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Haritha D. Samaranayake

Gene Therapy Strategies to Enhance Anti-Angiogenic Therapy, Chemotherapy and Radiotherapy in the Treatment of Malignant Glioma

A Pre-Clinical Evaluation

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HARITHA D. SAMARANAYAKE

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A Pre-Clinical Evaluation

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ABSTRACT

Malignant glioma (MG) is an aggressive cancer with a dismal prognosis. The latest clinical trials report an overall median survival (MS) of only around 15 months. Tumour recurrence is inevitable with an average survival of less than 10 months thereafter.

The objective of this thesis work was to evaluate the feasibility of using gene therapy strategies to enhance anti-angiogenic therapy, chemotherapy and radiotherapy in the treatment of MG. The first study demonstrated the ability of adenovirus-mediated *Herpes simplex* virus type-1 thymidine kinase (AdHSV-tk) and pro-drug ganciclovir (GCV) suicide gene therapy in combination with adenovirus-mediated soluble vascular endothelial growth factor receptor-1 (AdsVEGFR-1) anti-angiogenic gene therapy to enhance the therapeutic outcome in a syngeneic rat MG model. Anti-angiogenic gene therapy, in spite of not being able to significantly impair the angiogenesis in the tumours, was able to reduce the recruitment of tumour promoting CD68+ macrophages/microglia.

In the second study, a novel mechanism of enhancing the cytotoxicity of temozolomide (TMZ) by up-regulating the gene expression of mismatch repair (MMR) proteins MSH2 and MLH1, when combined with AdHSV-tk/GCV suicide gene therapy, was described. The study was further extended to delineate the optimal treatment protocol so that the combination could maximise the synergistic effect. This gene therapy-based local enhancement of the cytotoxicity of TMZ increased the therapeutic index, demonstrating the safety of the combination.

Addition of the histone deacetylase inhibitor (HDACi), valproic acid (VPA), was not able to further enhance the efficacy of suicide gene therapy-TMZ combination *in-vivo,* in spite of the promising *in-vitro* results, in the third study. The short plasma half-life of VPA *in-vivo* may have been the reason for the lack of efficacy. This study revealed an efficient *in-vivo* treatment protocol to combine suicide gene therapy with TMZ that would have potential clinical applications.

The fourth study described the *in-vivo* efficacy of an efficient gene therapy-based targeting system for inoperable tumours, where lentivirus-mediated low-density lipoprotein receptor (LDLR)-avidin fusion protein gene therapy was capable of binding biotinylated compounds *in-vivo* and significantly enhanced the survival of the animals when treated with biotinylated ^{90}yttruim. The study highlights the possibility of utilizing gene therapy to target radiotherapy into inoperable tumours.

In conclusion, this work shows that the combination of different gene therapy strategies or gene therapy with chemotherapy is a feasible way to achieve synergistic outcomes. Careful consideration of the therapeutic protocol is important in obtaining maximum synergy. Furthermore, gene therapy can be a powerful tool to target radiotherapy and chemotherapy into the tumours, reducing unwanted side effects.

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Medical Subject Headings: Glioma/therapy; Gene Therapy; Angiogenesis Inhibitors; Thymidine Kinase; Ganciclovir; Vascular Endothelial Growth Factor Receptor-1; Dacarbazine/analogs & derivatives; Valproic Acid; Receptors, LDL; Avidin; Yttrium Radioisotopes

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TIIVISTELMÄ

Maligni gliooma (MG) on aggressiivinen ja erittäin huonoennusteinen syöpä. Viimeisimpien kliinisten kokeiden mukaan keskimääräinen potilaan elinajanennuste diagnoosin jälkeen on ainoastaan noin 15 kuukautta ja vääjäämättä tapahtuvan kasvaimen uusiutumisen jälkeen elinajanennuste jää alle 10 kuukauden.

Tämän väitöskirjatyön tarkoituksena oli selvittää miten geeniterapia parantaa pahanlaatuisen aivokasvaimen hoitotuloksia, kun se yhdistetään verisuontenkasvua estäävään, solusalpaaja- tai säteilyhoitoon. Ensimmäisessä osatyössä osoitettiin, että adenovirusvälitteisen itsemurhageeniterapian (*Herpes simplex* virus-1 tymidiinikinaasi (AdHSV-tk) ja aihiolääke ganciclovirin (GCV)) yhdistäminen adenovirusvälitteiseen ihmisen liukoisen verisuonten kasvutekijän reseptori-1:n (AdsVEGFR-1) geeniterapiaan tehostaa hoitoa syngeenisessä pahanlaatuisen glioomen rottamallissa. Vaikka anti-angiogeenisellä geeniterapialla ei ollut merkittävää vaikutusta kasvainten uudisverisuonituksen määrään, se kuitenkin vähensi tuumorigeneesiä edistävien CD68-positiivisten mikrogliasolujen esiintymistä kasvaimessa.

Toisessa osatyössä yhdistettiin AdHSV-tk/GCV geeniterapiaan klassinen, glioomen rutiinihoitona käytetty solusalpaajahoito temotsolomide (TMZ). Geeniterapiahoito sai aikaan kahden DNA-korjausmekansimiin liittyvän proteiinin ilmentymisen lisääntymisen, joka taas vaikutti positiivisesti TMZ hoidon tehokkuuteen. Geeniterapian avulla TMZ:n sytotoksisuutta voitiin tehostaa paikallisesti ja siten parantaa hoitovastetta ja hoidon turvallisuutta.

Kolmannessa osatyössä käytetty edelliseen yhdistelmään lisätty histonien deasetylaasi-inhibiittori valproaatti (VPA) ei tehostanut hoitotehoa eläinmallissa vaikka soluviljelykokeissa saadut tulokset olivat lupaavia. Syynä tähän saattoi olla VPA:n lyhyt puoliintumisaika plasmassa, jolloin konsentraatio jäi terapeuttista tasoa alhaisemmaksi. Tutkimuksessa selvitettin myös paras hoitoaikataulu, jolla saataisiin maksimoitua hoitojen yhteisvaikutus.

Neljännessä osatyössä lähestyttiin aivokasvainhoidon tehostamista hoidon paikallisen kohdentamisen kautta. Tässä geeniterapiassa lentivirusvälitteinen LDL-kolesterolin reseptorin (LDLR) ja avidiinin muodostama fuusioproteiini pystyi sitomaan ja soluunottamaan biotinyloituja aineita. Kohdennettu hoito merkittävästi pidensi biotinyloidulla ^{90}Yttriumilla hoidettujen eläinten elinaikaa. Tutkimus korostaa geeniterapian luomaa mahdollisuutta kohdentaa radioterapiaa leikkauskelvottomia kasvaimia hoidettaessa.

Väitöstyössä osoitettiin, että erilaisten geeniterapioiden yhdistäminen sekä keskenään että kemoterapian kanssa voi tehostaa hoitotuloksia. Tarkka hoitoaikataulujen suunnittelu on kuitenkin tärkeää maksimaalisen hyödyn saavuttamiseksi. Geeniterapia soveltuu lisäksi hyväksi työkaluksi kohdennettaessa radio- ja kemoterapiaa kasvaimiin ei-toivottujen sivuvaikutusten vähentämiseksi.

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Abbreviations

AA	anaplastic astrocytoma
AAV	adeno-associated virus
ACNU	1-(4-amino-2-methyl-5-piryrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea
ADA	adenosine deaminase
AdHSV-tk	adenovirus-mediated *Herpes simplex* virus type-1 thymidine kinase
AdsVEGFR-1	adenovirus-mediated soluble vascular endothelial growth factor receptor-1
AED	anti-epileptic drug
AFOS	alkaline phosphatase
AKT	protein kinase B
ALT	alanine aminotransferase
AP	abasic/apurinic site
APE	AP endonuclease
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related
BBB	blood-brain barrier
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BDEPC	bone marrow-derived endothelial progenitor cells
BDHPC	bone marrow-derived haematopoietic progenitor cells
BER	base-excision repair
bsmAb	bispecific mAb
Bt-^{90}Y-DOTA	biotinylated ^{90}Y-DOTA
BV	Bevacizumab
C	cytosine
CAR	coxsackie and adenovirus receptor
CCNU	1-(2-chloroethyl)-1-nitrosourea
CD	cluster of differentiation
CDA	cytosine deaminase
CDK	cyclin-dependent kinase
CDKN	CDK inhibitor
cDNA	complementary DNA
CED	convection-enhanced delivery
CM	conditioned media
CMV	cytomegalovirus
CNS	central nervous system
COX	cyclooxygenase
CPA	cyclophosphamide
CRAd	conditionally replicating oncolytic adenovirus vector
CSF	cerebro-spinal fluid
CSLC	cancer stem-like cells

CT	computer-assisted tomography
DC	dendritic cell
DDR	DNA damage response
dGTP	deoxy-guanosine-triphosphate
DOTA	2-(p-nitrobenzyl)-1, 4, 7, 10-tetraazylododecane-N, N′, N′′, N′′′-tetraacitic acid
DSB	double-stranded break
EGF	epidermal growth factor
EGFR	EGF receptor
EGFR*vIII*	EGFR variant III
EIAED	enzyme-inducing AED
ELISA	enzyme-linked immunosorbant assay
ENU	ethylnitrosourea
EORTC	European Organisation for Research and Treatment of Cancer
EXO1	exonuclease-1
FasL	Fas ligand
FBS	foetal bovine serum
FC	fluorocytosine
FGF	fibroblast growth factor
FGFR	FGF receptor
fMRI	functional MRI
FU	fluorouracil
GBM	glioblastoma multiforme
GCV	Ganciclovir
GCVTP	GCV-triphosphate
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte macrophage colony-stimulating factor
Gy	Gray
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HGF/SF	hepatocyte growth factor/ scatter factor
HGFR/MET	HGF receptor
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HSPG	heparan sulphate proteoglycans
HSV	*Herpes simplex* virus
HSV-tk	HSV thymidine kinase
HNPCC	hereditary non-polyposis colorectal carcinoma
HR	homologous recombination
IARC	International Agency for Research on Cancer
ICP	intracranial pressure
IDH	isocitrate dehydrogenase
IDL	insertion/deletion loop
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFR	IGF receptor
IHC	immunohistochemistry
IL	interleukin
i.p.	intra-peritoneal

i.v.	intravenous
LDLR	low-density lipoprotein receptor
LOH	loss of heterozygosity
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MCP	mercaptopurine
MDR	multi-drug resistant
MG	malignant glioma
MGMT	methyl guanine methyl transferase
MHC	major histocompatibility complex
miR	micro RNA
MLH	MutL homologous
MMP	matrix metalloproteinase
MMR	mismatch repair
MNU	methylnitrosourea
MOI	multiplicity of infection
MSH	MutS homologous
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MS	median survival
MSI	microsatellite instability
MSP	methylation-specific polymerase chain reaction
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NCIC	National Cancer Institute of Canada
NER	nucleotide-excision repair
NF	neurofibromatosis
NF-κB	nuclear factor of κ-light polypeptide gene enhancer in B-cells
NFKBIA	NF- κB inhibitor-α
NHEJ	non-homologous end joining
NK	natural killer
NO	nitric oxide
NRP	neuropilin receptor
NSC	neural stem cell
N^3-mA	N^3-methyl adenine
N^7-mG	N^7-methyl guanine
OA	oligoastrocytoma
ODG	oligodendroglioma
O^6-mG	O^6-methyl guanine
OS	overall survival
PARP	poly-ADP ribose polymerase
PCV	procarbazine, CCNU/lomustine & vincristine
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PET	positron emission tomography

PFS	progression-free survival
p.i.	post-tumour implantation
PI3K	phosphatidylinositol 3-kinase
PlGF	placental growth factor
PKC	protein kinase C
PMS	post-mitotic segregation
PNP	purine nucleoside phosphorylase
PTEN	phosphatase and tensin homolog
RB	retinoblastoma
RNAi	RNA interference
RPA	replication protein A
RT	radiotherapy
RTK	receptor tyrosine kinase
RTKi	RTK inhibitor
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficiency
SDF	stromal-derived factor
SEM	standard error of the mean
SFM	serum-free medium
siRNA	small-interfering RNA
SPECT	single-photon emission computed tomography
SSB	single-strand break
STAT	signal transducer and activator of transcription
SV	simian virus
sVEGFR-1	soluble vascular endothelial growth factor receptor-1
T	thymine
TAM	tumour-associated macrophage
TERT	telomerase reverse transcriptase
TG	thioguanine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TP 53	tumour protein 53
TMZ	Temozolomide
uPA	urokinase plasminogen activator
uPAR	uPA receptor
US-FDA	United States Federal Drug Administration
VEGF	vascular endothelia growth factor receptor
VEGFR	VEGF receptor
VPA	valproic acid
VPC	vector producing cell
vp	viral particles
WHO	World Health Organisation

1 Introduction

Every year over 12 million new patients are diagnosed with cancer and over 750 000 succumb to this disease. It is said to be the cause of death in one in every three adults in the developed nations. Malignant glioma (MG) is a devastating brain cancer with a dismal prognosis. Limitations and inefficiencies of the standard care, which at present consists of surgery, radiotherapy (RT) and/or chemotherapy, are the main reasons for the poor clinical outcome. Tumour heterogeneity is the norm and this represents a formidable obstacle for therapeutic success. Temozolomide (TMZ) is the latest chemotherapy to be approved for the treatment of MG along with anti-angiogenic therapy bevacizumab (BV). Largely unmet clinical needs of MG patients have inspired the search for better therapies. Gene therapy is the delivery of genetic material into the body with a therapeutic intent, and is one of the most widely explored novel therapies for cancer including MG. *Herpes simplex* virus type-1 thymidine kinase (HSV-tk) suicide gene therapy with pro-drug ganciclovir (GCV) is being evaluated for MG and several other cancers in clinical trials, while soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) is a promising new anti-angiogenic gene therapy strategy. Valproic acid (VPA) is a commonly used anti-epileptic drug (AED) to control seizures in MG patients but it seems to possess diverse anti-cancer properties that could modulate the effects of chemotherapy as well as gene therapy. Combining therapies acting through different modes-of-action is an attractive way to overcome the resistance due to tumour heterogeneity and to reduce the dose-limiting toxicities.

The aim of this thesis work was to evaluate the feasibility of combining different treatment options available for MG in order to maximise the synergistic effects while maintaining or even reducing the adverse effect profiles, and to understand the possible mechanisms of synergisms. This work explores the possibility of combining adenovirus-mediated HSV-tk/GCV (AdHSV-tk/GCV) suicide gene therapy with adenovirus-mediated sVEGFR-1 (AdsVEGFR-1) anti-angiogenic gene therapy in the treatment of MG, describing the possible anti-tumour mechanisms. It identifies a novel mechanism of synergism between AdHSV-tk/GCV gene therapy and TMZ that enhances the therapeutic index of the latter, and delineates an optimal treatment protocol to maximise the synergistic effect with potential clinical applications in many cancers in combination with multiple chemotherapeutic agents. Addition of VPA to the AdHSV-tk/GCV and TMZ combination, even though apparently beneficial, failed to further improve the synergistic effect, warranting further in-depth investigations. Finally, the thesis work demonstrates the *in-vivo* efficacy of lentivirus-mediated low-density lipoprotein receptor (LDLR)-avidin fusion-protein gene therapy as a universal targeted therapy strategy to deliver targeted RT or chemotherapy into cancers including MGs; a strategy that could have widespread applicability, especially in inoperable MGs.

The results confirm the feasibility of combining AdHSV-tk/GCV suicide gene therapy in the adjuvant setting with either chemotherapy (TMZ) or with anti-angiogenic therapy (AdsVEGFR-1).

2 Literature review

2.1 CANCER

A tumour can be defined as an abnormal, uncontrolled, purposeless proliferation of cells. Benign tumours are generally confined to the tissue of origin with their growth limited to local expansion, and are usually treated by surgical excision. Malignant tumours or cancers on the other hand, are capable of invading and destroying the adjacent tissues, and many of them will eventually metastasise to organs distant from their site of origin via the vascular or lymphatic system [Yokota, 2000]. More than 90 % of cancer deaths are due to this metastatic phenomenon that often renders simple surgical resection ineffective as a therapeutic option [Fidler, 2003] [Hanahan and Weinberg, 2011]. A cancer in the site of origin is referred to as a primary tumour and a lesion in a distant organ is termed a secondary/metastatic tumour.

2.1.1 Global cancer burden

Cancer is a major global health problem. Annually more than 12 million new patients are diagnosed with cancer, and it accounts for over 750, 000 deaths throughout the world [GLOBOCAN 2008 International Agency for Research on Cancer (IARC) http://globocan.iarc.fr/factsheet.asp#BOTH]. According to statistics from United States, malignant neoplasms were ranked second after diseases of the heart, as the leading causes of death in 2009 [Kochaneck, 2011]. Moreover, the National Cancer Institute of USA has estimated the cost of cancer care in US in 2010 exceeded $ 124 billion [Mariotto et al., 2011].

2.1.2 Pathophysiology

Cancer is a complex, multifactorial disease involving dynamic changes in the genome [Hanahan and Weinberg, 2000], orchestrated by host and environmental interactions [Lichtenstein et al., 2000]. Alterations in the genome can be changes in DNA sequence and copy number, chromosomal rearrangements and modifications and epigenetic changes, together driving the process of initiation and progression of cancer. The hallmarks of cancer are self-sufficiency in growth signals, insensitivity to anti-growth signals, ability for tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis [Hanahan and Weinberg, 2000]. These properties render cancer cells with acquired functional capabilities during the course of multistep tumorigenesis [Vogelstein and Kinzler, 2004]. In a sequel, the authors highlighted the importance of deregulating cellular energy metabolism and avoidance of immune detection and destruction as emerging hallmarks [Hanahan and Weinberg, 2011]. Genomic instability and tumour-promoting inflammation have been identified as the major enabling characteristics that foster these hallmarks. Furthermore, the tumour microenvironment, which is composed of various non-malignant cells, their cellular products and the extracellular matrix, plays a pivotal role in the initiation and progression of cancers [Tlsty and Hein, 2001] [Hanahan and Weinberg, 2011].

2.2 MALIGNANT GLIOMA AS A PRIMARY BRAIN TUMOUR

The brain is important from an oncological point-of-view because many malignancies elsewhere in the body, such as lung, breast and colo-rectum, can metastasise to the brain resulting in secondary brain tumours, while many different cell types in the brain can

acquire a malignant phenotype, giving rise to primary brain tumours. Primary brain tumours are less frequent compared to metastatic brain tumours [Eichler and Loeffler, 2007]. Apart from their general dismal prognosis, out of the 17 cancers studied brain tumours have the highest average years of life lost i.e. more than 20 years, surpassing most of the common cancers, highlighting the heavy burden of brain tumours to the individual patient. Furthermore, the ratio of percentage years of life lost over percentage mortality from a particular cancer is also the highest in brain tumours, highlighting the higher population burden of the disease that exceeds the simple mortality [Burnet et al., 2005].

2.2.1 Classification and grading

Conventionally brain tumours are classified based on histopathological/ morphological features and their resemblance to the presumed cell type of origin in the embryo/foetus or adult. Figure 1 summarises the classification of brain tumours [Takei et al., 2007a].

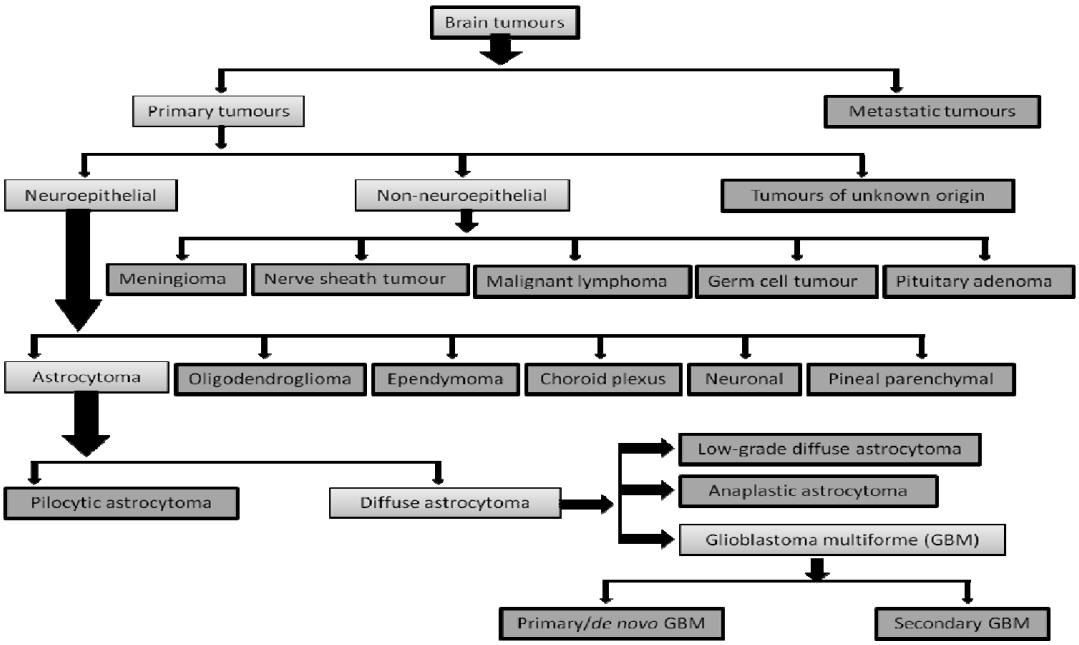

Figure 1: Classification of brain tumours

Approximately 60-70 % of all primary intracranial tumours are of neuroepithelial origin [Stewart and Kleihues, 2003] [Ohgaki and Kleihues, 2005a]. Glial cells comprising of astrocytes, oligodendrocytes and microglia are the most abundant cell in the nervous system. Neuroepithelial tumours of glial origin are collectively termed gliomas and these are the most common primary brain tumours in adults [Lonn et al., 2004]. Gliomas are further divided based on cytological and histological morphology, and immunohistochemical evidence of differentiation along the glial cell lineage [Nakamura et al., 2007]. When a glioblastoma multiforme (GBM) is diagnosed at the first biopsy without any clinical or histopathological evidence of a lesser malignant precursor, it is termed a *de novo* (primary) GBM. Secondary GBMs have a preceding histopathological, radiological or clinical evidence of a lower grade precursor [Ohgaki and Kleihues, 2005b]. The obstacles encountered in this classification system are that cell of origin is unknown in many brain tumours and that some tumour cells are so atypical making it impossible to compare them with any normal cell type [Collins, 2004].

Diagnostic classification based on histogenetics for brain tumours was originally proposed by Baily and Cushing [Collins, 2004]. Periodic refining of this morphological classification is reflected in the several editions of the World Health Organization's (WHO)

classification of central nervous system (CNS) tumours [Zulch, 1979] [Kleihues et al., 1993] [Kleihues and Cavenee, 1997] [Kleihues and Cavenee, 2000]. The aim of the WHO classification is to clearly define universal histopathological and clinical diagnostic criteria. The fourth edition of this series; i.e. the WHO classification of the tumours of the CNS, was published in 2007 [Louis et al., 2007a], and it described several new entities, histological variants, an updated WHO tumour grading scheme and genetic profiles [Louis et al., 2007b]. Nonetheless, these classifications still contain more than 100 clinico-pathological entities with a significant variation in biological behaviour, response to treatment and clinical outcome.

The objective of histological grading is to predict the biological behaviour and outcome of the tumour, and it is a key factor in determining adjuvant therapy [Louis et al., 2007b]. In the WHO tumour grading system, gliomas are graded into four grades of malignancy from grade I to IV based on morphological characteristics such as nuclear atypia, mitotic figures, microvascular proliferation and focal pseudopalisading necrosis [Nakamura et al., 2007], with increasing biological aggressiveness with increasing tumour grade [Collins, 2004] [Kleihues and Cavenee, 2000] (Table 1).

Table 1: WHO grading of primary brain tumours

WHO grading of primary brain tumours				
Grade	I	II	III	IV
Astrocytoma	Low-grade Pilocytic astrocytoma	Low-grade Low-grade diffuse astrocytoma	High-grade Anaplastic astrocytoma	High-grade GBM, Giant cell GBM, Gliosarcoma
Oligodendroglioma		Low-grade oligodendroglioma	High-grade/anaplastic oligodendroglioma	
Oligoastrocytoma		Low-grade oligoastrocytoma	High-grade/anaplastic oligoastrocytoma	
Malignant glioma	-	-	+	+
Anaplastic glioma	-	-	+	-

GBM-glioblastoma multiforme, WHO-World Health Organisation

Apart from the WHO grading, Kernohan and St-Ann/Mayo grading systems are used in some institutions. The term "malignant glioma" (MG) is usually reserved to describe WHO grade III and IV gliomas for which there are both histological evidence and biological behaviour of malignancy such as anaplasia, invasiveness and tumour recurrence; this group includes GBMs, anaplastic astrocytomas (AAs), anaplastic oligodendrogliomas (ODGs) and oligoastrocytomas (OAs), and anaplastic ependymomas [Louis et al., 2007b]. The term "anaplastic glioma" collectively describes WHO grade III gliomas [Wick and Weller, 2009]. The emphasis from here onwards will be on MG and GBM with only relevant references to important aspects about the lower grade gliomas.

The differentiation between grade II and III astrocytomas, classification of oligodendroglial tumours with necrosis and the diagnosis of mixed gliomas such as OAs, are areas not clearly defined in the current WHO classification leading to subjectivity in diagnostic neuropathology [Wick and Weller, 2009]. Overall the poor reproducibility of diagnostic criteria based on current classification and grading systems is reflected in the high discrepancy rate among different diagnostic reviews [Wick and Weller, 2009].

2.2.2 Epidemiology

According to GLOBOCAN 2008 (IARC), ~240,000 new cases of primary tumours of the brain and nervous system were diagnosed worldwide during that year (accessed 19/01/2012). This is less than 2 % of all newly diagnosed malignancies (excluding non-melanoma skin cancer) during that period. However, mortality due to brain and nervous system tumours during the same year was ~ 175,000, which is about 2.3 % of all cancer related mortality. A similar pattern of relatively higher mortality compared to the number of new cases is seen in many cancer registry databases for primary brain tumours,

emphasizing the aggressiveness of the disease. There is a slightly higher male preponderance with > 125, 000 new cases, as opposed to >110,000 new cases in women. The annual mortality was ~100,000 and ~75,000 for men and women, respectively (GLOBOCAN 2008 IARC).

Primary brain tumours show less geographic or population to population variation in incidence than other human neoplasms [Ferlay et al., 2001]. The higher age adjusted incidence observed in developed and industrialized countries [Parkin et al., 2002] and in Caucasians compared to African or Asian populations is attributed to socio-economic differences and underreporting rather than true genetic susceptibility [Ohgaki and Kleihues, 2005a]. Primary brain tumours have an overall incidence of 6-8 new cases per 100,000 population per year [Stewart and Kleihues, 2003]. Higher incidence rates for malignant brain tumours are found among men [Ohgaki and Kleihues, 2005b], while benign meningiomas are predominantly seen among women [Stewart and Kleihues, 2003]. The incidence for primary brain tumours in men varies between 6-11 new cases per 100,000 population per year in Western Europe, North America and Australia, and for females this figure varies between 4-11 new cases [Ferlay et al., 2001] [Parkin et al., 2002]. The overall incidence rate for glioma is in the range of 4-6 new cases per 100,000 population per year [Nano and Ceroni, 2005] [Larjavaara et al., 2007], whereas for MG (WHO grade III & IV) it is about 3 cases per 100,000 population per year [DeAngelis, 2001] (www.CBTRUS.org). A population based survey from Switzerland revealed an incidence of ~3.5 cases per 100,000 population per year for GBM [Ohgaki and Kleihues, 2005b].

The mortality from nervous system tumours varies from 4-7 deaths per 100,000 population per year in men and 3-5 for women in Western Europe, North America and Australia [Ferlay et al., 2001] [Parkin et al., 2002] and shows a similar worldwide pattern to the incidence, in both sexes and in most geographical areas [Ohgaki and Kleihues, 2005a]. Differences in the success of disease management are mostly attributed to the regional changes in the incidence to mortality ratios [Ferlay et al., 2001].

Brain tumours demonstrate a bimodal age distribution with a peak incidence in children aged 5-9 years [Salcman, 1980] and a second larger peak in adults aged 45-70 [Lantos et al., 2002]. The most frequent primary brain tumour types in adults are diffuse astrocytic tumours and meningiomas, whereas in children they are pilocytic astrocytomas, ependymomas and medulloblastomas [Collins, 2004]. Gliomas are the most common primary brain tumours accounting for more than 50 % in many series with an increase in the incidence with age [Lonn et al., 2004] but in some studies, the incidence is as high as 70 % [Ohgaki and Kleihues, 2005a]. According to some population based studies, GBMs accounted for ~70% of all gliomas [Ohgaki and Kleihues, 2005a] [Ohgaki and Kleihues, 2005b], while in others it was about 51 % [Larjavaara et al., 2007]. In general, GBMs have a male preponderance [Collins, 2004]. *De novo* and secondary GBMs account for 95 % and 5 % of GBMs, respectively [Ohgaki et al., 2004]. In some surveys, secondary GBMs have been as high as 20-25 % [Ohgaki and Kleihues, 2005a] [Biernat et al., 1997]. Men tend to have more *de novo* GBM, while in women secondary GBM is more frequent. *De novo* GBMs occur in the elderly (mean age at diagnosis 62 years), while for secondary GBMs, the mean age is 45 years [Ohgaki and Kleihues, 2005b]. The incidence of GBMs in the elderly has been increasing at a rate of 1-2 % per year over the last decades though some of this can be attributed to improvements in diagnostic facilities, especially neuro-imaging [Modan et al., 1992]. The mean age at clinical manifestation increases with increasing glioma grade with 72% of GBMs manifesting after 45 years of age, as opposed to 74% of pilocytic astrocytomas, which occur before the age of 20 [Stewart and Kleihues, 2003].

2.2.3 Aetiology

The aetiology of primary brain cancers remains largely unknown. Except for the rare brain tumours associated with familial cancer syndromes and those attributed to therapeutic irradiation, no causative environmental or lifestyle factors have been unequivocally

identified [Stewart and Kleihues, 2003]. Less than 5 % of gliomas have a hereditary risk [Filippini, 2012].

Several familial cancer syndromes are associated with CNS tumours. Tumour protein 53 (TP53) germline mutation; Li Fraumeni syndrome is associated with an increased incidence of astrocytomas, GBMs and medulloblastomas [Ohgaki and Kleihues, 2005a] [Li et al., 1995]. Neurofibromatosis-1 (NF1) is associated with an increase in optic nerve gliomas (pilocytic astrocytomas), diffuse astrocytomas and GBMs [Listernick et al., 1999], whereas NF2 is associated with meningiomas, ependymomas, astrocytomas and gliomas in medulla [Ohgaki and Kleihues, 2005a] [Collins, 2004]. Turcot syndrome type1 (B) with germline mutations in DNA mismatch repair (MMR) genes post-mitotic segregation-2 (PMS2), MutS homologous-2 (MSH2) and MutL homologous-1 (MLH1) presents with GBMs and hereditary non-polyposis colorectal carcinoma (HNPCC) [Hamilton et al., 1995], whereas the type 2 (A) with germline mutation of adenomatous polyposis coli (APC) gene is associated with medulloblastoma and familial adenomatous polyposis [Cavenee et al., 2000] [Hamilton et al., 1995]. Several other syndromes such as Basal cell nevus (Gorlin's), Cowden disease, Melanoma-astrocytoma [Collins, 2004] and Rhabdoid tumour predisposition are associated with brain cancer [Louis et al., 2007b].

Therapeutic irradiation is the only environmental factor unequivocally associated with brain cancer [Ohgaki and Kleihues, 2005a], i.e. both high-dose and low-dose irradiation have increased the risk for meningiomas [Ron et al., 1988]. Prophylactic brain irradiation for acute lymphoblastic leukaemia in children increased their risk for MG [Ohgaki and Kleihues, 2005a]. It is not clear whether diagnostic X-rays, irradiation due to atomic bombs and the Chernobyl accident have increased the brain cancer risk [Ohgaki and Kleihues, 2005a].

Though some epidemiological studies demonstrated an increased risk of brain cancer associated with certain occupations, these have not been confirmed by independent studies in other populations and none of them progressed to identify the putative carcinogen [Ohgaki and Kleihues, 2005a]. An increased risk of brain cancer has been associated with exposure to certain substances such as plastic, rubber products, vinyl chloride, arsenic, mercury, petroleum products, polycyclic aromatic hydrocarbons and lead [Ohgaki and Kleihues, 2005a]. The IARC Monograph on the Evaluation of Carcinogenic Risks in Humans (volume 1-88) reports a possible or weak association with nervous system tumours in humans with the exposure to beryllium, epichlorohydrin, chlordane/heptachlor, methylthiouracil thiouracil, propylthiouracil, lead, diisopropyl sulphate and dichloromethane [http://monographs.iarc.fr/monoeval/crthall.html]. A parental occupation in chemical industry (and exposure to polycyclic aromatic hydrocarbons) or agricultural work, has been claimed to increase the risk for brain cancer in the offspring [Ohgaki and Kleihues, 2005a].

A higher socio-economic status is associated with an increased risk of gliomas and meningiomas in women and many other studies have shown similar effect with "white-collar" jobs [Ohgaki and Kleihues, 2005a].

The role of diet as an aetiological agent for brain cancer is unclear but some reports suggest that a high intake of food containing *N*-nitroso compounds and protein can be associated with an increased glioma risk in adults, with an inverse association with a frequent intake of fresh fruits, vegetables, vitamin C, total fat, cholesterol, calcium, sodium [Ohgaki and Kleihues, 2005a] and total coffee and tea consumption [Michaud et al., 2010] [Holick et al., 2010], but available data in most studies were insufficient to establish any dose-response relationship [Blowers et al., 1997] [Boeing et al., 1993]. No causal link has been observed between smoking and adult gliomas but some studies implicated parental smoking in the risk of childhood gliomas [Ohgaki and Kleihues, 2005a]. On the other hand, some studies suggest that there is a causal link between alcohol consumption and risk of GBM [Baglietto et al., 2011].

Exposure to electromagnetic fields as a causative factor for glioma still remains controversial, with mixed results from different studies with insufficient evidence to draw a causal link [Ohgaki and Kleihues, 2005a] [Baldi et al., 2011]. In a similar manner, no consistent association has so far been found between the use of cellular phones and brain tumours, [Aydin et al., 2011] [Larjavaara et al., 2011] [Kheifets et al., 2010] [Deltour et al., 2012].

Epidemiological studies have shown a weak but inconsistent or non-significant association with traumatic head injury and brain cancer; however the causal relationship is difficult to prove [Ohgaki and Kleihues, 2005a]. A significant inverse association between glioma and allergic diseases such as asthma and eczema, and autoimmune diseases has been demonstrated [Ohgaki and Kleihues, 2005a]. There is evidence to suggest that female sex hormones, hormone replacement therapy and oral contraceptive use could be protective against glioma but may increase the risk of meningioma [Cowppli-Bony et al., 2011].

In countries where polio vaccines contaminated with simian virus 40 (SV40) were used, several studies have shown a higher occurrence of SV sequences in brain cancers when compared to surrounding normal tissue. However, there was no increase in the brain cancer incidence in the vaccinated populations [Ohgaki and Kleihues, 2005a]. Some studies suggest a possible link between human cytomegalovirus (CMV) infection and occurrence of MG [Straat et al., 2009].

There is evidence suggesting as well as disproving familial clustering of brain cancer [Malmer et al., 2001] [Scheurer et al., 2010]. The risk of glioma is thought to be increased with the polymorphism in the genes encoding enzymes involved in the metabolism of chemical carcinogens [Ohgaki and Kleihues, 2005a]. Genome-wide association studies have identified single-nucleotide polymorphisms in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *CDKN2B*, regulator of telomerase elongation helicase 1 (*RTEL 1*) and telomerase reverse transcriptase (*TERT*) loci with increased incidence of glioma [Huse and Holland, 2010] [Shete et al., 2009] [Wrensch et al., 2009] and many glioma-associated candidate genes [Yang et al., 2011].

2.2.4 Pathology

AAs often develop as progressions from low-grade diffuse astrocytomas. Mean age at diagnosis is 45-50 years [Ohgaki and Kleihues, 2005a]. Since they have a rapid growth, they often progress to GBMs within 2-3 years [Stewart and Kleihues, 2003]. Tumour cells are more pleomorphic with distinct nuclear atypia, mitotic activity and increased cellularity, and histological and immunohistochemical features of astrocytes. AAs do not show evidence of spontaneous tumour necrosis or abnormal microvascular proliferation [Collins, 2004]. The distinction between WHO grade II and III astrocytomas is primarily by assessment of tumour cell proliferation. However, WHO grading does not provide cut-off values to differentiate between the two groups. Proliferative markers such as phospho-histone H3 (PHH3) index ≤4 vs. >4 per 1000 cells, MIB-1 LI/Ki-67 ≤9 vs. >9 and mitoses per 10 high power fields ≤3 vs. >3 have been proposed as cut-offs between grade II vs. grade III, respectively [Colman et al., 2006] [Takei et al., 2007a].

The most malignant and most frequent CNS tumour in adults; GBM [Nakamura et al., 2007] [Ohgaki et al., 2004] is common in the elderly, with a mean age at diagnosis between 55-62 years [Ohgaki and Kleihues, 2005a]. In general, GBMs have cerebral hemisphere preponderance [Collins, 2004]. Usually there is a short clinical history of less than 3 months and GBM has a very poor prognosis despite progress in surgery and adjuvant therapy, largely due to incomplete resection and resistance to radio- and chemotherapy [Kleihues et al., 2002]. In general, high cellularity, increased mitosis, marked nuclear atypia and characteristic spontaneous tumour necrosis surrounded by pseudopalisading tumour cells, and florid endothelial proliferation [Stewart and Kleihues, 2003] [Kleihues and Cavenee, 2000] are hallmarks of GBM. However, they retain at least some phenotypic characteristics

of astrocytes such as expression of glial fibrillary acid protein (GFAP) [Kleihues et al., 1987] [Collins, 2004] [Takei et al., 2007a], which histologically places GBMs in astrocytic class. However, their precise origins remain elusive [Huse and Holland, 2010]. Imaging may reveal large areas of central necrosis with ring-like zones of contrast enhancement from viable tumour tissue [Collins, 2004]. As the name "multiforme" implies, GBMs have a wide spectrum of histological morphologies ranging from small-cell type to very pleomorphic giant-cell forms with poor differentiation [Collins, 2004] and gliosarcomas [Louis et al., 2007b]. Foci resembling ODG are seen in some GBMs [Louis et al., 2007b], especially in young patients, where necrosis is uncommon and has a favourable outcome [Vitucci et al., 2011]. The small cell subtype displays a monomorphic, small, round to slightly elongated, densely packed cell population with oval, mildly hyperchromatic nuclei containing occasional nucleoli [Perry et al., 2004], and increased nuclear: cytoplasmic ratio and mild atypia. Frequent mitoses, inconspicuous cytoplasmic borders, vascular endothelial proliferation and necrosis are other features. They have some resemblance to anaplastic ODG such as chicken-wire vasculature, clear haloes, perineuronal satellitosis and microcalcifications [Louis et al., 2007b]. Giant cell GBMs arise *de novo*, have a short clinical history and account for about 5 % of GBM, with a mean age at diagnosis of 42 years. Histologically there is giant cell predominance in conjunction with cohesion, distinct cell borders and reticulin-rich stroma with a strong GFAP reactivity [Louis et al., 2007a]. *De novo* and secondary GBM are morphologically indistinguishable [Godard et al., 2003] except for the relative rarity of necrosis in the latter type [Tohma et al., 1998].

The median age at diagnosis for ODG and OA is between 46-49 years [Ohgaki and Kleihues, 2005a]. Anaplastic ODGs display nuclear pleomorphism, hyperchromatism, hypercellularity, increased mitotic activity, microvascular proliferation, delicately branching chicken-wire vasculature, clear haloes, perineuronal satellitosis, microcalcification, microcysts and/or spontaneous necrosis, with uniform tumour cells containing round/oval nuclei and well-defined clear amphophilic cytoplasm [Brat et al., 2008] [Louis et al., 2007a]. Subependymal spread and cerebro-spinal fluid (CSF) seeding is possible with ODG [Collins, 2004]. Some of them harbour astrocytic features, hence they are termed OA. Some OA contain foci of necrosis [Louis et al., 2007b].

The anatomical location of MGs has a significant impact on the patient outcome. In adults, supratentorial tumours predominate [Inskip et al., 1995]. Most of the tumours (>85 %) are in cerebral lobes; in frontal (40 %), temporal (29 %), parietal (14 %) and occipital (3 %) in a decreasing order of frequency [Larjavaara et al., 2007]. GBMs follow a similar pattern [Simpson et al., 1993]. Deep cerebral structures (6.4 %), ventricles (2.2 %), cerebellum (1.5 %) and brainstem (4.1 %) accounted only a small percentage. There seems to be right hemisphere preponderance [Ali et al., 2003], with only a fraction of tumours in central structures [Larjavaara et al., 2007]. Less than 5 % of tumours are bilateral [Larjavaara et al., 2007], the majority of which were bifrontal [Inskip et al., 2003]. Precursor cell of tumour origin, differences in the microenvironment, structural and functional differences in different brain regions, and glial and neuronal interactions have been postulated for the observed spatial differences [Duffau and Capelle, 2004].

2.2.5 Molecular biology

The cardinal molecular-genetic events described in human MG/GBM are dysregulation of growth factor signalling by amplification and mutational activation of receptor tyrosine kinase (RTK) genes, activation of phosphatidylinositol 3-kinase (PI3K) pathway and inactivation of p53 and retinoblastoma (RB) tumour suppressor pathways [Ohgaki et al., 2004] (Figure 2).

RTK pathways commonly dysregulated in glioma are epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), ERBB2 (HER2/neu) and MET. It is suggested that at least one RTK pathway is altered in 88 % of GBMs [2008]. EGFR amplification or activating mutations are seen in ~45 % of GBM [2008] [Parsons et al., 2008]

especially in *de novo* GBM [Huse and Holland, 2010]. The EGFR variant III (EGFR*vIII*) mutation [Humphrey et al., 1990] is the commonest and is found in 20-30 % of *de novo* GBM. The majority (50-60 %) of these cases also exhibit EGFR amplification [Sugawa et al., 1990] [Huse and Holland, 2010]. Enhanced signalling through PDGFR-α is common in low-grade astrocytomas, ODG and some subtypes of GBM [Westermark et al., 1995]. Amplification of PDGFR-α is seen in 13 % of GBM, but activating mutations of the receptor are uncommon [Clarke and Dirks, 2003]. However, co-expression of receptor and ligand platelet-derived growth factor (PDGF)-B creates a possible autocrine/paracrine loop enhancing the oncogenic signaling [Huse and Holland, 2010]. Hepatocyte growth factor/ scatter factor (HGF/SF) and its receptor HGFR/MET seems to have a similar impact [Huse and Holland, 2010] with 4 % of GBMs bearing HGFR/MET amplification, whereas ERBB2 mutations were observed in 8 % of GBMs [2008]. Emerging evidence suggests that some GBMs are mosaic in RTK activation [Snuderl et al., 2011].

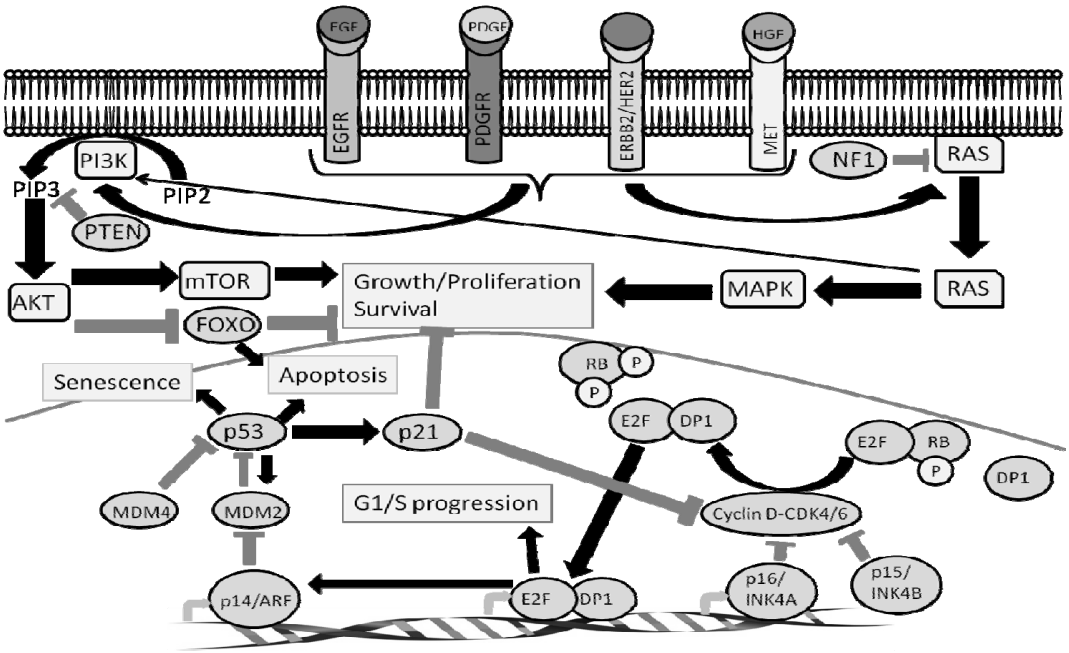

Figure 2: A schematic presentation of major molecular pathways dysregulated in malignant glioma. ↑ and ⊤ indicate activation and inhibition, respectively. ARF-alternate reading frame, CDK-cyclin dependent kinase, EGF-epidermal growth factor, EGFR-EGF receptor, ERBB-erythoroblastosis oncogene B, FOXO-forkhead box O, HER-human EGF receptor, HGF-hepatocyte growth factor, MAPK-mitogen-activated protein kinase, MDM-murine double minute, MET-HGF receptor, mTOR-mammalian target of rapamycin, NF-neurofibromatosis, PDGF-platelet-derived growth factor, PDGFR-PDGF receptor, PI3K-phosphatidylinositol 3-kinase, PTEN-phosphatase and tensin homolog, RAS-rat sarcoma, RB-retinoblastoma.

The effects of dysregulated RTK signalling in gliomas are mainly driven via intracellular signalling pathways, namely PI3K-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) and Ras- mitogen-activated protein kinase (MAPK). PI3K activating mutations are seen in 15 % of GBM, whereas its primary negative regulator phosphatase and tensin homolog (PTEN) is inactivated in 15-40 %, while AKT is amplified in only 2 % of GBM cases [2008] [Parsons et al., 2008] [Ohgaki and Kleihues, 2005b], leading to increased invasion, proliferation and cell survival. On the other hand, the negative regulator of Ras-MAPK pathway, *NF1* is inactivated by mutations or deletions in 15-18 % of sporadic GBM [2008] [Parsons et al., 2008], whereas mutational activation of Ras was seen in 2 % [2008]. Collectively, alterations in RTK, PI3K-AKT-mTOR and Ras-MAPK signallings contribute to proliferation and survival of MG cell.

TP53 tumour suppressor gene is frequently mutated in sporadic low-grade astrocytomas and secondary GBM [Louis, 1994]. The p53 pathway is altered in 64-87 % of GBMs, whereas 35 % had *TP53* inactivation by mutations or deletions [2008] [Parsons et al., 2008]. Furthermore, *TP53* inactivation for treated and untreated samples was 58 % and 37.5 %, respectively. In p53-intact GBMs, amplification of antagonist *MDM2* (14 %) and *MDM4* (7 %), and mutation or deletion of *CDKN2A* (~50 %) that encodes for p53 positive regulator p14/ARF was observed [2008] [Parsons et al., 2008]. These findings suggest that p53 inactivation is also a frequent event in *de novo* GBM.

RB tumour suppressor pathway is defective in a significant fraction of high-grade astrocytomas and ODGs and in 68-78 % of GBMs [2008] [Parsons et al., 2008]. They could be due to inactivation mutations or homozygous deletions of *RB1* gene itself (~11 %) [Parsons et al., 2008] or activation of negative regulators cyclin-dependent kinase 4 (*CDK4*) (18 %) and *CDK6* (1 %) or inactivations (9p21 deletion) of *CDKN2A* (52 %) [Parsons et al., 2008] that encodes for RB positive regulator $P16^{INK4A}$ and *CDKN2B* (47 %) [2008]. The RB pathway affects the cell-cycle controlling G1/S progression.

Mutations in isocitrate dehydrogenase 1 (IDH1) (the enzyme that converts isocitrate to α-ketoglutarate) and *IDH2* are seen in a high percentage of grade II and III astrocytomas and ODG (70-100 %) and secondary GBM (85 %), that are typical in young patients carrying a better prognosis, but is uncommon in *de novo* GBM (5 %) [Parsons et al., 2008] [Yan et al., 2009] [Hartmann et al., 2009]. They can be associated with TP53 mutations and 1p/19q deletions but not with EGFR amplification or loss of chromosome 10 [Yan et al., 2009] [Sanson et al., 2009]. The possible mechanisms of action are induction of hypoxia-inducible factor-1α (HIF-1α) [Zhao et al., 2009], by changing the DNA methylation profile and causing widespread epigenetic changes [Dang et al., 2009] [Ward et al., 2010] [Noushmehr et al., 2010].

Common molecular-genetic dysregulations and their frequencies of occurrence in gliomas of different WHO grades are presented in Figure 3.

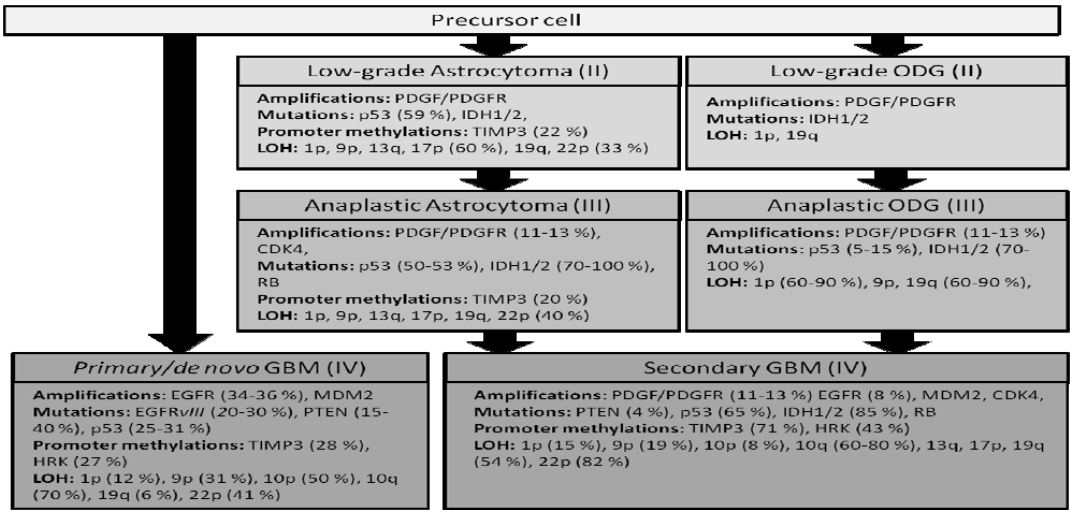

Figure 3: Frequent molecular-genetic dysregulations in gliomas. Frequencies of occurrence of the dysregulations and the WHO grades of the tumours are given in brackets. CDK-cyclin dependent kinase, EGFR-epidermal growth factor receptor, GBM-glioblastoma multiforme, HRK-hara-kiri, IDH-isocitrate dehydrogenase, LOH-loss of heterozygosity, MDM-murine double minute, ODG-oligodendroglioma, PDGF- platelet-derived growth factor, PDGFR-PDGF receptor, PTEN-phosphatase and tensin homolog, TIMP-tissue inhibitor of metalloproteinase, RB-retinoblastoma.

Aberrant constitutive activation of nuclear factor of κ-light polypeptide gene enhancer in B-cells (NF-κB) by activated EGFR [Habib et al., 2001] is reported in GBM [Nagai et al.,

2002] and implicated in migration and invasion [Raychaudhuri et al., 2007] [Tsunoda et al., 2005], and resistance to therapy [Bredel et al., 2006]. A repressor of NF-κB, NF-κB inhibitor-α (NFKBIA) is deleted especially in non-classical GBM (mesenchymal, neural and proneural) with a near mutual exclusivity with EGFR activation, resulting in a poor prognosis similar to EGFR activation [Bredel et al., 2011].

Recent studies have implicated several micro RNAs (miR) in glioma development, including overexpression of miR-26a, which represses PTEN, RB and MAP3K2, which is seen in 12 % of GBM [Huse et al., 2009] [Kim et al., 2010], miR-21, miR-7, miR-124a, miR-137, miR-221, miR-222 and miR-181 family [Novakova et al., 2009]. On the other hand, miR-34a, which is induced by p53, suppresses glioma growth by targeting MET, CDK6 and Notch receptors 1 & 2 [Li et al., 2009].

Molecular classification of MG

Numerous genome-wide expression profiling studies have shed light into the molecular classification and established the molecular heterogeneity of MG [Vitucci et al., 2011]. They established distinct molecular profiles between defined morphological subgroups (Table 2). Several studies identified prognostically distinct molecular subgroups, within defined morphological groups and among these groups, especially when the morphological diagnosis was ambiguous. In most instances, molecular grouping displayed a better correlation with outcome. These studies grouped gliomas/GBM into between 2-4 molecular subgroups. In spite of not having a standard nomenclature, subgroups from different studies displayed significant molecular overlap, similar molecular signatures and prognostic outcome [Vitucci et al., 2011]. However, most gliomas on recurrence shifted the mRNA profile towards a mesenchymal phenotype, in spite of retaining the original molecular subgrouping [Huse and Holland, 2010].

Table 2: Molecular classification of malignant gliomas

Features	Proneural (HC1A)	Classical	Proliferative (HC2A)	Mesenchymal (HC2B)
WHO grade	III or IV, majority non-GBM	IV	IV	IV
Mean age at diagnosis	40-51 years		50-55 years	50-55 years
Median survival	175 weeks/ 1.4-4.8 years		61 weeks/ 0.6-<1.3 years	65 weeks/ 0.6-<1.3 years
Tumour gene expression pattern	Neurogenesis		Haematopoietic/ neural stem cells/ cell proliferation	Extra-cellular matrix/ invasion, angiogenesis
Molecular-genetic features	Amplifications: PDGFR Mutations: p53, IDH1/2, PDGFR PIK3CA/PIK3R1, Activations: Notch signalling G-CIMP (29 %) EGFR normal PTEN intact	Amplifications: EGFR Mutations: EGFR*vIII,* No p53 mutations LOH: 9p (CDKN2A)	Amplification: EGFR (+/-) Mutations: PTEN LOH: 10q or 10 Gain: chromosome 7 Activations: AKT	Amplifications: EGFR (+/-) Mutations: NF1, p53, PTEN LOH: 10 Gains: chromosome 7 Deletions: NF1 Activations: STAT3, C/EBPβ, AKT
Histopathological features	Most differentiated, astrocytic or oligodendroglial, GBM with ODG features but without necrosis, High OLIG2, DLL3, BCAN	Small-cell GBM,	Astrocytic, necrosis is typical, High PCNA, Ki-67 & topoisomerase 2α,	Astrocytic, gliosarcomas, highly angiogenic, pseudopalisading necrosis, microvessel proliferation, necrosis only in 8 %, High CD44, VEGF, CHI3L1
Response to therapy	Respond well to chemo-radiation	Respond to chemo-radiation		Respond to chemo-radiation

References for the table are [Phillips et al., 2006] [Lee et al., 2008] [Vitucci et al., 2011] [Carro et al., 2010] [Freije et al., 2004] [Verhaak et al., 2010]. BCAN-Brevican core protein, CD-cluster of

differentiation, CDKN2A- cyclin-dependent kinase inhibitor 2A, C/EBPβ-CCAAT/enhancer binding protein-β, CHI3L1-chitinase 3-like protein 1, DLL-delta-like, EGFR-epidermal growth factor receptor, GBM-glioblastoma multiforme, G-CIMP-GBM CpG island methylator phenotype, IDH-isocitrate dehydrogenase, LOH-loss of heterozygosity, NF-neurofibromatosis, ODG-oligodendroglioma, OLIG-oligodendrocyte transcription factor, PCNA-proliferating cell nuclear antigen, PDGFR-platelet-derived growth factor receptor, PIK3CA/PI3KR1-phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit α/ PI3K regulatory subunit 1, PTEN-phosphatase and tensin homolog, STAT- signal transducer and activator of transcription, VEGF-vascular endothelial growth factor, WHO-World Health Organization

The molecular characterisation of MGs helps to provide a more precise subgrouping of tumours within an individual morphological category by accurately defining specific molecular, genetic and cellular features of gliomagenesis. This paves the way for successful augmentation of standard treatment regimens and to rationally design novel targeted therapies [Huse and Holland, 2010] enabling better prediction of clinical outcomes and response to therapy. Eventually this will provide the groundwork for personalised cancer therapy [Vitucci et al., 2011]. On the other hand, the molecular and cellular heterogeneity inherent in these tumours currently being unravelled, only highlights the formidable therapeutic challenges still remaining to be solved [Huse and Holland, 2010] [Vitucci et al., 2011].

2.2.6 Angiogenesis

During their progression from microscopic to macroscopic tumours with densely packed cells, cancers tend to outgrow their vascular supply. The resulting hypoxic environment restricts growth further, making angiogenesis an integral part in tumour development. For the tumour growth to continue, it is vital that it must develop new blood supplies to meet its metabolic demands [Singh and Agarwal, 2003]. It is unlikely that a tumour can grow beyond 2-3 mm^3 in size without angiogenesis [Folkman and Shing, 1992].

Vascularisation mechanisms in MG

MGs are highly vascular tumours [Furnari et al., 2007] with profound neovascularisation that employ multiple mechanisms of tumour vascularisation. Initial growth of brain tumours is aided by the growth of tumour cells along pre-existing blood vessels, which is termed vessel-co-option [Holash et al., 1999]. This represents the ability to initiate tumour growth independent of angiogenesis in experimental models, mediated by cancer stem-like cells (CSLC) [Sakariassen et al., 2006]. However, angiogenesis is the most important of these mechanisms. In sprouting angiogenesis, new blood vessels are formed from pre-existing vessels [Carmeliet and Jain, 2000] [Carmeliet, 2005]. Glomeruloid angiogenesis, which is especially seen in GBMs, occurs when tumour cells obtain a vascular supply from several closely associated microvessels surrounded by basement membrane of variable thickness within which are embedded a limited number of pericytes (Glomeruloid bodies) [Brat and Van Meir, 2001]. *De novo* formation of new blood vessels from bone marrow-derived endothelial progenitor cells (BDEPC) [Santarelli et al., 2006] or haematopoietic stem/progenitor cells (BDHSC/BDHPC) [De et al., 2005] incorporated into the tumour vessels from the circulation is called vasculogenesis, which is important during the early stages of tumour development [Nolan et al., 2007]. Its contribution to tumour neovascularisation is variable (1.5-58 %) [De et al., 2003] [Santarelli et al., 2006], and has even been questioned [Purhonen et al., 2008]. New blood vessel generation by septal formation inside the existing blood vessels is termed intussusception, which has been described in brain metastatic models. However, its contribution to primary brain tumour development is unclear [Jain et al., 2007]. Vasculogenic mimicry describes vascular channels that are devoid of endothelial cell lining, composed of tumour cells that lack endothelial cell markings, and the basement membrane [El et al., 2010] but their relevance is still unclear [McDonald et al., 2000]. Animal experiments have highlighted the possibility of

forming endothelial cells by tumour cell transdiferentiation [Soda et al., 2011] and recent studies have demonstrated the possibility of the creation of a tumour endothelium by GBM stem-like cells [Ricci-Vitiani et al., 2010] [Wang et al., 2010a].

Mediators of angiogenesis

Angiogenesis is a complex process, tightly regulated by the coordinated expression of a variety of stimulating and inhibiting mediators converging to trigger the "angiogenic switch" [Hanahan and Folkman, 1996]. The main angiogenic mediator, vascular endothelial growth factor (VEGF), is highly expressed in MGs by the tumour [Plate et al., 1992] and stromal cells [Fukumura et al., 1998], and infiltrating immune cells [Murdoch et al., 2008]. The VEGF family includes 6 secreted glycoproteins, namely VEGF-A (VEGF), -B, -C, -D, -E and placental growth factor (PlGF) (Figure 4). The main angiogenic mediator, VEGF-A (VEGF), has 4 main isoforms VEGF121, VEGF165, VEGF189 and VEGF206 of which VEGF165 is the predominant isoform expressed in tumours [Ellis and Hicklin, 2008] [Ferrara, 2004]. VEGF secretion is regulated by hypoxia through HIF-1 [Shweiki et al., 1992], acidosis [Fukumura et al., 2001], transcription factors (HIF-1, ETS-1, STAT-3), genetic alterations (EGFR activation, PTEN mutation), oncogenes (Ras, SRC), tumour suppressor genes (TP53), growth factors (epidermal growth factor (EGF), PDGF-B, basic fibroblast growth factor (bFGF)), hormones, cytokines (transforming growth factor-β (TGF-β)), and signalling molecules (nitric oxide (NO)) and MAPK [Jain et al., 2007] (Figure 4). VEGFs exert their effects through cognate VEGFR receptors (RTKs) VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4) aided by two co-receptors, neuropilin receptor-1 (NRP-1) and NRP-2 [Fischer et al., 2005a]. The angiogenic effects of VEGFs are exerted primarily through VEGFR-2 (KDR) [Stratmann et al., 1997] expressed mostly on endothelial cells [Ellis and Hicklin, 2008], by inducing their survival, proliferation and migration [Carmeliet and Jain, 2000] [Ferrara, 2004]. MGs over-express VEGFR-1 (Flt-1) and VGFR-2 (Flk-1) [Plate et al., 1994], which along with VEGF expression correlates with the tumour grade [Samoto et al., 1995] and may collectively create an autocrine/paracrine growth signalling loop [Kerbel, 2008]. VEGFs play a role in vasculogenesis by enhancing the recruitment of VEGFR-1 and/or VEGFR-2 expressing BDHPCs [Murdoch et al., 2008] and VEGFR-2 positive BDEPCs [Ellis and Hicklin, 2008]. VEGFs can inhibit the anti-tumour immune response [Ohm and Carbone, 2001] by impairing the differentiation of VEGFR-1 expressing progenitor cells into dendritic cells (DCs) [Gabrilovich et al., 1996], by affecting T-cell development [Ohm et al., 2003], by recruiting myeloid-derived suppressor cells (MDSC) that suppress anti-tumour T-cell and natural killer (NK) cells, by recruiting tumour promoting, VEGFR-1 or -2 expressing monocytes/macrophages [Kerber et al., 2008] [Barleon et al., 1996] [Dineen et al., 2008] and by inducing the pro-tumorigenic M-2 polarisation of tumour-associated macrophages (TAMs) [Murdoch et al., 2008] (Figure 4).

MGs frequently over-express other mediators of angiogenesis such as PlGF, NRP-1, FGF, plasminogen activation factor 1 (PAI-1), NO, cyclooxygenase 2 (COX-2), thrombospondin 2 (TSP-2), stem cell factor (SCF), PDGF and PDGFR, interleukin-8 (IL-8), IL-6, stromal-derived factor 1 (SDF1) and its receptor CXCR4, EGF, insulin-like growth factor 1 and 2 (IGF1 & 2), HGF, tumour necrosis factor α & β (TNF-α/β), interferons (IFN), cathepsin B, urokinase plasminogen activator receptor (uPAR), angiostatin, endostatin, soluble VEGFR-1 and platelet factor 4 (PF4), and bFGF, granulocyte macrophage-colony stimulating factor (GM-CSF), IGF1, SDF1 and angiopoietins that mediate vasculogenesis [Jain et al., 2007] [Fischer et al., 2005a] [Furnari et al., 2007]. Cancer stem-like cells also contribute to tumour angiogenesis by secreting pro-angiogenic factors [Bao et al., 2006b] [Salmaggi et al., 2006]. MGs also produce a variety of anti-angiogenic molecules; however the balance is in favour of angiogenesis [Lamszus et al., 2003].

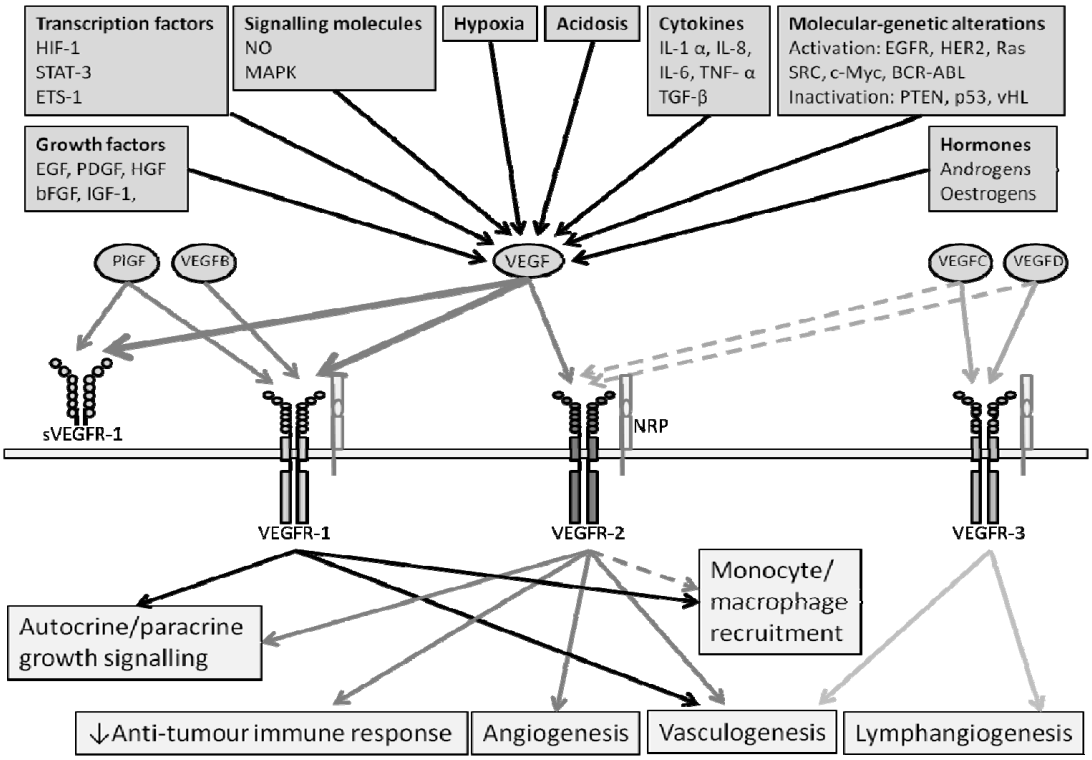

Figure 4: A schematic presentation of the regulation of VEGF production, VEGFs and VEGFR family interaction, and their contributions to cancer development. BCR-ABL-breakpoint cluster region-Abelson, bFGF-basic fibroblast growth factor, EGF-epidermal growth factor, EGFR-EGF receptor, ETS-E twenty six, HER-human EGFR, HGF-hepatocyte growth factor, HIF-hypoxia-inducible factor, IGF-insulin-like growth factor, IL-interleukin, MAPK-mitogen-activated protein kinase, NO-nitric oxide, NRP-neuropilin receptor, PDGF-platelet-derived growth factor, PlGF-placental growth factor, PTEN- phosphatase and tensin homolog, Ras-rat sarcoma, SRC-sarcoma, STAT- signal transducer and activator of transcription, TGF-transforming growth factor, TNF-tumour necrosis factor, sVEGFR-soluble VEGFR, VEGF-vascular endothelial growth factor, VEGFR-VEGF receptor, vHL-von-Hippel Lindau.

Blood-brain barrier

In the normal brain, the vasculature is highly organised and is composed of endothelial cells, pericytes and astrocytes which together form the blood-brain barrier (BBB). This structure selectively restricts the exchange of molecules between extracerebral and intracerebral circulatory compartments [Jain et al., 2007]. The lack of fenestrations between adjacent endothelial cells and pinocytotic and endocytotic endothelial vesicles, and the presence of continuously extending tight junctions connecting the adjacent endothelial cells limit passive diffusion. This barrier for passive diffusion is further increased by the presence of astrocytic processes enveloping the gap-junctions [Huber et al., 2001]. The passive entry of hydrophilic molecules larger than 500 kD is prevented by the endothelial cell tight junctions. The presence of active carrier-mediated transport systems on the endothelial cell surface compensates for the limited diffusion. Moreover, they contain active receptor-mediated efflux-proteins such as P-glycoprotein (P-gp)/multi-drug resistant protein (MDR), which transport compounds out of brain parenchyma contributing to drug resistance [Toth et al., 1996]. However, the growth of tumours beyond 1-2 mm in diameter within brain parenchyma compromises the BBB both structurally and functionally [Jain et al., 2007]. In large tumours, the loss of BBB function is not uniform. There may well be an intact BBB functioning at the infiltrating tumour edges with the transport across tumour vessels being heterogeneous [Jain et al., 2007].

Tumour vasculature

The intra-tumoural blood vessels of MGs are abnormally spaced, dilated, tortuous and aberrant in nature [Deane and Lantos, 1981a] [Deane and Lantos, 1981b] with heterogeneous flow rates [Wolf et al., 2005] and increased permeabilities [Takano et al., 1991], leading to elevated interstitial fluid pressure within the tumours [Boucher and Jain, 1992]. The increased VEGF levels within these tumours [Plate et al., 1992] through their action on VEGFR-2 [Ferrara and Bunting, 1996] generating immature, dysfunctional vessels along with impaired BBB, is mainly attributed to this property [Jain et al., 2007]. The increased vascular permeability and the lack of lymphatic drainage lead to an equilibrium in the oncotic and hydrostatic pressures between the tumour microvasculature and interstitial space [Boucher et al., 1997] and this stasis hinders chemotherapeutic delivery to the tumours [Netti et al., 1996]. VEGF induced vascular leakiness leads to a vasogenic oedema that contributes to the pathophysiology of these tumours [Berkman et al., 1993]. Tumour vessels also carry abnormalities in the endothelium, pericyte coverage and basement membrane, such as "Glomeruloid tufts" formed by multi-layered mitotic endothelial and perivascular cells [Plate and Mennel, 1995] [Benjamin et al., 1999], and increased open endothelial gaps and cytoplasmic vesicles [Deane and Lantos, 1981b] [Vajkoczy and Menger, 2000]. Collectively these abnormalities result in progressive tumour hypoxia [Valk et al., 1992], necrosis in spite of marked angiogenesis [Plate and Mennel, 1995] [Jain et al., 2007], microhaemorrhages and thrombosis [Cheng et al., 1996] [Kaur et al., 2004].

2.2.7 Cancer stem-like cells

GBMs were one of the first solid tumours in which CSLC were described [Singh et al., 2003] [Singh et al., 2004]. They constitute only a smaller fraction of the tumour mass (5-30 %) [Bao et al., 2006a]. These cells resemble neural stem cells (NSCs) and have the ability to proliferate over a long period of time, self-renew, differentiate into multiple cell types (multipotency) [Wang et al., 2010a] [Ricci-Vitiani et al., 2010] and are tumorigenic [Galli et al., 2004] [Yuan et al., 2004] [Johannessen et al., 2008]. Cluster of differentiation 133+ ($CD133^{+}$) has been considered as a marker for CSLC [Singh et al., 2003] [Singh et al., 2004] [Bao et al., 2006a], however $CD133^{-}$ cells with CSLC properties have been reported [Beier et al., 2007] [Wang et al., 2008] [Prestegarden et al., 2010]. Nestin, CD90, CD44, CXCR4, musashi1 (Msi1) and maternal embryonic leucine zipper kinase (MELK) [Johannessen et al., 2008] and many others have been reported representing as CSLC markers [Lathia et al., 2011] but none seems to be exclusive [Prestegarden et al., 2010]. They resist apoptosis by overexpressing anti-apoptotic genes such as Bcl-2, $Bcl\text{-}_{x}$ and survivin [Liu et al., 2006] [Johannessen et al., 2008]. Overexpression of DNA repair genes makes them resistant to chemotherapy and RT [Beier et al., 2007] [Bao et al., 2006a] [Svendsen et al., 2011], hence have been implicated in tumour recurrence. This is further aided by their overexpression of gene for multidrug resistance proteins [Salmaggi et al., 2006], ABCG2 [Chua et al., 2008] and VEGF [Bao et al., 2006b]. There does not seem to be any difference in the methyl guanine methyl transferase (MGMT) promoter methylation between CD133+ and CD133- CSLCs. However, CD133- cells have a more pronounced MGMT expression [Beier et al., 2008]. In contrast to the conventional MG cells, MG CSLCs with mutant p53 are more resistant to TMZ [Blough et al., 2011]. Survival and proliferation of CSLCs are dependent on the activation of several cellular signalling pathways, including NO-Notch-dill, wnt-β catenin, sonic-hedgehog-(HH)-Gli [Clement et al., 2007] and VEGF-VEGFR2-NRP [Beck et al., 2011]. A niche for the CSLC has been extensively discussed [Borovski et al., 2011]. There is evidence to suggest that CSLC reside in the perivascular niche [Calabrese et al., 2007] [Zhu et al., 2011] as well as adjacent to the hypoxic tumour areas [Mathieu et al., 2011]. The origin of CSLCs and MGs remains controversial [Visvader, 2011] [Bjerkvig et al., 2005]. There is some evidence to suggest that CSLC can arise from differentiated cells [Gupta et al., 2011], whereas the cell of origin for proneural type MG is believed to be an

oligodendrocyte progenitor cell [Liu et al., 2011] [Persson et al., 2010]. Inhibition of sonic-HH-Gli [Clement et al., 2007], wnt signalling [Shou et al., 2002], Notch signalling [Chen et al., 2010] and suppression of NO [Charles et al., 2010] [Zhu et al., 2011] has been attempted with the aim of suppressing CSLCs. Combination of anti-angiogenic therapy and cytotoxic therapy was able to reduce the GBM CSLC fraction [Folkins et al., 2007].

2.2.8 Tumour-associated macrophages/microglia

MGs are composed of tumour cells as well as intermingling parenchymal cells including cells of the tumour vasculature, microglia/macrophages, peripheral immune cells, stromal cells, astrocytes and neural precursor cells. The heterogeneity of MG is not only due to the heterogeneity of the tumour cells but also due to the heterogeneity of these tumour-associated cells [Charles et al., 2011]. Collectively these cells play a vital role in the tumorigenic process and progression of the tumour.

Microglia/macrophages form the bulk of tumour-associated cells, which can be up to 30 % of the tumour mass [Graeber et al., 2002] [Watters et al., 2005]. Whether TAMs are derived from resident microglia or from the peripheral cells remains elusive. The MG cells secrete monocyte chemotactic protein-3 [Okada et al., 2009], colony-stimulating factor-1 [Alterman and Stanley, 1994], granulocyte colony-stimulating factor (G-CSF), HGF/SF [Suzuki et al., 2008], VEGF [Rogers and Holen, 2011] and chemokines that act as chemoattractants. The EGF secreted by MG cells can also act as a chemoattractant and a proliferating signal for TAM through their expression of EGFR [Nolte et al., 1997]. There is evidence to suggest that tumour secreted VEGF-mediated monocyte/macrophage infiltration into tumours is dependent on the expression of VEGFR-1 by macrophages [Barleon et al., 1996] [Muramatsu et al., 2010] [Kerber et al., 2008]. However, there is evidence to point to a role for VEGFR-2 in other cancer models [Dineen et al., 2008]. TAMs are believed to reside in the hypoxic areas of the tumour. Within MGs, TAMs are suppressed by the secretion of IL-10, IL-4, IL-6 and TGF-β, prostaglandin E2 (PGE2) by the MG cells [Charles et al., 2011], reducing the production of TNF. It is believed that the majority of TAMs carry an M2-phenotype with CD163 and CD204 expression and the number of M2 TAMs correlates with the histological tumour grade [Charles et al., 2011] [Bingle et al., 2002]. Inhibition of Notch signalling also promoted the M2 phenotype [Wang et al., 2010b]. Once activated, TAMs release a variety of growth factors, cytokines, inflammatory mediators and proteolytic enzymes. The IL-1β secreted by TAMs enhances the production of TGF-β by MG cells [Naganuma et al., 1996]. TGF-β in turn suppresses the anti-tumour immune response by inhibiting lymphocyte proliferation, antigen presentation and immune cell activation [Letterio and Roberts, 1998]. Furthermore, an increase in the level of TGF-β enhances MG angiogenesis, proliferation and invasion through upregulation of VEGF, EGFR and matrix metalloproteinase 9 (MMP-9) production, respectively [Watters et al., 2005]. MG cell invasion is further aided by MMP-14 from TAMs activating MMP-2 produced by the MG cells [Charles et al., 2011]. TAMs have low major histocompatibility complex (MHC) class-II and B7 co-stimulatory molecule expression, aiding the suppression of anti-tumour immune response [Badie et al., 2002].

2.2.9 Role of DNA repair pathways

DNA repair pathways are essential for genomic integrity. Several DNA repair pathways have evolved to maintain genetic fidelity, to protect the genome from the wide range of DNA damaging agents causing diverse forms of DNA damages, and to function at different stages in the cell-cycle in a cell type-specific manner. The cellular mechanisms which have evolved to combat the threats of DNA damage are collectively termed as DNA damage responses (DDR), which include detection and repair of the DNA damage [Jackson and Bartek, 2009]. The major DNA repair pathways include direct reversal DNA repair by alkyltransferases, which include MGMT, MMR that repairs mismatches and insertion/deletion loops (IDLs), base-excision repair (BER) that repairs base damage such as

oxidations, alkylations or deaminations, nucleotide-excision repair (NER) that repairs helix-distorting lesions such as bulky chemical adducts or DNA cross-links, and homologous recombination (HR) and non-homologous end joining (NHEJ) pathways to repair DNA double-stranded breaks (DSB) [Helleday et al., 2008]. Sometimes these pathways act in concert, since they have some degree of redundancy towards some types of lesions [Jackson and Bartek, 2009]. Mammalian DDR signalling pathways include two protein kinases; ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), which are recruited and activated by DSBs and single-strand breaks, respectively [Shiloh, 2003]. ATM and ATR reduce the activity of CDK through protein kinases CHK1 and CHK2 by multiple mechanisms [Bartek and Lukas, 2007] [Kastan and Bartek, 2004]. Some of these activities are p53 dependent [Riley et al., 2008]. CDK inhibition arrests the progression of the cell-cycle at G1-S, intra-S or G2-M cell-cycle check points [Jackson and Bartek, 2009]. This provides ample time for DNA repair before the onset of replication or mitosis. If the damage is irreparable continuous DDR signalling triggers cell death by apoptosis or cellular senescence/autophagy [Halazonetis et al., 2008].

Impaired DNA repair is associated with embryonic lethality, short life span, rapid aging, impaired growth, immunodeficiency, neurological disorders and a variety of inherited syndromes including increased cancer susceptibility [Hakem, 2008] such as, HNPCC [Vasen et al., 2007], Turcot syndrome type 1 which can lead to GBM [Cavenee et al., 2000], and *hBRCA1* and *hBRCA2*-mediated familial breast and ovarian cancer syndromes [Narod and Foulkes, 2004]. Some sporadic cancers also harbour defects in DNA repair pathways [Hakem, 2008]. On the other hand, most of the commonly used chemotherapeutic agents and RT target DNA for their action [Jackson and Bartek, 2009]. Most DNA repair pathways allow cancer cells to survive DNA damage caused by cancer therapy, leading to resistance [Helleday et al., 2008]. For example, CSLC of GBM enhances DDR that protects them from RT [Bao et al., 2006a]. One notable exception is the MMR pathway, which induces MMR-mediated cytotoxicity following treatment with monofunctional alkylators [Karran and Marinus, 1982], such as TMZ [Kaina et al., 2007], cisplatin [Fram et al., 1985], and 6-thioguanine (6-TG) [Swann et al., 1996]. On the other hand, most cancer cells harbour defects in at least one DDR component but their survival is ensured by the function of other repair pathways due to redundancy. Inhibition of this pathway provides a selective anti-tumour effect since it makes cancer cells especially susceptible, while the normal cells are resistant [Jackson and Bartek, 2009]. This concept is termed "synthetic lethality" [Kinsella, 2009] [Helleday et al., 2008] and is under evaluation using single-strand break (SSB) repair protein poly-ADP ribose polymerase 1 (PARP1) inhibitors in HR DSB repair pathway defective cancers such as BRCA1 and BRCA2 impaired breast cancers [Jackson and Bartek, 2009].

MGMT direct repair pathway

Direct repair of DNA alkylating lesions is achieved by the DNA alkyltransferase MGMT. MGMT repair pathway is unique among DNA repair pathways because it is a single-step process involving a single protein that leads to irreversible inactivation of that repair protein [Kaina et al., 2007]. MGMT is able to remove alkylation adducts from O^6-positions of guanine. MGMT is capable of removing methyl, ethyl, propyl, chloroethyl and isopropyl groups. Methyl groups are exclusively removed by MGMT, whereas bulky ethyl groups can also be removed by NER pathway [Kaina et al., 2007]. Upon DNA alkylation, cytosolic MGMT is translocated into the nucleus where it will facilitate the transfer of alkyl groups from the O^6-position of guanine into its own cysteine residues (Cys145) [Lim and Li, 1996]. This transfer irreversibly inactivates MGMT leading to ubiquitylation [Srivenugopal et al., 1996] and proteasomal degradation [Xu-Welliver and Pegg, 2002]. Hence, one MGMT molecule can remove only one alkyl adduct. Phosphorylation increases nuclear translocation of MGMT [Srivenugopal et al., 2000]. MGMT can remove the methyl group

from O^6-methyl guanine (O^6-mG) irrespective of whether it is paired with cytosine (C) or thymine (T) [Lips and Kaina, 2001] (Figure 5).

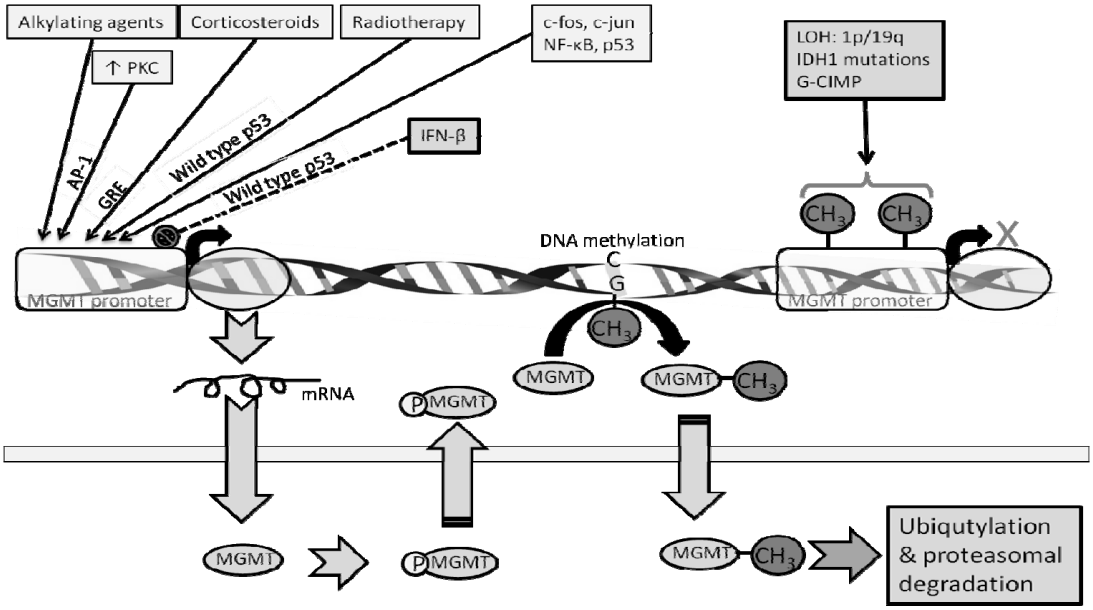

Figure 5: A schematic presentation of the regulation of MGMT gene expression and the mechanism of MGMT-mediated DNA repair. AP-activator protein, CH_3-methylation, G-CIMP-GBM CpG island methylator phenotype, GRE-glucocorticoid responsive element, IDH-isocitrate dehydrogenase, IFN-interferon, LOH-loss of heterozygosity, MGMT-methyl guanine methyl transferase, NF-κB- nuclear factor of κ-light polypeptide gene enhancer in B-cells P-phosphorylation, PKC-protein kinase C.

The human MGMT gene is located in chromosome 10q26 [Riemenschneider et al., 2010]. The expression of MGMT shows a tissue specific variation with high expressions in liver, spleen and colon but a low expression in brain [Kaina et al., 2007], and clear inter-individual variations [Janssen et al., 2001]. Breast, colon, lung and ovarian cancers have a higher MGMT expression [Chen et al., 1992] [Preuss et al., 1996], while MG, melanomas, pancreatic and testicular cancers display a very low expression. Roughly 17-30 % of MGs totally lack MGMT expression [Preuss et al., 1995] [Kaina et al., 2007]. Epigenetic silencing of the MGMT gene by methylation of CpG islands in the promoter region is a common mechanism of gene silencing [Bhakat and Mitra, 2003] (Figure 5). This is seen in 30-50 % of MG patients [Esteller et al., 2000] [Hegi et al., 2004] [Hegi et al., 2005] and ~70 % of secondary GBM patients [Weller et al., 2010]. However, there is no correlation between MGMT promoter methylation and protein expression [Maxwell et al., 2006] [Brell et al., 2005]. Furthermore, intratumoural variation in MGMT expression has also been reported [Lees et al., 2002] [Zaidi et al., 1996] [Mollemann et al., 2005] [Silber et al., 1996]. MGMT promoter methylation is associated with loss of heterozygosity (LOH) of 1p/19q and IDH1 mutation in ODG/OAs [Mollemann et al., 2005] [Brandes et al., 2006a] and with the latter in AA [Sanson et al., 2009]. The MGMT promoter methylation seems to be associated with methylation of many other cancer-related genes [Gerson, 2004] leading to a methylator phenotype [Noushmehr et al., 2010] (Figure 5). MGMT gene expression is induced by alkylating agent therapy, RT, and corticosteroid therapy [Kaina et al., 1991] [Grombacher et al., 1996] leading to increased production of MGMT protein that could have a negative effect on alkylating agent chemotherapy. Activation of protein kinase C (PKC) increases the MGMT expression through transcription factor AP-1, which has two binding sites in the MGMT promoter [Boldogh et al., 1998]. It can be transactivated by expression of c-Fos and c-Jun [Kaina et al., 2007]. NF-κB also increases MGMT expression by binding to its

promoter [Lavon et al., 2007]. The presence of wild-type p53 also tends to increase the MGMT gene expression by direct binding to MGMT promoter irrespective of its methylation status [Blough et al., 2007] [Hermisson et al., 2006] and dysfunctional p53 increases the cell sensitivity to TMZ [Xu et al., 2005a] [Xu et al., 2005b]. RT induced MGMT promoter activation is dependent on the presence of functional p53 [Rafferty et al., 1996]. However, IFN-β down-regulates MGMT expression in a p53 dependant manner [Natsume et al., 2005]. The presence of two glucocorticoid responsive elements (GRE) in the MGMT promoter makes it inducible by corticosteroids [Grombacher et al., 1996] [Biswas et al., 1999] (Figure 5). Furthermore, MG CSLCs tend to express more MGMT along with other DNA repair proteins [Johannessen et al., 2008], which makes them resistant to TMZ therapy [Chua et al., 2008] [Liu et al., 2006]. MGMT expression by glioma cells makes them resistant to monofunctional alkylators/O6-methylators such as TMZ, procarbazine and dacarbazine and O6-chloroethylators, lomustine/[1-(2-chloroethyl)-1-nitrosourea] (CCNU), carmustine/[1,3-bis(2-chloroethyl)-1-nitrosourea] (BCNU), nimustine/[1-(4-amino-2-methyl-5-piryrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea] (ACNU) and fotemustine [Pegg and Byers, 1992].

Mismatch repair pathway

The MMR pathway primarily targets base-substitution mismatches and small IDLs that are generated during DNA replication [Kunkel and Erie, 2005]. Defects in this pathway lead to accumulation of frameshifts in long stretches of mono- and di-nucleotide repeats (microsatellites) scattered throughout the genome that is termed microsatellite instability (MSI). Inherited germline mutations in MMR genes, and the subsequent inactivation of the other allele results in familial cancer syndromes such as HNPCC [Vasen et al., 2007] and Turcot syndrome type-1 [Cavenee et al., 2000]. It is reported that 5-15 % of sporadic colorectal, gastric, endometrial, cervical, ovarian, breast and lung cancers and MGs also carry MSI [Peltomaki, 2003]. There is evidence to suggest that p53 increases the gene expression of some MMR proteins [Chen and Sadowski, 2005]. In eukaryotic cells, mismatch recognition is undertaken by MSH2, MSH3 and MSH6 proteins. MSH2 and MSH6 form the MutSα heterodimer complex, which is the major human MMR complex to recognise base-substitution mismatches and IDLs up to one or two nucleotides. MutSβ complex formed by MSH2/MSH3 heterodimer recognises IDL up to 16 nucleotides [Kinsella, 2009]. MutSα forms a sliding clamp encircling the DNA strand in the vicinity of the mismatch. MutLα complex formed by MLH1 and PMS2 heterodimer or MutLβ formed by MLH1 and MLH3 is recruited to the ternary complex as the second step. MutSα, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) activate the latent MutLα endonuclease to provide the incision that serves as the entry point for MutSα activated exonuclease-1 (EXO1) to commence mismatch excision [Jiricny, 2006a]. The removal of the mismatch is done by EXO1 in an ATP and mismatch-dependant manner. EXO1 may be directed to the nascent strand by the presence of nicks or lack of methylation. EXO1-mediated excision removes >1000 nucleotides around the mismatch. EXO1 activity is inhibited by MutLα and replication protein A (RPA) once the mismatch is removed. The resulting single-strand gap is stabilised by RPA and the gap is filled by DNA polymerase-δ (pol δ). DNA ligase completes the repair process by ligating newly synthesised DNA stretch [Jiricny, 2006b] [Modrich, 2006] (Figure 6A).

MMR dysfunction [Friedman et al., 1997] can result from mutations in one of the MMR genes [Yip et al., 2009], by epigenetic silencing due to promoter methylation [Barvaux et al., 2004] or by loss of protein expression [Felsberg et al., 2011]. It is associated with an increased resistance to DNA monofunctional alkylating agents such as TMZ and dacarbazine, [Koi et al., 1994] [Cejka et al., 2003], platinum analogues cisplatin and carboplatin [Papouli et al., 2004] [Branch et al., 2000], antimetabolites such as 6-TG [Swann et al., 1996] and 6-mercaptopurine (6-MCP), and fluoroprymidines 5-fluorouracil (5-FU) and fluorodeoxyuridine [Kinsella, 2009], and increased cancer predisposition [Kunkel and

Erie, 2005]. MMR-mediated processing of the O^6-mG DNA lesions is essential for the cytotoxicity of these drugs and loss of MMR function impart 2-100 fold resistances to them [Kinsella, 2009] [Cejka et al., 2003]. There is evidence that loss of MMR function is more critical to TMZ cytotoxicity than MGMT [Liu et al., 1996] and the best response to TMZ was obtained with increased MMR and decreased MGMT protein levels [Friedman et al., 1998]. Treatment with these drugs places a selective pressure on the cancers to lose MMR function resulting in hypermutation phenotype recurrences. Development of MSI high acute myelogenous leukemia after treatment of lymphoblastic leukaemia with 6-TG and 6-MCP are examples of this phenomenon [Kinsella, 2009]. There is recent data suggesting that treatment with TMZ leads to loss of MMR function through mutations in MG patients [Cahill et al., 2007] [Hunter et al., 2006] [2008] and loss of MMR protein expression without affecting promoter methylation [Felsberg et al., 2011]. HSV-tk/GCV gene therapy was effective in eliminating MMR deficient cancer cells with high concentrations of the pro-drug GCV implying a possible therapeutic option for these multi-drug resistant phenotypes [O'Konek et al., 2009].

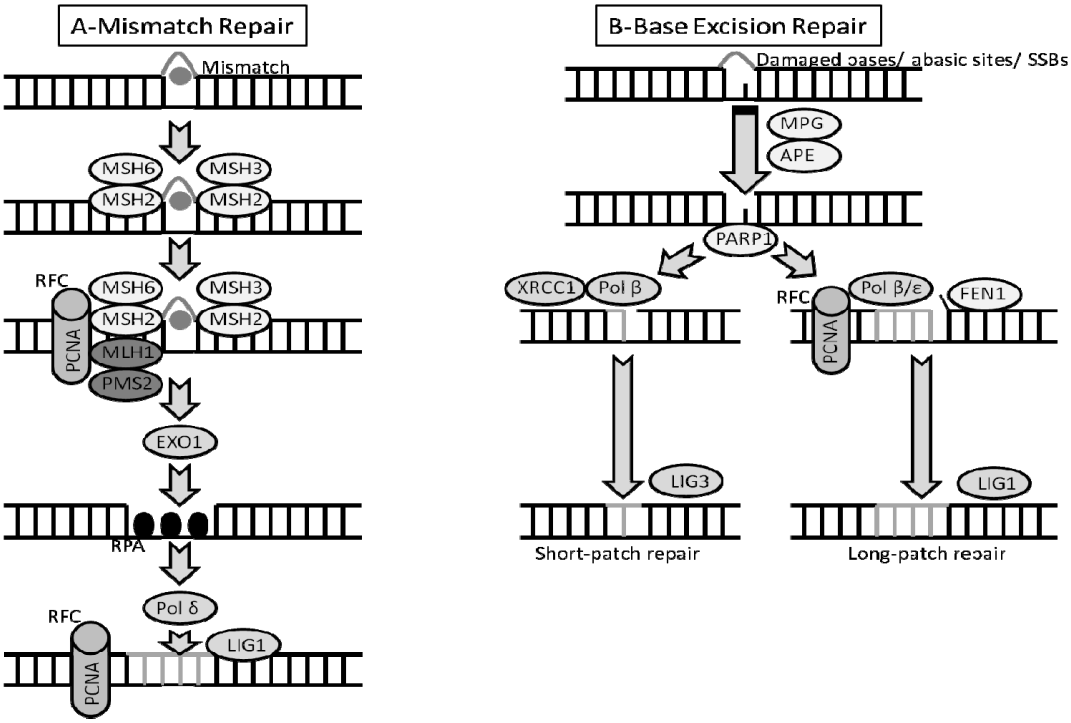

Figure 6: A schematic presentation of the mechanism of action of mismatch repair (A) and base-excision repair (B) pathways. APE-abasic/ apurinic site endonuclease, EXO-exonuclease, FEN-flap structure specific endonuclease, LIG-DNA ligase, MLH-mutL homologous, MPG-methylpurine glycosylase, MSH-mutS homologous, PCNA-proliferating cell nuclear antigen, PARP-poly-ADP ribose polymerase, PMS-post-mitotic segregation, Pol- DNA polymerase, RFC-replication factor C, RPA-replication protein A, SSB-single-strand break, XRCC-X-ray repair complimenting defective repair in Chinese hamster cells. Modified from http://www.nature.com/nrc/posters/dnadamage

Base-excision repair pathway

BER is the major repair pathway for endogenous and exogenous adduct-induced non-bulky base damages such as oxidative base modification, deamidations, hydroxylations [David et al., 2007], N^7-methyl guanine (N^7-mG) and N^3-methyl adenine (N^3-mA) caused by alkylating drugs, abasic/apurinic (AP) sites and DNA SSBs caused by RT, monofunctional alkylating agents and antimetabolites [Helleday et al., 2008]. BER is also a multi-protein, multi-step process. DNA glycosylases initiate the process by binding to the offending

lesions removing those by cleaving the N-glycosidic bond generating AP sites [Hitomi et al., 2007]. AP endonuclease-1 (APE1) recognises and removes the AP sites by cleaving the phosphodiester bond creating nucleotide gaps. It is reported that APE1 is frequently over expressed in MG [Bobola et al., 2001]. Subsequent repair process of either a short-patch repair for one nucleotide or a long-patch repair for 2-15 nucleotides, is initiated by DNA polymerase-β (polβ) and involves PARP1 (Figure 6B). The short-patch repair seems to be important in repairing RT and chemotherapy induces DNA damage, hence BER processing can lead to therapy resistance [Kinsella, 2009] [Trivedi et al., 2008] and inhibition of BER pathway components such as polβ [Frosina, 2000], APE1 [McNeill and Wilson, III, 2007] [Bobola et al., 2007] potentiated alkylating agent cytotoxicity. Furthermore, possible suppression of MSH6 as a result of APE1 overexpression may contribute to TMZ resistance [Chang et al., 2005a].

PARP1 is a zinc-finger DNA binding protein involved in SSB and DSB repair. PARP1 expression in MGs [Wharton et al., 2000] is increased by TMZ therapy [Cheng et al., 2005]. Moreover, inhibition of PARP1 increased the MG sensitivity to TMZ [Cheng et al., 2005].

Other DNA repair pathways

NER recognises helix-distorting lesions such as DNA crosslinks and bulky adducts and repairs them through two different sub-pathways, transcription-coupled NER or global-genome NER [Hoeijmakers, 2001]. DSB repair has two principle mechanisms; NHEJ and HR. In NHEJ, Ku proteins recognise DSBs and activated DNA protein kinase (DNA-PK) that recruits end-processing enzymes, DNA polymerases and ligases. MSH6 is believed to interact with Ku proteins in DSB repair [Shahi et al., 2011]. This is an error prone repair process that can operate in any phase of the cell-cycle [Lieber, 2008]. Expression of adenovirus E4orf6 inactivates DNA-PK and inhibited DSB repair by NHEJ in human glioma cells, and increased their radiosensitivity [Hart et al., 2005]. HR is restricted to S and G2 phase of cell-cycle. It is an error-free repair that leads to sister-chromatid exchange involving several sub-pathways [San et al., 2008] [Jackson and Bartek, 2009].

2.2.10 Clinical features

Intracranial tumours can produce general symptoms due to increased intracranial pressure (ICP) and local symptoms related to anatomical location of the tumour [Fine et al., 2005]. In general, the clinical features largely depend on the anatomical location of the tumour. Headache [Forsyth and Posner, 1993], focal or secondary generalised seizures, altered mental status and progressive neurological deficits including paresis, speech disturbances and personality changes are the most common clinical features [Stewart and Kleihues, 2003]. Symptoms are mostly attributed to infiltration and/or compression of normal brain tissue by the enlarging tumour, oedema and haemorrhage [Chamberlain and Kormanik, 1998].

Headache due to brain tumours is thought to originate from either dura or brain vasculature since the brain itself is not sensitive to pain. It can be mild and intermittent or constant and severe, typically occurring in early morning caused by raised ICP due to recumbency and hypoventilation during sleep. Although frontal and temporal lobe tumours can sometimes produce frontal, retro-orbital or temporal headaches, a focal headache localisation can be misleading [Forsyth and Posner, 1993]. Slow-growing tumours can grow to become large without causing headache, whereas their fast-growing counterparts can evoke headaches at very early stages. Apart from headache, nausea, vomiting, personality changes, slowing of psychomotor functions and somnolence can result from elevated ICP [Fine et al., 2005]. Increased ICP can eventually be life-threatening with visual disturbances, unconsciousness and respiratory arrest [Stewart and Kleihues, 2003].

It is estimated that around 50 % of patients with supratentorial tumours present with seizures, although only 6 % of the first seizures are due to brain tumours [Fine et al., 2005].

In particular, patients with ODGs can present with a long history of seizures [Stewart and Kleihues, 2003]. The effect of tumour size and location on their tendency to cause seizures varies with tumour grade. A low-grade tumour in temporal lobe can ultimately present with seizures after growing large asymptomatically. On the other hand, rapidly growing tumours in deeper structures appear to cause no-seizure symptoms due to a mass effect [Lee et al., 2010].

Focal neurological symptoms such as personality changes, loss of initiative, contralateral weakness, expressive aphasia, impairment of memory, urinary incontinence, dementia, visual changes, auditory hallucinations, receptive aphasia, sensory disorders, and ataxia can provide a clue for the probable tumour location [Fine et al., 2005].

2.2.11 Diagnostic methods

Imaging

Over the past few decades, advances in imaging techniques have vastly improved the early diagnosis, staging, and assessment of therapeutic response in MGs. From the conventional anatomical characterisation, the focus of imaging has recently shifted to functional and metabolic imaging of tumours [Hricak et al., 2005].

Computer-assisted tomography (CT) and magnetic resonance imaging (MRI) are the most widely used imaging methods in MG. Imaging helps in diagnosis, in defining the precise anatomical localisation, as well as an assessment of tumour volume for planning of surgery and RT, evaluating the residual tumour volume after therapy and in the detection of the therapeutic response, as well as late effects of therapy and recurrences [Fine et al., 2005]. The attenuation of the X-rays by the tissues is reflected as the image in CT, while in MRI proton density, T_1 and T_2 relaxation times and blood flow governs the pixel intensity. Gadolinium-enhanced MRI is gradually becoming the imaging modality of choice for MG imaging, replacing CT due to its superior anatomical resolution and its higher sensitivity to pathological changes in the brain tissue [Fine et al., 2005].

Vasogenic oedema caused by disruption of BBB is seen as a hypodense dark area in the CT and as an area of low signal intensity in T_1-weighted images and as an area of high signal intensity in T_2-weighted images in the MRI. It is believed that MRI is superior to CT in diagnosing hydrocephalus and its causes. In CT, acute haemorrhage appears as high attenuation, whereas sub-acute haemorrhage is isodense and hard to detect. Acute haemorrhage is seen as low signal intensity in the T_2 images whereas sub-acute haemorrhages produce a bright signal in both T_1 and T_2 MRI. Disruption of the BBB increases contrast enhancement of both CT and MRI. Contrast-enhanced CT and MRI scans are better capable of differentiating brain tumours from other pathological lesions, different types of brain tumours from one-another and high-grade brain tumours from low-grade ones [Fine et al., 2005]. Apart from its high sensitivity in lesion detection, MRI has the advantage of providing complete lesion delineation, high spatial resolution, and high accuracy in detecting surrounding peri-tumoural oedema, mass effect, cyst formation and intratumoural haemorrhage.

MRI within the first three days after surgery is considered as standard, although repeat imaging may be performed 4-6 weeks later, to obtain baseline values [Hricak et al., 2005]. Follow-up MRIs are indicated 2 months after RT and during chemotherapy after each cycle of 2 or 3 treatments. Continued follow-up is helpful in assessing response, detecting recurrence or other treatment complications [Bracard et al., 2006]. Macdonald criteria provide clear guidelines to monitor the treatment response by MRI [Macdonald et al., 1990a], but there is no consensus on tumour recurrence. Increase in radiographic tumour volume, development of new areas of contrast enhancement and increase in T_2-weighted changes are some of the parameters which need to be considered. Differentiating tumour progression from treatment-related effects such as radiation necrosis and MRI features suggestive of tumour progression in the absence of clinical deterioration, such as pseudoprogression seen in 20 % of patients treated with TMZ, are challenges in MRI

[Barbagallo et al., 2008]. MRI resolution is not sufficient to accurately demarcate distant tumour infiltration [Hricak et al., 2005]. Surgical biopsy is the gold-standard for differentiating late radiation necrosis from recurrence. Other techniques such as magnetic resonance spectroscopy (MRS), perfusion and diffusion MRI, and positron emission tomography (PET)/single-photon emission computed tomography (SPECT) as an adjunct to clinical status and conventional MRI can be helpful in making the diagnosis [Jenkinson et al., 2007]. The use of anti-angiogenic therapy poses a novel challenge for MRI. The dramatic response seen in oedema, mass effect and contrast enhancement with these drugs complicate the interpretation of conventional contrast enhanced MRI. Non-contrast enhancing tumour progression is common with these therapies making uncertain the validity of the Macdonald criteria for response assessment. Fluid attenuation inversion recovery (FLAIR) MRI and diffusion-MRI may be more helpful in this situation [Gurwara et al., 2010].

MRS provides data on the regional distribution of chemicals, such as choline, N-acetyl aspartate (NAA), lipids and lactate, associated with tumour metabolism [Hakumaki et al., 1998] [Hakumaki et al., 1999]. Choline and NAA are markers of cell proliferation and neuronal integrity, respectively, while lipids and lactate can be considered as markers of necrosis. Reduced levels of choline in areas of radiation necrosis compared to increased levels in areas of recurrence helps to differentiate the two conditions [Rabinov et al., 2002]. MRS is useful in assessing the treatment response and guiding biopsy but is unreliable when the lesions are less than 2 cm in size, or when they are adjacent to bone, fat or CSF due to signal contamination [Rees, 2003].

Perfusion MRI can measure the relative cerebral blood volume (rCBV), cerebral blood flow (CBF) and microvascular permeability. It is useful in assessing tumour vasculature especially after treatment with anti-angiogenic therapy [Batchelor et al., 2007] and in differentiating therapy-related necrosis from recurrences [Aronen and Perkio, 2002]. In addition it is useful in pre-operative planning and grading of gliomas [Aronen and Perkio, 2002].

With its ability to differentiate free and restricted water diffusion within brain, diffusion-weighted MRI too can be helpful in differentiating a malignant tumour from a radiation effects. The apparent diffusion coefficient is increased in response to treatment and decreased in tumour recurrence [Hein et al., 2004].

Functional MRI (fMRI) is useful in pre-operative planning to reduce the surgical morbidity and damage to vital brain areas [Hricak et al., 2005]. Intra-operative high-field MRI with integrated microscopy-based navigation helps to localise hidden tumour remnants, metabolic changes, tumour invasion and functional eloquent cortical and deep-seated brain areas, during surgery with the use of MRS, diffusion tensor imaging and fMRI modalities [Nimsky et al., 2006b].

PET and SPECT are the latest nuclear magnetic imaging modalities providing metabolic information about brain tumours [Amin et al., 2012]. These can be combined with CT or MRI to obtain anatomical images to differentiate necrosis, progression and pseudoprogression [Brandsma et al., 2008].

Biopsy

Histopathology is still the gold-standard for the diagnosis of MG [Brat et al., 2008], and in spite of the advances in non-invasive imaging modalities, their role remains complementary. Clearly, accurate tissue diagnosis and grading is vital in the determination of the optimal treatment regimen [Wick and Weller, 2009]. Furthermore, since the diagnosis is dependent on identification of characteristic histological patterns and initial focal progression of low-grade gliomas into high-grade gliomas with time, adequate sampling of the tumour tissue is of the utmost importance [Collins, 2004]. Resected-out surgical specimens are widely used in histological diagnosis. If the tumour is surgically inaccessible due to its anatomical location or situated in a diffuse non-focal manner, a needle biopsy can

be used for diagnostic purposes [Fine et al., 2005]. If the tumour is adjacent to a large blood vessel or brainstem, open biopsy is the method of choice. CT- or MRI-directed stereotactic biopsy is the most accurate and simplest technique [Apuzzo et al., 1987]. Metabolic imaging such as MRS along with anatomical imaging can be helpful in identifying the appropriate site for biopsy. However, the malignancy grading obtained by these types of limited biopsies is always the minimal grading due to the possible existence of more anaplastic areas elsewhere [Collins, 2004]. Prior chemotherapy or RT renders histological diagnosis extremely difficult or even impossible [Collins, 2004]. Local histopathological diagnosis and central review in several recently completed clinical trials revealed up to 40 % discrepancy and a high rate of disagreement among central review neuropathologists casting doubts on the reproducibility of the histopathological diagnostic criteria [Wick and Weller, 2009].

2.2.12 Treatment of malignant glioma

Standard therapy for newly diagnosed MG depends on the WHO grading. For WHO grade III anaplastic gliomas, surgery and adjuvant RT and/or chemotherapy have been shown to have equal benefits [Wick and Weller, 2009]. On the other hand, in many countries standard therapy for WHO grade IV GBMs include surgery followed by adjuvant RT with concomitant and adjuvant chemotherapy with TMZ [Stupp et al., 2005a] [Stupp et al., 2009]. However, WHO grade III tumours are often treated with regimens developed for GBM due to diagnostic uncertainties associated with tumour grading [Wick and Weller, 2009]. Older patients (>70 years) sometimes receive less aggressive therapy with either RT or chemotherapy [Keime-Guibert et al., 2007] [Glantz et al., 2003].

2.2.12.1 Surgery

Surgery is the oldest method of treating MG with a history dating back to the late 19th century [Pang et al., 2007]. Although the ultimate objective of surgery is complete tumour resection to achieve a potential cure, surgical cure alone is not possible for MGs. Curative total-resection of the tumour with microscopic clearance, is virtually impossible due to the invasive and infiltrative nature of MGs [Kelly et al., 1987], and due to the possible neurological deficit resulting from wide-margin of excision, especially in the eloquent cortex [Sawaya et al., 1998].

Under these circumstances, maximum de-bulking of the tumour with minimal risk for the patient seems to represent a reasonable compromise [Fine et al., 2005]. De-bulking reduces the secondary tumour effects such as oedema, hydrocephalus and increased ICP, achieving symptomatic control. It also provides tissue samples for accurate histological diagnosis, which is paramount for appropriate treatment planning [Hentschel and Lang, 2003]. Furthermore, surgical resection improves efficacy of adjuvant therapy [Pang et al., 2007] [Ryken et al., 2008]. Even though the recent advances in surgical approaches, techniques and instrumentation have made most tumours surgically amenable, the anatomical location and some histological subtypes increase the risk of surgery, making biopsy the only option.

Evidence suggests that patient outcome is best when the resection is near-total [Lacroix et al., 2001] [Sanai and Berger, 2008]. In some studies surgery alone has a MS of ~20 week (<6 months) as opposed to a MS of ~14 weeks with only supportive care for MG patients [Kelly et al., 1984] [Paoletti, 1984]. Several studies have revealed that the survival is better in patients undergoing surgical resection when compared to those having only biopsy [Hess, 1999] [Stark et al., 2005] [Vuorinen et al., 2003]. One study reported a 12-month survival of 47 % with total resection, 42 % with partial resection and 12 % with biopsy alone [Simpson et al., 1993]. Nonetheless, there is no clear evidence at present, suggesting that radical tumour resection imparts a clinical benefit in MG patients [Proescholdt et al., 2005] [Pang et al., 2007].

The benefit of re-operation for recurrent MG remains controversial [Barker et al., 1998]. Some studies suggest a MS of 3-5 months after re-operation [Barbagallo et al., 2008]. This

procedure improves symptoms and performance status, maintains quality of life [Barker et al., 1998], delays symptom progression, reduces corticosteroid doses and improves response to chemo/RT in selected patients [Barker et al., 1998] [Barbagallo et al., 2008].

Intra-operative critical functional cortical mapping by electrical cortical stimulation, pre-operative fMRI [Mueller et al., 1996], magneto-encephalography, motor and speech mapping [Berger et al., 1989] [Nimsky et al., 2006a], intra-operative ultrasonography, fluorescence-guided surgery with 5-aminolevulinic acid [Stummer et al., 2006] and frameless image-guided neuro-navigation systems have improved the safety of surgery [McDermott and Gutin, 1996]. In spite of the recent advances in imaging and surgical techniques, many CNS tumours still remain only partially resectable or are even unresectable [Fine et al., 2005]. For example, one study reported that only 54 % of the patients with GBM had undergone surgery at the population level [Ohgaki et al., 2004]. Moreover, the patients that received surgery were significantly younger (mean age 56 years) compared to those who did not (mean age 67 years) and they had better MS (7.9 vs. 2.5 months) [Ohgaki and Kleihues, 2005b].

2.2.12.2 Radiotherapy

Irrespective of the extent of surgical resection, MGs warrants further treatment with adjuvant therapy. Radiotherapy has been considered as the mainstay of treatment of MG for decades [Wen and Kesari, 2008]. Pooled meta-analysis data suggests that the addition of adjuvant RT at a dose of 45-60 Gray (Gy) after surgery can increase the MS of the MG patient from 3-4 months to 7-12 months [Laperriere et al., 2002] [Walker et al., 1978] [Walker et al., 1980]. With RT GBM patients achieved a 3-year survival of 6 % [Leibel et al., 1994]. GBM patients older than 70 years did not benefit from RT but some studies suggest that smaller doses and hypo-fractionated RT might still provide a survival benefit [Barker et al., 2001] [Chang et al., 2003].

In most centres, conventional fractionated external-beam radiation is administered at a total dose of 50-60 Gy in 1.8-2.0 Gy daily fractions five times a week over a period of 6-7 weeks, to cover the original tumour bed and a 2-3cm margin [Laperriere et al., 2002] [Leibel et al., 1994]. Typically, the response to RT is short-lived with recurrences emerging within one year [Hochberg and Pruitt, 1980]. Nowadays RT focuses on perilesional region rather than the conventional whole brain irradiation. When calculating the tumour volume for RT, enhancing volume containing the solid tumour tissue, surrounding oedema comprising of normal brain with microscopic tumour infiltration and a margin of normal brain is included in the treatment field [Fine et al., 2005]. Thus, the tolerance of normal brain becomes a vital limiting factor in RT and is dependent on the total radiation dose, size of the dose per fraction, overall treatment time, irradiated brain volume, host factors and adjuvant therapies [Fine et al., 2005]. The risk of brain injury increases with doses exceeding 60 Gy and with fractions exceeding 2.2 Gy. Radiation doses exceeding 60 Gy have not demonstrated any benefit in spite of increasing the incidences of side effects [Lee et al., 1999] [Salazar et al., 1979].

Side effects of cranial irradiation can be divided into three groups; 1) acute reactions occurring shortly after RT, 2) early-delayed reactions appearing a few weeks to 4 months after RT and, 3) late-delayed injuries which can occur a few months to years after irradiation. The clinical picture of radiation injuries can vary from asymptomatic white matter changes to fatal brain necrosis. Up to 4-9 % of the MG patients can develop clinically detectable radiation necrosis following 50-60Gy of conventional fractionated RT and this can be as high as 10-22 % at autopsy [Fine et al., 2005]. Exact pathological mechanisms of these injuries are unclear but damage to vascular endothelial cells and direct effects on oligodendroglial cells have been postulated. Cerebral cortical atrophy was reported in 17-39 % of patients undergoing whole brain irradiation for MG [Posner, 1995] that may cause symptoms of clinical encephalopathy [Swennen et al., 2004]. Cranial irradiation can result

in intellectual impairment in adults, recent memory loss, difficulties in attending to tasks, and hypothalamic-pituitary dysfunction [Sklar and Constine, 1995].

The poor response for standard RT in MG is mainly attributed to the inherent radioresistance of the tumours, tumour hypoxia [Hockel and Vaupel, 2001] and the radiosensitivity of the surrounding normal brain tissue [Fine et al., 2005]. The role of RT in recurrent MG is controversial. Some benefit is seen in younger patients with a good performance score when the gap between previous RT is >6 months. The use of radiosensitizers, dose-escalation schemes using altered fractions, interstitial brachytherapy, radio-surgery and three-dimensional conformal RT, boron (^{10}B) neutron capture therapy (BNCT), and proton RT are some of the newer methods to improve RT [Fine et al., 2005].

With the use of an imaging-compatible stereotactic devise for precise target localisation, stereotactic radio-surgery (SRS) delivers a highly focal, closed skull external irradiation. This can be administered either by gamma knife units made of multiple cobalt beams or by modified linear-accelerators. The ability to deliver a high dose of radiation to the precise intracranial target volume, in a single session without significant irradiation of the adjacent normal brain tissue in a non-invasive manner are the advantages of this method. Fractionated SRS (fSRS) is administered in fractions, for the improvement of the therapeutic ratio, which is important in the treatment of large gliomas and those adjacent to vital structures [Fine et al., 2005]. SRS may be applied for low volume recurrences [Hsieh et al., 2005] but data do not support the routine use in newly diagnosed GBM patients [Souhami et al., 2004].

In interstitial brachytherapy, a radioactive source is placed within the tumour either stereotactically or by free-hand [Fine et al., 2005]. ^{125}Iodine (^{125}I) and ^{192}iridium (^{192}Ir) are the commonly used radioactive sources as seeds or brachytherapy devices (GliaSite Radiation Therapy System) [Chan et al., 2005]. The application is limited to unilateral tumours of <5 cm diameter. Interstitial brachytherapy so far has not significantly improved the overall survival (OS) in GBM patients [Laperriere et al., 1998] [Gabayan et al., 2006].

In hyperfractionated-RT, multiple smaller-than-conventional RT fractions are used with the intention of delivering a higher total dose of radiation during the same time period with the aim of improving tumour control without increasing the risk of late complications [Fine et al., 2005]. Studies have not shown a significant improvement of survival in MG patients with this method [Prados et al., 2004]. Accelerated hypofractionated-intensity modulated RT with TMZ are under clinical evaluation [Panet-Raymond et al., 2009] [Chen et al., 2011a].

First evaluated for brain cancer therapy in 1950s, BNCT is being rejuvenated for the treatment of GBM and is now in the experimental stage [Joensuu et al., 2003]. ^{10}B has the ability to capture thermal neutrons after which they disintegrate into α-particles and Li nuclei. These heavily charged particles damage the cell causing cell death. Due to their limited ability to travel distances, the risk of damage to normal tissues is also less with this approach.

The use of radiation modifiers such as misonidazole, hydroxyurea, bromodeoxyuridine and difluoromethylornithine and radiosensitizers with RT has not improved the outcome of MG patients [Prados et al., 2001] [Prados et al., 2004].

2.2.12.3 Chemotherapy

The advantage of chemotherapy is that theoretically all tumour cells should be accessible to the chemotherapeutic agent through either the pre-existing or the tumour-associated microvasculature. Secondly, since the majority of normal cells within the CNS are post-mitotic, chemotherapeutic agents with a preferential action on dividing cells should have a high therapeutic index in the CNS. Neurotoxicity is minimal for many chemotherapeutic agents and in most instances; the limiting factor is their systemic toxicity. In spite of all these possible advantages, in reality chemotherapy if used at all is as an adjuvant to surgery and RT in a few CNS tumours [Fine et al., 2005]. Up until recently, chemotherapy was not

considered as effective therapy for newly diagnosed MG patients [Chang et al., 2005b]. The only place for chemotherapy was as salvage therapy in recurrent MG [Brandes and Fiorentino, 1996].

Meta-analyses revealed a modest survival advantage of 6-10 % at one year and 5-8 % at two years for newly diagnosed MG patients with adjuvant nitrosourea-based chemotherapy with an increase in the MS from 9.4 to 12 months. Furthermore, regardless of the type of chemotherapy, all patients with GBM and AA who had adjuvant chemotherapy demonstrated a significant survival advantage [Fine et al., 1993] [Stewart, 2002]. It was concluded from these studies that young patients with high-grade gliomas who had maximum surgical debulking and who had a good performance status should be offered adjuvant chemotherapy. On the other hand, older patients with large GBMs not amenable for surgical excision, with multiple neurological deficits and poor performance status did not experience a survival benefit with adjuvant chemotherapy. TMZ is the preferred chemotherapy for newly diagnosed GBM in a concomitant and adjuvant setting with RT. For AA, the usual regimen is RT with adjuvant TMZ. For anaplastic ODG and OA, the chemotherapy regimen of choice is the combination of procarbazine, lomustin (CCNU) and vincristine (PCV) [Wen and Kesari, 2008]. Other chemotherapeutics used as single-agents or in combination in the treatment of newly diagnosed MG are the nitrosoureas, carmustine (BCNU), lomustine (CCNU), nimustine (ACNU) and fotemustine, procarbazine, vincristine, cisplatin, etoposide and carboplatin.

Unlike in the treatment of newly diagnosed tumours, none of the chemotherapeutic agents have shown a significant survival benefit in recurrent MGs [Wen and Kesari, 2008] [Brandes and Fiorentino, 1996]. TMZ has shown some efficacy in recurrent anaplastic ODG and AA as second line agents as well as first line therapy [van den Bent et al., 2003a] [van den Bent et al., 2003b] and in recurrent GBM [Yung et al., 2000]. Apart from TMZ, BCNU [Brem et al., 1995], CCNU, procarbazine, tamoxifen [Ben Arush et al., 1999], irinotecan [Stupp et al., 2006], ifosfamide, etoposide and carboplatin [Schafer et al., 2011], are commonly used as single-agents or in combinations for the treatment of recurrent MG with minimal to moderate activity at the best [Wen and Kesari, 2008].

The primary reason for chemotherapeutic failure in CNS is intrinsic drug resistance partly due to the tumour heterogeneity at doses attainable due to systemic toxicity, as with tumours elsewhere. Secondly, there is impaired drug delivery into CNS tumours. The presence of the BBB is believed to be the culprit for impaired drug delivery. However, BBB function is almost completely disrupted in GBMs, especially in the contrast enhancing portion of the tumour. On the other hand, the leading front of the infiltrating tumour is located within normal brain parenchyma with an intact BBB. In spite of being highly angiogenic, tumour vasculature is aberrant, dilated, tortuous and leaky [Groothuis, 2000]. Disruption of BBB and increased permeability increases the interstitial fluid pressure, thereby effectively reducing not only the entry of O_2 and nutrients but also that of chemotherapeutics into the tumours. Furthermore, drug delivery is still reduced due to poor perfusion and non-patency of the tumour-induced neovasculature and the relatively long distance between tumour-induced angiogenic vessels and individual tumour cells. Thirdly, the resulting sub-lethal exposure of the tumour cell to the drug promotes acquired drug resistance [Fine et al., 2005]. Finally, the physiochemical characteristics of the drug itself determines its ability to cross the BBB, with smaller, ionically neutral, lipophilic drugs with a high octanol/water coefficient being able to better penetrate the BBB [Greig, 2001].

Alternative methods for delivering chemotherapeutic agents into brain tumours have been attempted to circumvent these problems. The disruption of BBB using agents such as mannitol or bradykinin has failed to live up to expectations [Kroll et al., 1998] [Emerich et al., 2001]. Intra-arterial drug delivery carried the drawback of increased drug delivery into adjacent normal brain areas leading to neurotoxicity and also tumours may draw their blood supply from multiple arteries, and there was significant morbidity associated with the procedure itself [Tfayli et al., 1999].

Convection-enhanced delivery (CED) using bulk fluid flow is another method of local drug delivery. In CED, a catheter implanted in the brain provides a continuous infusion of the drug under a constant pressure gradient. The advantages of this method include the ability to administer large macromolecules [Laske et al., 1997], liposomes and viral vectors, the possibility to achieve a homogenous concentration of the drug even at the far edge of the tumour, and the potential to target distinct anatomical zones of the brain [Lieberman et al., 1995]. The drawbacks of this method are the problems of repeated administration since it is an invasive procedure and the ultimate efficiency depends on the physiochemical characteristics of the administered drug [Fine et al., 2005].

Emerging evidence suggests that addition of chemotherapy into the standard care of MG along with surgery and RT increases the possibility of long-term survival in selected patients [Laws et al., 2003].

2.2.12.3.1 Temozolomide

TMZ (Temodal®, Temodar® Merck/Schering-Plough) is an orally available mono-functional alkylating agent with ~100 % bioavailability [Adhikari et al., 2008], that crosses well the BBB [Ostermann et al., 2004]. Plasma and CSF concentrations in patients are between 0.10-13.99 μg/ml (0.5-72 μmol/l) and 0.16-1.93 μg/ml (0.8-10 μmol/l), respectively [Ostermann et al., 2004]. VPA decreases the clearance of TMZ (www.temodar.com). It undergoes spontaneous hydrolysis at physiological pH to its active form 3-methyl-(triazen-1-yl) imidazole-4 carboxyamide (MTIC), which is further processed to form DNA methylating methyldiazonuim ions [Newlands et al., 1997] (Figure 7). The active form can methylate DNA at many sites, with the most significant being the methylations at O^6-position and N^7-position of guanine and N^3-position of adenine to form O^6-mG, N^7-mG and N^3-mA, respectively [Kaina et al., 2007] and AP sites [Adhikari et al., 2008]. Even though O^6-mG only accounts for 5-10 % of all methylations, it is the most important in terms of TMZ-mediated cytotoxicity [Kaina et al., 2007] [Hegi et al., 2008].

Mechanism of action

The mechanism of TMZ induced cytotoxicity has been studied extensively [Ochs and Kaina, 2000] [Roos et al., 2007b] [Roos et al., 2007a] and is schematically presented in Figure 7. The cytotoxicity of TMZ is dependent on the function of major DNA repair pathways; namely MGMT direct repair, MMR, BER and DSB repair pathways [Kaina et al., 2007]. O^6-mG itself is not toxic to the cells. MGMT acting in a suicide manner will repair the pre-toxic O^6-mG by transferring the methyl group from O^6-mG onto the cysteine residues of the protein and this will trigger its subsequent degradation [Pegg, 2000]. The expression of MGMT by the cells imparts a primary resistance to TMZ by removing the methyl group [Kaina et al., 1991]. If not removed, in subsequent DNA replication cycles O^6-mG will mispair with T [Toorchen and Topal, 1983] or pair with C. The O^6-mG/T mismatches promote nuclear translocation of MMR proteins [Christmann and Kaina, 2000]. Mismatches are recognised by MutSα [Duckett et al., 1996] that trigger the MMR pathway-dependent repair process by binding to the mismatch. However, the MMR pathway is only capable of removing the T in the nascent strand, not the offending O^6-mG [Hegi et al., 2008]. Repeated attempts of this kind of futile repair cycles [Karran and Bignami, 1994] will ultimately lead to block of replication, collapse of the replication fork and DNA DSB [Kaina et al., 2007]. DNA DSB repair pathways HR and/or NHEJ can still repair some of the DSBs [Roos et al., 2007a] [Roos et al., 2009]. This mechanism emphasizes the vital role of the MMR pathway in TMZ-mediated cytotoxicity [Kaina et al., 2007]. In fact, it has been reported that the loss of MMR function confers up to 100-fold resistance to TMZ as well as to other methylating agents [Stojic et al., 2004] [Branch et al., 1993]. Check-point activation and subsequent apoptosis is impaired in cells with reduced MMR protein levels [Lettieri et al., 1999] [Cejka et al., 2003]. DSBs will activate the ATM/ATR-Chk1/Chk2 signalling and G2/M cell-cycle arrest [Caporali et al., 2004] [Stojic et al., 2004] [Mirzoeva et al., 2006] that would ultimately

trigger the apoptotic pathway [Roos and Kaina, 2006] or autophagy [Kanzawa et al., 2004]. The pathway that will lead to apoptosis after DSB will depend on the p53 status of the cell [Kaina et al., 2007] and it will also determine the duration of cell-cycle arrest [Hirose et al., 2001]. In cells with mutant p53, apoptosis occurs via the mitochondrial pathway by decreasing B-cell lymphoma-2 (Bcl-2) and increasing cytochrome C [Ochs and Kaina, 2000]. In cells with wild-type p53, apoptosis is triggered via the death-receptor pathway (FasR/CD95/Apo-1) [Dunkern et al., 2003] [Roos et al., 2004]. Both mechanisms seem to contribute to MG cell apoptosis [Roos et al., 2007a]. Another school-of-thought suggests that binding of MutSα/MutLα complexes to the O^6-mG/T mismatch will directly trigger apoptosis by activating ATR/ATR-interacting protein (ATRIP) [Yoshioka et al., 2006] [Caporali et al., 2004]. The proposed mechanism indicates that cells have to undergo at least two cell divisions [Kaina et al., 2007], which may take up to 4-6 days [Roos et al., 2007a] before they die.

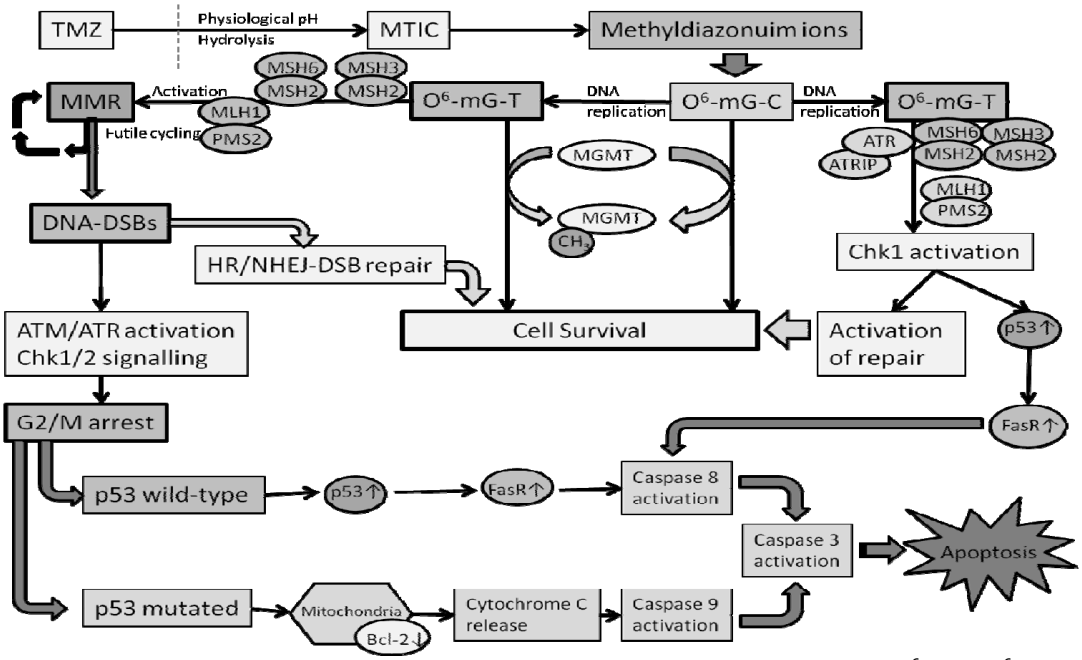

Figure 7: A schematic presentation of the mechanism of action of TMZ induced O^6-mG. O^6-mG-T mispairs can be further processed either by MMR-mediated futile cycling or by MMR and ATR/ATRIP-mediated direct signalling. ATM-ataxia-telangiectasia mutated, ATR-ataxia telangiectasia and Rad3 related, ATRIP- ATR-interacting protein, Bcl-B-cell lymphoma, C-cytosine, CH_3-methyl group, DSB-double-stranded break, FasR-Fas receptor, HR-homologous recombination, mG-methyl guanine, MGMT-methyl guanine methyl transferase, MLH-mutL homologous, MMR-mismatch repair, MSH-mutS homologous, MTIC-3-methyl-(triazen-1-yl) imidazole-4 carboxyamide, NHEJ-non-homologous end joining, PMS-post-mitotic segregation, T-thymine, TMZ-temozolomide.

N^7-mG and N^3-mA does not play a significant role in the cytotoxicity of dividing cells but may be important in non-dividing cells [Briegert and Kaina, 2007]. Further processing of N^7-mG and N^3-mA is undertaken by the BER pathway [Engelward et al., 1996]. N^7-mG account for 80-85 % of alkylation adducts, which are generally stable and non-toxic to the cells. They can be depurinated spontaneously or by N-methyl purine glycosylase (MPG) into cytotoxic AP, which are cleaved by APE1 and repaired by the downstream BER mechanisms [Kaina et al., 2007]. N^3-mA is toxic to the cells and is processed in the same manner as N^7-mG. Thus, a functional BER pathway seems to protect the cells from alkylating agents but imbalances in the pathway may increase cytotoxicity independent of the cellular MMR or p53 status [Kinsella, 2009] [Trivedi et al., 2008]. Inhibition of tumour

cell invasiveness [Wick et al., 2002], angiogenesis [Kim et al., 2006] [Mathieu et al., 2008] and regulatory T-cells (Tregs) at metronomic doses [Banissi et al., 2009] are other postulated anti-tumour mechanisms of TMZ.

The European Organisation for Research and Treatment of Cancer (EORTC) trial 26981-22981 and National Cancer Institute of Canada (NCIC) trial CE.3 randomised 573 newly diagnosed GBM patients into TMZ+RT (n=287) and RT alone (n=286) arms. TMZ+RT arm received concomitant TMZ (75 mg/m^2) daily along with RT for a maximum of 42-49d, followed by adjuvant TMZ monotherapy (150-200 mg/m^2) on days 1-5 of every 28-day cycle for up to 6 cycles [Ataman et al., 2004] [Stupp et al., 2005a] [Stupp et al., 2009]. The results revealed an impressive overall MS of 14.6 months for the combination group as opposed to 12.1 months for the RT only group. An OS of 27.2 % vs. 10.1 % at 2-years, 16 % vs. 4.4 % at 3-years, 12.1 % vs. 3 % at 4 years and 9.8 % vs. 1.9 % at 5-years was observed for the combination vs. RT only groups, respectively. This led to the accelerated regulatory approval of concomitant and adjuvant TMZ for the management of newly diagnosed GBM [Stupp et al., 2009] [Cloughesy, 2010]. The progression-free survival (PFS) was 11.2 % vs. 1.8 % at 2-years, 6 % vs. 1.3 % at 3-years, 5.6 % vs. 1.3 % at 4-years and 4.1 % vs. 1.3 % at 5-years for the combination vs. RT only arms. However, the MS after progression was equal at 6.2 months for both groups [Stupp et al., 2009]. The combination proved superior in all prognostic subgroups irrespective of the MGMT methylation status. However, MGMT promoter methylation seems to achieve a better response in both arms [Hegi et al., 2005] [Stupp et al., 2009].

TMZ has demonstrated efficacy in recurrent AA [Yung et al., 1999] and recurrent ODG [van den Bent et al., 2003a] [van den Bent et al., 2003b] but for recurrent GBM the outcome was not encouraging [Yung et al., 2000] [Brada et al., 2001]. TMZ was not superior to PCV in recurrent MG [Brada et al., 2010].

The adverse effects of TMZ include alopecia, anorexia, nausea, vomiting, constipation, diarrhoea, headache, fatigue, convulsions, hemiparesis, opportunistic infections, skin reactions and myelosuppression with thrombocytopenia (26d), neutropenia and lymphocytopenia (28d) (www.temodar.com). In melanoma studies, it was revealed that TMZ selectively depleted CD4+ lymphocytes [Su et al., 2004]. The combination of TMZ with RT required prophylaxis for *Pneumocystis carinii* pneumonia [Stupp et al., 2009].

Resistance to TMZ

Resistance to TMZ can occur through modulations in the MGMT, MMR and BER pathways, by activation of PI3K/AKT pathway through PTEN inactivation [Jiang et al., 2007], by activation of NF-κB [Bredel et al., 2006] and by promoting/selective overgrowth of the CSLC phenotype [Gaspar et al., 2010]. Epigenetic silencing of the MGMT gene by promoter methylation of MG cells [Esteller et al., 1999] was associated with an increase in the sensitivity to TMZ and other alkylating agents and a better outcome in MG patients [Hegi et al., 2005]. The extent of MGMT promoter methylation is variable in MG patients, and is dependent on the assay in use, tissue sampling technique, method of quantification, prior treatment and state of disease progression [Hegi et al., 2008]. The methylation status and MGMT expression can be heterogeneous within the same tumour [Zaidi et al., 1996]. Immunohistochemistry (IHC) [Zaidi et al., 1996] and high-performance liquid chromatography (HPLC) [Dolan et al., 1989] are often used to assess the MGMT status in pre-clinical work, whereas the methylation-specific polymerase chain reaction (MSP) is used in clinical studies [Galm and Herman, 2005]. Other methods in use are methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), quantitative real-time MSP (qMSP) and bisulphite sequencing [van Nifterik et al., 2010]. Standardisation and validation of the assay technique is vital for correct interpretation and comparison of data [Vlassenbroeck et al., 2008]. It was reported that there was no correlation between MGMT promoter methylation and protein expression [Maxwell et al., 2006] [Brell et al., 2005] but some evidence existed that it would be more predictive of the outcome than MGMT

promoter methylation [van Nifterik et al., 2010]. MGMT gene expression may be induced by alkylating agents, RT, corticosteroid therapy [Kaina et al., 1991] [Grombacher et al., 1996] and downregulated by IFN-β [Natsume et al., 2008]. The presence of wild-type p53 increased the MGMT gene expression in MG cells independent of MGMT methylation status [Blough et al., 2007] and reduced the sensitivity to TMZ [Hermisson et al., 2006] [Blough et al., 2011] or vice versa [Xu et al., 2005a] [Xu et al., 2005b]. However, according to other studies, the presence of wild-type p53 in MG cells sensitised them to TMZ [Batista et al., 2007] [Roos et al., 2007a]. Most of the evaluations have reported a MGMT methylation rate of 30-50 % [Esteller et al., 2000] [Hegi et al., 2004]. MG CSLCs tend express more MGMT, which makes them resistant to TMZ therapy [Liu et al., 2006]. In contrast, another study suggested that TMZ reduced the proliferation, abolished tumourigenicity and preferentially depleted CSLC [Beier et al., 2008].

Attempts have been made to deplete the cellular MGMT in cancer cells by non-toxic pseudosubstrate inhibitors of MGMT such as O^6-benzylguanine (O^6-BG), RNA interference (RNAi)-mediated MGMT gene silencing and MGMT depletion by dose-dense TMZ therapy. However, attempts at systemic inhibition of MGMT have been hampered by haematological toxicity [Quinn et al., 2005]. It is unclear whether alternative TMZ dosing schedules would be able to deplete MGMT in the tumour to achieve a therapeutic benefit without significantly affecting the drug-associated toxicities [Tolcher et al., 2003] [Weiler et al., 2010]. Liposome-mediated MGMT small-interfering RNA (siRNA) gene therapy has been evaluated as a way to enhance TMZ cytotoxicity [Kato et al., 2010]. MGMT methylation is associated with the LOH of 1p and 19q in ODG [Dong et al., 2001], AA, OA [Sadones et al., 2009] and GBM [Ishii et al., 2007] that are particularly sensitive for RT and chemotherapy [Stupp et al., 2006] [Brandes et al., 2006], and with pseudoprogression [Brandes et al., 2008].

MMR dysfunction [Friedman et al., 1997] can result from mutations of one of the MMR genes [Yip et al., 2009] or by epigenetic silencing due to promoter methylation [Barvaux et al., 2004] [Esteller, 2002]. However, it has been claimed that MMR dysfunction is uncommon in newly diagnosed MGs [Martinez et al., 2004] [Cahill et al., 2007]. There is recent data suggesting that treatment of GBM with TMZ results in the selection for MMR inactivation by mutations [Cahill et al., 2007] [Hunter et al., 2006] or loss of expression [Felsberg et al., 2011] leading to hypermutation phenotype recurrences. These TMZ resistant cell populations in tumours give rise to recurrences that have to be treated with different approaches than TMZ.

Enhancing the efficacy of TMZ

Multiple pre-clinical attempts have been made to enhance the efficacy of TMZ by combining the drug with other therapeutic agents. *In-vitro* data have pointed to a possible synergism between TMZ and RT [Wedge et al., 1997] but *in-vivo* results in MGMT expressing models were only positive when MGMT was inhibited [Chakravarti et al., 2006] [Carlson et al., 2009]. Moreover, there is more evidence that the enhanced efficacy when RT and TMZ are combined is dependent on the treatment schedule, where TMZ has to be started 3 days before to achieve the maximum benefit [Chalmers et al., 2009]. TMZ has been combined with cisplatin [D'Atri et al., 2000], irinotecan [Houghton et al., 2000], imatinib [Ren et al., 2009], ribonucleotide reductase inhibitors [Figul et al., 2003], IFN-β [Motomura et al., 2010], PARP1 inhibitors [Cheng et al., 2005] suicide gene therapy [Rainov et al., 2001] and conditionally replicative adenoviruses [Ulasov et al., 2009]. The combination of TMZ with oncolytic viruses is intriguing. Since expression of their early-gene proteins inhibit DNA repair pathways such as MGMT, BER and HR, oncolytic viruses could enhance the effects of TMZ [Alonso et al., 2007]. On the other hand, oncolytic *Herpes simplex* virus (HSV) takes advantage of cellular DNA repair machinery and thus it may benefit by TMZ induced upregulation of DNA repair and cell-cycle regulation genes [Jiang et al., 2006].

2.2.12.3.2 Other chemotherapy options

Nitrosoureas such as BCNU and CCNU are commonly used in the second-line treatment of brain tumours [Rosenthal et al., 2004] due to their excellent BBB penetration properties [Levin, 1980]. However, their therapeutic utility is severely hampered by haematological, pulmonary and renal toxicities [Brandes et al., 2004]. Moreover, their therapeutic benefit has been marginal [Walker et al., 1978] [Walker et al., 1980]. BCNU is equally effective as TMZ in recurrent GBM but with a worse adverse effect profile [Brandes et al., 2004].

The BCNU impregnated Gliadel® wafer is a biodegradable synthetic polymer that can be surgically implanted into the tumour or surgical cavity thus bypassing the BBB [Brem et al., 1991]. It is the first of this kind of treatments to be approved by the United States Federal Drug Administration (US-FDA) for the treatment of recurrent (1996) and newly diagnosed GBM (2003). The drug is released in a controlled manner over several days or weeks providing high-dose local chemotherapy. The sufficiency of the BCNU dose at 3.8 % could be a concern due to the lack of proper dose-escalation studies [Stupp et al., 2006]. Lipid solubility of BCNU allows bi-directional penetration of BBB leading to systemic toxicities and increased risk of cerebral oedema and seizures. In addition, the limited diffusion of BCNU from the implanted site prevents the drug from reaching distant infiltrating tumour cells [Strasser et al., 1995]. High cost is another drawback associated with this therapy. However, clinical trials in primary GBM patients have demonstrated a marginal but statistically significant prolongation of survival and the maintenance of neurological function and performance status [Westphal et al., 2003] although the data on recurrent GBM is not convincing [Hart et al., 2008].

PCV is the most widely used chemotherapeutic combination for the treatment of anaplastic ODG and OA, and has been the standard care for over a decade [Stupp et al., 2005b]. However, in recent trials, PCV in the neo-adjuvant or adjuvant setting prolonged the PFS but had no impact on OS [Cairncross et al., 2006] [van den Bent et al., 2006] and the efficacy of PCV regimen has been questioned [2001]. Many studies comparing PCV with a single-agent nitrosourea for AA did not reveal superiority of either treatment [Levin et al., 1990] [Prados et al., 1999]. Furthermore, there is no convincing evidence to support the use of PCV over the single-agent nitrosourea in GBM patients [Fine et al., 2005]. A recent phase III trial revealed a similar efficacy with RT, PCV or TMZ in WHO grade III MGs [Wick et al., 2009]. PCV is generally a well-tolerated regimen but myelosuppression is not uncommon [van den Bent et al., 2003a].

Carboplatin and cisplatin have not proven superiority over BCNU as single-agents [Dropcho et al., 1992] or in combination with BCNU in GBM patients [Grossman et al., 2003]. Carboplatin was not effective in recurrent ODG [Soffietti et al., 2004]. Carboplatin in combination with etoposide displayed some efficacy in recurrent MG [Franceschi et al., 2004] [Scopece et al., 2006].

The combination of nimustine (ACNU) and cisplatin in the neo-adjuvant setting was unsuccessful due to adverse effects [Kim et al., 2011a] [Han et al., 2009]. In contrast, ACNU and cisplatin as neo-adjuvant therapy prior to RT gave promising results for newly diagnosed GBM patients in a small phase II trial [Choi et al., 2002] and the combination had some effect against the recurrent disease [Gwak et al., 2005].

2.2.12.4 Anti-angiogenic therapy

Anti-angiogenic therapy was classically expected to act by targeting angiogenic mediators, receptors and their signalling pathways to inhibit new vessel formation leading to tumour regression or dormancy [Folkman, 1971]. However, the current concept of anti-angiogenic therapy includes transient vessel normalisation, restoration of BBB and a reduction of vascular permeability that decreases the interstitial pressure and vasogenic oedema. The overall action of these functions is to increase the delivery of chemotherapeutic agents by improving the vascular flow and drug penetration during the therapeutic window [Jain, 2005]. Some argue that it is the improvement in tumour blood flow and oxygenation [Jain,

2005] that would sensitise tumours to chemotherapy and RT [Winkler et al., 2004], while others have postulated that there is a possible reduction in tumour blood flow and increase in hypoxia [Keunen et al., 2011] [Wachsberger et al., 2005]. There is also evidence for a reduction in the delivery of chemotherapeutic agents secondary to BBB restoration [Claes et al., 2008] [Varallyay et al., 2009]. Direct anti-tumour effects may be achieved by other anti-tumour mechanisms e.g. interrupting growth factor and their receptor-mediated autocrine/paracrine loops, disruption of CSLC niche and their reduction in combination with cytotoxic therapy [Calabrese et al., 2007] [Folkins et al., 2007], sensitising endothelial cells to chemotherapy and RT [Kerbel, 2006], counteracting the surge of VEGF following RT [Gorski et al., 1999] and counteracting anti-tumour immune responses of VEGF [Ellis and Hicklin, 2008].

Bevacizumab

Bevacizumab (BV) is a humanised murine monoclonal antibody (mAb) that binds VEGF-A preventing its interaction and activation of the cognate receptors. Previously it has been approved for the treatment of colorectal and non-small cell lung cancers in combination with other forms of chemotherapy. The results from a phase II trial combining BV with irinotecan for recurrent MG demonstrated a 63 % radiographic response rate and 32 % 6-month PFS [Vredenburgh et al., 2007a]. In the extended study, recurrent GBM patients had a 6-month PFS of 46 %, 6-month OS of 77 % with 57% of patients showing at least a partial response (PR) [Vredenburgh et al., 2007a]. In a phase II randomised, multi-centre, non-comparative trial for recurrent GBM patients, BV alone achieved a median OS of 9.2 months, 6-month PFS of 42.6 % and an objective response rate of 28 %, and a median OS of 8.7 months, a 6-month PFS of 50.3 % and objective response rate of ~38 % for BV and irinotecan group [Friedman et al., 2009], leading to an accelerated US-FDA approval [Tabatabai and Stupp, 2009]. A corticosteroid sparing effect of BV has been proposed [Vredenburgh et al., 2010]. BV in combination with metronomic chemotherapy was not effective and showed increased toxicities [Reardon et al., 2011a] [Reardon et al., 2009]. CNS haemorrhage, thromboembolic events, cerebral infarctions, hypertension, bowel perforations, hypophosphatemia, convulsions, confusion, headache, blindness, fatigue, lethargy, reversible posterior leukoencephalopathy syndrome (RPLS), and thrombotic thrombocytopenic purpura were encountered as adverse effects in these trials [Jain et al., 2007]. Up to 30 % of patients treated with BV have experienced grade 3 and 4 toxicities [Thompson et al., 2010] [Vredenburgh et al., 2007a] [Vredenburgh et al., 2007b]. A pre-clinical study combining BV with carboplatin in a rat MG model demonstrated synergism in survival. In spite of having larger tumour volumes, animals treated with BV lived longer, indicating that the reduction in tumour related oedema had been a major component in the response [Jahnke et al., 2009].

Anti-angiogenic therapies in development

Tables 3 and 4 summarise the anti-angiogenic therapies in clinical and pre-clinical development, respectively, for the treatment of MG.

Table 3: Anti-angiogenic therapies in clinical development for the treatment of MG

Name	Description	Mode of action	Clinical phase	Efficacy	References
Aflibercept/ VEGF trap (Zaltrap)	Fusion of VEGFR-1 & -2 with Fc portion of IgG	High affinity binding of VEGF and PlGF	II	Minimal efficacy in recurrent GBM patients.	[Gerstner and Batchelor, 2012]
Enzastaurin LY317615	Serine/threonine kinase inhibitor	Inhibit PKC and PI3K/AKT	III II I	No benefit in recurrent GBM patients compared to lomustine. Efficacy in combination with TMZ in newly diagnosed GBM.	[Wick et al., 2010] [Rampling et al., 2012]
Cediranib/	Multi-kinase	Pan-VEGFR,	III	No survival benefit as a	[Gerstner

AZD2171 (Recentin)	RTK inhibitor	PDGFR-β, c-KIT inhibitor	I/II	monotherapy or in combination with lomustine	and Batchelor, 2012]
Pazopanib/ GW786034 (Votrient)	Multi-kinase RTK inhibitor	VEGFRs, c-KIT, PDGFRs	II	Not effective in recurrent GBM patients	[Gerstner and Batchelor, 2012]
Sorafenib/ BAY 439006 (Nexavar)	Multi-kinase RTK inhibitor	VEGFR-2, Flt-3, PDGFR-β, RAF, c-KIT, RET, FGFR-1 inhibitor	I/II	Minimal efficacy in newly diagnosed GBM patients. Under evaluation for recurrent GBM	[Nabors et al., 2011]
Sunitinib/ SU11248 (Sutent)	Multi-kinase RTK inhibitor	VEGFRs, Flt-3, PDGFRs, CSF-1R, RET inhibitor	II I	No efficacy in recurrent HGG/ MG patients as single agent or when combined with irinotecan	[Neyns et al., 2011] [Reardon et al., 2011b]
Vandetanib/ ZD6474 (Zactima)	Multi-kinase RTK inhibitor	VEGFRs 2 & 3, EGFR, RET inhibitor	I/II	Safe in combination with RT and TMZ in newly diagnose GBM, under evaluation in recurrent MG with radiosurgery	[Drappatz et al., 2010] [Fields et al., 2012]
Cabozantinib/ XL-184/ (BMS907351)	Multi-kinase RTK inhibitor	VEGFR-2, MET, RET inhibitor	II	Promising results in progressive GBM patients	[Wen et al., 2010b]:
Vatalanib/ PTK787/ ZK222584	Multi-kinase RTK inhibitor	VEGFRs, c-KIT, PDGFR-β inhibitor	I I/II	Evaluated in combination therapy for newly diagnosed and recurrent GBM patients	[Gerstner et al., 2011] [Brandes et al., 2010]
AEE788	Multi-kinase RTK inhibitor	Inhibits EGFR and VEGFR-2	IB/II	Under evaluation in recurrent GBM patients	[Reardon et al., 2012a]
Tandutinib/ MLN-518	Multi-kinase RTK inhibitor	Inhibits Flt-3, PDGFR-β, c-KIT	I/II & II	Under clinical evaluation for GBM	[Lehky et al., 2011]
Lenvatinib E7080	Multi-kinase RTK inhibitor	VEGFR-2/3, FDFR-1, PDGFR- β	II	Under evaluation in recurrent GBM	[Gerstner and Batchelor, 2012]
Cilengitide/ EMD-121974	Integrin inhibitor	αvβ5 inhibitor	I/II II	Under evaluation with standard therapy. Modest efficacy as a single agent in recurrent GBM patients	[Stupp et al., 2010] [Gilbert et al., 2012]
Thalidomide (Thalomid) Lenalidomide/ CC-5013 (Revlimid)	Immunemodulator with anti-angiogenic properties, inhibit integrin-mediated signalling	Inhibit VEGF & FGF pathways ↑PDGFR-β, inhibit NO-mediated endothelial cell migration	II I	Limited efficacy in combination with chemotherapy or radiotherapy in recurrent and/or newly diagnosed GBM patients	[Rahman et al., 2010]
ABT-510	Thrombospondin-1 mimetic	Block VEGF, bFGF & IL-8, CD36R	I	Well tolerated with standard therapy in newly diagnosed GBM patients	[Nabors et al., 2010]
Rilotumumab/ AMG102	Fully human IgG2 mAb	Block HGF/SF	II	Modest effect as a monotherapy in recurrent GBM patients	[Wen et al., 2011]
Ramcirumab/ IMC-1121B	Fully human IgG2 mAb	VEGFR-2 inhibition	I/II	Under evaluation for recurrent GBM	[Rahman et al., 2010]
Astrasentan (Xinlay)	Endothelin-A receptor antagonist	By ↓HIF-1α	I	Modest effect as a monotherapy in recurrent MG patients	[Phuphanich et al., 2008]
IMC-3G3	Human IgG1 mAb	Inhibit PDGFR-α	I/II	Under evaluation for recurrent GBM	[Gerstner and Batchelor, 2012]
Celecoxib (Celebrex)	COX-2 inhibitor	By ↓VEGF & FGF, & ↑endostatin	II	Effective in combination in recurrent, but not in newly diagnosed MG	[Chi et al., 2009]
CT-322	Fibronectin-based inhibitor	Inhibit VEGFR-1-3	II	Under evaluation in recurrent GBM patients	[Chi et al., 2009]

2-methoxy estradiol/ 2ME2 (Panzem)	Oestrogen metabolite	↓HIF-1α	II	Under evaluation in recurrent GBM patients	[Chi et al., 2009]

bFGF-basic fibroblast growth factor, c-KIT-receptor for stem cell factor, COX-cyclooxygenase, CSF-1R-colony stimulating factor 1 receptor, EGFR-epidermal growth factor receptor, FGFR-FGR receptor, Flt-Fms-like tyrosine kinase, GBM-glioblastoma multiforme, HGF-hepatocyte growth factor, HIF-hypoxia-inducible factor, IgG-immunoglobulin G, IL-interleukin, MET-receptor for HGF, mAb-monoclonal antibody, MG-malignant glioma, NO-nitric oxide, PDGFR-platelet-derived growth factor receptor, PKC-protein kinase C, PlGF-placental growth factor, PI3K-phosphatidylinositol 3-kinase, RET-rearranged during transformation, RAF-rapidly accelerated fibrosarcoma, RT-radiotherapy, RTK-receptor tyrosine kinase, SF-scatter factor, TMZ-temozolomide, VEGF-vascular endothelial growth factor, VEGFR-VEGF receptor.

Table 4: Selected anti-angiogenic therapies in pre-clinical development for the treatment of MG

Name	Description	Mode of action	Efficacy	References
Brivanib alaninate/ BMS-582664	RTK inhibitor	Inhibits VEGFR-2 & FGFR-1 and -2	Anti-tumour and anti-angiogenic effects	[Bhide et al., 2010]
CEP-7055	RTK inhibitor	Pan-VEGFR inhibitor	Efficacy in GBM xenografts with TMZ	[Jones-Bolin et al., 2006]
Endostatin	C-terminal fragment of type XVIII collagen	Binding to integrins & inhibition of VEGFR-2	Prolonged survival. Enhanced efficacy with chemotherapy. Reduced tumour cell migration	[Grossman et al., 2011]
DC101	Murine mAb	Inhibit VEGFR-2	Reduced tumour growth and angiogenesis	[Kunkel et al., 2001] [Verhoeff et al., 2009]

FGFR-fibroblast growth factor receptor, GBM-glioblastoma multiforme, IgG-immunoglobulin G, mAb-monoclonal antibody, MG-malignant glioma, PDGFR-platelet-derived growth factor receptor, RTK-receptor tyrosine kinase, TMZ-temozolomide, VEGFR-vascular endothelial growth factor receptor

Challenges for anti-angiogenic therapy

Several caveats in response assessment following anti-angiogenic therapy using contrast enhanced imaging have been reported. The reduced contrast enhancement due to vascular normalisation [Pope et al., 2006] [Ali et al., 2008] [Bokstein et al., 2008] [Kang et al., 2008] [Narayana et al., 2009] [Vredenburgh et al., 2007a] [Vredenburgh et al., 2007b] [Ananthnarayan et al., 2008] [Varallyay et al., 2009] is often difficult to disentangle from the anti-tumour response to therapy [Jain et al., 2007], especially in the case of misdiagnosed pseudoprogression, which also decreases contrast enhancement after anti-angiogenic therapy [Brandsma et al., 2008] [Weinstein et al., 2010] [Wong and Brem, 2007]. Thus, a reduction in oedema may also be interpreted as a response to therapy, while the progression of non-enhancing tumours may not be detected by this method [Norden et al., 2008] [Iwamoto et al., 2009]. Finally the lack of uniformity among different response assessment criteria, and the use of different response assessment criteria in different studies make comparisons difficult [Wen et al., 2010a] [Thompson et al., 2011].

In general, the long-term survival benefits gained from anti-angiogenic therapy have not been encouraging, and the tumour shrinkage and dormancy has not been spectacular [Norden et al., 2008] [Paez-Ribes et al., 2009]. Once the transient response is overcome by resistance mechanisms [Bergers and Hanahan, 2008], the tumours have been reported to become more aggressive with increased invasion and vessel co-option or to cause multi-focal recurrences [Rubenstein et al., 2000] [Kunkel et al., 2001] [Gomez-Manzano et al., 2008] [Fischer et al., 2008] [Norden et al., 2008] [Narayana et al., 2009] [Iwamoto et al., 2009] [Zuniga et al., 2009] [de Groot et al., 2010] [Keunen et al., 2011]. The need for long-term high-dose drug administration, systemic toxicities, immunogenicity, short serum half-life, high cost, their inherent cytostatic nature and inability to affect the pre-existing stable

microvasculature are some of the other drawbacks of anti-angiogenic therapy [Kirsch et al., 2000] [Puduvalli and Sawaya, 2000] [Furnari et al., 2007].

2.2.12.5 Other treatments

Supportive care

General medical management is of the utmost importance for the success in MG patient care. This includes the appropriate management of possible complications, such as seizures, peri-tumoural oedema, venous thromboembolism, fatigue and cognitive dysfunction [Wen et al., 2006].

Approximately 30-50 % of GBM patients experience seizures [Wen and Schiff, 2011]. The release of glutamate by the MG cells has been thought to be responsible for the seizures [Buckingham et al., 2011]. AEDs have to be used in patients with seizures in order to control the symptoms. AEDs that induce hepatic cytochrome P-450 enzyme (EIAED), such as phenytoin and carbamazepine carry the risk of increasing the metabolism of chemotherapeutic drugs and their use in MG patients is controversial [Jaeckle et al., 2009] [Oberndorfer et al., 2005]. Non-enzyme inducing anti-epileptics (non-EIAED) such as levetiracetam (Keppra) are preferred as the first-line therapy. A recent EORTC/NCIC study reported a better survival in patients who received both VPA (a non-EIAED) and TMZ [Weller et al., 2011]. However, adding VPA increased the haematological toxicities. The use of prophylactic anti-epileptics is controversial [Glantz et al., 2000].

Peri-tumoural oedema and subsequent elevated ICP is a major cause of morbidity in MG patients. The corticosteroid, dexamethasone, is frequently used to alleviate these symptoms. Long-term high-dose use of corticosteroids can lead to Cushing's syndrome, corticosteroid myopathy and the risk of opportunistic infections such as *Pneumocystis* pneumonia, since corticosteroids prevent immune defence against infection. Other late complications of high-dose steroids include osteoporosis and fractures although vitamin D, calcium supplementation and bisphosphonates may be useful in preventing these complications [Wen and Kesari, 2008]. Furthermore, administration of dexamethasone is known to inhibit chemotherapeutic induced apoptosis of MG cells by blocking mitochondrial cytochrome *c* release, abolition of caspase-3 activity and inhibition of caspase-9 and PARP cleavage. It is also thought to increase the level of the anti-apoptotic molecule Bcl-X_L [Ni et al., 2006].

MG patients have a 20-30 % risk of developing venous thromboembolism from leg and pelvic veins [Wen et al., 2006] [Gerber et al., 2006]. Anti-coagulants are generally prescribed unless contraindicated due to intratumoural haemorrhage or other contraindication. Low-molecular weight heparin is preferred over warfarin for anti-coagulation. Intratumoural haemorrhage due to anti-coagulation is rare in these patients [Wen et al., 2006] [Ruff and Posner, 1983].

Novel anti-tumour therapies

Rationally-developed targeted therapies are an emerging field with promising options for MG therapy. With the better understanding of genetics and molecular pathogenesis of MG, new therapeutic targets have been identified enabling the development of novel therapeutic approaches that have a better tumour specificity and lower adverse effect profile. Tables 5, 6 and 7 summarise some of the novel targeted therapies, immunotherapies and targeted toxin therapies, respectively, in clinical development for the treatment of MG.

Table 5: A summary of novel targeted therapies for MG in clinical development

Name	Description	Target/s	Efficacy	References
Gefitinib (Iressa)	RTKi	EGFR	Unimpressive as monotherapy and in combination with mTOR inhibitors or bevacizumab in recurrent and newly diagnosed GBM patients	[Desjardins et al., 2009]
Erlotinib (Tarceva)	RTKi	EGFR	Unimpressive as monotherapy	[Desjardins

			and in combination with mTOR inhibitors or bevacizumab in recurrent and newly diagnosed GBM patients	et al., 2009]
Lapatinib (Tykerb/Tyverb	RTKi	EGFR/HER-2	Unimpressive as a monotherapy in recurrent GBM	[Thiessen et al., 2010]
Rindopepimut (CDX-110)	Vaccine	EGFR*vIII*	Promising results in newly diagnosed GBM patients	[Del Vecchio and Wong, 2010]
Cetuximab (Erbitux)	Chimeric mouse/ human mAb	EGFR	Unimpressive as a monotherapy or in combination with bevacizumab +/- irinotecan in recurrent GBM	[Neyns et al., 2009] [Hasselbalch et al., 2010]
Nimotuzumab	Humanised IgG1 mAb	EGFR	Moderate activity as a monotherapy Under evaluation as a radio-immunotherapy in recurrent MG	[Rahman et al., 2010]
Imatinib (Gleevec/ Glivec)	RTKi	PDGFR-β, c-KIT, BCR-ABL,	Limited efficacy as a monotherapy in recurrent and newly diagnosed MG, mixed results in combinations	[Chi et al., 2009] [Rahman et al., 2010]
Dasatinib/ BMS-354825 (Sprycel)	RTKi	PDGFR, SRC, BCR-ABL, c-KIT	Unimpressive in recurrent GBM patients	[Lu-Emerson et al., 2011]
Tipifarnib R115777 (Zarnestra)	Farnesyl transferase inhibitor	Ras/ MAPK	Limited success in newly diagnosed and recurrent MG/ GBM patients	[Rahman et al., 2010]
Lonafarnib SCH66336	Farnesyl transferase inhibitor	Ras/ MAPK	Under evaluation with TMZ in an adjuvant setting in newly diagnosed GBM patients	[Desjardins et al., 2011]
Sirolimus (Rapamune)	mTOR inhibitor	PI3K/AKT/ mTOR	Under evaluation in newly diagnosed and recurrent GBM patients as a monotherapy and in combinations	[Rahman et al., 2010]
Temsirolimus/ CCI-779 (Torisel)	mTOR inhibitor	PI3K/AKT/ mTOR	Under evaluation as a monotherapy and in combinations	[Rahman et al., 2010]
Everolimus RAD-001 (Zortress/Certican)	mTOR inhibitor	PI3K/AKT/ mTOR	Under clinical evaluation as a monotherapy and in combinations	[Rahman et al., 2010]
Ridaforolimus AP23573/MK-8669	mTOR inhibitor	PI3K/AKT/ mTOR	Under evaluation for recurrent MG	[Reardon et al., 2012b]
Tamoxifen (Nolvadex/Valodex)	ER agonist/ antagonist	PKC inhibitor	Under evaluation in newly diagnosed and recurrent MG patients	[Patel et al., 2012]
Enzastaurin	Small molecular inhibitor	PKC inhibitor	Under evaluation in newly diagnosed and recurrent MG patients	[Rahman et al., 2010]
Phenyl butyrate (Buphenyl)	Aliphatic acid-Zn chelator	HDACi	Under evaluation in recurrent GBM patients	[Butowski et al., 2006]
Valproic acid	Aliphatic acid-Zn chelator	HDACi	Under evaluation	[Weller et al., 2011]
Romidepsin/ FK228 (Istodax)	Zn-binding thiol prodrug	HDACi	Modest effect in recurrent GBM patients	[Iwamoto et al., 2011]
Panobinostat/ LBH589	Hydroxamic acid-Zn chelator	HDACi	Under evaluation for recurrent MG in combination with bevacizumab	[Drappatz et al., 2012]
Vorinostat /SAHA (Zolinza)	Hydroxamic acid-Zn chelator	HDACi	Modest activity as a monotherapy in recurrent GBM, Under evaluation in combinations	[Galanis et al., 2009]
Bortezomib/ PS-341 (Velcade)	Boronic acid-dipeptide	Proteasome inhibitor	Under evaluation as a monotherapy and in combinations in recurrent and newly diagnosed GBM	[Kubicek et al., 2009] [Phuphanich et al., 2010]
Pioglitazone	Thiazolidine dione	PPAR-γ agonist	Modest efficacy in recurrent MG patients in combination therapy	[Hau et al., 2007]

BCR-ABL-breakpoint cluster region-Abelson, c-KIT-receptor for stem cell factor, EGFR-epidermal growth factor receptor, GBM-glioblastoma multiforme, HDACi-histone deacetylase inhibitor, HER-human epidermal growth factor receptor, IgG-immunoglobulin G, mAb-monoclonal antibody, MAPK-mitogen-activated protein kinase, MG-malignant glioma, mTOR-mammalian target of rapamycin, PDGFR-platelet-derived growth factor receptor, PI3K- phosphatidylinositol 3-kinase, PKC-protein kinase C, PPAR- peroxisome proliferator-activated receptor, RTKi-receptor tyrosine kinase inhibitor, TMZ-temozolomide, Zn-zinc.

Table 6: A summary of the immunotherapies in clinical development for MG

Approach	Method	Efficacy	Reference
Cytokine modulation	TNF-α (TNF-SAM2)	Modest therapeutic benefit in newly diagnosed MG	[Oshiro et al., 2006]
	IFN-α	Some efficacy in newly diagnosed GBM but a modest benefit with increased adverse effects in recurrent GBM	[Jackson et al., 2011]
	IFN-β	Mixed results in newly diagnosed and recurrent GBM patients	[Jackson et al., 2011] [Wakabayashi et al., 2011]
	IFN-γ	Disappointing results, increased adverse effects	[Jackson et al., 2011]
	IL-2	Modest therapeutic benefit, marked adverse effects	[Jackson et al., 2011]
	IL-12	Mixed results in clinical trials	[Jackson et al., 2011]
	Poly-ICLC-synthetic	Some efficacy in newly diagnosed GBM patients receiving standard care	[Rosenfeld et al., 2010]
	TGF-β anti-sense oligonucleotide Trabederson	Significant benefit in recurrent AA but some increase in adverse effects	[Jackson et al., 2011]
	GM-CSF	Under evaluation in a vaccine trial	[Jackson et al., 2011]
Active-specific	Dendritic cell-based vaccination (e.g. CDX-110)	Encouraging results in both newly diagnosed and recurrent MG/ GBM patients	[Jackson et al., 2011] [Chang et al., 2011] [Fadul et al., 2011],
	Autologous tumour cell vaccination	Encouraging results in both newly diagnosed and recurrent MG/ GBM patients	[Muragaki et al., 2011], [Clavreul et al., 2010] [Jackson et al., 2011]
	Personalised peptide vaccination	Evaluated in recurrent GBM patients, safety established	[Terasaki et al., 2011]
Passive-nonspecific	Lymphokine-activated killer cells (LAK)	Encouraging results in newly diagnosed and recurrent GBM	[Jackson et al., 2011] [Dillman et al., 2009]
Passive-specific	Effector T-cell therapy from peripheral blood or TILs	Disappointing early trials, encouraging results in some later studies with newly diagnosed GBM	[Jackson et al., 2011]
Antibody-mediated radio immuno--therapy	Anti-EGFR mAb labelled ^{125}iodine (^{125}I)	Some efficacy in newly diagnosed GBM patients with TMZ or RT	[Li et al., 2010]
	Anti-tenascin mAb labelled ^{131}iodine (^{131}I)	Encouraging results in newly diagnosed and recurrent GBM patients with standard therapy	[Reardon et al., 2008]
	Anti-tenascin mAb labelled ^{211}Astatine (^{211}At)	Encouraging results in recurrent GBM patients	[Zalutsky et al., 2008]
	Anti-DNA-histone 1 mAb labelled ^{131}iodine (^{131}I)	Evaluated in newly diagnosed and recurrent MG patients, established safety	[Hdeib and Sloan, 2011]

EGFR-epidermal growth factor receptor, GBM-glioblastoma multiforme, IFN-interferon, ICLC-polyinocinic-poly cytidylic acid stabilized with polylysine and carboxymethylcellulose, IL-interleukin, mAb-monoclonal antibody, MG-malignant glioma, RT-radiotherapy, TGF-transforming growth factor, TMZ-temozolomide, TNF-tumour necrosis factor.

Table 7: A summary of the targeted toxin therapies in clinical development for MG

Target	Ligand	Efficacy	References
IL-13 receptor α2	IL-13 tagged with pseudomonas exotoxin (Cintredekin besudotox)	Well tolerated. No survival benefit in recurrent GBM patients	[Jackson et al., 2011] [Castro et al., 2011]
IL-4 receptor	IL-4 fused with pseudomonas exotoxin (NBI-3001)	Evaluated in recurrent MG patients. Well tolerated	[Jackson et al., 2011] [Castro et al., 2011]
EGFR	TGF-α fused with pseudomonas exotoxin (TP-38)	Modest effect in recurrent GBM/ MG patients	[Castro et al., 2011]
Transferrin receptor	Mutated transferrin fused with diphtheria toxin (Tf-CRM107)	Encouraging results in recurrent MG patients	[Castro et al., 2011]

EGFR-epidermal growth factor receptor, GBM-glioblastoma multiforme, IL-interleukin, MG-malignant glioma, TGF-transforming growth factor

2.2.13 Prognosis and outcome

On many occasions, the survival and outcome data for MG patients are derived from controlled clinical studies where patients are carefully selected and the trials are conducted under strictly controlled circumstances. The true prognosis and outcome at the population level seems much bleaker.

Many different combinations of criteria are employed to predict the response to therapy and clinical outcome of MG patients. Some of the parameters taken into consideration include histopathological diagnosis with WHO tumour grading, age of the patient at diagnosis, gender, neurological performance status, tumour location, contrast enhancement in radiology, extent of surgical resection, proliferation indices and genetic and epigenetic alterations [Simpson et al., 1993] [Jeremic et al., 1994] [Louis et al., 2007b]. Even though these parameters do not allow accurate prediction of outcome for individual patients, they account for the vast majority of prognostic variability in MG [Vitucci et al., 2011]. The outcome of high-grade tumours is also largely dependent on the availability of effective treatment regimens [Louis et al., 2007b].

Histopathological features such as ischemic necrosis, cellular undifferentiation [Nakamura et al., 2007], increased microvascular proliferation [Leon et al., 1996] [Birlik et al., 2006] carry a poor prognosis in GBM. Molecular characteristics such as LOH of 10q (but not PTEN mutations as such) [Ohgaki et al., 2004] [Ohgaki and Kleihues, 2005b], activation of EGFR, EGFR*vIII* mutation [Desjardins et al., 2009], PI3K-AKT-mTOR [Phillips et al., 2006] pathways and deletion of NFKBIA [Bredel et al., 2011] [Chakravarti et al., 2002], upregulation of YKL40 [Pelloski et al., 2005] are associated with poor prognosis, while IDH1 mutations [Hartmann et al., 2010] and activation of Notch signalling [Phillips et al., 2006] carry a better prognosis in GBM. AA patients with wild-type IDH1 have a worse prognosis as compared to IDH1-mutated GBM patients [Hartmann et al., 2010]. MGs with an oligodendroglial component respond better to the PCV combination [Macdonald et al., 1990b]. In ODGs, subjects with LOH at 1p and 19q have a better prognosis with robust response to chemotherapy [Cairncross et al., 1998].

Overall MS for grade III AAs vary between ~1.6-3.5 years [Ohgaki and Kleihues, 2005a] [Vitucci et al., 2011] [Collins, 2004], with a 5-year survival of 11 % and a 10-year survival of 7 % [Ohgaki and Kleihues, 2005a]. For grade III ODG this time varied between 3.5-8.8 years [Ohgaki and Kleihues, 2005b] [Vitucci et al., 2011], with a 5-year and 10-year survival of 30 and 7.5 % respectively, while anaplastic OA have a MS between 1.5-3.9 year, with a 5-year survival of 12.5 % [Ohgaki and Kleihues, 2005a] [Vitucci et al., 2011]. Clinical trials reveal that the presence of necrosis is associated with significantly worse prognosis in OA [Miller et al., 2006] [van den Bent et al., 2006]. GBMs have a MS between 0.4-0.9 years and an average survival of ~8-11 months, with a six-month survival of 42.4 %, one-year survival of 17.7 %, 2-year survival of 3.3 %, 5-year survival of 1.2 % and 0.2 % survival at 10 years [Ohgaki and Kleihues, 2005a] [Ohgaki and Kleihues, 2005b] [Vitucci et al., 2011] [Simpson et al., 1993]. Overall 5-year survival for GBM in most surveys is less than 3 % [Stewart and Kleihues, 2003] [Ohgaki et al., 2004]. Moreover, age displayed an inverse correlation with

survival with GBM patients below 50 years having a MS of 8.8 months as opposed to 1.6 months for those over 80 years old [Ohgaki and Kleihues, 2005b]. MS time of *de novo* (primary) and secondary GBM is 4.7 and 7.8 months, respectively, but after age adjustments, there was no significant difference detected [Ohgaki and Kleihues, 2005b]. Most of these surveys were carried out before the rime when TMZ was added to standard therapy for GBM.

Historical data suggests that newly diagnosed MG patients who receive only supportive care have had a MS of ~14 weeks [Avgeropoulos and Batchelor, 1999]. Surgery alone increased the MS to ~20 weeks (3-4 months) [Kelly et al., 1984] [Paoletti, 1984]. Post-operative RT increased the MS up to 7-12 months [Laperriere et al., 2002] [Walker et al., 1978]. Addition of chemotherapy (mainly nitrosoureas) further increased the MS from 9.4 to 12 months [Fine et al., 1993] and one year survival from 40 to 46 % [Stewart, 2002].

Recurrence is considered as virtually universal in MG patients with the location often found within 2-3 cm of the resection margin [Burger et al., 1983] [Rostomily et al., 1994] in >80 % of patients [Wallner et al., 1989] [Hochberg and Pruitt, 1980]. Multi-focal recurrences are seen in ~5 % of the patients. The median time for progression after standard therapy is 6.9 months [Stupp et al., 2005a]. Re-operation provides a further 3-5 months of MS for these patients [Barbagallo et al., 2008]. The objective response rate for recurrent MG patients with salvage chemotherapy is ~6 % and 6-month PFS is 15 % [Wong et al., 1999] with a MS between 4-7 months [Chamberlain and Tsao-Wei, 2004] [Chua et al., 2004]. The majority of grade III MGs on recurrence has progressed to grade IV GBM [Ohgaki and Kleihues, 2005b]. Finally, recurrences that are deep-seated, contralateral and diffuse, with wide dissemination have a worse prognosis [Rostomily et al., 1994].

2.3 GENE THERAPY FOR MALIGNANT GLIOMA

2.3.1 Introduction

Gene therapy by definition is the delivery of genetic material into cells or tissues with a therapeutic intent. It encompasses a wide range of treatment types, all of which use genetic material to modify cells to attain the desired therapeutic effect [Mulligan, 1993]. Technical developments for the manipulation of genetic material, development of efficient vectors and advances in knowledge and better understanding of many disease processes at the molecular level, over the last few decades paved the way for gene therapy. The actual concept of gene therapy dates back to 1960s but since then, gene therapy has proven itself as a safe and acceptable therapeutic option and has demonstrated therapeutic efficacy in many disease conditions with several gene therapy drugs entering clinical trials.

Gene therapy has the advantage over conventional therapies due to the fact that it can be specifically targeted into the required location, thereby delivering locally a high therapeutic dose without risking systemic adverse effects. Furthermore, since most gene therapies are single-time applications, they can be cost-effective in the long-run.

2.3.2 History of gene therapy

The fundamental basis for gene therapy was laid with the demonstration in the early 1960s that, mammalian cells could be modified to incorporate and express foreign DNA. By the late 1960s, it was apparent that exogenous genetic information could be used for efficient genetic transformation [Friedmann, 1992]. The use of viruses/pseudovirions to transport therapeutic genes was proposed in this pre-recombinant DNA era [Friedmann, 1992]. The demonstration of the ability of modified tumour viruses to transfer a foreign genetic sequence to mammalian cells was a major milestone in this long journey [Friedmann, 1992]. The discovery of the calcium phosphate chemical transfection method laid the foundation to introduce therapeutic genes efficiently and functionally into mammalian cells and to the first human gene therapy study in 1980. In this study, Cline

and colleagues unsuccessfully attempted to treat thalassaemia by re-infusing bone marrow cells, *in-vitro* transfected with the human globin gene [Friedmann, 1992]. Though criticized on scientific, administrative and ethical grounds, Cline's human study triggered discussion on the scientific, ethical and public policy aspects of this new field, giving hope that these problems could be surmountable [Friedmann, 1992].

The discoveries of the presence of integrated proviruses from the viral RNA genome in the infected cells and reverse-transcriptase lead to the development of retroviral vectors that overcame the barriers of chemical transfection [Friedmann, 1992]. Several experiments demonstrated the utility of retrovirus vectors with the possibility of transducing many human cell types [Friedmann, 1992]. The vector armoury was further enlarged with the development of adenoviral vectors [Van et al., 1984] and adeno-associated virus vectors (AAV) [Friedmann, 1992].

Attention of gene therapy at this early stages was focussed towards monogenic disorders such as Lesch-Nyhan syndrome, adenosine deaminase (ADA) deficiency, familial hyper cholesterolaemia of LDLR deficiency, α1-antitrypsin deficiency, clotting factor deficiencies, and Gaucher disease [Friedmann, 1992].

US-FDA approved first gene therapy protocol was carried out in 1989, where tumour-infiltrating lymphocytes collected from advanced melanoma patients were *ex-vivo* transduced with a marker gene, expanded *in-vitro* and re-infused to the patients [Rosenberg et al., 1990]. The first gene therapy trial with a therapeutic intent was initiated in 1990 to treat an inherited immunodeficiency disorder: ADA deficiency, where patients' T-lymphocytes were *ex-vivo* transfected with the missing gene using retroviral vectors and re-infused back to the patients [Blaese et al., 1993] [Blaese et al., 1995]. Since then, multiple gene therapy clinical trials have been conducted for diseases such as cancer, vascular diseases, infectious diseases and inherited genetic disorders. The first proof of therapeutic cure with gene therapy came in year 2000 when Fisher and colleagues reported the successes in patients who underwent gene therapy for X-linked severe combined immunodeficiency (SCID) [Cavazzana-Calvo et al., 2000] and Aiuti *et al.* reporting success with ADA-SCID in 2002 [Aiuti et al., 2002]. Recent success stories for gene therapy were in the treatment of adrenoleukodystrophy [Cartier et al., 2009], Leber's congenital amaurosis [Maguire et al., 2009] [Bennett et al., 2012], Wiskott-Aldrich syndrome [Boztug et al., 2010] and haemophilia B [Nathwani et al., 2011].

Gene therapy was not without its setbacks. The death of Jesse Gelsinger in 1999 during a gene therapy clinical trial to treat ornithine transcarbamylase deficiency, due to multi-organ failure was the first such event [Lehrman, 1999] [Raper et al., 2003]. The next major setback was the three cases of leukemia occurring after gene therapy for SCID-X1 in France, most probably due to insertional mutagenesis caused by the retroviral vector, making a reality of the most feared complication of integrating vectors [Hacein-Bey-Abina et al., 2003a] [Hacein-Bey-Abina et al., 2003b]. Since then, one more patient has developed leukemia with one death due to leukemia [Hacein-Bey-Abina et al., 2008]. A similar study in UK also had one leukemia case out of 10 treated patients [Howe et al., 2008] In spite of the setbacks, the two trials were successful in terms of long-term disease control [Hacein-Bey-Abina et al., 2010]. The death of Jolee Mohr in a gene therapy clinical trial for rheumatoid arthritis was not attributed to gene therapy [Kaiser, 2007].

The first gene therapy drug approval for human use was Gendicine (Shenzhen SiBiono GeneTech Co. Ltd), which is an adenovirus vector expressing p53 for the treatment of advanced cancer, approved by Chinese regulatory authorities [Peng, 2005]. The second commercial gene therapy product, an oncolytic adenovirus H101 (Shanghai Sunway Biotech Co. Ltd) was also approved in China [Lu et al., 2004a].

By January 2012 a total of 1786 gene therapy clinical trial were registered at the Journal of Gene Medicine website (http://www.abedia.com/wiley/genes.php). More than 60 % of the trials were in clinical phase I and only 3.5 % were in phase III. Approximately 65 % of the

trials were targeting cancer, with monogenic, cardiovascular and infectious diseases being the other common indications (Figure 8).

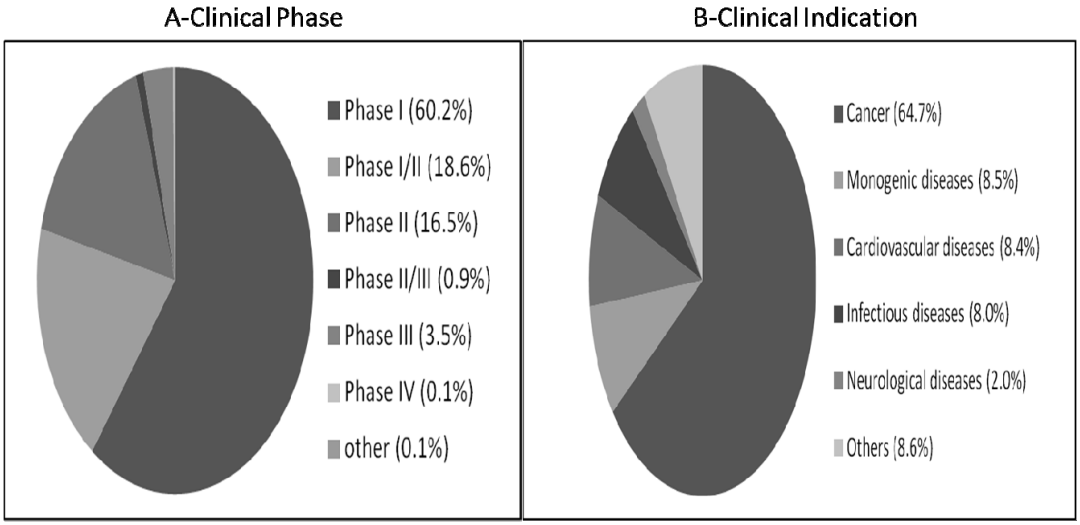

Figure 8: Gene therapy clinical trials grouped according to the phase of clinical evaluation (A) and the indication (B). Modified from http://www.abedia.com/wiley/genes.php

2.3.3 Gene therapy for cancer

The fact that gene therapy is viewed as a treatment strategy for cancer is not surprising considering that cancer is a major global health problem. Cancer is the second most common cause of death among adults in most developed counties, indicating a largely unmet need for the development of novel therapies. Conventional cancer therapies are usually hampered by their systemic toxicities, often leading to treatment discontinuation and failure. Gene therapy with its ability to specifically target the tumour has a clear advantage over conventional therapies. Many cancer types have been targeted in both pre-clinical and clinical settings with gene therapy, including lung [Roth et al., 1996], breast [Stewart et al., 1999], pancreatic [Chawla et al., 2010], liver [Guan et al., 2011], colo-rectal [Sobol et al., 1999], brain [Immonen et al., 2004], prostate [Herman et al., 1999], bladder [Malmstrom et al., 2010], head and neck [Clayman et al., 1999], ovarian [Kim et al., 2011b], renal cancer [Rini et al., 1999] and melanoma [Stewart et al., 1999]. Different gene therapy strategies have been employed for cancer such as pro-drug activating suicide gene therapy, anti-angiogenic gene therapy, oncolytic virotherapy, gene therapy-based immune modulation, correction/compensation of gene defects, genetic manipulation of apoptotic and tumour invasion pathways, antisense and RNAi strategies [Devi, 2006] and protection of bone marrow from chemotherapy [Sikora, 1995]. The two main problems to be overcome by gene therapy in cancer care are the need to target metastatic cancers, which account for 90 % of cancer mortality and the need to find the best way to combine gene therapy with conventional and novel targeted therapies in order to address the inherent heterogeneities of cancers that make them resistant to most therapies.

2.3.4 Gene transfer vectors

The greatest challenges encountered by gene therapy are to deliver an adequate amount of genetic material into the target tissue and to maintain the gene expression for the desired period of time. In gene therapy, vectors are used to deliver genes into the target tissue. An ideal vector should not have any limitations in the transgene capacity, be able to efficiently and specifically transduce dividing and non-dividing target cells and be able to express the transgene in an adequate amount for a sufficient duration of time. Manufacturing of the

vector should be easy and cost-effective and should be possible in high concentrations. In addition, the vector should not induce an immune response within the host, enabling repeated, safe vector administrations without any adverse effects [Romano et al., 2000] [Verma and Weitzman, 2005]. Sadly, none of the existing vectors match this ideal. At present, both viral, non-viral and cell based vectors are being used for this purpose [Lawler et al., 2006]. Viral vectors are being used in the majority of clinical trials and pre-clinical work. Adeno (23 %) and retrovirus vectors (20 %) are the most commonly used viral vectors in clinical trials. Vaccinia virus, poxvirus, AAV, HSV and lentivirus vectors are the examples of other viral vectors in use. Plasmid transfection and lipofection are the most common non-viral gene delivery methods in clinical trials.

Gene delivery into target tissue can be achieved by two methods. Direct *in-vivo* gene delivery means that the gene is delivered directly into the target tissue, tumour cavity after surgical resection, or into the systemic circulation. In *ex-vivo* gene transfer, the gene transduction is conducted outside the living organism, into previously isolated cells, which are re-introduced back to the target recipient. Direct intratumoural gene delivery has the limitation that it is possible only with accessible solid tumours and practically feasible only when the number of tumours is limited. Moreover, increased intratumoural pressure limits the amount of vector volume that can be injected and permeation of the vector within the tumour is variable, and is dependent on the multiple factors including the tumour cellular density and the size of the vector particles. The advantage of direct vector delivery is that transductional or transcriptional targeting of the vector is not required. Systemic delivery allows the vector to reach almost any part of the body. Hence, targeting of the vector specifically to the tumour cells by transductional targeting or restricting the transgene expression to tumour cells by transcriptional targeting is essential [Palmer et al., 2006]. Transductional targeting can be achieved either by modifying or blocking the vector cell surface molecules that interact with their natural cell surface receptors or by targeting novel specific receptors [Douglas et al., 1996] [Weitman et al., 1992]. Transcriptional targeting can be achieved by using tumour or tissue-specific promoters [Miller and Whelan, 1997], or by creating a biological difference in enzyme activity between tumour cells and normal cells, such as that of ribonucleotide reductase [Yoon et al., 2000]. Nestin [Dahlstrand et al., 1992], human TERT (hTERT) [Komata et al., 2002], E2F-1 [Parr et al., 1997], midkine [Kohno et al., 2004], survivin [Das et al., 2002], and COX-2 [Joki et al., 2000], are some of the promoters that are active in human gliomas. In addition, the use of radiation inducible promoters that can be induced by RT has been investigated [Rasmussen et al., 2002]. CED has been used to increase the gene transfer efficacy in the tumours [Hadaczek et al., 2005].

2.3.4.1 Viral vectors

Viral vectors are considered as the most effective of all gene delivery methods for *in-vivo* gene transfer [Chiocca et al., 2003]. The commonly used viral vectors for MG gene therapy include retroviruses, HSV [Shah et al., 2003], adenovirus [Wirth et al., 2009] [Maatta et al., 2009] and AAV virus [Mizuno et al., 1998]. In addition, baculovirus [Wang et al., 2006], Newcastle Disease virus (NDV) [Csatary and Bakacs, 1999], poliovirus [Gromeier et al., 2000], Semliki Forest virus [Ren et al., 2003], measles virus [Phuong et al., 2003], lentivirus [Naldini et al., 1996a], reovirus [Wilcox et al., 2001], and vaccinia virus [Gridley et al., 1998] have been used as vectors. In most of these vectors, the viral genome is genetically modified to make them replication defective and non-pathogenic, and to make space for the transgene. However, in oncolytic viruses, the genome is modified to provide it with the ability to replicate only within tumour cells that would lead to tumour destruction [Hurtado et al., 2005]. Some of the new viral vectors have the ability to replicate only once within the target cells in order to increase the dissemination and transgene copy number within the transduced cells without the ability to disseminate systemically [Bourbeau et al., 2007]. General features of commonly used viral vectors are summarised in Table 8.

Table 8: A summary of the general features of viral vectors in gene delivery

Vector	Genome	Envelope	Size (nm)	Insert size (kb)	Transduction	Integration	Expression
Adeno virus	DS DNA	No	70-100	4.7-30	Dividing and non-dividing cells	Episomal	Transient but high
Lenti virus	SS RNA	Yes	100-150	<10	Dividing and non-dividing cells	Integrating	Stable long term
AAV	SS DNA	No	20-25	4.5	Dividing and non-dividing cells	Episomal with low integration	Stable long term
HSV	DS DNA	Yes	100-200	30-150	Dividing and non-dividing cells	Episomal	Transient but longer duration

AAV-adeno-associated virus, DS=double stranded, HSV-herpes simplex virus, SS=single strand

2.3.4.1.1 Adenovirus vectors

First discovered in 1953, adenoviruses are pathogenic to humans as well as many animals. The human adenovirus family (*Adenoviridae*) has more than 50 serotypes that have the ability to infect both dividing and non-dividing human cell types including cells of the respiratory tract, eye, urinary bladder, gastrointestinal tract and liver resulting in mild infections [Verma and Weitzman, 2005] [Douglas, 2007]. They have a linear double-stranded DNA genome of 36 kb [Chroboczek et al., 1992]. Icosahedral viral particles (vps) are 70-100 nm in size. The capsid of the non-enveloped virus is made of hexons, pentons, protein IX and fibre proteins that facilitate receptor attachment. The majority of the adenovirus serotypes use the coxsackie and adenovirus receptor (CAR) to enter cells [Bergelson et al., 1997] [Tomko et al., 1997]. Other receptors such as MHC class-I [Hong et al., 1997], heparan sulphate glycosaminoglycans [Dechecchi et al., 2000], vascular cell adhesion molecule-1 (VCAM1) [Chu et al., 2001] also play a role in the receptor-mediated endocytosis [Zhang and Bergelson, 2005] of the virus through clathrin-coated vesicles [Meier et al., 2002]. Internalisation is facilitated by the pentons interacting with cellular integrin $\alpha v\beta 5$ that act as a co-receptor [Wickham et al., 1993]. The virus is then transported into the endosomes [Glasgow et al., 2006] [Verma and Weitzman, 2005]. Subsequent acidification of the endosomes results in disassembly of the viruses and release of the capsid, which docks at the nuclear pore complex, releasing the viral DNA into the nucleus, where it starts replicating in an episomal manner without integration into host genome [Glasgow et al., 2006] [Verma and Weitzman, 2005].

The ability to make the virus replication-deficient was crucial in the development of adenovirus vectors [Van et al., 1984]. Serotypes five and two are most commonly used as gene therapy vectors but other serotypes and nonhuman viruses have also been used. In the first-generation adenovirus vectors, the region encoding for early viral protein E1 is replaced by the transgene making the vectors replication defective [Danthinne and Imperiale, 2000]. By deleting the E1 gene, a transgene insertion capacity of 4.7-4.9 kb is achieved [Bett et al., 1993], which is further increased upon 8.3 kb by deleting the non-essential region E3 [Bett et al., 1994] [Verma and Weitzman, 2005]. Second-generation adenovirus vectors were created by deleting E2 and E4 regions to circumvent the problem of host cellular immune response against the viral proteins [Armentano et al., 1995] [Wang et al., 1995]. Cre/loxP system based site-specific DNA excision of 25 kb of adenovirus genome created high-capacity third-generation vectors [Parks et al., 1996] [Lieber et al., 1996] [Hardy et al., 1997]. A so-called "gutted" adenovirus vector was designed when all viral sequences except for the inverted terminal repeats (ITRs) and *cis*-acting packaging signals, were removed [Kochanek et al., 2001]. Conditionally replicating oncolytic

adenoviruses (CRAd) that replicate and lyse the tumours were created by deleting the E1B region [Bischoff et al., 1996]. Adenovirus vectors have a non-integrating, transient but high transgene expression in both dividing and non-dividing cells of many different tissues including lung, skeletal muscle, heart, liver, blood cells, vasculature and CNS [Kozarsky and Wilson, 1993] [Huard et al., 1995]. The systemic administration of the vector has a predilection to liver, spleen, heart, lung and kidney irrespective of the CAR expression status [Glasgow et al., 2006]. They can be produced at very high concentrations of 10^{12}-10^{13} particles/ml [Verma and Weitzman, 2005] and have been used in human studies at doses up to $3x10^{12}$ vps per ml (vp/ml) [Barzon et al., 2006]. Dose escalating studies have revealed toxicity with CNS symptoms, fever, leucocytosis, and hyponatremia with doses of 10^{12} vp/ml [Trask et al., 2000]. Many studies have demonstrated that the toxicities to adenoviral vectors are transient, mild inflammatory reactions, which are well tolerated [Barzon et al., 2006]. Since adenoviruses do not integrate their transgene into the host genome, their use does not carry the risk of insertional mutagenesis and the transient gene expression is not a major concern in cancer gene therapy, especially with suicide gene therapy. Their ability to transduce non-dividing cells increases the transduction efficacy especially in those tumours with a relatively low mitotic index. They gave a transduction efficacy from 0.01-10% with some areas of the tumour having an efficacy of 20% in one human glioma study with the LacZ marker gene [Puumalainen et al., 1998]. Some studies have demonstrated a transduction efficacy of 95-100% around the injection site with a mean maximum distance of about 5 mm [Lang et al., 2003].

A significant proportion of the human population can possess pre-existing antibodies that could rapidly inactivate the systemically administered adenoviral vectors [Nazir and Metcalf, 2005]. Some serological surveys have shown this to be as high as 40-60% for serotypes 1, 2 and 5 in children [Verma and Weitzman, 2005]. Vector injection results in an initial non-specific host response with the release of the cytokines TNF-α, IL-1 and IL-6, followed by a specific response directed against the infected cells mediated by cytotoxic T-lymphocytes, monocytes and NK cells, and a humoral response through activated B-cells and CD4+ T-lymphocytes giving rise to a major obstacle for the efficiency and a safety concerns for the use of adenovirus vectors [Verma and Weitzman, 2005] [Driesse et al., 2000]. This risk would be even greater with repeated vector administrations; making efficacy of repeated gene transfer very limited [Yang et al., 1994]. In spite of the brain being considered as an "immune privileged" region, intracerebral injection of adenovirus vectors is known to cause an immune reaction through a poorly understood mechanism [Lowenstein, 2002] [Dewey et al., 1999]. However, if the vectors are injected carefully into the brain parenchyma, avoiding the ventricles or systemic vasculature, as in the case of brain cancer gene therapy, it will only result in a transient innate immune response without any adaptive immune response [Stevenson et al., 1997b] [Stevenson et al., 1997a]. This is probably due to the lack of functional professional antigen presenting cells such as lymphoid DCs within the brain, the lack of classical lymphatics and the presence of the BBB [Lowenstein, 2002]. The ability of adenovirus vectors to mount an immune response within the host could be considered as an advantage in cancer gene therapy, especially in brain [Kajiwara et al., 1997].

Variable expression of the CAR receptor can limit the use of adenovirus vectors in MG gene therapy [Einfeld et al., 2001] [Mori et al., 1999]. However, in spite of the low CAR expression by MGs [Lawler et al., 2006], most of them were successfully transduced by adenovirus vectors [Fueyo et al., 2003] [Mori et al., 1999]. Chemical agents have been identified that increase the level of CAR expression such as HDACi/VPA [Goldsmith et al., 2007] [Kothari et al., 2010].

The wide tropism of recombinant adenovirus vectors could lead to reduced specificity of gene therapy with unwanted toxic effects. By targeting vectors to the appropriate cell type, these risks can be minimized, in addition to reducing the required vector dose. The use of serotypes with different tropisms [Chillon et al., 1999] and chemical attachment of different

ligands [Fisher et al., 2001] [Lanciotti et al., 2003] were some early attempts at targeting the vectors though without much success. Adopter-based adenovirus targeting uses bi-specific antibodies, antibody fragment chemical conjugates [Douglas et al., 1996] [Gu et al., 1999] [Dmitriev et al., 2000] [Kashentseva et al., 2002], cell-selective ligands and direct introduction of a targeting ligand onto the fibre knob, as a molecular bridge between the vector and the target cell surface receptor, bypassing the native receptor and creating novel tropism with the new receptors [Glasgow et al., 2006]. Pseudotyping with different fibre proteins [Krasnykh et al., 1996], expression of chimeric fibre proteins and other capsid proteins [Vigne et al., 2003], ligand incorporation into the fibre knob [Lamfers et al., 2002] and de-knobbing of fibre coupled with ligand addition, are some of the other attempts at transductional targeting of adenovirus vectors [Glasgow et al., 2006]. Overexpressed EGFR in MG [Libermann et al., 1985] has been used to target the adenovirus vectors to glioma cells, capitalising on the fact that ligand bound EGFR is internalized by activation of PI3K-dependent pathway [Li et al., 1998]. Bispecific antibody conjugates [Miller et al., 1998], bispecific single-chain antibodies [van, V et al., 2002], chimeric fusion molecules [Dmitriev et al., 2000] and bFGR-2 [Wang et al., 2005] have been used successfully to retarget adenoviral vectors to the EGFR. Adenoviral vectors with the ability to replicate only once within the target cells without the ability to disseminate have increased the transgene copy number and expression within the transduced cells. This has been shown to improve survival in a murine glioma model when compared to standard adenoviral vectors [Bourbeau et al., 2007]. Another method that has been used to target gene therapy is transcriptional targeting, where a tissue specific or tumour specific promoter regulates the transgene expression only in tumour cells, or regulatable promoters which can be switched on and off on-demand [Palmer et al., 2006]. The use of the survivin or midkine promoters in a CRAd-5/3 has demonstrated significant tumour cell specificity towards glioma cell lines [Ulasov et al., 2007] [Kohno et al., 2004]. Blocking the binding of adenovirus vectors to the native cell surface receptors and the bridging interactions formed by coagulation factor is termed detargeting. This can be achieved by genetic ablation of native receptor-binding determinants, blocking bridging interactions or by using polymer-coated "stealth" vectors to avoid these interactions [Coughlan et al., 2010]. The immunogenicity and toxicity of the vector can be reduced by blocking the interaction with natural receptors [Palmer et al., 2006], increasing the safety and bypass the pre-existing anti-vector immunity, thus increasing its efficacy [Verma and Weitzman, 2005].

2.3.4.1.2 Lentivirus vectors

Lentiviruses belong to the *Retroviridae* family and are often derived from human immunodeficiency virus-1 (HIV-1) [Goff, 2001]. Other non-HIVs have also been used as gene therapy vectors. General features of lentivirus vectors are summarised in Table 8 [Naldini et al., 1996b]. The wild-type virus binds to the cell surface receptors such as heparan sulphate proteoglycans (HSPG), CD4 and chemokine receptors and are internalised into CD4 cells, macrophages and DCs. The nucleoprotein complex is released into the cytoplasm [Freed and Mouland, 2006] where it is reverse-transcribed into a complementary DNA (cDNA) by the reverse transcriptase complex. The resulting cDNA incorporated pre-integration complex enters the nucleus and integrates into the host genome [Anderson and Hope, 2005]. Lentivirus vectors only have about 5 % of the original wild-type genome. They are considered as low-immunogenic vectors. Usually the lentivirus vectors are pseudotyped by vesicular stomatitis virus glycoprotein G (VSV-G) [Burns et al., 1993]. Pseudotyping increases the tropism as well as the immunogenicity of the vector [Baekelandt et al., 2003]. The vector has the ability to transduce quiescent cells such as neurones [Cockrell and Kafri, 2007]. Drawbacks of lentivirus vectors are low vector titres, risk of insertional mutagenesis and possible replication-competency. Lentivirus vectors have been used in clinical trials for the treatment of HIV [Dropulic and June, 2006] [Levine et al., 2006], β-thalassemia [Bank et al., 2005] and adrenoleukodystrophy [Cartier et

al., 2009]. They have been used in several pre-clinical MG gene therapy experiments [Naldini et al., 1996a] [Stein and Davidson, 2002] [Guo et al., 2011] [Lopez-Ornelas et al., 2011]. Lentiviruses pseudotyped with lymphocytic choriomeningitis virus (LCMV) envelope glycoprotein have demonstrated a better transduction in MG models [Huszthy et al., 2009] [Miletic et al., 2007] [Miletic et al., 2004].

2.3.4.1.3 Other viral vectors

AAV belongs to the *Parvoviridae* family. These are non-enveloped, single-stranded DNA viruses, sized 20-25 nm with an icosahedral capsid [Xie et al., 2002]. More than 10 serotypes of this virus have been identified [Mori et al., 2004]. AAV, with the aid of capsid proteins, binds to host cells surface proteins such as HSPG, FGF receptor-1 (FGFR-1), integrin $\alpha_v\beta_5$ and HGFR leading to clathrin-mediated endocytosis [Kashiwakura et al., 2005] [Qing et al., 1999] [Summerford and Samulski, 1998]. Wild-type viruses have a specific predilection to integrate into chromosome 19 [Miller et al., 2002]. AAV serotype-2 (AAV-2) vectors with a transgene insertion capacity of 4.5 kb and a tropism to muscle, lung, retina, neurons, vascular smooth muscles and hepatocytes, are most commonly used in gene therapy applications [Wu et al., 2006] [Verma and Weitzman, 2005]. They can be produced in titres of 10^{10} pfu/ml [Conway et al., 1999]. AAV vector-mediated gene transfer can result in both episomal gene expression [Afione et al., 1996] and integration [Nakai et al., 2001]. The AAV vectors have the ability to transduce non-dividing cells. Due to their small size, AAVs have the ability to penetrate solid tumours effectively [Raj et al., 2001]. They are non-pathogenic and non-toxic in humans, but carry the risk of insertional mutagenesis and have a limited transgene insertion capacity. The possibility of pre-existing host immunity to the vector is also a concern [Tenenbaum et al., 2003]. There is evidence to suggest that AAV can trigger an immune response within the host [Kok et al., 2005]. AAV vectors have been used in many pre-clinical studies on brain cancer, with mixed success [Okada et al., 1996] [Hadaczek et al., 2005] [Harding et al., 2006].

Herpes viruses are human pathogens belonging to *Herpesviridae* family consisting of eight members [Brady and Bernstein, 2004]. HSV-1 is a large, enveloped, double stranded DNA virus, pathogenic to humans causing cytolytic replication in mucosal or epithelial cells, with a neuronal predilection and the ability to induce latent infection due to the persistence of the viral genome [Verma and Weitzman, 2005]. Two types of HSV vectors are in use, non-replicating vectors and replicating oncolytic vectors. In the non-replicating group, recombinant HSV-1 vectors have a non-integrating transient gene expression with a transgene insertion capacity of 30 kb [Burton et al., 2002] and a relatively longer transgene expression in neurones. These vectors can be produced in titres of 10^{10} pfu/ml. Due to the expression of viral proteins, they can be cytopathic and cytotoxic, and can induce an immune response within the host [Miller and Fraser, 2000] [Verma and Weitzman, 2005]. HSV-1 amplicon vectors have a minimal remaining viral genome and theoretically a large transgene insertion capacity (150 kb) with the ability to transduce both dividing and non-dividing cells [Sena-Esteves et al., 2000], but need the helper virus for production [Verma and Weitzman, 2005]. HSV-G207 and HSV-1716 are conditionally replicating HSVs with specific gene mutations and a selective ability to replicate only within dividing cancer cells, leading to oncolysis [McKie et al., 1996] [Mineta et al., 1994]. Both of these viruses have been tried on MG in both pre-clinical as well as in clinical studies [Rampling et al., 2000].

2.3.4.2 Non-viral vectors

Due to their low toxicity, low immunogenicity, and lack of infectivity, non-viral gene delivery methods are considered as safe alternatives for viral vectors that have a long-term stability and a large transgene carrying capacity [Barzon et al., 2006]. Furthermore, production and manipulation of these delivery methods are relatively easy when compared to viral vectors [Schmidt-Wolf and Schmidt-Wolf, 2003].

Direct intra-lesional or systemic injections of naked/plasmid DNA are the simplest of these methods but have the disadvantages of low transduction efficacy *in-vivo* and the risk of degradation by nucleases and phagocytes [Kawabata et al., 1995]. Calcium phosphate precipitation facilitates the gene transfer by disrupting the electrochemical barrier between cell membrane and the DNA, but is no longer very popular [Wells, 2004]. Electroporation, gene-gun, ultrasound and laser techniques can be used to increase the cell membrane permeability to facilitate transduction by naked/plasmid DNA [Mehier-Humbert and Guy, 2005]. The non-integrating episomal nature of plasmid DNA with low transduction efficiency and transient gene expression, have been limiting factors encountered with this method.

In order to protect the plasmid DNA from endogenous degradation and to facilitate entry through cell membrane, DNA can be packed into liposomes made of lipid bi-layers, polymers or micelles creating synthetic vectors [Yoshida and Mizuno, 2003]. Cationic-lipid/DNA complexes (lipoplex) [Felgner et al., 1987], cationic polymer/DNA complexes (polyplex) [Wu and Wu, 1987] and lipid-polymer hybrid systems have also been developed. Lipoplex- and polyplex-plasmid complexes [Thomas and Klibanov, 2003] have significantly improved transduction efficacy when compared to the traditional plasmids [Schatzlein, 2001]. Monoclonal antibodies have been used successfully to target liposomes to glioma cells [Zhang et al., 2004].

Attempts have been made to create plasmid DNA vectors capable of transgene integration and long-term gene expression using the Sleeping Beauty transposable element with positive results in GBM pre-clinical models [Wu et al., 2007] [Ohlfest et al., 2005]. The main disadvantage of this method is that these retro-transposons have a predilection to integrate into actively transcribed genes leading to possible problems with cellular function [Hackett et al., 2005]. In another attempt, phiC31 integrase system was used to integrate plasmid DNA into preferred locations in the mammalian genome for long-term gene expression [Calos, 2006].

Direct introduction of synthetic oligonucleotide [Kamiyama et al., 2002] or plasmid-based siRNA [Kang et al., 2005] into the target cells with the aim of gene silencing has been another approach for non-viral gene delivery that has been successful, especially in cancer gene therapy [Loke et al., 1989].

Bacteria also have been utilised as gene therapy vectors. They have the ability to carry the transgene into many cell types and are easy to produce but carry the risk of immunogenicity [Higgins and Portnoy, 1998].

Cellular gene delivery method is an up-and-coming gene delivery method, where NSCs [Yip et al., 2003] [Ehtesham et al., 2002], bone marrow-derived stem cells [Lee et al., 2003], mesenchymal stem cells [Nakamura et al., 2004] and endothelial progenitor cells [Moore et al., 2004] are used as vectors for their tumour homing ability, to carry a therapeutic payload to the primary tumours as well as metastatic lesions including micrometastasis [Thorne, 2007]. NSCs have an inherent tumour tropism and tumour killing ability, and are tolerated by the immune system. They can be genetically modified to carry anti-tumour transgenes and can be administered systemically [Lawler et al., 2006]. This method has the potential to develop as an alternative to viral gene delivery or to enhance viral gene delivery [Herrlinger et al., 2000].

Non-viral gene delivery methods have been used in ~25 % of registered clinical trials by January 2012 (http://www.abedia.com/wiley/vectors.php). Both naked/plasmid DNA and lipofection using cationic liposomes have been used in several gene therapy clinical trials for brain cancer [Yoshida et al., 2004].

2.3.5 Gene therapy strategies

The dismal prognosis without an appreciable improvement in the clinical outcome over the last few decades has made MG an ideal candidate for novel therapeutic development. Conventional cancer therapies have been mostly unsuccessful due to the diffusely

infiltrative nature of MGs, preventing complete surgical resection, the inherent radioresistance and limitations in the radiation dose to the brain, limited delivery of chemotherapeutics into the brain and the resistance of the tumours to most of the chemotherapies. On the other hand, most of the MGs are single, localized lesions of rapidly dividing cells in a background of non-dividing brain parenchyma, that rarely metastasise, and recurrences are most often close to the original lesion, making them highly amenable for gene therapy [Lawler et al., 2006]. Brain tumours have been a target for gene therapy for many years, with the first clinical trials being registered in 1992. In this first trial, Brenner *et al.* used autologous tumour cells, *ex-vivo* modified to express the IL-2 gene using retroviral vectors, in neuroblastoma patients. In the same year, Oldfield *et al.* tested HSV-tk suicide gene therapy using retrovirus vector producing cells (VPCs) with intravenous (i.v.) GCV therapy in brain cancers [Oldfield et al., 1993] [Ram et al., 1997].

Many gene therapy strategies are under extensive evaluation for MG therapy at the pre-clinical and clinical trial levels. Table 9 summarises the gene therapy strategies under development for MG. Though most of these methods have demonstrated immense success in *in-vitro* and pre-clinical level, rather few approaches have progressed up to phase III clinical trials [Pulkkanen and Yla-Herttuala, 2005] [Barzon et al., 2006]. In most instances, gene therapy has been attempted as a monotherapy approach in both pre-clinical and clinical trials with only a few attempts at combining different therapy strategies.

Table 9: Gene therapy strategies for MG

Strategy	Stage of development	Reference
Pro-drug activating suicide gene therapy	Clinical trials	[Castro et al., 2011]
Anti-angiogenic gene therapy	Pre-clinical	[Samaranayake et al., 2010]
Oncolytic virotherapy	Clinical trials	[Castro et al., 2011]
Gene therapy-based immune modulation	Clinical trials	[Castro et al., 2011]
Correction of tumour genetic defects	Clinical trials	[Castro et al., 2011]
Inhibition of tumour invasion	Pre-clinical	[King et al., 2005] [Castro et al., 2003]
Induction of apoptosis	Pre-clinical	[King et al., 2005] [Castro et al., 2003]
Gene therapy to enhance chemo- and RT	Pre-clinical	[King et al., 2005] [Castro et al., 2003]
Myeloprotective gene therapy	Clinical trials	http://www.abedia.com/wiley/index.html
Antisense and RNAi-based strategies	Clinical trials	http://www.abedia.com/wiley/index.html

RNAi-RNA interference, RT-radiotherapy

2.3.5.1 Pro-drug activating suicide gene therapy

Pioneering concepts of employing this innovative approach for cancer therapy dates back to 1960s, where endogenous enzymes highly expressed in tumour cells were used for pro-drug activation [Connors and Whisson, 1966] [Cobb et al., 1969]. The principle of pro-drug activating suicide gene therapy, which is also known as cytotoxic gene therapy, is to introduce a transgene encoding for an enzyme, that is either absent in mammalian cells or present in a very inactive form, into the tumour cells, or into the surgical cavity of resected tumours. Enzyme produced by the transduced cells will convert the subsequently administered inactive pro-drug into its active form, evoking the death of cells expressing the therapeutic gene. The phenomenon that kills the neighbouring non-transduced cells is termed the "bystander effect" [Freeman et al., 1993], which is fundamental for the therapeutic success. It can result from the transfer of the active metabolite into the neighbouring cells via gap-junctions [Elshami et al., 1996] [Mesnil et al., 1996], apoptotic vesicles [Freeman et al., 1993] or through simple diffusion [Huber et al., 1994]. Activation of the anti-tumour host immune system by cell killing also contributes to the bystander effect [Barba et al., 1994]. The lipophilicity of the pro-drug and the active metabolite also influence the bystander effect [Wilson et al., 2002]. The bystander effect compensates for the limited transduction efficacy of gene delivery vectors. The potent and highly targeted

tumour-specific nature of this approach places it far ahead of conventional chemotherapy. The possibility of avoiding toxic effects of systemic drug administration is the other major advantage of this method. For the pro-drug activating suicide gene therapy to be successful, the suicide enzyme has to be selectively expressed in the tumour cells, the pro-drug used should be selective to the activating enzyme, which should metabolise the pro-drug rapidly and effectively into the potent cytotoxic form that kills cancer cells in all stages of the cell-cycle with a good bystander effect [Denny, 2003]. Both the pro-drug and the active metabolite should have good biodistribution properties, and the pro-drug or the expression of suicide gene itself should not be toxic to the cells.

This is a widely used gene therapy approach for the treatment of cancer involved in 144 registered clinical trials commonly using retrovirus and adenovirus vectors. It is the most frequently employed gene therapy application for the treatment of MG with over 20 registered clinical trials by January 2012 (http://www.abedia.com/wiley). Multiple pro-drug activating suicide gene therapy strategies have been described in the literature [Niculescu-Duvaz and Springer, 2005]. HSV-tk/GCV, cytosine deaminase (CDA)/5-fluorocytosine (5-FC), cytochrome P450/cyclophosphamide (CPA), *Escherichia coli (E.coli)* purine nucleoside phosphorylase (PNP)/6-methyl-purine-2'-deoxynucleoside, and carboxypeptidase G2 (CPG2)/methotrexate-α-phenylalanine are some of the pro-drug activation systems evaluated for MG treatment [King et al., 2005]. Apart from the commonly used retrovirus vectors/VPC and adenovirus vectors [Pulkkanen and Yla-Herttuala, 2005], HSV and lipofection have been used as the vector to introduce the suicide gene in clinical trials [Barzon et al., 2006]. Research at the pre-clinical level has demonstrated successful delivery of HSV-tk transgene replication-deficient retrovirus vectors [Vincent et al., 1996], retroviral packaging cells [Takamiya et al., 1993], replication-deficient adenovirus vectors [Chen et al., 1994], replication competent adenovirus [Nanda et al., 2001], HSV [Boviatsis et al., 1994a] [Boviatsis et al., 1994b], and AAV vectors [Okada et al., 1996]. Neural stem cells have also been used to deliver pro-drug converting enzymes [Barresi et al., 2003] [Uhl et al., 2005], and to enhance the transduction efficacy of viral vectors [Herrlinger et al., 2000].

Most suicide gene therapy strategies depend on the ability of the systemically delivered pro-drug to reach the target site, limiting the efficacy of this method. Non-specific activation of the pro-drug in unintended tissues and the diffusion of the active drug into the systemic circulation may lead to toxicities. Limitations in the transduction efficacy of currently available vectors are a major hurdle to overcome. Gene silencing can result in non-expression of the transgene or expression for a limited duration of time, even in effectively transduced cells [Frank et al., 2004].

Development of more efficient gene delivery vectors, increased transgene copy number [Kim et al., 2000], novel/mutated suicide enzymes with better substrate affinity [Fuchita et al., 2009] [Black et al., 2001], fusion suicide genes [Erbs et al., 2000], pro-drugs with better pharmacokinetic and pharmacodynamics properties [Davies et al., 2005], enhancing the bystander effect, combination with standard and novel therapeutics [Boucher et al., 2000] and better treatment protocols to maximise the synergistic effects represents the ways forward for suicide gene therapy [Wirth et al., 2009] [Maatta et al., 2009].

2.3.5.1.1 Overview of suicide gene therapy strategies

An overview of the suicide gene therapy strategies are presented in Table 10.

Table 10: Suicide gene therapy strategies for MG

Suicide enzyme	Pro-drug	Active metabolite	Mechanism/s of action	By-stander effect	References
HSV-tk	GCV	GCVTP	Inhibits incorporation of dGTP into DNA and DNA polymerase leading to mitochondrial or death receptor-mediated apoptosis	Cell contact dependent tumour regression with 10 % transduction	[Castro et al., 2011] [King et al., 2005]
CDA- *(E. coli & S. cerevisiae)*	5-FC	5-FU	Inhibits thymidylate synthase, reduces dTTP; inhibits DNA synthesis & repair. Cell-cycle independent mitochondrial apoptosis, independent of p53 or death receptor status	Independent of cell-cell contact. Significant tumour regression with 2-4 % transduction	[Castro et al., 2011] [King et al., 2005]
Cytochrome P450	CPA	Mustard-like toxin	DNA crosslinking & protein alkylation leading to apoptosis	Independent of cell-cell contact	[Castro et al., 2011]
PNP (*E. coli*)	Purine analogue	Toxic adenine analogue	Inhibits mRNA and protein synthesis leading to cell-cycle dependent apoptosis	Cell contact independent. Tumour elimination with 2-5 % transduction	[Castro et al., 2011] [King et al., 2005]
CPG2-	CMDA	Mustard-like toxin	DNA crosslinking leading to cell-cycle independent apoptosis	Transduction of 10-12 % leading to 50-100 % cell killing	[Castro et al., 2011] [King et al., 2005]

CDA-cytosine deaminase, CMDA-4-benzoyl-L-glutamic acid CPA-cyclophosphamide, CPG2-carboxypeptidase G2, dTTP-deoxy thymine triphosphate, FC-fluorocytosine, FU-fluorouracil, GCV-ganciclovir, GCVTP-GCV triphosphate, HSV-tk-herpes simplex virus thymidine kinase, PNP-purine nucleoside phosphorylase

2.3.5.1.2 *Herpes simplex* virus type-1 thymidine kinase/GCV

The HSV-tk gene was cloned in 1980 [McKnight, 1980] and its potential as a suicide enzyme for cancer treatment soon became established [Moolten, 1986] [Moolten, 1987]. The HSV-tk suicide gene therapy with pro-drug GCV approach is the most extensively studied and widely used suicide gene therapy approach for cancer including MG [Pulkkanen and Yla-Herttuala, 2005]. It is by far the most advanced of all MG gene therapy strategies in clinical trials to date having completed two multi-centre randomised phase III clinical trials [Rainov, 2000] (Press release by Ark Therapeutics PLC 18/12/2009). Apart from MG, the HSV-tk/GCV approach has been evaluated in several cancer types including prostate cancer [Nasu et al., 2007], malignant mesothelioma [Sterman et al., 2005], ovarian cancer [Link, Jr. et al., 1996] and liver cancer [Li et al., 2007].

The proof-of-concept for the use of HSV-tk/GCV gene therapy for the treatment of cancer was established in 1990 [Moolten and Wells, 1990] [Moolten et al., 1990]. Several pre-clinical studies demonstrated a tumour regression [Culver et al., 1992] [Izquierdo et al., 1995] and an increased survival [Ram et al., 1993] [Rainov et al., 1996] by treating orthotopic MGs by intratumoural implantation of HSV-tk producing VPCs in rodent models. In the initial studies, a mixture of glioma cell and VPCs were inoculated at the same time followed by GCV treatment [Culver et al., 1992], whereas in others, VPCs were inoculated one week later [Ram et al., 1993]. Other studies showed tumour regression as well as improved survival [Barba et al., 1994] and some even demonstrated complete cure [Izquierdo et al., 1995]. There are also reports suggesting to only a limited response using this approach [Benedetti et al., 1997] [Poptani et al., 1998] [Sandmair et al., 1999]. One comparison study

gave similar response with retrovirus VPCs and adenovirus vectors [Vincent et al., 1996], but later it was shown that adenovirus vectors have a better transduction efficacy than retrovirus vectors in human MG [Puumalainen et al., 1998]. The use of adenovirus vector-mediated HSV-tk gene delivery has resulted in regression of tumour volume [Chen et al., 1994] [Maron et al., 1996] including complete responses [Perez-Cruet et al., 1994] and increased survival [Vincent et al., 1996] [Tyynela et al., 2002] in MG animal models. In comparative studies, using both types i.e. adenovirus vectors and retrovirus VPCs, adenovirus vectors showed a better response [Sandmair et al., 2000a] and this was found to be curative when the transduction efficacy was high enough, [Sandmair et al., 2000b] suggesting that the low therapeutic efficacy with retroviral vectors could mainly be due to their low transduction efficacy [Sandmair et al., 1999]. Toxicity studies have not revealed any serious adverse effects except for mild reactive gliosis at the injection site, with the use of retrovirus vectors [Raffel et al., 1994] and adenovirus vectors [Langford et al., 2009], as well as with adenoviral vectors in non-human primates, suggesting that this is a safe therapeutic option [Driesse et al., 1998]. However, there is evidence that long-term expression of HSV-tk can lead to chronic inflammation [Dewey et al., 1999] [Thomas et al., 2000] and virus entry into CSF can lead to toxicities [Driesse et al., 2000].

Mechanisms of action of HSV-tk/GCV

The HSV-tk homodimer is capable of phosphorylating T, deoxycytidine, deoxythymidylate and several pyrimidine and guanosine analogues into their monophosphates. The antiviral drugs, acyclovir and GCV, can act as substrates for HSV-tk, which has a 1000-fold higher affinity for GCV compared to its mammalian counterpart [Palmer et al., 2006]. GCV is a synthetic acyclic analogue of deoxy-guanosine, which was developed in the early 1980s [Smith et al., 1982] [Ashton et al., 1982] [Martin et al., 1983] for the treatment and prevention of CMV infection. The chemical formula of GCV is $C_9H_{13}N_5O_4$ with a molecular mass of 255g/mol and is a hydrophilic compound with poor pharmacokinetic properties [Denny, 2003]. Oral bioavailability is poor, hence it is administered parenterally. The drug has a renal clearance and a serum half-life of 2.5 to 5 hours [Noble and Faulds, 1998]. Adverse effects of GCV include neutropenia, thrombocytopenia, headache, convulsions and coma [Faulds and Heel, 1990]. GCV enters the mammalian cells via nucleoside transporters [Buursma et al., 2004], where it is phosphorylated into 5'monophosphate (GCVMP) by HSV-tk with a substrate K_m of 47μM [Field et al., 1983] (Figure 9). This is considered as the rate-limiting step of GCV phosphorylation. Mammalian guanylate kinase and nucleoside diphosphokinase further phosphorylates GCVMP into GCV-diphosphate (GCVDP) and GCV-triphosphate (GCVTP), respectively [Cheng et al., 1983] [Boehme, 1984]. GCVTP competitively inhibits the incorporation of deoxy-guanosine-triphosphate (dGTP) into DNA. GCVTP is highly cytotoxic due to its ability to incorporate into the nascent DNA strand causing DNA double-strand destabilisation, termination of strand elongation [Mesnil and Yamasaki, 2000], SSBs of DNA [Moolten, 1986], and inhibiting the progression of cell-cycle through S-phase [Halloran and Fenton, 1998] leading to a G2/M arrest [Tomicic et al., 2002] and cell death [Rubsam et al., 1998]. There is some evidence to suggest that GCVTP can inhibit DNA polymerase [Ilsley et al., 1995] (Figure 9). The exact mechanism of GCV induced cell killing is yet to be revealed. There are reports suggesting that cell death occurs by apoptosis [Hamel et al., 1996] [McMasters et al., 1998] induced by declining Bcl-2 levels [Tomicic et al., 2002] or phosphorylation of Bcl-x_L [Fischer et al., 2005b] and activating caspases triggering the mitochondrial apoptotic pathway [Glaser et al., 2001] [Tomicic et al., 2002], independent of p53 and death receptors [Fischer et al., 2005b], while others claim that there is a p53-dependent death receptor-mediated apoptosis [Beltinger et al., 1999]. Evidence suggests that GCV can induce chromosomal aberrations and sister-chromatid exchange, an indication of a possible involvement of HR DNA DSB repair pathways [Thust et al., 1996] [Helleday, 2003] [Sonoda et al., 1999] and a role for the BER pathway in reducing the

efficacy of HSV-tk/GCV [Tomicic et al., 2001] (Figure 9). HSV-tk/GCV-mediated cytotoxicity is cell-cycle dependent and affecting mainly dividing cells. This is an advantage in brain cancer gene therapy when tumour cells are dividing amidst non-dividing normal brain cells. Fourteen-day treatment with GCV has been shown to kill HSV-tk transduced glioma cells in pre-clinical studies [Barba et al., 1993].

Local and systemic immune responses contribute to the HSV-tk/GCV-mediated anti-tumour effect [Barba et al., 1994] [Rainov et al., 2000] by enhancing the tumour infiltration of, CD4+ and CD8+ T-cells, NK cells and macrophages. This was accompanied with an increased expression of cytokine IL-12 and Fas ligand (FasL), by the host [Perez-Cruet et al., 1994] [Rainov, 2000]. Induction of the immune system resulted in tumour regression in local as well as at distant sites in both normal and immune-compromised animals [Dilber et al., 1996] [Bi et al., 1997], with long-term immunity against subcutaneous tumours [King et al., 2005]. Due to their ability to transduce and kill dividing endothelial cells [Puumalainen et al., 1998] HSV-tk gene therapy is also thought to elicit an anti-angiogenic effect [Lawler et al., 2006] (Figure 9).

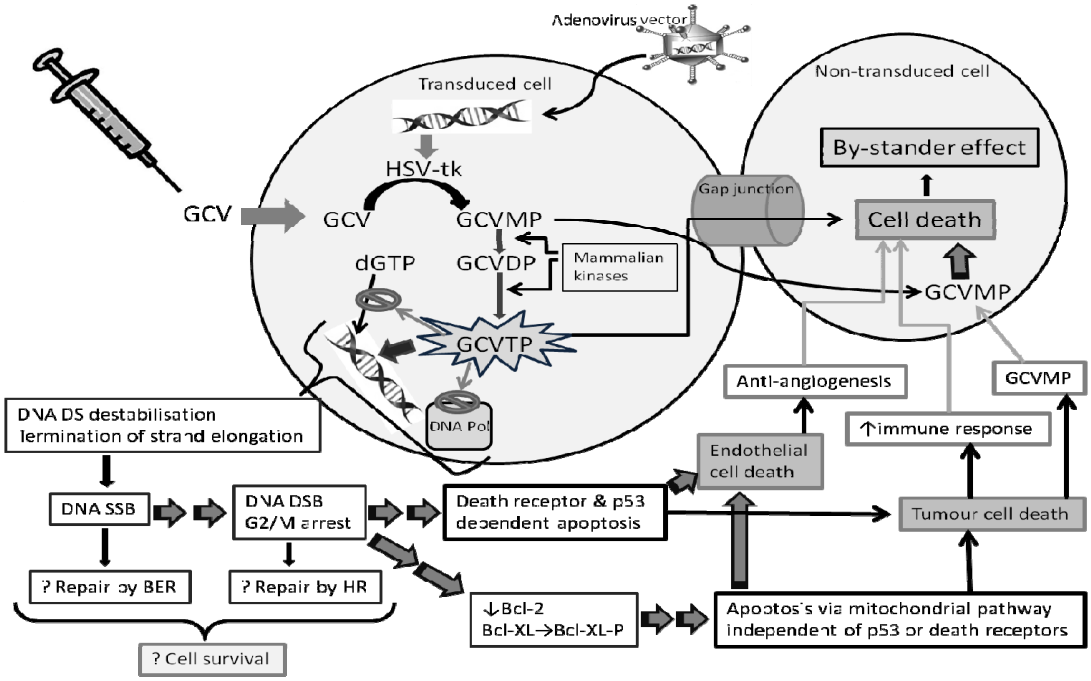

Figure 9: Principle of HSV-tk/GCV therapy including the bystander effect. Bcl-2 B-cell lymphoma-2, Bcl-XL-P-phosphorylated Bcl-XL, BER-base-excision repair, dGTP-deoxy guanosine triphosphate, DNA pol-DNA polymerase, DSB-double-stranded break, GCV-ganciclovir, GCVMP-GCV-monophosphate, GCVDP-GCV-diphosphate, GCVTP-GCV-triphosphate, HR-homologous recombination, HSV-tk-Herpes simplex virus thymidine kinase, SSB-single strand break. Refer to the text for the relevant references.

Bystander effect of HSV-tk/GCV

The bystander effect plays an important role in HSV-tk/GCV-mediated cytotoxicity [Freeman et al., 1993] even though the mechanism is not completely understood. Free diffusion across cell membrane of GCVDP and GCVTP is unlikely due to the negative charge of the molecules. The most widely accepted mechanism is the diffusion of toxic GCVTP via gap-junctions in a cell-to-cell contact-dependent manner, to neighbouring non-transduced cells, and thus triggering their apoptosis [Elshami et al., 1996] [Mesnil et al., 1996] (Figure 9). However, there is also evidence suggesting that cell-to-cell contact is not essential for HSV-tk/GCV-mediated bystander effect [Bai et al., 1999] [Rubsam et al., 1999]. Many experiments in murine models have suggested that 10 % of the tumour cells have to

be transduced with the HSV-tk gene for HSV-tk/GCV system to completely or significantly regress the tumour both *in-vitro* and *in-vivo* [Sandmair et al., 2000b] [Culver et al., 1992] [Freeman et al., 1993]. In addition, the effect seems to be dependent on the MG cell type [Burrows et al., 2002] [Ishii-Morita et al., 1997]. For example, C6 gliomas seem to be more sensitive to the treatment when compared to human glioma cell lines [Sturtz et al., 1997]. Evidence points to an inhibition of HSV-tk/GCV bystander effect by co-administration of dexamethasone, possibly due to a reduction of gap-junction-mediated intercellular communication, inhibition of gap-junction synthesis or to some kind of modulation of the apoptotic cascade [Robe et al., 2005]. Other possible mechanisms responsible for the bystander effect could be the stimulation of the host immune system to attack the tumour [Colombo et al., 1995] [Kianmanesh et al., 1997], endothelial cell transduction leading to disruption of the tumour vasculature [Ram et al., 1994], and phagocytosis of apoptotic vesicles by neighbouring non-transduced cells [Freeman et al., 1993] (Figure 9).

Methods to enhance HSV-tk/GCV efficacy

A major limiting factor for HSV-tk/GCV gene therapy is the limited transduction efficacy [Puumalainen et al., 1998] [Sandmair et al., 1999] [Rainov, 2000]. Increasing the number of vector administrations has increased the therapeutic efficacy of AdHSV-tk and GCV suicide gene therapy [Tyynela et al., 2002]. In clinical trials, multiple vector injections into the tumour cavity have been used [Immonen et al., 2004]. CED of vectors has been attempted to increase the transduction efficacy [Hadaczek et al., 2005] and other improvements of the vectors have also been investigated to enhance the transgene expression [Maatta et al., 2006].

Even though the affinity of HSV-tk to GCV is high (K_m= 47μM) [Balzarini et al., 1993] its affinity for the thymidine is much higher (K_m=0.5 μM) [Munir et al., 1994]. Several mutated forms of HSV-tk with increased enzymatic activity have shown enhanced effects in MG and other cancer models [Black et al., 2001].

Better GCV formulations with enhanced bioavailability have been developed in attempts to increase substrate availability [Miura et al., 2002]. The use of bradykinin, RMP-7 along with GCV, has improved the delivery of GCV across the BBB with enhanced therapeutic efficacy [LeMay et al., 1998] and several other pro-drugs have been tried with HSV-tk. GCV elaidic acid ester is more lipophilic than GCV and is converted to GCV by non-specific hydrolysis [Denny, 2003]. Penciclovir is closely related to GCV with low lipophilicity but it is less genotoxic than GCV making it a safer alternative [Thust et al., 2000]. Valganciclovir is an orally available pro-drug of GCV. Acyclovir (ACV) is chemically related to GCV but more lipophilic and less genotoxic. It is less potent than GCV and also has a lower bystander effect [Denny, 2003]. The relatively more lipophilic valacyclovir is a valine ester of ACV and is administered orally [Denny, 2003].

Attempts have been made to enhance the bystander effect of HSV-tk by combining it with connexin 43 [Marconi et al., 2000] and connexin 30, which are gap-junction protein subunits expressed by astrocytes [Mesnil et al., 1996]. The combination of the Cx43 gene with HSV-tk gene therapy showed an enhanced efficacy in C6 gliomas [Dilber et al., 1997], but not in U87 gliomas [Cirenei et al., 1998]. Another study using HSV-tk/GCV with Cx43 and TNF-α along with RT revealed a high level of tumour regression [Niranjan et al., 2003]. Treatment with retinoids has also been able to increase the gap-junctions by induction of connexin expression and this augmented the efficiency of GCV in HSV-tk transduced cell lines [Park et al., 1997].

Inhibition of polyamine biosynthesis by difluoromethylornithine (DFMO) has been shown to enhance the efficacy of the HSV-tk/GCV anti-tumour effect by causing an accumulation of the cells in S-phase [Pasanen et al., 2003].

Combination therapy strategies with HSV-tk/GCV

The inherent heterogeneity of MG poses a formidable challenge to single-agent therapeutics to achieve a successful disease control and in this respect gene therapy does not seem to be an exception. Combination of different therapies makes it possible to capitalise on the multitude of genetic weaknesses in MGs and this can prevent or delay the occurrence of resistance to each individual therapeutic agent. However, most of the early work with gene therapy evaluated its efficacy as a single-agent. Demonstrating the efficacy as a single-agent is important as a proof-of-concept but combination strategies have to be considered for ultimate clinical success. Few studies have evaluated the efficacy of combining different gene therapy strategies and gene therapy with conventional therapies.

Some attempts have been made to enhance the cytotoxicity of HSV-tk by combining this approach with other therapeutic options. In most occasions, the combinations were chosen to enhance the host immune system against the tumours. This is logical because cellular antigens released by HSV-tk/GCV therapy can help stimulate the host anti-tumour immunity. HSV-tk/GCV gene therapy has been combined safely with IL-2 in clinical trials but the therapeutic efficacy is yet to be proven [Colombo et al., 2005]. In pre-clinical studies, HSV-tk has been combined with other gene therapy strategies such as IL-2 [Palu et al., 2000], TNF-α [Moriuchi et al., 1998] [Niranjan et al., 2003], IL-4 [Benedetti et al., 1997], Flt3L [King et al., 2008], macrophage colony stimulating factor (M-CSF) [Tyynela et al., 2002] and GM-CSF [Jones et al., 2000].

HSV-tk/GCV has also been successfully combined with other suicide gene therapy strategies such as CD/5-FC [Aghi et al., 1998], uracil phosphoribosyltransferase (UPRT)/5-FU [Desaknai et al., 2003], and cytochrome P450/CPA [Aghi et al., 1999], p53 [Huang et al., 2007],anti-angiogenic endostatin gene therapy [Pulkkanen et al., 2002], oncolytic viruses [Zhang et al., 2010], I-kappa B-alpha [Moriuchi et al., 2005] and BimS [Yamaguchi et al., 2003].

The combination of AdHSV-tk/GCV suicide gene therapy with RT has been utilised to up-regulate promoters and increase gene expression [Marples et al., 2002], and to enhance the cytotoxicity [Nishihara et al., 1997]. Synergistic effects were observed when HSV-tk/GCV was combined with chemotherapeutic agents such as hydroxyurea [Boucher et al., 2000], gemcitabine [Boucher and Shewach, 2005], 5-FU [Wildner et al., 1999a], topotican [Wildner et al., 1999b] and TMZ [Rainov et al., 2001]. The study of Rainov *et al.* demonstrated synergism between HSV-tk/GCV and TMZ but failed to provide a clear mechanism for this synergism. It was speculated that inhibition of DNA polδ by the suicide gene therapy could be one reason for synergism [Rainov et al., 2001]. There is accumulating evidence to suggest that HSV-tk/GCV suicide gene therapy can make cancer cells more sensitive to chemotherapy and RT, and *vice versa*, suggesting an promising adjuvant role for this gene therapy strategy in cancer care [Kim et al., 1997].

2.3.5.1.3 AdHSV-tk/GCV gene therapy clinical trials

Early clinical trials used retrovirus VPCs as the gene delivery vector in the treatment of MG [Oldfield et al., 1993] [Ram et al., 1997] [Klatzmann et al., 1998]. These trials established the safety of MG gene therapy but in terms of efficacy they were unsuccessful [Palu et al., 1999], except for the occasional long-term survivors [Ram et al., 1997] [Klatzmann et al., 1998]. The main reason for the failure has been attributed to the poor transduction efficacy of VPCs [Ram et al., 1997] [Sandmair et al., 2000a]. Puumalainen *et al.* in 1998 demonstrated a low transduction efficacy (0.01-4.0 %) of VPCs [Puumalainen et al., 1998]. The phase III trial published by Rainov *et al.* failed to detect any survival benefit using retrovirus VPC-mediated HSV-tk/GCV gene therapy as compared to standard care [Rainov, 2000].

Sandmair *et al.* in 2000 published the first clinical trial where adenovirus vectors and retrovirus VPCs were compared side-by-side as ways to deliver the HSV-tk suicide gene in the surgical resection cavity of MG glioma patients in a phase I/IIa study [Sandmair et al., 2000a]. This was the one of the first studies to be published where adenovirus vectors were

used as the gene delivery vectors to treat MG. A mean survival of 15 months was observed in the AdHSV-tk/GCV group compared to 7.4 months for the retrovirus VPA group, establishing the superiority of the adenovirus vector.

The phase I trial of Trask *et al.* in 2000, where recurrent MG patients were treated with intratumoural AdHSV-tk and GCV, reported a mean survival of 9.4 months and a MS of 4 months [Trask et al., 2000]. Eck *et al.* (2002) reported a median time to progression of 3 months and a MS of 10 months in MG patients treated in a phase I study with AdHSV-tk/GCV and demonstrated the safety of the procedure [Maatta et al., 2009]. In their phase-I study; Germano *et al.* reported an average survival of 58.9 weeks after AdHSV-tk/GCV gene therapy in recurrent MG patients with the majority of patients having unchanged neurological performance scores [Germano et al., 2003]. A median PFS of 2.3 months and an OS of 4 months were reported by Smitt *et al.* in a phase-I trial in recurrent MG patients with AdHSV-tk/GCV. Vector doses up to $4x10^{11}$ vp/ml were well tolerated by the recipients [Smitt et al., 2003].

In 2004, Immonen *et al.* first published a phase-IIb randomised controlled clinical trial results for AdHSV-tk/GCV gene therapy for MG, where 36 patients were randomised to standard care (surgery and adjuvant RT) and standard care and gene therapy arms. Mean survivals of 70.6 and 36.0 weeks and MSs of 62.4 and 37.7 weeks were reported for the gene therapy and standard care arms, respectively [Immonen et al., 2004].

Recently, Ark Therapeutics PLC completed a phase-III randomised, controlled, parallel group, multi-centre clinical trial (ASPECT study/Ark Study 904)), where the efficacy of AdHSV-tk/GCV, Sitimagene cerodenovec (Cerepro®) was compared against standard care in operable MG [van Putten et al., 2010]. Final results have not yet been published but a significant effect on the primary end-point was reported in a subgroup of patients (Press release by Ark Therapeutics PLC 18/12/2009).

2.3.5.2 Anti-angiogenic gene therapy

The major limitations of current anti-angiogenic therapies have been discussed in the anti-angiogenic therapy section. Anti-angiogenic gene therapy has the potential to overcome some of the disadvantages of systemic therapy. Gene therapy based targeting of anti-angiogenic agents into the tumours circumvents the systemic toxicities, permitting attainment of a high concentration of the therapeutic compound in the tumour vicinity over a desired period of time [Dell'Eva et al., 2002]. Once started on, it has been questioned whether anti-angiogenic therapy can ever be stopped safely [Zuniga et al., 2010], with evidence suggesting that it would have to be continued for a long-period of time [Kirsch et al., 2000] placing enormous economic strain on the health care systems. As a one-time therapeutic application, gene therapy could be more economical when compared to classical pharmaceuticals [Morishita, 2004]. Due to local administration, anti-angiogenic gene therapy can overcome natural barriers for conventional drug delivery such as the BBB. The low toxic profile of gene therapy makes it a potential candidate to be combined with cytotoxic chemotherapies in contrast to pharmaceutical agents that have adverse effect profiles on their own.

Anti-angiogenic cancer gene therapy has demonstrated promising results in pre-clinical studies in many cancer types [Chen et al., 2001] [Sallinen et al., 2009]. However, the studies have so far been limited to the pre-clinical phase. Anti-angiogenic gene therapy has been intended to inhibit pro-angiogenic pathways as well as stimulating anti-angiogenic pathways, in tumour-directed and systemic gene therapy approaches using viral and non-viral vectors [Samaranayake et al., 2010].

Gene therapy-based inhibition of pro-angiogenic pathways

VEGF-VEGFR signalling pathway has been the main target of this approach for decades. PDGFR, FGF-FGFR, Tie-2, HIF-1α and HGF are the other main pro-angiogenic pathways inhibited by gene therapy. Most studies have evaluated a single anti-angiogenic gene

therapy and only a few attempts have been made to combine anti-angiogenic gene therapy with other gene therapy strategies or with chemotherapy or RT.

Successful VEGF inhibition and therapeutic benefit in MG models have been achieved by anti-sense VEGF constructs [Saleh et al., 1996] and VEGF siRNA delivery [Niola et al., 2006], and by expressing a dominant negative VEGFR-1 mutant [Heidenreich et al., 2004] and VEGFR-2 [Machein et al., 1999]. Intratumoral injection of a plasmid vector encoding VEGF siRNA reduced the tumour vascularity but failed to stop tumour growth in a MG xenograft model. However, retrovirus mediated stable transduction of the same cell lines with VEGF siRNA and IL-4 gene completely prevented the growth of tumours [Niola et al., 2006]. MG cells transfected with a dominant-negative HIF-1α and siRNA against HIF-1α [Gillespie et al., 2007] reduced *in-vivo* tumour growth and proliferation but not the microvessel density suggesting that mechanisms other than anti-angiogenesis are responsible for the effects, in spite of the reduction in VEGF secretion [Jensen et al., 2006] [Fujiwara et al., 2007]. Inhibition of FGF-2/FGFR-2 signalling pathway by dominant-negative FGFR-2 or FGFR-1 in C6 glioma cells decreased the tumour growth in both immunodeficient and immunocompetent animal models [Auguste et al., 2001].

Soluble VEGFR-medicated anti-angiogenic gene therapy

One option to target the VEGF-VEGFR signalling pathways is to deliver soluble forms of VEGFRs (sVEGFR) that lack the transmembrane domain, hence are incapable of initiating intracellular signal transduction cascades. Due to its high affinity, sVEGFR-1 [Kendall and Thomas, 1993] sequesters VEGF and inhibits angiogenesis by making it unavailable for the transmembrane receptors. It also inhibits VEGF-mediated intracellular signalling through a dominant negative homo/hetero-dimerisation with trans-membrane VEGF receptors [Barleon et al., 1997] [Kendall et al., 1996].

Human sVEGFR-1 is synthesized by alternative splicing of the VEGFR-1 mRNA [Thomas, 1996] or by proteolytic processing of the transmembrane VEGFR [Olsson et al., 2006]. The resulting sVEGFR-1 is truncated on the C-terminal side of the sixth extracellular IgG-like domain and contains a unique 31-amino acid C-terminal residue [Kendall and Thomas, 1993] [Thomas, 1996]. The sVEGFR-1 retains its high affinity binding (Kd of 2-10 pM) ability to VEGF [Kendall et al., 1994], which is one order of magnitude higher than the binding affinity of VEGFR-2 [Shibuya, 2006]. Extracellular immunoglobulin-like (Ig-like) domains 2 and 3 are essential for high affinity VEGF binding [Keyt et al., 1996] [Tanaka et al., 1997] and domain 4 for the receptor dimerisation [Barleon et al., 1997]. The sVEGFR-1 is implicated in placental insufficiency and pre-eclampsia [Shibuya, 2006].

Several different forms of the sVEGFR-1 have been used in cancer gene therapy, including the natural form of the receptor with six Ig-like domains and the 31 amino acid C-terminal residue [Kong et al., 1998], truncated extracellular portion of the VEGFR-1 with all seven Ig-like domains fused to Fc portion of human IgG [Mori et al., 2000], the secreted form of sVEGFR-1 with the first-six Ig-like domains fused to Fc portion of human IgG [Shiose et al., 2000], the first-three Ig-like domains with C-terminal 6x His-tag [Kuo et al., 2001], the first-three Ig-like domains [Yang et al., 2001], the first-three Ig-like domains fused to Fc portion of IgG [Harding et al., 2006], the first-five Ig-like domains fused to Fc portion of human IgG [Sallinen et al., 2009], and the first-five Ig-like domains [Ramachandra et al., 2009]. Human as well as murine forms of sVEGFR-1 have been studied.

Soluble VEGFR-1 has been the most extensively studied sVEGFR in cancer gene therapy, being successful in inhibiting angiogenesis in pre-clinical models of colon cancer [Kong et al., 1998], fibrosarcoma [Goldman et al., 1998], melanoma [Shiose et al., 2000], gastric cancer [Mori et al., 2000], lung cancer [Takayama et al., 2000], pancreatic cancer [Kuo et al., 2001], ovarian cancer [Mahasreshti et al., 2001], breast cancer [Cuadros et al., 2003], thyroid cancer [Ye et al., 2004], renal cancer [Yoshimura et al., 2004], liver cancer [Schmidt et al., 2005], GBM [Ohlfest et al., 2005], oral cancer [Gao et al., 2007] and osteosarcoma [Yin et al., 2008].

The therapeutic benefits of sVEGFRs are attributed to the inhibition of tumour angiogenesis, growth, metastasis, ascites formation, and increases in survival and apoptosis. AAV-mediated delivery of sVEGFR-1/-2 fusion protein gene (sVEGFR-1/R-2) in murine colon carcinoma and melanoma models lead to an increase in the number of activated CD4+ and CD8+ tumour infiltrating effector T-cells and activated DCs, while reducing the numbers of regulatory T-cells. On the other hand, tumours modified to overexpress VEGF had a higher number of regulatory T-cells in the lymphocyte population infiltrating the tumour [Li et al., 2006] suggesting that blockade of VEGF improves anti-tumour immunity as an anti-tumour mechanism.

The delivery of sVEGFR-1 has been successful with different viral [Sallinen et al., 2009] and non-viral vectors [Mori et al., 2000]. Some studies have employed retrovirus-mediated *ex-vivo* transduced cells [Goldman et al., 1998]. Replication defective adenovirus vectors are the preferred vector for sVEGFR-1 delivery. A few studies have used AAV [Hasumi et al., 2002], oncolytic adenovirus [Zhang et al., 2005] and vaccinia virus vectors [Guse et al., 2010]. Expression of sVEGFR-1 was detectable for up to three weeks after adenovirus-mediated gene delivery into skeletal muscle [Takayama et al., 2000].

The routes of vector administration have been diverse with intratumoral [Kong et al., 1998], intra-muscular (i.m.) [Takei et al., 2007b], i.v. [Mahasreshti et al., 2003], intra-tracheal [Kong et al., 1998], intra-peritoneal (i.p.) [Sako et al., 2004], intra-nasal [Mae et al., 2005] and pleural [Mae and Crystal, 2002] *in-vivo* gene delivery as well as *ex-vivo* gene transfer [Shiose et al., 2000], depending on the disease model and the vector used. The therapeutic efficacy seems to depend on the mode of vector delivery and the location of the target organ. For example, in a colon cancer model i.v. administration of sVEGFR-1 inhibited the growth of primary and the liver metastasis but not the lung metastasis, which responded to intratracheal vector administration. In the same model, intratumoral gene therapy suppressed the tumour growth in a subcutaneous model [Kong et al., 1998].

Several studies demonstrated toxicities with i.v. administration of adenovirus-mediated sVEGFR-1 [Mahasreshti et al., 2003] [Mahasreshti et al., 2001] [Kuo et al., 2001]. These include haemorrhage and focal necrosis of liver, congestion in lungs, spleen and kidneys, and reduced survival of the animals [Mahasreshti et al., 2003]. One study reported increased alveolar inflammation [Mae and Crystal, 2002].

Most of the studies evaluated the efficacy of sVEGFR-1 as a monotherapy. A few studies have combined sVEGFR-1 with chemotherapies such as 5-FU [Zhang et al., 2005], cisplatin [Gao et al., 2007] and paclitaxel [Sopo et al., 2012]. Combination with other anti-angiogenic agents like endostatin [Graepler et al., 2005] and angiostatin-endostatin fusion-protein (statin AE) [Ohlfest et al., 2005] have shown synergy with sVEGFR-1. In two studies, sVEGFR-1 was successfully combined with an oncolytic adenovirus [Zhang et al., 2005] and oncolytic vaccinia virus [Guse et al., 2010]. Two studies reported success with the combination of sVEGFR-2 and -3 in an ovarian cancer model [Sallinen et al., 2009] [Sopo et al., 2012], while another study successfully combined sVEGFR-1, -3 and soluble Tie2 receptor in the same cancer model [Sallinen et al., 2011]. AAV-mediated delivery of sVEGFR-1/-2 fusion protein gene (sVEGFR-1/R-2) demonstrated significant anti-tumour efficacy in a murine glioma model, but tumour progression was seen later suggesting the presence of escape mechanisms [Harding et al., 2006].

Several studies have used a soluble form of VEGFR-2 as the anti-angiogenic agent in many tumour models [Lyons et al., 2007] such as prostate cancer [Jin et al., 2005] pancreatic cancer [Reinblatt et al., 2005] and liver cancer [Pin et al., 2004]. HSV, oncolytic adenovirus and Semliki Forest virus vectors have been used in these studies. The putative anti-tumour mechanisms include reduced tumour growth, tumour volume, angiogenesis, ascites formation and metastasis. Adenovirus-mediated sVEGFR-2 gene therapy was far superior in inhibiting tumour growth as compared to angiostatin, endostatin and neuropilin gene therapies [Kuo et al., 2001]. However, adding adenoviruses expressing soluble PDGFR-β or Tie2-Fc provided no added therapeutic benefit, suggesting that targeting multiple anti-

angiogenic pathways is only needed when one is not blocked adequately [Kuhnert et al., 2008]. Combination of adenovirus-mediated sVEGFR-1, -2 and -3 in a mouse ovarian cancer model resulted in reduced tumour growth, less vascularity and lower ascites formation compared to the individual gene therapies hinting at the superiority of anti-angiogenic gene therapy combinations [Sallinen et al., 2009]. In combination with chemotherapy, this combination increased the survival of the animals [Sopo et al., 2012].

Gene therapy-based stimulation of anti-angiogenic pathways

Genetic delivery of endogenous inhibitors of angiogenesis and cytokines has been commonly used to potentiate anti-angiogenic pathways in cancer gene therapy. Endostatin is a by-product of type XVIII collagen that inhibits VEGF and bFGF. Non-viral gene delivery methods [Chen et al., 1999] and viral vectors [Subramanian et al., 2005b] have been tried in the treatment of several cancer models [Bjerkvig et al., 2003] [Pulkkanen et al., 2002] with endostatin. Increased animal survival and apoptosis of cancer cells, and decreased tumour growth and angiogenesis were reported as the main anti-tumour effects of endostatin. Inhibition of tumour metastasis and vessel co-option has been observed with endostatin therapy [Yoon et al., 1999] [Subramanian et al., 2005b]. The ability of endostatin to inhibit tumour dissemination is attributed to a para-endothelial action due to its binding to integrin $\alpha 5\beta 1$ [Yokoyama et al., 2007]. However, endostatin was not as effective in human xenograft models as it was in murine cancer models [Jin et al., 2001]. Adenovirus-mediated systemic administration achieved endostatin concentrations between 936 ng/ml [Sauter et al., 2000] and 1.34μg/ml [Wen et al., 2001]. Serum endostatin levels peaked three days after intratumoural gene therapy but declined to almost half by day 7 [Li et al., 2004]. The effect of i.v. adenovirus vector administration was lost after sometime due to the impact of anti-adenoviral antibodies [Jin et al., 2001]. Adverse effects such as weight-loss, bleeding and death of animals were seen with high dose i.v. administration of adenovirus vectors [Wen et al., 2001]. On the other hand, single i.m. administration of AAV vectors achieved a sustained secretion of endostatin up to nine weeks [Subramanian et al., 2005b]. *Ex-vivo* transduction demonstrated that therapeutic efficacy persisted even when the percentage of transduced cells was only 25% [Yoon et al., 1999]. A possible synergistic effect was detected when endostatin gene therapy was combined with RT [Luo et al., 2005], chemotherapy [Subramanian et al., 2005b] and other gene therapy strategies such as GM-CSF [Tai et al., 2003] and AdHSV-tk/GCV suicide gene therapy [Pulkkanen et al., 2002].

Angiostatin is a plasmin degradation product that inhibits endothelial cell proliferation and migration, inducing cell apoptosis. Stable transduction of liver cancer cells with angiostatin gene suppressed the tumour growth [Schmidt et al., 2006] in murine models but failed to inhibit tumour angiogenesis, implicating that multifactorial effects of gene therapy can contribute to the anti-tumour effects [Schmidt et al., 2006]. However, intratumoural gene therapy was successful in a MG model [Ma et al., 2002].

Several attempts have been made to combine different angiogenic inhibitors. Transduction with endostatin and angiostatin genes either separately [Isayeva et al., 2007] or as a fusion gene [Raikwar et al., 2005] are some such examples. These combinations had a better therapeutic effect compared to the individual therapies but the fusion genes were even superior to the combinations [Scappaticci et al., 2001]. The further addition of paclitaxel [Isayeva et al., 2007] and adenovirus encoding a soluble form of Tie2 gene therapy [Raikwar et al., 2005] enhanced the efficacy in some studies [Kim et al., 2004].

Intramuscular delivery of plasmid DNA encoding vasostatin (N-terminal domain of calreticulin) gene inhibited tumour angiogenesis and growth, and increased survival and tumour cell apoptosis in tumour bearing mice [Xiao et al., 2002].

Several cytokine gene therapy strategies such as IFN-α [Persano et al., 2009], IFN-β [Streck et al., 2005], IFN-γ [Saleh et al., 2000], IL-12 [sselin-Paturel et al., 1999] and IL-4 [Saleh et al., 1999] have also demonstrated anti-angiogenic effects.

2.3.5.3 Other gene therapy strategies

Oncolytic virotherapy

The concept of using viruses to attack tumours dates back to early 20th century [Dalba et al., 2005]. The principle of oncolytic virotherapy is that the virus has the ability to replicate only within tumour cells leading to tumour cell lysis. The released vps then spread within the tumour infecting and destroying new tumour cells. The second gene therapy drug to be approved in China H101 is an oncolytic adenovirus, with an E1B-55kD deletion [2006]. Next to suicide gene therapy, oncolytic virotherapy has been the most widely studied gene therapy strategy for MG in clinical trials. In spite of promising pre-clinical results oncolytic viruses so far have failed to live up to expectations in clinical trials [Sonabend et al., 2006]. Oncolytic HSV 1716 [Harrow et al., 2004] and G207 [Markert et al., 2009], replication competent adenovirus: delta-24-RGD [Gomez-Manzano et al., 2004] and ONYX-015 [Bischoff et al., 1996] [Chiocca et al., 2004], NDV-HUJ (OV001) [Freeman et al., 2006], recombinant polio/rhino virus, measles virus [Phuong et al., 2003], reovirus [Forsyth et al., 2008] and retroviruses [Wang et al., 2003] are currently under development. Oncolytic virotherapy have been successfully combined with CDA/5-FC [Conrad et al., 2005], cytokine gene therapy [Cerullo et al., 2010] [Koski et al., 2010], anti-angiogenic therapy [Zhang et al., 2012], p53 gene therapy [Geoerger et al., 2004], chemotherapy [Alonso et al., 2007] [Gomez-Manzano et al., 2006] and RT [Geoerger et al., 2003].

Gene therapy-based immune modulation

The objective of this therapeutic approach is to boost the host immune system to eradicate the tumour. Like most other malignancies, gliomas are able to suppress the host immune system by secreting immunosuppressive factors such as TGF- β [Platten et al., 2000] and IL-10, and by decreased expression of MHC molecules and increased expression of FasL [Waziri, 2010]. The paucity of antigen presenting cells in the brain parenchyma, the immunologically privileged state of the brain and general impairment of host immune function are also believed to contribute [Hussain and Heimberger, 2005]. With the aim of enhancing cell-mediated immunity, both adoptive and active (vaccine) immunotherapy strategies have been attempted on glioma, with varying successes [Barzon et al., 2006] [Lawler et al., 2006]. Autologous tumour cells/fibroblasts genetically modified to secrete cytokines IL-2 [Sobol et al., 1995], IL-4 [Okada et al., 2007] and GM-CSF [Parney et al., 2006] have achieved some success in pre-clinical work. Genetic delivery of cytokine genes into the tumours has been attempted with IL-2 [Lichtor and Glick, 2003], IL-4 [Benedetti et al., 2000], IL-12 [Ren et al., 2003], IFN-α [Tsugawa et al., 2004], IFN-β [Chiocca et al., 2008], IFN-γ [Smith et al., 2007], TNF-α and GM-CSF [Smith et al., 2007], alone and in combination with vaccine therapy [Lawler et al., 2006]. The genetic delivery of DC growth factors such as Flt-3 in combination with AdHSV-tk/GCV suicide gene therapy showed promising pre-clinical results and is being evaluated in a clinical trial [King et al., 2005].

Gene therapy-based correction/compensation of tumour genetic defects

The development of MGs is commonly associated with dysfunction of several genes and intracellular signalling pathways [Ohgaki and Kleihues, 2005b]. Correction/compensation of these genetic defects by delivering the wild-type gene via gene therapy seems to be a logical approach for cancer therapy [Rao and James, 2004]. Several studies that attempted to correct non-functional p53 [Lang et al., 1999] and its downstream pathway [Wang et al., 2001] have demonstrated multiple anti-tumour effects in pre-clinical MG models and limited effects in clinical trials [Lang et al., 2003]. In addition, p53 gene therapy increased chemo- and radio-sensitivity in tumours [Dorigo et al., 1998] [Badie et al., 1998]. Gene therapy-based targeting of defects in RB and cell-cycles regulation pathways has conferred some benefits in pre-clinical studies [Fueyo et al., 2000]. Similar results were seen with PTEN gene therapy [Abe et al., 2003]. Activated EGFR signalling in MG has been targeted by gene therapy using ribozymes [Halatsch et al., 2000], antisense oligonucleotides [Zhang

et al., 2004], RNAi-based strategies [Vollmann et al., 2006] and dominant-negative EGFR [Lammering et al., 2003].

Gene therapy targeting tumour invasion

One of the biggest challenges in MG therapy is the extensive invasion of the surrounding normal brain parenchyma by the tumours [Lawler et al., 2006]. MG invasion is mediated by extracellular matrix proteases and protease inhibitors, cytoskeleton reorganization, growth factors, and cytokines [Rao, 2003]. The genetic delivery of PTEN and tissue inhibitor of metalloproteinase (TIMP) [Lu et al., 2004b] [Baker et al., 2000], pigment epithelium-derived factor [Guan et al., 2004], anti-sense uPAR and cathepsin B, [Gondi et al., 2004b], and siRNA targeting urokinase plasminogen activator (uPA)/uPAR [Gondi et al., 2004a], TGF-β [Friese et al., 2004]) and ADAM17 [Zheng et al., 2007] have inhibited MG invasiveness in pre-clinical work.

Induction of apoptosis by gene therapy

The escape from the normal apoptotic control is one of the salient features that confer a survival advantage on cancer cells. Apoptosis-specific signalling molecules and cell surface bound death receptors/ligands such as Fas/FasL and Apo2L/TNF-related apoptosis-inducing ligand (TRAIL), regulate the apoptotic pathways. Many gene therapy strategies including suicide gene therapy, cytokine gene therapy and p53 gene therapy have been able to induce apoptosis in the MG cells. Gene therapy with FasL [Frei et al., 1998], TRAIL [Ehtesham et al., 2002], pro-apoptotic Bim [Yamaguchi et al., 2003] and Bax [Arafat et al., 2003], and RNAi/anti-sense oligonucleotide mediated knock-down of anti-apoptotic molecules [Uchida et al., 2004] and IGF receptor (IGFR)-1R (IGFR-1R) [Andrews et al., 2001] have shown positive results in MGs *in-vitro* and *in-vivo*.

Myeloprotective gene therapy

The myelotoxicity of the chemotherapeutic agents is a major dose-limiting factor in cancer chemotherapy and is a common cause for premature termination of treatment and sub-optimal dosing regimens. The genetic delivery of the MDR gene into haematopoietic cells can reduce the impact of chemotherapy on bone marrow and allow the usage of high-dose chemotherapy by increasing the therapeutic index [Palmer et al., 2006]. Bone marrow transplantation with haematopoietic stem cells transduced with MGMT was able to protect against high-dose chemotherapy [Cai et al., 2008].

Gene therapy to enhance chemotherapy and radiotherapy

Chemotherapy and RT are still the major therapeutic arms in oncology. Any novel therapy in clinical trials will have to be compared at some-point with these approaches to prove their potential efficacy. Hence, the impact of the novel therapy on the efficacy of chemotherapy and RT will determine the final outcome. Several gene therapy strategies have been shown to enhance the chemo- and radio-sensitivity of MGs *in-vitro* and *in-vivo*. Recent work has highlighted the fact that oncolytic adenovirus vectors enhance the sensitivity to TMZ by suppressing MGMT expression [Alonso et al., 2007] and to irinotecan by increasing topoisomerase-1 expression [Gomez-Manzano et al., 2006]. Gene therapy based inhibition of cell-cycle regulators [Ruan et al., 1999], pro-apoptotic gene therapy [Arafat et al., 2003], p53 gene therapy [Dorigo et al., 1998] [Badie et al., 1998], PTEN gene therapy [Lawler et al., 2006], dominant-negative EGFR gene therapy [Lammering et al., 2003] and inhibition of c-Met [Chu et al., 2006] has increased the chemo- and radio-sensitivity of MG cells. It is also known that HSV-tk/GCV suicide gene therapy can sensitise gliomas to chemotherapy and RT [Kim et al., 1997] [Rainov et al., 2001].

2.3.6 Valproic acid

Discovered in the late-1800s VPA has been in clinical use for the treatment of seizures for over 40 years [Loscher, 2002]. It is one of the most widely used, well-established first-line AEDs, well tolerated by patients [Wolff et al., 2008]. Multiple mechanisms have been proposed for its anti-epileptic actions [Loscher, 2002]. It is an orally available drug with plasma and brain concentrations of 40-100 μg/ml (280-690 μmol/L) and 6-27 μg/g (42-190 μmol/L), respectively, in humans after 15-20 mg/kg oral dose [Loscher, 2002].

Anti-tumour mechanisms

VPA-mediated modulation has been implicated in tumour angiogenesis, immunogenicity, invasion, metastasis, differentiation, proliferation and apoptosis [Gotfryd et al., 2010]. With the discovery of its HDACi action, VPA has received a new lease-of-life in pharmaceutical research. The organisation of DNA into nucleosomes is undertaken by histone proteins depending on their acetylation status, which is regulated by histone acetyl transferase (HAT) and histone deacetylase (HDAC). Acetylation opens the chromatin increasing gene expression. HDAC and HAT activity is dysregulated in GBM [Marks et al., 2001]. VPA can hyperacetylate histones H3 and H4 and inhibit HDAC activity [Phiel et al., 2001] [Kramer et al., 2003] and furthermore, HDACi inhibits tumour growth, evokes cell-cycle arrest at G1/S [Yeow et al., 2006], as well as increasing differentiation [Benitez et al., 2008] and apoptosis in MG cells [Loscher, 2002]. Other possible anti-tumour mechanisms include impairment of DSB processing [Robert et al., 2011], stimulation of autophagy [Fu et al., 2010], acetylation of Ku proteins inhibiting NHEJ DSB repair [Subramanian et al., 2005a] and increased the cytotoxicity of NK cells [Chavez-Blanco et al., 2011]. One study revealed a 13-fold increase in histone acetylation with VPA [Gotfryd et al., 2010]. Other mechanisms such as phosphorylation of intracellular signalling proteins may contribute the VPA-mediated actions in a cell type specific manner [Gotfryd et al., 2010].

Some pro-tumour mechanisms of VPA have also been proposed. HDACi involving VPA through PI3K/AKT [Mayo et al., 2003] and/or PKC-mediated [Kim et al., 2003] pathways transcriptionally activate NF-κB, which inhibits drug-induced apoptosis by upregulating p21 [Burgess et al., 2001]. There is some evidence to suggest that VPA can increase the HR repair frequency [Defoort et al., 2006] and cancer cell motility [Gotfryd et al., 2010]. Some HDACi have the ability to impair the innate immune system mediated through toll-like receptors [Roger et al., 2011].

Effects on chemo- and radiotherapy

There is some pre-clinical data suggesting an additive/synergistic effect for HDACi with chemotherapy and RT [Yin et al., 2007] [Chinnaiyan et al., 2005] via stimulation of autophagy [Fu et al., 2010] in cancer cells, possibly by impairing DSB processing [Robert et al., 2011]. However, other studies have failed to demonstrate any synergy between VPA and chemotherapy [Stander et al., 1998]. There is evidence that VPA in spite of being an HDACi does not increase MGMT expression [Sasai et al., 2007]. A recent study demonstrated that VPA could sensitise MG cells to TMZ and γ-radiation, and more importantly, VPA did not antagonise the cytotoxicity of TMZ [van Nifterik et al., 2012]. Another study claimed that redox regulation could be a possible mechanism for the synergism between TMZ and VPA [Chen et al., 2011b]. Increased cytotoxicity was observed when VPA was combined with doxorubicin [Catalano et al., 2006], etoposide [Das et al., 2007], cisplatin, gemcitabine and adriamycin [Chavez-Blanco et al., 2006]. Evaluation of data from EORTC/NCIC phase III trial has revealed that patients who received both VPA and TMZ enjoyed a better survival. However, no benefit was seen with VPA and RT without TMZ, suggesting the VPA-mediated enhanced efficacy is TMZ dependent [Weller et al., 2011].

Effects on adenovirus-mediated HSV-tk/GCV gene therapy

VPA by increasing the expression of CAR receptors [Goldsmith et al., 2007] on tumour cell surfaces can increase the transduction efficacy of adenovirus vectors and the transgene expression after adenovirus-mediated gene transfer [Kothari et al., 2010]. Some HDACi have increased the bystander effect of HSV-tk/GCV by increasing gap-junction communication [Ammerpohl et al., 2004] [Robe et al., 2004] by increasing levels of connexin 43 [Asklund et al., 2004]. HSV-tk/GCV-mediated cytotoxicity seems to be influenced by DNA DSB repair, especially by HR [Thust et al., 1996] [Helleday, 2003] [Sonoda et al., 1999]. VPA-mediated impairment of DSB processing [Robert et al., 2011] could possibly enhance the cytotoxicity of HSV-tk/GCV.

2.3.7 Gene therapy-mediated targeted therapy

2.3.7.1 Introduction to targeted therapy

Systemic adverse effects are one of the major drawbacks encountered in cancer therapy. They are common with both RT and chemotherapy, and often limit the therapeutic dose and frequency. Sometimes adverse effects can even prevent the completion of treatment courses, contributing to the unfavourable outcome of cancer patients. The main aim of targeted cancer therapy is to limit the treatment effect to the tumours and thereby to reduce systemic adverse effects and increase the therapeutic window. Furthermore, it may be possible to reduce the required drug dosage, volume and the amount of drugs in the systemic circulation [Goodwin and Meares, 2001].

Targeting can be achieved by either direct targeting or pre-targeting methods [Paganelli et al., 1990]. Direct targeting is a single-step process where the therapeutic payload is directly linked to a targeting moiety, which could be a mAb, peptide antigen, growth factor, or cytokine that would have a higher affinity towards the tumour due to expression of a specific target such as cell surface receptors. mAbs labelled with iodine 131 (^{131}I) tositumomab (Bexxar, Corixa & Glaxo-SmithKline) and yttrium 90 (^{90}Y) labelled ibritumomab tiuxetan (Zevalin, Biogen IDEC) directed against CD20 antigen are examples of direct targeted therapies approved for the treatment of non-Hodgkin's lymphoma. So far no targeted therapy has been approved for MG but radionuclide-labelled mAbs and growth factor-labelled toxins are under clinical development [Li et al., 2010] [Kunwar et al., 2010] [Hockaday et al., 2005]. The toxicities due to these agents gaining access to the circulation are still a problem although that can be overcome by loco-regional application of the therapy [Goetz et al., 2003]. Apart from their therapeutic use, the direct targeting radioconjugates are useful in tumour imaging [Hockaday et al., 2005]. Chemotherapeutics encapsulated in liposomes directed against a tumour antigen are another method of direct targeting [Chen et al., 2011c] [Fondell et al., 2010].

Pre-targeting is a multi-step process involving sequential administration of targeting constructs and finally the targeted therapeutic payload [Goodwin et al., 1986]. Pre-targeting can be achieved by using bispecific mAbs (bsmAb) that can bind to a tumour specific antigen. These bsmAbs have a specific binding site for hapten-bound targeted therapeutics, streptavidin or avidin conjugated antibodies that can trap biotinylated therapeutics, biotinylated antibodies that can complex with therapeutics bound to avidin or streptavidin, as well as DNA/oligonucleotide sequence conjugated antibodies that can bind oligonucleotide-labelled therapeutics or enzyme conjugated antibodies that can activate pro-drugs [Goldenberg et al., 2006]. The mAbs have to be modified so that they can be cleared rapidly from the circulation before administration of the targeted therapeutic in order to prevent its binding in the circulation. Due to their smaller size (~100kD), rapid clearance, high tissue permeation and diffusion, bsmAbs are more suitable for the purpose. The binding affinity of bsmAb-based pre-targeting is much less (10^{-9}M) compared to avidin/biotin-based systems (10^{-15}M) [Goldenberg et al., 2006]. The greatest obstacle faced by antibody-based pre-targeting systems is the need to identify targets exclusive to the

tumours, and the development of antibodies/peptides that are specific for that target. Unfortunately, most of the tumour targets identified so far is not unique to tumours.

2.3.7.2 Avidin-biotin technology

Avidin is a 66 kD tetrameric glycoprotein in chicken egg-white that can bind one biotin molecule into each of its identical monomers with extremely high affinity i.e. Kd values of 10^{-13}-10^{-15} M [Green, 1990]. The bacterial analogue to avidin, streptavidin is derived from *Streptomyces avidinii* and it is also a tetrameric protein of 60 kD with similar binding characteristics to biotin. Avidin and streptavidin have a ~60 % sequence homology, similar secondary, tertiary and quaternary structures [Argarana et al., 1986], and are stable against heat, pH, denaturants and enzymes [Wilchek et al., 2006]. In spite of the apparent similarities between them, there are some crucial differences that give these two molecules distinct pharmacokinetic and bio-distribution patterns [Schechter et al., 1990]. Due to its high lysine and arginine content, avidin is positively charged with an isoelectric point (iP) of ~10.5 and is heavily glycosylated, leading to the possibility of non-specific binding into tissues, rapid clearance from circulation and accumulation in liver [Sakahara and Saga, 1999]. Hence, avidin is frequently used as a clearing agent to remove biotinylated mAbs from the circulation. On the other hand, streptavidin has an iP of ~5-8 and is not glycosylated, which reduces both non-specific binding and increases its serum half-life [Sakahara and Saga, 1999]. Therefore, streptavidin has a better permeation into the tumour. Deglycosylation and neutralisation increase the serum half-life of avidin, while binding to galactose increases the clearance and liver accumulation of streptavidin [Rosebrough and Hartley, 1996]. Both molecules seem to have a natural tendency to accumulate in tumours [Yao et al., 1998] [Hnatowich et al., 1993]. However, their repeated use is prevented by their immunogenicity in humans [Knox et al., 2000] [Hytonen et al., 2003]. Attempts have been made to improve the utility of these proteins by chemical and genetic modifications [Laitinen et al., 2006].

Biotin is a 244 Da water soluble vitamin of the B-complex group (B_7/ H) that acts as a coenzyme in carboxylation/decarboxylation reactions. It plays an important role in several cellular functions including metabolism of carbohydrates, fatty acids and amino acids, and citric acid cycle [Knowles, 1989] [Samols et al., 1988]. It also plays a role in mammalian gene regulation, cell proliferation and DDR [Zempleni, 2005]. The presence of a carboxylic acid moiety allows the biotinylation of molecules by chemical or biological processes, without affecting their affinity to avidin/streptavidin [Sakahara and Saga, 1999]. The presence of high amounts of biotinidase in human serum can affect the stability of biotinylated substances [Sakahara and Saga, 1999]. Biotin can be radiolabelled with ^{90}yttrium (^{90}Y) and a wide variety of radionuclides using diethylenetriamaniepentaacitic acid (DTPA), ethylinediaminetetraacitic acid (EDTA) or 2-(p-nitrobenzyl)-1, 4, 7, 10-tetraazylododecane-N, N', N'', N'''-tetraacitic acid (DOTA) [Sakahara and Saga, 1999]. DOTA is superior to DTPA in its suitability for biotinylation for therapeutic purposes due to its high stability [Chinol et al., 1997]. It has a *in-vitro* human serum stability for up to 96h with ~85 % *in-vivo* clearance within 24h [Urbano et al., 2007]. ^{90}Y has physical half-life of 2.7 days (64h) providing a maximum energy of 2.3 MeV via pure beta emission and is widely used in radio-immunotherapy [Sakahara and Saga, 1999].

Avidin/biotin-based pre-targeting

Avidin/biotin-based pre-targeting systems can involve several steps in the targeting of the therapeutic payload. In two-step pre-targeting systems, a biotinylated mAb with specificity to a tumour antigen is administered first, followed by the therapeutic payload bound to avidin as the second step after allowing sufficient time to clear unbound antibody from the circulation [Paganelli et al., 1992]. Avidin/streptavidin and biotin can be used in the reverse manner in this method. Biotinidases in the serum can cleave biotinylated therapeutic conjugates. On the other hand, conjugates labelled with either avidin or

streptavidin have unfavourable bio-distribution properties and are rapidly removed from circulation by liver for avidin and kidney for streptavidin [Goldenberg et al., 2006]. In three-step pre-targeting, the biotinylated tumour specific mAb is administered first followed by avidin or streptavidin that binds the tumour-bound biotinylated mAb. The third step is the administration of a biotinylated therapeutic payload [Paganelli et al., 1991]. Glycosylated avidin has a short serum half-life due to rapid clearance by the liver, and is able to remove the unbound mAb from the circulation. Another method of three-step pre-targeting is first to administer avidin or streptavidin conjugated tumour specific mAb. In the second step, a clearing agent to remove the unbound avidin/streptavidin mAb conjugates from circulation is given, followed by the biotinylated therapeutic payload [Shen et al., 2005]. By increasing the number of steps, one can reduce off-target adverse effects. Number of clearance steps can be incorporated into the three-step targeting method to further reduce the unwanted systemic effects. In the four-step pre-targeting method, the biotinylated mAb step is followed by administration of avidin. Streptavidin which is not glycosylated and has a better tissue penetration and a longer serum half-life than avidin is administered before the biotinylated therapeutic payload with the aim to increase the possible binding sites for the therapeutic agent. In a five-step pre-targeting method, biotinylated albumin is administered between streptavidin and the therapeutic payload, in order to saturate the unbound streptavidin in the circulation. It is essential to have an adequate time in-between administrations to clear the unbound mAb, avidin and streptavidin. Pre-targeting provides a highly specific tissue targeting that reduces the adverse effects by lowering the amount of drug/radiation in the circulation and other tissues, thereby increasing the therapeutic index, ultimately leading to improved efficacy. The disadvantages of avidin/biotin-based pre-targeting are the larger size of the mAb construct (170-220 kD) with poor diffusion and tissue permeation, the immunogenicity of streptavidin, the binding of avidin to various other tissues and uptake of biotinylated therapeutic payload by untargeted tissues due to biotinylation leading to toxicities [Goldenberg et al., 2006]. Loco-regional pre-targeted radio-immunotherapy with biotinylated ^{90}Y in combination with chemotherapy has been evaluated in GBM patients [Grana et al., 2002] [Boiardi et al., 2005].

The extremely high affinity of avidin/streptavidin towards biotin forms the basis of avidin-biotin technology, which has wide applications in many diverse fields such as molecular biology, biotechnology, IHC, diagnostic imaging, therapeutic targeting and gene therapy.

Avidin-biotin technology in gene therapy

Avidin-biotin technology has been exploited in various gene therapy applications. Firstly, this system has shown promising results in overcoming the major obstacle of gene therapy; the poor transduction efficacy of current vectors. Thus, the transduction efficacy can be increased by targeting gene therapy vectors into the desired site. Pioneering studies used streptavidin to bridge antibody against retroviral envelope protein and antibody against cell surface antigen [Roux et al., 1989] [Etienne-Julan et al., 1992]. The principle of present avidin-biotin based vector targeting is based on coating the vector surface with either biotin or avidin/streptavidin by chemical or metabolic techniques. Chemical biotinylation has been used to create biotinylated adeno [Smith et al., 1999], retro, AAV [Ponnazhagan et al., 2002], vaccinia [Purow and Staveley-O'Carroll, 2005] virus vectors that demonstrated better transduction efficacy [Zhong et al., 2001] and improved transduction properties. Using metabolic biotinylation adeno [Parrott et al., 2003], AAV [Stachler and Bartlett, 2006], lenti [Nesbeth et al., 2006] and baculo [Kaikkonen et al., 2008] virus vectors have been made. On the other hand, non-viral [Wojda and Miller, 2000], adeno [Park et al., 2008] and baculo [Raty et al., 2004] virus vectors expressing surface avidin and lentivirus vectors expressing surface streptavidin and avidin [Kaikkonen et al., 2009] have been described.

The use of avidin-biotin technology for in-vivo imaging was exemplified by the use of avidin/streptavidin expressing baculovirus vectors conjugated with biotinylated iron oxide particles or radionuclides in MRI and SPECT imaging, respectively [Raty et al., 2006] [Raty et al., 2007]. A lentivirus vector expressing avidin and streptavidin has been created with the same principle using biotinylated radionuclide for SPECT imaging [Kaikkonen et al., 2009].

2.3.7.3 LDL receptor-avidin fusion protein gene therapy

The main limitation of using avidin-biotin pre-targeting in therapy is the need to develop mAbs or bsmAbs that are specific to a particular tumour. Taking into account the inherent heterogeneity of tumours, this means that antibody development may have to be tailored to the individual patient, or in the worst case, to the different stages of tumour progression in the same patient. Gene therapy can be combined with avidin-biotin technology to overcome this problem. The avidin transgene can be delivered into the target tissue by gene therapy applications followed by the delivery of a biotinylated therapeutic payload [Walker et al., 1996]. Lehtolainen *et al.* described the development of a fusion-protein composed of endocytotic LDLR and avidin (Lodavin®, Ark Therapeutics PLC), which is expressed on the cell surface after local gene transfer using Semliki Forest virus vectors. The monomeric version of the fusion protein has an affinity of Kd 2 x 10^{-8} M for biotin. After local gene transfer into orthotopic rat MGs using the same vector, the construct was capable of binding locally and systemically (intra-arterially) administered biotinylated molecules [Lehtolainen et al., 2003]. This construct utilizes a two-step process for targeted therapy. The principle of LDLR-avidin fusion protein gene therapy is presented in Figure 10.

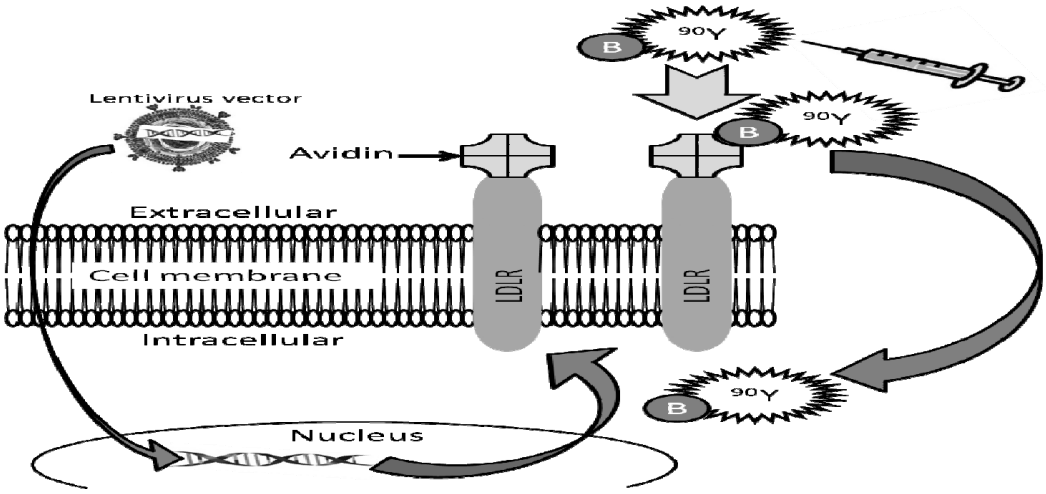

Figure 10: Principle of LDLR-avidin fusion protein gene therapy-based targeted therapy. LDLR-avidin fusion protein is expressed on the cell surface after lentivirus-mediated local gene transfer. Systemically administered biotinylated yttrium binds to avidin with high affinity and is internalised. B-biotin, LDLR-low-density lipoprotein, ^{90}Y-^{90}yttrium

A recent study demonstrated long-term expression of this fusion-protein gene after lentivirus-mediated local gene transfer in an orthotopic rat MG model and its ability to bind biotinylated compounds *in-vitro*. The study also revealed the development of antibodies against the fusion-protein and the viral vector in animals after repeated vector injections that could affect the binding of the therapeutic payload [Lesch et al., 2009].

A fusion-protein has also been described, which is composed of macrophage scavenger receptor and avidin (Scavidin) and it was shown to possess the ability to bind biotinylated compounds [Lehtolainen et al., 2002a] and could be used in imaging applications [Mantyla et al., 2006].

2.4 ANIMAL MODELS FOR MALIGNANT GLIOMA STUDIES

Experimental tumour models are vital for studying tumorigenesis, basic tumour biology, and to evaluate new preventive, diagnostic and therapeutic strategies for cancer [Workman et al., 2010] [Bulnes-Sesma et al., 2006]. Historically, tumours were created in animals by administration of chemical carcinogens or by infecting the animals with oncogenic viruses. These methods are tedious and hampered by the long waiting times, unpredictable tumour incidence, and the lack of reproducibility [Barth and Kaur, 2009], even though they do resemble better the natural occurrence of cancers in humans. However, tumours thus formed can be propagated into tumour cell lines, which can be injected into the same strain of animals to create tumours with a much higher tumour take, predictable growth patterns and less biological variability within a much shorter time. These tumours are syngeneic in the original animal strain; hence they do not evoke significant immune responses in the host, having some resemblance to the usually immunosuppressive human tumours [Barth, 1998]. There are drawbacks of this method, i.e. the tumour cells are of non-human origin and thus the results obtained cannot be directly extrapolated into human cancers and the biological behaviour of these tumours may not exactly resemble those encountered in human tumours. Moreover, since a large number of cells have to be injected to generate tumours by this method, from a point-of-view of human tumorigenesis, they do not totally resemble the malignancy process in humans. However, syngeneic tumour models are easy to handle and extremely useful in proof-of-concept studies. The rodent MG tumour cell lines induced by nitrosourea are typical examples of this method [Barth and Kaur, 2009].

The second method of creating tumours for experimental purposes is to inject human or murine cancer cells directly into immune-deficient mice (Nude/SCID) [Fogh et al., 1977] or nude rats to create xenograft/xenogeneic tumours [Candolfi et al., 2007]. An advantage of this technique is that tumours are of human origin. However, the types of immune-deficiencies in these animals do not resemble those found in humans with cancer. Hence, these models are not suitable for the evaluation of the host immune response to the growing tumours. Furthermore, due to the same reason, the growth kinetics, vascularisation, invasiveness and metastatic patterns of these tumours may not completely resemble their human counterparts [Workman et al., 2010] [Candolfi et al., 2007]. Another point of concern is that long-term passaging of tumour cell lines means that the cell lines will lose some of the original features and may acquire novel molecular genetic characteristics [Workman et al., 2010]. This can be avoided by using low-passage number cell lines. Another way to circumvent this problem is to use tumour cell lines from fresh patient samples directly or after passaging in the relevant animal model a few times. Tumours thus created tend to resemble the original human tumours far more than the other methods but the reproducibility with different patient samples may be a concern [Workman et al., 2010] [Dong et al., 2010] [Wang et al., 2009].

The third method is to use viral vectors and transgenic technologies to introduce specific genetic abnormalities into the tumour cells and into the host enabling the development of tumours with a specific genetic background [Chow et al., 2011] [Hager and Hanahan, 1999] [Crabtree et al., 2003]. The genetic modifications can be done either *ex-vivo* or *in-vivo*. A further modification of this method is to introduce a multitude of gene abnormalities that can be activated in a sequential manner [Zhu et al., 2005], simulating the multistep process of carcinogenesis [Workman et al., 2010].

One can divide the experimental tumours on the basis of tumour location into two groups. Orthotopic tumours are tumours located in the natural anatomical location, as opposed to heterotopic tumours which are located elsewhere than the natural location, such as subcutaneous tumours of internal organ malignancies. There is a growing body of evidence suggesting that the microenvironment of the tumours plays a critical role in their behaviour [Barth and Kaur, 2009]. For example, there is evidence to suggest that brain tumours growing as subcutaneous xenografts do not display the same growth kinetics or

respond to therapies in the same way as their intracranial counterparts. However, the subcutaneous xenograft tumours are convenient to create and easy to follow-up.

Rats and mice are the most widely used species to create brain tumour models in experimental studies [Peterson et al., 1994]. Since the mid-1970s, several rat brain tumour models have been described and characterised [Ramadan and Wechsler, 1975] [Ishida et al., 1975]. Apart from the rodent models, cat, dog, rabbit and nonhuman primate brain tumour models have been used to a lesser extent [Chopra and Mikkelsen, 1998]. Exposure to chemical carcinogens such as methylnitrosourea (MNU) or ethylnitrosourea (ENU) results in the development of brain tumours in rodents. Transplantation of these tumour cells after culture, into the same rodent strain results in a syngeneic tumour model [Barth, 1998]. Rat brain tumours have the advantage that they can be more precisely implanted into the desired location than in the mouse brain, are easy to manipulate, can accommodate larger injection volumes, permit a longer follow-up time and are more amenable for imaging than their mouse counterparts. There are reports claiming that nitrosourea-induced rat MGs do not exhibits the same genetic changes typical to human MG [Schlegel et al., 1999]. However, other studies have revealed that the rodent tumours induced by N-ENU, carry mutations in p53, neu/erbB-2 and Ras pathways, and thus resemble human MGs [Bulnes-Sesma et al., 2006]. The general consensus for which features a valid brain tumour model should contain are as follows; glial cell origin of the tumour, ability to grow *in-vitro* as a continuous cell line and propagate *in-vivo* by serial transplantation, predictable and reproducible growth pattern, glioma-like characteristics within the brain, sufficient survival time for therapeutic intervention, non-immunogenic or weakly immunogenic in the syngeneic host, confinement to the brain and the possibility to extrapolate the therapeutic outcome to human tumours [Barth and Kaur, 2009]. Unfortunately, none of the existing animal models exactly mimic the human MGs.

BT4C rat MG model

BT4C rat glioma cell lines are derived from the foetal BDIX rat brain cells exposed to N-ENU *in-utero*. Shortly after exposure, the cells were transferred to cell culture and selected for the tumorigenic potency [Laerum and Rajewsky, 1975]. BT4C gliomas are syngeneic in BDIX rats with almost 100 % tumour take [Visted et al., 2000], and do not induce immunological reactions in the host thus mimicking human gliomas [Laerum and Rajewsky, 1975] [Laerum et al., 1977] and contain a mixture of multipolar glia-like cells and flattened cells with fewer and shorter cytoplasmic processes and occasional giant cells [Laerum et al., 1977]. BT4C cells produce high amounts of type IV collagen and fibronectin and can have a high turnover of type I collagen depending on the growth conditions [Bjerkvig et al., 1989]. Moreover, these tumours are highly cellular with pleomorphic nuclei, a sarcomatous growth pattern, possessing numerous mitotic figures, scattered giant cells, irregular and dilated blood vessels, areas of proliferation, a propensity for invasion into surrounding normal brain parenchyma along perivascular tracts, occasional satellite lesions (tumour cell nests) in the brain parenchyma, neo-vascularisation, especially in the periphery with frequent microhaemorrhages and occasional scattered necrosis. On the other hand, the incidence of central necrosis is rare [Stuhr et al., 2007] [Barth and Kaur, 2009]. One study has shown that 98% of the tumours developed using this method, were neurogenic in origin resembling ODGs, astrocytomas, mixed gliomas, anaplastic gliomas, glioependymomas, ependymomas and neurinomas [Ramadan and Wechsler, 1975]. BT4C tumours overexpress S100, VEGF, tissue plasminogen activator (tPA), uPA, and have a high microvessel density in the tumour periphery [Barth and Kaur, 2009]. The BT4C model has been used in multiple therapeutic approaches [Tyynela et al., 2002] [Raty et al., 2004]. $BT4C_n$ cells that were derived after repeated *in-vivo* passaging [Mella et al., 1990] form more invasive tumours due to the lack of neural cell adhesion molecule (NCAM) protein synthesis [Andersson et al., 1991].

Other rat MG models

C6 rat glioma (ATCC#CCL-107) is induced in outbred Wistar rats by repetitive administration of N-MNU. Since it is not syngeneic in any of the inbred strains, this leads to a significant anti-tumour immune response in the host, which is a major drawback with this model [Barth, 1998] i.e. there is the possibility of an immune mediated spontaneous tumour regression [Beutler et al., 1999]. C6 gliomas have mutant p16/CDKN2A/Ink4a locus but wild-type p53 and overexpress PDGF-β, IGF-1, EGFR and Erb3/Her3 genes [Barth and Kaur, 2009]. Weakly immunogenic, F98 (ATCC#CRL-2397) gliomas have a very infiltrative growth pattern with microsatellites. These glioma cells were created in a similar way as BT4C tumours, by administration of ENU to pregnant Fischer rats [Barth, 1998]. They overexpress PDGF-β, Ras, EGFR, cyclinD1 and D2 [Barth and Kaur, 2009].

The widely used 9L gliosarcoma (T9 glioma) was induced by repetitive administration of MNU to inbred Fischer rats. The high immunogenicity of these tumours makes them less suitable for survival studies in immunocompetent rats [Barth, 1998]. These tumours carry a mutant p53 and overexpress EGFR and TGF-α [Barth and Kaur, 2009]. Since they are non-immunogenic in the syngeneic host, the RG2 (D74) (ATCC#CRL-2433) glioma model is considered a useful model mimicking human GBM due to its high invasiveness [Barth, 1998] [Barth and Kaur, 2009].

Mouse models of glioma

Mouse models of glioma can be syngeneic or xenogeneic. Several mouse models are in use for glioma studies. SMA-497 and SMA-560 glioma cell lines have been characterised from an inbred VM/Dk mouse strain, which spontaneously developed astrocytomas [Serano et al., 1980] and they have been used in pre-clinical gene therapy experiments. GL26 gliomas in C57BL/6 mice are syngeneic [Candolfi et al., 2007]. The glioma model 4C8 is also syngeneic in B6D2F1 mice, and has been used in glioma studies [Weiner et al., 1999].

Glioma xenograft models produced by subcutaneous tumour cell implantation in immunodeficient athymic or nude mice, although widely used in research, seem to display a significant animal to animal variation in their tumour morphology limiting their use in research [Jones et al., 1981].

2.5 ETHICAL CONSIDERATIONS IN CANCER GENE THERAPY

Public discussions on the ethical issues concerning human gene therapy started many years before the commencement of proper gene therapy clinical trials [Anderson and Fletcher, 1980], especially following the Cline experiment, where Cline and colleagues unsuccessfully attempted to treat thalassaemia by re-infusing bone marrow cells, *in-vitro* transfected with the human globin gene [Friedmann, 1992]. Deliberate genetic manipulation of the human beings even for therapeutic purposes raises difficult ethical and public policy problems i.e. trying to balance the improvement of care for some serious medical conditions on one hand with the possible adverse impact on the environment and human genome on the other [Anderson, 1998]. Continued public discussion, technical advances and clarification of the ethical differences between somatic gene therapy and germ line genetic modification paved the way for somatic gene therapy clinical trials [Friedmann, 1992].

There are several ethical questions surrounding gene therapy, for example, distinguishing the use of gene therapy in “good” and “bad” terms, distinguishing between “normal” and “abnormal” traits, social acceptance to gene therapy, use of gene therapy for performance enhancement, unknown long-term consequences of gene modification and the possible discrepancies in the access of gene therapy. The Bioethics Directive (Council of Europe 1998) and Clinical Trials Directive (Council of Europe 2001 and 2004) regulates gene therapy related activities within the European Union, while individual countries have their

own legislations, such as the Gene Technology Law and Medicines Act in Finland [Gonin et al., 2005]. Preventive, diagnostic and therapeutic genetic manipulation of somatic cells is permitted based on these directives and laws, but germ line genetic manipulation is not allowed. Approval by the institutional and local ethical committees has to be obtained before the commencement of gene therapy clinical trials and also for pre-clinical research work. Gene therapy products have to be manufactured under "good manufacturing practices" (GMP) conditions for human use. Another point of concern is the use of genetically modified microorganisms as gene therapy vectors and the possible consequences of accidental release of these vectors into the environment. However, proper risk assessments and implementation of appropriate risk reduction methods have addressed this issue to a great extent. Another issue that needs to be addressed is the discrepancy between the potential of gene therapy and social expectations of gene therapy. Overenthusiastic interpretations of gene therapy successes may be contributing to this difference in perception; raising the question of how critical should one be when interpreting gene therapy successes.

Gene therapy for brain cancer targets somatic tumour cells and does not involve germ line cells. By virtue of its action, all the cells that are transduced with the new gene in suicide gene therapy will be eliminated by the activated pro-drug if they attempt to divide, thus preventing the possibility of transmitting the transgene into subsequent generations.

The possibility of transducing unwanted cells, the risk of insertional mutagenesis with the use of integrating vectors, the risk of having infective recombinant helper viruses and the possible immune reactions against the vector strains are some of the factors limiting the widespread use of human gene therapy that need to be addressed. The death of Jesse Gelsinger due to a systemic inflammatory response, following an adenovirus-mediated gene therapy in a clinical trial [Raper et al., 2003] [Lehrman, 1999] and the development of leukaemias in children after retrovirus-mediated gene therapy clinical trial for X-linked SCID-X1 [Hacein-Bey-Abina et al., 2003a], highlight the possible risks associated with this therapeutic approach. Nonetheless, only a few severe adverse-events have been reported in gene therapy clinical trials suggesting that it has a general overall good safety profile. In a recent comparison, it was revealed that the incidence of serious adverse events among the patients subjected to adenovirus-mediated gene therapy clinical trials was 0.9 and 4.1/10000 patient-days in cardiovascular and MG clinical trials, respectively, which can be favourably compared to the randomised control patients who had 0.5 and 2.1/10000 patient days, respectively [Wirth et al., 2006].

3 Objectives of the study

1. To evaluate the pre-clinical feasibility and efficacy of combining adenovirus-mediated HSV-tk/GCV suicide gene therapy with adenovirus-mediated sVEGFR-1 anti-angiogenic gene therapy for the treatment of malignant glioma.

2. To unravel the underlying mechanisms of synergism when adenovirus-mediated HSV-tk/GCV suicide gene therapy is combined with the chemotherapeutic agent, TMZ, in MG therapy and to delineate the optimal treatment protocol to maximise the synergistic effect.

3. To determine the effects of adding VPA to adenovirus-mediated HSV-tk/GCV suicide gene therapy and TMZ combination in the treatment of malignant glioma.

4. To evaluate the *in-vivo* efficacy of lentivirus-mediated LDLR-avidin fusion protein gene as a gene therapy-based targeting strategy to deliver therapeutic compounds into inoperable malignant gliomas.

4 Materials and methods

4.1 MATERIALS

4.1.1 Viral vectors

All adenovirus vectors used were replication deficient, first generation E1-E3 deleted, derived from serotype 5 adenoviruses carrying either sVEGFR-1 (1-5)-Ig (AdsVEGFR-1), HSV-tk (Gene Bank V00470) (AdHSV-tk) or LacZ (AdLacZ) cDNA, driven by a CMV promoter. The production of adenovirus vector encoding cDNA for the first five immunoglobulin-like domains of human VEGFR-1 fused in-frame with the sequence encoding for Fc-portion of human IgG (AdsVEGFR-1) was achieved by cloning the VEGFR-1 (1-5)-Ig expression cassette into the *Eco*RV site of pAdCMV plasmid [Bhardwaj et al., 2005]. The functional titre of AdsVEGFR-1 was 2.7 x 10^{10} pfu/ml. Adenoviruses were manufactured in HEK-293 cells [Graham et al., 1977] and purified by density gradient centrifugation [Sandmair et al., 2000a] [Immonen et al., 2004]. AdHSV-tk (Cerepro®) was supplied by Ark Therapeutics PLC. The titre of AdHSV-tk vector stock used in this study was 1.5 x 10^{12} vp/ml. Adenovirus vector carrying the LacZ marker-gene used for gene transfer controls had an OD260-titer of 3.1 x 10^{12} vp/ml [Puumalainen et al., 1998].

Lentivirus vectors encoding for LDLR-avidin fusion gene (Lodavin®, Ark Therapeutics PLC) [Lehtolainen et al., 2003] were produced by cloning into a third generation self-inactivating lentivirus transfer plasmid under a CAG promoter. The lentiviruses were manufactured by calcium phosphate transfection in 293T cells as described before [Follenzi and Naldini, 2002]. The lentivirus vectors had a titre of 4.5 x 10^9 transducing units (TU)/ml.

4.1.2 Cell lines

BT4C rat MG cell line [Laerum et al., 1977] was a generous gift from University of Bergen, Norway. Human MG cell lines T98G (ATCC, CRL-1690) and U87-MG (ATCC, HTB-14) were purchased from American Type Culture Collection (ATCC). Adherent MG cells were grown at 37° C in the presence of 5 % CO_2 in high-glucose Dulbecco´s modified Eagle's medium (DMEM) (BT4C and U87MG) or in minimum essential medium (MEM) (T98G), containing L-glutamine and glucose, supplemented with 10 % foetal bovine serum (FBS) (Hyclone).

4.1.3 Experimental animals

Six to eight-weeks old, immunocompetent, inbred, male BDIX rats weighing 175-200 g were purchased from Charles Rivers Laboratories, France and 4-6 weeks old male BALB/cA nude mice were purchased from Taconic, Denmark. Animals were housed at the National Laboratory Animal Centre, University of Eastern Finland's Snellmania animal facilities. A twelve-hour period of light per day with a constant temperature of 24° C was maintained at the housing facility and food and water were provided *ad libitum*. Animals were quarantined for 5 days before experimental procedures.

4.1.4 Pharmaceutical compounds

GCV (Cymevene® 500 mg, Roche, Finland) was diluted in 10 ml of distilled water for injection to achieve a 50 mg/ml stock solution. TMZ (Temodar®/Temodal®, Schering-Plough-Merck), 100 mg capsule was dissolved in 10 % dimethyl sulphoxide (DMSO)/0.9 % saline solutions daily before the injections. VPA solution (Deprakine 200mg/ml) was purchased from Sanofi Winthrop Industrie Ambarés, France. ^{90}Yttrium (^{90}Y) was purchased from PerkinElmer Finland. Labelling of ^{90}Y with biotinylated DOTA was done with

ammonium acetate buffer at 95° C for over 35 minutes, according to a protocol by Paganelli *et al.* [Paganelli et al., 1999]. Biotinylated DOTA was provided by University of Eastern Finland.

4.2 METHODS

4.2.1 *In-vitro* studies

4.2.1.1 Viability/cytotoxicity assays

Sub-confluent BT4C cells were transduced with AdsVEGFR-1 vector at multiplicity of infection (MOI) 0, 0.1, 1, 5, 10 and 25 to assess the effect of the vector and VEGF inhibition on the viability and proliferation of BT4C cells. Cell viability was measured 24, 48, 72, 96, 120 and 144h after transduction using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit) according to the manufacturer's instructions. Absorbance was detected at 490nm with a Microplate reader.

To determine the *in-vitro* synergy between AdHSV-tk/GCV and TMZ, 2500 BT4C cells were plated per well onto 96-well plates on day 3, after transduction with AdHSV-tk at MOIs 1, 5 and 10 in 6-well plates on day 2. Cells were treated with 1 μg/ml GCV and TMZ at concentrations of 10 and 100 μmol/L on days 4 and 6. Cell viability was measured on day 10 using a MTS assay.

The effect of cellular MGMT status on the synergistic effect was evaluated by using the known MGMT expressing T98G [Park et al., 2006] and MGMT negative U87MG [Chahal et al., 2010] human MG cell lines. Cells at 4000 per well were plated onto 96-well plates on day 3 after transduction with AdHSV-tk at MOIs 5 and 20 on day 2. Cells were treated with 1 and 10 μg/ml of GCV and 100 μmol/L of TMZ on days 4 and 6. Cell viability was measured 5 days later with a CellTiter-Glo® Luminescent cell viability assay (Promega) according to the manufacture's instructions. To further confirm the findings, T98G and U87MG cells 100,000 per well were plated onto 6-well plates on day one. AdHSV-tk transduction was undertaken with MOI 5 on day 2. On days 4 and 6, the cells were treated with 1μg/ml of GCV and 100 μmol/L of TMZ. Microscopic examination and photographing was conducted on day 10.

In order to determine whether the timing of GCV in relation to TMZ therapy has an impact on the synergy, 2500 BT4C cells per well were plated onto 96-well plates on day 3, after transduction with AdHSV-tk/GCV at MOIs 30, 50 and 75 on day 2. GCV therapy at 1 μg/ml was given on days 4 and 6 followed by TMZ therapy at 100 μmol/L either on day 9 or day 14. Cell viability was measured by MTS assay 4 days after the last treatment. The findings were confirmed by crystal violate staining done on 6-well plates after plating 10000 BT4C cells per well and following the same protocol as used in the microscopic examination except that TMZ therapy was given at doses 1, 10 and 100 μmol/L on day 9. Crystal violet staining (Sigma-Aldrich, St. Louis, MO, USA) was done on fixed cells according to the manufacturer's protocol, 4 days after last treatment.

Synergy between two therapies was determined by using fractional product method of Webb [Webb, 1961] [Verrier et al., 2001] [Das et al., 2007] [Chou, 2002] where, $\gamma 1$ and $\gamma 2$ are the fraction of cell survival after treatment with drug-1 and drug-2, respectively. The predicted survival after combining both drugs is derived by multiplying the fraction of survival with drug-1 and that of drug-2 ($\gamma 1\gamma 2$). The observed fraction of cell survival after the drug combination is denoted by $\gamma 12$. If $\gamma 1\gamma 2 > \gamma 12$, this indicates a synergic cytotoxicity with the two drug combination, $\gamma 1\gamma 2 < \gamma 12$ indicates antagonism with the combination. An additive effect is seen if $\gamma 1\gamma 2$ is equal to $\gamma 12$.

The effect of adding VPA into the AdHSV-tk/GCV and TMZ combination was evaluated in BT4C cells grown in VPA concentrations of 0.1, 0.5 and 1 mmol/L. BT4C cells were plated

onto 6-well plates, 50 000 cells per well (day 0). On the following day (day 1) the cells were treated with VPA daily until cell viability measurement or were left untreated. On day 2, the cells were either transduced with AdHSV-tk with MOI 5 or not transduced (NT). On the following day (day 3) cells were transferred to 96-well plates, 1500 cells per well. The cells were treated with 100 μmol/L of TMZ and 1 μg/ml GCV once a day on days 4 and 6. Cell viability was measured on day 8 with the MTS assay.

All cytotoxicity measurements were done in triplicate and are presented as averages.

4.2.1.2 ELISA

In-vitro production of VEGF and sVEGFR-1 by BT4C cells ($5x10^5$), transduced with AdHSV-tk and/or AdsVEGFR-1 at MOIs 0 and 25, was measured 1 week after transduction by enzyme-linked immunosorbant assays (ELISAs) specific for rat VEGF (R&D Systems, Germany) and human sVEGFR-1 (R&D Systems, Germany), according to the manufacturer's instructions. The conditioned media (CM) were collected after 24h serum starvation and stored at -70°C until the ELISAs were performed. The objective was to evaluate whether simultaneous transduction with two vectors had any effect on the sVEGFR-1 and VEGF production by BT4C cells.

4.2.1.3 Tubule formation assays

The functionality of AdsVEGFR-1 was tested by the tubule formation assay using a V2a Kit (TCS Cellworks, Buckingham, UK) according to the manufacturer's instructions using 1 to 10 dilutions of the CM from BT4C cells transduced with AdsVEGFR-1, AdHSV-tk, the combination and the control cells collected previously, with the optimised growth medium provided by the manufacturer. Digital images of 10x magnification were obtained using an inverted phase microscope on day 15. Tubular formations were quantified in a blinded manner using CellF imaging software (Olympus Soft Imaging Solutions).

4.2.1.4 Cell migration assays

The invasiveness of BT4C cells, grown in 1:10 and 1:1 dilutions of the four CM collected before and serum-free media (SFM), or transduced 5 days before with MOI 5 of AdsVEGFR-1, AdHSV-tk and the combination (MOI 5+5) growing in SFM, was measured using QCM™ 96-Well Cell Invasion Assay (Millipore, Germany) according to the manufacturer's instructions. The invasion of non-transduced BT4C cells growing in SFM, into a chamber containing chemoattractant (media with 10 % FBS) was the positive control, with migration into a chamber containing SFM as the negative control. In the reverse experiment, the invasiveness of non-transduced cells into the chemoattractant media in 1:1 dilution with CM was also measured.

4.2.1.5 DNA repair status

4.2.1.5.1 RT-PCR

To evaluate the effect of AdHSV-tk/GCV and TMZ on the MGMT and MMR DNA repair pathways, 50000 BT4C cells were plated per well onto to a 6-well plate on day 1, transduced with AdHSV-tk at MOI 5 on day 2 and treated with 1 μg/ml GCV and 100 μmol/L TMZ on days 4 and 6. The first samples for RT-PCR were collected on day 5 (1 day after starting treatment) and the second samples on day 8 (5 days after starting treatment). Total RNA was extracted from treated cells by TriReagent (Invitrogen) according to the manufacturer's protocol, its purity was confirmed and concentration measured by spectrophotometer (Nanorop ND-1000). RNA was reverse transcribed to cDNA by M-MuLV reverse transcriptase (MBI Fermentas). Target gene mRNA levels were measured by real-time PCR (StepOnePlus Real-Time PCR system, Applied Biosystems) using specific Taqman® gene expression assays (Applied Biosystems) for rat MLH1 (Rn00579159_m1), MSH2 (Rn00579198_m1), MGMT (Rn00563462_m1) and beta actin (4352931E) for normalization.

4.2.1.5.2 Western blot for MGMT

Cultured BT4C cells were lysed and protein concentrations measured by the BCA assay (Thermo Scientific, Rockford, IL, USA). About 20-30 μg of total proteins were run on a polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Blocked blots were incubated with a primary MGMT antibody (MT3.1 Santa Cruz Biotechnology) followed by the corresponding secondary antibody (Biotinylated anti-mouse IgG-HRP Pierce Biotechnology). Blots were visualised using Super Signal® West Dura Extended Duration Substrate kit (Pierce Biotechnology) with Kodak X-OMAT 2000 Processor.

4.2.2 *In-vivo* studies

4.2.2.1 BT4C rat malignant glioma in BDIX rats

All animal experiments were approved by the Animal Welfare and Ethics Committee of University of Eastern Finland. Immunocompetent, inbred, male BDIX rats (Charles Rivers Laboratories, France) weighing 175-200 g, bearing orthotopic BT4C rat MGs were created as described elsewhere [Sandmair et al., 2000b] [Tyynela et al., 2002]. Briefly, a total of 10,000 BT4C cells in 5 μl of Opti-Mem (Invitrogen) was slowly administered into the brain of an anaesthetised (fentanyl citrate & midazolam) rat at 1 cm posterior to the bregma and 2 cm to the right of sagittal suture [Paxinos et al., 1985], at a depth of 2.5 mm (above the right corpus callosum) over 2-3 min, using a 27G, 25 μl Hamilton-syringe (Hamilton, Bonaduz Ab, Switzerland) placed in a stereotactic device (David Kopf Instruments, California, USA). The needle was left in place for 5 min and then slowly withdrawn to avoid backflow.

4.2.2.2 *In-vivo* imaging

The presence of intracranial tumours was verified on post-tumour implantation (p.i.) day 12 or 13 in the adenovirus studies and on p.i. day 14 in the lentivirus study, by MRI. Anaesthesia was induced with 5 % isoflurane in a mixture of 70 %: 30 % N_2O:O_2, and was maintained at 1.5 % isoflurane. MRIs were conducted using a 4.7T small animal MRI scanner (Magnex Scientific Ltd, Abington, UK) interfaced to a Varian Unity Inova (Palo Alto, CA, USA) console. Non-contrast enhanced, T2-weighted spin-echo sequence with an echo time of 0.08 seconds and repetition time of 2.5 seconds was used to generate 17 coronal images of 1 mm thickness with no gap between slices to cover the tumour area in the rat brain. Images were from 256 x 256 matrices with a field of view of 4.0 x 4.0 cm. Total tumour volume was calculated by delineating the tumour area in all the image slices, processed and analysed with Matlab version 7.1b (MathWorks Inc. USA) using pre-made macros. Follow-up MRIs were done for the adenovirus-mediated studies on p.i. days 28 and 42.

4.2.2.3 *In-vivo* gene transfers

Table 11 summarises the study protocol for the sVEGFR-1study. On p.i. days 14 and 15 intratumoural gene transfers were conducted using the multiple-site vector injection technique. Depending on the study group (Table 11), 15 μl of AdHSV-tk and/or 10 μl of AdsVEGFR-1 were injected using a Hamilton-syringe placed in the stereotactic device. Vector injections of 7.5 μl of AdHSV-tk and/or 5 μl of AdsVEGFR-1, per each location were carried out at vertical depths of 2.0 and 2.5 mm from the bregma, 10 min apart. The needle was left in place for 10 min at each depth to allow proper dissipation of the vector solution. For the group receiving both vectors, AdHSV-tk gene transfer was done first.

Tables 12 and 13 summarise the study protocol for AdHSV-tk/GCV and TMZ, and AdHSV-tk/GCV with TMZ and VPA studies, respectively. All gene transfers were conducted using multiple-site vector injection technique on p.i. days 14 and 15. A total volume of 20 μl of AdHSV-tk or AdLacZ was administered as described above. Vector injections of 10 μl per location were carried out at vertical depths of 2.0 and 2.5 mm from the bregma, 10 min apart.

Table 14 summarises the treatment protocol for the lentivirus-mediated LDLR-avidin fusion-protein gene therapy and biotinylated ^{90}Y-DOTA (Bt-^{90}Y-DOTA) study. Intratumoral gene transfer was done on p.i. days 17 and 18 with a volume of 10 μl/day (2 μl at 5-different locations) using a Hamilton-syringe placed in the stereotactic device.

Table 11: Study Protocol for the AdHSV-tk/GCV and AdsVEGFR-1 Study

Study Groups	n	Protocol (in days from tumour implantation)		
		MRI	Gene Transfer	GCV
Control-survival	13	13	-	-
AdsVEGFR-1-survival	9	13	14-15	-
AdHSV-tk/GCV-survival	19	13	14-15	19-32
Combination-survival	8	13	14-15	19-32
Control-histology	7	13	-	-
AdsVEGFR-1-histology	4	13	14-15	-
AdHSV-tk/GCV-histology	3	13	14-15	19-32
Combination-histology	3	13	14-15	19-32

n = number of animals

Table 12: Study Protocol for the AdHSV-tk/GCV and TMZ Study

Study Groups	n	Protocol (in days from tumour implantation)					
		MRI	Gene Transfer	Gap	GCV	Gap	TMZ
AdLacZ	3	12	14-15	-	-	-	-
Control	9	12	-	-	-	-	-
TMZ	10	12	-	-	-	-	19-23
AdHSV-tk/GCV	19	12	14-15	16-18	19-25	-	-
AdHSV-tk/GCV+TMZ (a)	11	12	14-15	16-18	19-25	26-29	30-34
AdHSV-tk/GCV+TMZ (b)	7	13	14-15	-	16-22	-	23-27

n = number of animals

Table 13: Study Protocol for the AdHSV-tk/GCV with TMZ and VPA

Study Groups	n	Protocol (in days from tumour implantation)					
		MRI	VPA	Gene Transfers	GCV	VPA	TMZ
Control	11	12	-	-	-	-	-
VPA	6	13	14-15	-	-	19-32	-
TMZ+VPA	7	13	14-15	-	-	19-32	28-32
AdHSV-tk/GCV	10	13	-	14-15	19-32	-	-
AdHSV-tk/GCV+VPA	10	13	14-15	14-15	19-32	19-32	-
AdHSV-tk/GCV+TMZ	7	13	-	14-15	19-32	-	28-32
AdHSV-tk/GCV+TMZ+VPA	9	13	14-15	14-15	19-32	19-32	28-32

n = number of animals

Table 14: Study Protocol for the Lentivirus-LDLR-Avidin and Bt-^{90}Y-DOTA Study

Study Groups	n	Protocol (in days from tumour implantation)		
		MRI	Gene Transfers	Bt-^{90}Y-DOTA
Control	8	14	-	-
Bt-^{90}Y-DOTA	8	14	-	24-25
Lenti-LDLR-avidin and Bt-^{90}Y-DOTA	8	14	17-18	24-25
Lenti-LDLR-avidin-histology	3	14	17-18	-

n = number of animals

4.2.2.4 Pharmacological therapy

In the sVEGFR-1 study (Table 11), AdHSV-tk transduced animals received i.p. GCV treatment starting 5 days after gene transfer for 14 days, at a dose of 50 mg/kg/day given in two divided doses. Table 12 describes the treatment protocol for the different groups in the AdHSV-tk/GCV and TMZ study. The GCV dose was similar as above but the duration of GCV therapy in this study was 7 days. TMZ therapy was given at a dose of 60 mg/kg/ day as an i.p. injection once a day on 5 consecutive days. The dose and the duration of TMZ treatment were determined based on values in the literature [Friedman et al., 1995] [Newlands et al., 1997]. The treatment protocol for VPA study is summarised in Table 13. I.e. GCV and TMZ doses were as above. VPA (Deprakine 200mg/ml) was injected as subcutaneous (sc) injections at a dose of 200 mg/kg twice-a-day for the indicated durations (Table 13). Treatment protocols for the lentivirus studies are summarised in Table 14. Bt-^{90}Y-DOTA was administered into the tail-vein in 500 µl of saline at a dose of 20 mCi/kg/day, on 2 consecutive days.

4.2.2.5 Survival analyses

Survival was calculated in days from tumour cell implantation to the sacrifice or death of an animal. In the sVEGFR-1 study, the primary end-point was to follow-up the animals in the survival study for 50 days after tumour implantation. In the other three studies the animals were followed-up until the criteria for euthanasia were met. The animals were observed daily and body weight measured regularly, and they were sacrificed with CO_2 if the criteria for euthanasia as defined by the Animal Welfare and Ethics Committee were met. Animals from histology groups were sacrificed 10 days after completing each therapy or at a corresponding point in the tumour progression for histological analyses. Rats were transcardially perfused with 100 ml of 1 % PBS. The samples from liver, spleen, kidney and the whole brain were fixed in 4 % PFA at 4 ˚C overnight and then changed into 15 % sucrose solution having a pH of 7.4 at 4 ˚C. Samples were processed and embedded in paraffin blocks, and were sectioned into 5 µm thick slices. In the AdHSV-tk/GCV and TMZ study the AdLacZ transduced animals were sacrificed 5 days after gene transfer and the brains frozen in isopentane and stored at -70 ˚C. Frozen brains were embedded directly into Tissue-TEK®OCT compound and sectioned into 10µm thick slices. Histology animals in LDLR-avidin fusion-protein gene therapy study were sacrificed 5 days after gene transfer and brains collected for paraffin samples as described before.

4.2.2.6 Blood and serum analyses

Serum samples were collected from the animals after the completion of therapy from sVEGFR-1 study and AdHSV-tk/GCV and TMZ study, and analysed for bilirubin, alkaline phosphatase (AFOS), alanine aminotransferase (ALT) and creatinine at Kuopio University Hospital laboratory. In the AdHSV-tk/GCV and TMZ study, blood samples were also collected for full blood count (FBC) analyses. From the same samples CD3, CD4 and CD8 cell counts were analysed by flow cytometer (FACSanto II, BD Biosciences) using rat

leucocyte antibodies (APC Mouse anti-rat CD3, PE Mouse anti-rat CD4 and FITC Mouse Anti-Rat CD8, BD Biosciences).

4.2.2.7 Histology and immunohistochemistry

Randomly selected paraffin embedded tumour sections from the sVEGFR-1 histology animals were stained with haematoxylin and eosin for general tumour morphology. Immunohistochemical stainings were done using anti-rat primary antibodies for CD34 (AF4117-R&D Systems-1/100), rat VEGF (ab1316-AbCam-1/200), human sVEGFR-1 (ab9540-AbCam-1/100), CD68 (MCA341R-AbDSerotec-1/100), CD8 (MCA48R-AbDSerotec-1/50), CD4 (MCA55R-AbDSerotec-1/50) and CD25 (MCA273R-AbDSerotec-1/50). Kidney, liver and spleen samples were chosen as positive controls. Secondary antibodies were biotinylated horse anti-mouse IgG or goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Vectastain ABC-kit (Vector Laboratories) and DAB Plus (Zymed Laboratories, San Francisco, CA, USA) were used to form the colour precipitate. Counter staining was done with haematoxylin (Merck KGaA). Stainings for apoptosis were done using ApoTag® apoptosis detection kit (Chemicon International) according to the manufacturer's instructions. The stained sections were examined under microscope and imaged using Olympus Bx41 inverted phase microscope under 20x magnification. Quantitative analysis of tumour angiogenesis was done in a blinded manner using CellF image analysis software (Olympus Soft Imaging Solutions).

Frozen sections from the LacZ gene transfer control animals were stained by incubating for 18h with X-gal (MBI Fermentas) as previously described [Lehtolainen et al., 2002b] to assess the gene transduction efficacy.

Brain sections from LDLR-avidin fusion-protein gene therapy study were stained with goat anti-avidin primary antibody (Vector Laboratories, Burlingame, CA, USA-1/250) as described before [Lehtolainen et al., 2002a] in order to determine the transgene expression.

4.2.2.8 BT4C rat malignant glioma nude mice study

In the experiment designed to determine the duration of therapeutic efficacy of AdHSV-tk/GCV and AdsVEGFR-1 gene therapy combination in reducing tumour growth rate, male BALB/cA nude mice (Taconic, Denmark) at 4-6 weeks (n=11) were injected with 10^5 BT4C cells suspended in 100 µl of Opti-Mem (Invitrogen) into the right flank to create tumour xenografts. Three weeks later tumour bearing mice were randomised into control (n=6) and gene therapy group (n=5). The gene therapy group received intratumoural gene transfer of both AdHSV-tk/GCV and AdsVEGFR-1 vectors 15 µl each, on two consecutive days (day 21 & 22 p.i.). Intraperitoneal GCV was given for one week starting on day 22 p.i. at a dose of 100 mg/kg/day per mouse in two divided doses. Tumour dimensions in 3 planes perpendicular planes to each other (A, B & C) were measured using a calliper and tumour volumes calculated using the formula $4/3*\pi*A*B*C$.

4.2.3 Statistical analyses

Results were analysed with GraphPad Prism Version 5.01 statistical software (GraphPad Software Inc. USA). Survival data analyses were done using Kaplan-Meier survival plots and the curves were compared using Mantel-Cox Log-rank test. Column statistics were analysed using one-way ANOVA or Kruskal-Wallis test with Bonferroni or Dunn's post-hoc test to compare groups. Similarly, mice tumour volumes were compared using either t-test or Mann-Whitney test. Grouped statistics were compared using two-way ANOVA and Bonferroni post-hoc test.

5 Results and discussions

5.1 SUICIDE AND ANTI-ANGIOGENIC GENE THERAPY

5.1.1 Viability of BT4C cells after AdsVEGFR-1 gene transfer

The results reveal that transduction with AdsVEGFR-1 did not affect the viability or growth pattern of BT4C cells over a wide range of MOIs (0.1-25) up to 6 days after transduction (Figure 11). Maximum gene expression by adenovirus vectors was achieved 4-5 days after gene transfer. Hence, lack of growth inhibition even after 6 days indicates that the vector at these MOIs was not toxic to the cells and the degree to which VEGF was inhibited by sVEGFR-1 did not affect the growth pattern of BT4C rat MG cells. Generally MGs are considered highly vascular tumours with high VEGF expression [Plate et al., 1992]. Previous work in BT4C rat MG model has described high-expression of VEGF by these cells [Barth and Kaur, 2009]. However, it is not clear the extent to which VEGF acts as an autocrine/paracrine growth factor on MG cells [Weindel et al., 1994], especially on BT4C rat MG cells, as previously been described in breast cancer [Lee et al., 2007].

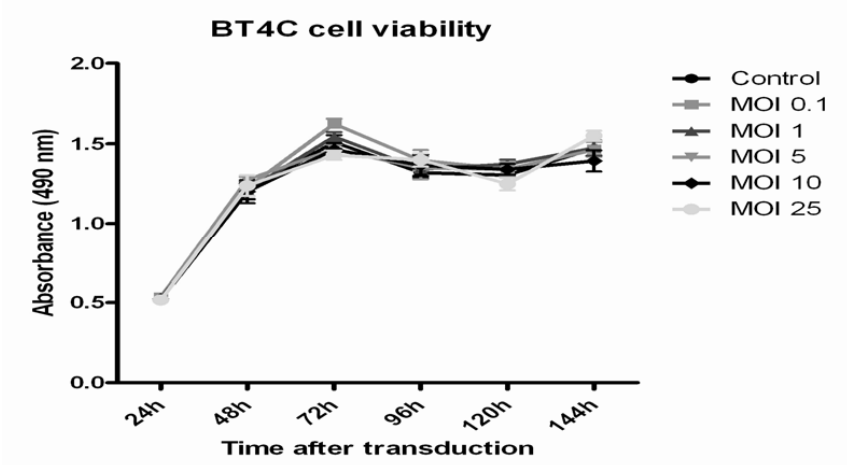

Figure 11: Cell viability measured over time by absorbance at 490nm using an MTS assay after transducing BT4C cells with different MOIs (0, 0.1, 1, 5, 10 and 25) of AdsVEGFR-1 vector. AdsVEGFR-1 had no significant impact on the viability of BT4C cells within the range of MOIs tested.

5.1.2 Expression of soluble VEGFR-1 after gene transfer

Transduction of BT4C cells with AdsVEGFR-1 alone and in combination with AdHSV-tk led to the production of human sVEGFR-1 at almost equal concentrations of >700pg/ml. Simultaneous transduction by two-different vectors did not have any significant impact on the production of the transgene construct. This *in-vitro* data supports the simultaneous gene transfer methodology applied in the *in-vivo* experiment to be effective in expressing the transgene constructs without interfering with each other (Figure 12A). It is known that MG produce high amounts of sVEGFR-1 but the VEGF/sVEGFR-1 ratio is increased tipping the balance towards an angiogenic phenotype [Plate et al., 1994] [Lamszus et al., 2003]. The transgene construct used in the study was from the human sVEGFR-1 and the ELISA technique was specific for human sVEGFR-1. Accordingly, no human sVEGFR-1 was detected from the control and AdHSV-tk transduced cells (Figure 12A).

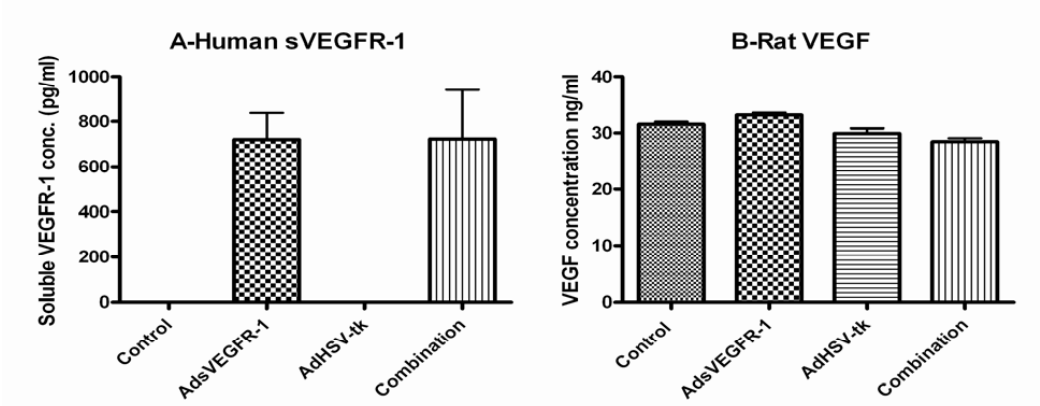

Figure 12: Transduction of BT4C cells simultaneously with 2 different vectors had no effect on the production of sVEGFR-1 since almost equal concentrations were produced by AdsVEGFR-1 transduced cells and combination transduced cells (A). Secretion of VEGF and sVEGFR-1 by BT4C cells after gene transfer with AdHSV-tk, AdsVEGFR-1 or the combination of the two vectors. No significant impact on VEGF secretion by the gene transfers can be demonstrated (B). Error bars indicate standard error of the mean (SEM).

5.1.3 Effect of human sVEGFR-1 on VEGF production

In accordance with the literature [Barth and Kaur, 2009], BT4C rat MG cells produced VEGF in high concentrations (>30ng/ml). Transduction with either vector alone or the vector combination did not have any major effects on the VEGF secretion by the BT4C cells (Figure 12B). We have not differentiated which of the VEGF isoforms are being secreted by BT4C cells. However, $VEGF_{165}$ is typically produced by cancers [Ellis and Hicklin, 2008] [Ferrara, 2004] and in human MGs isoforms 121 and 165 have been reported [Berkman et al., 1993]. Moreover, VEGFR-1 can bind all isoforms of VEGF [Maes et al., 2004]. The ELISA technique used in this study is specific to the rat $VEGF_{164}$ isoform. It was not clear whether this antibody may still detect sVEGFR-1 bound VEGF, which could explain the non-reduction of VEGF levels after AdsVEGFR-1 gene therapy in spite of producing sVEGFR-1. On the other hand, in MGs, VEGF expression far exceeds that of sVEGFR-1 [Lamszus et al., 2003]. Increasing the sVEGFR-1 production in the tumours by gene therapy helps to shift this balance in favour of anti-angiogenesis, even though the VEGF levels are not reduced remarkably. The affinity of the human sVEGFR-1 towards rat VEGF does not seem to be a major reason for the result observed because some studies have confirmed that human sVEGFR-1 is capable of inhibiting angiogenesis in rodent models and achieving therapeutic effects [Yang et al., 2001] [Schmidt et al., 2005] [Graepler et al., 2005] [Harding et al., 2006] and the sequence homology between the two species is 90 % [Keck et al., 1989]. It must also be highlighted at this point that apart from sequestering VEGF, sVEGFR-1 can also act through dominant negative heterodimerisation with VEGFR-2 [Barleon et al., 1997] [Cebe-Suarez et al., 2006] [Kendall et al., 1996] [Thomas, 1996].

5.1.4 The functionality of AdsVEGFR-1

Analysis of the tubule formation revealed that the sVEGFR-1 produced and secreted into the CM by the transduced cells is functional. The area covered by the tubes (Figure 13A), total tube length (Figure 13B), total tubes (Figure 13C) and total branch points (Figure 13D) were less in AdsVEGFR-1 and the combination groups, where the CM contained sVEGFR-1 as compared to the control and AdHSV-tk groups (Figure 13). Analysis of these parameters revealed that the areas covered by the tubes in AdsVEGFR-1 group, and total tube length in AdsVEGFR-1 and the combination groups, were significantly ($p<0.05$) less than the respective control groups. Representative images from each group are presented in Figure 14. These results indicate that the sVEGFR-1 is functional and capable of equally

suppressing VEGF-mediated angiogenesis when transduced either on its own or in combination with AdHSV-tk into BT4C cells.

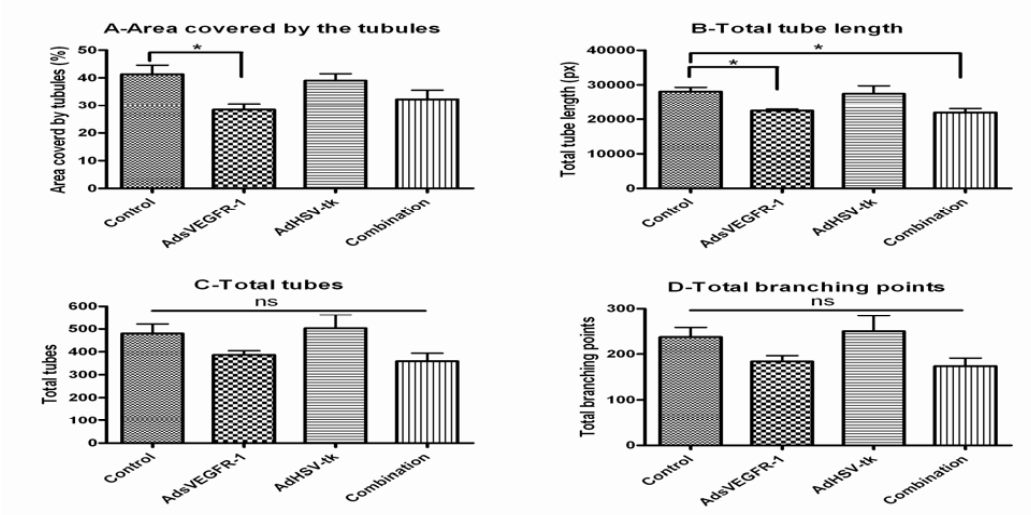

Figure 13: Quantifications of tubule formation assay under different CM, indicating the area covered by tubules (A), total tube length (B), total tubes (C) and total branch points (D). All the parameters demonstrate a similar trend towards lower values in the groups having sVEGFR-1. * = p value <0.05. ns = no statistical significance. Error bars are SEM.

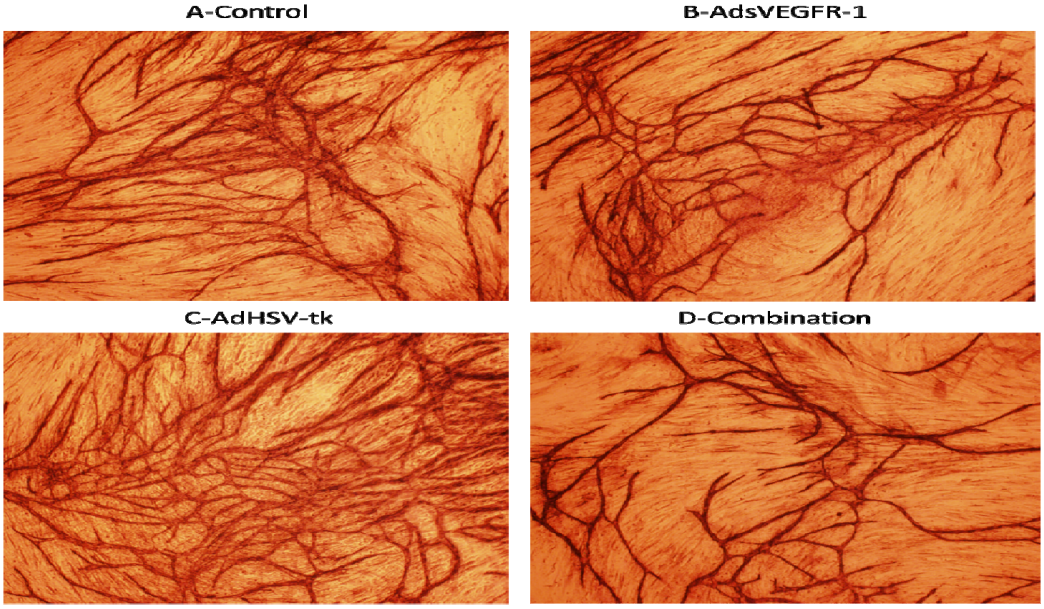

Figure 14: (A) Representative images of tubule formation assay from the control (A), AdsVEGFR-1 (B), AdHSV-tk (C) and the combination (D) groups are presented. Magnification 10x.

5.1.5 The effect of gene transfer on BT4C cell migration

Evidence suggests that treatment of MGs with anti-angiogenic therapies can increase the invasiveness of the tumours [Narayana et al., 2009] [Iwamoto et al., 2009] [Zuniga et al., 2009] [de Groot et al., 2010] [Keunen et al., 2011]. Supplementation of the SFM, where BT4C cells were growing, with the different CM in 10:1 (Figure 15A) or 1:1 (Figure 15B) dilutions

generally increased the invasiveness of BT4C cells in all groups. There was no obvious differential increase among the groups or between any of the groups with the positive control group. One possibility for this observation could be the general depletion of growth factors and nutrients in the CM promoting the invasiveness of the cells towards the chemoattractant. However, addition of AdsVEGFR-1 CM (1:10) significantly increased the invasiveness as compared to the negative control group (Figure 15A). Prior transduction of the cells with viral vectors increased their invasiveness compared to the non-transduced positive control cells but the differences were not statistically significant (Figure 15C). No significant difference was observed among the transduced groups. However, transduction by AdsVEGFR-1 increased the invasiveness significantly compared to the negative control (Figure 15C).

In the reverse experiment, which assessed the invasiveness of the BT4C cells growing in SFM towards the chemoattractant was diminished to the level of negative control by diluting the chemoattractant 1:1 with the CM from control, AdsVEGFR-1, AdHSV-tk and combination groups (Figure 15D). There was no significant differential suppression of the invasiveness among the groups confirming the previous findings that the migration was being affected more by the generalised loss of growth factors and nutrients. However, the inhibition by AdHSV-tk CM was significant as compared to the positive control (Figure 15D).

The results point to a possible increase in the invasiveness of BT4C cells after transduction with adenovirus vectors or secondary to transgene expression by the cells. However, the effect of the individual transgene product in the CM on cell migration could not be evaluated properly due to the potentially superior effect of growth factor and nutrient deprivation in CM. Furthermore, the changes in the levels of VEGF due to the presence of sVEGFR-1 may not have been sufficient to influence the migration potential of the tumour cells.

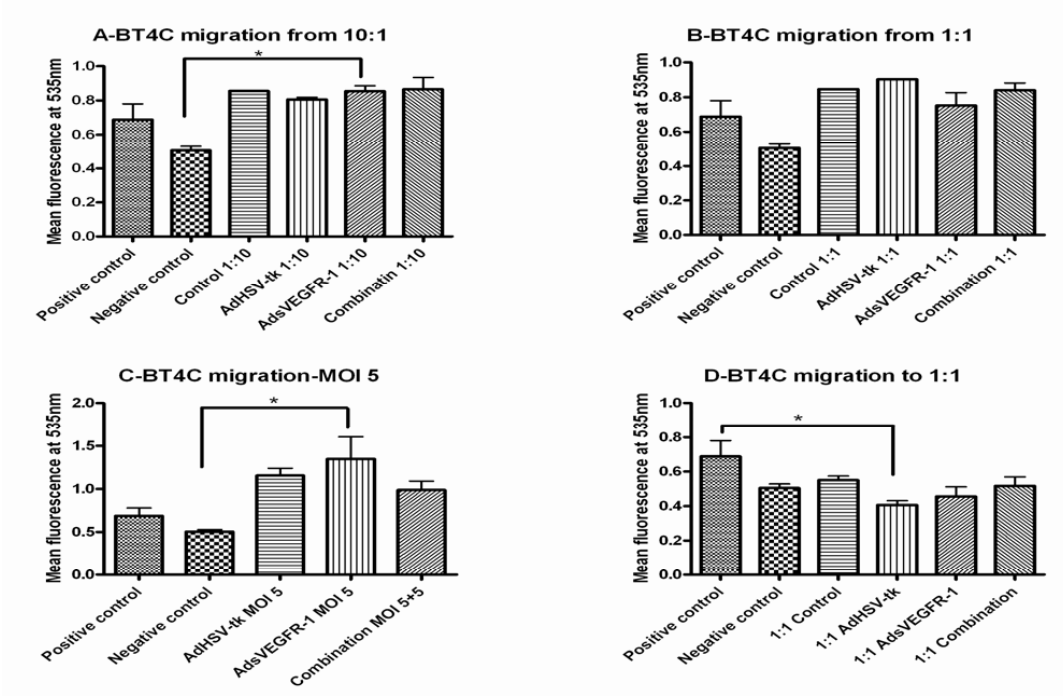

Figure 15: Migration ability of BT4C cells cultured in SFM supplemented 10:1 (A) and 1:1 (B) with CM collected after transduction with AdHSV-tk, AdsVEGFR-1 or the combination compared to the CM from non-transduced cells. No differential increases in the invasiveness were observed among the groups and there were no differences when compared to the positive

control group. Transduction of BT4C cells with the two vectors or the vector combination increased the invasiveness of the cells with a statistically significant increase between the negative control and AdsVEGFR-1 group (C). Migration of BT4C cells towards the chemoattractant supplemented with 1:1 CM was impaired in general in all groups to the level of negative control (D). * = p value <0.05. Error bars are SEM.

5.1.6 The impact of gene therapy combination on tumour volume

There was no significant difference in mean tumour volumes between the groups before starting treatment on 13d (data not shown). MRI on 28d p.i. revealed that AdsVEGFR-1 gene therapy had no effect in controlling tumour growth in this aggressive MG model (Figure 16A). AdHSV-tk/GCV and the combination groups displayed significantly lower ($p<0.001$) mean tumour volumes compared to control and AdsVEGFR-1 only groups, but not compared to each other, suggesting that the addition of AdsVEGFR-1 did not confer a further benefit in impairing tumour growth rate, and that AdHSV-tk/GCV is solely responsible for the effect (Figure 16A). This is in contradiction to what would be expected of an anti-angiogenic therapy, which is to reduce the tumour growth rate [Folkman, 1971], even though they are incapable of reducing the tumour volume [Norden et al., 2008] [Paez-Ribes et al., 2009]. MRI 42d p.i. could not be compared to control or AdsVEGFR-1 groups due to the lack of surviving animals. However, tumour volumes of AdHSV-tk/GCV and the combination groups were not significantly different (Figure 16B). It is surprising that animals in the combination group lived significantly longer (data presented below) compared to the AdHSV-tk/GCV group that had a smaller mean tumour volume on p.i. 42d. Similar results have been reported in the literature with the use of anti-angiogenic therapies [Jahnke et al., 2009], highlighting the possibility that indirect mechanisms such as reduction in peri-tumoural oedema may be contributing to the therapeutic efficacy. This study did not use MRI techniques to detect changes in tumour perfusion and oedema which would have been useful in interpreting the results.

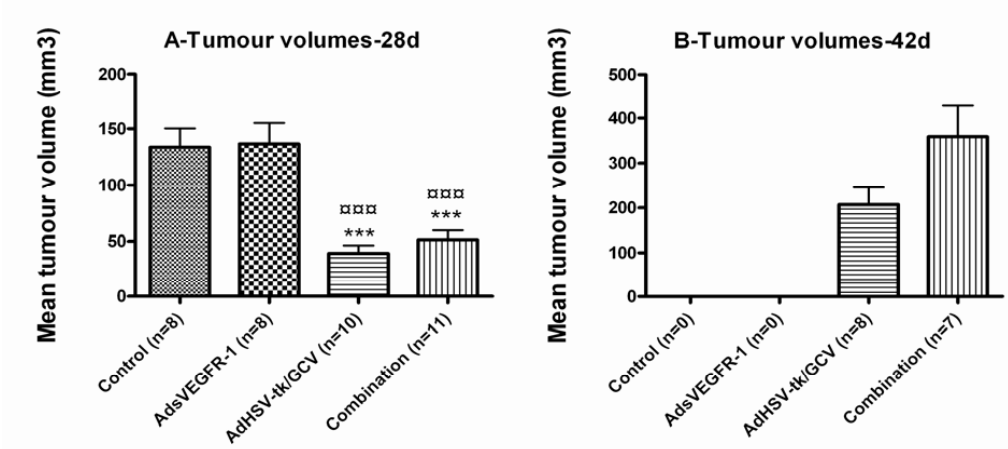

Figure 16: Mean tumour volumes measured by MRI on p.i. 28d (A) and 42d (B). *** and ¤¤¤ denote significances ($p<0.001$) compared to control and AdsVEGFR-1 groups, respectively. No survivors in control and AdsVEGFR-1 groups by p.i. 42d. Error bars are SEM.

5.1.7 The impact of gene therapy combination on survival

AdsVEGFR-1 alone was unable to improve the survival of tumour bearing rats (Figure 17A). In fact, compared to control group, the MS was reduced by 13.5 % in this group. AdHSV-tk/GCV increased the MS by ~11 % and by 28 % compared to control and AdsVEGFR-1 groups, respectively. The combination of AdHSV-tk/GCV and AdsVEGFR-1 was able to increase the MS by 27 % and ~47 % compared to the control and AdsVEGFR-1 groups, respectively (Table 15). Moreover, when compared to AdHSV-tk/GCV group this

was a ~15 % increase. Survival in the combination group was significantly better ($p<0.01$) compared to control (hazard ratio 0.24 (0.08-0.7)) and AdsVEGFR-1 groups (hazard ratio 0.2 (0.06-0.68) (Figure 17D and E). These findings indicate a 76 % and 80 % risk reduction, respectively. However, in spite of having a better trend towards survival, the difference did not reach statistical significance as compared to AdHSV-tk/GCV group (Figure 17F). AdHSV-tk/GCV increased the survival significantly compared to the control group (Figure 17B) ($p<0.05$) but not compared to the AdsVEGFR-1 group (Figure 17C). Furthermore, at the primary end-point of 50d p.i., the combination group had 50 % of the animals still alive as opposed to 16 % in AdHSV-tk/GCV group, further emphasising that combination is superior to AdHSV-tk/GCV alone in enhancing survival in this model. Neither control nor AdsVEGFR-1 groups had any animals surviving at this time (Table 15).

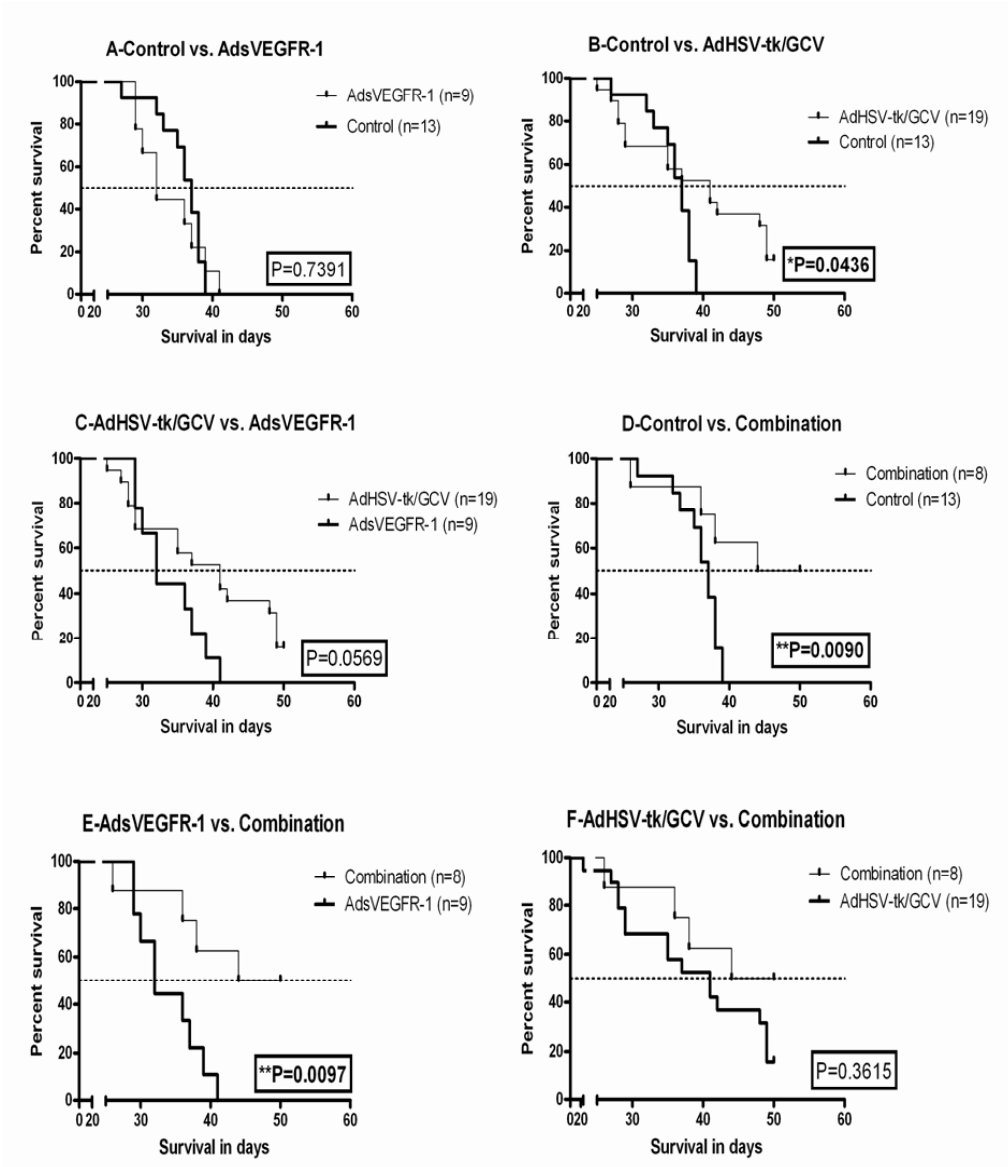

Figure 17: Kaplan-Meier survival plots comparing the survival of the control group with AdsVEGFR-1 (A), AdHSV-tk/GCV (B) and the combination group (D), AdsVEGFR-1 group with AdHSV-tk/GCV (C) and the combination group (E), and AdHSV-tk/GCV group with the

combination group (F). Data was censored at the primary end-point (50d p.i.) and plotted on separate figures to improve the readability of the results. * and ** indicate p values <0.05 and <0.01, respectively.

Table 15: A summary of the survival data for AdHSV-tk/GCV and AdsVEGFR-1 study

Study Groups	n	Survival Data			
		Median Survival	% Change (Control)	% Change (AdsVEGFR-1)	% Survival Day 50
Control	13	37	-	15.6	0
AdsVEGFR-1	9	32	13.5*	-	0
AdHSV-tk/GCV	19	41	10.5	28.1	16
Combination	8	47	27.0	46.8	50

n= number of animals, * percentage decrease from control

The overall results of the *in-vivo* experiments indicate that the combination of AdHSV-tk/GCV and AdsVEGFR-1 is feasible in an orthotopic syngeneic MG model and that the combination achieves a better therapeutic outcome compared to the individual therapy. In spite of having larger tumour volumes at the last imaging the animals in the combination group fared better than those in the other groups. In intracranial tumour models animals often have to be sacrificed due to poor physical wellbeing at least partly due to elevated ICP, obstruction of the ventricular system, peri-tumoural oedema and pressure effects. Several clinical studies have demonstrated a reduction in the peri-tumoural oedema in patients after anti-angiogenic therapy that may have contributed to the general wellbeing of the patients even though it did not have a significant impact on the PFS or OS of the patients [Batchelor et al., 2007]. In the present study both therapies were combined at the same time. However, the optimal protocol for combination still remains untested.

5.1.8 The safety of gene therapy combination

Liver function tests, AFOS and bilirubin were within the normal range for rats and did not display any significant differences among the groups (Figure 18B and C). However, ALT levels were significantly lower compared to the AdHSV-tk/GCV group in AdsVEGFR-1 ($p<0.01$) and combination ($p<0.001$) groups, and when compared to the control groups, in the combination group ($p<0.05$) (Figure 18A).

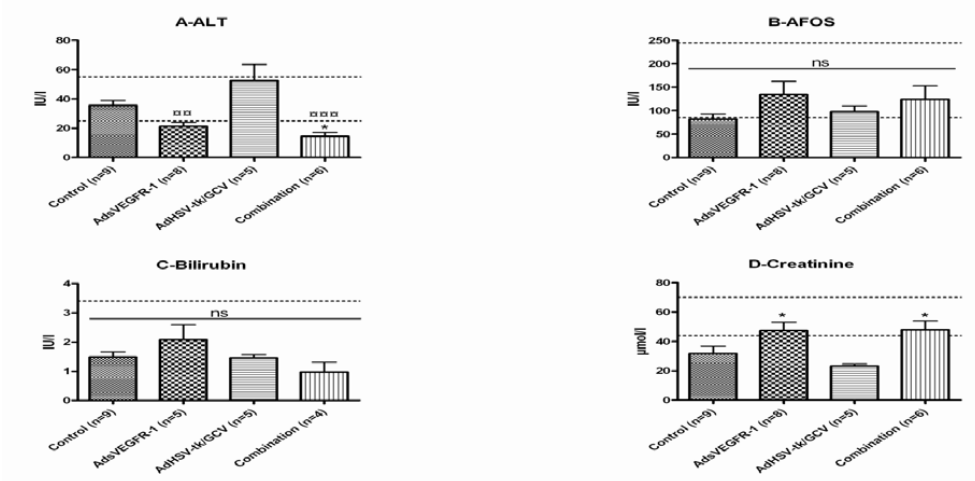

Figure 18: Serum clinical chemistry analyses of alanine aminotransferase (ALT) (A), alkaline phosphatase (AFOS) (B), bilirubin (C) and creatinine (D). Range of normality for rats are

indicated with horizontal lines and the error bars indicate SEM. ns = no significance. * = $p<0.05$, ¤¤ = $p<0.01$, and ¤¤¤ = $p<0.001$.

The ALT levels were within the upper-limits of normality in the AdHSV-tk/GCV groups. Transient elevation of ALT levels has been reported with AdHSV-tk/GCV suicide gene therapy [Immonen et al., 2004] (Figure 18A). The renal function test, serum creatinine, was within the normal range in AdsVEGFR-1 and combination groups and below the normal range in the control and AdHSV-tk/GCV groups. The differences were significant ($p<0.05$) in AdsVEGFR-1 and combination groups compared to AdHSV-tk/GCV group (Figure 18D). Previous studies have reported adverse effects such as liver and renal toxicities with systemic administration of adenovirus-mediated sVEGFR-1 [Mahasreshti et al., 2003] [Mahasreshti et al., 2001] [Kuo et al., 2001], but not with local gene transfer. Our results support the fact that AdsVEGFR-1 gene therapy is safe with local gene delivery.

5.1.9 Immunohistochemistry

As expected, human sVEGFR-1 expression was limited to AdsVEGFR-1 and combination gene therapy groups (Figure 20, sVEGFR-1 B and D). VEGF expression by the BT4C tumours seems to be diminished in those two groups (Figure 20, VEGF: B and D) providing *in-vivo* confirmation for the functionality of the AdsEVGFR-1. However, the CD34 staining for angiogenesis did not show a significant difference among the study groups (Figure 20, CD34). The sVEGFR-1 exerts its therapeutic effect mainly through inhibition of tumour angiogenesis secondary to sequestration of VEGF [Barleon et al., 1997] [Cebe-Suarez et al., 2006] [Kendall et al., 1996] [Thomas, 1996]. AdHSV-tk/GCV can also kill dividing endothelial cells and have anti-angiogenic properties [Ram et al., 1994] [Floeth et al., 2001]. Quantification of the angiogenesis confirmed that there was no difference in the mean capillary number (Figure 19A) and density (Figure 19B) between the groups. In this study, contrary to the popular belief, inhibition of angiogenesis was not a major anti-tumour mechanism. Even though MGs secrete high amounts of VEGF, several other angiogenic mediators have been described, that may well have contributed to the tumour angiogenesis [Jain et al., 2007] [Fischer et al., 2005a] [Furnari et al., 2007]. Furthermore, several mechanisms of resistance to VEGF-targeted anti-angiogenic therapies have been reported [Bergers and Hanahan, 2008] that could have led to the escape of tumour from this therapy. When the samples were collected 10 days after therapy, human sVEGFR-1 was still being expressed by the tumours, excluding the possibility that the lack of gene expression is the reason for failure to inhibit angiogenesis. Moreover, adenovirus-mediated gene expression in the rat brain has been reported for up to 6 months [Geddes et al., 1997].

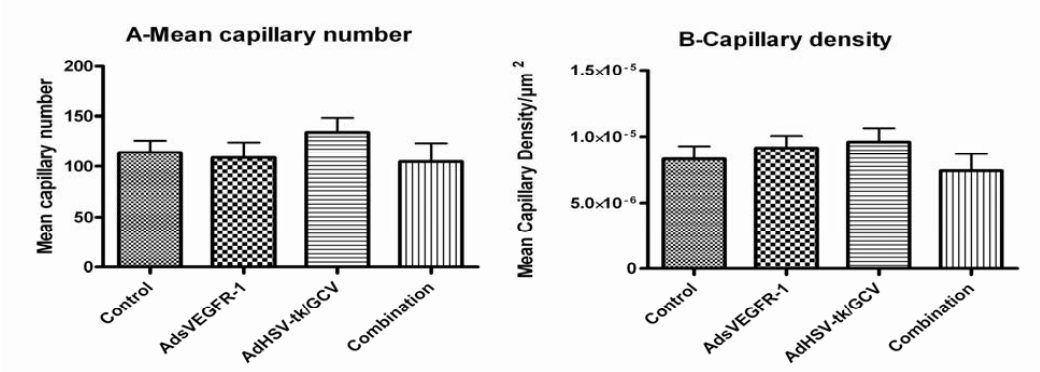

Figure 19: Quantification of tumour angiogenesis from CD34 stainings. The mean capillary number (A) and the mean capillary density (B) did not show any significant difference among the groups. Error bars are SEM.

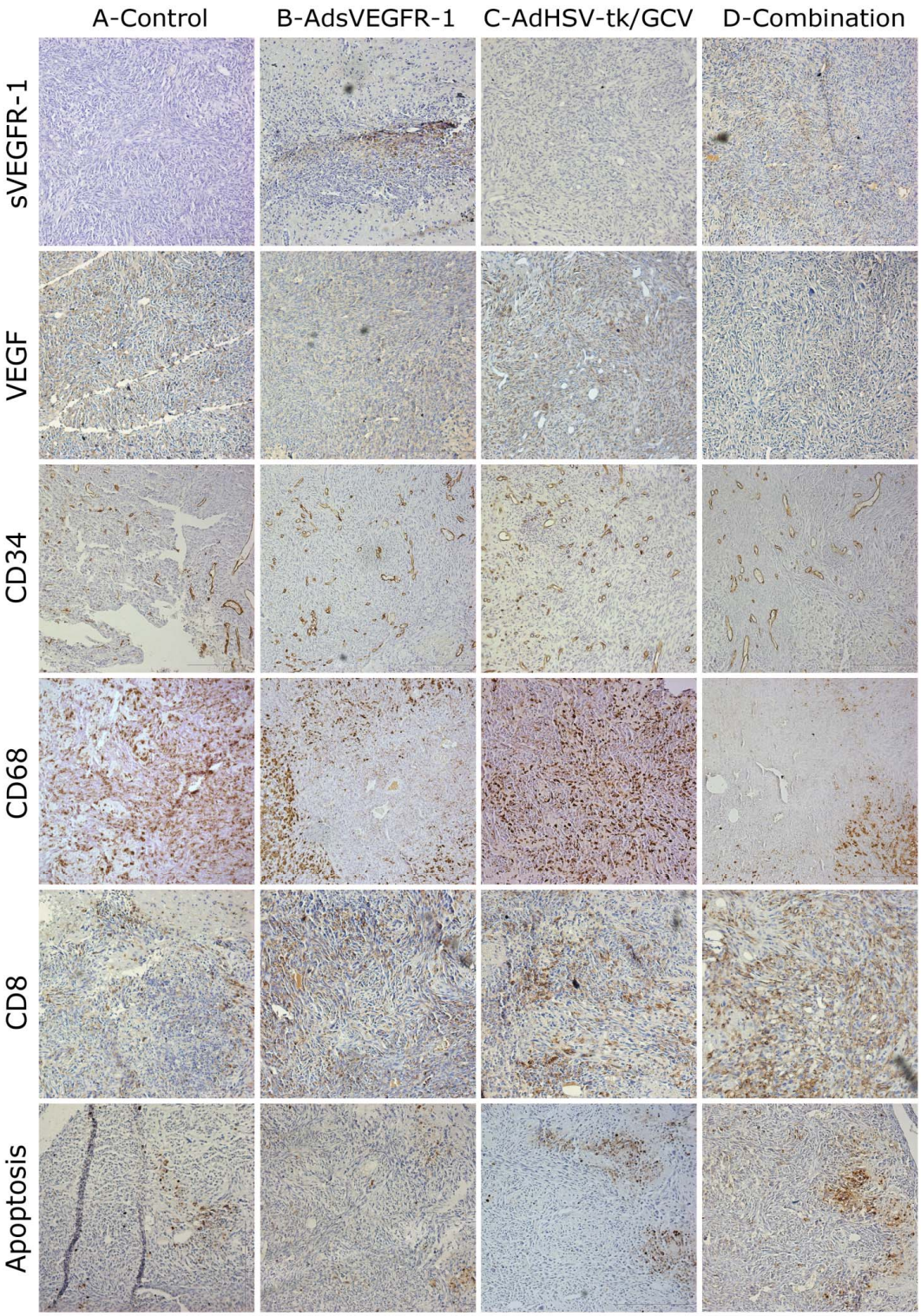

Figure 20: Immunohistochemical stainings for human sVEGFR-1, rat VEGF, angiogenesis by CD34, tumour infiltrating macrophages/microglia by CD68, tumour infiltrating cytotoxic T-lymphocytes by CD8 and apoptosis by ApoTag® staining in control (A), AdsVEGFR-1 (B), AdHSV-tk/GCV (C) and Combination (D) groups. Magnification 20x.

Since viral vectors were used to deliver the transgenes into the tumours, the triggering of an immune response against the virus vectors that would evoke an anti-tumour immune response is a possibility. Thus, the tumour sections were stained for CD68, CD8, CD4 and CD25 expressing cells but no expression of CD4 or CD25 was observed (data not shown). On the other hand, tumours were grossly infiltrated by CD68 positive cells (Figure 20, CD68; A-D). There is evidence to suggest that CD68 positivity in MG can be as high as 30 % [Graeber et al., 2002] [Watters et al., 2005]. CD68 has been described by some to be a marker of both M1 and M2-phenotype macrophages [Ohri et al., 2009]; while others propose that it is a marker of the M2 phenotype [Murdoch et al., 2008]. However, recent evaluation of human MG samples suggests that CD68 is also expressed by both microglia and tumour cells. Furthermore, the high CD68 positivity was associated with a higher malignancy grade and poor survival [Strojnik et al., 2009]. Multiple pro-tumour mechanisms have been attributed to TAMs [Naganuma et al., 1996] [Letterio and Roberts, 1998] [Watters et al., 2005]. On close inspection, it is clear that a specific area around the gene transfer needle-track in AdsVEGFR-1 and combination groups was devoid of CD68+ cells (Figure 20, CD68; B and D) as opposed to diffuse uniform TAM infiltration in the control and AdHSV-tk/GCV groups (Figure 20, CD68; A and D). VEGF is a known chemoattractant for TAMs due to their expression of VEGFR-1 and/or VEGFR-2 [Barleon et al., 1996] [Muramatsu et al., 2010] [Kerber et al., 2008] [Dineen et al., 2008]. Hence, it is apparent that the action of sVEGFR-1 in sequestering VEGF has inhibited the CD68+ pro-tumorigenic TAM infiltration into some areas of the tumour, which could possibly aid in the anti-tumour response. Similar results have been reported with anti-VEGF therapies in other cancers [Di et al., 2011] [Roland et al., 2009]. All groups had some CD8+ T-lymphocyte infiltration (Figure 20, CD8) and as described previously, the AdHSV-tk/GCV treated groups seem to have a higher number of CD8+ cells (Figure 20, CD8; C and D) [Perez-Cruet et al., 1994] [Aguilar et al., 2011]. Apoptotic cells were observed in all groups (Figure 20, Apoptosis), but were more pronounced in AdHSV-tk/GCV treated groups (Figure 20, Apoptosis; C and D).

5.1.10 Duration of efficacy

The gene therapy combination started to reduce the tumour growth rate 4 days after gene transfer (3 days after starting GCV) on p.i. 25d, when adenovirus vector-mediated gene expression was at its highest. Mean tumour volumes were significantly lower ($p<0.05$) compared to the controls between p.i. 28-32d (Figure 21). In the treatment group, tumour growth rates started accelerating 14 days after gene transfer (7 days after stopping GCV) and were no longer significant from p.i. 35d onwards. The therapeutic efficacy lasted for only a few days after the stopping of GCV and was not capable of completely eliminating the established tumours. Possible limiting factors in the study could be the duration of GCV therapy and transgene expression.

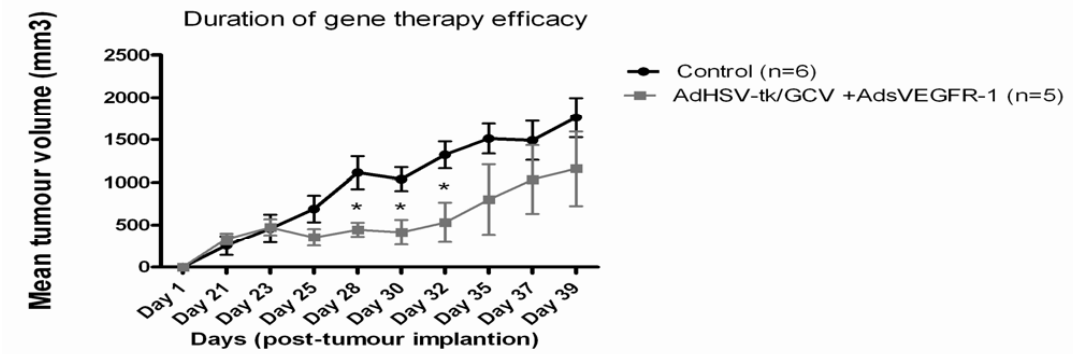

Figure 21: Comparison of mean tumour volumes of control and combination gene therapy groups at gene transfer (21d), during (23-28d) and after (30-39d) GCV therapy. * indicate significant ($p<0.05$) differences. Error bars are SEM.

AdHSV-tk/GCV is a well-established suicide gene therapy strategy, which is in clinical trials for MG [Immonen et al., 2004]. Soluble VEGFR-1 has been extensively studied as an anti-angiogenic gene therapy strategy against a variety of cancers including MG [Ohlfest et al., 2005] [Harding et al., 2006] [Goldman et al., 1998]. The high vascularity of MGs theoretically makes them highly amenable to anti-angiogenic therapy. With the approval of BV, anti-angiogenic therapies have entered the MG therapeutic arena. However, previously AdHSV-tk/GCV and AdsVEGFR-1 gene therapies have not been combined for the treatment of MG or for that matter, any other cancer. AdHSV-tk/GCV has been successfully combined with endostatin gene therapy for the treatment of renal cell cancer [Pulkkanen et al., 2002], while sVEGFR-1 has been combined with oncolytic viruses [Zhang et al., 2005], [Guse et al., 2010], other sVEGFRs [Sallinen et al., 2009], soluble Tie2 [Sallinen et al., 2011] and chemotherapy [Sopo et al., 2012]. All previous studies with sVEGFR-1 gene therapy for MG were conducted on nude mice models. Therefore, this is the first study to test the efficacy of sVEGFR-1 in an immunocompetent orthotopic rat MG model using adenovirus vectors to deliver sVEGFR-1 gene therapy. It was possible to successfully demonstrate the feasibility of combining these two gene therapy strategies together for the treatment of MG and to examine the putative anti-tumour mechanisms in operation. One possible mechanism for the better therapeutic outcome of this combination could be the increased GCV delivery into the tumour due to vascular normalisation by anti-angiogenic therapy and the reduced peri-tumoural oedema and ICP improving the general wellbeing of the animals. However, the sVEGFR-1 gene therapy did not demonstrate any significant anti-angiogenic effects in spite of enhancing the efficacy of AdHSV-tk/GCV suicide gene therapy. The study was not intended to differentiate between whether there was no inhibition of angiogenesis from the beginning or whether resistance mechanisms subsequently masked the effect later. As a single therapy AdsVEGFR-1 did not confer any therapeutic benefit, in line with the concept that anti-angiogenic therapy should be combined with a cytotoxic therapy for optimal benefit [Ibrahim et al., 2012]. AdsVEGFR-1 therapy in this study was given to the established tumours on p.i. 14d. Since the presence of tumours needed to be confirmed before therapy, which could not be done by MRI before p.i. 12-13d, it was not possible to undertake earlier commencement of the therapy. Early treatment by anti-angiogenic therapy may have led to a different outcome. The underlying reasons for the better therapeutic effect needs further evaluation and the optimal therapeutic protocol, which would maximise the beneficial effect, remains to be determined. AdsVEGFR-1 as an anti-angiogenic agent has some advantages over the currently approved BV. Being a parenteral therapy, BV has the disadvantage of evoking systemic adverse effects. On the other hand, tumour-directed AdsVEGFR-1 anti-angiogenic therapy does not carry this risk. Furthermore, BV has failed to confer an OS benefit to the MG patients [Gerstner and Batchelor, 2012], whereas the present findings suggest that AdsVEGFR-1 in combination with cytotoxic therapies may enhance survival. The major limitation in combination gene therapy strategies, especially in the brain, is the limited vector volume that can be injected into the tumours. The development of concentrated vectors and vectors expressing multiple transgene constructs could be one way forward to improve therapeutic effects.

5.2 SUICIDE GENE THERAPY AND TEMOZOLOMIDE STUDY

5.2.1 Synergism between AdHSV-tk/GCV and TMZ in BT4C cells

The cytotoxicity was markedly enhanced when AdHSV-tk/GCV was simultaneously combined with TMZ in BT4C cells (Figure 22). TMZ alone, even at therapeutic plasma concentrations of 100 μmol/L [Ostermann et al., 2004], was not very efficient in killing BT4C cells. TMZ at 10 μmol/L had no effect alone, except when combined with MOI 10 of

AdHSV-tk/GCV. However, at 100 µmol/L TMZ shows a clear dose response with increasing MOIs of AdHSV-tk/GCV. The cell viability in this group was less than that of the corresponding AdHSV-tk/GCV only groups, indicating a possible synergistic or an additive effect of the combination. Interestingly, TMZ at 10 µmol/L. in combination with AdHSV-tk/GCV at MOI 10, showed much better cytotoxicity than the 100 µmol/L concentration of TMZ on its own. The results confirm the hypothesis that, the therapeutic efficacy is markedly enhanced by simultaneously combining AdHSV/tk/GCV with TMZ. The ability of AdHSV-tk/GCV to potentiate the cytotoxicity of TMZ is worth pursuing because AdHSV-tk/GCV is a targeted gene therapy affecting only the tumour. The combination was evaluated as being synergistic or additive by using the fractional product method of Webb [Webb, 1961] (Table 16). TMZ at 10µmol/L was only synergistic at MOI 10, whereas at 100 µmol/L TMZ demonstrated a marked synergistic cytotoxic effect with all 3 MOIs of AdHSV-tk/GCV. This finding is important because TMZ is the standard care of chemotherapy for GBM and AdHSV-tk/GCV is under clinical evaluation for the same condition.

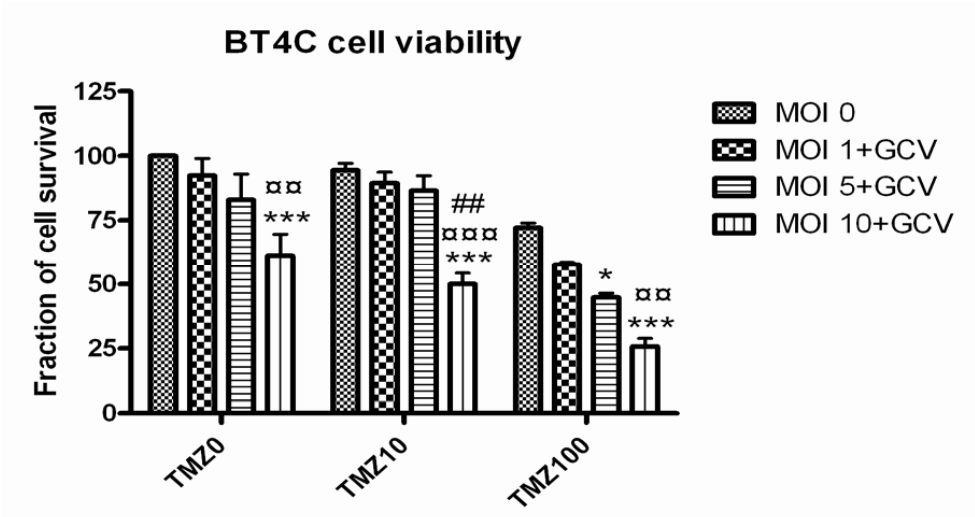

Figure 22: BT4C cell viability after combining MOIs 1, 5 and 10 of AdHSV-tk and 1 µg/ml GCV with 10 and 100 µmol/L of TMZ. The triplicate values were normalized to the average of untreated cells. The significances compared to MOI 0, MOI 1 and MOI 5 are indicated in *, ¤ and #, respectively. P values <0.05, <0.01 and <0.001 are indicated as */¤/#, **/¤¤/## and ***/¤¤¤/###, respectively. The viability of BT4C cells was significantly reduced by adding TMZ to AdHSV-tk/GCV, even at a low concentration of 10 µmol/L. Error bars indicate SEM.

Table 16: Evaluation of the synergism between simultaneous combination of AdHSV-tk/GCV and TMZ in BT4C cells by fractional product method of Webb

Viability with AdHSV-tk/GCV		**Viability with TMZ**		**Predicted viability**	**Observed viability**	
MOI	**γ1**	**Dose**	**γ2**	**γ1γ2**	**Combination**	**γ12**
1	0.924	10	0.944	0.872	MOI-1+10	0.894
1	0.924	100	0.721	**0.666**	MOI-1+100	**0.577**
5	0.830	10	0.944	0.783	MOI-5+10	0.865
5	0.830	100	0.721	**0.599**	MOI-5+100	**0.449**
10	0.599	10	0.944	**0.565**	MOI-10+10	**0.543**
10	0.599	100	0,721	**0.432**	MOI-10+100	**0.252**

GCV dose 1 µg/ml and TMZ dose was 10 or 100 µmol/L. Points of synergism are shown in bold**.**

5.2.2 The impact of MGMT repair status on the synergism

To this end, the known MGMT expressing human MG cell line T98G [Park et al., 2006] and the MGMT non-expressing cell line U87MG [Blough et al., 2011] were evaluated. The expression of MGMT by MG confers a primary resistance to methylating agents such as TMZ [Hegi et al., 2005]. AdHSV-tk/GCV demonstrated some cytotoxicity in T98G cells, whereas TMZ had absolutely no effect (Figure 23A) and the comparison did not reveal any synergy (Table 17). On the other hand, the MGMT negative U87MG cell line was efficiently killed by both AdHSV-tk/GCV and TMZ (Figure 23B), and there was profound synergism in this interaction (Table 18). The TMZ dose of 100 μmol/L was chosen because it is a physiologically achievable dose [Ostermann et al., 2004] that had shown synergy in BT4C cells. Previous work has shown that T98G cells are not very sensitive to AdHSV-tk/GCV therapy; hence the GCV dose was increased up to 10 μg/ml while AdHSV-tk MOI was increased up to 20 to examine whether a therapeutic effect could be achieved. TMZ at 100 μmol/L demonstrated a clear dose-response with AdHSV-tk/GCV in U87MG cells (Figure 23B).

Results from microscopic examination of T98G and U87MG cells 4 days after the last treatment are shown in Figure 24. The results highlight the lack of efficacy of TMZ in MGMT positive T98G cells, which demonstrated some sensitivity to AdHSV-tk/GCV. However, MGMT negative U87MG cells were sensitive to both TMZ and AdHSV-tk/GCV but the cytotoxicity of the gene therapy was clearly superior (Figure 24).

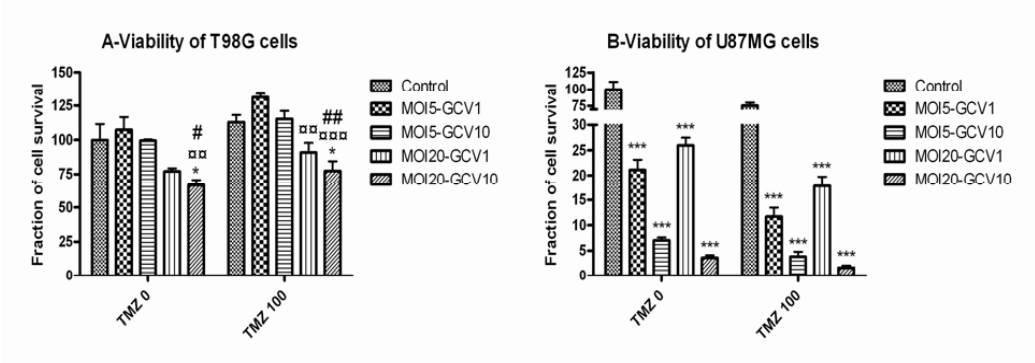

Figure 23: T98G (A) and U87MG (B) cell viability measured by luminescence assay after combining MOIs 5 and 20 of AdHSV-tk/GCV and GCV at doses of 1 or 10μg/ml with 100 μmol/L of TMZ. The triplicate values were normalized to the average of untreated cells. Significances compared to the control, MOI5-GCV1 and MOI5-GCV10 groups are indicated by */***, ¤/¤¤/¤¤¤ and #/##, respectively. P values <0.05, <0.01 and <0.001 are indicated as */¤/#, **/¤¤/## and ***/¤¤¤/###, respectively. Error bars are SEM.

Table 17: Evaluation of the synergism between simultaneous administration of AdHSV-tk/GCV and TMZ in MGMT positive T98G human MG cells

Viability with AdHSV-tk/GCV		Viability with TMZ		Predicted viability	Observed viability	
MOI/GCV	**γ1**	**Dose**	**γ2***	**γ1γ2**	**Combination**	**γ12**
MOI-5/GCV-1	1.073	100	1.129	1.211	MOI-5/GCV-1+TMZ	1.312
MOI-5/GCV-10	0.995	100	1.129	1.124	MOI-5/GCV-10+TMZ	1.154
MOI-20/GCV-1	0.771	100	1.129	0.871	MOI-20/GCV-1+TMZ	0.908
MOI-20/GCV-10	0.664	100	1.129	0.750	MOI-20/GCV-10+TMZ	0.775

No synergism was observed. *The cell viability with TMZ alone was more than that of controls.

Table 18: Evaluation of the synergism between simultaneous administration of AdHSV-tk/GCV and TMZ in MGMT negative U87MG human MG cells

Viability with AdHSV-tk/GCV		Viability with TMZ		Predicted viability	Observed viability	
MOI/GCV	**γ1**	**Dose**	**γ2**	**γ1γ2**	**Combination**	**γ12**
MOI-5/GCV-1	0.211	100	0.753	**0.159**	MOI-5/GCV-1+TMZ	**0.117**
MOI-5/GCV-10	0.072	100	0.753	**0.053**	MOI-5/GCV-10+TMZ	**0.038**
MOI-20/GCV-1	0.247	100	0.753	0.186	MOI-20/GCV-1+TMZ	0.189
MOI-20/GCV-10	0.033	100	0.753	**0.025**	MOI-20/GCV-10+TMZ	**0.015**

GCV doses of 1 and 10 μg/ml with TMZ dose of 100 μmol/L. Points of synergism are shown in bold.

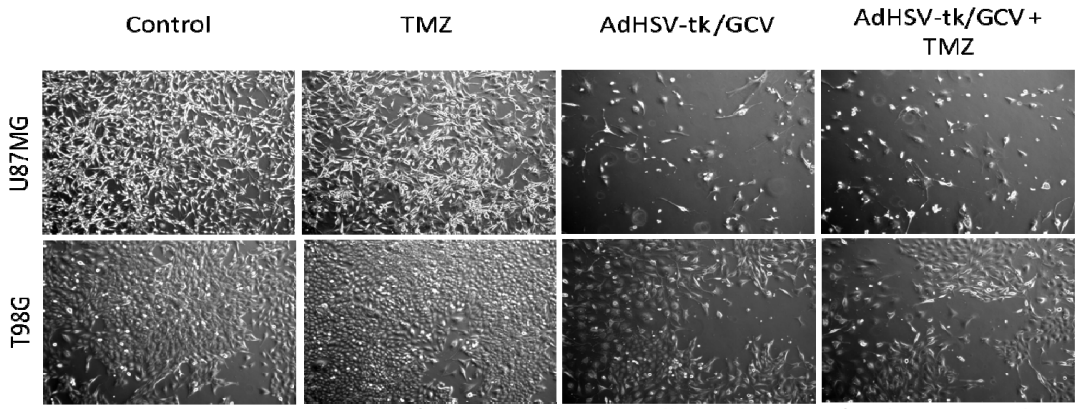

Figure 24: Microscopic examination of U87MG and T98G cells on day 10, after being transduced with AdHSV-tk MOI 5 and treated with 1 μg/ml of GCV and/or 100 μmol/L TMZ on days 4 and 6. Magnification 10x.

These findings confirm the observations in BT4C rat MG cells in human MG cells, where the simultaneous combination of AdHSV-tk/GCV with TMZ was synergistic. However, the synergism is dependent on the cellular MGMT status. MG cells expressing MGMT were not sensitive to TMZ; hence no synergism was observed. Whether synergy can be achieved in MGMT positive cell lines by depleting MGMT needs to be evaluated. On the other hand, MGMT negative MG cells demonstrated marked synergism when treated with AdHSV-tk/GCV and TMZ. Expression of MGMT by MG cells confers primary resistance to the alkylating chemotherapeutic agents such as TMZ [Kaina et al., 2007]. The MGMT status of BT4C cells was evaluated by Western blot and RT-PCR but no protein or RNA amplification was observed, respectively (data not shown), in spite of the cell line being extremely resistant to TMZ treatment. Apart from changes in MGMT expression, several other mechanisms of resistance to TMZ have been described, including MMR dysfunction [Branch et al., 1993].

5.2.3 The effect of treatment protocol on synergism

Next aim was to evaluate whether the treatment protocol has an impact on the synergism. To this end, BT4C cells were treated with TMZ either 5 (Figure 25A) or 10 days (Figure 25B) after starting GCV therapy. When administered alone, TMZ evoked only a marginal cytotoxicity in BT4C cells at 100 μmol/L dose, whereas 10 μmol/L dose was totally ineffective (Figure 25A and Table 19). However, both doses of TMZ induced synergistic cytotoxicity with increasing MOIs of AdHSV-tk/GCV when added 5 days after the first dose of GCV (Table 19), even though TMZ alone caused only a marginal cytotoxicity in BT4C cells. When TMZ was added 10 days after starting GCV, it was still able to demonstrate a synergistic cytotoxic effect with AdHSV-tk/GCV in spite of failing to exert

any cytotoxicity in BT4C cells as a monotherapy even at 100 μmol/L dose (Figure 25B and Table 20). Interestingly, AdHSV-tk/GCV was able to maintain anti-tumour effects even after 12 days from the last GCV dosing with extremely high cytotoxicity (Figure 25B and Table 20). These findings indicates that the synergistic effect can still be achieved by administering TMZ even 10 days after starting GCV therapy. However, the synergism observed between AdHSV-tk/GCV and TMZ is protocol dependent and may even be lost if administration of TMZ is delayed for too long.

The crystal violet stainings, which were done with the same experimental protocol, where TMZ was added 5 days after starting AdHSV-tk/GCV, confirms that there had been a profound synergistic effect between these two combinations with even a 5 day gap between the therapies (Figure 26). TMZ at 100 μmol/L had a marginal cytotoxic effect on BT4C cells, whereas AdHSV-tk/GCV was much more efficient in killing the cells.

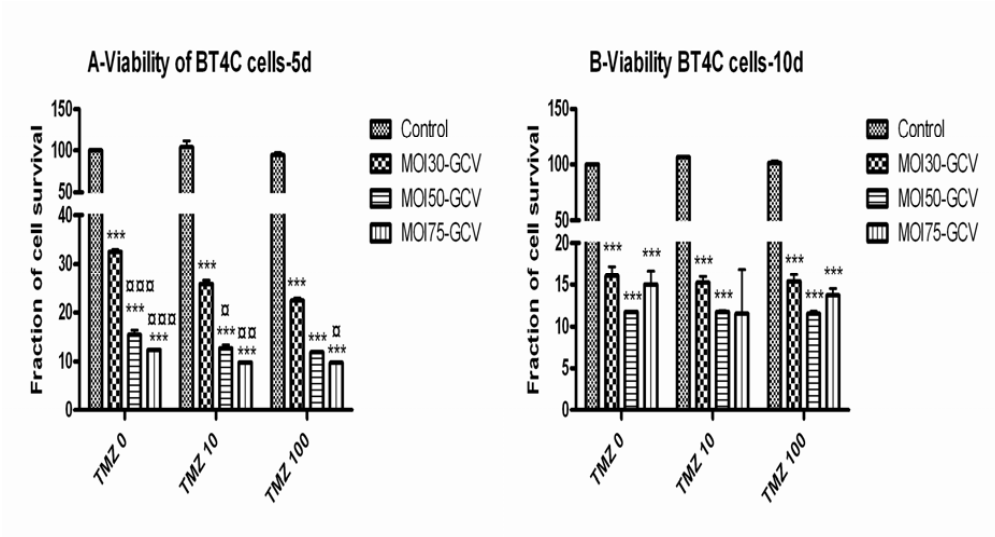

Figure 25: BT4C cell viability after combining with 10 or 100 μmol/L of TMZ at 5 (A) and 10 days (B) after starting GCV at 1 μg/ml following transduction with AdHSV-tk at MOIs 30, 50 and 75. The triplicate values were normalized to the average of untreated cells. All MOIs had *** significance ($p<0.001$) compared to the controls. Significances compared to the control and MOI30-GCV groups are indicated by *** and ¤/¤¤/¤¤¤, respectively. P values <0.05, <0.01 and <0.001 are indicated as */¤, **/¤¤ and ***/¤¤¤, respectively. Error bars are SEM.

Table 19: Evaluation of the synergism when TMZ is added 5 days after AdHSV-tk/GCV in BT4C cells

Viability with AdHSV-tk/GCV		**Viability with TMZ**		**Predicted viability**	**Observed viability**	
MOI	**γ1**	**Dose**	**γ2**	**γ1γ2**	**Combination**	**γ12**
30	0.325	10	1.038*	**0.337**	MO1-30+10	**0.258**
30	0.325	100	0.950	**0.309**	MOI-30+100	**0.224**
50	0.154	10	1.038*	**0.160**	MOI-50+10	**0.127**
50	0.154	100	0.950	**0.147**	MOI-50+100	**0.119**
75	0.124	10	1.038*	**0.128**	MOI-75+10	**0.098**
75	0.124	100	0.950	**0.117**	MOI-75+100	**0.097**

Points of synergism are indicated in bold. * The fractional survival with TMZ 10 μmol/L alone is more than one indicating lack of cytotoxicity.

Table 20: Evaluation of the synergism when TMZ is added 10 days after AdHSV-tk/GCV in BT4C cells

Viability with AdHSV-tk/GCV		Viability with TMZ		Predicted viability	Observed viability	
MOI	**γ1**	**Dose**	**γ2**	**γ1γ2**	**Combination**	**γ12**
30	0.160	10	1.064*	**0.170**	MO1-30+10	**0.152**
30	0.160	100	1.012*	**0.162**	MOI-30+100	**0.154**
50	0.117	10	1.064*	**0.125**	MOI-50+10	**0.117**
50	0.117	100	1.012*	**0.119**	MOI-50+100	**0.115**
75	0.150	10	1.064*	**0.160**	MOI-75+10	**0.115**
75	0.150	100	1.012*	**0.152**	MOI-75+100	**0.137**

Points of synergism are indicated in bold. * The fractional survival with TMZ alone is more than one indicating lack of cytotoxicity.

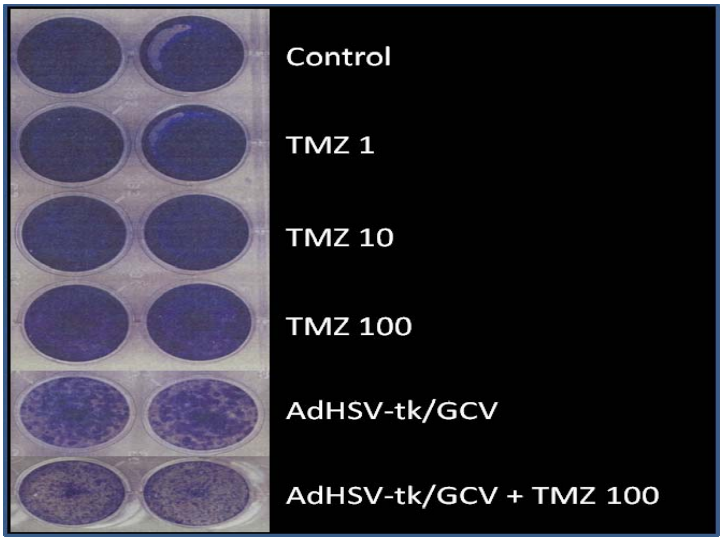

Figure 26: The crystal violet staining conducted 4 days after TMZ therapy of BT4C cells treated with AdHSV-tk (MOI 5)/GCV (1 μg/ml) on day 4 and 6 and TMZ (1, 10 and 100 μmol/L) on day 9.

Previous work by Rainov *et al.* has detected *in-vitro* synergism between these two therapies when they are combined simultaneously [Rainov et al., 2001]. This study demonstrated that the synergism between AdHSV-tk/GCV and TMZ was protocol dependent and most likely to be lost if the TMZ administration were to be delayed beyond 10 days after GCV therapy. This suggests there could be a specific therapeutic window for the synergism between these two therapies. What was even more interesting was that treatment with AdHSV-tk/GCV sensitised both human and rat MG cells to TMZ in a MGMT status and protocol-dependent manner. The use of fractional product method to evaluate synergism is valid only in pure, mutually non-exclusive conditions, when dose-effect relationships are of first-order kinetics. In other words, synergism calculated by this method may not be consistent with the classical isobologram method, since it only takes into account potency ignoring the dose-effect curve of each drug and may underestimate the synergism compared to classical isobologram method. There are other more advanced and sensitive methods to determine synergy [Chou, 2002].

5.2.4 The mechanism of synergism

The mechanism behind the synergistic effects of TMZ and AdHSV-tk/GCV was further studied in BT4C cell culture experiments. It is known that the efficacy of TMZ is highly dependent on the status of several DNA repair pathways such as MGMT and MMR in the cancer cells [Kaina et al., 2007]. The previous experiments had revealed that the synergism was dependent on the MGMT repair status of the cells. Hence, it was hypothesised that in MG cells that do not express MGMT, the synergism could be related to the status of MMR pathway. The MMR status of the BT4C cells was analysed by RT-PCR of MLH1 and MSH2, after treating the cells simultaneously with AdHSV-tk/GCV and TMZ. The MLH1 (Figure 27A) and MSH2 (Figure 27B) gene expressions were significantly ($p<0.001$) increased after AdHSV-tk/GCV therapy and the increase was more than four and three folds, respectively compared to the untreated control BT4C cells. The increase was persistent when AdHSV-tk/GCV was combined with TMZ (Figure 27). No such increase was seen with TMZ therapy alone. A significant up-regulation of RNA levels was observed only four days after the start of GCV treatment.

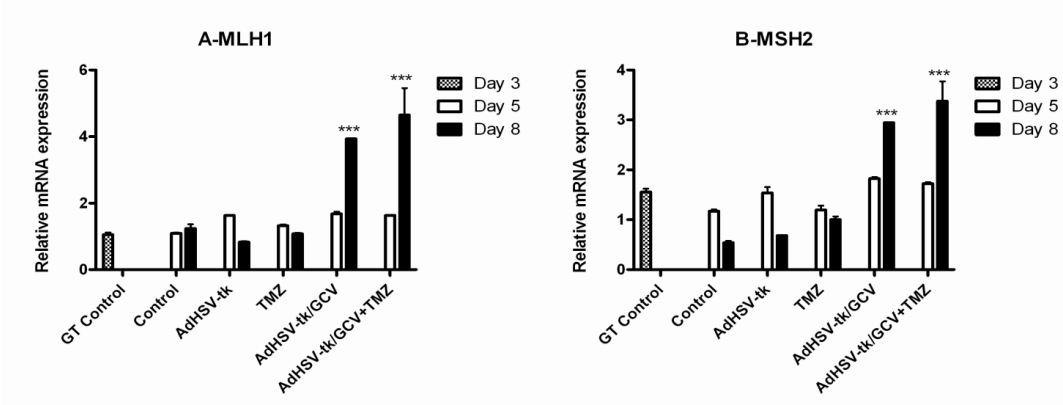

Figure 27: Relative mRNA expression of MLH1 (A) and MSH2 (B) MMR proteins by BT4C cells measured by RT-PCR. *** indicates a significant ($p<0.001$) increase compared to the control cells. Error bars are SEM.

It is already known that impairment of MMR pathway confers almost 100-fold resistance to methylating agents such as TMZ [Branch et al., 1993] [Koi et al., 1994]. A functional MMR pathway is critical to allow the TMZ induced O^6-mG lesions to be converted into cytotoxic DSBs [Kaina et al., 2007]. Even though increased nuclear translocation of MMR proteins has been reported after methylating agent therapy [Christmann and Kaina, 2000], up-regulation of the gene expression of the MMR pathway after AdHSV-tk/GCV suicide gene therapy has not been reported before. This is the first report of up-regulation of MMR pathway gene expression after AdHSV-tk/GCV therapy and is being proposed as the mechanism by which AdHSV-tk/GCV enhances the cytotoxicity of TMZ administered within the therapeutic window. The study by Rainov *et al.* demonstrated a synergism between HSV-tk/GCV and TMZ when combined simultaneously. However, they failed to provide a mechanism for the synergism. It was postulated that inhibition of DNA polδ by the suicide gene therapy could be one reason for synergism [Rainov et al., 2001]. Even though we and others [Rainov et al., 2001] have shown synergism with simultaneous administration of the two therapies, for the maximum synergistic effect, the TMZ should be administered during the MMR pathway up-regulation, which occurs a few days after starting GCV therapy. AdHSV-tk/GCV-mediated enhancement of the cytotoxicity of TMZ is unique because this enhancement is limited to the tumour, where the gene therapy is effective. Hence, theoretically this would not increase the adverse effects of TMZ, and in this way could possibly increase the therapeutic index of TMZ. The finding that AdHSV-tk/GCV suicide

gene therapy can enhance the MMR pathways is intriguing and may have far-reaching consequences. Apart from TMZ, MMR dysfunction is associated with an increased resistance to the many cytotoxic agents e.g. DNA monofunctional alkylating agents, such as procarbazine and dacarbazine, [Branch et al., 1993] [Koi et al., 1994] [Goldmacher et al., 1986] [Cejka et al., 2003], platinum analogues cisplatin and carboplatin [Papouli et al., 2004] [Branch et al., 2000], antimetabolites, such as 6-TG [Aquilina et al., 1989] [Swann et al., 1996] [Aquilina et al., 1993] and 6-MCP, and fluoropyrimidines 5-FU and fluorodeoxyuridine [Kinsella, 2009]. Whether AdHSV-tk/GCV can enhance the cytotoxicity of these chemotherapeutics needs to be fully evaluated. Furthermore, loss of MMR function has been reported as a mechanism of resistance to TMZ [Felsberg et al., 2011] leading to hypermutator phenotype recurrences [Cahill et al., 2007] [Hunter et al., 2006]. It would be interesting to determine whether the resistance can be prevented or delayed by combining TMZ with AdHSV-tk/GCV suicide gene therapy.

The exact role of the MMR pathway in AdHSV-tk/GCV gene therapy is not known but MMR deficiency enhances tumour sensitivity to GCV at high GCV concentrations [O'Konek et al., 2009]. It is not clear whether activation of the MMR pathway by AdHSV-tk/GCV gene therapy leads to repair of the DNA damage evoked by suicide gene therapy. The incorporation of GCV triphosphate into DNA is known to cause GC to TA transversions, errors in DNA replication and an increased mutation frequency that can contribute to the activation of the MMR pathway. It is also possible that the generation of GCVTP, which competes with dGTP for the incorporation into DNA, could lead to misincorporations resulting in the activation of the MMR pathway [Martomo and Mathews, 2002;Kunz, 1982;Bebenek et al., 1992].

5.2.5 The effect of therapy combination on tumour volume

Next it was decided to determine whether it would be possible to extrapolate the *in-vitro* results into an *in-vivo* experimental MG situation. The other aim of the study was to mimic the treatment protocol in the phase III clinical trials where TMZ had been administered after a variable gap after completing GCV therapy (Press release by Ark Therapeutics PLC 18/12/2009). Orthotopic BT4C rat MGs in immunocompetent BDIX rats was chosen as the animal model due to previous experience [Tyynela et al., 2002]. The LacZ gene transfer using the same technique demonstrated transduction of the tumours by the adenovirus vectors (data not shown). The MS of untreated animals in this model is around 35 days. MRI confirmation of the tumour presence is possible only around p.i. day 12 or 13. Hence, this meant that gene transfers had to be completed in 2 days, allowing a gap for gene expression, 14 days of GCV therapy, a gap after GCV and TMZ and 5 days for TMZ to be completed within 22 days. A decision was made to reduce the duration of GCV therapy to 7 days even though 14 days has been the traditional duration that has demonstrated efficacy [Tyynela et al., 2002].

The therapeutic efficacy was determined non-invasively by MRI by calculating the tumour volumes on p.i. days 28 (Figure 28A) and 42 (Figure 28B). The MRI findings confirmed the *in-vitro* data that TMZ alone had no effect in this model (Figure 28A). There was no significant difference between the mean tumour volumes between AdHSV-tk/GCV and combination group-A, which also had only completed GCV therapy by that time-point (Figure 28A), confirming the uniformity of the findings. On the other hand, combination group-B that had completed both GCV and TMZ therapies, had a significantly lower mean tumour volume as compared to control ($p<0.001$), TMZ ($p<0.001$) and AdHSV-tk/GCV ($p<0.05$) groups (Figure 28A). Combination group-A had a significantly ($p<0.05$) lower mean tumour volume compared to control and TMZ groups (Figure 28A). The difference between the combination groups A and B did not reach statistical significance in spite of the latter having a lower mean tumour volume on p.i. days 28 (Figure 28A) and 42 (Figure 28B). Both the combination group-A ($p<0.05$) and –B ($p<0.01$) displayed significantly lower

mean tumour volumes compared to AdHSV-tk/GCV group on p.i. day 42 (Figure 28B). A summary of mean tumour volumes is given in Table 21.

Table 21: The mean tumour volumes on days 28 and 42 after tumour implantation

	Mean Tumour Volumes (mm^3)+/- SEM for the study groups				
MRI	**Control**	**TMZ**	**AdHSV-tk/GCV**	**Combination-A**	**Combination-B**
Day 28	102.3 +/- 17.9	107.2 +/-10.6	66.5 +/- 11.4	44.6 +/- 10.2	8.3 +/- 1.8
Day 42	428.0 +/- 180.7	370.0*	294.5 +/- 59.2	120.4 +/- 37.6	8.5 +/- 0.7

* Only one surviving animal

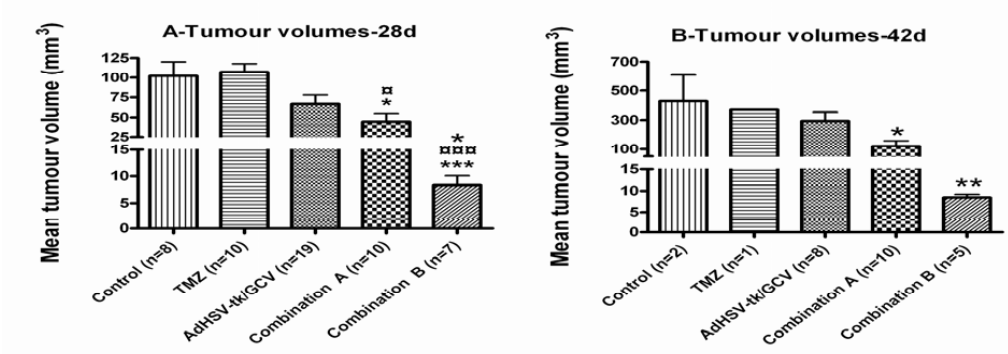

Figure 28: Mean tumour volumes measured by MRI on p.i. 28d (A) and 42d (B). By day 28 combination group-B had a significantly lower mean tumour volume compared to the control *** ($p<0.001$), TMZ ¤¤¤ ($p<0.001$) and AdHSV-tk/GCV * ($p<0.05$) groups. Combination group-A had a significantly ($p<0.05$) lower mean tumour volume as compared to the control (*) and TMZ (¤) groups (A). Still at day 42 the mean tumour volumes of the combination groups-A (*) ($p<0.05$) and -B (**) ($p<0.01$) were significantly lower compared to the AdHSV-tk/GCV group (B). Error bars are SEM.

The mean tumour volumes in the control group increased >30 fold between first and second MRIs and in TMZ group the increase was > 15 fold. During the same period, the mean tumour volumes of both AdHSV-tk/GCV group and combination-A group increased > 8 fold. However, in combination-B group, which completed both GCV and TMZ therapies during this period, the increase in mean tumour volume was only 79% (i.e. <1 fold). Between the second and third MRIs, the mean tumour volume increase in this group was less than 2 %, suggesting that this protocol was able to maintain stable disease for a prolonged period. The efficacy of AdHSV-tk/GCV group cannot be compared to the previous studies due to the shorter duration of GCV therapy. The results suggest an *in-vivo* synergism between the two therapies and favour a protocol where TMZ is administered soon after completing GCV therapy, once again in line with the *in-vitro* findings.

5.2.6 The effect of therapy combination on survival

The comparisons of the animal survivals are given in Figure 29 with a summary of survival data in Table 22. A significant survival enhancement was seen in the combination-A ($p<0.01$) and combination-B ($p<0.001$) groups as compared to the control (Figure 29C and D) and TMZ alone (Figure 29F and G) groups. Combination-B also achieved a significant survival benefit ($p<0.05$) compared to the AdHSV-tk/GCV group (Figure 29J). None of the other comparisons reached statistical significance. Neither TMZ nor AdHSV-tk/GCV alone was able to enhance survival compared to controls. In spite of having a better trend for survival in combination-B group compared to the combination-A, the difference did not reach statistical significance (Figure 29K).

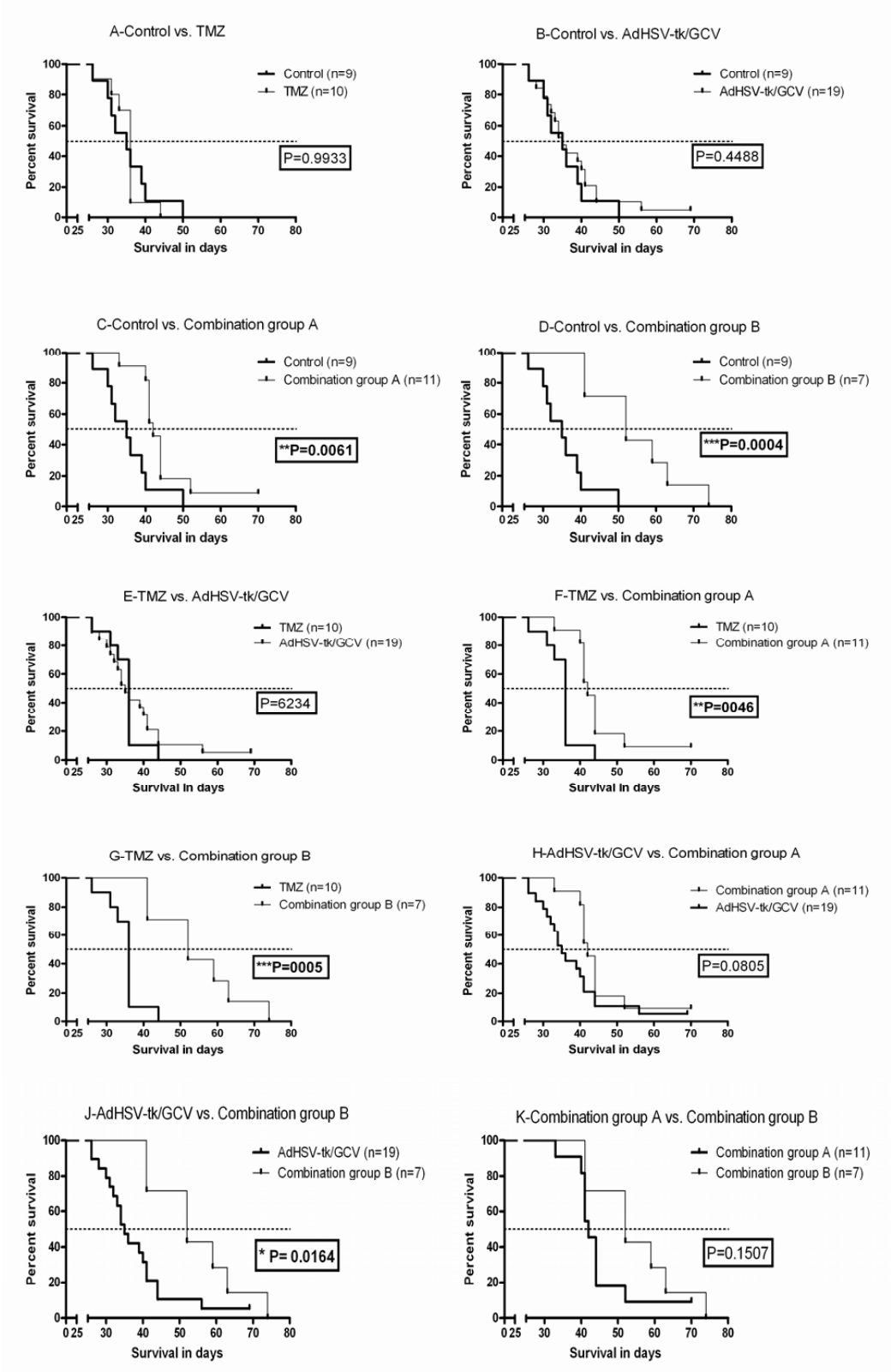

Figure 29: Kaplan-Meier survival plots comparing the control group with TMZ (A), AdHSV-tk/GCV (B), combination-A (C) and combination-B (D), TMZ group with AdHSV-tk/GCV (E),

combination-A (F) and combination-B (G), AdHSV-tk/GCV group with combination-A (H) and combination-B (J), and combination-A with combination-B (K). The results are shown in separate graphs to improve readability. P values <0.05, <0.01 and <0.001 are indicated by *, ** and *** respectively.

Table 22: A summary of the survival data

Survival in days	Study Groups				
	Control	**TMZ**	**AdHSV-tk/GCV**	**Combination-A**	**Combination-B**
Median	35	36	35	42	52
Mean+/-SEM	35.4+/-2.4	35+/-1.5	37.8+/-2.4	44.7+/-2.9	54.6+/-4.5

AdHSV-tk/GCV-adenovirus-mediated herpes simplex virus thymidine kinase and ganciclovir, SEM-standard error of the mean, TMZ-temozolomide

The control, TMZ and AdHSV-tk/GCV groups had a MS of around 35 days. The MS was increased by 20 % (up to 42 days) and 48 % (up to 52 days) in the combination-A and –B groups, respectively. In spite of the combination-B group having a MS more than 20 % compared to the combination-A group, the difference did not reach statistical significance. However, the results once again confirm the findings of the tumour volumes and *in-vitro* studies, where the combination was superior to either of the therapies alone and that the efficacy of the combination could be enhanced by reducing the gap between GCV and TMZ therapies.

5.2.7 The safety of therapy combination

Since both TMZ and GCV are known suppressors of bone marrow [Raez et al., 1999] [Dario and Tomei, 2006], full blood count analyses were done after the completion of the treatment in all groups (Figure 30). All groups that received TMZ had leukocytopenia (Figure 30A) and thrombocytopenia (Figure 30B). In the combination-B group, the values seem to be smaller compared to combination-A group, even though not statistically significant. Interestingly, AdHSV-tk/GCV therapy alone did not show this adverse effect. Red blood cell (RBC) and haemoglobin counts were within normal limits in all groups (data not shown). The FACS analysis (Figure 31) of the T-cell populations for CD3 (general T-lymphocyte marker), CD4 (T-helper cell marker) and CD8 (cytotoxic T-cell marker) revealed a significant ($p<0.05$) reduction in the number of CD3+ cells in the TMZ treated group (Figure 31A). AdHSV-tk /GCV alone did not exert this adverse effect. The CD4:CD3 population ratio did not differ significantly in the treatment groups when compared to controls (Figure 31B). However, the CD8:CD3 ratio was significantly lower (** $p < 0.01$) in the TMZ treated group when compared to the controls (Figure 31C).

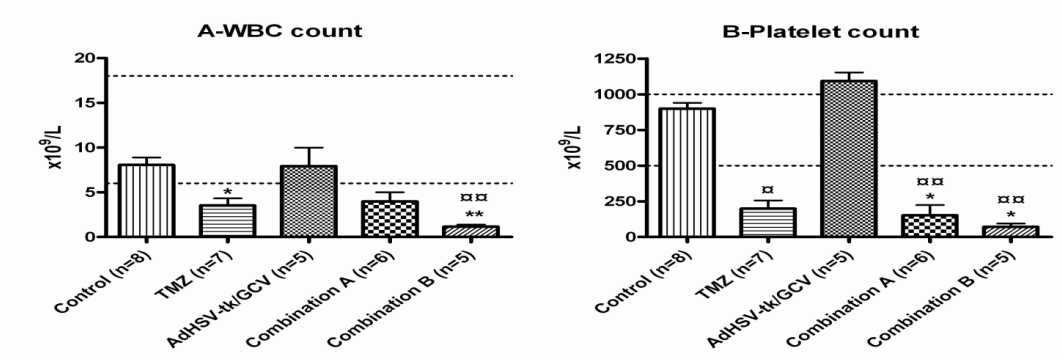

Figure 30: The white blood cell counts (WBC) (A) and platelet counts (B) after treatment in each group. Significances compared to control and AdHSV-tk/GCV groups are indicated by */** and ¤/¤¤, respectively. P values <0.05 and <0.01 are indicated by */¤ and **/¤¤, respectively.

All groups that received TMZ had lower values compared to those that did not receive TMZ. Error bars are SEM.

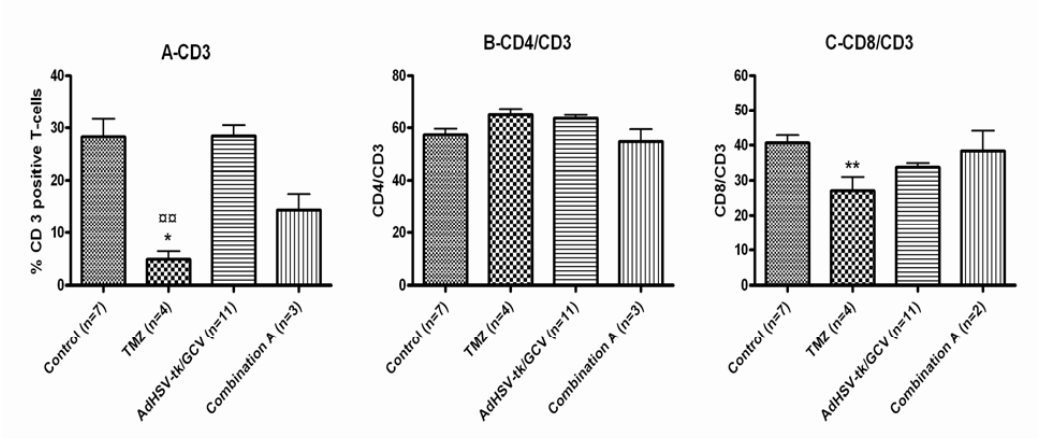

Figure 31: The proportion of CD3+ cells out of the lymphocyte cell population (A), CD4:CD3 population ratio (B) and CD8:CD3 population ratio (C) in different treatment groups. Significances compared to the control and AdHSV-tk/GCV groups are indicated by */** and ¤/¤¤, respectively. P values <0.05 and 0.01 are indicated by */¤ and **/¤¤, respectively. Error bars are SEM.

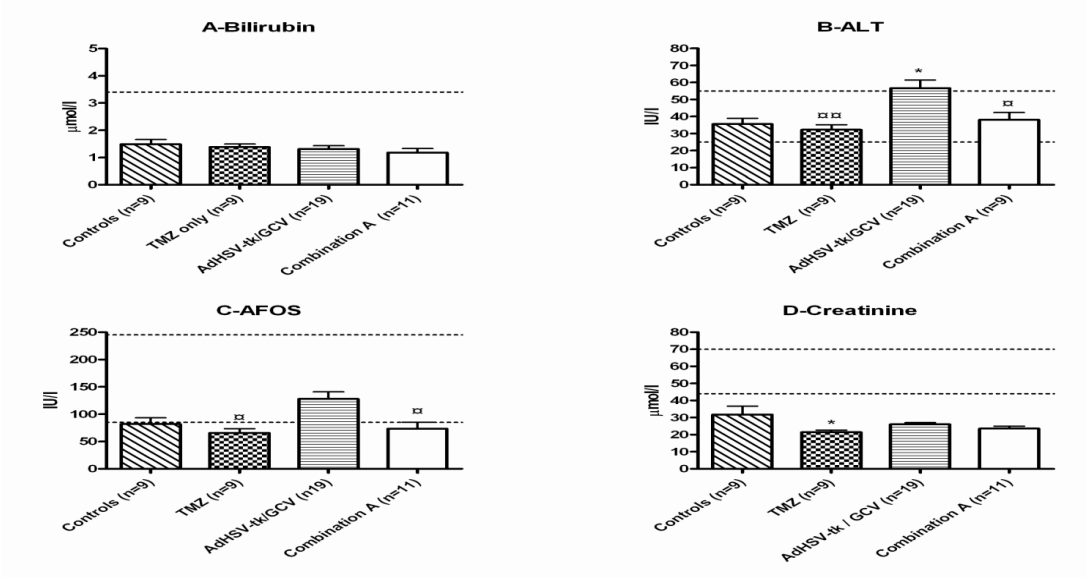

Figure 32: Serum clinical chemistry analyses for bilirubin (A), ALT (B), AFOS (C) and creatinine (D). Significances compared to the control and AdHSV-tk/GCV groups are indicated by * and ¤/¤¤, respectively. P values <0.05 and 0.01 are indicated by */¤ and **/¤¤, respectively Error bars are SEM.

Analyses of the serum samples (Figure 32) indicated that none of the parameters differed significantly between treatment and control animals, except for an elevation in ALT levels in the AdHSV-tk/GCV group (Figure 32B) and a decrease in serum creatinine in the TMZ group. However, some of these values were below the normal limits for rats. A transient elevation of ALT level has been reported previously with AdHSV-tk/GCV therapy [Immonen et al., 2004]. AFOS levels were significantly lower in the TMZ and combination-

A group compared to the AdHSV-tk/GCV group (Figure 32C). FACS and serum analyses were not done from the combination-B group. Careful analyses of the full blood counts may be necessary to obtain a true perspective of the myelotoxicities when administering TMZ and GCV simultaneously or close to each other.

5.3 SUICIDE GENE THERAPY, TMZ AND VPA STUDY

5.3.1 The effect of VPA on AdHSV-tk/GCV and TMZ

VPA is a commonly used AED in MG patients that has HDACi properties and is known to enhance the efficacy of HSV-tk/GCV suicide gene therapy and some chemotherapies [Wen and Schiff, 2011]. *In-vitro* combination of VPA with the AdHSV-tk/GCV and TMZ enhanced the cytotoxicity of both AdHSV-tk/GCV and TMZ, alone and in combination (Figure 33), in a dose-dependent manner. The differences in the BT4C cell viability of the control group (GCV-TMZ-), when compared to GCV-TMZ+ and GCV+TMZ+ groups in all VPA doses in both non-transduced and transduced cells, and compared to GCV+TMZ- group in VPA 0.5 and 1 mmol doses in transduced cells, were statistically significant ($p<0.05$) (Figure 33B). The combination of TMZ with GCV in non-transduced cells did not increase the cytotoxicity further (Figure 33A), suggesting that the synergistic effect was dependent on both AdHSV-tk and GCV. The VPA dose between 0.5 and 1.0 mmol is close to the therapeutically achievable doses when VPA is used as an AED [Chen et al., 2011b] [Catalano et al., 2006] where the therapeutic enhancements were more pronounced. The enhancement of the cytotoxicity of AdHSV-tk/GCV and AdHSV-tk/GCV and TMZ combination by VPA was more marked than the enhancement in TMZ (Figure 33B). Possible mechanisms for the better efficacy could be the VPA-mediated increase in the CAR receptor expression and enhanced adenovirus-mediated gene expression, as reported before [Goldsmith et al., 2007] [Kothari et al., 2010]. In addition it is reported that VPA can also increase the bystander effect of HSV-tk/GCV therapy by increasing the expression of gap-junctions [Ammerpohl et al., 2004] [Robe et al., 2004] [Asklund et al., 2004].

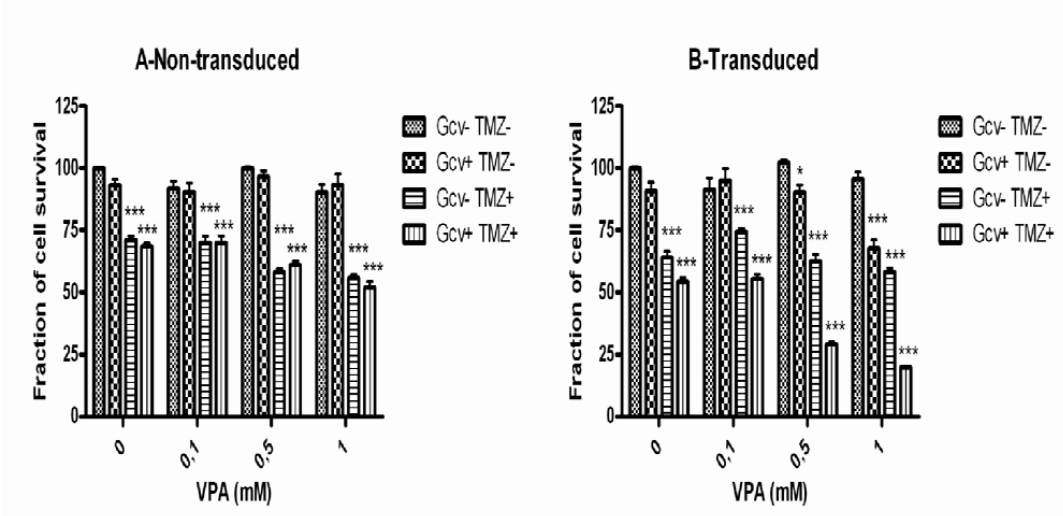

Figure 33: Viability of BT4C cells non-transduced (A) and transduced with AdHSV-tk (B) with GCV and TMZ at different VPA concentrations. The triplicate values were normalized to the average of untreated cells. Significances compared to the GCV-TMZ- group are indicated by */**/***. P values <0.05, <0.01 and <0.001 are indicated by *, ** and ***, respectively. Error bars are SEM.

5.3.2 The effect of adding VPA on tumour volume

In-vivo evaluation of the impact of addition of VPA into AdHSV-tk/GCV and TMZ combination in the BT4C rat MG model failed to demonstrate any reduction in the tumour growth rate (Figure 34). VPA failed to reduce the tumour growth rate alone or in combination with TMZ, AdHSV-tk/GCV or AdHSV-tk/GCV+TMZ. By day 28 (Figure 34A) none of the AdHSV-tk/GCV and TMZ combination groups had received TMZ yet. Hence, the impact on the tumour volumes is purely due to AdHSV-tk/GCV. There were no significant differences in the mean tumour volumes among these groups, again emphasising the uniformity of the findings. However, the two groups that received VPA did seem to have slightly higher mean tumour volumes compared to those that did not receive VPA (Figure 34A). By day 42 the groups that received AdHSV-tk/GCV and TMZ had lower mean tumour volumes compared to the AdHSV-tk/GCV groups even though this was not statistically significant. However, the differences were significant when compared to the AdHSV-tk/GCV+VPA group (Figure 34B). There were no animals surviving in the control group by this time; and no comparisons were possible with the VPA and TMZ+VPA groups due to the small animal numbers. Addition of TMZ to AdHSV-tk/GCV made it possible to maintain a slower tumour growth rate over a prolonged period of time. The mean tumour volumes for the groups are given in Table 23.

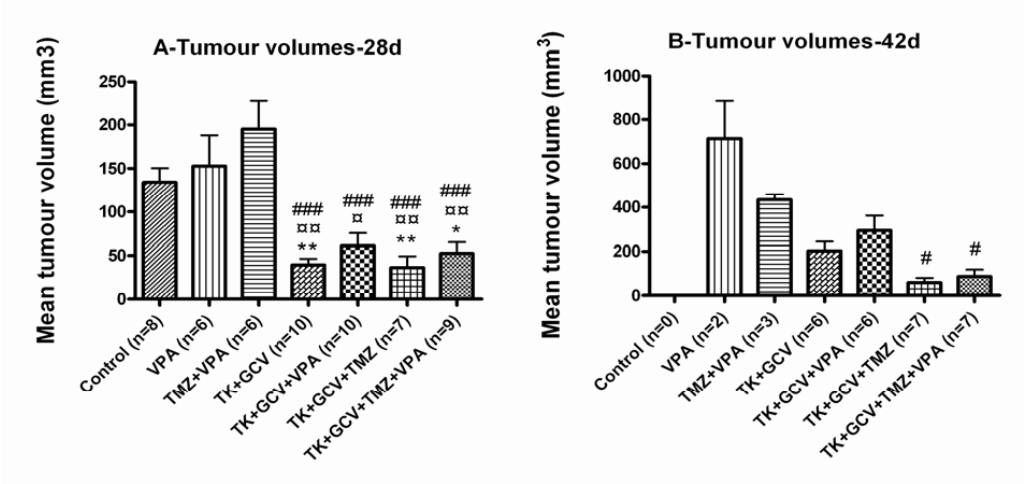

Figure 34: Mean tumour volumes on days 28 (A) and 42 (B) after tumour implantation. Significances compared to control, VPA and TMZ+VPA groups are indicated by */**, ¤/¤¤ and #/##/###, respectively. P values <0.05, <0.01 and <0.001 are indicated by */¤/#, **/¤¤/## and ***/¤¤¤/###, respectively. Error bars are SEM.

Table 23: A summary of the mean tumour volumes

Study Groups	Mean Tumour Volumes (mm^3) +/- SEM			
	n	Day 28	n	Day 42
Control	8	134.2 +/- 16.2	0	-
VPA	6	152.9 +/- 34.8	2	713.0 +/- 172.5
TMZ+VPA	6	195.2 +/- 32.6	3	434.7 +/- 24.0
TK+GCV	10	39.4 +/- 6.9	6	203.1 +/- 42.5
TK+GCV+VPA	10	61.3 +/- 14.5	6	294.7 +/- 68.6
TK+GCV+TMZ	7	36.4 +/- 12.8	7	59.8 +/- 21.3
TK+GCV+TMZ+VPA	9	52.6 +/- 12.7	7	87.0 +/-31.8

n = animal number

5.3.3 The effect of adding VPA on survival

VPA failed to show a therapeutic benefit alone or in combination with TMZ and AdHSV-tk/GCV+TMZ (Figures 35 and 36). However, addition of VPA to AdHSV-tk/GCV was able to enhance the survival significantly compared to the control group (Figure 35B), where AdHSV-tk/GCV alone was not significantly better (Figure 35D) indicative of a possible therapeutic enhancement. The comparison between AdHSV-tk/GCV and AdHSV-tk/GCV+VPA was not statistically significant (data not shown). The results from this study revealed that in AdHSV-tk/GCV+TMZ group where GCV was given for the usual 14 days, the last five days of which was in combination with TMZ, displayed a significantly better survival compared to the control (Figure 35A) ($p<0.001$), AdHSV-tk/GCV (Figure 36A) ($p<0.05$), TMZ+VPA (Figure 36B) ($p<0.001$) and AdHSV-tk/GCV+VPA (Figure 26D) ($p<0.01$) groups. This group had the best survival outcome in the entire study and this represents the first clear *in-vivo* evidence for a demonstration of a superior survival benefit compared to either of the therapies given alone. Previous work by Rainov *et al.* did not demonstrate any clear *in-vivo* benefit with these two combinations [Rainov et al., 2001]. The 14-day GCV therapy is commonly used in patients in MG clinical trials [Immonen et al., 2004]. Hence, it would be worthwhile to evaluate the therapeutic benefit of adding TMZ either simultaneously with GCV or immediately following GCV in MG patients after a through toxicological evaluation of this approach. The AdHSV-tk/GCV+VPA group achieved a significant ($p<0.01$) survival benefit compared to the control group (Figure 35B), while AdHSV-tk/GCV+TMZ+VPA group had a significant survival benefit compared to the control ($p<0.001$) (Figure 35C) and TMZ+VPA ($p<0.05$) group (Figure 36C). Table 24 summarises the survival data of the study. AdHSV-tk/GCV+TMZ combination increased the MS by 54 %, 46 % and 34 % compared to the control, TMZ+VPA and AdHSV-tk/GCV groups, respectively.

One reason for the failure of VPA to enhance the efficacy of TMZ and HSV-tk/GCV therapy *in-vivo* could be the reduced availability of VPA in the target site after subcutaneous administration. There is evidence to suggest that rodents tend to eliminate VPA ten-times faster than humans [Loscher, 1999]. According to the literature, in mice 80-100 mg/kg i.p. dosing has resulted in plasma and brain concentrations of 120-150 μg/ml (830-1040 μmol/L) and 25-40 μg/g (170-280 μmol/L), respectively [Loscher, 2002]. The twice-a-day dosing used in the study may not have been frequent enough to maintain the therapeutic level. Thus, the lack of efficacy with VPA alone is not surprising because the dose used in this study was the much smaller AED dose.

Table 24: A summary of the survival data

Study Groups	n	Survival in days	
		Mean +/- SEM	Median
Control	11	36.3 +/- 0.5	36.0
VPA	6	36.7 +/- 2-1	37.5
TMZ+VPA	7	37.1 +/- 3.3	35.0
TK+GCV	19	39.7 +/- 2.4	41.0
TK+GCV+VPA	10	42.8 +/- 2.3	42.0
TK+GCV+TMZ	7	60.7 +/- 7.0	55.0
TK+GCV+TMZ+VPA	9	49.9 +/- 3.6	48.0

n = number of animals

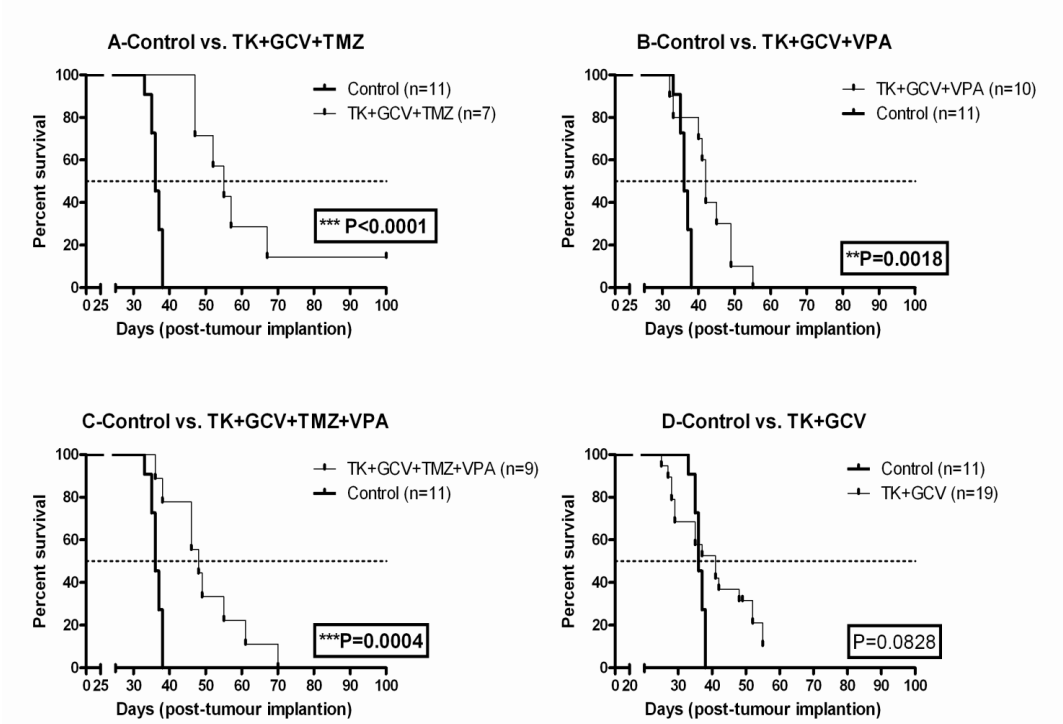

Figure 35: Kaplan-Meier survival plots comparing the control group with TK+GCV+TMZ (A), TK+GCV+VPA (B), TK+GCV+TMZ+VPA (C) and TK+GCV (D). The results are shown in separate graphs to improve readability. P values <0.05, <0.01 and <0.001 are indicated by *, ** and *** respectively.

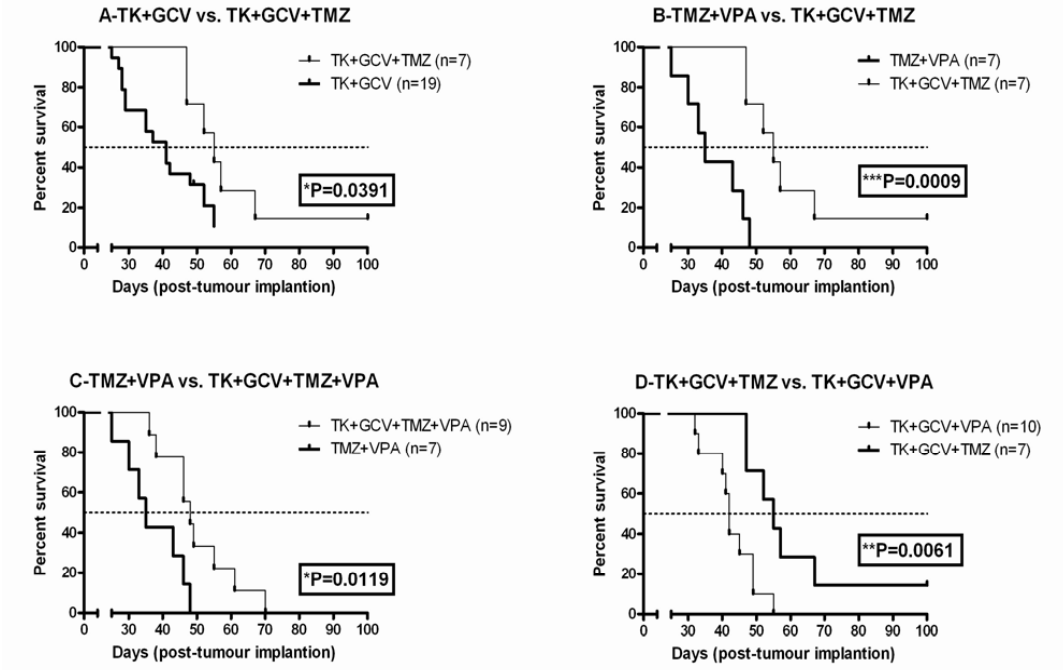

Figure 36: Kaplan-Meier survival plots for the AdHSV-tk/GCV+TMZ group which demonstrated a significant survival benefit compared to the AdHSV-tk/GCV (p<0.05)(A), TMZ+VPA (p<0.001)

(B) and AdHSV-tk/GCV+VPA ($p<0.01$) (D) groups but not compared to AdHSV-tk/GCV+TMZ+VPA group (data not shown), which had a significantly better survival compared to TMZ+VPA ($p<0.05$) (C) group. The results are shown in separate graphs to improve readability. P values <0.05, <0.01 and <0.001 are indicated by *, ** and *** respectively.

5.4 TARGETED GENE THERAPY STUDY

5.4.1 The effect of targeted radiotherapy on survival

IHC stainings confirmed the expression of LDLR-avidin fusion-protein targeting construct within the intracranial rat MGs after lentivirus-mediated local gene transfer (Figure 37).

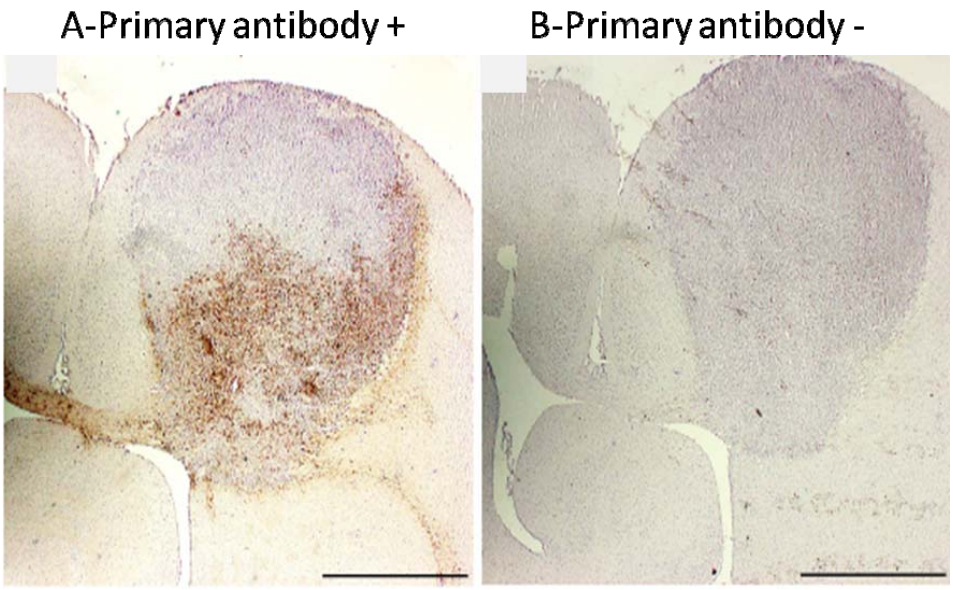

Figure 37: Expression of LDLR-avidin fusion-protein targeting construct within the tumours after lentivirus vector-mediated local gene transfer was confirmed by IHC using anti-avidin antibody (A). Control staining without primary antibody (B). Scale bar 1 mm.

In order to evaluate the *in-vivo* efficacy of lentivirus-mediated LDLR-avidin fusion-protein gene therapy, Bt-^{90}Y-DOTA was injected i.v. into BT4C rat MG bearing BDIX rats. The results revealed that the animals treated with Bt-^{90}Y-DOTA following transduction with the targeting construct using lentivirus vectors survived longer (MS 44.5 days) compared to the two control groups; untreated and Bt-^{90}Y-DOTA only groups (MS = 37 and 33.5 days, respectively). The survival benefits were statistically significant when compared to the two control groups (Figure 38B and C). The MS of the animals in the lentivirus-mediated LDLR-avidin fusion-protein gene therapy group was 7.5 days (~20%) longer when compared to the control group that received no treatment ($p = 0.0213$), and 11 days (~33%) longer when compared to the group that received only Bt-^{90}Y-DOTA ($p = 0.0001$). The administration of Bt-^{90}Y-DOTA alone did not improve survival (Figure 38A).

This study demonstrated the *in-vivo* feasibility of a gene therapy-based targeting system that can be used for any type of solid tumour, independent of the tumour type. Targeting strategies based on the use of mAbs utilise a particular target expressed on the tumour cells (e.g. the EGFR on cancer cells). Clinical trials have already been performed in MG patients employing these pre-targeting methods without evoking any significant toxicity [Paganelli et al., 2006]. However, the pre-targeting systems are greatly dependent on the affinity and specificity of the primary targeting mAb. For that reason, it would be a major advantage if

the targeting strategy would be independent of the tumour type. Compared to mAb-based pre-targeting systems that require multiple steps, gene therapy-based system requires only two steps: 1) Gene transfer of the targeting construct and 2) administration of the biotinylated therapeutic molecule. The advantage of this gene therapy-based targeting system over pre-targeting systems is that no specific targeting mAb is required. Whereas mAbs are specific for only a particular antigen (and hence can be used only in particular tumours expressing the antigen), this novel targeting system is universal and can be applied to all tumours. The results confirm that the targeting construct is capable of high affinity binding of biotinylated molecules *in-vivo,* and that it can be used for therapeutic purposes. Generally, any molecule that can be biotinylated can be used with this present system for targeting purposes, including nanoparticles and liposomes paving the way to gene therapy-based targeted chemotherapy.

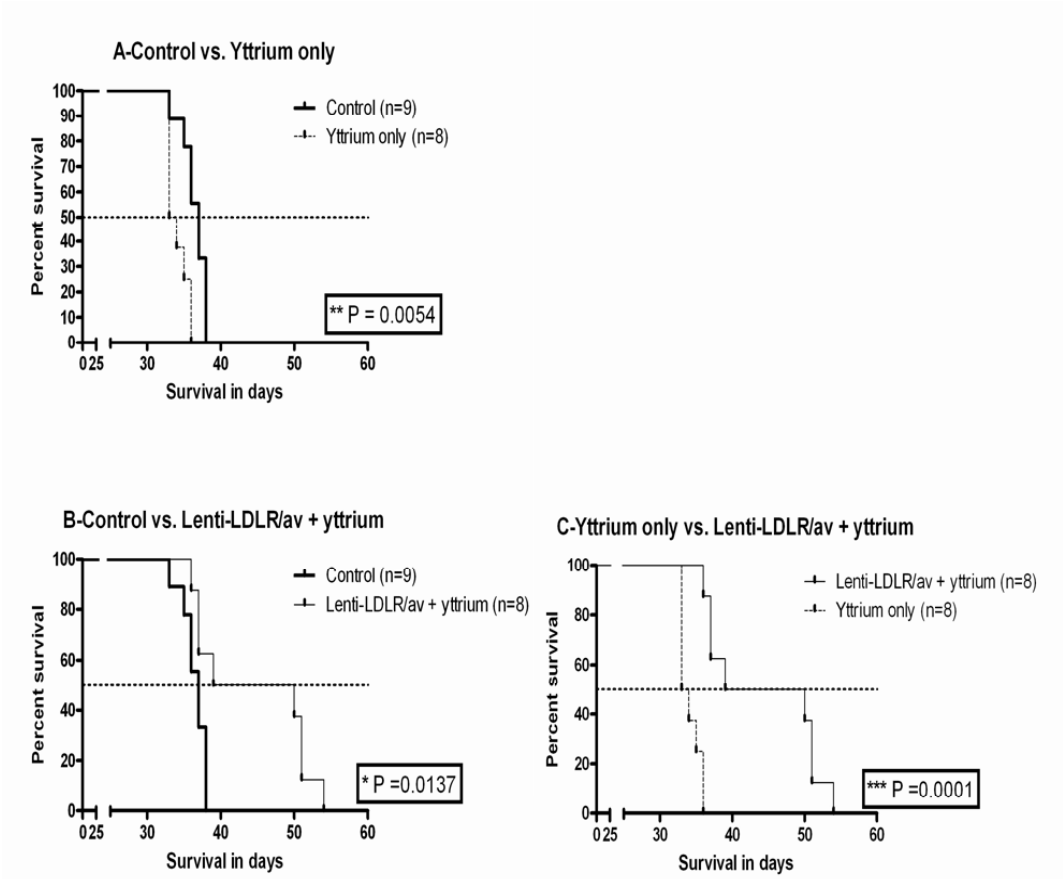

Figure 38: Kaplan-Meier survival plots comparing the survival of the control group to Bt-^{90}Y-DOTA alone (A) and lentivirus-mediated gene therapy with Bt-^{90}Y-DOTA group (B), and Bt-^{90}Y-DOTA alone group to lentivirus-mediated gene therapy with Bt-^{90}Y-DOTA group (C). The results are shown in separate graphs to improve readability. P values <0.05, <0.01 and <0.001 are indicated by *, ** and *** respectively.

6 Summary and conclusions

On the basis of the salient findings of this thesis work, the following conclusions can be made:

1. The simultaneous combination of adenovirus-mediated HSV-tk/GCV suicide gene therapy with adenovirus-mediated soluble VEGFR-1 anti-angiogenic gene therapy is feasible for the treatment of cancers such as MG. Apart from inhibiting angiogenesis; other anti-tumour mechanisms such as reduced infiltration by tumour associated macrophages/microglia and reduced oedema may contribute to the therapeutic benefit.

2. Adenovirus-mediated HSV-tk/GCV suicide gene therapy enhances the cytotoxicity of TMZ by locally up-regulating the expression of the MMR pathway genes in a treatment protocol-dependant manner in MGMT negative MG. This novel mechanism of synergism between these two therapies can be maximised by administering these two therapies within a specific therapeutic time window. The therapeutic index of TMZ is increased due to the local enhancement of cytotoxicity without increasing the systemic adverse effect profile.

3. Addition of VPA did not further enhance the synergistic effect of AdHSV-tk/GCV and TMZ combination. However, simultaneous combination of TMZ with GCV during the last five days of the routine 14-day GCV course in AdHSV-tk suicide gene therapy provided long-term control of tumour growth and improved survival in an orthotopic immunocompetent rat MG model, pointing to a potential clinical use after toxicological evaluation.

4. *In-vivo* feasibility of lentivirus-mediated LDLR-avidin fusion-protein gene therapy to bind systemically administered biotinylated therapeutics and confer a therapeutic benefit was demonstrated in a syngeneic, orthotopic and immunocompetent rat MG model. This universal gene therapy-based targeting system is free from the need for developing tumour specific antibodies for each tumour type, and is capable of targeting any biotinylated therapeutic compound including radiopharmaceuticals and chemotherapeutic agents, which may be particularly advantageous in inoperable tumours.

The development of treatment resistance is a major obstacle in cancer care that can be overcome, to a certain extent, by combining different therapies. In summary, the outcome of this thesis work confirms the feasibility of successfully combining AdHSV-tk/GCV suicide gene therapy with AdsVEGFR-1 anti-angiogenic gene therapy, which lacks the systemic adverse effects of parenteral therapies, for the treatment of aggressive MGs. Moreover, a novel mechanism of synergism is described between AdHSV-tk/GCV and TMZ that does not increase the adverse effect profile, which is the other main drawback associated with chemotherapy. These findings have direct implications when planning future clinical trials with these combinations. Furthermore, the results also highlight the feasibility of using a novel gene therapy-based targeted therapy strategy with wide therapeutic potentials.

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Haritha D. Samaranayake

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A Pre-Clinical Evaluation

Malignant glioma is a devastating cancer with a dismal prognosis. The outcome of this thesis work confirms the feasibility of successfully combining anti-angiogenic gene therapy with suicide gene therapy to treat this condition. It also describes a novel mechanism to account for the synergism and a treatment protocol that does not increase the adverse effect profile when suicide gene therapy is combined with temozolomide. Furthermore, the results also highlight the efficacy of a novel gene therapy-based targeted therapy strategy with wide therapeutic potential.

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