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HANNA STEDT

Gene Therapy of Malignant Glioma

Alternative Strategies and Combination Therapies

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Author's address: Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
P.O. Box 1627
FI-70211 Kuopio
FINLAND
E-mail: hanna.stedt@uef.fi

Supervisors: Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
FINLAND

Ann-Marie Määttä, Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
FINLAND

Mikko Turunen, Ph.D.
Department of Biotechnology and Molecular Medicine
A. I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
FINLAND

Reviewers: Docent Pirjo Laakkonen, Ph.D.
Research Programs Unit
Translational Cancer Biology
Biomedicum Helsinki, University of Helsinki
P.O. Box 63
FI-00014 Helsinki
FINLAND

Associate Professor Hrvoje Miletic, M.D., Ph.D.
Department of Pathology
Haukeland University Hospital
Jonas Lies Vei 65
5021 Bergen
NORWAY

Opponent: Professor Heikki Minn, M.D., Ph.D.
Department of Oncology and Radiotherapy
Turku University Hospital
P.O. Box 51
FI-20521 Turku
FINLAND

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ABSTRACT

Malignant glioma (MG) is the most common malignant brain tumor; its most malignant form, glioblastoma multiforme has a dismal prognosis of ~14 months from diagnosis. Little progress has been made with traditional therapies which consist of a combination of surgery, radiotherapy and chemotherapy. MG is an ideal target for gene therapy due to its spatially restricted localization and rarely metastasizing character. The aims of this thesis were to develop novel gene therapy approaches as well as combination therapies in preclinical *in vitro* and *in vivo* MG models.

In the first study, Src kinase, a central signaling mediator of oncogenesis, was targeted with lentiviral delivery of small hairpin RNAs (shRNAs). In subcutaneous nude mice xenografts, the inhibition of Src was able to significantly reduce tumor growth and vascularity, whereas in immunocompetent orthotopic rat MG model tumor growth reduction and enhanced survival was only seen with *ex vivo* transduced tumors. A modest *in vivo* therapeutic effect was seen in combination with a chemotherapeutic temozolomide (TMZ) and a histone deacetylase inhibitor valproic acid (VPA).

In the second study, adenoviral suicide gene therapy of Herpes simplex virus-1 thymidine kinase with the prodrug ganciclovir (HSV-TK/GCV) was combined with TMZ and VPA. *In vitro* efficient viability reduction, enhanced transduction and bystander effect were demonstrated. *In vivo* HSV-TK/GCV+TMZ improved the survival and reduced the tumor growth of rats with orthotopic MG but VPA administration conferred no additional benefit. An improved combination therapy schedule was also introduced.

In the third study, HSV-TK/GCV therapy was compared with tomato thymidine kinase combined with its specific prodrug, azidothymidine (ToTK/AZT). Both enzymes demonstrated efficacy and substrate specificity *in vitro* but only mice receiving ToTK/AZT had improved survival compared to non-treated control mice with intracranial MG. No significant differences were observed between the two suicide gene therapies *in vivo*.

In conclusion, Src is a potential target for gene therapy of MG. The efficacy of *in vivo* therapy was hampered by the low transduction efficiency, which has been recognized as one of the major challenges in gene therapy. Simultaneous administration of GCV with TMZ further enhanced HSV-TK/GCV+TMZ efficacy being of potential value for MG patients. Despite the conflicting findings in the literature, in the current experimental setting, VPA was not able to achieve any further therapeutic benefits warranting for careful optimization of the treatment schedule. ToTK/AZT was found to be an equally efficient alternative for HSV-TK/GCV therapy with favorable therapeutic characteristics. In summary, gene therapy is a potential therapeutic approach for MG although it requires a carefully optimized treatment protocol.

National Library of Medicine Classification: QU 470, QU 550, QU 560, QZ 380
Medical Subject Headings: Glioma/therapy; Gene Targeting; Transduction, Genetic; Genetic Vectors; Genes, Suicide; src-Family Kinases/antagonists & inhibitors; RNA, Small Interfering; Lentivirus; Adenoviridae; Thymidine Kinase; Valproic Acid; Ganciclovir; Zidovudine; Disease Models, Animal; Mice; Rats

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TIIVISTELMÄ

Maligni glioma (MG) on yleisin pahanlaatuinen aivokasvaintyyppi, ja elinajanodote sen pahimmanlaatuisen kasvaimen, glioblastooman, osalta on ainoastaan noin 14 kuukautta. Perinteisesti käytetyistä leikkaushoidosta, sädehoidosta ja kemoterapiasta huolimatta potilaiden ennuste on parantunut ainoastaan vähän viime vuosikymmeninä. MG sopii hyvin geeniterapian kohteeksi paikallisen sijaintinsa ansiosta, minkä lisäksi sillä ei ole taipumusta lähettää etäpesäkkeitä. Tämän väitöskirjatyön tavoitteena oli kehittää MG:an uusia geenihoitoja ja niiden yhdistelmiä soluviljely- ja eläinkoemalleissa.

Ensimmäisessä osatyössä syövän kannalta keskeistä Src kinaasia kohdennettiin lentivirusvälitteisillä shRNA-molekyyleillä. Tällä hoidolla kyettiin rajoittamaan ihonalaisten kasvainten kasvua sekä heikentämään niiden verisuonitusta immuunipuutteisissa hiirissä. Immuunivasteeltaan normaalien rottien aivokasvaimissa kyseinen hoito yksinään ei ollut tehokas, mutta sen tehoa pystyttiin hieman parantamaan liittämällä siihen kemoterapialääke temozolomidi (TMZ) sekä histonideasetylaasiestäjä valproaatti (VPA).

Toisessa osatyössä hyödynnettiin Herpes simplex virus-1 tymidiinikinaasin ja aihiolääke ganciclovirin (HSV-TK/GCV) itsemurhageeniterapiaa. Tämä geeniterapia yhdistettiin TMZ:iin ja VPA:iin. Solukokeissa hoito oli tehokas parantaen solujen transduktiotehoa sekä myös hoitovastetta viereisissä soluissa. HSV-TK/GCV+TMZ-hoito pienensi rottien aivokasvaimia pidentäen myös elinaikaa merkittävästi. VPA:lla ei saatu hoitovastetta. Kyseisessä osatyössä parannettiin myös yhdistelmäterapian aikataulua.

Kolmannessa osatyössä HSV-TK/GCV-terapiaa verrattiin tomaatin tymidiinikinaasi ja aihiolääke azidotymidiini (ToTK/AZT) -hoitoon. Solukokeissa molemmat hoidot osoittautuivat tehokkaiksi ja kinaasit aihiolääkkeilleen spesifisiksi. Hiirten aivokasvainmallissa ainoastaan ToTK/AZT-hoidolla oli hiirten elinaikaa merkittävästi pidentävä vaikutus verrattuna kontrollihiiriin. Hoitojen välillä ei todettu merkittävää eroa.

Yhteenvetona voidaan todeta Src kinaasin olevan keskeinen malignin glioman geeniterapiakohde, vaikkakin eläinmalleissa hoitotehoa rajoittaa matala transduktioteho, jonka on todettu olevan yksi geeniterapian suurimpia haasteita. GCV:n ja TMZ:n yhtäaikainen annostelu tehosti HSV-TK/GCV+TMZ-hoitoa, mistä saattaisivat hyötyä myös MG potilaat. Ristiriitaisesta kirjallisuudesta huolimatta kyseisessä tutkimuksessa VPA:sta ei saatu lisähyötyä, mikä korostaa tutkimusasetelman tarkan optimoinnin tärkeyttä. ToTK/AZT osoittautui mahdolliseksi vaihtoehdoksi HSV-TK/GCV-hoidolle edullisten ominaisuuksiensa ansiosta. Geeniterapian voidaan todeta olevan potentiaalinen malignin glioman hoitomuoto hoitoprotokollan ollessa tarkkaan optimoitu.

Luokitus: QU 470, QU 550, QU 560, QZ 380
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"If we knew what it was we were doing, it would not be called research, would it?"
- Albert Einstein -

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List of the original publications

This dissertation is based on the following original publications:

I Stedt H, Alasaarela L, Samaranayake H, Pikkarainen J, Määttä A-M, Kholová I, Parker AS, Ylä-Herttuala S. Specific inhibition of SRC kinase impairs malignant glioma growth *in vitro* and *in vivo*. *Molecular Therapy Nucleic Acids 1: 1-10, 2012*.

II Stedt H, Samaranayake H, Pikkarainen J, Määttä A-M, Alasaarela L, Airenne K, Ylä-Herttuala S. Improved therapeutic effect on malignant glioma with adenoviral suicide gene therapy combined with temozolomide. *Gene Therapy 20: 1165-1171, 2013*.

III Stedt H, Samaranayake H, Kurkipuro J, Wirth G, Christiansen LS, Vuorio T, Määttä A-M, Piškur J, Ylä-Herttuala S. Tomato thymidine kinase-based suicide gene therapy for malignant glioma – an alternative for Herpes Simplex virus-1 thymidine kinase. *Accepted for publication in Cancer Gene Therapy, 2014*.

Contents

Abbreviations

AAV	Adeno-associated virus
AcMNPV	Autographa californica multiple nucleopolyhedrovirus
ADA-SCID	Adenosine deaminase severe combined immunodeficiency
Ad. / AV	Adenovirus
AED	Antiepileptic drug
AGO2	Argonaute 2
APC	Antigen presenting cell
AZT	Azidothymidine
AZT-DP	Azidothymidine diphosphate
AZT-MP	Azidothymidine monophosphate
BBB	Blood brain barrier
BCA	Bicinconic acid
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea (Carmustine)
BNCT	Boron neutron capture therapy
BV	Baculovirus
CAR	Coxsackie-adenovirus receptor
CCNU	N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (Lomustine)
CD	Cluster of differentiation
cDNA	Complementary DNA
CHI3L1	Chitinase-3-like protein 1
CNS	Central nervous system
CT	Computer tomography
CTLA-4	Cytotoxic T lymphocyte antigen-4
Cx43	Connexin 43
DMSO	Dimethyl sulfoxide
dNK	Deoxynucleosidekinases
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EMA	European Medicines Agency
EU	European Union
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
FLT1	Fms-like tyrosine kinase 1, Vascular endothelial growth factor receptor 1 (VEGFR-1)
Flt3L	Fms-like tyrosine kinase 3 ligand
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
GCP	Good clinical practices
GCV	Ganciclovir
GDEPT	Gene-directed enzyme prodrug therapy
GFP	Green fluorescent protein
GLP	Good laboratory practices
GMP	Good manufacturing practices
GSC	Glioma stem cells
HAT	Histone acetyl transferase

HDAC	Histone deacetylase
HDI	Histone deacetylase inhibitor
HIF-1α	Hypoxia inducible factor-1α
HIV-1	Human immunodeficiency virus 1
HNSCC	Head and neck squamous cell carcinoma
HSV-TK	Herpes Simplex virus-1 thymidine kinase
HUVEC	Human umbilical vein endothelial cells
I.c.	Intracranial
IDH	Isocitrate dehydrogenase
Ig	Immunoglobulin
ILR2G	Interleukin 2 receptor gamma
IL-2	Interleukin 2
IL-13	Interleukin 13
IMPD	Investigational Medicinal Product Dossier
I.p.	Intraperitoneal
KDR	Kinase insert domain receptor, Vascular endothelial growth factor receptor 2 (VEGFR-2)
LOH	Loss of heterozygosity
LV	Lentivirus
MET	Mesenchymal epithelial transition factor
MG	Malignant glioma
MGMT	O^6-methylguanine-DNA methyltransferase
MHC	Major histocompatibility complex
miRNA	Micro RNA
MMP-2	Matrix metalloproteinase 2
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
ncRNA	Non-coding RNA
NF1	Neurofibromin 1
nt	Nucleotide
oHSV1	Oncolytic Herpes Simplex virus-1
OS	Overall survival
OTC	Ornithine transcarbamylase
PCV	Procarbazine-lomustine-vincristine
PDGFR	Platelet derived growth factor receptor
PFS	Progression free survival
PI3K	Phosphoinositide-3 kinase
PKC-β	Protein kinase C beta
P.o.	Per oral
PTGS	Post-transcriptional gene silencing
QoL	Quality of life
qPCR	quantitative real-time polymerase chain reaction
Rb	Retinoblastoma
RCT	Randomized controlled trial
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Radiotherapy
RTK	Receptor tyrosine kinase

RTKI	Receptor tyrosine kinase inhibitor
RV	Retrovirus
RV-VPC	Retroviral viral packaging cells
S.c.	Subcutaneous
SEM	Standard error of mean
SFK	Src family kinase
shRNA	Small hairpin RNA
SIN	Self-inactivating
siRNA	Small interfering RNA
TGS	Transcriptional gene silencing
TK1	Thymidine kinase 1
TMZ	Temozolomide
TN-C	Tenascin-C
ToTK	Tomato thymidine kinase
TSP-1	Thrombospondin-1
VEGF-A	Vascular endothelial growth factor A
VPA	Valproic acid/Sodium valproate
v-Src	Viral Src
VSV-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organisation
XPO5	Exportin 5
5-ALA	5-amino-laevulinic acid

1 Introduction

According to World Health Organization (WHO) cancers are significant causes of death worldwide with 8.2 million cancer deaths in 2012 while 14.1 million new cancer cases were diagnosed in the same year (Stewart, Wild 2014). Malignant gliomas (MGs) are the most common malignant primary brain tumors; the most malignant of these tumors, glioblastomas (GBMs), have a prognosis of less than 15 months with standard treatment (Omuro, DeAngelis 2013, Stupp et al. 2014). The standard treatment consists of a combination of surgery, radiotherapy and chemotherapy, but it is merely palliative in the case of GBM (Stupp et al. 2005, Stupp et al. 2009). The efficacy of the current treatment strategies is limited by the toxicity associated with radio- and chemotherapy, and the invasive nature of GBM preventing the total resection of tumor. Therefore novel therapeutic approaches are urgently needed.

In gene therapy, genetic material is delivered into a target cell with the intent of achieving a therapeutic outcome. Most of the gene therapy clinical trials are being undertaken against cancer and this is reflected in the broad spectrum of therapeutic targets (Wirth, Parker & Yla-Herttuala 2013). Src is a tyrosine kinase located downstream of several growth factor receptors mediating a wide spectrum of cellular functions. Src has been shown to be important for oncogenesis and thus it is a major therapeutic target of MG (Ahluwalia et al. 2010, Alvarez, Kantarjian & Cortes 2006). In this thesis, lentiviral delivery of shRNAs was used to achieve Src inhibition. The most widely studied suicide gene therapy based on Herpes simplex virus-1 thymidine kinase and prodrug ganciclovir (HSV-TK/GCV) was combined with the first-line chemotherapeutic agent for MG, temozolomide (TMZ). This treatment was further combined with valproate (VPA), a histone deacetylase inhibitor previously shown to enhance gene therapy, radiotherapy and chemotherapy (Fan et al. 2005, Van Nifterik et al. 2012). VPA is an anticonvulsant drug which has long been utilized in MG patients (Loscher 1999, Chateauvieux et al. 2010). HSV-TK/GCV therapy was also compared with a novel suicide gene therapy of tomato thymidine kinase combined with a prodrug azidothymidine (ToTK/AZT). AZT has been shown to easily penetrate blood brain barrier (BBB) improving tumor accessibility (Denny 2003).

The aim of this thesis was to assess the aforementioned therapeutic strategies and their combinations *in vitro* and *in vivo* in preclinical MG models. The overall purpose of these experiments was to gain knowledge to further develop and optimize treatments for MG.

2 Review of literature

2.1 CANCER

Cancer is a major cause of death all around the world with an increasing incidence as the individual ages. It is the leading cause of death in the developed countries and the second most common in the developing countries (Umar, Dunn & Greenwald 2012). Tumors, manifestations of cancer, can be classified as being either benign or malign. The primary tumor exists at the site of occurrence while malign tumors have the capability of sending metastases to distant locations in the body. It is these metastases which are the cause of cancer deaths in over 90 % of the cases (Hanahan, Weinberg 2011, Sporn 1996).

Independent of tissue of origin, malignant tumors display certain common characteristic alterations in their physiological behavior, the so called hallmarks of cancer (Figure 1). These include self-sufficiency in growth signals, insensitivity to antigrowth signals, resistance to cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. In addition to these well-established hallmark characteristics, more recently deregulation of cellular energetics and avoidance of immune destruction have been proposed as emerging hallmarks. In order to acquire these hallmarks, the concept of tumor enabling characteristics, namely genomic instability and tumor-promoting inflammation, has been established. (Hanahan, Weinberg 2000, Hanahan, Weinberg 2011)

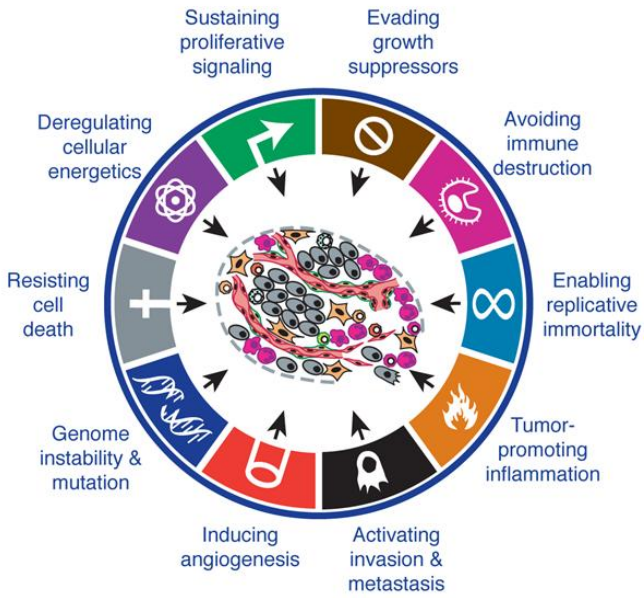

Figure 1. Hallmarks of cancer. The eight hallmarks and two enabling characteristics (genome instability & mutation and tumor-promoting inflammation) of cancer. Modified from Hanahan & Weinberg 2011.

2.2 MALIGNANT GLIOMA

2.2.1 Epidemiology and etiology

Malignant gliomas (MGs) are the most common type of malignant brain tumors. The yearly incidence is ~3-5/100 000 with a slight male predominance and peak incidence in the fifth and sixth decades of life (Stupp et al. 2014). There is a tendency towards higher incidence rates in the highly developed, industrialized countries (Ohgaki, Kleihues 2005a). In the Finnish population, 950 primary central nervous systems (CNS) tumors were diagnosed in 2009, 40 percentage of these being gliomas (Joensuu et al. 2013). Most tumors develop in the cerebral hemispheres and have an unknown etiology. The only well-known risk factor is a previous course of radiotherapy. In addition, some rare genetic disorders, for example Li-Fraumeni syndrome and neurofibromatosis, are thought to be associated with 5 % of brain cancers. MG does not seem to show any clear associations with diet, smoking, environmental factors or viruses (Ohgaki, Kleihues 2005a).

2.2.2 Classification and grading

Tumors of the CNS, including MGs, are histopathologically heterogeneous. The latest WHO classification of CNS tumors dates from 2007 (Table 1). Tumors are given histological grades from I to IV reflecting their biological behavior (Table 2) and the grading influences the choice of therapies, particularly the use of adjuvant radiation and specific chemotherapy (Louis et al. 2007, Maenpaa 2010). Grade I and II tumors are considered as low-grade whereas grades III and IV represent high-grade tumors. MGs belong to grades III and IV. Glioblastoma (multiforme) (GBM) is a grade IV tumor accounting for ~80 % of MGs. It is characterized by high cellularity and mitotic activity, vascular proliferation and necrosis (Omuro, DeAngelis 2013).

Table 1. The most common gliomas

Tumor based on cell origin	Grade	Histological appearance
Astrocytoma	II	Astrocytoma
	III	Anaplastic astrocytoma
	IV	Glioblastoma (multiforme) (GBM)
Oligodendroglial glioma	II	Oligodendroglioma, oligoastrocytoma
	III	Anaplastic oligodendroglioma, anaplastic oligoastrocytoma
	(IV	Glioblastoma with oligodendroglial component)

Modified from Louis et al. 2007 and Maenpaa 2010.

Table 2. Characteristics of different brain tumor grades

Grade	Characteristics
I	Benign tumor with slow growth, defined margins, often operable, does not infiltrate brain parenchyma, prognosis good if tumor is fully removed
II	Infiltrating brain parenchyma and cannot normally be fully removed, tendency to recur and progress to higher grades
III	Infiltrating brain parenchyma and cannot normally be fully removed, margins undefined, high tendency to recur, histological evidence of malignancy
IV	Infiltrating brain parenchyma and cannot normally be fully removed, histological and cytological evidence of malignancy, fast progression, fatal outcome

Modified from Louis et al. 2007.

2.2.3 Pathogenesis and molecular biology

Tumors of MG originate from the CNS supportive glial cells or their precursors, neural stem cells. They are not only pleomorphic in terms of size and shape, but also highly heterogeneous from a molecular standpoint (Tanaka et al. 2013). On the other hand, as shown by The Cancer Genome Atlas (TCGA) project, there are common genetic alterations in the three main signaling pathways in most GBMs: receptor tyrosine kinase (RTK), Ras, phosphoinositide-3 kinase (PI3K) (88%); P53 (87%) and retinoblastoma protein (Rb) (78%) (Cancer Genome Atlas Research Network 2008). Primary GBMs develop without a previous lesion (*de novo*) and account for most tumors in older patients. Secondary GBMs, on the other hand, are more likely to occur in younger patients and progress from a pre-existing lower-grade glioma. Primary and secondary GBMs differ also significantly in their genetic profiles (Nobusawa et al. 2009).

The MG types of tumors have been divided into 4 subclasses: classical, mesenchymal, proneural and neural, based on their transcriptional profile (Verhaak et al. 2010). Recently it has been acknowledged that different subclasses can exist even within a single tumor, therefore affecting the choice of an individual therapy and the treatment outcome (Patel et al. 2014, Sottoriva et al. 2013). The classical subtype displays the most common genetic aberrations in GBM, namely chromosome 7 amplification and 10 deletion, epidermal growth factor receptor (EGFR) amplification and Ink4a/ARF locus spanning deletion. Characteristic to the mesenchymal subtype is prominent necrosis and the associated inflammation, high expression of CHI3L1 and MET as well as neurofibromin 1 (NF1) mutations/deletion and low level of NF1 mRNA expression. The proneural subtype has been associated with younger age of patients and most secondary GBMs are classified as proneural. Abnormalities in platelet-derived growth factor receptor-A (PDGFR-A), isocitrate dehydrogenase (IDH) and mutations in TP53 are characteristics encountered with this subtype. The neural subtype has expression patterns rather resembling normal brain tissue indicative of a differentiated cell phenotype (Murat et al. 2008, Verhaak et al. 2010, Phillips et al. 2006). While this subtyping may help to understand the pathogenesis of GBM, and to develop new diagnostic assays as well as assisting in the choice of different therapeutic approach, it also emphasizes the vast molecular pathogenesis underlying MG. (Colman, Aldape 2008, Huse, Holland 2010)

Clinically significant molecular markers include genetic loss on chromosomes 1p/19q, mutations of IDH and methylation of O^6-methylguanine-DNA methyltransferase (Mgmt) gene promoter. Co-deletion, resulting in loss of heterozygosity (LOH) 1p/19q, is caused by

the chromosomal translocation often seen in oligodendrogliomas. This results in increased sensitivity to radio- and chemotherapy and prolonged survival of patients. IDH mutations are common in low-grade gliomas and are associated with a better prognosis. The observation of IDH mutations in higher-grade gliomas suggests that they have developed from a lower grade precursor tumor. Epigenetic silencing of the Mgmt promoter by methylation proposes weakened cellular repair capacity towards DNA damage (Hegi et al. 2008). In retrospective analyses, this property was found to be correlated with a beneficial response to alkylating agent chemotherapy (Hegi et al. 2005, Reifenberger et al. 2012). The need for analyses of Mgmt methylation status is however dependent on the diagnostic and therapeutic context of an individual patient, and it has not been shown to vary within the molecular subclasses of MGs (Verhaak et al. 2010). However, it has been shown to be an important biomarker of treatment in the elderly (Malmstrom et al. 2012, Wick et al. 2012). Other molecular markers include GBM-associated epidermal growth factor receptor variant III (EGFRvIII) which is a possible biomarker for vaccination. (Stupp et al. 2014, Weller et al. 2013, Thon, Kreth & Kreth 2013, Louis 2006)

2.2.4 Clinical features and diagnostics

The symptoms of a patient with MG depend largely on tumor location, its size and growth speed. Increased intracranial pressure can cause general symptoms whereas local symptoms are related to anatomical tumor location via infiltration and/or compression of normal brain structures. The most typical first symptom is an epileptic seizure and the life-time risk for this symptom is in the range of 30-50 % (van Breemen, Wilms & Vecht 2007). New-onset persistent or recurrent headache, nausea and vomiting, problems with vision as well as various cognitive problems also warrant further examination of the patient. Cognitive problems may include problems in memory functions, deduction, observation, ability to concentrate and maintain attention. In addition, aphasia, hemiparesis and urinary incontinence may be encountered. (Omuro, DeAngelis 2013)

The diagnosis of MG is based on histopathology complemented with imaging findings (Figure 2). Most often computer tomography (CT) is the primary imaging method due to its widespread availability, but magnetic resonance imaging (MRI) is the most accurate and versatile, especially when imaging the spinal cord or hypophysis (Omuro, DeAngelis 2013). In addition, obtaining a histological sample from the tumor, usually taken during surgery, is crucial for planning the treatment and follow-up. Primary CNS tumors, including MGs, rarely metastasize elsewhere in the body, but can spread within CNS. Therefore the evaluation of the proliferation index from histological sample helps to estimate the growth potential and further to select the most appropriate treatment (Joensuu et al. 2013). The response to treatment as well as differential diagnosis between tumor recurrence and treatment-induced unspecific changes may be clarified further by undertaking magnetic resonance spectroscopy, positron emission tomography or single-photon emission computed tomography with a tracer (Stupp et al. 2014, Minn 2005).

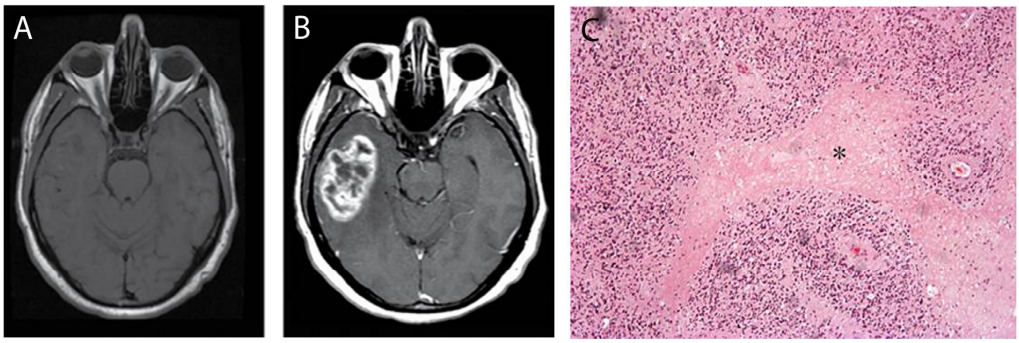

Figure 2. Appearance of GBM in MRI and histology. Axial T1-weighted MRI without (A) and with (B) gadolinium contrast agent demonstrating a GBM tumor in the right temporal lobe. The cystic/necrotic core of the tumor is well illustrated in figure 2B as well as in 2C revealing the histological appearance of GBM. The necrotic core of the tumor is marked with an asterisk (C). High cellularity and endothelial proliferation are seen surrounding the capillaries. The tumor in C is from a different patient from figures A and B. Modified from Omuro & DeAngelis 2013 (A and B) and www.solunetti.fi (C).

2.2.5 Treatment

There is no curative treatment for MG. The standard care of a patient with MG consists of a combination of surgery, radiotherapy and chemotherapy. Concomitant and adjuvant TMZ chemotherapies in addition to radiotherapy are the current standard of care for GBM patients up to age 70 (Stupp et al. 2005, Stupp et al. 2009), even older if fit (Gilbert et al. 2013). This has significantly improved the median survival (benefit of 2.5 months in comparison to radiotherapy only). The location and size of a tumor, primary therapy versus recurrent disease and overall situation of a patient including age, health condition and other diseases are some of the factors affecting the choice of the therapy. Symptoms of a patient and life estimate are also factors that need to be considered. Treatment is planned individually, but although the aim might be permanent recovery, often the best that can be achieved is either slowing down the disease progression or minimizing the injurious effects caused by the tumor and the treatments (Joensuu et al. 2013). With GBM, almost all patients will experience a recurrent disease manifested as a local progression usually within 2-3 cm margins from the original tumor area after 7-10 months progression free survival (PFS). Systemic therapy is considered essential for recurrent tumors. Nevertheless, post-recurrence survival is commonly only 6-9 months (Thon, Kreth & Kreth 2013).

2.2.5.1 Surgery

When applicable, surgery with full or partial resection is the primary mode of treatment. An emergency operation is only needed when there is a massive hydrocephalus or a tumor is very large. A histological sample is taken during the surgery and the cryosection can be analyzed immediately to decide on the resection margins. If the tumor is small and located deep in the brain, a stereotactic biopsy can be taken and analyzed prior to possible surgery. The anatomy of nearby brain structures and physiological functions of brain areas are evaluated and necessary imaging conducted before the surgery. Assisting equipment such as a stereotactic device or neuronavigator may be used in the operation. The use of the fluorescent marker, 5-amino-laevulinic acid (5-ALA), has been shown to increase the

complete resection rate as well as improving PFS (Stummer et al. 2006). Complete removal of the tumor is the clinical goal with non-malignant, grade I gliomas which have well defined margins. However, with grade II-IV tumors, surgical resection is almost always partial and suboptimal, and therefore further treatment with radio- and chemotherapy often will be necessary (Joensuu et al. 2013). Postoperative imaging with MRI should be done within a couple of days to evaluate the extent of resection and residual disease. With recurrent MG, survival benefits of surgical resection are unclear, but it can be used to relieve the symptoms caused by the tumor mass, cytoreduction and update of tumor characteristics (Omuro, DeAngelis 2013). Only approximately one in every four patients has a recurrent GBM tumor, which is amenable to repeated surgery (Mandl et al. 2008).

2.2.5.2 Radiotherapy

Radiotherapy (RT) is given either as an adjuvant treatment after the surgery or as a stand-alone option for inoperable tumors. RT can also be used to alleviate the symptoms caused by the tumor as a palliative treatment. It is usually given over 5-6 weeks up to 60 Gy total dose in 1.8-2.0 Gy fractions (Joensuu et al. 2013). Doses beyond 60 Gy have not been found to offer any additional benefits (Stupp et al. 2014). Shorter, hypo-fractionated regimens may be beneficial for elderly patients or patients with a low performance status (Malmstrom et al. 2012). Furthermore, elderly patients with an unmethylated Mgmt promoter should be treated with RT alone (Malmstrom et al. 2012, Wick et al. 2012). Pseudoprogression is a phenomenon seen usually 4-12 weeks after RT in 20-30 % of patients with increased tumor size and a mass effect corresponding to RT effects rather than treatment failure (de Wit et al. 2004, Wen et al. 2010). The common practice is to continue the ongoing treatment with close imaging follow-up, and to attempt to alleviate any possible symptoms suffered by the patient (Omuro, DeAngelis 2013, Stupp et al. 2014). For recurrent MG, several technically different radiation approaches are available: fractionated 3D-conformal-, fractionated stereotactic and hypofractionated stereotactic/radiosurgical approaches have been used (Combs, Debus & Schulz-Ertner 2007). There are some other less extensively used alternatives for example brachytherapy, radio-immunotherapy and boron neutron capture therapy (BNCT) (Niyazi et al. 2011, Joensuu et al. 2003). However, re-irradiation is an option only for selected patients due to the increased risk of radiation-evoked brain injury and cognitive side effects due to large, cumulative doses (Juratli, Schackert & Krex 2013).

2.2.5.3 Chemotherapy

TMZ is an alkylating agent which adds methyl groups to the O^6-position of guanine. It is the first line chemotherapy for MG and can be given concomitantly with RT following surgery, as an adjuvant therapy as well as in palliative care. When it has been given both concomitantly and in the adjuvant phase for primary GBM, it has been shown to increase OS as well as PFS when compared to radiotherapy alone (Hart et al. 2013). TMZ has good BBB penetration, and the treatment efficacy is based on the prevention of replication by crosslinking DNA. It is orally administered at doses of 75 mg/m^2/day daily during radiotherapy and 150-200 mg/m^2/day on five consecutive days in six cycles of 28 days as an adjuvant treatment (Stupp et al. 2014, Stupp et al. 2005). The most common toxicities associated with the drug are neutropenia and thrombocytopenia. Mgmt gene promoter methylation has proved to be the strongest prognostic marker for treatment outcome, and the benefit of TMZ chemotherapy is largely restricted to this patient subgroup (Hegi et al.

2005). There have been attempts to improve the efficacy with dose-dense TMZ regimens (for example 21-of-28-days schedule) which aim at reducing MGMT levels and therefore exhausting its repair activity. However, these trials have not demonstrated improved efficacy with dose-dense regimens indicating that it is peak TMZ concentrations, rather than prolonged exposure, which seems to be crucial for treatment efficacy (Gilbert et al. 2013, Brada et al. 2010).

Carmustine (BCNU) containing biodegradable polymers is another US Food and Drug Administration (FDA) approved agent for the first-line treatment of MG (Lin, Kleinberg 2008). These can be implanted into the tumor cavity after tumor resection resulting in a modest survival benefit as compared with radiotherapy alone (Westphal et al. 2003). However, in the setting of recurrent MG, no additional benefit was observed either in terms of PFS or quality of life (QoL) (Hart et al. 2008). Other chemotherapeutics used in MG include for example a combination of procarbazine, lomustine and vincristine (PCV), carboplatin and irinotecan (Omuro, DeAngelis 2013, Stupp et al. 2014).

For recurrent MG, the salvage chemotherapy options include TMZ re-challenge, other alkylating agents (e.g. nitrosoureas and carboplatin) or bevacizumab (Omuro, DeAngelis 2013). Carmustine and lomustine (CCNU), belonging to the nitrosoureas, were previously traditional treatment options for recurrent tumors. However, their efficacy is modest and the risk of hematotoxicities is high (Batchelor et al. 2013). In recurrent GBM, TMZ has not been shown to improve OS in comparison to radiotherapy (Hart et al. 2013) and neither did PCV, although PFS and QoL were significantly improved with TMZ as compared to PVC (Brada et al. 2010). It has been reported that relapsing low-grade astrocytoma, anaplastic astrocytomas and oligodendrogliomas are more likely than GBM to respond to TMZ (Yung et al. 1999). The treatment efficacy is generally compromised by the chemoresistance of GBM cells, systemic toxicities and the limited bioavailability of most of these drugs in CNS (Thon, Kreth & Kreth 2013).

2.2.5.4 Other treatments

In addition to surgery, radiotherapy and chemotherapy, symptomatic medication is a part of the standard treatment of MG patients; e.g. corticosteroids for tumor-associated edema and anticonvulsants to prevent seizures (Omuro, DeAngelis 2013). New alternative treatment modalities are constantly being developed. MG vaccination strategies will be discussed in more detail in the immunotherapy chapter 2.3.3.3. A novel therapeutic approach using aligned polymeric nanofibers to guide intracortical brain tumor cells to an extracortical cytotoxic hydrogel was recently described for the treatment of inoperable tumors (Jain et al. 2014).

2.2.5.4.1 Targeted therapies

The pathology and gene expression of MGs are extremely heterogenous even within a single tumor, and therefore various therapeutic modalities such as antibodies and kinase inhibitors have been developed against several different targets (Rich, Bigner 2004, Sathornsumetee et al. 2007). Small molecule kinase inhibitors have been some of the most widely studied approaches with the main kinase targets being EGFR, mammalian target of rapamycin (mTOR), vascular endothelial growth factor receptor-2 (KDR/VEGFR-2), vascular endothelial growth factor receptor-1 (FLT1/VEGFR-1), protein kinase C beta (PKCβ) and platelet-derived growth factor receptor (PDGFR). Some of these have reached

clinical trials up to phase III, but the results so far have not been so impressive to warrant any change in standard clinical practice (De Witt Hamer 2010). A few examples of targeted therapies will be discussed below.

Since the treatment response of TMZ is strongly correlated with Mgmt gene promoter methylation, alternative treatment strategies for patients with unmethylated Mgmt have been sought. Bevacizumab (Avastin®), a humanized murine vascular endothelial growth factor A (VEGF-A) monoclonal antibody, has FDA approval for the treatment of recurrent GBM. Bevacizumab inhibits VEGF-A binding to its receptors and this results in down-regulation of angiogenesis, a prominent feature in GBM (Ferrara, Hillan & Novotny 2005). Recently, in two phase III trials, bevacizumab was shown to improve PFS by 3.4 and 4.4 months as compared to placebo group in newly diagnosed GBM. However, neither trial demonstrated any improvement in OS and adverse events, such as a neurocognitive decline, were attributed to bevacizumab (Chinot et al. 2014, Gilbert et al. 2014). The over-expression of EGFR and its amplification are prominent features in approximately half of the GBMs. Therefore different approaches to inhibit EGFR have been investigated: two small molecular inhibitors, erlotinib and gefitinib, the monoclonal antibody cetuximab and their combinations. However, these approaches have mainly shown efficacy in subsets of MG patients. (Schwer et al. 2008, Preusser et al. 2008, Neyns et al. 2009, de Groot et al. 2008, Prados et al. 2006, Wen et al. 2014)

Cilengitide is an example of a targeted treatment approach for newly diagnosed GBM that has reached phase III. Cilengitide is a selective peptide antagonist of α_v integrin receptors and these receptors which are expressed on endothelial cells and tumor cells in GBM transmit signals for angiogenesis, attachment, migration, invasion and viability (Maurer et al. 2009, Schnell et al. 2008). Cilengitide therapy was shown to improve PFS and OS in a phase I/IIa study (Stupp et al. 2010). However, although being well-tolerated, Cilengitide failed to prolong PFS or OS in a phase III multicenter study (Stupp et al. 2013). CooP is another, more recently discovered tumor homing peptide, which was shown to reduce the number of tumor satellites in the brain when conjugated with the chemotherapeutic agent, chlorambucil. It was therefore postulated to target the invasive tumor cells by functioning as a drug carrier for otherwise difficult-to-reach tumor areas (Hyvonen et al. 2014).

2.2.5.4.2 Histone deacetylase inhibitors, Valproic acid

Epigenetic mechanisms are important regulators of gene functions and they have been shown to be involved also in MG development and progression (Nagarajan, Costello 2009). One of the functional regulators is histone acetylation, which is the result of the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of histones by HATs promotes the appearance of open chromatin and active transcription whereas HDACs function as transcriptional repressors by deacetylating histones (Beumer, Tawbi 2010). Thus histone deacetylase inhibitors (HDIs) promote hyperacetylation and alter gene expression by inhibiting HDACs, an event which has been shown to preferably occur in transformed cells as compared with normal cells (Karagiannis, El-Osta 2006).

HDIs have been shown to modulate tumor angiogenesis, immunogenicity, invasion, metastasis, differentiation, proliferation and apoptosis (Bolden, Peart & Johnstone 2006, Berendsen et al. 2012). These compounds sensitize cancer cells to radiation and DNA damaging agents, for example chemotherapeutics, by promoting open chromatin and

repressing DNA damage response by prolonging the expression of damage response markers (Chen et al. 2007). Therefore, they have emerged as a new class of potential anti-cancer compounds. HDIs have been shown to alter, both up- and down-regulate, possibly over 20 % of the transcriptome in a cell type-specific manner (Chateauvieux et al. 2010, Gotfryd et al. 2010). Their functions have been only partially elucidated and some of them have also impaired the efficacy of drug therapy such as the increased expression of multi-drug resistance proteins leading to increased cellular efflux of chemotherapeutics (Kim et al. 2008, Tabe et al. 2006).

Valproic acid (VPA) is a HDI belonging to the class of short chain fatty acids (Beumer, Tawbi 2010). It has traditionally been used as an anticonvulsant for epileptic seizures (AED), also for MG patients, and as a mood-stabilizer in bipolar disorders (Chateauvieux et al. 2010, Loscher 1999). VPA, and its sodium salt valproate, have been shown to upregulate coxsackie-adenovirus receptor (CAR) (Segura-Pacheco et al. 2007), enhance transduction efficiency (Stedt et al. 2013) as well as upregulate transgene expression (Fan et al. 2005). VPA has been reported to sensitize glioma cells to radiation and TMZ without antagonizing its effect (Van Nifterik et al. 2012). The latter is mediated by downregulation of Mgmt expression in TMZ-resistant cells (Ryu et al. 2012). Patients receiving VPA with TMZ/RT were shown to have an improved survival compared to those treated with other AEDs or no AEDs in the EORTC/NCIC phase III trial (Weller et al. 2011). Furthermore, the improved survival with VPA together with RT (Barker et al. 2013) or TMZ/RT has been demonstrated (Kerkhof et al. 2013). However, contradictory results have also been obtained (Tsai et al. 2012, van Breemen et al. 2009) and the discrepancies will need to be clarified in further prospective studies.

2.2.6 Prognosis

The prognosis of a patient with MG is influenced not only by clinical findings such as age of the patient, neurologic performance status and tumor location but also other factors i.e. radiological features such as contrast enhancement, extent of surgical resection, proliferation indices and genetic alterations. In addition, tumor grade is a key factor when predicting the therapeutic response and outcome of a patient (Louis et al. 2007). An older age of an MG patient has been associated with shorter survival at the population level (Ohgaki, Kleihues 2005b). Aggressive treatment was shown to significantly reduce the mortality in classical and mesenchymal subtypes and was claimed to do so in neural type tumors. However, survival was not altered in the proneural subtype (Cancer Genome Atlas Research Network 2008, Murat et al. 2008, Verhaak et al. 2010). One of the reasons for poor treatment response may be the presence of glioma stem cells (GSC) in tumors (Singh et al. 2004). Although their role in MG is still controversial, GSCs have been shown to be resistant to the conventional therapies, i.e. radiotherapy (Bao et al. 2006) and chemotherapy (Liu et al. 2006).

MG has a poor prognosis: historically the median survival of patients on supportive care has been only ~14 weeks (Avgeropoulos, Batchelor 1999). This was increased to ~20 weeks with surgery only and further up to 7-12 months with post-operative RT (Avgeropoulos, Batchelor 1999, Laperriere et al. 2002). Chemotherapy (mainly with nitrosoureas at the time) increased the median survival up to 12 months (Fine et al. 1993). To date, the overall median survival with GBM with standard therapy is 14.6 months after diagnosis, the 2-year survival rate is 26.5 % and the 5-year survival rate is 9.8 % (Stupp et al. 2009, Stupp et al.

2005). With grade II tumors, survival can exceed over 10 years as is the case with grade III anaplastic oligodendrogliomas. On the other hand, the expected survival of grade III anaplastic astrocytoma patients is 3.5 years (Stupp et al. 2014). In addition to the short life estimate, there is also a substantial reduction in QoL.

2.2.7 Animal models of malignant glioma

Ideally, brain tumor models should fulfill the following criteria: they should originate from glial cells, grow *in vitro* as continuous cell lines and be capable of propagation by serial transplantations *in vivo*. Tumor growth rates should be predictable and reproducible, and glioma-like growth characteristics (e.g. neovascularization, necrosis and invasive growth) should be present. The tumors should grow intracranially and their growth kinetics should be susceptible to therapeutic interventions and to allow monitoring of their efficacy. In the therapeutic evaluation, tumors should be non- or weakly immunogenic in syngeneic hosts, and the response to conventional treatments should be predictive of the response in human tumors (Barth, Kaur 2009, Castro et al. 2011, Candolfi et al. 2007). Although there are several preclinical models available, none of them fulfills all these criteria (Candolfi et al. 2007, Barth, Kaur 2009).

Most of the models use rodents having tumors either subcutaneously (heterotopically) or intracranially (orthotopically). Tumors are often introduced by inoculation of a tumor cell line or transplanting tumor tissue into the target location. Chemicals such as nitrosoureas have been used as mutagens to create these models (Barth, Kaur 2009, Laerum et al. 1977). Xenograft models using human MG cells demand an immunocompromised host in order to avoid immune responses whereas in syngeneic models immunocompetent rodents can be used (Candolfi et al. 2007). These implantation models often have efficient gliomagenesis, reproducible growth rates and accurate knowledge of the tumor location, whereas endogenous spontaneous tumor initiation is lacking. The glioma-like growth characteristics depend on the model used (Candolfi et al. 2007, Chen et al. 2013). However, the lack of an intact immune system in xenograft models prevents their use in immunotherapeutic applications and this should be kept in mind with other applications (Candolfi et al. 2007, Kroeger et al. 2010). Genetically engineered mouse models obtained with insertion of oncogenes or knockout of tumor suppressor genes either in germline or somatic cells have also been applied (Chen et al. 2013, Marumoto et al. 2009). The genetic glioma models are intended to mimic molecular and histological features of human MGs as well as the tumorigenic process itself. Their weaknesses are costly, time-consuming and slower tumor development, and relatively poor prediction of drug therapeutic response (Chen et al. 2013). The only spontaneous GBM model in use is a canine model which displays a pathogenic resemblance to humans enabling also easier resection of tumors as a large animal model (Chen et al. 2013). Individual animal models have been reviewed elsewhere for example by Candolfi et al. (Candolfi et al. 2007) and Barth & Kaur (Barth, Kaur 2009).

2.3 GENE THERAPY

2.3.1 Concepts

Gene therapy can be defined as delivering genetic material into a target cell with a therapeutic intent. Cancer is nowadays the most common disease being treated by gene therapy (Figure 3), although originally gene therapy was applied mainly to monogenic

diseases (Verma, Somia 1997). Gene therapy can either be targeted to somatic cells or germline, latter being transmitted to next generations. Only somatic cell gene therapy has been permitted up to date (Wirth, Parker & Yla-Herttuala 2013). Foreign genetic material, i.e. a transgene, can be taken to its target in several ways. When introduced in cell culture, the term *in vitro* is used. The introduction of a transgene into a patient can be done directly *in vivo* or alternatively *ex vivo*. When the *ex vivo* approach is used either autologous or heterologous cells are transduced outside the patient followed by introducing them into the patient. In the case of heterologous cells, the recipient's immune system may become activated and destroy the cells unless they are protected in some way, for example by encapsulation. (Verma, Weitzman 2005)

The first FDA approved gene therapy clinical protocol was initiated in 1989. The aim of this protocol was to track the movements of tumor infiltrating lymphocytes in melanoma patients after *ex vivo* gene transfer of the neomycin marker gene (Rosenberg et al. 1990). One year later, the first human gene therapy with therapeutic intent was conducted. Two children with a monogenetic immunodeficiency, adenosine deaminase severe combined immunodeficiency (ADA-SCID), were treated with white blood cells which were *ex vivo* transduced with a normal ADA gene. One of the children had a temporary treatment response, however debatable because of a simultaneous enzyme replacement therapy (Blaese et al. 1995). Subsequently the number of human gene therapy trials has expanded. The first gene therapy product to be approved was Gendicine in China in 2003. This product contains a recombinant adenovirus-p53 and was approved for the treatment of head and neck squamous cell carcinoma (HNSCC) (Pearson, Jia & Kandachi 2004, Wilson 2005). (Wirth, Parker & Yla-Herttuala 2013, Wirth et al. 2009)

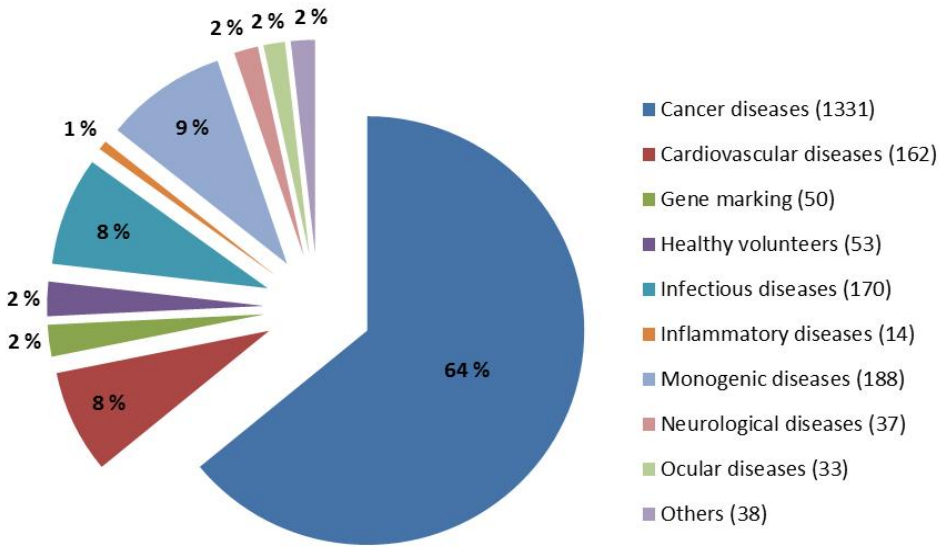

Figure 3. Indications of gene therapy clinical trials (modified from http://www.abedia.com/wiley/index.html, updated June 2014)

2.3.2 Gene therapy vectors

There are different methods that can be exploited to deliver genetic material into a cell; those can be classified as physical, non-viral and viral. The vehicle used for delivery is called a vector. Adenoviral vectors are the most commonly used gene delivery vectors followed by retroviral vectors and plasmids (Figure 4). Each type of vector has its own advantages and disadvantages, and can be chosen to best meet the demands of the application. Varying properties include target preferences, efficacy of gene transfer, toxicity and immune response and duration of the gene transfer. A low gene transfer efficacy is still one of the major problems associated with limited therapeutic efficacy (Pulkkanen, Yla-Herttuala 2005). In oncological applications, this is at least partly explained by inability to reach a large number of tumor cells as well as limited vector spreading within the tumor mass (Lawler, Peruzzi & Chiocca 2006). The beneficial properties of gene therapy vector include high efficiency and specificity in target cells whether dividing or non-dividing, sufficient duration of expression, easy and cost-effective manufacturing at high concentrations with no limitations due to insertion capacity and safe re-administrations without adverse effects or immune response unless this is a desired property (Wirth 2011).

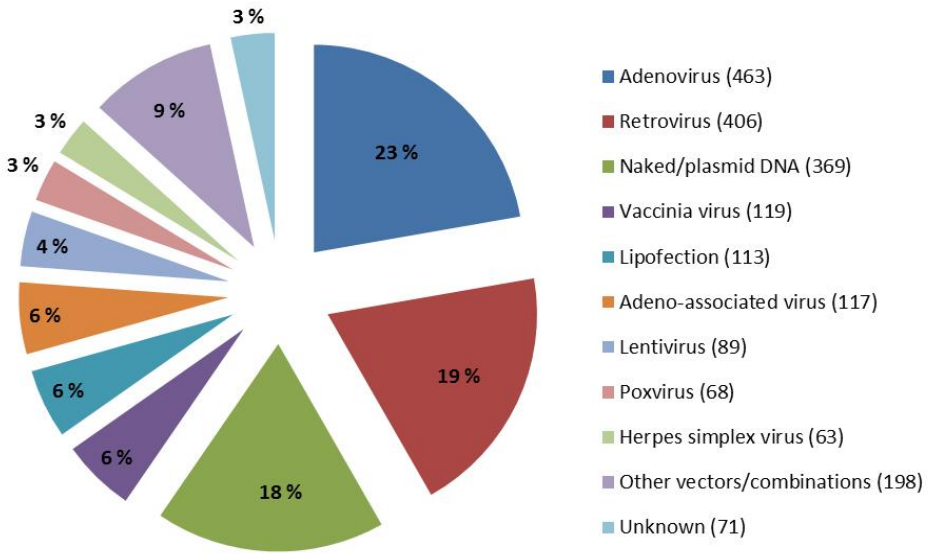

Figure 4. Vectors used in gene therapy clinical trials (modified from http://www.abedia.com/wiley/index.html, updated June 2014)

2.3.2.1 Adenoviral vectors

Adenoviruses (AVs) are DNA viruses causing mainly respiratory tract, eye, intestine and urinary tract membrane infections in humans (Verma, Weitzman 2005). The gene therapy vectors created based on AV genome are non-pathogenic and most often made replication-deficient by deletion of E1 and E3 genes. Further removal of viral genes in the second generation vectors increases the transgene capacity and with the so-called "gutless", 3rd generation vectors having all the viral genes removed, the packaging capacity is up to 36 kb (Alba, Bosch & Chillon 2005). Oncolytic AVs retaining their replicative capability in tumor

cells have also been used in MG applications (Jiang et al. 2009, Castro et al. 2014). Although there are over 50 existing serotypes, most vectors used in clinical trials belong to serotypes 2 and 5 (Giacca, Zacchigna 2012). AV vectors do not integrate into the host genome and have a transient expression. AV vectors can transduce both dividing and non-dividing cells and they can be produced in high titers up to 10^{13} particles/ml (Kootstra, Verma 2003). The side effects of AV gene transfer include the potential immunogenicity, which prevents re-administration of the same vector (Kafri et al. 1998, Yang et al. 1994). The prevalence rates of neutralizing antibodies against serotype 5 AVs in some human populations are close to 90 % (Chen et al. 2010). The primary AV receptor, CAR is often down-regulated in several cancers including MG (Kim et al. 2003). On the other hand, AV vectors in clinical use have proven to be safe (Hedman et al. 2009, Immonen et al. 2004, Muona et al. 2012). (Lawler, Peruzzi & Chiocca 2006, Verma, Weitzman 2005)

2.3.2.2 Retroviral and lentiviral vectors

Retroviruses (RVs) are a large group of RNA viruses. They have a common property of reverse transcribing their single stranded-RNA into double stranded-DNA and subsequently integrating into the host genome to allow long-term expression. Each RV has a characteristic pattern of integration within the mammalian genome (Cavazza, Moiani & Mavilio 2013). All RVs have three common genes: *gag* for the structural proteins, *pol* for the viral enzymes and *env* for the envelope glycoproteins (Singer, Verma 2008). In addition, more complex viruses have additional regulatory proteins. During the development of safer vector constructs, most of these regulatory proteins have been removed and the necessary ones expressed *in trans* from helper constructs in vector production (Dropulic 2011). The transgene capacity of RV vectors is 8-10 kb and they integrate into host genome having therefore persistent expression. Traditional RVs infect only dividing cells excluding lentiviruses (LVs), which can also infect non-dividing cells (Naldini et al. 1996b). Most LV vectors are based on human immunodeficiency virus-1 (HIV-1) genome, although non-HIV LVs have been used as well (Valori et al. 2008). The tropism of these viruses is largely dependent on envelope glycoprotein and can be modified to broaden the tropism and strengthen the virus for purification process during vector production. The most common envelope glycoprotein in pseudotyping is the vesicular stomatitis virus glycoprotein (VSV-G) (Singer, Verma 2008). LV vectors have been shown to efficiently transduce most cell types in the brain, resulting in long-term transgene expression (Jakobsson, Lundberg 2006, Naldini et al. 1996a). For example, they have been used in functional genomics applications such as libraries expressing cDNAs or shRNAs, and animal model applications like transgenesis (Dropulic 2011, Wiznerowicz, Trono 2005). Unlike AVs, RVs have lower toxicities and they are less immunogenic. Another advantage is the lack of pre-existing immunity to vector components in most subjects unlike with AV and adeno-associated virus (AAV) vectors (Matrai, Chuah & VandenDriessche 2010). However, the potential disadvantage in their use is the possibility of insertional mutagenesis caused by the vector integration into a susceptible genomic site causing for example proto-oncogene activation as will be discussed further in chapter 2.3.4. (Lawler, Peruzzi & Chiocca 2006, Sinn, Sauter & McCray 2005)

2.3.2.3 Other viral vectors

Herpes simplex-1 viruses (HSV1) are double-stranded, neurotropic DNA viruses capable of accommodating large or multiple transgene cassettes when used as gene transfer vectors. They have a broad host range with a variety of cell types, can infect both dividing and non-dividing cells, and express transgenes long term via latent neuronal infection. There are two types of HSV1 based gene transfer vectors: recombinant and amplicon vectors with both having the safety advantage of deletion of viral replication genes. In addition, there are oncolytic HSV1 vectors (oHSV1) capable of selective replication in tumor cells based on the deletion of nonessential gene(s) conferring tumor-restricted replication and lysis. (Frampton et al. 2005, Gatson, Chiocca & Kaur 2012, Grandi et al. 2009)

AAVs are single-stranded DNA viruses belonging to the family of *Parvoviridae*. There are currently 14 known serotypes with varying tissue preferences, AAV-2 being the predominant one. AAVs need a helper virus, adeno-, herpes-, papilloma- or vaccinia virus, to facilitate efficient infection and replication. They transduce both dividing and non-dividing cells but are non-pathogenic to humans. They persist mainly in episomal forms, but also sometimes in integrative forms resulting in long-term stable expression. The small capacity for transgenic DNA (<5 kb) and laborious production process are factors limiting the clinical utility of this transgene vector although AAV has been shown to be safe and to trigger a very limited immune response. (Buning et al. 2008, Goncalves 2005, Atchison, Casto & Hammon 1965)

Baculoviruses (BV) are large double-stranded DNA viruses infecting mainly insect cells and thus are non-pathogenic to humans. The *Baculoviridae* family consists of about 700 known members Autographa californica multiple nucleopolyhedrovirus (AcMNPV) being the prototypic baculovirus. As gene transfer vectors, they are easily manipulated with the possibility of incorporating large inserts (> 38 kb), they can be readily produced and purified, and can be transduced into most of the cell types. Their safety features include their inability to replicate in mammalian cells as well as their low cytotoxicity. However, *in vivo* they are quickly cleared from the circulation by the complement system. (Airenne et al. 2013, Hu 2008)

Some other less commonly used viral gene transfer vectors have been summarized in the review by Räty et al. (Raty et al. 2008).

2.3.2.4 Non-viral vectors and physical methods

The most commonly used non-viral gene therapy vectors include naked DNA, cationic lipids, polymers and peptide-based gene delivery systems (Glover, Lipps & Jans 2005, Schatzlein 2001). These have a safety advantage over viral vectors, less restrictions of transgene size, cheaper production with better scalability and no risk of insertional mutagenesis as with integrating viral vectors. However, their transfection efficacy is lower and their expression is transient (Raty et al. 2008). There are also physical methods of gene transfer e.g. electroporation, hydrodynamic delivery, ultrasound, microinjection and ballistic delivery (Glover, Lipps & Jans 2005, Al-Dosari, Knapp & Liu 2005, Hosseinkhani et al. 2003, Wells 2004).

2.3.3 Gene therapy of malignant glioma

MG is an ideal target for gene therapy due to its local occurrence in spatially restricted environment in CNS and rarely has a metastasizing character (Pulkkanen, Yla-Herttuala

2005). The incidence of distant metastases has been shown to be 0.4-0.5 %, bone, lymph nodes, lungs or liver being the target tissues (Fonkem, Lun & Wong 2011, Pasquier et al. 1980). On the other hand, the recent observation by Müller et al. claimed that circulating tumor cells could be found in peripheral blood of ~20 % of GBM patients (Müller et al. 2014). However, no extracranial metastases or correlation to OS were detected in these patients, warranting further investigation of their significance.

There is a need for alternative treatment options like gene therapy since there has been only little progress in the prognosis of MG patients during the last decades. Several different approaches for MG gene therapy have been evaluated including suicide gene therapy, anti-angiogenic gene therapy, immunotherapy and oncolytic virotherapy.

2.3.3.1 Suicide gene therapy

Suicide gene therapy is synonymously also called gene-directed enzyme prodrug therapy (GDEPT), molecular chemotherapy, enzyme-activating prodrug therapy and gene prodrug activation therapy (Denny 2003, Portsmouth, Hlavaty & Renner 2007). It is a process where the expression of an exogenous enzyme catalyses the formation of a toxic product from a nontoxic prodrug substrate. Optimally, the suicide gene should either be absent from the human genome or expressed at very low levels, exhibit high catalytic activity, be necessary and sufficient for full prodrug activation, and be easily modified and included into transgene vectors (Portsmouth, Hlavaty & Renner 2007). The ideal prodrug should have a high affinity for the suicide enzyme but low affinity for endogenous enzymes, have good ability to penetrate tumors, and be efficiently taken up by the tumor cells, as well as being highly toxic only upon activation (Portsmouth, Hlavaty & Renner 2007). Several different suicide gene prodrug combinations have been described, but none of these fulfills all of these criteria (Aghi, Hochberg & Breakefield 2000, Denny 2003, Portsmouth, Hlavaty & Renner 2007).

2.3.3.1.1 Herpes simplex virus-1 thymidine kinase / ganciclovir therapy

Herpes Simplex virus-1 thymidine kinase combined with the prodrug ganciclovir (HSV-TK/GCV) has been the most extensively studied and well-known suicide gene therapy system (Portsmouth, Hlavaty & Renner 2007, Aghi, Hochberg & Breakefield 2000). Its potential for cancer treatment was postulated already in late 1980s (Moolten 1986) and the first proof-of-concept for its application *in vivo* in murine sarcomas was demonstrated in 1990 (Moolten, Wells 1990). GCV is a synthetic acyclic analogue of 2'-deoxy-guanosine and it was originally used as an antiviral drug (Maatta et al. 2009). In cytotoxic gene therapy (Figure 5), GCV is phosphorylated to its monophosphate form by HSV-TK and to di- and tri-phosphates by guanylate kinase and further by other cellular kinases such as phosphoglycerate kinase (Aghi, Hochberg & Breakefield 2000). GCV-triphosphate is a poor substrate for DNA polymerase since it does not possess a 3' hydroxyl group resulting in chain termination and cell death soon after its incorporation into DNA (Ilsley et al. 1995). HSV-TK/GCV cytotoxicity is cell cycle dependent and for this reason, it is mainly dividing cells that are affected. This is a benefit in CNS where the tumor is largely surrounded by non-dividing brain parenchyma cells. The exact mechanism of HSV-TK/GCV cytotoxicity remains obscure and has been shown to be cell-type dependent (Fillat et al. 2003). The GCV-triphosphate interaction with DNA results in the appearance of non-functional DNA fragments inducing apoptosis in cancer cells (Freeman et al. 1993). Cell death is also

induced by increased accumulation of p53 and death receptors, and finally caspase activation, resulting in loss of mitochondrial membrane potential and release of cytochrome c (Beltinger et al. 1999). It has also been speculated that activated antitumoral immune response against nontransduced cells (Kianmanesh et al. 1997, Vile et al. 1997), and immune cells infiltration locally and systemically are contributors to HSV-TK/GCV mediated antitumor effect (Barba et al. 1994, Dewey et al. 1999, Rainov et al. 2000, Immonen et al. 2004). Over the years, the efficacy of HSV-TK/GCV treatment has been improved by introducing novel splice-corrected (Chalmers et al. 2001) and mutated (Black, Kokoris & Sabo 2001, Preuss et al. 2011) HSV-TK variants. Their enhanced GCV affinity enables for less immunosuppressive GCV doses to be used.

The bystander effect is an important phenomenon further enhancing the efficacy of several suicide gene therapies including HSV-TK/GCV. Although observed in early studies by Moolten et al., Culver et al. 1992 and Freeman et al. 1993 were the first researchers to report that even low amounts of TK-positive tumor cells could achieve tumor growth restriction (Culver et al. 1992, Freeman et al. 1993). It has been estimated that only about 10 % of the tumor cells need to express HSV-TK gene in order to prevent the tumor growth with GCV administration (Sandmair et al. 2000b, Freeman et al. 1993). Several mechanisms have been postulated to account for the bystander effect, although the transfer of toxic GCV metabolites through gap junctions from TK-positive to TK-negative cells leading to cell death is the most widely acknowledged mechanism (Asklund et al. 2003, Touraine et al. 1998). Other mechanisms have been proposed e.g. induction of apoptosis of nontransduced cells by accumulation of GCV-triphosphates in the surrounding cells (Hamel et al. 1996), killing of tumor endothelial cells (Ram et al. 1994), and activation of a host immune response against the tumor cells due to HSV-TK expression and exposure of tumor antigens after surrounding cell death (Vile et al. 1997). Normal brain cells have been shown to contribute to the bystander effect without being eliminated by GCV themselves (Miletic et al. 2007).

Soon after the first proof-of-concept study in 1990, several clinical phase I/II studies applying HSV-TK producing retroviral packaging cell (RV-VPC) intratumoral injections combined to systemic GCV for recurrent GBM were conducted (Klatzmann et al. 1998, Oldfield et al. 1993). However, in phase III multicenter randomized controlled trial (RCT) in newly diagnosed GBM patients, RV-VPC treatment of HSV/GCV with standard treatment failed to improve PFS as well as OS (Rainov 2000). In the same year, Sandmair et al. published a phase I/IIa study where AV HSV-TK/GCV therapy was shown to significantly increase survival in comparison to RV as well as control group (15 vs 7.4 vs 8.3 months). In addition, AV vectors were shown to have a higher transduction efficacy and transgene expression than could be obtained with RV vectors (Sandmair et al. 2000a). AV vectors were also found to be superior over RV vectors *in vivo* in MG patients after β-galactosidase gene transfer (Puumalainen et al. 1998). The phase IIb trial, conducted by Immonen et al., was the first RCT to achieve increased survival of patients administered sitimagene ceradenovec (Cerepro®, Ark Therapeutics Ltd., AV vector encoding for HSV-TK) compared with patients receiving standard care (Immonen et al. 2004). A significant increase in survival was maintained even in a separate analysis with GBM patients only excluding lower grade MGs. Encouraged by these results, a phase III multicenter, standard care RCT with Cerepro® was conducted in patients with operable primary MGs. When compared with standard care, Cerepro® was found to prolong the time to re-intervention

or death after surgery in GBM patients. However, there was no significant effect on OS. Although adverse effects, most commonly transient hemiparesis, hyponatremia and seizures, were more common in the treatment group, overall the treatment was considered to be safe being in line with preclinical safety assessment (Westphal et al. 2013, Langford et al. 2009). Based on the trial results, European Medicines Agency (EMA) proposed a further clinical evaluation of Cerepro® to provide sufficient evidence of clinical benefit over current standard treatment.

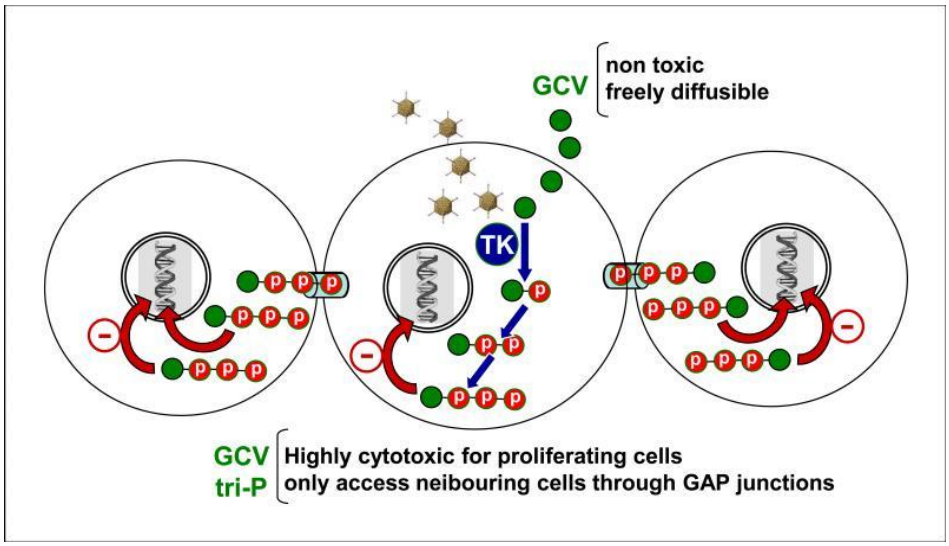

Figure 5. Mechanism of HSV-TK/GCV suicide gene therapy. Herpes simplex virus-1 thymidine kinase (TK) is introduced into target cells via gene transfer with adenoviral vectors. TK phosphorylates non-toxic prodrug ganciclovir (GCV) into GCV-monophosphate (1P), which is further phosphorylated into di- (2P) and triphosphate (3P) forms by cellular kinases. In proliferating cells, GCV-3P is incorporated into DNA resulting in chain termination and apoptosis of the cell. The cell death is further propagated in nontransduced neighboring cells by GCV-3P travelling through gap junctions. Modified from Castro et al. 2014.

2.3.3.1.2 Tomato thymidine kinase / azidothymidine therapy

In CNS diseases like MG, the chemotherapeutic agent or a prodrug should be readily able to penetrate through the BBB in order to reach its target and interact with the intracranially administered suicide gene (Portsmouth, Hlavaty & Renner 2007). AZT (azidothymidine, Zidovudine®), an analogue of thymidine and a drug originally used to treat HIV patients, has been shown to easily penetrate BBB and it has a well-known safety profile obtained after several years of clinical experience (Denny 2003). Human deoxynucleoside kinases (dNK) poorly phosphorylate the parent compound into the diphosphate form (AZT-DP), presenting a safety advantage while preventing unwanted activation (Lavie et al. 1997). A thymidine kinase 1 (TK1) –like enzyme from tomato plant (ToTK) has been so far the only TK1 enzyme found to be able to efficiently phosphorylate AZT into its mono- (AZT-MP) and diphosphate (AZT-DP) form and the enzyme displays high substrate specificity for AZT (Khan et al. 2010). ToTK has been shown always to exist in a highly active form, independent of ATP availability (Khan et al. 2010, Mutahir et al. 2011). The enzymatic efficiency is at least partly contributed to the inefficient negative feedback regulation of

thymidine triphosphates (Larsen, Munch-Petersen & Piskur 2014). The ToTK/AZT combination has shown to be an efficient suicide gene therapy in MG cells and neural stem cells have been utilized as a suicide gene vehicle in rat MG application (Khan et al. 2010).

2.3.3.2 Anti-angiogenic therapy

In angiogenesis, new capillaries are formed from the pre-existing vasculature and this is considered as one of the hallmarks of cancer, GBM being one of the most angiogenic cancers (Hanahan, Weinberg 2011, Louis 2006). The importance of tumor angiogenesis was hypothesized by Dr. Judah Folkman in 1971 (Folkman 1971). In tumor angiogenesis, cells have to acquire an angiogenic phenotype, turning on a so-called "angiogenic switch" in order to be able to maintain their supply of nutrients and oxygen (Hanahan, Folkman 1996). The balance between pro- and anti-angiogenic factors is important and those factors can be targeted in therapeutic applications, for example with neutralizing antibodies and receptor tyrosine kinase inhibitors (RTKIs). Anti-angiogenesis can be introduced either by down-regulating the proangiogenic factors like vascular endothelial growth factor A (VEGF-A) (Ellis, Hicklin 2008, Ferrara 2002), fibroblast growth factor (FGF) or platelet-dericed growth factor (PDGF), or via the upregulation of anti-angiogenic factors like thrombospondin-1 (TSP-1), angiostatin or endostatin (Carmeliet, Jain 2000, Samaranayake et al. 2010). So far, the only clinically approved anti-angiogenic agent is Bevacizumab, which is discussed briefly in the "Targeted therapies" section 2.2.5.4.1 (Tabatabai, Stupp 2009).

The effects of anti-angiogenic therapies are partly mediated by vascular normalization in which peri-tumoral vasogenic edema is reduced, therefore alleviating adverse symptoms and improving QoL (Batchelor et al. 2007, Jain 2005). Vascular normalization has also been thought to temporarily improve chemotherapy penetration into the tumor bed as well as enhancing the intratumoral distribution of the injected viral vectors (Jain et al. 2007, Gatson, Chiocca & Kaur 2012). Resistance to antiangiogenic therapies may develop through the activation of compensatory angiogenic mechanisms, protection of the tumor vasculature by proangiogenic inflammatory cells and increased pericyte coverage and enhanced tumor invasiveness, vessel co-option and metastasis (Bergers, Hanahan 2008, Samaranayake et al. 2010).

2.3.3.3 Immunotherapy

The brain was long considered a niche where the immune system had only a limited detection and elimination capacity towards foreign antigens, a state referred to as "immune privilege". This was thought to be due to the low number of dendritic cells in the brain, the lack of lymphatic drainage and the production of anti-inflammatory mediators (Assi et al. 2012, Lowenstein et al. 2007). However, recently there has been increasing evidence of CNS immunocompetence and active interactions with the peripheral immune system, and therefore an increase in the interest of developing immunotherapy for GBM (Jackson, Lim & Drake 2014, Reardon et al. 2013, Reardon et al. 2014). The most extensively studied and well-established immunotherapy for GBM has been vaccination either with tumor lysate or synthetic tumor antigens (Jackson, Lim & Drake 2014, Reardon et al. 2014). In vaccination, target specific antigen(s) along with an immunostimulatory adjuvant is taken up by antigen presenting cells (APC), most often *ex vivo* propagated dendritic cells, and presented in antigen-MHC (major histocompatibility complex) to elicit a T-cell response in the patient. Several glioma-associated antigens have been recognized and immunotherapy trials are

ongoing, with EGFRvIII vaccine strategies being the most extensively studied (Jackson, Lim & Drake 2014, Reardon et al. 2013). Vaccination strategies can further be enhanced by adopting other immunomodulatory approaches, for example AV TK/GCV + Flt3L (fms-like tyrosine kinase 3 ligand) gene therapy (Mineharu et al. 2011). The T-cell response can also be elicited by directly introducing immune mediators such as interferons or interleukins into the patient for example by using gene transfer vectors (Castro et al. 2011). Some other immunotherapy strategies include immune checkpoint inhibitors, like CTLA-4 (Cytotoxic T lymphocyte antigen-4) blocking antibodies enabling immunological elimination of tumor cells (Hodi et al. 2010), and a reduction in the number or activity of immunosuppressive regulatory T cells (Curtin et al. 2008).

2.3.3.4 RNA interference

RNA interference (RNAi), a universal mechanism of gene silencing, was first discovered in *C. elegans* by Fire et al. in 1998 (Fire et al. 1998) and a few years later found to be functional also in mammalian cells (Elbashir et al. 2001). The mediators of RNAi are approximately 20-30 nucleotides (nt) long RNA molecules, either exogenously transfected small interfering RNAs (siRNAs) or small hairpin RNAs (shRNAs) encoded by gene transfer vectors within the cell (Dykxhoorn, Novina & Sharp 2003). ShRNAs are transcribed within the nucleus, exported to cytoplasm by Exportin-5 and processed by the Dicer complex into ~21-25 nt siRNA fragments. These siRNAs, either processed from shRNA or exogenously introduced, are loaded into RISC (RNA-induced silencing complex) where the guide RNA strand mediates either a direct sequence specific cleavage or translational repression and RNA degradation. The former is induced by full complementarity of the siRNA with the mRNA target sequence, whereas the latter is a result of partial complementarity. In addition to these post-transcriptional gene silencing (PTGS) mechanisms, promoter-complementary siRNAs can mediate transcriptional gene silencing (TGS) in nucleus triggering chromatin remodeling and histone modifications (Castanotto, Rossi 2009) (Figure 6).

Several RNAi based applications have been developed (Castanotto, Rossi 2009, Dillon et al. 2005) and the first human clinical trial started in 2004 for the wet age-related macular degeneration (Whelan 2005). In MG, impaired tumor growth has been achieved for example in murine models after RNAi against hypoxia inducible factor-1α (HIF-1α) (Gillespie et al. 2007), Src (Gillespie et al. 2007, Stedt et al. 2012) and Mgmt combined with TMZ (Viel et al. 2013). RNAi against Tenascin-C (TN-C), an extracellular matrix protein, has been claimed to improve OS of patients with MG (Rolle et al. 2010). However, RNAi based therapeutics have also raised some safety concerns, namely induction of specific side effects through partial sequence complementarity and unspecific side effects like triggering an interferon response (Bridge et al. 2003). In addition, siRNA-mediated saturation of the above described cellular machinery processing also microRNAs (miRNAs) may cause perturbations in essential cellular functions (Grimm et al. 2006, Khan et al. 2009).

MiRNAs are a group of endogenous non-coding RNAs (ncRNAs) regulating various cellular processes through the mechanisms described above (Visone, Croce 2009, Castanotto, Rossi 2009). Deregulation of miRNAs has been linked to initiation, progression and metastasis of many cancers including MG (Hermansen, Kristensen 2013, Palumbo et al. 2014). Several miRNAs have been shown to be either upregulated or downregulated in MG, although some of the findings are somewhat contradictory. The potential of miRNAs as

cancer biomarkers and treatment options as well as their roles in predicting treatment response and survival is only beginning to be elucidated (Hermansen, Kristensen 2013).

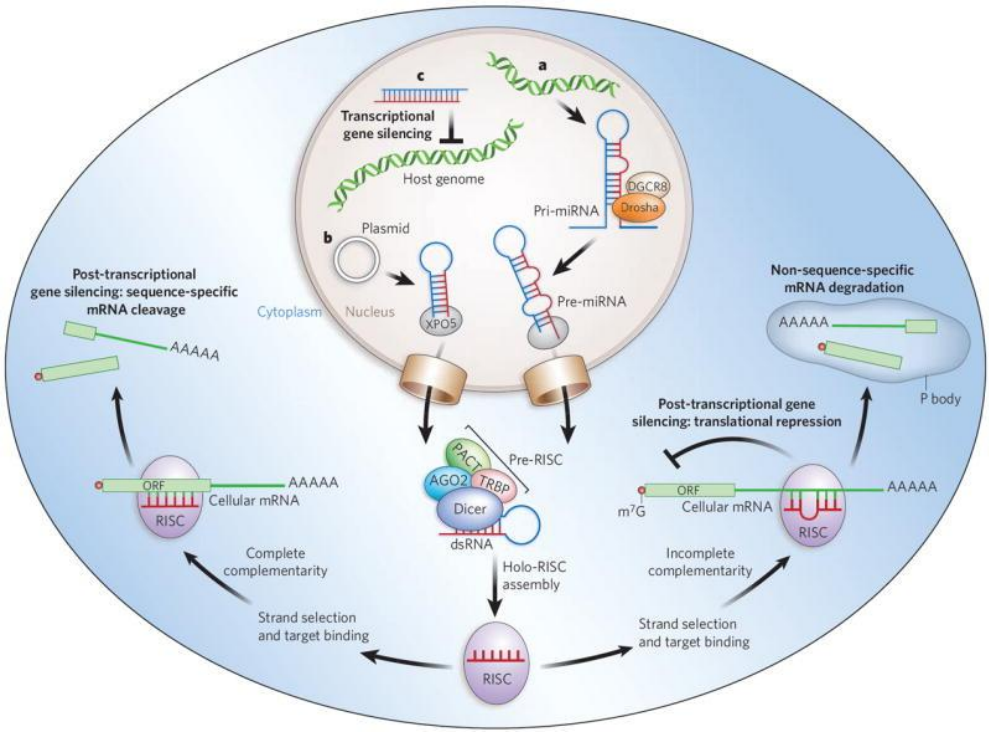

Figure 6. Mechanism of RNA interference. **a**, Primary microRNAs (pri-miRNAs) are processed to precursor miRNAs (pre-miRNAs) by the Drosha complex and transported to cytoplasm by Exportin 5 (XPO5). **b**, Similarly artificially transcribed shRNAs, either from plasmid or other vectors, are transported to cytoplasm by XPO5. In the cytoplasm, these double stranded RNAs (dsRNAs) are processed by Dicer containing the pre-RISC complex and the processed guide strand in RISC (RNA-induced silencing complex) transports the complex to complementary mRNA sequence. With full complementarity (left pathway), the target mRNA sequence is degraded through Argonaute 2 (AGO2)-mediated cleavage. With partial complementarity (right pathway), translational inhibition occurs accompanied by non-sequence-specific degradation of mRNA in P bodies. **c**, In addition, transcriptional gene silencing via chromatin remodeling and histone modifications can occur in the nucleus with promoter-complementary siRNAs. Modified from Castanotto et al. 2009.

2.3.3.4.1 Src

Src is a nonreceptor tyrosine kinase belonging to a family of Src family kinases (SFKs) with eight other members (Summy, Gallick 2006). It is located downstream of several growth factor receptors (receptor tyrosine kinases) and it functions by phosphorylating specific tyrosine residues in other proteins (Alvarez, Kantarjian & Cortes 2006). Src was the first tyrosine kinase to be characterized in 1979 (Oppermann et al. 1979) and since then it has been shown to be a central mediator of various physiological and pathological processes such as proliferation, migration, angiogenesis, invasion and survival via various signaling pathways (Ahluwalia et al. 2010) some of which are illustrated in Figure 7. These processes are also considered to be necessary for tumourigenesis, and therefore the role of Src has been studied in detail in various cancers including MG (Alvarez, Kantarjian & Cortes 2006,

de Groot, Milano 2009). Transgenic mice expressing v-Src (viral homologue of cellular Src) develop glioblastoma tumors closely resembling human tumors, evidence for a role for Src in glioma development and progression (Weissenberger et al. 1997). Src activity has been shown to be elevated in human GBM likely due to increased activation of cell surface growth factor receptors and integrins since no gene amplification or mutations have been detected in GBM tumors (Cancer Genome Atlas Research Network 2008).

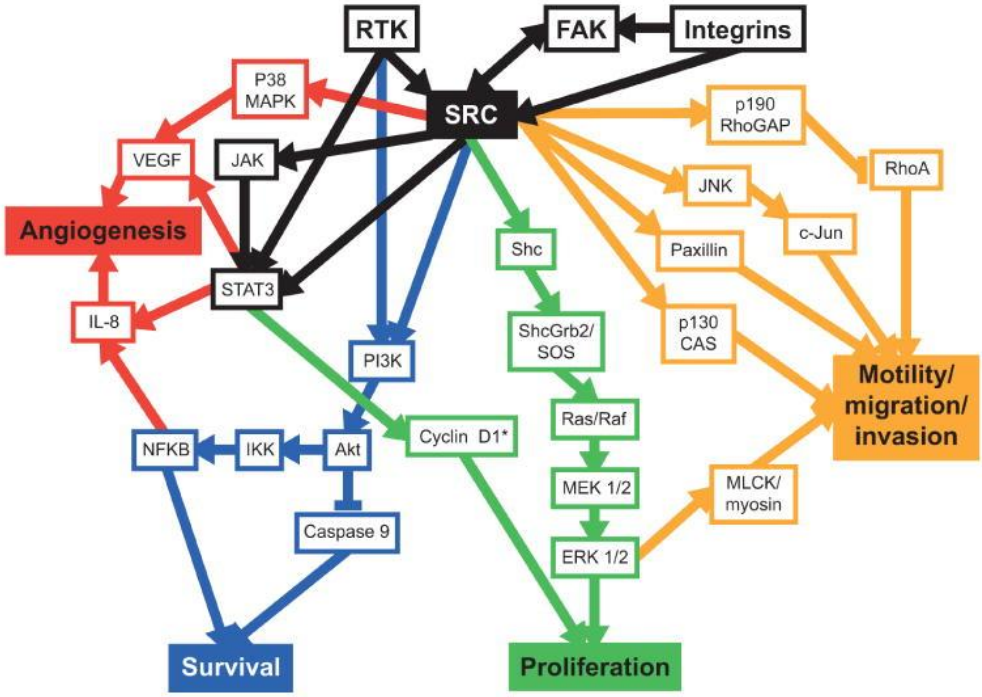

Figure 7. Signaling network of Src-mediated tumor progression. Interaction of Src with receptor tyrosine kinases (RTK), integrins and focal adhesion kinase (FAK) activates the Src kinase activity and further downstream signaling resulting in cellular responses like angiogenesis, survival, proliferation and motility/migration/invasion. Some of the signaling intermediates in the corresponding pathways are shown. However, not all signaling pathways are well characterized with unknown mediators and connections. In addition there is considerable "crosstalk" in the illustrated pathways not demonstrated by arrows. For more details of individual signaling molecules, please refer to the original publications. Modified from Ahluwalia et al. 2010 and Summy & Gallick 2006.

Src deficient ($src^{-/-}$) mouse models have exhibited reduced tumor-induced vascular permeability, resulting in anti-invasive and anti-metastatic properties (Criscuoli, Nguyen & Eliceiri 2005, Lund et al. 2006, Weis et al. 2004), and therefore Src inhibitors have been proposed to be combined with treatments aimed at reducing the tumor bulk itself (Brunton, Frame 2008). Different therapeutic agents capable of inhibiting SFKs in MG have been studied including small molecule inhibitors like Dasatinib, PP2 and SU6656 (Araujo, Logothetis 2010, Zhang, Yu 2012, Ahluwalia et al. 2010) as well as shRNAs (Stedt et al. 2012). None of the SFK inhibitors in clinical testing (Dasatinib, Saracatinib, Bosutinib) have received approval from FDA for the treatment of solid tumors because the results have not

been encouraging. Dasatinib (SFK/ABL dual inhibitor) has been clinically assessed for GBM therapy both as a monotherapy and in treatment combinations. It has received FDA-approval for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (Zhang, Yu 2012). In addition to combination treatments, the discovery of effective response biomarkers and pre-selection of patients based on these markers have been suggested to improve the success of SFK inhibitors (Zhang, Yu 2012).

2.3.3.5 Other gene therapy strategies

In addition to the above strategies, there are several other gene therapy options available including targeted toxins, oncogene and tumor suppressor gene therapy, and oncolytic virotherapy, examples of which are given below.

Targeted toxins are formed from two components: a ligand of the targeted receptor exclusively overexpressed on the target cells and a radioisotope or a toxin moiety consisting of catalytic and translocation domains of highly toxic bacterial products such as *Pseudomonas* or *Diphteria* exotoxin. Once the ligand-toxin fusion has been selectively internalized into the target cell, the toxin inhibits protein synthesis triggering cell death (Castro et al. 2011). An example of a ligand-receptor couple is interleukin-13 (IL-13) and its receptor IL-13Rα2 overexpressed on glioma cells. IL-13 has been fused to mutated *Pseudomonas* exotoxin to create IL-13 toxin, which has been further improved and shown to possess both high safety and efficacy in murine *in vivo* GBM applications (Candolfi et al. 2010).

Mutations in oncogenes and tumor suppressor genes create the basis for a multistep process of cancer development. These pathways have been partly discussed in "Pathogenesis and molecular biology" section 2.2.3. Mutations in tumor suppressor p53 are the most common alterations seen in 25-30 % of primary and 60-70 % of secondary GBMs (England, Huang & Karsy 2013). Therapeutic approaches aiming at replacing mutant p53 by gene transfer of wild-type p53 or reactivation of wild-type p53 have been investigated. The AV gene delivery of p53 was evaluated in a phase I clinical trial of MG which demonstrated the safe expression of functional protein, however, within a limited distance from the injection site (Lang et al. 2003). Although inactivation of p53 occurs early in gliomagenesis (Castro et al. 2011), its role as a molecular marker remains controversial since most studies have failed to show any association with prognosis (England, Huang & Karsy 2013).

Oncolytic virotherapy is based on the concept in which a replicationcapable virus selectively replicates within tumor cells resulting in cell lysis and further propagation of viral progeny into the nearby tumor cells. Tumor selectivity can be achieved for example by retargeting towards tumor-associated receptors like EGFRvIII (Wollmann, Ozduman & van den Pol 2012). Several oncolytic viruses have been examined in GBM, and at least HSV, AV, Newcastle disease virus, reovirus, parvovirus, measles virus and poliovirus have advanced to clinical trials (Wollmann, Ozduman & van den Pol 2012). HSV-based oncolytic viruses were the first (Martuza et al. 1991) and the most extensively studied oncolytic viruses. The AV-based oncolytic virus, ONYX-015, demonstrated good safety in a phase I clinical trial of MG, although it was concluded that potential for efficacy warranted further investigation (Chiocca et al. 2004). In 2005, China approved an almost identical AV-based therapy for HNSSC as the world's first oncolytic virus therapy for cancer (Garber 2006).

2.3.4 Safety and ethics

Over the years, continuous efforts have been made to enhance the safety of gene therapy. Safety aspects in vector development are reflected in the different generations of gene transfer vectors. In AV vectors, deletion of replication genes E1 and E3 created the first generation vectors whereas in third generation "gutless" vectors, all of the viral genes have been removed. A similar safety evolution has been achieved in LV progressing up to third generation self-inactivating vectors from which all unnecessary accessory genes have been removed. For the production purposes, necessary genes, including a transgene, are coded by separate plasmids further reducing the likelihood of generating replication competent viruses, the major safety concern. Despite the large-scale production and testing of LVs, no replication competent viruses have been detected (Cornetta et al. 2011, Manilla et al. 2005). Other examples of ensuring safety include self-inactivating (SIN)-vectors, optimization of vector purification and vector administration locally, if possible, to avoid systemic side effects. (Raty et al. 2008)

Concerns have been raised regarding some properties connected to gene transfer vectors, namely the risk of an insertional oncogenesis with RV vectors and the possibility for immune responses mainly with AV vectors. In 1999, a patient suffering from a partial ornithine transcarbamylase (OTC) deficiency died during a phase I clinical trial aiming at treating OTC-deficiency with a high dose AV gene transfer. The suspected cause of the patient's death was an AV vector-related immune response manifested as acute respiratory system collapse and subsequent multiorgan failure (Fox 2000, Lehrman 1999). Initially, innate immune system is activated and after repeated exposures, the adaptive immune response neutralizes the vector and eliminates transduced cells, possibly preventing the use of the same vector again. However, in the case of AV vectors, prevalent antibodies from previous exposure to wild type AVs, are detectable in 97 % of individuals (Chirmule et al. 1999). On the other hand, in diseases like MG, immune activation might also serve as an enhancer of the treatment response and therefore it could be considered as a beneficial response (Immonen et al. 2004, Sandmair et al. 2000b).

In the early years of this century, four patients with severe combined immunodeficiency (SCID-X1) participated in gene therapy trials transferring the missing interleukin 2 receptor gamma (ILR2G) gene into CD34+ bone marrow precursor cells. These patients developed leukemias after a few years delay subsequent to the gene therapy and the cause was considered to be genomic integration of the RV vector used in the trials predisposing the subjects to leukemia through insertional mutagenesis activated proto-oncogenes (Hacein-Bey-Abina et al. 2008, Hacein-Bey-Abina et al. 2003). This risk has mainly been associated with the use of gamma RV vectors (Modlich, Baum 2009, Montini et al. 2006) although its existence is acknowledged also with LV vectors (Cavazzana-Calvo et al. 2010). As a solution to insertional oncogenesis, chromatin insulators isolating the viral promoter, directed integration to a predetermined genomic site and integrase-defective LV vectors have been proposed (Vargas et al. 2004, Yi, Hahm & Lee 2005, Schenkwein et al. 2013). However, problems such as random integration can still occur (Tan et al. 2006).

These single, but tragic deaths connected to gene therapy have raised several questions about safety and ethics of gene therapy in general. On the other hand, in three RCTs of cardiovascular diseases and MG, the incidences of serious adverse events in AV-treated patients were 0.9 and 4.0 per 10 000 patient days in cardiovascular diseases and MG trials as compared to 0.5 and 2.1 in the randomized control patients, respectively (Wirth et al.

2006). It has to be remembered that the current treatments for MG, i.e. radiotherapy and chemotherapy, also are subject to severe adverse effects.

The procedure of obtaining approval for a clinical trial in the European Union (EU) is standardized and includes the completion of an IMPD (Investigational Medicinal Product Dossier) which contains basic data about clinical study, clinical objectives, vector description, manufacturing, supply and import, preclinical data and risk assessment, and information about patients as well as a mandatory informed consent. The IMPD is evaluated by national agencies as well as the ethical committee, and Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP) and if approved, Good Clinical Practices (GCP) are presumed to be followed (Gonin et al. 2005). These gene therapy related activities are regulated at the EU level by the Bioethics Directive and Clinical Trials Directive, as well as being subject to national legislation, such as Gene Technology Law and Medicines Act in Finland.

Ethical issues concerning gene therapy are vast and complex, and are often loaded with emotional charge in dialogue. What kind of gene therapy is acceptable, what is the price and to whom should it be available? Should the applications be limited to defined medical conditions or could human traits also be altered? Nevertheless, the active discussion and open interaction between investigators, regulatory authorities and the general public needs to be maintained.

3 Aims of the study

MG is a cancer with a poor prognosis. There is a clear need for development of new therapeutic strategies to treat these patients. MG is well suited for gene therapy due to its local occurrence in an anatomically restricted location and non-metastasizing character. Therefore, the aims were to investigate and evaluate different therapeutic options and their combinations *in vitro* and *in vivo* in preclinical MG models to better understand and further develop new and current treatment modalities.

The specific aims for this thesis were as follows:

I To study whether it is possible to inhibit tumor cell growth with shRNAs targeting Src kinase and to translate these *in vitro* findings into *in vivo* inhibition of tumor growth in two separate MG models

II To assess whether VPA could enhance the treatment efficacy of HSV-TK/GCV+TMZ combination and to further optimize the efficacy of the latter combination

III To compare the efficacy of HSV-TK/GCV therapy with a novel suicide gene therapy of ToTK/AZT *in vitro* and *in vivo*

4 Materials and methods

The materials and methods used in this study are briefly summarized in the following tables 3-7. More information about materials and detailed description of the methods can be found in the original publications (I-III) referred to in the tables. The statistical analyses used are listed in the original publications.

Table 3. Methods used in original publications I-III

Method	Description	Original publication
In vitro		
DNA cloning	Construction of Ad.ToTK vector	III
Lentiviral production	Calcium phosphate transfection, viral concentration by ultracentrifugation and titer determination with FACS and p24 assay	I
Adenoviral production	Calcium phosphate transfection, plaque selections, viral concentration by ultracentrifugation and titer determination by spectrophotometric analysis	II, III
Cell culture	Isolation of human umbilical vein endothelial cells	I
	Cell line and primary cell culture	I, II, III
Microscopy	Light microscopy	I, II, III
	Fluorescence microscopy	I, II
FACS	Transduction efficiency measurement	I, II, III
Cell viability	CellTiter-Glo luminescent cell viability assay, CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay	I, II, III
In vitro angiogenesis	Tubulogenesis assessment on Matrigel	I
Kinase activity assay	Radiolabel enzymatic activity measurement	III
Methods to study gene expression	RNA isolation and concentration measurement, quantitative real-time polymerase chain reaction (qPCR)	I
Methods to study protein expression	Total protein extraction and concentration measurement by BCA assay, western blot	I
	Immunohistochemistry (paraffin or frozen sections)	I, II, III
Ex vivo / In vivo		
Tumor cell inoculation	Subcutaneous (s.c.)	I
	Intracranial (i.c.)	I, II, III
Gene transfer	Intracranial (i.c.)	I, II, III
Manual tumor measurement	3-dimensional tumor measurement and volume analysis	I
MRI, Matlab	Tumor confirmation or follow-up, tumor volume analysis	I, II, III

Blood sampling	Full blood counts, clinical chemistry parameters, VPA concentration measurements	I, II, III
Medication	Treatment medications to rodents i.p. / p.o. / s.c.	I, II, III

Ad.ToTK, Adenoviral vector including tomato thymidine kinase insert; FACS, Fluorescence-activated cell sorting; MRI, Magnetic resonance imaging; VPA, Valproic acid; i.c., intracranial; i.p., intraperitoneal; p.o., per oral; s.c., subcutaneous.

Table 4. Cell lines and rodent strains

Cell line	**Description**	**Source**	**Original publication**
HUVEC	Human umbilical vein endothelial cells	Isolated from umbilical cords obtained from maternity ward of Kuopio university hospital	I
BT4C	Rat glioma cells	Laerum et al. (Laerum et al. 1977)	I, II, III
GL261	Mouse glioma cells	A kind gift from Prof. Hinkkanen	III
U118MG	Human glioma cells	ATCC: HTB-15	I, III
U87MG-Fluc	Human glioma cells expressing luciferase	A kind gift from Prof. Hinkkanen	III
Rodent strain	**Description**	**Producer**	**Original publication**
NMRI	Nude mice	Taconic, Ejby, Denmark	I
Hsd:AthymicNude-Foxn1nu	Nude mice	Harlan Laboratories, Horst, Netherlands	III
BDIX	Immunocompetent rats	Charles River Laboratories, Lille, France	I, II

ATCC, American type culture collection.

Table 5. Viral vectors

Viral vector	**Description**	**Original publication**
LV.shSRC1	Lentiviral vector, Src shRNA insert 1	I
LV.shSRC2	Lentiviral vector, Src shRNA insert 2	I
LV.shGL3	Lentiviral vector, Luciferase shRNA insert	I
Ad.GFP	Adenoviral vector, Green fluorescent protein marker gene insert	II
Ad.LacZ	Adenoviral vector, LacZ marker gene insert	II
Ad.HSV-TK (Cerepro®)	Adenoviral vector, Herpes simplex virus-1 thymidine kinase insert	II, III
Ad.ToTK	Adenoviral vector, Tomato thymidine kinase insert	III
Ad.control	Adenoviral vector, no insert	III

Table 6. Pharmaceutical products

Product	**Description**	**Original publications**
AZT	Azidothymidine	III
DMSO	Dimethyl sulfoxide (TMZ dilutions)	I, II
dThd	Deoxythymidine	III
GCV	Ganciclovir hydrochloride (Cymevene®)	II, III
rEGF	Recombinant Epidermal Growth Factor protein	I
rVEGF-A	Recombinant Vascular Endothelial Growth Factor protein	I
TMZ	Temozolomide (Temodal®)	I, II
VPA	Valproic acid sodium salt (Deprakine®)	I, II
Isoflurane	Inhalation anesthetic with N_2O/O_2 carrier	I, II, III
Ketamine	Ketalar®, injection anesthetic	I, II, III
Medetomidine	Domitor®, injection anesthetic	I, II, III
Atipamezole	Antisedan®, antisedative	I, II, III
Carprofen	Rimadyl®, analgesic	I, II, III
Oftagel	Moisturizing eye drops	I, II, III

Table 7. qPCR assays and antibodies

Product	**Description, Source**	**Original publications**
Hs00178494_m1	qPCR assay for human Src, Applied Biosystems	I
4333764F	qPCR assay for human GAPDH, Applied Biosystems	I
β-actin	Primary ab, Abcam (#ab8227)	I
CD34	Primary ab, HyCult Biotechnology (#HM1015)	I
Ki-67	Primary ab, Dako (#M7240)	I
LYVE-1	Primary ab, ReliaTech (#103-PA50AG)	I
MMP-2	Primary ab, R&D Systems Inc.	I
Muscle actin	Primary ab, Enzo Life Sciences (#ENZ-C34931)	I
MxA	Primary ab, A kind gift from Prof. Julkunen	I
Src	Primary ab, Cell Signalling Technology (#2108)	I
Goat anti-mouse IgG-HRP	Secondary ab, Thermo Fisher Scientific (#PI-31430)	I
Goat anti-rabbit IgG-HRP	Secondary ab, Thermo Fisher Scientific (#PI-31460)	I
Connexin 43	Primary ab, Sigma Aldrich (#C6219)	II
Biotinylated goat anti-rabbit	Secondary ab, Vector Laboratories (#BA-1000)	II
F4/80	Primary ab, AbD Serotec (#MCA497R)	III
Biotinylated rabbit anti-rat	Secondary ab, Vector Laboratories (#BA-4000)	III

ab, antibody; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HRP, Horseradish peroxidase; MMP-2, Matrix metalloproteinase 2

5 Results and Discussion

In the following sections, the results of each study (I-III) will be briefly summarized and some of them illustrated by figures followed by discussion. More detailed results and further discussion are provided in the corresponding original publications.

5.1 SRC INHIBITION IN MG (I)

5.1.1 Src shRNAs mediate efficient inhibition *in vitro*

The central role of Src in tumorigenesis has been acknowledged in studies using Src-deficient mice as well as its pharmacological inhibitors (Criscuoli, Nguyen & Eliceiri 2005, Lund et al. 2006, Weis et al. 2004). It was decided to study the role of specific Src inhibition on MG growth and the potential of this inhibition as a therapeutic approach. Local tumor delivery was used to avoid systemic side effects and to evaluate the responses within the tumor tissue. GBM is one of the most angiogenic cancers and Src is important not only for the tumor cells but also in the endothelial cells lining the vessels (Hanahan, Weinberg 2011, Werdich, Penn 2005). Therefore, preliminary *in vitro* testing was conducted in HUVECs. The LV delivery of shRNAs against Src was shown to inhibit the corresponding mRNA and protein levels up to 90 % in comparison to nontransduced cells (Figure 8). The functionality was further proven by inhibition of downstream signaling demonstrated by reduced MMP-2 known to be important for Src-mediated invasion and metastasis (I). Src inhibition was also shown to decrease VEGF-A-mediated cell viability as well as to reduce tubulogenesis mimicking angiogenesis *in vitro* (I). Altogether, the Src shRNAs used in this study exhibited high efficacy in several *in vitro* measurements.

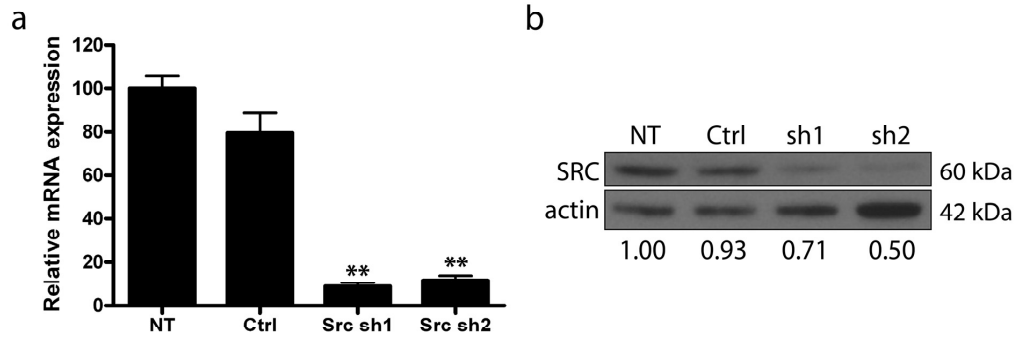

Figure 8. Functionality of shRNAs *in vitro*. (a) The extent of Src inhibition in HUVECs was measured at the mRNA level by quantitative real-time RT-PCR. Target gene expression was normalized to GAPDH mRNA expression. **P<0.01 vs. nontransduced cells. (b) Inhibitory effects of shRNAs against Src kinase were analysed also on protein level by Western blotting. β-actin was used as a control for sample loading. Quantifications of the Western blots are shown below each lane. NT, nontransduced; Ctrl, transduced with a control vector expressing shRNA against luciferase; sh1, shRNA sequence 1 against Src; sh2, shRNA sequence 2 against Src. Error bars = SEM.

5.1.2 Effect of transduction efficiency on *in vivo* tumor growth and survival

The preliminary *in vivo* efficacy assessment of Src shRNAs was conducted in nude mice in subcutaneous tumor xenografts to allow convenient follow-up. Those tumors having only 10 % or even 50 % of cells *ex vivo* transduced with Src shRNAs did not show reduced growth whereas tumors with almost 100 % of the cells being transduced exhibited a marked growth reduction, being almost 50-times smaller than the control vector-transduced tumors (Figure 9a). The maintenance of transduction over 6 weeks follow-up was confirmed post-sacrifice (I). The tumors having ~100 % cells transduced with Src shRNAs demonstrated reduced Src protein levels (I) and fewer capillaries (Figure 9b), whereas the proliferation indexes were not significantly different (I).

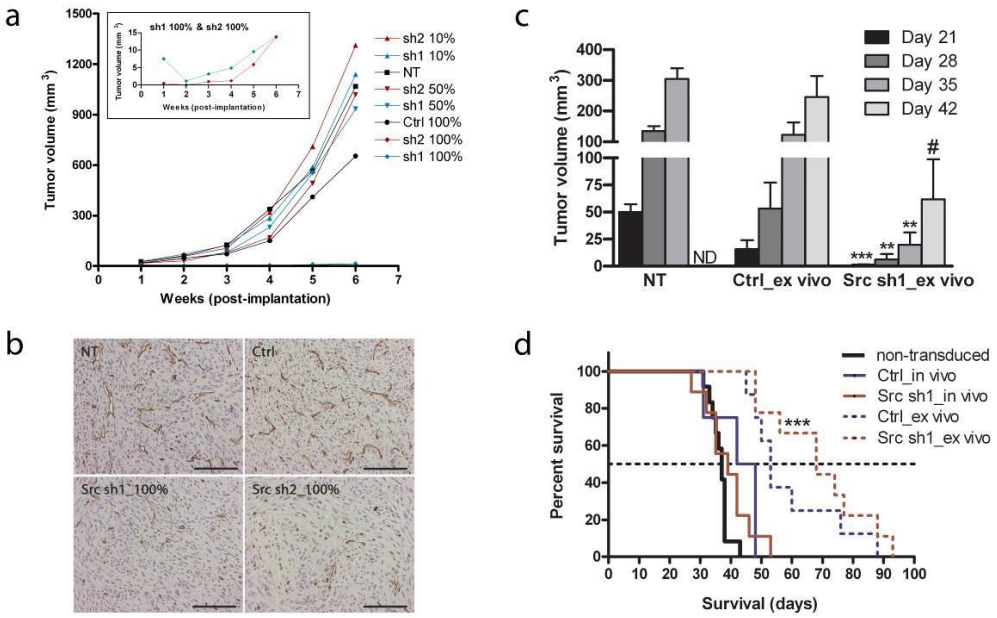

Figure 9. Functionality of shRNAs *in vivo* in mouse subcutaneous and rat intracranial glioma models. (a) Tumor growth was measured during a 6 week follow-up period in nude mice glioma xenografts. U118MG glioma cells were transduced with lentiviral vectors encoding shRNAs against Src and luciferase as a control. Different proportions of transduced cells were implanted subcutaneously into the flanks of nude mice and tumor growth was measured weekly. The insert shows tumor growth of sh1 100 % and sh2 100 % groups over the same follow-up period with a more detailed scale on y axis. P<0.05 Ctrl versus sh1/sh2 100 %. (b) CD34 capillary immunostaining from mouse tumors, 200x magnification with 100 µm scale. (c) Rat intracranial MG *ex vivo* tumor volumes measured by MRI at post-inoculation follow-up. **P<0.01, ***P<0.001 versus nontransduced tumors, #P<0.05 versus control vector-transduced tumors. ND = not detected (no survivors remaining). (d) Rat MG survival proportions in days after tumor inoculation, ***P<Src sh1_*ex vivo* versus nontransduced. Ctrl, transduced with a control vector expressing shRNA against luciferase; NT, nontransduced; sh1/sh2, shRNA sequence 1 or 2 against Src. Error bars = SEM.

The efficacy of Src shRNA1 construct was further studied in a rat orthotopic MG model. *Ex vivo* Src shRNA1 transduced tumors were significantly smaller than nontransduced or control vector-transduced tumors conferring a survival benefit on these rats (Figure 9c-d).

However, with *in vivo* gene transfers, there were no differences between Src-inhibited versus controls in either tumor volumes or survival (I). *In vivo* treatment with Src shRNA1 was further combined with two drugs already in clinical use, VPA and TMZ, to improve the treatment efficacy. This combination could reduce the tumor volume to 59 % of Src shRNA1 tumors only (I). However, the differences in tumor volumes or survival of rats with the combination treatment were not statistically significant (I).

Src inhibitors have displayed contradictory findings in terms of proliferation inhibition, but have been claimed to be beneficial in inhibition of invasion and metastasis (Brunton, Frame 2008, Criscuoli, Nguyen & Eliceiri 2005, Lund et al. 2006). Since Src is located in the immediate vicinity of several growth factor receptors, it has a wide and complex downstream signaling network (Figure 7). Therefore, its inhibition is likely to affect several cellular responses; this may be an advantage if one wishes to restrict a broad spectrum of cellular actions as often is the case in cancer treatment. However, the same broad inhibition can cause systemic side effects as well as creating a selection pressure towards other signaling pathways (Rich, Bigner 2004, Sathornsumetee et al. 2007).

The importance of transduction efficiency for therapeutic success was proposed already several years ago (Pulkkanen, Yla-Herttuala 2005) and is still one of the major challenges of gene therapy. In the present study, Src shRNAs demonstrated efficient *in vitro* inhibition as well as marked tumor growth restriction in nude mice only when almost 100 % of tumor cells had been transduced. Based on the results, it can be concluded that level of Src inhibition needs to reach over 50 % for sustained growth inhibition.

Another interesting aspect is the role of immune response for the treatment response. Tumors in nude mice retained their high GFP positivity as a mark of high transduction efficiency throughout the follow-up. However, in the immunocompetent rats used in the intracranial MG model, the GFP-positivity of *ex vivo* transduced tumor cells had decreased from ~94 % down to 9.7 % in 20 days. *In vivo* gene transfer efficacy was 6.0 % at 5 days post-transduction. Similar to the results obtained in the nude mice experiment, it is therefore unlikely that these transduction efficiencies could achieve a significant treatment response with a single gene therapy treatment. There could be several reasons for decreased transduction efficiency but these remained outwith the scope of this study. However, one possible explanation is the activity of the immune system towards the gene therapy vector. Although LV is known to be weakly immunogenic, the construct includes green fluorescent protein (GFP) known to cause immune reactions (Stripecke et al. 1999). In addition, the role of an activated interferon response cannot be ruled out based on a single target, namely MxA, analysis. Another explanation for the overgrowth of nontransduced cells could be the saturation of cellular RNAi machinery in transduced cells processing both miRNAs and vector-delivered shRNAs (Khan et al. 2009).

Src inhibitors have been suggested for combination therapies (Brunton, Frame 2008). In this study, a trend towards a beneficial treatment response was detected on tumor volumes with combination of Src shRNA and TMZ + VPA, however this was not statistically significant. This further emphasizes the importance of careful optimization of each individual treatment and their combinations for a maximal treatment response.

5.2 HSV-TK/GCV+TMZ+VPA COMBINATION TREATMENT FOR MG (II)

5.2.1 VPA enhances HSV-TK/GCV+TMZ treatment in *vitro*

VPA has a long history as an anticonvulsant and mood-stabilizer, but more recently it has been utilized for gene therapy as a HDI (Bolden, Peart & Johnstone 2006, Chateauvieux et al. 2010). VPA has been shown to enhance transduction efficiency and upregulate transgene expression (Fan et al. 2005, Segura-Pacheco et al. 2007) as well as sensitizing MG cells to chemotherapeutic agents like TMZ (Ryu et al. 2012). It was therefore combined with already clinically evaluated HSV-TK/GCV gene therapy and the first-line MG chemotherapeutic, TMZ. This present study investigated whether VPA could enhance the treatment efficacy of TK+GCV+TMZ combination and further optimize this combination therapy. VPA was shown to enhance the cytotoxicity of TK+GCV and TMZ both alone and in combination in a dose-dependent manner (Figure 10a). It enhanced AV transduction efficiency (Figure 10b), relocated Cx43 protein to cell junctions (II) and enhanced the bystander effect (II).

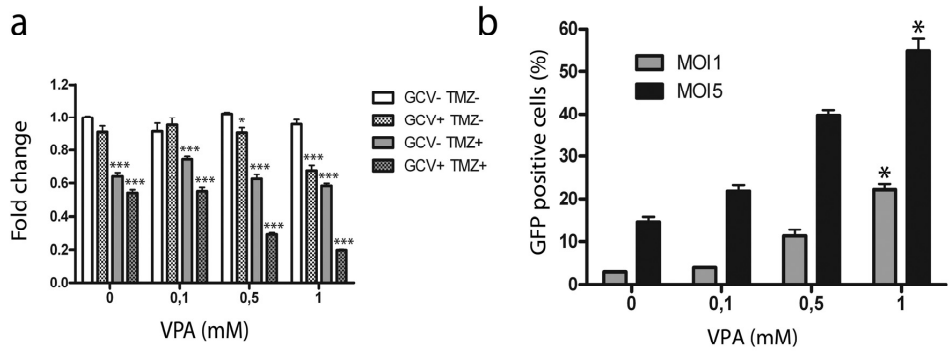

Figure 10. Effect of VPA on glioma cell viability and adenoviral transduction efficiency. (a) Cell viability of ganciclovir-, temozolomide- and/or valproate-treated BT4C cells was measured using the MTS assay in Ad.HSV-TK MOI 5 transduced cells. Significances are shown compared with the GCV-TMZ- group. (b) Transduction efficacy (percentage of GFP-positive cells) measured by FACS. BT4C cells were transduced with MOI 1 or 5 and treated with increasing concentrations of VPA. Significances are given compared with no VPA with corresponding MOI. GFP, green fluorescent protein; MOI, multiplicity of infection; GCV, ganciclovir; TMZ, temozolomide; VPA, sodium valproate; ±, administered/not administered; *P<0.05 and ***P<0.001. Error bars = SEM.

5.2.2 No additional treatment benefit with VPA on HSV-TK/GCV+TMZ *in vivo*

For *in vivo* testing, route of delivery and dosing of VPA were optimized ending up with subcutaneous dosage of 200 mg/kg twice a day (II). VPA, given prior to transductions, was shown to enhance the *in vivo* transduction efficacy based on beta-galactosidase staining of frozen tumor sections. Nevertheless, VPA failed to reduce tumor growth either on its own or in combination with TK+GCV, TMZ or combination of these compounds (Figure 11a,c, II). However, adding VPA to TK+GCV with or without TMZ was able to enhance survival of rats significantly compared to control group (Figure 11b). Furthermore, TK+GCV+TMZ treated rats had significantly (33 %) increased survival in comparison to TK+GCV+VPA

treatment and still 14 % increased survival compared to full combination of TK+GCV+TMZ+VPA, although this was not statistically significant (II). TMZ and VPA were both shown to cause thrombocytopenia and TMZ also leukocytopenia (II) being in line with observations made in patients.

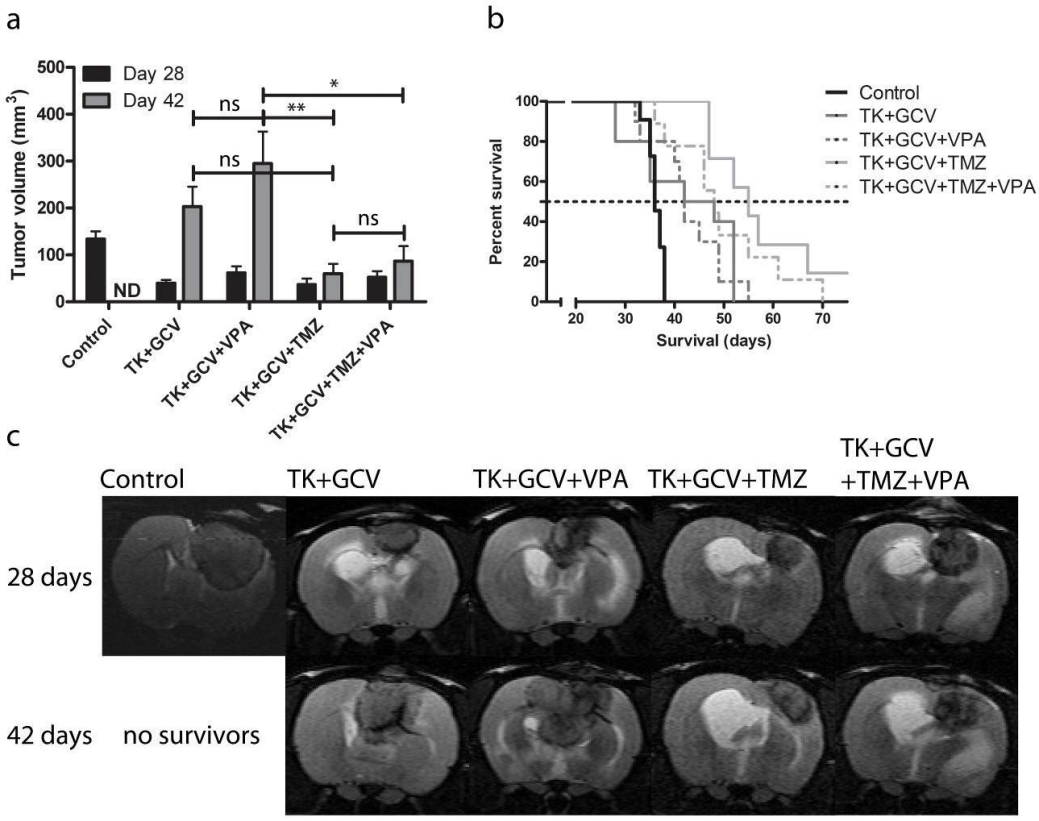

Figure 11. Effect of VPA on tumor volumes and survival of rats with MG. (a) Tumor volumes in magnetic resonance imaging (MRI) at day 28 and 42 post-inoculation for the main study groups. Significances at day 42 are indicated. (b) Survival proportions of the same study groups in days post-inoculation. (c) Representative MRI data for the corresponding groups. Control, non-operated rats; TK, Ad.HSV-TK; GCV, ganciclovir; TMZ, temozolomide; VPA, sodium valproate; ND, not detected (no survivors remaining); ns, non-significant. *P<0.05 and **P<0.01. Error bars = SEM.

The treatment protocol for TK+GCV+TMZ used in this study (14-day GCV with the last 5 days combined to TMZ) proved to be superior over a previously used protocol, where TMZ was given separately from GCV (7-day GCV, 5-day gap, 5-day TMZ) resulting in a significant benefit in tumor volumes (Figure 12a) as well as survival (Figure 12b).

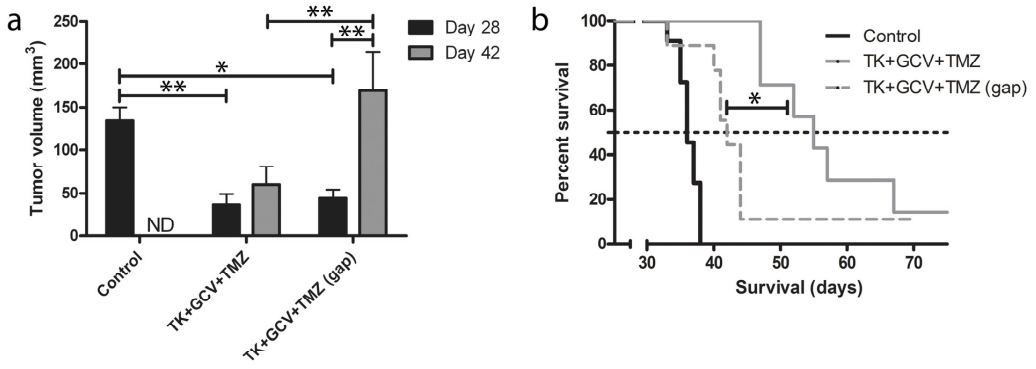

Figure 12. Comparison of treatment protocols. (a) Tumor volumes in magnetic resonance imaging (MRI) at day 28 and 42 post-inoculation for the control group and TK+GCV+TMZ treatment groups with two separate protocols. (b) Survival proportions of the corresponding groups in days post-inoculation. The TK+GCV+TMZ group was treated with GCV for 14 days including TMZ on the last 5 days. The TK+GCV+TMZ (gap) group received GCV for 7 days followed by a 5-day gap and then TMZ for the 5 following days. Otherwise treatment protocols were the same. Control, non-operated rats; TK, Ad.HSV-TK; GCV, ganciclovir; TMZ, temozolomide; ND, not detected (no survivors remaining); *P<0.05 and **P<0.01. Error bars = SEM.

It has been claimed that HDIs are useful anticancer compounds as they have been shown to enhance the efficacy of gene therapy, radiotherapy and chemotherapy (Chen et al. 2007, Fan et al. 2005, Van Nifterik et al. 2012). On the other hand, HDIs have demonstrated both anti- and protumorigenic properties and many of their effects are still unknown (Burgess et al. 2001, Gotfryd et al. 2010, Weller et al. 2011). Generally, they have been more efficient in hematological malignancies where the dedifferentiation is a primary carcinogenic event that can be reversed by the epigenetic functions of HDIs. Success in solid tumors has not been so uniform (Wagner et al. 2010), and no general correlation has been reported between VPA and survival of MG patients (Tsai et al. 2012, van Breemen et al. 2009).

VPA has been shown to impair the expression of Mgmt DNA repair gene sensitizing MG cells to TMZ (Ryu et al. 2012). This mechanism could at least partially explain the enhanced treatment effect observed with the combination of VPA and TMZ *in vitro*. VPA has also been shown to increase CAR receptor expression and enhance AV-mediated gene expression, which could explain the enhanced transduction efficiency *in vitro*. Cx43 is a gap-junctional protein and therefore its relocation to cell junctions could enhance the bystander effect through increased gap junctional communication of GCV metabolites (Asklund et al. 2003, Asklund et al. 2004). The level of VPA stays more constant *in vitro*, providing longer and more stable exposure and therefore better foundation for efficient treatment responses, even when using physiological drug concentrations.

Modeling of the VPA responses *in vivo* has additional challenges such as reaching the target cells with adequate concentrations and exposure time. Rodents have been shown to eliminate VPA 10 times faster than humans (Chateauvieux et al. 2010, Stout et al. 2001), and therefore, twice a day dosing may not have been frequent enough to maintain the therapeutically effective concentrations although single value measurements were at a desirable level. Other factors being responsible for the weak *in vivo* treatment response

could have been the lower transduction efficiency compared to that observed in *in vitro* experiments and unknown, possibly even protumorigenic responses originating from the tumor microenvironment not present *in vitro*.

Despite the encouraging *in vitro* results, VPA was not able to confer further benefits on the TK+GCV+TMZ treatment combination *in vivo*. Nevertheless, the TK+GCV+TMZ combination demonstrated clearly, for the first time, an *in vivo* survival benefit in comparison to single treatments. The protocol for TK+GCV+TMZ used in this study proved to be superior over its previous version with shorter GCV administration and non-overlapping TMZ dosing. The encouraging results obtained with the current protocol (14-day GCV with the last 5 days combined to TMZ) could be the basis for evaluating the effects of simultaneous GCV and TMZ dosing in MG patients.

5.3 ToTK/AZT – AN ALTERNATIVE SUICIDE GENE THERAPY FOR MG (III)

5.3.1 Substrate specificity and efficiency of suicide gene therapies *in vitro*

HSV-TK/GCV has been the most studied suicide gene therapy (Fillat et al. 2003, Maatta et al. 2009). While it has proven to be safe, the lipophobicity of GCV and relatively low enzymatic activity of HSV-TK hamper its efficacy (Khan et al. 2010). A novel suicide gene therapy based on ToTK/AZT has demonstrated high enzymatic activity and substrate specificity, as well as the beneficial lipophilicity of AZT would be considered to improve drug BBB penetration (Denny 2003, Khan et al. 2010, Mutahir et al. 2011). This study investigated whether ToTK/AZT could be used as an alternative to HSV-TK/GCV suicide gene therapy. *In vitro* both enzymes, HSV-TK and ToTK, were shown to prefer their own substrates, GCV and AZT respectively, in a concentration dependent manner in U87MG (Figure 13a-b) as well as in U118MG human MG cells (III). A similar concentration dependent viability reduction was also seen in murine glioma cells (BT4C and GL261) for HSV-TK/GCV (Figure 13d, III) whereas ToTK showed no viability reduction with AZT (Figure 13c, III). The highest concentrations of AZT and GCV were included as toxicity controls in viability experiments. In the kinase activity measurements, ToTK exhibited the high activity towards both deoxythymidine (dThd) and AZT in comparison to the minimal activity seen with HSV-TK in U87MG. In BT4C rat glioma cells, no notable activity was detected with either of the enzymes towards AZT or dThd (Figure 13e, III).

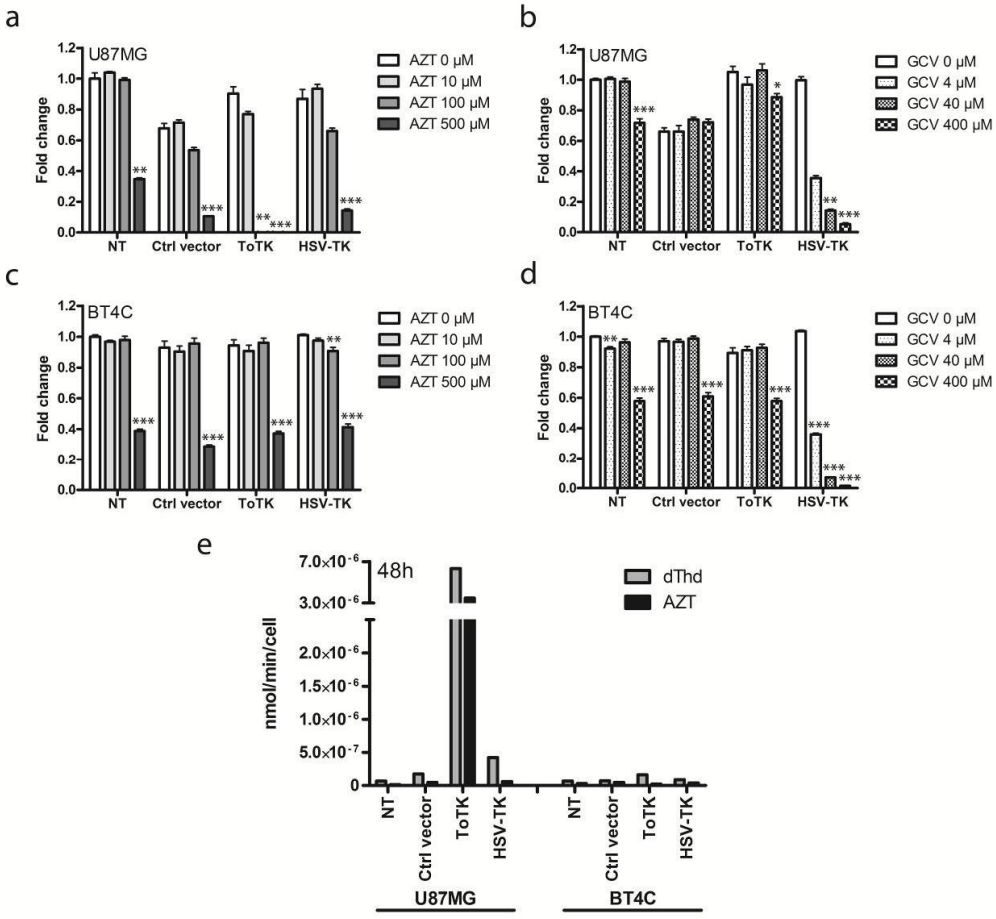

Figure 13. Functionality of suicide gene therapies *in vitro***.** Cell viabilities of U87MG human glioma cells after AZT (a) and GCV (b), and BT4C rat glioma cells after AZT (c) and GCV (d) treatments. Cells were either nontransduced, transduced with either control vector or one of the treatment vectors. Cell viability was measured by the MTS assay. Significances compared to similarly transduced cells with no nucleoside analogue are shown. (e) For kinase activity measurement cells were transduced as above and the enzymatic activity towards radiolabelled dThd or AZT was measured at 48 h. Results are expressed in nanomoles of phosphorylated substrate per minute per cell (nmol/min/cell). NT, nontransduced; Ctrl vector, transduced with control vector containing no insert; ToTK, transduced with vector containing tomato thymidine kinase; HSV-TK, transduced with vector containing Herpes Simplex virus-1 thymidine kinase; AZT, azidothymidine; GCV, ganciclovir; dThd, deoxythymidine. *P<0.05; **P<0.01; ***P<0.001. Error bars = SEM.

5.3.2 No significant differences between suicide gene therapies *in vivo*

A preliminary *in vivo* experiment was conducted to establish the mouse model, to set-up the MRI monitoring and to determine the AZT concentration. The efficacies of suicide gene therapies were then evaluated in nude mice intracranial glioma model by tumor growth measurements and survival follow-up. The HSV-TK/GCV-treated tumors were the smallest and grew the least according to consecutive MRI measurements (Figure 14a). A similar trend was also seen with ToTK/AZT tumors. However, no statistically significant

differences among groups were detected in tumor volumes (Figure 14a). The survival of ToTK/AZT-treated mice was significantly (*P<0.05) longer than in the group of control mice (Figure 14b). Survival of HSV-TK/GCV-treated mice was not significantly increased, but half of the mice were still alive with detectable tumors at the end of the follow-up at day 70 post-inoculation. No significant differences were detected between the ToTK/AZT and HSV-TK/GCV groups (Figure 14b). Macrophages were abundant in almost all tumors with no clear correlation with the tumor size, the mode of the treatment or the survival time.

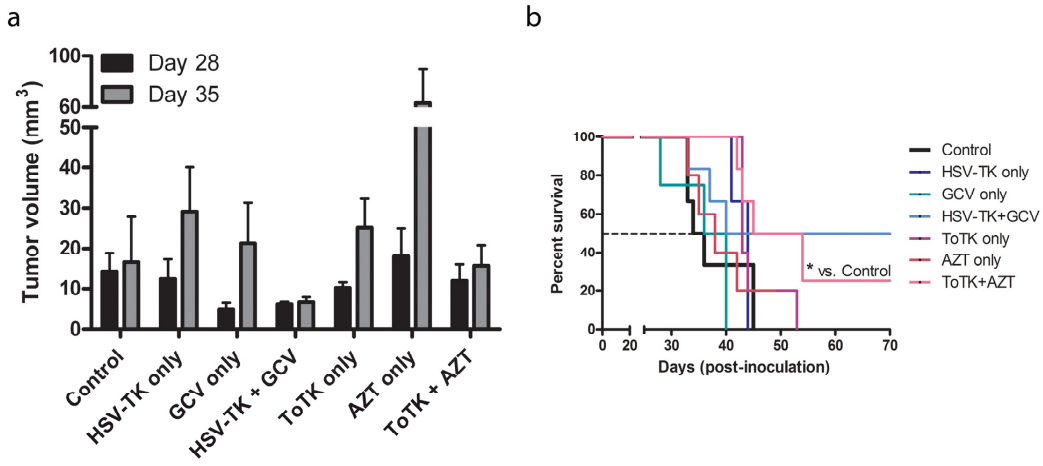

Figure 14. Functionality of suicide gene therapies *in vivo*. (a) Tumor volumes measured by MRI at day 28 and 35 post-inoculation given in mm^3 for different treatment groups. (b) Mice survival proportions in days post-inoculation for the corresponding groups. Significance (*P<0.05) of ToTK+AZT compared with Control is shown. Control, mice with no gene transfer or medication; HSV-TK, Herpes Simplex virus-1 thymidine kinase; GCV, ganciclovir; ToTK, tomato thymidine kinase; AZT, azidothymidine. Error bars = SEM.

The HSV-TK/GCV suicide gene system has been used for over two decades and improvements have been made in order to enhance HSV-TK enzymatic activity towards GCV. Splice-corrected versions and mutated variants have made it possible for less immunosuppressive doses of GCV to be used (Black, Kokoris & Sabo 2001, Chalmers et al. 2001, Preuss et al. 2011). On the contrary, the potential benefits of tomato thymidine kinase have been recognized only recently and further functional refinements still lie ahead. In this study "native" enzymes were compared, both expressed from an identical AV vector. HSV-TK and ToTK, were shown to prefer their own prodrugs, GCV and AZT respectively, with only marginal activity towards the other prodrug. Therefore, although impeding the comparison, different prodrugs were used for both enzymes in order to achieve maximal functionality.

Both HSV-TK and ToTK were shown to reduce cell viability with their preferred prodrugs in human MG cells. However, only HSV-TK demonstrated the same activity in murine MG cells. This was confirmed not only in the cell viability assessment but also in kinase activity measurements where ToTK displayed no activity in rat glioma cells. The explanation for this difference is so far unknown since it has previously been shown that

murine MG cells are readily transduced with AVs (Stedt et al. 2013) and functionality of ToTK construct was proven in human MG cells. As a result, for the *in vivo* study, human MG cells were chosen and this meant that an immunocompromised rodent model had to be used. Although the orthotopic location made it possible to study the tumors in their natural environment, the role of the intact immune system on tumor growth and treatments could not be examined. In addition, possible interactions with tumor microenvironment of mouse origin were likely influenced.

In vivo both of the suicide gene therapies exhibited the least tumor growth and the mice showed a trend towards increased survival. However, only the survival of ToTK/AZT-treated mice was statistically significantly longer than in control mice, with no statistical differences being detected between the suicide gene therapies. There were no differences in macrophage immunostainings in terms of survival. One of the reasons for the modest *in vivo* treatment response could have been the limited transduction efficacy compared to the *in vitro* situation. These constructs did not contain a marker gene and transduction efficacy was not separately studied in the current experiment. However, this is a generally known phenomenon which was also encountered in the first study of this thesis (Stedt et al. 2012). Furthermore, the small number of mice per group (n = 5-7) could have reduced the power to detect statistically significant differences.

Another aspect which needs to be considered is the concentration of nucleoside analogues. The concentrations used in this study were chosen based on the previous experience with GCV (Leinonen et al. 2012, Sandmair et al. 2000b, Tyynela et al. 2002) and the published literature with AZT (Danesi et al. 1998, Dobrovolsky et al. 2005, Sato et al. 2007). However, although a preliminary experiment was carried out to determine a suitable *in vivo* concentration for AZT, differences in experimental conditions in literature such as the mouse strain may have affected the treatment outcome. It is known that the most common adverse effects with GCV are neutropenia and thrombocytopenia (Denny 2003, Faulds, Heel 1990). AZT has been well tolerated with low concentrations but high concentrations have been shown to exert toxic effects e.g. hematotoxicity and hepatotoxicity (Sato et al. 2007, Danesi et al. 1998). Therefore, full blood counts as well as liver and kidney functions were analysed. No adverse effects were detected with the administered treatments.

In this third study, neither of the treatments, HSV-TK/GCV or ToTK/AZT, demonstrated clear superiority over the other. Both were found to be efficient *in vitro* while no significant differences in *in vivo* efficacy were detected during the follow-up period. Therefore, it can be concluded that ToTK/AZT is a potential alternative for HSV-TK/GCV treatment but further comparative studies with increased animal numbers will be needed before more in depth conclusions can be made.

6 Summary and conclusions

The following conclusions can be made based on the individual studies (I-III) of this thesis:

I Src is a key player in tumorigenesis and a potential target for RNA interference–mediated treatment of MG. Due to the limited gene transfer efficacy *in vivo* and finite possibilities associated with single-target treatments, Src shRNAs are proposed to be used as part of combination therapies.

II Despite promising *in vitro* results, VPA did not further enhance the therapeutic effect of the HSV-TK/GCV+TMZ combination *in vivo*. The current protocol of administering TMZ simultaneously with the last five days of 14-day GCV treatment following HSV-TK gene transfer restricted the tumor growth and improved rat survival. This finding may be of potential clinical value for MG patients.

III ToTK/AZT and HSV-TK/GCV were shown to be efficient in human MG cells, and the latter combination also in rodent MG cells. There was no significant difference in the *in vivo* treatment efficacy observed between the suicide gene therapies. ToTK/AZT is a potential alternative for HSV-TK/GCV due to its beneficial therapeutic properties.

Traditional treatments of MG, surgery, radiotherapy and chemotherapy, are merely palliative for GBM. Due to the infiltrative growth pattern of GBM, complete surgical resection is practically impossible. Brain tumors are often resistant to radiotherapy and the maximal dose for brain is 60 Gy, higher doses increase the risk of adverse effects without confering any further therapeutic benefit. In addition to the toxicity of chemotherapy itself, the BBB represents an obstacle to achieve sufficient concentrations within the treatable area. For these reasons, alternative strategies including gene therapy are needed.

This thesis has explored alternative strategies as well as the combinations discussed above. The different agents in combination therapies can exert different modes of action as well as targeting different cellular functions. In addition, adverse effects may be reduced as lower drug concentrations or doses of each individual drug can be administered. On the other hand, one should keep in mind that while increasing the number of different therapies, the optimization of their administration protocols becomes more challenging and there is an increasing possibility of unwanted interactions. Therefore, a careful preclinical assessment of individual therapies and their combinations is a prerequisite before these protocols can enter into clinical practice.

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Hanna Stedt

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Alternative Strategies and Combination Therapies

Malignant glioma (MG) is a cancer with a dismal prognosis. Novel gene therapy strategies and their combinations with two clinically used drugs, temozolomide and valproate, were studied in preclinical *in vitro* and *in vivo* MG models. Src kinase was shown to be a key therapeutic target in gliomagenesis, and ToTK/AZT an alternative suicide gene therapy to HSV-TK/GCV. It is hoped that the new enhanced treatment schedule for HSV-TK/GCV with temozolomide will be of potential value for MG patients.

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