EPHA2 siRNA SILENCING ON MALIGNANT GLIOMA CELLS

By

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PERENCATAN EPHA2 siRNA KE ATAS SEL TUMOR OTAK MALIGNAN

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LIST OF ABBREVIATIONS

GBM	:	Glioblastoma Multiforme
CNS	:	Central Nervous System
U87 MG	:	Uppsala 87 Malignant Glioma
RTK	:	Receptor tyrosine kinase
siRNA	:	Small interfering RNA
dsRNA	:	Double stranded RNA
Eph	:	Erythropoietin-producing human hepatocellular carcinoma Receptors
EphA2	:	Eph receptor A2
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
NT	:	Non-Targeting
WHO	:	World Health Organization
SPSS	:	Statistical Package for the Social Sciences
FBS	:	Fetal Bovine Serum
MTS	:	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 (4 sulfophenyl)-2H-tetrazolium)
PBS	:	Phosphate-buffered saline

LIST OF SYMBOLS

%	:	Percentage
nM	:	Nano molar
µL	:	Micro litre
p	:	P- value
SD	:	Standard deviation

PERENCATAN EPHA2 siRNA KE ATAS SEL TUMOR OTAK MALIGNAN

ABSTRAK

Tumor otak malignan adalah salah satu jenis tumor yang paling ganas dan membawa maut dengan kadar jangka hayat hanya 9-12 bulan. Kadar ekspresi gen EphA2 yang tinggi telah dikaitkan dengan perkembangan dan percambahan tumor otak malignan. Untuk mengkaji peranan EphA2 dalam pembiakan sel tumor otak malignan manusia, perencatan ekspresi gen EphA2 telah dijalankan dengan menggunakan siRNA. Dalam kajian ini, tiga kepekatan siRNA 12.5 nM , 25 nM dan 37.5 nM telah digunakan untuk mensasarkan gen EphA2 di dalam sel U87 secara in vitro. Real-Time PCR telah dijalankan untuk menganalisis tahap ekspresi gen EphA2 diikuti oleh ujian MTS untuk mengkaji perubahan daya tahan sel U87. Berdasarkan hasil pemerhatian dari ekspresi gen EphA2 menunjukkan bahawa kadar perencatan sangat signifikan dalam kumpulan sel yang dirawat dengan ketiga-tiga kepekatan siRNA ($p < 0.05$). Peratusan tertinggi kadar perencatan gen EphA2 dapat diperhatikan pada kepekatan 12.5 nM (73.6%), diikuti oleh 37.5 nM (63.1%) dan 25 nM (45%). Berdasarkan analisis MTS, kadar penurunan daya tahan sel U87 dapat diperhatikan dalam kumpulan yang dirawat dengan EphA2 siRNA 25 nM dan 37.5 M selepas 48 jam proses transfeksi. Pengurangan sebanyak 50.1% dan 22.2% kadar daya tahan sel U87 masing-masing dapat dilihat dalam sel yang dirawat oleh EphA2 siRNA 25 nM dan 37.5 nM. Kajian sebelum ini menunjukkan bahawa EphA2 secara signifikannya terlibat didalam aktiviti daya tahan sel tumor otak malignan. Walaubagaimanapun, di dalam kajian ini, hubungan diantara kadar perencatan daya tahan sel U87 dengan kadar

perencatan ekspresi gen EphA2 adalah tidak signifikan. Kesimpulannya, kajian ini menunjukkan bahawa transfeksi yang dijalankan oleh ketiga-tiga kepekatan EphA2 siRNA adalah signifikan untuk merencatkan ekspresi EphA2 pada tahap mRNA, namun begitu, ianya tidak konsisten dengan kadar perencatan aktiviti daya tahan sel U87.

Kata kunci: tumor malignan, EphA2, siRNA, percambahan sel

EPHA2 siRNA SILENCING ON MALIGNANT GLIOMA CELLS

ABSTRACT

Malignant glioma is one of the most aggressive type of solid tumours which is highly fatal with median survival rate of only 9-12 months. Recently, overexpression of EphA2 has been correlated with the development and tumour progression of malignant glioma. In order to examine the role of EphA2 in malignant glioma cells, inhibition of EphA2 gene expression was carried out using small interfering RNA (siRNA). In this study, siRNA targeting EphA2 gene was used to transfect U87 cells in vitro at three different concentration 12.5 nM, 25 nM and 37.5 nM. Real-Time PCR was used to analyse the EphA2 gene expression levels followed by MTS assay in order to examine the changes in cell viability of U87 cells. Evaluation of the gene expression showed that, significant inhibition of the EphA2 gene expression was observed in groups of cells treated with all 3 concentration of siRNA ($p < 0.05$). The highest knockdown percentage of EphA2 gene was observed at concentration of 12.5 nM (73.6%), followed by 37.5 nM (63.1%) and 25 nM (45%). Based on MTS assay analysis, a decline in cell viability of U87 cells was observed in groups treated with EphA2 siRNA 25 nM and 37.5 nM after 48 hours post transfection. Reduction of 50.1 % and 22.2 % of U87 cells viability was observed in cells treated with EphA2 siRNA 25 nM and 37.5 nM respectively. Recent evidences have shown that EphA2 significantly involves in mediating viability activities of malignant glioma cells. Nevertheless, in this study, the correlation between inhibition of U87 cells viability and EphA2 gene expression knockdown was found not to be significant. In conclusion,

this study demonstrated that transfection with all three concentration of EphA2-siRNAs significantly inhibited EphA2 expression at the mRNA level, however it did not consistently suppresses the viability of U87 cells.

Keyword: malignant glioma, EphA2, siRNA, cell proliferation

CHAPTER 1

INTRODUCTION

Study background

Malignant glioma is known as the most severe form of primary brain tumour (Price and Chiocca, 2014). Every year, around 1 ~ 5 cases in 100,000 people are diagnosed with malignant glioma (Dolecek et al., 2012) with poor prognosis and low median survival rate (Hamisch et al., 2017). Despite various advancements in the treatment strategy for treating malignant glioma, prognosis of the patients remains poor. Most of the patients die due to recurrence of the tumour.

In normal condition, erythropoietin-producing hepatocellular receptor A2 (EphA2) receptor tyrosine kinase plays an important role in controlling multiple physiological processes to maintain homeostasis inside the cells (Zhou and Sakurai, 2017). However, in malignant glioma tumours, it has been reported that, elevated expression of EphA2 was frequently observed, and plays a critical role in oncogenic signalling involving proliferation, angiogenesis, metastasis of tumour (Hafner *et al.*, 2004; Shen *et al.*, 2014). It is also positively correlated with poor diagnosis of the patients (Diamond *et al.*, 2017).

Growing evidences show that gene silencing via siRNA approach has a great therapeutic potential for treating malignant glioma in order to improve the survival rate of the tumour patients. siRNA can improve the median survival rate of malignant glioma patients by blocking targeted EphA2 mRNA and subsequently silencing the expression of EphA2 in the tumour cells. Silencing of EphA2 will leads to lower expression of EphA2 resulting in the decrease of cell proliferation of malignant glioma. Due to siRNA sensitivity and feasibility technique to knock down the expression of a wide range of genes, it provided a suitable platform for various studies to be carried out. Thus, EphA2 comes into sight as a future therapeutic target to curb the development of malignant glioma tumours.

CHAPTER 2

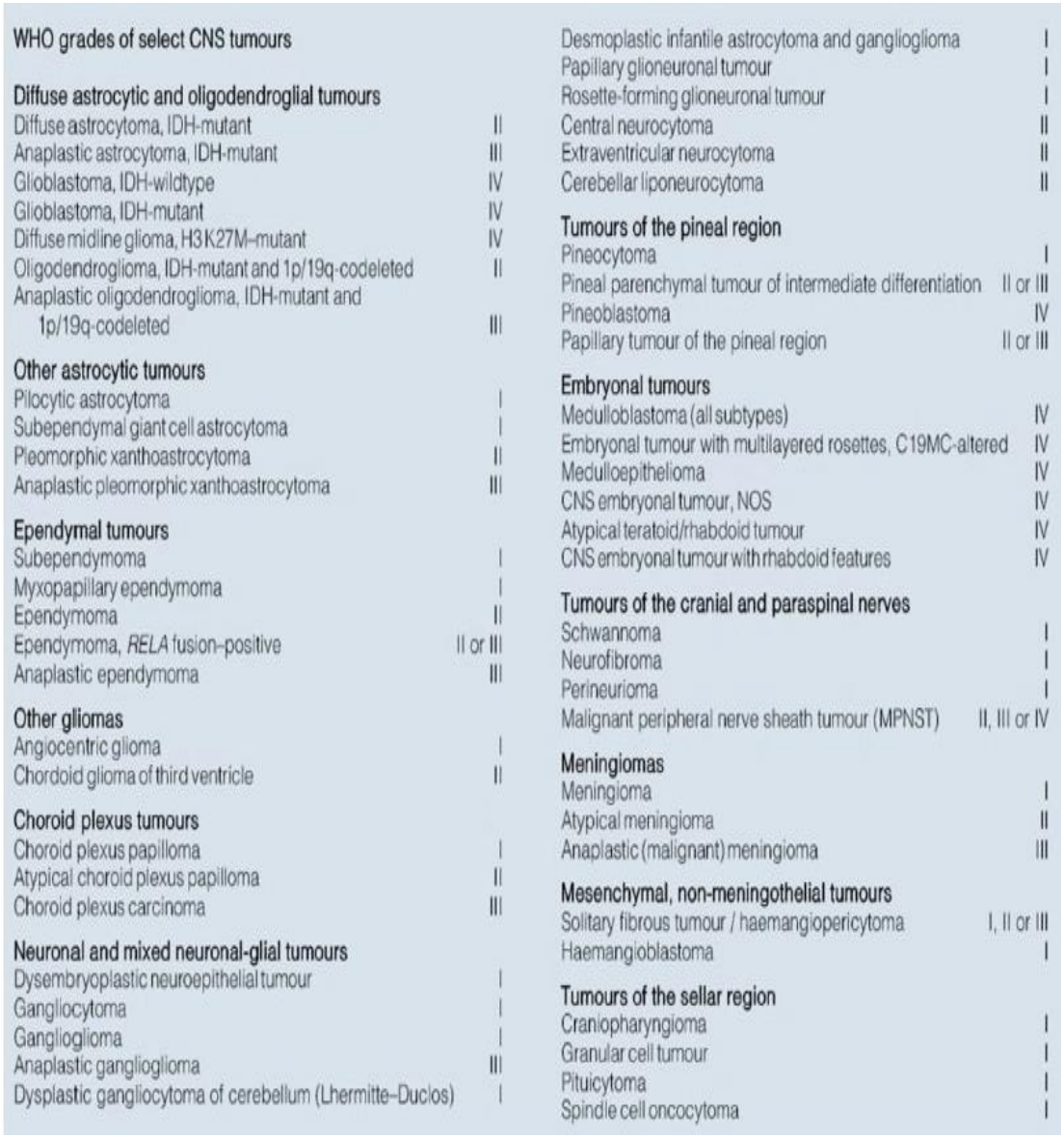
LITERATURE REVIEW

2.1 Brain tumour

2.1.1 Classification, risk factors and clinical presentations of brain tumours.

Brain tumour can be defined as an abnormal growth of cells that occurs in any part of tissue inside the cranium including the brain, skull, meninges, cranial nerves, pituitary gland and pineal gland (Ness, 2013). Brain tumour can be classified into two types which are primary and secondary (metastatic) brain tumours. Primary brain tumour generally develops from the brain cells itself and does not spread to other parts of the body. The tumour appears in either malignant or benign form. Meanwhile for secondary brain tumour, it spreads to the brain from other parts of the body and always in malignant form. According to World Health Organization (WHO), brain tumours can be classified into four grades and it is based on the rate of tumour growth, similarity tumour to normal cells, presence or vascularity and necrotic cell in the tumour cells (Louis *et al.*, 2016) . Usually, grade I tumours grow very slow and can be cured by surgery alone, whereas grade II tumours have the potential to invade adjacent normal cells and it may be reoccur again with higher grades of malignancies (Banerjee and Nicolaides, 2017). In contrast, for grade III tumours, it usually actively produce abnormal cells that can penetrate adjacent cells. Meanwhile, grade IV tumours are the

most malignant with rapid infiltration and proliferation to adjacent cells and have a central area of necrosis (Chang *et al.*, 2017). Besides that, these tumours will form new blood vessel in order to maintain their rapid growth and the most common example of highly angiogenic brain tumours are grade III and grade IV tumour. The example of grade IV tumour is glioblastoma multiforme (GBM) which referred as malignant glioma. Brain tumours classification is illustrated below in Figure 2.1.



WHO grades of select CNS tumours

Diffuse astrocytic and oligodendroglial tumours	
Diffuse astrocytoma, IDH-mutant	II
Anaplastic astrocytoma, IDH-mutant	III
Glioblastoma, IDH-wildtype	IV
Glioblastoma, IDH-mutant	IV
Diffuse midline glioma, H3K27M–mutant	IV
Oligodendroglioma, IDH-mutant and 1p/19q-codeleted	II
Anaplastic oligodendroglioma, IDH-mutant and 1p/19q-codeleted	III
Other astrocytic tumours	
Pilocytic astrocytoma	I
Subependymal giant cell astrocytoma	I
Pleomorphic xanthoastrocytoma	II
Anaplastic pleomorphic xanthoastrocytoma	III
Ependymal tumours	
Subependymoma	I
Myxopapillary ependymoma	I
Ependymoma	II
Ependymoma, *RELA* fusion–positive	II or III
Anaplastic ependymoma	III
Other gliomas	
Angiocentric glioma	I
Chordoid glioma of third ventricle	II
Choroid plexus tumours	
Choroid plexus papilloma	I
Atypical choroid plexus papilloma	II
Choroid plexus carcinoma	III
Neuronal and mixed neuronal-glial tumours	
Dysembryoplastic neuroepithelial tumour	I
Gangliocytoma	I
Ganglioglioma	I
Anaplastic ganglioglioma	III
Dysplastic gangliocytoma of cerebellum (Lhermitte–Duclos)	I
Desmoplastic infantile astrocytoma and ganglioglioma	I
Papillary glioneuronal tumour	I
Rosette-forming glioneuronal tumour	I
Central neurocytoma	II
Extraventricular neurocytoma	II
Cerebellar liponeurocytoma	II
Tumours of the pineal region	
Pineocytoma	I
Pineal parenchymal tumour of intermediate differentiation	II or III
Pineoblastoma	IV
Papillary tumour of the pineal region	II or III
Embryonal tumours	
Medulloblastoma (all subtypes)	IV
Embryonal tumour with multilayered rosettes, C19MC-altered	IV
Medulloepithelioma	IV
CNS embryonal tumour, NOS	IV
Atypical teratoid/rhabdoid tumour	IV
CNS embryonal tumour with rhabdoid features	IV
Tumours of the cranial and paraspinal nerves	
Schwannoma	I
Neurofibroma	I
Perineurioma	I
Malignant peripheral nerve sheath tumour (MPNST)	II, III or IV
Meningiomas	
Meningioma	I
Atypical meningioma	II
Anaplastic (malignant) meningioma	III
Mesenchymal, non-meningothelial tumours	
Solitary fibrous tumour / haemangiopericytoma	I, II or III
Haemangioblastoma	I
Tumours of the sellar region	
Craniopharyngioma	I
Granular cell tumour	I
Pituicytoma	I
Spindle cell oncocytoma	I

Figure 2.1: Classification of brain tumours based on World Health Organization (WHO). (Adapted from Louis et al., 2016).

There are many factors that can lead to development of brain tumours like ionizing radiation, virus infections, genetic abnormalities and other environment exposures. As reported by Ellor et al (2014), ionizing radiation is known as one of the factor that can enhanced risk of glioma development. For example, after several years of therapeutics radiation for another tumour like astrocytoma, radiation-induced glioblastoma might be occurred (Ellor *et al.*, 2014; Johnson *et al.*, 2015). Besides that, exposure toward other environmental condition such as smoking, pesticides, vinyl chloride and synthetic rubber manufacturing have been less associated with the development of glioma. Whereas, non-ionizing radiation from cell phones and formaldehyde have not been proven yet to caused glioblastoma multiform (Bondy et al., 2008; Alifieris and Trafalis, 2015). It has been reported that, increased risk of glioma development can be found in certain genetic diseases such as retinoblastoma, Turcot syndrome, Li-Fraumeni syndrome, tuberous sclerosis, neurofibromatosis 1 and 2, however less than 1% of glioma patients aware about their hereditary disease (Ellor *et al.*, 2014).

Brain tumour patients may have a variety of signs and symptoms. Primary brain tumours symptoms are based on type, rate of growth and location of the tumour (Stupp *et al.*, 2014). Gliomas symptoms can occur directly which is by compressing the area of the brain or indirectly by enhancing the intracranial pressure which may lead to alteration of mental function, vomiting, nausea, seizure and headache (Chang *et al.*, 2005). About 15-95% brain tumours patients have at least one seizure symptoms during their illness and most of low-grade gliomas patients will easily get seizures

rather than glioblastoma multiform (Grade IV) patients. (Chan *et al.*, 2016). The histology of the tumours, location and patient age are usually correlated with seizure condition. For example, patient with age 30 years and above will commonly get seizures often and based on histological diagnosis, about 62% mixed gliomas, 53% oligodendrogliomas and 42% astrocytomas patients experienced seizures (Liigant *et al.*, 2001; ENGLOT *et al.*, 2016)

2.2 Malignant glioma

2.2.1 Incidence and symptoms of malignant glioma

Malignant glioma may lead to a progressive destruction of brain tissue due to its characteristics which are highly aggressive and invasive growth of tumours (Louis *et al.*, 2016). The most common primary malignant glioma consisting of 16% of all primary brain and central nervous system neoplasm is known as glioblastoma multiforme (GBM) (Thakkar *et al.*, 2014). Based on a study reviewed in Sarawak from January 2009 until December 2012, the incidence rate for malignant glioma in Malaysia was found to be 4.6 per 100,000 population per year (Lee *et al.*, 2014). Meanwhile, the incidence rate of malignant glioma in United States of America is about 3.2 per 100,000 population (Davis, 2016). Until now, malignant glioma still remains as untreated diseases with low median survival rate which is around 10 - 15 months (Koshy *et al.*, 2012; Omuro and DeAngelis, 2013). Besides brain, malignant glioma can also emerged in other parts of the central nervous system including brain stem, cerebellum and spinal cord. Almost sixty-one percent of all primary malignant gliomas develop in the four lobes of the brain such as 25% in frontal lobe, 20% in

temporal lobe followed by 13% in parietal lobe and 3 % in occipital lobe (Davis, 2016). Malignant glioma is originally thought to derive from glia cells. However, based on the evidences provided by recent studies, it is suggested that the tumours appear from multiple cell types with neural stem cell properties and they are differentiated from stem cell to neuron to glia (Phillips *et al.*, 2006; Ligon *et al.*, 2017)

Symptoms of malignant glioma depend on the type, location and rate of tumour growth which can occur directly or indirectly. Malignant glioma can cause symptoms directly by invading or compressing certain area of the brain. Stress on the pariental lobes or sensory cortex may lead to disability to process complex sensory input which may results in agraphesthesia, astereognosis, or difficulties two- point discrimination, tactile localization, loss of word recognition and language comprehension (Hill *et al.*, 2002). At the same time, symptoms of malignant glioma can occur indirectly by increasing the intracranial pressure in the brain which may lead to headaches, blurred vision, nausea and vomiting. Besides that, pressure in frontal lobe of malignant glioma may lead to changes of personality and mental status (Antunes *et al.*, 2017) which result in poor attention span, failure of memory, lack of concern and judgement and social inappropriateness. Compression in frontal lobe of dominant hemisphere can give impact in Broca'a area and then lead to motor dysphasia, whereas, glioma on Wernicke's area result in aphasia or dysphasia. In addition, malignant glioma in temporal lobes may contribute to partial complex seizures, contralateral hemianopsia and loss of consciousness. Presence of malignant glioma in occipital lobes areas usually associated with visual disturbances like alexia, disability to focus on objects and inability to recognize faces or colours (Omuro and DeAngelis, 2013).

2.2.2 Molecular pathogenesis of malignant glioma

Malignant gliomas originate from neural progenitor cells and likely occur due to alteration of chromosomal regions and the deregulation of growth-factor signalling pathways (Ocvirk and Mesti, 2016). Glioblastomas which is regarded as the most common type of malignant glioma can be divided into two main subtypes which are based on basis of biological and genetic differences. Generally, primary malignant gliomas occur in patients older than 50 years old and are identified by amplification or mutation of EGFR, deletion on chromosome 10 of the phosphatase and tensin homologue (PTEN), deletion of p16 and loss of heterozygosity of chromosome 10q (Aldape *et al.*, 2015). Meanwhile, secondary malignant gliomas develop in younger patients as low-grade or anaplastic astrocytomas and it take several years before it convert into malignant gliomas. It can be described based on loss of heterozygosity of chromosome 10q (LOH), overexpression of the platelet derived growth factor receptor (PDGFR), mutations of tumour-suppressor gene (p53) and abnormalities in retinoblastoma (Rb) pathways and p16 (Wen and Kesari, 2008). The characteristics of high-grade oligodendrogliomas are recognized by the loss of chromosomes 1p and 19q in 50% to 90% of patients and the progression from low-grade to anaplastic oligodendroglioma is related with defects in cell cycle pathways involving Rb, p53 and PTEN (Louis *et al.*, 2007; Louis *et al.*, 2016). The alterations of genes that are mostly discovered in malignant gliomas are summarized in Table 2.1 (Schwartzbaum *et al.*, 2006).

Table 2.1: The mostly discovered genes alterations in malignant gliomas. (Adapted from Schwartzbaum et al., 2006).

Type of alteration	Candidate gliomas genes
Gains	-Receptor interacting protein kinase 5 (RIPK5) -Mouse double minute 4 (MDM4), Mouse double minute 2 (MDM2), -Phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta (PIK3C2B) -Epidermal growth factor receptor (EGFR) -Cyclin-dependent kinase 4 (CDK4) and others
Deletions	-NIMA-related kinase 1 (NEK1), -Tumour protein 53 (p53) -BRAF -Neurofibromatosis type I (NF1) -Isocitrate dehydrogenase 1 (IDH 1) -Cyclin-dependent kinase inhibitor 2A (CDKN2) -Phosphatase and tensin homolog (PTEN) -O-6-methylguanine-DNA methyltransferase (MGMT) -Retinoblastoma 1 (RB1) -Glioma tumor suppressor candidate region gene 1 (GLTSCR1), GLTSCR2, ligase I, DNA, ATP-dependent (LIG1), cytohesin 2 (CYTH2), integrase interactor 1 (INI1) -between cyclin-dependent kinase inhibitor 1C (CDKN1C) and related RAS viral (r-ras) oncogene homolog 2 (RRAS2)

Dysregulation of growth-factor signalling pathways are commonly involving EGFR and PDGFR. It can occur via genetic and epigenetic mechanisms such as amplification, deletion, gene mutation, methylation or demethylation, gains or losses of whole or partial chromosome and transcriptional interference (Figure 2.2). Both of

these ligands commonly expressed in malignant glioma and actively involved in glial development by creating an autocrine loop that trigger proliferation of the tumour (Jones and Holland, 2011). Besides that, growth factor-receptor signalling via intermediate signal transduction generators activated transcriptional programs for survival, invasion, angiogenesis and proliferation. Usually, it will be activated by growth factors like phosphatidylinositol 3-kinase (PI3K)-Akt- mammalian target of rapamycin (mTOR) pathways which involved in the inhibition of apoptosis and cellular proliferation and Ras-mitogen activated protein (MAP) kinase pathways which involved in cell cycle progression and proliferation. On the other hands, PDGFR, EGFR and VEGF-receptor (VEGFR) pathways also involved in promoting multipotent stem cells proliferation through normal development of nervous system (Thakkar *et al.*, 2014).

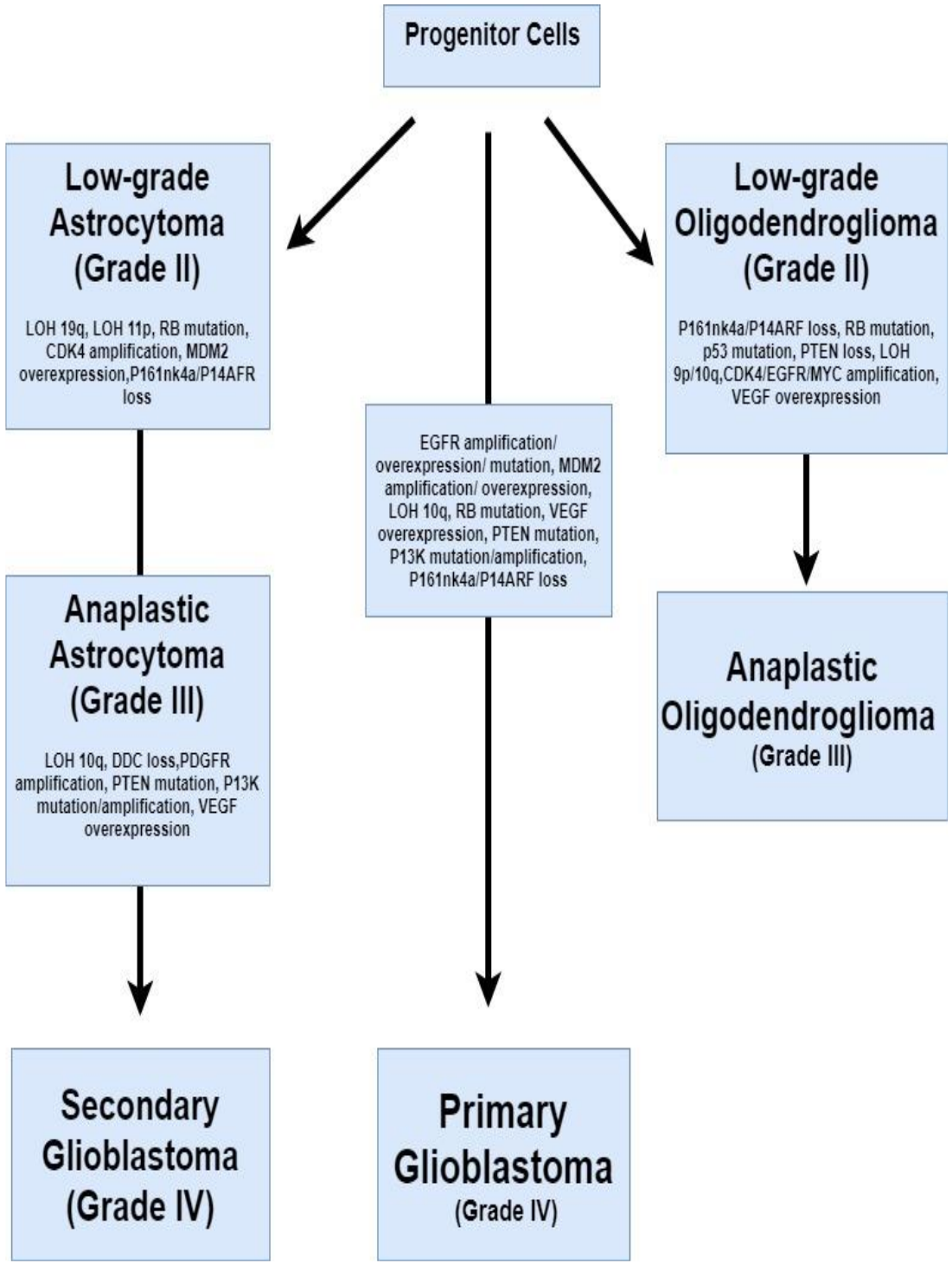


Figure 2.2: Dysregulation of growth-factor signalling pathways. Vascular endothelial growth factor (VEGF) are expressed in all high-grade gliomas. DCC indicates deleted in colorectal carcinoma, EGFR- epidermal growth factor receptor, LOH- loss of heterozygosity, MDM2 –murine double minute 2, PDGFR- platelet-derived growth factor receptor, PI3K- phosphatidylinositol 3-kinase, PTEN-phosphatase and tensin homologue and RB- retinoblastoma.(Modified from Cohen et al., 2013; Aldape et al., 2015).

2.2.3 Current treatments of malignant glioma

Surgical resection, radiotherapy and chemotherapy are known as the standard treatments for malignant gliomas. The advantages of maximal surgical resection are, it can decrease the symptoms from mass effect and provides tissue for molecular studies and histological diagnosis (Ahmed *et al.*, 2014). In addition, advancement in surgical resection like functional MRI, CT scans, diffuse tensor imaging (DTI) and fluorescence-guided surgery can enhance the safety of surgery and improved the extend of resection than can be achieved. However, with advances in surgery procedures, the prognosis for malignant glioma patients especially glioblastoma multiform remain poor with the median survival rate less than 15 months (Thakkar *et al.*, 2014). This is due to some limitations such as surgical procedure are likely to cause damage to normal tissue, misinterpretation of fMRI results due to neurovascular uncoupling and the location of the tumour is unreachable (Bush *et al.*, 2017).

Occasionally, when surgical resection is impossible, radiotherapy with an external beam were chosen as the initial treatment for malignant glioma (Juratli *et al.*, 2013). Besides that, in 1990, DNA alkylating agents which known as temozolomide (TMZ) has been approved as a chemotherapy agent for malignant glioma treatment by Food and Drug Administration (FDA) (Price and Chiocca, 2014). TMZ has the ability to pass through the blood brain barrier (BBB). Several studies involving several chemotherapeutic agents claimed that, TMZ is a drug that can increased median survival rate in malignant glioma patients (Urbańska *et al.*, 2014). According to randomized phase III trial shows that, the combination of radiotherapy treatment with

TMZ enhanced median survival rate from 12.1up to 14.6 months compared with radiotherapy alone (Ocvirk and Mesti, 2016). Moreover, phase III study involving 573 patients and 85 centres conducted by National Cancer Institute of Canada Clinical Trials Group (NCIC) and European Organisation for Research and Treatment of Cancer (EORTC) shows that TMZ can be used as a treatment for malignant glioma due to improvement in the survival rates from 10 % (radiotherapy alone) up to 27 % (radiotherapy with TMZ) (Stupp *et al.,* 2005; Ellor *et al.,* 2014).

Another chemotherapy agent for malignant glioma is known as nitrosourea (e.g: carmustine, lomustine and nimustine). They are alkylating agents and lipid soluble which have the ability to pass the BBB successfully. They can be used through oral intake, intra- arterial delivery and acts as a wafer in treatment of malignant glioma. For example, the biodegradable polymers containing carmustine were implanted into the tumour bed after malignant glioma resection. The aim of carmustine treatment is to kill the residual tumour cells. Based on a randomized, placebo-controlled trials of carmustine treatment in malignant glioma patients, the median survival increased from 11.6 months up to 13.9 months and this survival rate was maintained around 2-3 years (Westphal *et al*., 2006; Chen *et al.,* 2017).

However, there are some disadvantages of radiotherapy and chemotherapy treatment of malignant gliomas. The most common and immediate side effects of radiotherapy are hair loss, weakness, headache, fever, vomiting, nausea and scalp redness (Charnley *et al.,* 2009; Ellor *et al.,* 2014; Pourgholi *et al.,* 2016). Meanwhile, cytotoxic drugs for chemotherapy usually lead to gastrointestinal effects, headaches,

dizziness and fatigue. Besides that, intra-arterial administration of nitrosoureas like carmustine has also associated with acute neurotoxicity such as confusion and seizures (Dropcho, 1999; Philip-Ephraim *et al.*, 2012).

2.3 Eph family receptors

The biggest subfamily of receptor tyrosine kinases (RTKs) known as Eph receptors (Ephs) (Himanen and Nikolov, 2003; Tandon *et al.*, 2011). Eph receptors consists of 15 members and they are classified into two groups which is EphA (EphA1-EphA9) and EphB (EphB1-EphB6). EphA receptors normally tether all A-type ephrins, meanwhile EphB receptors tether to all B-type ligands. However, there are some exceptions, for example EphA1 mainly binds to ephrinA 1and EphA4 binds with both ephrinA and ephrinB, followed by ephrinA5 binds to EphA and EphB receptors (Figure 2.3) (Mosch *et al.*, 2010). Eph receptors depend on their binding affinity for their ligands, Ephrins (Eph receptor interaction protein) (Clifford *et al.*, 2008; Xi *et al.*, 2012). According to Hirai et al (1987), the first cloned of Eph gene was encountered in 1985 and a few years later, the first ephrin ligands was found in cancer cells (Grumolato and Aaronson, 2017). The connection between Ephs and its ephrin ligands is occurred due to the activation of bidirectional and transducer signalling cascades. Eph receptors and its ligands plays an important roles in biological functions such as involved in cell proliferation, angiogenesis, synaptic plasticity, tissue-border formation, axon guidance, development of the nervous system and embryonic patterning (Kullander and Klein, 2002; Lodola *et al.*, 2017).

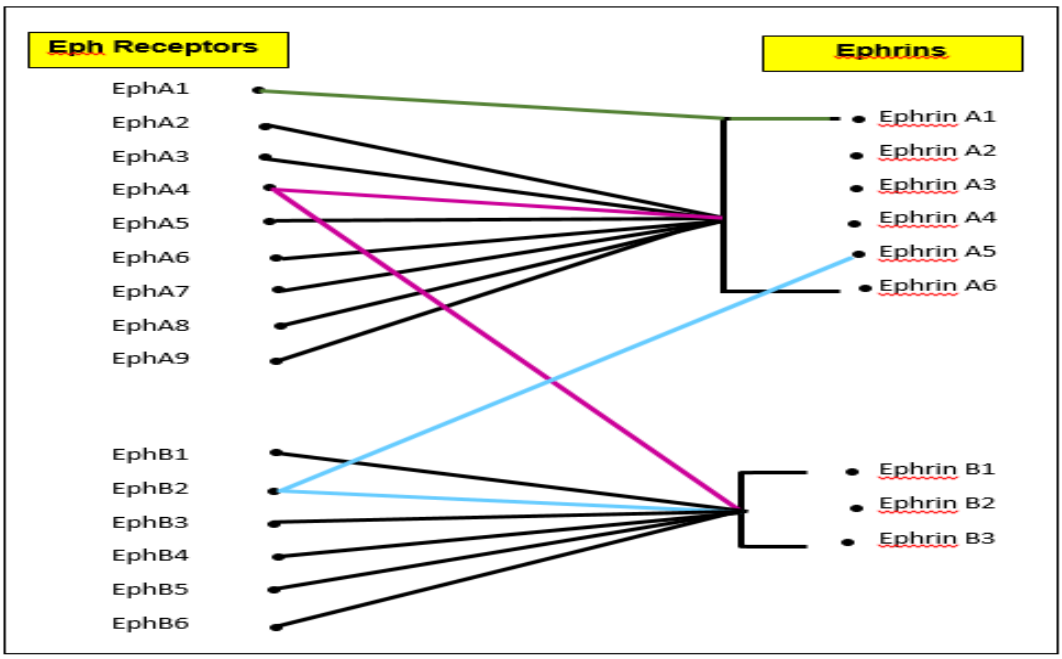


Figure 2.3: Interactions of Eph receptors and ephrin ligands. (Modified from Mosch et al., 2010).

2.3.1 EphA2 receptor

Several studies demonstrated that increased level of EphA2 can be found in various human tumours such as in carcinoma of prostate, colon, breast, pancreas (Chang *et al.*, 2008), ovaries, gastrointestinal tract oesophagus, liver, thyroid (Karidis *et al.*, 2011) and malignant glioma (Wykosky *et al.*, 2007). According to Lindberg and Hunter (1990), EphA2 was discovered in 1990 via screening process of human epithelial (Hela cells) cDNA library by using a degenerative oligonucleotide probe against the amino acid sequence HRDLAAR. Amino acid sequence HRDLAAR is a highly conserved region among tyrosine kinases (Lindberg and Hunter, 1990). At first, EphA2 was known as epithelial cell kinase (eck) due to its expression in the majority of epithelial cells. The human EphA2 gene consists of 90% amino acid sequence

homology to the mouse EphA2. It encodes for a receptor tyrosine kinase of 976 amino acids with molecular weight of 130 kDa and located on chromosome 1.

Ruiz and Robertson (1994) reported that, EphA2 gene was expressed in various regions during human development and it was supported with the study by Mori et al (1995). During early embryogenesis, expression of EphA2 gene was detected in the hindbrain and distal area of primitive streak especially in rhombomere 4 , while the EphA2 expression was decreased in the nervous system during late embryogenesis (Mori *et al.*, 1995). In adult stage, the EphA2 gene expression was observed in the spleen and brain with moderate levels (Lindberg and Hunter, 1990). However, no EphA2 expression was found in thymus, skeletal muscle, heart, liver or testes.

2.3.1.1 Structures of EphA2 receptor

The structure of extracellular domain Eph receptors contains two fibronectin type III repeats, also known as ligand-binding globular domain and cysteine-rich region. Besides that, the cytoplasmic part of Eph receptors can be classified into four functional units such as sterile α-motif (SAM), PDZ-Domain binding motif and two tyrosine residues of juxtamembrane. SAM domain contains about ~ 70 amino acids and it can form dimers and oligodimers, whereas, PDZ-binding motif contains binding sequence like hydrophobic residue and located in the carboxy-terminal 4-5 amino acids residues. During binding, ephrin A generally attaches to the cell membrane through a glycosyphosphatidylinositol (GPI) anchor (Tandon *et al.*, 2011; Xi *et al.*,

2012). The examples of the structures of EphA2 receptor and its ephrin ligands (Ephrin A1) are illustrated below in figure 2.4.

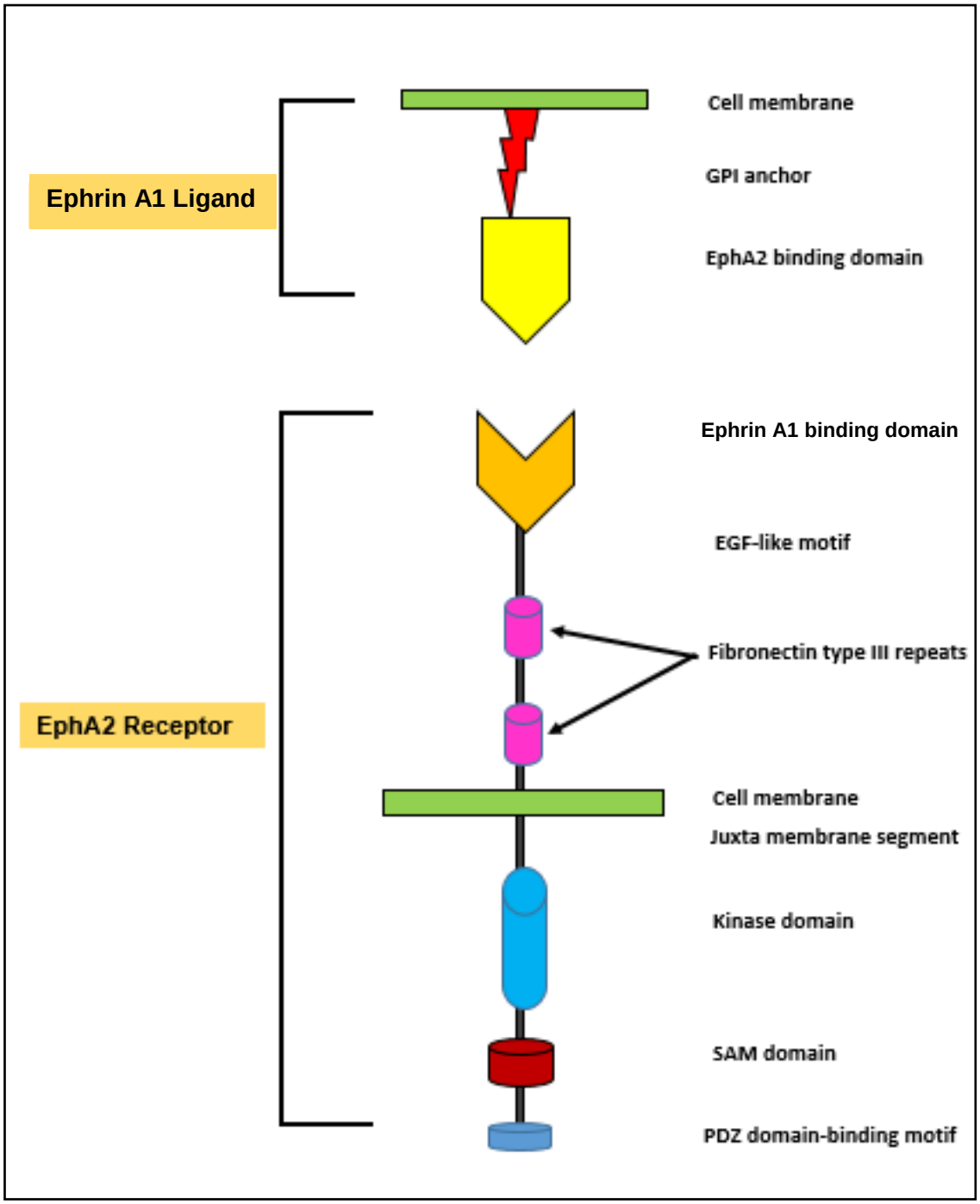


Figure 2.4: Binding of Eph receptor (EphA2 receptor) and its ephrin ligands (Ephrin A1). (Modified from Xi et al., 2012).

2.3.2 Roles of EphA2 in cancer development

EphA2 receptors and its ligand (ephrin A1) play important roles in physiological and pathophysiological process like proliferation, migration and invasion. Besides that, they are also involved in angiogenesis and tumour neovascularization (Xi *et al.*, 2012). Angiogenesis can be defined as the growth and formation of new blood vessels from existing blood vessels (Hanahan and Folkman, 1996; Coelho et al., 2017). It is important for metastasis and tumour growth processes because it provide the tumour with oxygen, growth factors and nutrients. Previous study reported that, angiogenesis process in malignant glioma is modulated by EphA2 (Maragoudakis *et al.*, 2012). According to *in vitro* and *in vivo* studies conducted in 2008 and 2009, EphA2 enhanced vascular (blood vessels) permeability via phosphorylation of claudins (Larson *et al.*, 2008; Miao and Wang, 2009a). Claudins are tight junction membrane proteins which plays a role as paracellular barriers to control the movement of molecules in the intracellular space between the epithelium cells (Günzel and Alan, 2013). Besides, further study revealed that, high expression of EphA2 was positively correlated with VEGF expression during in angiogenesis process (Sawamiphak, 2010) in human breast cancer, Kaposi sarcoma, and squamous cell carcinoma of the tongue (Shao *et al.*, 2008).

Up-regulation of EphA2 are found in most invasive tumour cells such as in glioblastoma, ovarian cancer , melanoma, prostate cancer, breast cancer, liver cancer, and oesophageal squamous cell carcinoma (Tandon *et al.*, 2011). Some study stated that, high expression of EphA2 is sufficient to cause tumorigenesis by increasing the

proliferation and migration activities of the cells (Zelinski *et al.*, 2001). It is also reported that, high expression of EphA2 receptors lead to high in cancer tumour invasive behaviour (Ruoslahti, 1999; Ribatti and Nico, 2016). Previous studies in 2005 reported that, EphA2 was highly expressed in malignant glioma especially in glioblastoma multiforme (Wykosky *et al.*, 2005) which suggested that EphA2 plays an important role in neovascularisation (Wu *et al.*, 2011). In addition, overexpression of EphA2 has also been correlated with proliferation, pathological grade and apoptosis in astrocytic brain tumours (Li *et al.*, 2007).

Previous studies claimed that, the inhibition of EphA2 caused delay in tumour growth of mice xenografts (Li *et al.*, 2010). Besides that, inhibition of EphA2 also decreased FAK phosphorylation which led to inhibition of integrin-mediated cell adhesion, migration, proliferation and cell spreading (Miao and Wang, 2009b; Mosch *et al.*, 2010). It is known that integrins plays a crucial roles in the mediation of cell adhesion, fibronectin deposition and cell anchorage. Moreover, integrins directly affect cell motility, proliferation and invasion which contribute to metastasis and formation of tumours (Maulik *et al.*, 2002; Surawska *et al.*, 2004; Wykosky and Debinski, 2008).

The roles of EphA2 in normal versus cancer cell are shown below in Figure 2.5. In normal condition, binding between EphA2 receptor and its ligands led to EphA2 phosphorylation and caused degradation of EphA2 resulting in lower expression of EphA2. This phosphorylation process was important for normal signalling pathways such as MAPK and Akt which involved in cell differentiation, proliferation and

migration. Meanwhile, in cancer condition, EphA2 failed to interact with its ligands led to accumulation of the un-phosphorylated form of EphA2 in the cells. This stimulated oncogenic signalling pathways which increased angiogenesis, proliferation and metastasis process of the cells and promote tumorigenicity (Tandon *et al.*, 2011).

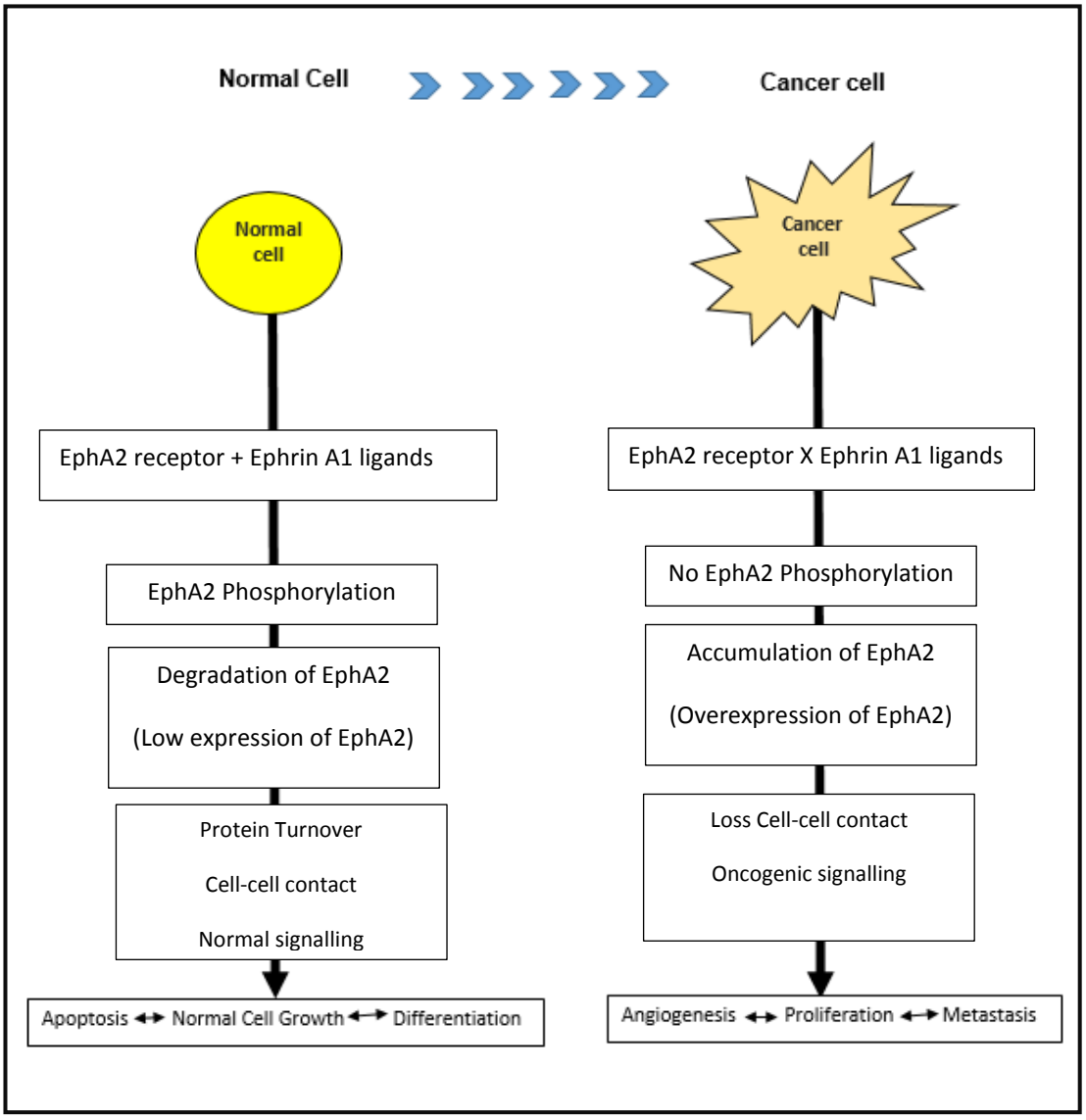


Figure 2.5: The role of EphA2 in normal versus cancer cell. (Modified from Tandon et al., 2011).

2.4 Small interfering RNA (siRNA)

2.4.1 Structure of siRNA

Small interfering RNA (siRNA) is known as silencing RNA or short interfering RNA. It is double-stranded RNA (dsRNA) molecules with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides and it contain 20-25 base pairs in length (Guo *et al.*, 2013). The structure of siRNA are illustrated below in Figure 2.5. siRNA are generally produced from long dsRNAs and small hairpin RNAs by a catalytic process mediated by Dicer enzyme (Huang *et al.*, 2008). Besides gene silencing, siRNA also plays a role in RNAi related pathway via shaping of the chromatin structure or by acting as antiviral mechanism (Agrawal *et al.*, 2003). siRNAs can be introduced into cells via several methods of delivery such as transfection, electroporation, viral-mediated delivery or modified siRNA (Xu and Wang, 2015). Nowadays, siRNAs have known as an important tool to validate gene function and drug targeting in the post-genomic area. The sensitivity and feasibility of the technique to knockdown any gene by using synthetic siRNAs has provided a suitable platform for various studies to be carried out (Guo *et al.*, 2013).

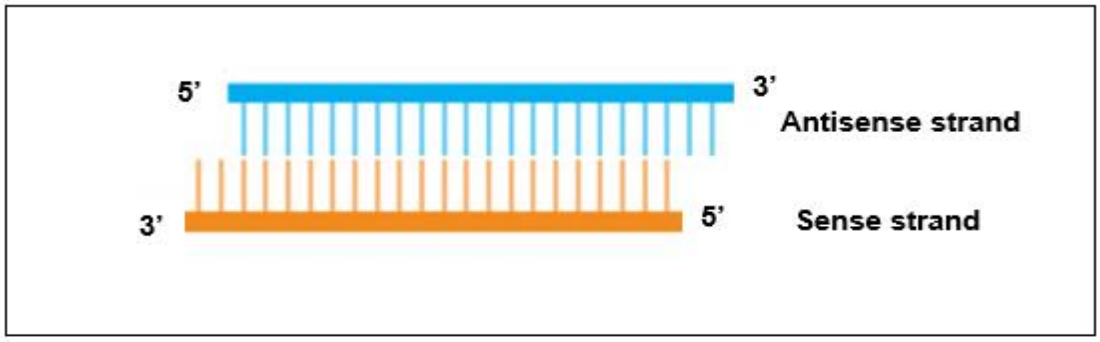


Figure 2.6: General structure of siRNA. (Adapted from Guo et al., 2013)

2.4.2 Gene silencing mechanism using siRNA

RNA interference is valuable in drug development and gene silencing because it has high specificity, easy to synthesis, significant effect and less side effects (Davis *et al.*, 2010). There are three approaches for RNA interference; endogenous microRNA (miRNA), short hairpin RNA (shRNA) and small interfering RNA (siRNA) (Xu and Wang, 2015). Among these types, siRNA is the most commonly used RNAi tool for introducing a synthetic double-stranded RNA to target a specific mRNA for degradation. The interference leads to prevention of protein translation in the cells.

Gene silencing mechanism of siRNA occurred when long double stranded of RNA (dsRNA) (e.g: complementary RNAs, hairpin, RNA dependent RNA polymerases) is cleaved by endo-ribonuclease enzyme known as Dicer to form a short interfering RNA or silencing interfering RNA (siRNA). Dicer enzyme delivers siRNA to a group of protein called as RNA-inducing silencing complex (RISC), where the catalytic component argonaute (Ago) is able to degrade siRNA into single strand and

bind with the target mRNA to induce mRNA cleavage. Further mRNA degradation lead to gene silencing or knockdown process (Wittrup and Lieberman, 2015). Figure 2.7 showed the major steps involved in mechanism of siRNA gene silencing.

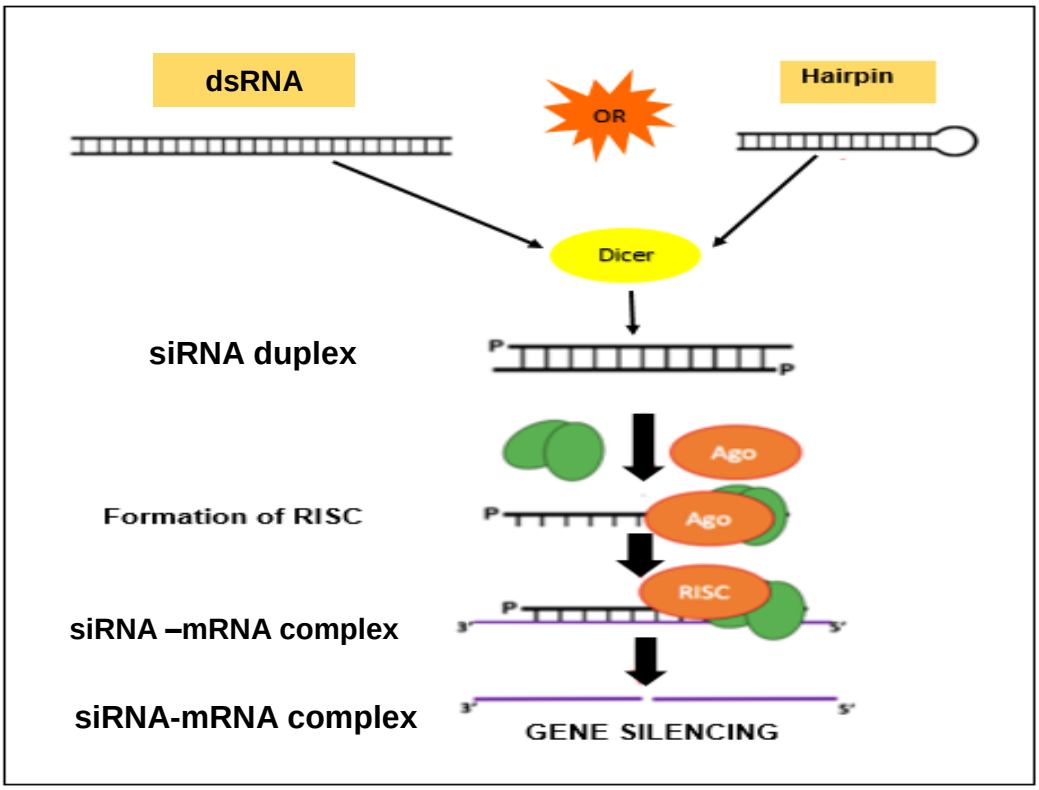


Figure 2.7: Summary mechanism of siRNA gene silencing. (Adapted from Wittrup, A., & Lieberman, J., 2015).

2.5 Rationale of study

Around 1~ 5 cases in 100,000 people are diagnosed with malignant glioma every year. According to World Health Organization (WHO), the most aggressive malignant glioma is referred to glioblastoma multiforme which is classified into grade IV. Overexpression of EphA2 was found in malignant glioma cases, and correlated with the tumour progression and poor diagnosis of patients. Increasing evidences have suggested EphA2 involvement in mediating various biological activities supporting tumorigenesis including tumor proliferation, angiogenesis, invasion and metastasis. Nowadays, siRNA is known as an important tool to detect the drug targeting and gene function in the genomic area. Due to it's feasibility and sensitivity technique to inhibit the expression of a wide range of genes, siRNA has been used as a platform for various study to be carried out. In this study, siRNA targeting EphA2 was used to determine its efficiency to inhibit EphA2 gene expression *in vitro.* Furthermore, the effects of EphA2 gene silencing on malignant glioma cells proliferation was also determined. The findings from this study will help to evaluate the roles of EphA2 in mediating proliferation activity of malignant glioma cells.