THE EFFECTS OF EPHA2 INHIBITION ON VEGF, VEGFR-1 AND VEGFR-2 IN HUMAN MALIGNANT GLIOMA CELLS

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by

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LIST OF ABBREVIATIONS

ACTB	β-Actin
cDNA	Complementary DNA
Ct	Cycle threshold
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
EGF	Epidermal Growth Factor
EphA2	Eph-A2 Receptor
FBS	Fetal Bovine Serum
GBM	Glioblastoma Multiforme
GPI	Glycosylphosphatidylinositol linkage
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
IDH	Isocitrate Dehydrogenase
LB	Lithium Boric acid buffer
LBD	Ligand-binding Domain
mRNA	Messenger RNA
PVDF	Polyvinylidene Difluoride
RBD	Receptor-binding Domain
RTK	Receptor Tyrosine Kinases
SAM	Sterile Alpha Motif
siRNA	Small interfering RNA
TBS	Tris-buffered Saline
TM	Transmembrane region

TMZ	Temozolomide
UV	Ultra Violet
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor-Receptor 1
VEGFR-2	Vascular Endothelial Growth Factor-Receptor 2

KESAN-KESAN PERENCATAN EPHA2 KEPADA VEGF, VEGFR-1 DAN VEGFR-2 DALAM SEL MALIGNAN GLIOMA MANUSIA

ABSTRAK

Kanser otak glioblastoma (GBM) dikelaskan sebagai kanser astrositik tahap ke-4. Walaupun terdapat pelbagai jenis kecanggihan rawatan, tahap median survival pesakit GBM masih rendah iaitu di antara 12 hingga ke 14 bulan sahaja di samping prognosis klinikal yang lemah. Gen EphA2 manusia memainkan peranan penting dalam proses angiogenesis dan ekspresi EphA2 yang tinggi di dalam glioblastoma sudah dikenalpasti. Pola kenaikan ekspresi EphA2 telah dikaitkan dengan kelemahan prognosis pesakit, pengurangan kadar survival dan risiko penyakit yang berulang. Kajian ini adalah bertujuan untuk mengenal pasti peranan EphA2 dalam proses angiogenesis yang dikawalatur oleh VEGF, VEGFR-1 dan VEGFR-2. Di dalam kajian ini, dua jenis sel yang diperolehi daripada sumber yang berlainan telah digunakan. U-87MG ialah sel yang diperolehi daripada pesakit dengan GBM tahap ke-4, manakala DBTRG-05MG pula ialah sel yang diperolehi daripada pesakit GBM tahap ke-4 yang telah menerima rawatan kemoterapi dan radioterapi. Kesan-kesan perencatan gen EphA2 selepas transfeksi oleh *small interference* RNA *(siRNA)* ke atas ekspresi gen dan protein EphA2 dan molekul penanda angiogenesis; VEGF, VEGFR-1 dan VEGFR-2 telah dijalankan menggunakan kaedah *Real Time-PCR* serta *Western blot*. Hasil kajian ini menunjukkan penurunan ekspresi mRNA EphA2 (p=0.006) telah menyebabkan penurunan ekspresi mRNA VEGF (p=0.0123) dan VEGFR-2 (p=0.0299) yang signifikan di dalam sel U-87MG. Seterusnya, pada peringkat ekspresi protein, tiada perubahan yang signifikan dicatatkan bagi protein VEGF (hanya 0.98-kali berbanding 1-kali kumpulan kawalan), tetapi ekspresi protein VEGFR-2 menurun

sebanyak 0.51-kali. Manakala bagi sel DBTRG-05MG, perencatan ekspresi mRNA EphA2 (p=0.0137) telah menyebabkan peningkatan ekspresi mRNA VEGF (p=0.2131), tetapi menyebabkan penurunan ekspresi mRNA VEGFR-2 (p=0.1453). Namun, kedua-dua perbezaan adalah tidak signifikan. Di peringkat translasi protein, tiada perbezaan dicatatkan bagi ekspresi protein VEGF (hanya 0.92-kali berbanding 1-kali kumpulan kawalan), tetapi terdapat penurunan ekspresi protein VEGFR-2 dengan nilai 0.61-kali. Kajian ini juga telah mengenalpasti bahawa sel malignan glioma U-87MG dan DBTRG-05MG tidak mengekspresi gen dan protein VEGFR-1. Oleh yang demikian, kajian ini telah berjaya menemui bahawa EphA2 memainkan peranan di dalam tindak balas VEGF/VEGFR-2 sel GBM. Kesimpulannya, kajian ini membuktikan bahawa perencatan ekspresi mRNA EphA2 dengan menggunakan EphA2-*siRNA* telah mengakibatkan perencatan ekspresi gen dan protein VEGFR-2 tanpa menjejaskan ekspresi VEGF di dalam sel malignan glioma. Menariknya, kajian ini telah berjaya menunjukkan bahawa EphA2 memainkan peranan penting dalam angiogenesis yang dikawalatur melalui isyarat VEGFR-2.

THE EFFECTS OF EPHA2 INHIBITION ON VEGF, VEGFR-1 AND VEGFR-2 IN HUMAN MALIGNANT GLIOMA CELLS

ABSTRACT

Brain tumor "glioblastoma multiforme (GBM)" is assigned under fourth pathologic grades (grade IV) of astrocytic tumor. Despite various advances in the treatment modalities, median survival rate of GBM patients remains low between 12 to 14 months with a poor clinical prognosis. Human EphA2 gene plays an important role in regulating angiogenesis and known to be over expressed in GBM tumors. The elevated trend of EphA2 was associated to the poor prognosis of GBM patients, shorter survival rate and recurrence of the disease. This study aims to investigate the roles of EphA2 in angiogenesis signaling pathway governed by VEGF, VEGFR-1 and VEGFR-2. In this study, two types of cell lines derived from different origins were utilized. U-87MG cells were isolated from a patient with GBM type 4 diagnosis, whereas DBTRG-05MG was isolated from a GBM type 4 patient who underwent chemotherapy and radiotherapy. The effects of EphA2 gene silencing following transfection of small interfering RNA (siRNA) on the gene and protein expression of EphA2 and tumor angiogenesis related markers; VEGF, VEGFR-1 and VEGFR-2 were evaluated by using quantitative Real Time-PCR and Western blot analysis. This study showed downregulation of EphA2 mRNA (p=0.0061) had significantly downregulated VEGF (p=0.0123) and VEGFR-2 (p=0.0299) mRNA expression in U-87MG cells. Consequently, at protein level there was no difference in VEGF expression (0.98-fold relative to 1-fold of non-transfected group), but decrement of VEGFR-2 expression by 0.51-fold was observed. As for DBTRG-05MG cells, downregulation of EphA2 mRNA (p=0.0137) had led to an increasing trend of VEGF

mRNA expression (p=0.2131), but decreasing trend of VEGFR-2 (p=0.1453). Both differences were not statistically significant. At protein translational level, there was no difference of VEGF protein expression (0.92-fold relative to 1-fold of non-transfected group), but reducing VEGFR-2 protein by 0.61-fold decrease. This study also had shown that there was no VEGFR-1 gene and protein expression in both U-87MG and DBTRG-05MG malignant glioma cells. Therefore, it was discovered that EphA2 plays an important role in VEGF/VEGFR-2 signaling in GBM cells. In conclusion, this study demonstrated that silencing EphA2 mRNA expression by siRNA led to the downregulation of gene and protein expression of VEGFR-2 without affecting VEGF secretion in malignant glioma cells. Interestingly, this study has successfully elucidated that EphA2 plays important roles in mediating angiogenesis through VEGFR-2 signaling.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Glioblastoma, also known as glioblastoma multiforme (GBM) is the most prevalent brain tumor with the incidence rate of 12.76 per 100 000 population among adults in the United States. It is classified as the most malignant type of brain tumor based on World Health Organization (WHO) 2016 Classification of Tumors of the Central Nervous System (Louis *et al.*, 2016; Zhang *et al.*, 2017). GBM accounts for 14.9% of all primary brain tumors, and 55.4% of all gliomas. The tumor has the maximum number of cases of all malignant tumors, with a prediction of 12 390 new cases in 2017 (Ostrom *et al.*, 2015). GBM is very aggressive, highly infiltrative, has unrestricted cellular proliferation, necrosis, and shows a lot of abnormal blood vessels growth around the tumor (Jones and Holland, 2011).

GBM commonly occurs among adults aged between 45–65, and frequently to be found among men rather than women (Siegel *et al.*, 2016). The clinical responses of patients are generally poor with the maximum patient survival of only 5 years, and median survival rate of only 12 to 14 months (Smoll *et al.*, 2013; Dima Suki., 2016). Despite various advancements in the medical care and treatment approaches, a lot of patients die from recurrent tumors due to its resistance to therapy (Osuka and Erwin, 2017). The first quarter of the second year (5^{th} quarter) post-diagnosis has been evaluated as the topmost death incidence with an excess hazard ratio of 7.58 which indicates poor prognosis of this grade IV gliomas (Smoll *et al.*, 2013; Ostrom *et al.*, 2015).

Taken together with the highest incidence of malignant brain tumors in adults, its highly aggressive behavior and therapy-resistance nature, which subsequently lead to a very poor prognosis of GBM patients, scientists are struggling to understand and investigate the finest characteristics of GBM at the cellular and molecular levels to combat the disease. Recent evidences have shown that EphA2 is highly expressed in GBM (Wang *et al.*, 2008a; Binda *et al.*, 2012; Day *et al.*, 2013). It is encoded by a gene that is located on chromosome 1. This 130kDa EphA2 transmembrane receptor protein, plays numerous roles in cellular functions. It acts as a regulator for promoting cellular interaction, cell migration, cell adhesion and neuronal development (Miao *et al.*, 2000; de Saint-Vis *et al.*, 2003). The elevated trend of EphA2 expression was associated to the poor prognosis of GBM patients, shorter survival rate and recurrence of the disease (Liu *et al.*, 2006; Wang *et al.*, 2008a).

EphA2 was found to act as a critical regulator for tumor neovascularization and angiogenesis. For instance, antagonizing EphA signaling resulted in inhibition of tumor neovascularization and tumor growth, thus providing the first functional evidence for the role of Eph A class receptors in tumor angiogenesis, blood vessel formation and remodeling during vascular development (Brantley *et al.*, 2002). This indicates that Eph signaling is critical for angiogenesis. Importantly, one of the dominant features of GBM is highly angiogenic and known as angiogenic dependent tumor (Würdinger and Tannous, 2009; Tchaicha *et al.*, 2011). However, the underlying roles and function of EphA2 in angiogenesis signaling pathway especially in GBM remains to be investigated.

CHAPTER 2

LITERATURE REVIEW

2.1 Brain Tumor and its Classification

2.1.1 Glioblastoma multiforme (GBM)

Brain tumor is a central nervous system (CNS) tumor that is characterized by an abnormal growth of tissue in the brain or central spine. Its growth damages areas of normal brain tissue and interrupts proper brain functions if left untreated. Primary brain tumors begin in the cells of the brain itself, while secondary brain tumors start in another part of the body and metastasize to the brain. In Malaysia, brain tumor is the eleventh most common cancer in males and in females, thirteenth. The highest incidence rate was reported among Chinese followed by Malay and Indian (Manan *et al.*, 2016). Like the United States, brain tumor is also the second leading cause of cancer death among children in Malaysia (Manan *et al.*, 2016; Siegel *et al.*, 2016).

According to 2016 WHO classification, brain tumors are grouped into 4 grades relative to their growth rate and prognosis. Grades I and II are benign tumors that have low proliferative potential, while Grades III and IV are malignant tumors with significant proliferative activity, but poorly differentiated with the presence of cell necrosis (Louis *et al.*, 2016). The latest 2016 grading of brain tumor are based on histology feature with the addition of molecular parameters to distinguish the exact tumor entities (Louis *et al.*, 2016).

Out of all brain and CNS tumors, 27% are gliomas out of which, majority 80% of the gliomas are malignant. Gliomas arise from glial cells progenitors which in

normal condition act as non-neuronal supportive cells to provide protection and support for neurons. Gliomas are including; astrocytoma and GBM that arise from astrocytes, ependymoma that arise from ependymal cells, oligodendroglioma and oligoastrocytoma that both originated from oligodendrocytes and mixed glial cells.

Importantly, GBM accounts for the majority of gliomas by 55.1% (Ostrom *et al.*, 2015). It was assigned under fourth pathological grade (grade IV) of astrocytic tumor subclasses which originated from astrocyte glial cells (Louis *et al.*, 2016). Based on histology characteristics, GBM is classified as tumor of neuroepithelial tissue due to cytology feature of large epithelioid cells with a lot of eosinophilic cytoplasm, vesicular chromatin and distinctive nucleoli (Ostrom *et al.*, 2015; Louis *et al.*, 2016).

Apart of histology features of GBM, an addition of molecular parameters have been included in WHO 2016 Classification to provide an integrated diagnosis for GBM patients (Louis *et al.*, 2016). More than 80% of Grades II and III tumors contain mutations in isocitrate dehydrogenase (IDH) and it presence has been used as GBM diagnostic marker. The IDH gene encodes for IDH enzyme that catalyses isocitrate to α-ketoglutarate within the Krebs cycle (Narayanan *et al.*, 2012). The IDH-wildtype is often identified in primary GBM which accounts for almost 90% cases. While, IDH-mutant GBM accounts for 10% cases correspond closely to secondary GBMs which undergoes transformation from low-grade tumor into GBM (Louis *et al.*, 2016; Gessler *et al.*, 2017). Then GBM is highly vascularized but with abnormal vasculature. It has uneven vessel diameter and thickened basement membranes.

2.1.2 Clinical presentation and pathophysiology of GBM patients

GBM can invade any part of central nervous system including brain stem, cerebellum and spinal cord. However, it occurs almost exclusively in the brain by which 61% of all primary gliomas occur in the brain's four lobes: frontal (25.3%), temporal (19.6%), parietal (12.7%) and occipital (3.3%) (American Association of Neuroscience Nurses., 2016). In adults, most tumors are observed in supratentorial parts of the brain (Stark *et al.*, 2005; Choi *et al.*, 2015) which is, in contradiction to children, with tumor rates are higher in the infratentorial part including the brain stem and cerebellum (Jurkiewicz *et al.*, 2010; Louis *et al.*, 2016).

General GBM symptoms are including cognitive-behavioural deteroriation, worsening level of responsiveness, fatigue and dizziness (Narayanan *et al.*, 2012). Seizures is the most frequent presentation of GBM by which 25% of GBM patients suffer from it and can increase to as many as 50% of patients at a later stage. Thus, antiepileptic drugs (AED) are prescribed to ease the patient suffer (Wali *et al.*, 2017). Additionally, GBM leads to an increase in intracranial pressure which will induce symptoms such as headache, nausea, vomiting, papilledema or for the worst; focal or progressive neurologic deficits (Hammoud M.A. *et al.*, 1996; Narayanan *et al.*, 2012).

Localized symptoms also occur based on GBM anatomic location. For instance, speech deficit and hemiparesis occur if GBM develop in frontal and temporal lobes. Visual impairment is a distinct symptom if the disease occurs inside occipital lobes. In certain cases of GBM invading cerebellum, patients will lose voluntary coordination of the muscle movements. GBM in brain stem can be detected by lower cranial nerves deficit (Reni *et al.*, 2017). Thus, most GBM patients are prescribed with corticosteroids to control vasogenic edema and alleviate accompanying signs and symptoms (American Association of Neuroscience Nurses., 2016).

2.1.3 Current treatments for GBM

Patients with symptoms of brain tumor will initially undergo medical screening for the tumor confirmation. The standard imaging procedures are including Magnetic Resonance Imaging (MRI), Computed Tomography (CT) Scan and Brain Positron Emission Tomography (PET). MRI uses combination of magnetic field and radio waves without producing any radiation to define lesion boundaries including size, shape and location of the tumor (Elsadway and Ibrahim Ali, 2017). CT Scan captures images from different angels using X-rays to make a detailed and cross-sectional picture. Meanwhile, PET is where a sugar radiotracer delivered to the bloodstream through injection. Appearance of the tumor is detected after few hours using a special camera during the scan (Giglio and Puduvalli, 2017). All these technologies have allowed for full navigation of patient-specific data for suitable treatment plan.

Multidisciplinary GBM treatment approaches are including neurosurgery followed by radiation and chemotherapy. Maximally safe surgical resection is applied for tumor removal, which is depending on the tumor size and shape, brain blood vessels and artery location, as well as any vulnerable brain sites (Giglio and Puduvalli, 2017). Because of the high degree of invasiveness and infiltrative nature of the tumor cells, complete removal of the tumor is often impossible. Most of the time, this condition leads to the tumor recurrence.

Following optimal surgical resection, patients commonly wait for as long as four weeks time to allow the craniotomy wound healing before another therapeutic approach is given. Radiotherapy is high energy and ultimately focused rays; whether X-rays, protons or photons that are delivered to disrupt tumor DNA. Radical and palliative regimes are two standard modes of radiotherapy for GBM treatment. Radical regime radiotherapy uses 60 Gy dose over 30 daily fractions. Meanwhile, palliative

regime radiotherapy uses 30 Gy dose over two weeks in six fractions (Narayanan *et al.*, 2012). However, one of this treatment challenge is radiotherapy resistance. The tumor cells could resist the treatment by upregulating the DNA double-stranded break repair machinery (Mukherjee *et al.*, 2009). Thus, the radiation treatment generally should include concurrent or subsequent chemotherapy to significantly increase the survival benefit of the patients (Stupp *et al.*, 2005).

Chemotherapy is a drug-based GBM treatment which intent to disrupt the life cycle of tumor cells by damaging their DNA or interfering with DNA synthesis. Two modes of chemotherapy delivery are systemic and local (American Association of Neuroscience Nurses., 2016). Local chemotherapy delivery is given at a specific site of the tumor. For instance, up to eight Carmustine wafers are placed inside the brain within the space left by the removed tumor. They remain embedded in the resection cavity and will dissolve slowly over time. Whereas, systemic chemotherapy drugs are taken orally in capsulated or liquid form, and travel throughout the whole body via bloodstream. It is given in cycles of "treatment days" followed by "days of rest". Common cycles are 14, 21 or 28 days long. The drugs are including Carboplatin, Cisplatin, Etoposide, Lomustine and Temozolomide (TMZ) (Stupp *et al.*, 2005).

The most widely chosen drug for chemotherapy is TMZ; an imidazotetrazinone methylating agent that methylates purines (A or G) in DNA and induces cells apoptosis (Friedman *et al.*, 2000). This anti-tumor drug permeabilizes more easily without any cumulative toxicity reports for patients treated with TMZ (Tolcher *et al.*, 2003; Hainsworth *et al.*, 2010). Chemotherapy resistance also become a challenge to this treatment modulated by secretion of DNA repair enzyme O^6-methylguanine DNA methyltransferase (MGMT) by tumor cells (Wick *et al.*, 2009). Above all, these vast

strategies of treatment intent exclusively to combat GBM invasion and its recurrence, as well as for the betterment of patient's quality of life.

2.2 Receptor Tyrosine Kinases (RTKs)

RTKs are essential components mediating various cellular processes of cell growth, differentiation, metabolism and motility. RTKs are forms of protein tyrosine kinase enzyme that catalyzes the transfer of phosphate group from adenosine triphosphate (ATP) to tyrosine residues on protein substrates that lead to tyrosine phosphorylation. This process further modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins.

RTK is a big family that includes the Eph receptor as the largest family of RTK in human, insulin receptors and growth factor receptors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (Hubbard and Till, 2000).

2.2.1 Eph receptors

Historically, the first Eph gene was found to be expressed in erythropoietin producing hepatocellular cancer cell line and therefore being called as *eph* (Hirai *et al.*, 1987). EphA2 was next found in 1990 as *eck* (epithelial cell kinase) because of their expression in most of epithelial cells (Lindberg and Hunter, 1990).

Eph receptors are divided into Eph-A and Eph-B sub-classes (pronounced eff-A and eff-B) (Eph Nomenclature Committee, 1997). The name has been standardized based on the sequence relations and attachments to the corresponding class of Ephrin ligands. Eph-A receptors interact to the ephrin-A ligands that bound to the membrane via glycosylphosphatidylinositol (GPI) linkage (Figure 2.1). There are 9 types of EphA receptors (EphA1-EphA9) that bind to 5 types of ephrin A (ephrin A1-A5). Whereas, Eph-B receptors interact to the ephrin-B ligands that attached to the membrane via transmembrane proteins with an intracellular domain and it has cytoplasmic tail extended to intracellular space which composed of transmembrane domain and an intracellular PDZ domain for phosphorylation (P). (Figure 2.1).

Figure 2.1 shows Eph receptor and its binding to Ephrin A and B. Eph-receptor are extended between two parts: intracellular and extracellular regions. The intracellular region contains transmembrane region (TM), the sterile alpha motif (SAM) and the PDZ domain. Abundant tyrosines present within this region which are important for phosphorylation and protein binding sites (P). While in the extracellular region, the receptor contains globular ephrin-ligand binding domain (LBD), Cysteine-rich domain (C) encompassing sushi and Epidermal Growth Factor (EGF), and two repeated fibronectin (FN) domains. LBD of Eph-receptor binds to receptor binding domain (RBD) of Ephrin ligand.

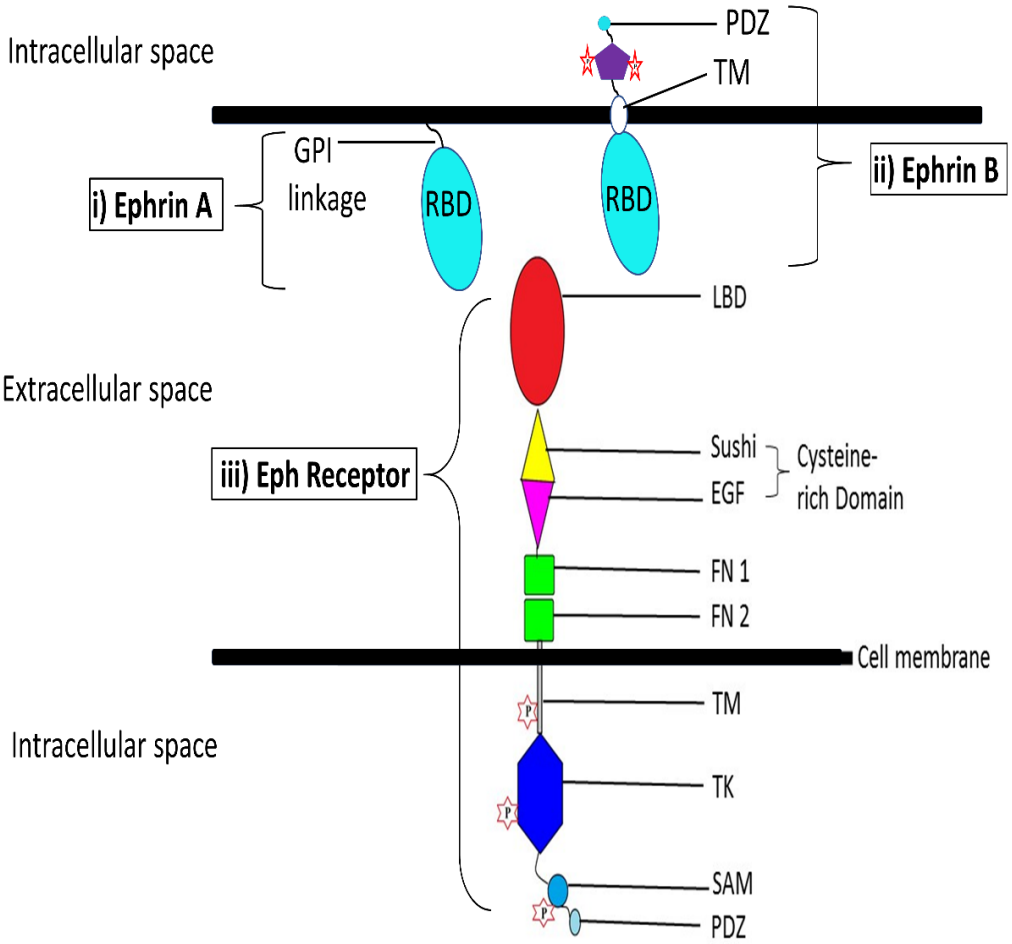

Figure 2.1: Binding of ephrin A (i) and ephrin B (ii) to Eph receptor (iii). (Modified from Himanen and Nikolov, 2003a; Himanen and Nikolov, 2003b, Seiradake *et al*., 2013; Kania and Klein, 2016)

Eph receptors and ephrin ligands are unique because the receptors and the ligands are bound to the membrane, unlike other ligands that only act as soluble mediators. The receptors are extended between the two sides of cell; extracellular and cytoplasmic side. Extracellular region is the place where it will interact and bind to the ephrin ligand, whereas in cytoplasmic region is where phosphorylation of the tyrosine residues (Tyr) occurs following Eph–ephrin interaction (Himanen and Nikolov, 2003a).

In extracellular region, the ligand binding domain (LBD) of Eph receptors binds and interacts to ephrins with the highest affinity, followed by the cysteine rich region and fibronectin domain repeats with lower binding affinity. Three parts of cytoplasmic portions Eph receptor are juxtamembrane region, the kinase domain, and SAM domain. They are the critical parts for Eph kinase activity**,** phosphorylation of Tyr and for communications with signaling proteins that induce many biological responses. Tyr phosphorylation also occurs in intracellular region of Ephrin-B ligand, as in Eph receptors. The end portion carboxyterminus of both Eph receptors and ephrinB ligands are PDZ domains which play important function in cell surface localization, clustering and signaling (Kayser *et al.,* 2006).

2.2.2 Signaling of EphA2 and ephrinA1 in tumor cells

Human EphA2 gene is located on chromosome 1 that encodes for 130kDa EphA2 protein with approximately 976 amino acids. It is known that 90% of it sequences are homologous to the EphA2 mouse (Himanen and Nikolov, 2003a). EphA2 receptor can interact with all types of ephrin from types 1 to 5 (Himanen and Nikolov, 2003b). However, the most common ligand that interact to EphA2 receptor is ephrinA1 (Lema Tomé *et al.,* 2012). Different types of its interaction mode are shown in Figure 2.2.

Forward signaling is signal transduction originated from ephrin ligands directed to Eph receptors. It is also called as Ephrin:EphA2 forward. Meanwhile, reverse signaling is signal transduction originated from Eph receptors to ephrin ligands. It is also called as EphA2:Ephrin reverse. Due to their membrane localization, the signal can also be simultaneously activated in both forward and reverse directions and known as Ephrin-EphA2 bidirectional signaling.

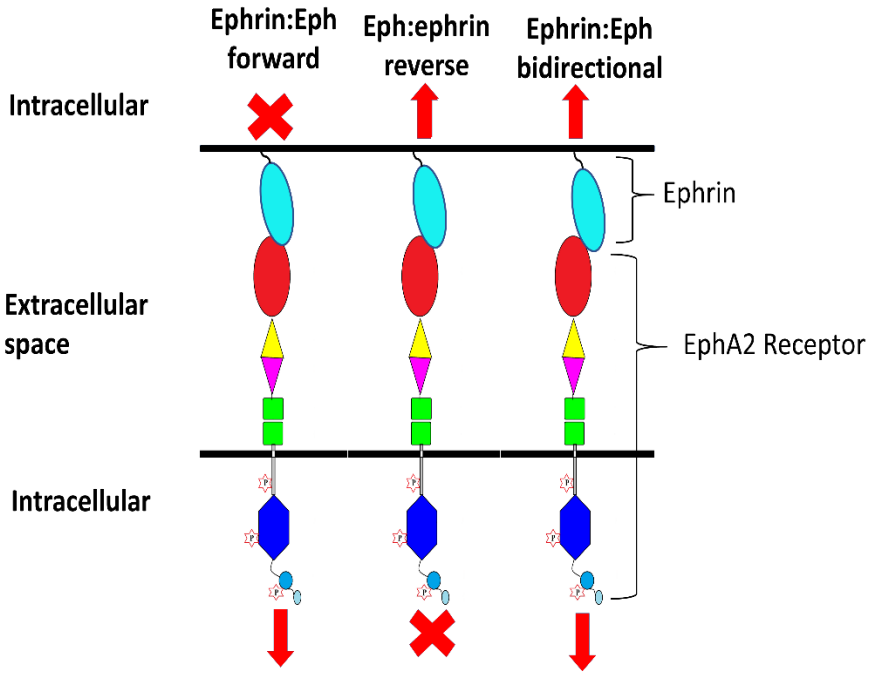

Figure 2.2 EphA2-ephrin types of interactions. (Modified from Kania and Klein, 2016)

In a situation of two or more Eph-Ephrin interaction occuring simultenously, the signaling can be classified into; parallel or anti-parallel. Parallel signaling is induced when forward and reverse signaling occur simultenously and in parallel to each other. While anti-parallel signaling is a simultaneously occurring forward signaling, where forward ephrin–EphA2 signals are conveyed toward different directions of Eph receptor. Figure 2.3 shows parallel and anti-parallel signaling for EphA2-ephrin interaction.

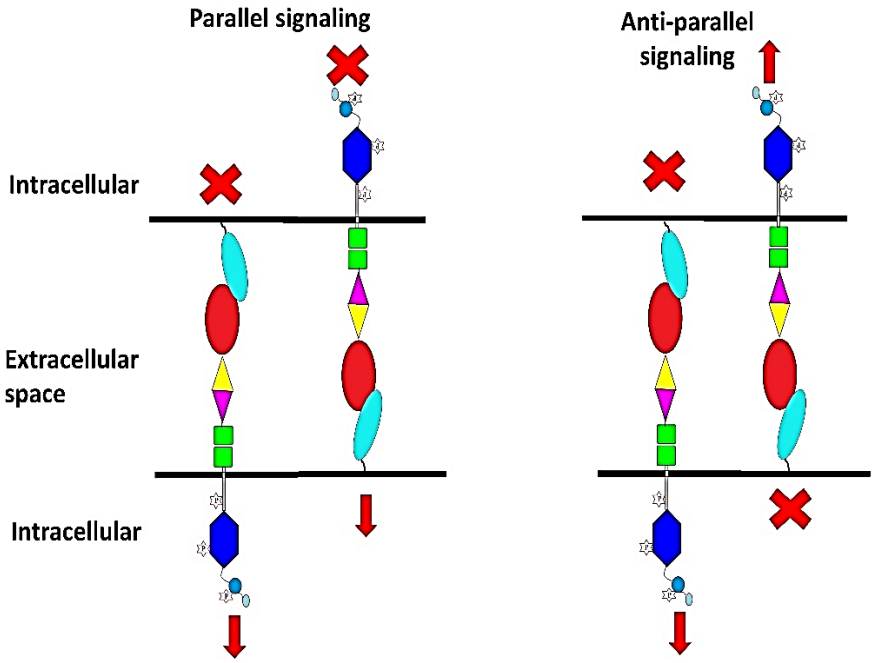

Figure 2.3: Parallel and anti-parallel signaling of EphA2-ephrin interaction. (Modified from Kania and Klein, 2016)

The EphA2 receptors could be triggered and activated upon binding to ephrinA1 ligand. The membrane attachment of both EphA2 and ephrinA1 provides various mode mechanism of interactions, unique from other receptor tyrosine kinases family. EphA2 and ephrinA1 can function independently of each other, through interchange with other signaling systems (Barquilla and Pasquale, 2015). Primarily, interaction Eph receptors with cell surface tethered-ephrin ligands can activate Eph receptor kinase-dependent signaling. Additionally, the ephrins itself can also convey signals, leading to bidirectional signaling. In this case, Eph receptor can act as ligand in the same way the ligand can act as receptor (Tandon *et al.*, 2011) and the ephrin cytoplasmic tail enables recruitment of further signaling effectors (Klein, 2004).

EphA2 receptor phosphorylation and kinase activity have been proved to induce tumor malignancy and confer cells oncogenic potential (Fang *et al.*, 2005). EphA2 are kinase enzymes that signal by transferring a phosphate group to a protein substrate (Garber, 2010). Another mechanism mediating the interactions is ligand/receptor endocytosis. During endocytosis, EphrinA1 is cleaved from the cell membrane and functionally interacts with EphA2 leading to signal transduction and receptor activation for the next molecular response (Kuijper *et al.*, 2007).

Stimulation of EphA2 causes powerful changes in tumor cells behavior. Interaction between EphA2 and ephrinA1 with other guidance molecules will navigate developmental guidance that lead sheets of cell layers to become tumors. The contact of ephrinA1 and EphA2 receptor in neighboring cells conveys signal in forward signaling will cause the cells to repel from each other. If the EphA2 receptor isn't activated or called as reverse signaling, then the interaction with the ligand will cause either cell adhesion or repulsion (Kania and Klein, 2016). As a result, adhesion leads to tissue formation, and repulsion leads to boundary separation between tissues. This same mechanistic action is also being used for tumor formation and invasion.

On top of that, Ephrin-EphA2 receptors interaction can induce short distance signaling and mediate cellular process like cell proliferation, migration, formation of tissue boundaries, axon guidance and platelet aggregation (Wykosky *et al.*, 2005; Arvanitis and Davy, 2008; Kania and Klein, 2016). Above all, a systematic interaction between EphA2 and ephrin ligands can initiate the formation of a complex network of regulatory pathways that must act coherently to control multifunction of biological responses.

2.2.3 Clinical and biological impacts of EphA2 overexpression

EphA2 overexpression has been detected in many types of tumors including ovarian (Thaker *et al.,* 2004), breast (Zelinski *et al.,* 2001), prostate (Nakamura *et al.,* 2005) and also in GBM (Hatano *et al.,* 2005; Wykosky *et al.,* 2005). A study by Wykosky and Debinski showed that overexpression of EphA2 protein was detected in 61% of GBM tumor patients (2008). EphA2 overexpression was significantly associated with higher grade of tumor and advanced stage of the disease. For instance, higher EphA2 expression was detected in advanced tumor anaplastic astrocytomas (Grade III) and GBM (Grade IV) (Wykosky *et al.*, 2005). Analysis of NOD/SCID mice glioma tissues attained by surgical aspirate revealed that EphA2 expression was significantly higher in glioma tissue specimens ($p = 0.021$) compared to normal brain tissues (Day *et al.*, 2013).

The elevated trend of EphA2 is responsible for adverse clinical impact of the tumors. For instance, Liu et al. (2006) proved that EphA2 mRNA overexpression worsen the patient survival in a panel of 21 GBMs.60% of primary GBM manifest high-level expression of EphA2 and significantly ($p<0.01$) associated with the adverse overall survival rate of the GBM patients (Wang *et al.,* 2008a). Highly expressed EphA2 has been associated with the aggressiveness of ovarian carcinoma (Thaker *et al.*, 2004). EphA2 has been identified as an oncoprotein that responsible to transform mammary epithelial cells (MCF-10A) to become malignant (Zelinski *et al.*, 2001). Overexpression of EphA2 has been related to malignancy in squamous cell carcinoma of oral tongue (Shao *et al.*, 2008). EphA2 overexpression in endometrial tumor patients has been associated with increased depth of myometrial invasion and its aggressiveness (Merritt *et al.,* 2014). EphA2 overexpression in ovarian cancer patients

has been significantly correlated to the increase of microvasvular density (MVD) and decrease of median patients survival (Lin *et al.*, 2007).

The elevated EphA2 expression has triggered malignant phenotypes including to promote cell growth and proliferation. A study has positively correlated between EphA2 overexpression to promote higher proliferation rate of brain astrocytic tumors (Li *et al.*, 2007). The higher EphA2 expression, the higher the level of endometrial tumor proliferation which was demonstrated by positive Ki-67 staining (Merritt *et al.*, 2014). Overexpression of EphA2 has also significantly (p<0.01) enhanced cells proliferation of Lymph Node Carcinoma of the Prostate (LNCaP) tumor (Chen *et al.*, 2014). Most recently, inhibition of EphA2 expression reduced cell cycle progression and growth of the most aggressive form of breast tumors; basal-like/triple-negative breast cancers (Song *et al.*, 2017).

EphA2 overexpression was also responsible for tumor migration and metastasis. It was found that blocking of EphA2 receptor signaling inhibited lung metastasis *in vivo* (Fang *et al.*, 2005). Silencing EphA2 expression in colorectal tumor has suppressed its basal migration and invasion rates which was assessed using invasive colorectal cancer cell line models (Dunne *et al.*, 2016). EphA2 overexpression and Akt activation has regulated GBM migration and invasion through Akt-EphA2 signaling (Miao *et al.*, 2009). Downregulation of EphA2 had significantly inhibited mammary adenocarcinoma metastasis to the lung in transgenic mouse model of mammary carcinoma (Brantley-Sieders *et al.*, 2008). Histologically, microvessel density (MVD) was relatively higher in the highly expressed EphA2 of GBM (Wang *et al.*, 2008a). EphA2-deficient has impaired the respond of pulmonary microvascular endothelial to VEGF in transgenic mice (Youngblood *et al.*, 2015). These are the evidences that EphA2 act as a regulator for tumor angiogenesis.

2.3 Angiogenesis Signaling Pathway in Tumors

Angiogenesis, the formation of new blood vessels from the pre-existing vasculature, has been shown to be a critical step in the progression and metastasis of solid tumors. This process involves endothelial cell proliferation, migration, tubulogenesis, and perivascular supporting cell recruitment (Zetter, 1998; Takano, 2012). At the beginning of the process, endothelial cells form lateral pseudopodia transforms to become a hollow tube. The hollow tube becomes the new capillary sprout networks and continue expanding and fusing with other capillary sprouts, resulting in a continuous blood flow in the tumors (Zetter, 1998).

As a highly ordered process, angiogenesis is governed under tight regulation of angiogenic factors and inhibitors known as pro- and anti-angiogenic mediators. More than 25 different growth factors and cytokines have been identified to involve in the process of angiogenesis in tumors (Ono, 2008). The pro-angiogenic molecules must be in balance with anti-angiogenic molecules to compel changes in metabolic demands for maintaining adequate oxygen and nutrient delivery and avoiding any pathological conditions. It is known that, GBM is one of the most angiogenic tumors with the highest rate of vascular smooth muscle proliferation which enables the tumor cells to generate its own vasculature (Takeuchi *et al.*, 2009). The abnormal vascularization is also supported by neurovascular unit created by the endothelial cells, pericytes, and astrocytes (Charles *et al.*, 2011). On top of that, GBM has high rate of microvascular proliferation, endothelial cytogenetic instability and hyperplasia (Takano, 2012). Such characteristics are generally used to detect high-grade gliomas and to signify regions of angiogenesis. Such regions, also called perivascular niches (Charles *et al.*, 2011).

To date, a number of different signaling pathways have been shown as contributors to tumor angiogenesis. Signaling networks involving ligand-receptor complex in angiogenesis process include; Angiopoietin ligands with the Tie receptors (Eklund and Olsen, 2006), Delta-like and Jagged ligands with Notch receptors (Purow *et al.*, 2005), Slit ligand with the Robo receptors (Legg *et al.*, 2008) VEGF ligand with receptors VEGFR-1, VEGFR-2, and NRP1 (Ferrara, 2004) and Ephrin ligands with the Eph receptors (Cheng *et al.*, 2002a). However, the mechanism through which VEGF ligand with receptors and Ephrin ligands with the Eph receptors pathways intersect remains unclear.

Angiogenesis process in tumors are mainly governed by VEGF and its receptors; VEGFR-1 and VEGFR-2 glycoproteins that act directly in the signaling pathway. As an important regulator of angiogenesis, lost of VEGF-A allele during an embryonic development has resulted in death (Carmeliet *et al.*, 1996). VEGF binds to two receptor tyrosine kinases (RTK); VEGFR-1 (Flt-1/fms-like tyrosine kinase) and VEGFR-2 (KDR,Flk-1/kinase domain region) (Safiyyah Ziyad and M. Luisa Iruela-Arispe, 2011). However, the signaling and biological characteristics of these two receptors are different. VEGFR-1 has the higher affinity for VEGF compared to VEGFR-2 which has lower affinity (de Vries *et al.*, 1992; Terman *et al.*, 1992).

VEGF ligands bind to either VEGFR-1 or VEGFR-2 and lead to the formation of VEGF-R homodimers. Both VEGFR-1 and R-2 are composed of the extracellular domain for VEGF binding region. The extracellular domain composed of approximately 750-amino-acid-residue, that is organized into seven immunoglobulin (Ig)-like folds. It is followed by a single transmembrane region, a juxtamembrane domain, a split into 2 tyrosine-kinase domain that is interrupted by a 70-amino-acid

kinase insert, and a C-terminal tail. Figure 2.4 shows the molecular structure and interaction of VEGF ligand and its receptors.

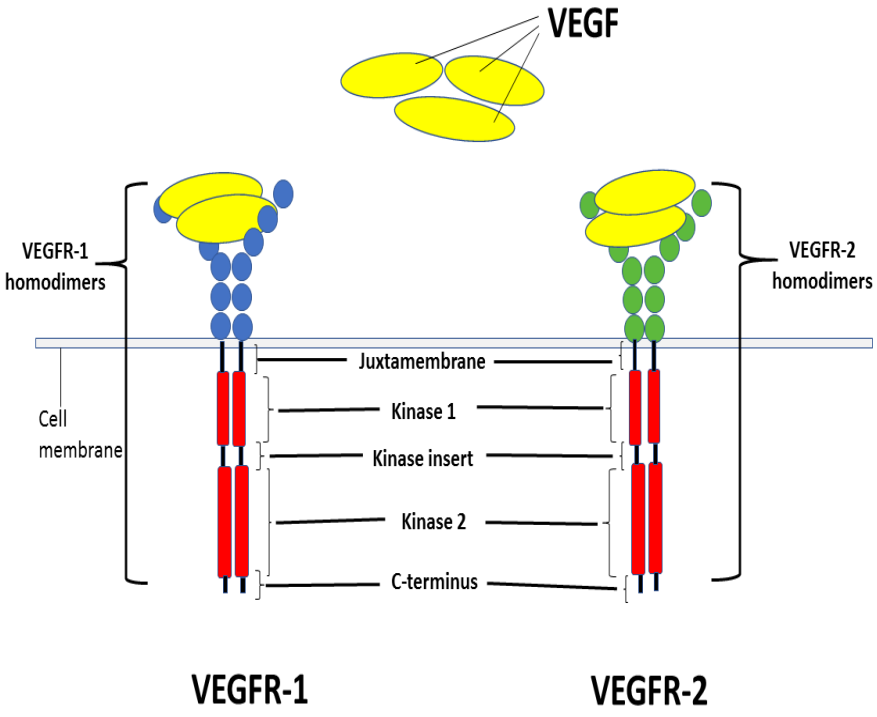

Figure 2.4: VEGF binding to its receptor, VEGFR-1 and VEGFR-2.
(Modified from Osson *et al*., 2006).

As a dimeric glycoprotein, VEGF act as a mitogen for vascular endothelial cells that are released from arteries, veins, and lymphatics. This mitogen is the major pro-angiogenic and survival factor that induces both proliferation and sprouting of endothelial cells. Six glycoproteins of VEGF family are VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF) (Makrilia *et al.*, 2009). The VEGF family members are secreted in approximately 40 kDa of protein size (Olsson *et al.*, 2006).

Regulation of VEGFR-1 and VEGFR-2 is controlled by the presence of VEGF ligands. Upon VEGF binding to VEGFRs, it leads to dimerization of the receptors. Dimerization is accompanied by activation of the receptor-kinase activity to initiate autophosphorylation of the receptors. The phosphorylated receptors then employ interacting proteins and initiate the activation of signaling pathways.

To promote tumor angiogenesis, VEGFR-1 involves by contributing to vascular sprouting and metastasis (Wong *et al.*, 2009). It is also a positive regulator of monocyte and macrophage migration, and has been described as a positive and negative regulator of VEGFR-2 signaling capacity. When negative regulation is exerted, it will prevent VEGF from binding to VEGFR-2 (Ferrara, 2004). On the other hand, VEGFR-2 is the receptor for mediating the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. VEGFR-2 mediates recruitment of circulating endothelial precursors to sites of active angiogenesis in vascular endothelial cells (Olsson *et al.*, 2006).

2.3.1 Roles of ephrinA1-EphA2 interaction in tumor angiogenesis

Upregulation of ephrinA1-EphA2 in tumors may affect tumor growth and vascularization. A survey on the expression patterns of Eph molecules in tumor vasculature revealed that the ephrinA1-EphA2 complex is consistently overexpressed in endothelial cells of tumor-associated vessels in a variety of tumors, including tumor xenografs of MDA-MB-435 human breast cancer and KS1767 human Kaposi's sarcoma, ovarian, prostate, lung, and skin tumor (Ogawa *et al.*, 2000; Ronca *et al.*, 2017). This implicates that ephrinA1-EphA2 expression corresponds to enhance angiogenesis.

Additional studies discovered the significant of the interaction related to angiogenesis pathways. The role of ephrin/Eph signaling in angiogenesis is depending on the phase of vascular development, receptor class, and cancer type (Safiyyah Ziyad and M. Luisa Iruela-Arispe, 2011). Antagonizing EphA signaling resulted in inhibition of tumor neovascularization and tumor growth, thus providing the first functional evidence for the role of Eph A class receptors in tumor angiogenesis (Brantley *et al.*, 2002). The use of EphA2-Fc as a blocker of the ephrinA1-EphA2 inhibited angiogenesis in a transgenic model of pancreatic carcinoma as well as in a model of metastatic mammary adenocarcinoma (Wykosky and Debinski, 2008).

Interaction of ephrinA1 with EphA2 induced endothelial cells aggregation and migration after downstream enzymes; PI3 kinase and Rac1 GTPase were activated (Brantley-Sieders *et al.*, 2004). In human brain microvascular endothelial cell line (HBMEC), EphA2 involves in the regulation of endothelial tight junction formation. Inactivation of EphA2 had resulted in the inhibition of HBMEC angiogenesis via reduction of the capillary-like tubular structures formation (Wu *et al.*, 2011).

In addition, EphA2 was also found to be related to angiogenesis through VEGF signaling (Shao *et al.*, 2008). Interaction of EphA2 and VEGF in angiogenesis signaling pathway was proven when soluble EphA2-Fc receptors inhibited VEGF-induced survival, migration, sprouting of endothelial cells and corneal angiogenesis (Cheng *et al.*, 2002b). In a different study, angiogenic factors such as VEGF or TNF-α in the tumor microenvironment had induced the expression and activation of ephrinA1 leading to endothelial cells migration and blood vessel assembly (Cheng *et al.*, 2002a). EphA2 was required for VEGF-induced endothelial cell migration and assembly into capillary-like structures. It was found that EphA2 inhibition could block VEGF-induced angiogenic response. An *in vivo* data that showed administration of soluble EphA receptor systemically blocks VEGF-induced corneal angiogenesis and VEGF-dependent RIP-Tag angiogenic islet formation and development of solid islet cell carcinoma (Cheng *et al.*, 2003).

The presence of functional interaction between EphA2 and VEGF signaling was further supported via stimulation of EphA2 receptor by ephrinA1. This interaction had inhibited VEGF-induced phosphorylation of VEGFR-2 which subsequently reduced the angiogenic cell activity in bovine retinal endothelial cells (Ojima *et al.*, 2006) . A study by Chen *et.al* (2006) suggested a specific relation between EphA2 and VEGF when EphA2 appears to be required for VEGF-induced endothelial cell migration and assembly into capillary-like structures when soluble Eph receptor has successfully inhibited pathologic retinal angiogenesis. Kucharzewska *et al.* (2013) found that hypoxic condition induced higher microvascular sprouting and angiogenesis compared to normotic condition. Hypoxia is also known to activate various cell surface receptors including VEGFR-2 and EphA2. An *in vitro* study showed that endothelial migration in RIP-Tag tumor were inhibited in the presence of

VEGF-neutralizing antibodies or soluble EphA2 receptor. These results reflect that VEGF is the primary angiogenic factor that induces endothelial cell migration. (Cheng *et al.,* 2003).

Miao *et al*., (2014) reported that EphA2 expression in HBMEC was upregulated by VEGF through its binding to VEGFR-2 resulting an increment of endothelial cells permeability. From the growing evidences, it is known that EphA2 is one of the regulators for angiogenesis in various pathological conditions through mechanism that involves VEGF and its' receptors. Nevertheless, its role in GBM angiogenesis is yet to be elucidated.

2.4 Small Interference RNA (siRNA) for Inhibition of Gene Expression

RNA interference (RNAi) technology has been developed as a mechanism to silent the expression of specific gene of interest. Double stranded RNA (dsRNA) will be introduced into cells to degrade its complementary messenger RNA (mRNA). Consequently, silencing gene expression will lead to the knockdown of protein synthesis. Silencing of gene expression using the RNAi has emerged as the method of choice for studying gene function in mammalian cells and disclose new approaches for therapeutic intervention.

There are various forms of RNAi including micro-RNA (miRNA); a short single-stranded RNA with 19–25 nucleotides which are expressed naturally in all higher eukaryotes. Then, short-hairpin-RNA (shRNA) has a tight hairpin turn shape that is artificially manufactured and designed based on miRNAs. Small modulatory RNA (smRNA) is a short, dsRNA which can be found in the nucleus of neural stem cells of mice. Piwi-interacting RNA (piRNA), is a single-stranded 25–31 nucleotides RNA which have recently been found in mouse, rat, and human testes. Finally, "short" RNA inhibitors or known as small interfering RNAs (siRNA).

siRNA is a short duplexes product from dsRNA or shRNA. siRNA molecular structure is composed of short length of 21–23 nucleotides with 5' phosphorylated end, 19-nucleotides duplexed region and 2-nucleotides unpaired and unphosphorylated 3' end. They overhang symmetrically as shown in Figure 2.5.