour, rather than the tumour itself, is a promising new approach to cancer therapy as the target cells are much more stable genetically, and therefore less likely to develop mutations that would lead to drug resistance (Kerbel and Folkman, 2002). Not only could anti-angiogenic drugs inhibit the growth of new microvessels surrounding the tumour, they also could induce the regression of recently-developed microvessels, which leads to either the arrestment of growth or apoptotic death of the tumour cells (Holmgren et al., 1995). Anti-angiogenic therapy could also be useful in the prevention of cancer in patients with high-risk of developing cancer or the recurrence of cancer, as well as in the prevention of cancer metastasis.

The antiangiogenic drug bevacizumab is a monoclonal antibody against VEGF. It is often added to the first line therapy of colorectal cancer, and has been shown to both prolong progression-free survival and overall survival of the patients (Wagner et al., 2009). Slow-growing tumours are harder to treat using chemotherapy, but was shown to respond better to anti-angiogenic therapies (Kerbel and Folkman, 2002). With the addition of bevacizumab to fluorouracil-based combination chemotherapy in the treatment of metastatic colorectal cancer, an improvement of response rate, median time to disease progression, and patient survival was recorded (Hurwitz et al., 2004).