“Patient-individual models of gliomas: establishment, characterization and applications”

Dissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

Christina Susanne Mullins

aus Rostock

Rostock, 27.02.2013

urn:nbn:de:gbv:28-diss2013-0132-4

Gutachter

1. Gutachter: Prof. Dr. Schröder
Institut für Biowissenschaften
Abt. Genetik

Albert-Einstein-Str. 3
D-18059 Rostock

2. Gutachter: PD Dr. Linnebacher
AG Molekulare Onkologie und Immuntherapie
Abteilung für Allg., Thorax-, Gefäß- und Transplantationschirurgie

Universitätsmedizin Rostock
Schillingallee 69
D-18057 Rostock

3. Gutachter: Prof. Dr. Van Gool
UZ Leuven, Kindergeneeskunde
Campus Gasthuisberg

Herestraat 49
B-3000 Leuven

Verteidigung

Verteidigt am 28.06.2013

Table of content

1. List of Abbreviations

bFGF	basic fibroblast growth factor
B-Raf	v-raf murine sarcoma viral oncogene homolog B1
BTSC	brain tumor stem cells
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CGH	comparative genomic hybridization
CIN	chromosomal instability
CMV	cytomegalovirus
CSC	cancer stem cells
DC	dendritic cells
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
EGFRvIII	epithelial growth factor receptor variant III
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
HER2/neu	human epidermal growth factor receptor 2
HIP 1	huntingtin interacting protein 1
HLA	human leukocyte antigen
IDH	isocitrate dehydrogenase
IDO	indoleamine-2,3-dioxygenase
IFNγ	interferon gamma
IL	interleukin
IL-13Rα	interleukin-13 receptor alpha
K-Ras	Kirsten rat sarcoma viral oncogene homolog
MGMT	O-6-methylguanine-DNA methyltransferase
MMP	metallopeptidase
MMR	mismatch repair
MRI	magnetic resonance imaging

MSI	microsatellite instability
mTOR	mammalian target of rapamycin
NF1	neurofibromin 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFβR	platelet-derived growth factor beta receptor
PTEN	phosphatase and tensin homolog
RB	retinoblastoma
RGD	R=arginine, G= glycine, D=aspartic acid
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
T reg	regulatory T cells
TAA	tumor associated antigen
TBP	TATA box binding protein
TGF-β	transforming growth factor-beta
TMZ	Temozolomide
TNFα	tumor necrosis factor alpha
TP53	tumor protein p53
TSA	tumor specific antigen
VEGF	vascular endothelial growth factor
WHO	world health organization

2. Introduction

2.1 Glioblastoma multiforme

2.1.1 Definition

Glioblastoma multiforme (GBM) is defined as a “malignant, invasive, rapidly growing pulpy or cystic tumor of the cerebrum (or the spinal cord). The lesion spreads with pseudopod-like projections. It is composed of a mixture of monocytes, pyriform cells, immature and mature astrocytes, and neural ectodermal cells with fibrous or protoplasmic processes; also called anaplastic astrocytoma or glioma multiforme” [Mosby's Medical Dictionary, 8th edition 2009, Elsevier].

The world health organization (WHO) classifies tumors of the central nervous system according to their histology, morphology and malignancy into four grades I - IV[1]. Grade I is assigned to low proliferative lesions which may be cured by surgical resection alone. Neoplasms with low-level proliferative activity but a generally infiltrative nature are designated grade II; in these cases recurrence is frequent. WHO grade III is generally reserved for lesions with histological evidence of malignancy, including nuclear atypia and brisk mitotic activity. The designation grade IV is assigned to cytologically malignant, mitotically (highly) active, necrosis-prone neoplasms with typically rapid pre- and postoperative disease progression and a fatal outcome[2,3]. Although therapeutically not relevant these tumors are divided into primary (arise *de novo* as grade IV tumors) and secondary (progress from low grade to grade IV tumors) GBM[4].

2.1.2 Epidemiology

GBM is the most common malignancy of the brain in adults; it accounts for 12 - 15% of all brain tumors [Pschyrembel] and makes up for half of the gliomas[5]. The yearly incidence is 7 newly diagnosed cases per 100 000 adults and only 0.1 per 100 000 in children[6]. Although the disease may be present at any age, the incidence of primary GBM peaks at 50 - 70 years [Pschyrembel], with a median age of 64 years at diagnosis[6]. In contrast, secondary GBM, which develop from relapses of lower-grade precursor malignancies, more frequently appear in younger patients at a median age of 45 years[7].

There is a slight preponderance of GBM in male, with a male to female ratio of 1.5 to 1[8]. Interestingly the incidence is twice as high in European descendants as

compared to African American or Asian descendants[9]. These types of studies (comparing large ethnic populations under the “same” living conditions) are only feasible in larger populations where different ethnicities are domiciled, e.g. the United States.

2.1.3 Etiology

Although most GBM appear to be sporadic, several genetic disorders are associated with an increased incidence: tuberous sclerosis, neurofibromatosis 1 and 2, von Hippel Lindau disease, Turcot and Li-Fraumeni syndrome[8] [Pschyrembel]. The only well-established risk factors for GBM are exposure to ionizing radiation and a genetic predisposition[9]. More recently, the contribution of several common low-penetrance susceptibility alleles to the development of gliomas was discovered[10,11]. In contrast to many other tumor entities, no connection between GBM and smoking, a particular diet or the use of mobile phones could be made[8,12]. Only very inconclusive evidence for a potential association with occupational risk factors (such as working as physician, fire fighter or farmer), the exposure to electromagnetic fields and brain traumas was obtained[9].

2.1.4 Clinical presentation and diagnosis

The initial clinical presentation is highly variable and depends primarily on the localization (cerebrum, frontal lobe, corpus callosum) and size of the tumor[5]. Very occasionally, a tumor is asymptomatic until it reaches an enormous size[8]. Common symptoms include neurological symptoms (aphasia, paresthesia, hemiparesis and visual as well as sensory disturbances), mood and personality changes, seizures or symptoms of increased intracranial pressure such as nausea, vomiting or headache[5,8]. For diagnosis, magnetic resonance imaging (MRI) is the imaging technique of first choice. In selected cases positron-emission tomography or advanced MRI modalities (e.g. perfusion imaging, diffusion imaging magnetic resonance spectroscopy) may be useful (e.g. for selection of biopsy targets or differentiation of recurrent tumor from treatment-related changes)[5,8].

2.1.5 Prognosis

The prognosis for patients presenting with a GBM is dismal, given the fact that up to date no curative therapy could be established[5,13]. The two year survival rate is 10%

when irradiation is the sole treatment; for combined radio-chemotherapy with Temozolomide (TMZ) the two year survival rate can be augmented to 14% for patients with an active (unmethylated) O-6-methylguanine-deoxyribonucleic acid (DNA) methyltransferase (MGMT) promoter or even to 46% for patients with inactivation (methylation) of this promoter[14,15].

Despite aggressive irradiation and chemo-treatment, the median overall survival remains low: 15 months for newly-diagnosed GBM and 5-7 months for recurrent or relapsed GBM[13].

2.1.6 Pathobiology and molecular biomarkers

Cell of origin

For the most part, it remains unclear what initiates gliomagenesis and in which cell type malignant transformation is initiated. Several studies, especially those using murine models, addressed this question and revealed that any cell in the hierarchy with proliferative capacity can serve as cell of origin[16]. Hence, not only neuronal stem cells and the eponymous (glia) cells of the disease but also mature neurons, astrocytes and oligodendrocyte precursors may initiate GBM tumors[16-18].

Cancer stem cells (brain tumor stem cells)

Most cancers comprise a heterogeneous population of cells with different proliferative potential[19]. There is increasing evidence that the tumor bulk mass contains a population of cells with stem-like characteristics, so called cancer stem cells (CSC). These CSC are defined as cells with the ability for self-renewal, extensive proliferative capacity, the potential for multilineage differentiation and tumor initiation[3,20,21]. CSC are not only responsible for tumor maintenance but are also thought to be the key players in recurrence and therapy resistance[22].

Tumor entities recently described as having CSC populations include malignancies of the hematological system, breast, brain, pancreas, neck, prostate and colon[23-29]. In glioblastoma, brain tumor stem cells (BTSC), also often referred to as glioma-initiating or glioma-propagating cells, are thought to represent a small subpopulation of cells giving rise to all types of GBM cells and seem exquisitely resistant to conventional therapeutic interventions[3,20,30]. The lack of robust markers allowing the identification of BTSC is an obstacle in the development of specific treatments. Frequently proposed cell surface markers for BTSC (alone or in combination) include CD133, CD15, Nestin, CD34 and CD44[22,30-32].

Hallmarks of GBM tumors (angiogenesis, invasion and heterogeneity)

The dismal prognosis of GBM is largely due to a highly invasive phenotype, the extensive (neo-) angiogenesis and great heterogeneity of these tumors. These hallmarks have very frequently been analyzed and described by the scientific community[1,3,33-36]. Angiogenesis facilitates tumor progression[35]. Therapy failure often is pinned to the heterogeneous composition of the tumors[22]. Finally, extensive invasion into the surrounding brain tissue regularly accounts for recurrence or relapse of the tumors[3]. In contrast to most other malignancies, GBM tumors very rarely metastasize[37].

Mutations

Common mutations in GBM comprise general tumor mutations (e.g. tumor protein p53 (TP53) and phosphatase and tensin homolog (PTEN)) as well as GBM specific alterations (e.g. epidermal growth factor receptor (EGFR), isocitrate dehydrogenase (IDH) 1 and 2)[1,38,39]. The most frequently mutated genes are TP53 (35-42%), PTEN (24-37%), neurofibromin 1 (NF1) (15-21%), EGFR (14-45%), retinoblastoma (RB) 1 (8-13%), phosphoinositide-3-kinase, regulatory subunit 1 (8-10%), phosphatidyl-inositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (7-10%) and IDH 1 (11-20%)[40-43]. Notably mutations in the genes IDH 1 and 2 are GBM specific mutations; often taken into consideration to distinguish between primary and secondary GBM in especially the most common mutation R132H for IDH 1[4]. Further a GBM specific mutation of the EGFR is the variant III (EGFRvIII). This mutation comprises the deletion of exons 2-7, encoding for the extracellular domain of the receptor and thus leading to a weak but constitutively active receptor signaling[44,45]. The EGFRvIII is frequently present in primary GBM and only scarcely found in secondary GBM[46,47].

Chromosomal changes

Genomic instability is one key characteristic of malignancies[48]. Generally two types are discerned; chromosomal instability (CIN) and microsatellite instability (MSI). MSI is rare in non-hereditary newly diagnosed GBM[49,50]. However, after long term chemotherapy inactivating mutations in mismatch repair (MMR) genes may be observed more frequently and the frequency of MSI in relapsed GBM tumors is increased[49]. Also the loss of the MMR system leads to mutations especially in repetitive sequences, so called microsatellites, and specifically in the alteration of their length[51].

Evolving new techniques have set a market for detailed chromosomal alteration analyses. Popular methods include single nucleotide polymorphism (SNP) arrays and comparative genomic hybridization (CGH), which allow not only detecting copy number alterations but also structural changes[43]. A broad spectrum of copy number alterations and gene abnormalities have been discovered for GBM tumors. Frequently amplified gene loci include 1q32, 1q44, 3q26, 4q12, 7p11-12, 7q21-22, 7q31, 12q13-15 and 12p13; whereas often deleted loci are 1p32-36, 2q21-22, 6q26-27, 9p (complete or at least 21-23), 10p and q, 13q14, 17p13, 17q11 and 19q[43,52]. Loss of heterozygosity or loss of chromosome 10 is the most common genetic alteration in GBM tumors and is associated with poor survival[53]. In contrast the co-deletion of 1p and 19q is associated with a prolonged survival[39].

Epigenetics (methylation)

The most prominent epigenetic marker in GBM tumors is the methylation status of the MGMT promoter. The MGMT gene encodes for a DNA repair protein, which can reverse the DNA-damage of alkylation by chemotherapeutic agents such as TMZ. The methylation status is thus a predictive marker for success of chemotherapy with alkylating agents. A methylated (inactive) promoter correlates with a better response to TMZ, whereas an unmethylated (active) promoter is associated with a weaker response[15]. The methylation status of the MGMT promoter is not only a predictive marker but also has prognostic value. Patients with MGMT promoter methylation in the tumors have a better prognosis[15]; so their clinical outcome is better independent of the treatment they receive.

Beside methylation of the MGMT promoter other gene loci have been described to frequently be hypermethylated in GBM tumors: fms-related tyrosine kinase 3, frizzled family receptor 9, GATA binding protein 6, homeobox A11, homeobox A3, homeobox A5, homeobox A9, 5-hydroxytryptamine (serotonin) receptor 1B, Moloney murine sarcoma viral oncogene homolog, neurofilament light polypeptide Ras association (RalGDS/AF-6) domain family member 1, retinol binding protein 1, slit homolog 2, T-cell acute lymphocytic leukemia 1, transcription factor AP-2 alpha, transcription factor AP-2 beta, transcription factor AP-2 gamma, tumor suppressor candidate 3 and zinc finger protein 215. Other gene loci are often hypomethylated in GBM: chemokine (C-X-C motif) ligand 3, interleukin (IL) 8, matrix metallopeptidase (MMP) 9, protease serine, 1, prostate stem cell antigen, S-100 calcium binding protein A2 and tumor necrosis factor (ligand) superfamily member 10[54-56].

Aberrant signaling pathways

Mutations, chromosomal and epigenetic alterations ultimately lead to altered signaling processes in GBM cells. The main pathways affected hereof are the RB pathway, the p53 pathway, the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha / mitogen activated kinase-like protein pathway, the EGFR pathway, the PTEN / serine/threonine protein kinase pathway and the platelet-derived growth factor (PDGF) pathway[5]. All of these pathways are involved in major cell processes such as cell growth, proliferation, replication and cell cycle control.

Molecular sub-classification

Different approaches have been undertaken to classify and thus better stratify these tumors - i.e. to design optimal treatment strategies. The most prominent and established classification by the WHO differentiates tumors (WHO grade I - IV) according to their histology, morphology and degree of malignancy (as described above). Further, these tumors are divided into primary and secondary GBM[4]. Primary tumors are characterized by amplification and/or mutation of the EGFR, whereas secondary tumors are associated with mutations in the genes IDH 1 and 2[4].

The most recent approach classifies these tumors by the origin of the tumor initiating cell type and specific molecular markers: pro-neural (oligodendrocytic cells), neural (neurons), mesenchymal (astroglia, microglia) and classical (astrocytic cells) GBM[39,40,57]. Especially for the latter classification a variety of molecular characteristics is taken into account; characteristic for the proneural subtype are mutations in the genes TP53 and IDH 1, in the neural subtype no representative mutations have been identified so far, characteristic mutations for the mesenchymal subtype are found in NF1 and PTEN and the classical subtype is associated with EGFR amplification and mutation[39,40,57]. The importance of molecularly defining the tumors is affirmed by the increasingly extensive molecular pathological profiling.

2.1.7 Therapy

Conventional (standard) Therapy

Since the seminal work by Stupp et al. was published in 2005, the standard therapy for patients with newly diagnosed GBM consists of (sub-) total resection followed by radio-chemotherapy. This consists of fractionated focal irradiation that is given e.g. at 2Gy per cycle. The treatment is administered five days per week over a period of six

weeks adding up to a total dose of 60Gy with concomitant TMZ of 75mg/square meter per day from the first to the last day of irradiation but no longer than 49 days. After a four week break adjuvant TMZ treatment consists of a five day schedule every 28 days with a 150-200mg/square meter dose which is given in addition[14]. In case of relapse surgery is repeated in 30% of cases. Alternatively, hypofractionated stereotactic irradiation or chemotherapy is applied; Fotemustine or Nimustine (alkylating substances) are alternative second-line agents after TMZ failure[58-60].

Despite optimal combination therapy, the poor prognosis remains. This enduring dismal prognosis clearly underlines the necessity for new therapeutic interventions. The wide field of immunotherapy represents one major novel approach. In common with another emerging strategy, the so called targeted therapies, is defining (tumor specific) molecular structures for such therapeutic interventions.

Immunotherapy

Tumors develop when the immune system cannot recognize or eliminate malignant cells; many different influences often render a tumor either invisible to the immune system or resistant to its cytolytic functions[61]. The ultimate goal of immunotherapy is to make these malignant cells visible to the immune system and overcome that resistance.

In 2011 Ralph M. Steinman (together with Bruce A. Beutler and Jules A. Hoffmann) was awarded The Nobel Prize in Medicine "For his discovery of the dendritic cell and its role in adaptive immunity" [“Ralph M. Steinman - Biographical" Nobelprize.org.20 Jan2013]. Dendritic cells (DC) are professional antigen presenting cells, capable of stimulating $CD4^+$ and $CD8^+$ T cells[62]. The principle of a DC vaccination is to load the DC with tumor antigen(s), have them present the antigen(s) to T cells and thus stimulate tumor-specific cytotoxic T cells, which can eliminate residual tumor cells in the patients[62]. A common strategy to generate such antigen-specific DC is to isolate monocytes from patient blood, differentiate them with the help of cytokines to immature DC and finally load and mature the DC with a cocktail of cytokines and antigens (in form of peptides, tumor lysate or nucleic acid)[62]. Using tumor lysate as the source of antigens is one very promising approach and already under investigation in clinical trials. The success in patients with recurrent GBM but also in patients with newly diagnosed brain tumor is enormous[63-65]. Tumor-derived ribonucleic acid (RNA) is another successful source of antigens[66]. Finally, a variety of

tumor-antigen derived peptides selected for highest immunogenicity are analyzed in clinical studies as well[67,68].

Cytomegalovirus (CMV) proteins confer both oncogenic and tumor-modulating mechanisms and numerous entities have been described to be CMV positive[69].

Recent reports have demonstrated the presence of CMV also in GBM tumors. CMV may promote the malignant phenotype of GBM cells by enhancing cell invasiveness, activating telomerase and inducing tumor-suppressive monocytes[70,71]. CMV is a common immunological target after conditioning, stem cell transplantation and subsequent CMV reactivation. A direct T cell transfer is only rarely performed in GBM patients, however there are few case reports on autologous *ex vivo* expanded CMV specific or γδ T cells prolonging survival of GBM patients[72].

Targeted therapy

The aim of targeted therapy is defining molecular structures for drug applications. Therapeutic antibodies bind to molecules or receptors that are involved in key signaling processes of tumor cells [73]. The monoclonal antibody Bevacizumab inhibits angiogenesis by depriving the cells of vascular endothelial growth factor (VEGF)[73]. Cetuximab, also a monoclonal antibody, binds to the EGFR and prevents binding of the ligand epidermal growth factor (EGF) to the receptor and disrupts the pathway[73].

Beside monoclonal antibodies, small molecules are capable of interrupting cell signaling. In GBM therapy, tyrosine kinase inhibitors are popular. They can inhibit specific kinases or tyrosine kinases in general. Erlotinib and Gefitinib inhibit the EGFR, Cediranib inhibits VEGF, Sorafinib and Sunitinib inhibit the VEGFR and PDGFR and Imatinib is a general tyrosine kinase inhibitor[74-78]. Enzastaurin inhibits the protein kinase C and Etoposide is an inhibitor of the topoisomerase[79,80]. Frequently targeted is the mammalian target of rapamycin (mTOR) pathway by inhibiting mTOR through substances as Temsirolimus, Everolimus and Rapamycin[81]. Celecoxib reduces GBM cell viability *in vitro* and combined with irradiation increases tumor necrosis and reduces tumor microvascular density[82].

Cilengitide (CGT), a cyclic RGD pentapeptide (R=arginine, G= glycine, D=aspartic acid) antagonist of the integrins αvβ3 and αvβ5, which are over expressed both on GBM and on tumor invasive endothelial cells[83], is a novel promising compound for the treatment of solid cancers, and various clinical trials have been performed or are still on-going[84-86]. Integrins are dimeric membrane proteins composed of alpha and beta subunits[87,88]. The classic role of integrins is anchoring cells to the extra cellular matrix

but they also participate in a variety of signaling processes. They are involved in malignant transformation, migration, and metastasis[89]. The integrins αvβ3 and αvβ5 play key roles in different angiogenic pathways. The αvβ3 integrins are involved in the basic fibroblast growth factor/tumor necrosis factor alpha (TNFα) induced pathway while αvβ5 integrins regulate the VEGF/transforming growth factor alpha dependent one[90]. In addition to anti-angiogenesis, CGT displays a broad anti-neoplastic effect which is not yet fully understood, but likely involves both integrin-expressing tumor cells and the surrounding stroma. *In vitro*, CGT treated GBM cells detach from the surface and undergo cell death by anoikis (or a similar mechanism)[91,92], and *in vivo* CGT has strong anti-GBM activity as monotherapy[93]. Recent studies showed beneficial effects of CGT in treatment of GBM either as monotherapy[94] or as add-on to standard irradiation plus TMZ treatment[85,86].

Novel / rediscovered therapeutic approaches

DNA alkylating agents, apart from TMZ, were neglected after the ground breaking results by Stupp and colleagues with TMZ. More recently, the substance class of nitrosourea agents (Nitrosourea and Fotemustine) has had a renaissance and was reestablished as alternative therapy for GBM. At surgery Carmustine may be implanted as wafers directly into the tumor bed[95].

Agents originally used to treat other complaints such as Disulfiram to treat alcoholism and Thalidomide as a sedative have proven efficacy in treating GBM tumors[96,97].

Transforming growth factor-beta (TGF-β) plays a role in all major tumor processes. It retains stemness of BTSC, contributes to aberrant vascularization, functions as an important player in invasion, is a potent immunosuppressant cytokine secreted by GBM tumors and is also involved in chemo- and radio-resistance[98-100]. Therefore, TGF-β is an ideal target for GBM therapy. The TGF-β2 inhibitor Trabedersen is as effective or even slightly more potent compared to standard therapy[101]. A TGF-β receptor I inhibitor (LY2109761) is under current investigation in clinical trials. A safety study in Glioma has just been completed and the data should be published soon (NCT01472731); while the trial investigating the combination of LY2109761 with TMZ-based radio-chemotherapy in patients with newly diagnosed malignant Glioma is still recruiting (NCT01220271).

Closely related to immunotherapy is the oncolytic virus therapy which is based on using live viruses to selectively infect and subsequently replicate in cancer cells, with minimal destruction of non-neoplastic tissue[102]. The idea of using oncolytic viruses

goes back to the early 1900s, when an Italian physician could demonstrate spontaneous regression of cervical carcinoma after injection with a live rabies vaccine[103]. Since then many clinical trials have been performed demonstrating safety and efficacy[102,103]. Just recently a phase I/IIa study administering Parvovirus H-1 (ParvOryx) in patients with progressive primary or recurrent GBM has started and the study design was published[104].

In summary of the above, it seems likely that a combination of conventional and novel therapy forms is most likely the way to go und numerous strategies are currently under investigation[105].

2.2 Glioblastoma models

2.2.1 Definition

A model is a “thing used as an example to follow or imitate” [Oxford dictionary: http://oxforddictionaries.com].

The main purpose for generating models of brain tumors is to identify mechanisms contributing to oncogenesis or tumor maintenance, uncovering distinct molecular patterns and defining or evaluating potential therapeutic strategies. “Targeted” therapies “only” need molecular testing but for functional analyses, such as response prediction, vital and proliferating malignant cells are indispensable[106,107]. Consequently, the wide heterogeneous spectrum must be considered in drug development and preclinical testing. Patient individual tumor models provide ideal material for such studies. There are two types of patient individual tumor models: *in vitro* (primary cell cultures) and *in vivo* (patient derived xenografts in immunodeficient animals)[108,109]. These models should be passaged as little as possible preventing epigenetic or genetic alterations and thus keeping them close to the original tumor[110,111]. Moreover, it is important to establish models from individual tumors in order to cover a broad spectrum and to ensure that the genetic heterogeneity of a given tumor entity is fully represented. These individual models allow the most accurate response and resistance prediction outside the patient. The high precision of therapy prediction with such individual models in carcinomas could be demonstrated by Voskoglou-Nomikos and colleagues as well as by Fiebig and co-

workers with 90% and even 97% accuracy rates for prediction of response and resistance, respectively[112,113].

2.2.2 *In vitro* models

Monolayer culture

Establishing cell cultures from GBM tumors was popular in the 1970s to 1990s (this becomes apparent when performing a pub med search: 1973 – 1998: 16 publications describing the establishment and characterization of glioma cell lines)[114]. Tumor tissue is minced and single cell suspension transferred to culture dishes; outgrowing cells grow in monolayers[115,116]. This method of modeling is easily feasible, crowned with success and provides models for highly reproducible analyses[114]. Frequently, the cell lines are according to their highly malignant nature immortal and readily expandable to a vast number of cells for experimental approaches[114]. However, limitations lie in genotypic and phenotypic drift or even clonal selection over time of culturing[117]. An over proportionally high frequency of the mesenchymal subtype is observed *in vitro*[57] and not all molecular characteristics are maintained under standard culture conditions[118-121].

Neurosphere culture

To overcome the limitations of monolayer culture, new strategies have been developed. For so called spheroid cultures, single cell suspensions are transferred to culture dishes made of ultralow adhesion plastic[122]; outgrowing cells cluster as spheroids. The aggregates are multicellular and maintain DNA ploidy, a similar percentage of proliferating cells as *in situ*, clonal sub-populations and thus the heterogeneity of the patient tumor[123,124]. On the down side the level of apoptosis is increased (compared to monolayer cultures), the expression of differentiation markers is up-regulated (compared to stem cell cultures), the spheroids are hard to disaggregate and finally it is difficult to obtain a sufficient number of cells for experimental analysis[125].

Stem cell culture

Establishing tumor cell lines that retain cancer-initiating stem cell properties would provide a valuable and accurate model of the human disease and give insight into

the origin of tumor heterogeneity as well as enable detailed analysis of molecular mechanisms regulating transformation, self-renewal and differentiation[125].

Neurosphere cultures enable isolation and identification of BTSC from human adult GBM, which possess the capacity to establish, sustain, and expand these tumors *in vitro* and *in vivo*[24,126]. Also, more recently specific monolayer culture conditions have been identified which allow propagation of BTSC[117,125]. The cells are grown in serum free media (as opposed to the classical monolayer cell culture) but key growth factors EGF and basic fibroblast growth factor (bFGF) are supplemented[125]. The advantage of monolayer BTSC culture, as opposed to the spheroid version, is an increased efficacy of establishing and propagating GBM cells. Lower apoptosis rates and decreased differentiation may be the attributes leading to the advantage[125].

2.2.3 *In vivo* models

Chemically induced tumors

Since the 1970s several chemically induced brain tumor models have been developed[127]. Murine, canine and feline models exist but are less popular[114]. These experimental tumors may be induced by local, oral, intravenous or transplacental exposure to N-nitroso compounds of adult or pregnant animals[128,129]. Chemically induced brain tumors appear to differ largely from human gliomas and are frequently referred to as “gliosarcomas” or “gliomas-like tumors”[114]. The histological characteristics are mainly lost; infiltrative growth may be observed but no single cell infiltration[114].

Genetically engineered mice

Increased understanding of genomic alterations in human brain tumors has led to the development of highly defined and well characterized genetically engineered mouse models[114]. Multiple gene gains and losses are possible as well as a cell type and developmental specific manipulation[130-133]. Conditional strategies include tet-regulation (on- off switch: by adding tetracyclines to the water the expression or inhibition of a gene cloned behind a tet-regulated promoter can be induced) and cre-inducible alleles (the cre recombinase is expressed in a promoter specific manner and thus induces exclusively in cells with activated promoter the excision of a gene between two lox sequences)[71,134]. Genetically engineered models allow addressing specific molecular events responsible for tumor initiation and progression[114]. Further modeling

of tumor stroma interaction is possible e.g. analyzing (neo-) angiogenesis processes in tumors[114]. Since these models include immune competent animals, influences of the immune system may be determined[114]; not only in therapy settings. The major drawback of the system is the loss of heterogeneity since the experimental tumor is composed of cells with a number of specific homogenous genetic changes[114]. These models cannot properly reflect the complete intratumoral genomic and phenotypic variations of a natural human GBM.

Xenograft models

The *in vivo* model representing patient individual tumors are so called xenografts. The strategy is based on implanting fresh (brain) tumor cells or pieces into immunodeficient animals[135]. The engraftment can be in an orthotopic manner (into the organ of which the patient tumor originated from) or a heterotopic one (mostly subcutaneous implantation into the flanks). The latter often is a subcutaneous implantation into the flanks of mice. This method is easily feasible since it does not require great surgical skills and the procedure is fast to perform[136]. However, the take rate is rather low and if tumors grow it is outside of their normal environment[136]. For the orthotopic GBM model, tumor cells are implanted into the brain of mice. The success rate is nearly 100% and the tumor growth takes place in a more familiar micromilieu. Moreover, the invasive phenotype is preserved (as opposed to the heterotopic model) and the tumors show histological features similar to the patients´ tumors[137]. However, the tumors depend on the host vasculature for oxygen and nutrition[114]. As a major drawback, orthotopic modeling of brain tumors is technically challenging.

2.3 The aim of the study

GBM tumors are associated with a very poor prognosis despite optimal aggressive combinational therapy regimens; thus highlighting the urgency of developing novel most optimally effective therapies. Here tumor models may enter the picture either as *in vitro* (cell lines) or *in vivo* models (engraftment into immunodeficient mice). Their crucial role would lie in the ability of drug testing on a cellular functional level and the identification of novel prognostic and predictive markers. A feasible solution might be the establishment of patient-individual tumor models out of a small resection specimen sample of each patient and thus not interfering with the necessity to obtain

complete pathological diagnosis. These patient-derived cell lines would definitely provide a nearly unlimited and readily available source of antigens for immunotherapeutic interventions and allow basic and translational research at the same time.

In this study, preferably a tumor sample of every resection specimen of GBM patients that had been operated on in Rostock should be collected, a proportion stored in a GBM biobank and model establishment attempted. Any established models should then be added to the biobank.

The biobank, envisioned in this project, should not only store tumor samples but include patients´ peripheral lymphocytes and sera as well as all established patient-derived models. All samples (patient tumors and models) should undergo molecular pathological characterization.

The morphology of outgrowing *in vitro* models should be documented. The cell lines should be analyzed for their expression of GBM and neuronal markers by flow cytometry and the proportion of BTSC-like cells in the cultures should be determined. With regard to immunological approaches the presence of human leukocyte antigen (HLA) class I and II molecules is to be assessed and the specific alleles (two-digits encompassing) are to be identified by HLA typing. Because the presence of tumor antigens is of great interest, their expression should also be analyzed by flow cytometry. The molecular composition of GBM tumors is of great relevance. Thus, molecular pathological analyses such as assessing MGMT promoter methylation, mutations in typical tumor suppressor genes and (EGFR) amplifications are to be performed and compared to the patients´ tumors. The secretion of cytokines, relevant for tumor growth and immunosuppression, should be quantified by enzyme-linked immunosorbent assay (ELISA). In a first step towards individualized therapy and response prediction an extensive drug sensitivity screening is planned. Finally, CGH arrays of the models shall complete the characterization and allow for the identification of novel tumor suppressor candidate genes which shall be validated in subsequent analyses.

For *in vivo* models (xenografts) tumor pieces should be implanted subcutaneous into the flanks of immunodeficient mice. Successful outgrowth should be documented, the mice sacrificed and xenograft models added to the biobank.

The cell lines shall be analyzed concerning their *in vitro* sensitivity to a broad panel of drugs (used in standard or experimental GBM therapies) and drug screening

potential should be assessed. These findings might be subsequently verified in the *in vivo* models.

Given the time consuming, and not seldom complex logistic of patient-individual tumor modeling the aim of this study is to establish a broad variety of these patient-derived tumor models, both, *in vitro* and *in vivo* and most possibly representing the full clinical repertoire. This way, establishment of *in vitro* and *in vivo* models from fresh and frozen vital tumor material could be compared. The goal is to improve the take rates and simplify logistics to ultimately provide an easily feasible method and thus also enable decentralized sample collection.

This work should substantially support the attempt of providing an individual, tumor-specific therapy for every GBM patient.

3. Material and Methods

3.1 Material

Disposable material

Cell strainer (100µM)	BD Falcon
CryoPure tube (1.6ml)	Sarstedt
Culture flasks (T25, T75, T175)	Greiner bio-one
PP-tubes (1.3ml)	Greiner bio-one
PP-tubes sterile (15ml, 50ml)	Greiner bio-one
FACS tubes (5ml)	Sarstedt
Insulin Syringes U-100	BD Diabetes
Microtiter plates (6-Well, 24-Well, 96-Well)	Greiner bio-one
Reaction tube (0.5ml, 1.5ml, 2.0ml)	Sarstedt
Scalpels sterile (Figure 10)	Dahlhausen
Serological pipette (5ml, 10ml, 25ml)	Greiner bio-one
Serum monovette (7.5ml)	Sarstedt
Surgical thread Vicryl 6.0	Ethicon
Syringe (20ml)	BD Plastipak

Reagents

Agarose	Biozym
Betaisodona solution	Mundipharma GmbH
Calcein AM	eBioscience
Collagen	secret composition
Co-trimoxazol	Ratiopharm
Dimethyl sulfoxide (DMSO)	AppliChem
DMEM/Ham´s F-12	PAA
Ethanol	AppliChem
Eye and nose ointment	Bayer Vital GmbH
Exonuclease I	Fermentas
FastAP alkaline phosphatase	Frementas
Fetal calf serum (FCS)	PAA
Formafix 4%	Grimm med. Logistik GmbH

Glutamin	PAA
Heparin	Roche
Hepes	Sigma-Aldrich
Ketamin 10%	Belapharm
Lymphocyte	PAA
MyTaq HS DNA polymerase	Bioline
Phosphate buffered saline (PBS)	PAA
Penicillin	Jenapharm
Rompun 2%	Bayer Vital GmbH
Saponin	Sigma-Alrich
Streptomycin	InfectoPharm
SssI enzyme	Fermentas
SYBR Green master mix	Applied Biosystems
Trypan blue	Fluka
Trypsin	PAA

Therapeutic agents

BCNU	Bristol-Myers Squibb
Bevacizumab	Roche
CCNU	Sigma-Aldrich
Celecoxib	Molekula
Cetuximab	GlaxoSmithKline
Cilengitide	Merck KGaA
Cisplatin	Teva GmbH
Cytarabine	Cell Pharm GmbH
Imatinib	Novartis
Irinotecan	Pfizer
Methotrexate	Teva GmbH
Nilotinib	Novartis
Procarbazine	Sigma-tau
Rapamycin	Pfizer
Thalidomide	Sigma-Aldrich
Temozolomide	Sigma-Aldrich
Topotecan	GlaxoSmithKline

Vincristine	Hexal

Kits

Wizard Genomic DNA Purification Kit	Promega
GeneMATRIX universal RNA purification Kit	EURx
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
EpiTect Bisulfite Kit	Qiagen
SensiFAST Probe Kit	Bioline
BigDye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems
BigDye XTerminator Purification Kit	Applied Biosystems
SNP Array 6.0	Affymetrix

IL-6 ELISA (matched pair)	Immunotools
IL-8 ELISA (matched pair)	Immunotools
TNFα ELISA (set pair)	Immunotools
AssayMax Human TGF-β1 ELISA Kit	Assaypro
Human CEA ELISA Kit	RayBio

Antibodies

Isotype controls for flow cytometry

All isotype controls were obtained from Immunotools.

Specific antibodies for flow cytometry

Species	Target	Clone	Isotype	Label	Manufacturer
Rat	pan αv	RMV-7	IgG1	PE	eBioscience
Mouse	αvβ3	LM609	IgG1	none	Merck Millipore
Rabbit	αvβ5	EM09902	IgG	none	Merck KGaA
Mouse	CD15	MEM-158	IgM	PE	Immunotools
Mouse	CD24	SN3	IgG1	PE	Immunotools
Mouse	CD34	-581-	IgG1	PE	Immunotools
Mouse	CD44	MEM-85	IgG2b	APC	Immunotools
Mouse	CD90	AS02	IgG1	FITC	Dianova
Mouse	CD133	AC133	IgG1	PE	Miltenyi
Mouse	CEA	IH4Fc	IgG1	PE	Immunotools

Mouse	HIP 1	1B11	IgG1	none	Abcam
Rabbit	IL-13Rα	polyclonal	IgG	none	Assay bio Tech
Rabbit	TGF-β	polyclonal	IgG	none	Abcam
Mouse	GFAP	GA5	IgG1	FITC	eBioscience
Mouse	Nestin	10C2	IgG1	FITC	eBioscience
Mouse	S-100	B32.1	IgG1	None	Abcam
Mouse	Vimentin	V9	IgG1	None	Abcam

Secondary antibodies for flow cytometry

Species	Target	Label	Manufacturer
Goat	Mouse	PE	Dako Cytomation
Swine	Rabbit	FITC	Dako Cytomation

Primer

Target	Primer Sequence	
	forward	reverse
IDH 1 (Exon 4)	5'-GCACGGTCTTCAGAGAAGCC-3'	5'-CACATTATTGCCAACATGAC-3'
IDH 2 (Exon 4)	5'-GCCCACACATTTGCACTCTA-3'	5'-CAGAGACAAGAGGATGGCTAGG-3'
B-Raf (Exon 15)	5'-TCATAATGCTTGCTCTGATAGGA-3'	5'-CTTTCTAGTAACTCAGCAGC-3'
K-Ras (Exon 2)	5'-GTACTGGTGGAGTATTTGATAGTGTATTAA-3'	5'-TCAAAGAATGGTCCTGCACC-3'
K-Ras (Exon 3)	5'-CTTTGGAGCAGGAACAATGTCT-3'	5'-TACACAAAGAAAGCCCTCCCC-3'
TP53 (Exon 5)	5'-(GC40)TTCCTCTTCCTACAGTACTC-3'	5'-CTGGGCAACCAGCCCTGTCGT-3'
TP53 (Exon 6)	5'-(GC40)GACGACAGGGCTGGTTGCCCA-3'	5'-AGTTGCAAACCAGACCTCAG-3'
TP53 (Exon 7)	5'-(GC40)TCTCCTAGGTTGGCTCT-3'	5'-GCAAGTGGCTCCTGACCTGG-3'
TP53 (Exon 8)	5'-CCTATCCTGAGTAGTGGTAATC-3'	5'-(GC40)CCGCTTCTTGTCCTGCTTGCTT-3'
PTEN (segment 1)	5'-TTCCATCCTGCAGAAGAAGC-3'	5'-GCTGTGGTGGGTTATGGTCT-3'
PTEN (segment 2)	5'-ACCGCCAAATTTAATTGCAG-3'	5'-CGCCACTGAACATTGGAATA-3'
PTEN (segment 3)	5'-GTGGCACTGTTGTTTCACAAG-3'	5'-CTGCACGCTCTATACTGCAAA-3'
PTEN (segment 4)	5'-ACCAGGACCAGAGGAAACCT-3'	5'-AAGGTCCATTTTCAGTTTATTCAAG-3'
EGFR	5'-TCCCATGATGATCTGTCCCTCACA-3'	5'-CAGGAAAATGCTGGCTGACCTAAG-3'
LINE1	5'-TGCTTTGAATGCGTCCCAGAG-3'	5'-AAAGCCGCTCAACTACATGG-3'
MGMT methylation	5'-GCGTTTCGACGTTCGTAGGT-3'	5'-CACTCTTCCGAAAACGAAACG-3'
Probe (MGMT)	5'-6FAM-CGCAAACGATACGCACCGCGA-TMR-3'	
MGMT expression	5'-CCGAGGCTATCGAAGAGTTC-3'	5'-TCCGAATTTCACAACCTTCA-3'
COL2A1	5'-TCTAACAATTATAAACTCCAACCACCAA-3'	5'-GGGAAGATGGGATAGAAGGGAATAT-3'
Probe (COL2A1)	5'-6FAM-CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-TMR-3'	
PTEN expression	5'-ACCAGGACCAGAGGAAACCT-3'	5'-CTGCACGCTCTATACTGCAAA-3'
TBP	5'-TCGGAGAGTTCTGGGATTGT-3'	5'-CACGAAGTGCAATGGTCTTT-3'

Buffer

Buffer P: PBS with 1% FCS, 0.03% Saponin and 0.01M Hepes

Instruments

Accu pipet	Integra
Centrifuges	Eppendorf (5415 D)
	Hettich (Rotina 38)
CO_2 Incubator	Memmert
FACS Calibur	BD
Freezer (-20°C)	Bosch
Freezer (-80°C)	Kryotech
Freezing container	Nalgene
Laminaflow bench	Nunc
NanoDrop	Thermo-Scientific
Microplate reader Infinite M200	Tecan
Microscope (Primo Vert)	Zeiss
Mikro-Dismembrator S	Sartorius
Neubauer chamber	Marienfeld
Nitrogen tank (Espace 661)	Air Liquide
Polymerase chain reaction (PCR) Cycler	BioRad
Pipette	Eppendorf
Refrigerator	Liebherr
Sliding rule	Asculap
StepOne™ Real-Time PCR System	Applied Biosystems
USB Camera (AxioCam)	Zeiss
Water bath	VEB MLW Prüfgerätewerk
3500 Series Genetic Analyzer	Applied Biosystems

Animals

NMRI nu/nu

Software and Programs

Office 2010	Microsoft
SigmaStat 3.5	Systat
SigmaPlot 10.0	Systat
SeqScape Software v2.7	Applied Biosystems
AxioVision 4.8.2	Carl Zeiss
Photoshope CS3	Adobe
Papers	Mekentosj
Genotyping Console 4.1.2	Affymetrix

3.2 Methods

Tumor specimen collection and cryopreservation

Between August 2009 and October 2012, clinical samples from patients with WHO grade I - IV GBM tumors were collected from the Neurosurgery department at the University hospital Rostock. Prior informed consent was obtained in written form from all patients, and all procedures were approved by the institutions' Ethics Committee (reference number: A 2009/34) in accordance with general accepted guidelines for the use of human material. Resection specimens of GBM tumors were received sterile and freshly from surgery. Tumor tissue samples were snap frozen in liquid nitrogen and stored in the gas phase above liquid nitrogen. Additionally, tumor tissue cubes (3 x 3 x 3 mm) were frozen vitally. For this procedure, tumor pieces were cut with a sterile scalpel blade, and 4 tumor pieces were transferred into one sterile cryo-tube in 1.5ml freezing medium (FCS containing 10% DMSO), sealed in a freezing container, and placed immediately at -80°C. Until unthawing, tubes were kept at -80°C (for a maximum of 6 weeks) or, after overnight cooling, transferred into a nitrogen tank (for longer storage periods). For subsequent modeling procedures, cryo-preserved tumor pieces were thawed at 37°C.

Xenografting into immunodeficient mice

Tumor xenograftings were done by one of the following approaches: (I) xenografting of primaries on the day of surgery; (II) xenografting of primaries after

cryo-preservation; and (III) re-transplantation of xenografts. Tumor pieces were implanted subcutaneously bilaterally into the flanks of six to eight week old female NMRI nu/nu mice under anesthesia consisting of Ketamin/Xylazin (90/25 mg/kg body weight) injected intraperitoneal. Mice were kept in the animal facilities of the medical faculty of the University of Rostock and maintained in specified pathogen-free conditions. Animals were exposed to 12-h light/12-h darkness cycles and standard food and water including antibiotics (Co-trimoxazol) ad libitum. Their care and housing were in accordance with guidelines as put forth by the German Ethical Committee and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, vol.25, no.28, 1996). Growth of tumors to volumes of 1 - 1.5 cm^3 was taken as evidence of successful xenografting, and the animals were then sacrificed for collection of tumor tissues for further studies.

Tissue culture and cell line establishment

Tumor tissue was minced (by crossed scalpels) in DMEM/Ham´s F-12 cell culture media supplemented with 10% FCS, 2mM L-glutamine and penicillin-streptomycin and passed through a cell strainer to obtain a single cell suspension. Cells were washed with PBS and seeded in 6 well plates coated with collagen. Outgrowing cells were detached with trypsin and transferred to T25 cell culture flasks. Cells passaged 2-3 times in this manner were transferred to T175 culture flasks and expanded for subsequent analyses.

In general, all cell culture processes were performed under a sterile lamina flow bench. The cell lines were cultured in DMEM/Ham´s F-12 media supplemented with 10% FCS and 2mM L-glutamin (penicillin-streptomycin was only added to fresh cultures). All cells were kept in an incubator at 37°C and 5% CO_2. Cells were detached by incubation with trypsin for 5min at 37°C. Cell number and viability was assessed by trypan blue staining and counting using a Neubauer chamber. For cryo-storage cells were washed with PBS and 1×10^6 cells were frozen in 1.5ml freezing media (FCS containing 10% DMSO) per aliquot. Cryo-tubes were placed in a freezing container and frozen down at -80°C.

Phenotypic characterization (microphotography)

Cells were cultured in T25 flasks to a confluence of 60 – 80% and photographed using the AxioVision 4.8.2 software. Photographs were edited subsequently with Photoshop CS3.

Growth kinetics

Cells (5 x 10^5 cells) were plated in 5ml media in quintuplicate T25 culture flasks per cell line and allowed to attach and grow for 48h. Cells were detached by trypsinization and the amount of vital cells was assessed by trypan blue staining using a Neubauer chamber. One flask was counted every 24h for five consecutive days.

Isolation of nucleic acids

Genomic DNA (gDNA) from snap frozen tumor tissue and cell culture cell pellets (3 x 10^6 cells) was isolated using the Wizard Genomic DNA Purification kit according to the manufacturer's instructions. Total RNA from cell culture pellets (3 x 10^6 cells) was isolated using the GeneMATRIX universal RNA purification kit also according to the manufacturer´s instructions. Concentration of isolated nucleic acids was determined with the NanoDrop1000.

Complementary DNA (cDNA) synthesis

10µl total RNA was used for reverse transcription applying the High Capacity cDNA Reverse Transcription Kit according to the manufacturer´s instructions.

Molecular characterization

MGMT promoter methylation

For analyzing the MGMT promoter methylation, the MethyLight method was applied. Briefly, gDNA was subject to bisulfite conversion using the Epitect Bisulfite Kit according to the manufacturer's recommendations. A primer / probe combination specific for methylated MGMT promoter sequence was used, with the SensiFast

Probe Kit. CpG Methylase (SssI) treated DNA served as calibrator, since it is considered to be fully methylated. The collagenase gene 2A1 (COL2A1), was used as endogenous control. The percentage of methylated reference (PMR) value was calculated by dividing the MGMT / COL2A1 ratio of the sample by the MGMT / COL2A1 ratio of the SssI-treated DNA, and multiplying by 100. Samples with a PMR value > 4 were considered as methylated[138]. All reactions were performed in triplicates.

cDNA expression

The relative expression of MGMT and PTEN cDNA was assessed by real time PCR. The desired regions were amplified by PCR using the specific primers for either MGMT or PTEN and TATA box binding protein (TBP; housekeeping gene) and the Fast SYBR Green master mix on a StepOne Realtime PCR system. The amount of incorporated SYBER Green was assessed at the end of each cycle by measuring the absorbance at 260nm. Quantitative values (Ct value = threshold cycle number at which the increase in the signal associated with an exponential growth of PCR products starts to be detected) were expressed as x-fold differences in target gene expression relative to the reference gene TBP and were determined as follows:

$$2^{-\Delta Ct} \text{ [}\Delta Ct = Ct\text{-value (TBP)} - Ct\text{-value (target gene)]}$$

Mutations (TP53, IDH1 & 2, K-Ras, B-Raf, PTEN)

Samples underwent analyses for the following loci: IDH 1 R132 (exon 4), IDH 2 R172 (exon 4), v-raf murine sarcoma viral oncogene homolog B1 (B-Raf) V600 (exon 15), Kirsten rat sarcoma viral oncogene homolog (K-Ras) G12, G13 (exon 2) and Q61 (exon 3), TP53 (exons 5 to 8) and full length PTEN (cDNA). The desired regions were amplified by PCR using the specific primers. The PCR was performed using MyTaqHS polymerase according to the manufacturer's recommendations. The PCR reaction was controlled by agarose gel electrophoresis and 15µl of the products were purified using 3 units of FAST AP Alkaline Phosphatase and 30 units of Exonuclease I by incubation at 37°C for 15min and subsequent heat inactivation at 85°C for 15min.

One microliter of the PCR product was used as template for Sanger sequencing using BigDye Terminator v1.1 Cycle Sequencing kit and the primers used for PCR according to the manufacturer's protocol. The sequencing products were purified

using the BigDye XTerminator Purification kit. The sequence was analyzed using the 3500 genetic analyzer system and the SeqScape Software v2.7.

EGFR amplification

For determination of EGFR copy number, quantitative PCR was performed. 30 ng gDNA were used as template. The run was performed on a StepOne Realtime PCR system using Fast SYBR Green Mastermix. Commercial normal human gDNA was used as calibrator and the repetitive element LINE1 as endogenous control. The calculation of the EGFR copy number was performed using the ΔΔCt-algorithm. All reactions were performed in triplicates.

CIN was assessed using SNP Array 6.0 from Affymetrix according to manufacturer's instructions. The analyses were performed by the core facility of the Department for Immunology under supervision of Dr. Koczan.

HLA typing

A 2-digit resolution typing of the following HLA loci was performed by the Transfusion Medicine at the University Medicine in Rostock: HLA-A, -B and -C and HLA-DR and -DQ.

Flow cytometry

Cells were harvested by incubation with trypsin; the enzymatic reaction was stopped by adding cell culture media. Cells were washed with PBS, counted and 5 x 10^5 cells were stained with respective antibodies or isotype controls for an extra-cellular staining. Cells were washed with PBS and resuspended in final volume of 200µl PBS. In case of unlabeled primary antibodies, excess antibody was washed out with PBS and respective secondary antibodies were added and final wash step was performed as above.

Similarly, 5 x 10^5 cells were treated with buffer P for 10min to permeabilize the cell membrane for an intra-cellular staining. Cells were incubated with the antibodies and washed with buffer P. After a second 10min incubation period respective secondary antibodies were added in buffer P. Cells were washed and resuspended in Formafix at a final volume of 200µl.

For the staining method with unlabeled primary antibodies, cells handled the same way with no primary antibody served as negative controls. All incubations were performed on ice for 30min.

Cytokine secretion (ELISA)

Cells (5 x 10^4 cells) were plated in 5ml media per well in duplicates in 6 well culture plates and allowed to attach for 24h. The media was replaced by fresh media or media without FCS (for TGF-β secretion). 1ml samples of supernatant were collected on days 3 and 5 and stored at -80°C. For detection of cytokine production samples were unthawed on ice and 100µl supernatant was used for each ELISA assay. The ELISA assays were performed according to the manufacturer's instructions.

Drug response

Cells (5 x 10^3 cells) were plated in 150µl media per well in triplicate in 96well flat bottom culture plates and allowed to attach for 24h. The following concentration ranges of drugs were tested (given are final concentrations in the experimental wells):

BCNU: 500µM – 32nM

Bevacizumab: 2.5mg/ml – 39ng/ml

CCNU: 500µM – 32nM

Celecoxib: 1mM – 64nM

Cetuximab: 20µg/ml – 313ng/ml

Cilengitide: 40µM – 10nM

Cisplatin: 30µM – 30nM

Cytarabine: 500µM – 125nM

Imatinib: 250µM – 60nM

Irinotecan: 1mM – 244nM

Methotrexate: 1mM – 1µM

Nilotinib: 10µM – 2.5nM

Procarbazine: 50µM – 3.2 nM

Rapamycin: 30µM – 30nM

Thalidomide: 40µM – 10nM

Temozolomide: 2mM – 128nM

Topotecan: 5µM – 320pM

Vincristine: 244nM – 300pM

Equal volumes DMSO (for cells treated with BCNU, CCNU, Celecoxib, Procarbazine, Thalidomide and Temozolomide) were added to cells serving as live control. Cells were incubated with the substances for 72h, and media was replaced together with substances in the same concentrations as before. After another 72 hour incubation period cells serving as dead control were incubated with 70% ethanol for 30min and viability was assessed by using the viability dye calcein AM in a final concentration of 0.7µM in fresh medium:PBS (2:1). Cells were incubated at 37°C in the dark for 20min; fluorescence intensity was assessed using the microplate reader Infinite M200 with 485nm excitation, 535nm emission and a constant gain of 160. Values were normalized (1= value live control; 0= value dead control).

Statistics

All statistical analyses were performed using the software program SigmaPlot. Success rates of model establishment from newly diagnosed tumors and relapses were compared by performing a Chi square test and a Fishers exact test. A T-test was performed to identify correlations between drug responses and molecular characteristics of the tumors. In order to analyze differences (or not) between the cell line pairs a paired T-test was performed. Finally, differences in the response of tow cell lines (in pairs) to a drug were assessed by Mann-Whitney U test.

The IC_{50} values were calculated using the Standard Curves macro in SigmaPlot.

All survival curves and box plot diagrams were generated using the SigmaPlot software.

4. Results

4.1 Biobanking and Model Generation

Having the big buzz words “individualized therapy”, “response-” and “resistance-prediction” in mind, the establishment and detailed characterization of patient-derived models and biobanking of patient material is a logical scientific reaction.

The assessed patient cohort was operated on in the Department of Neurosurgery at the University Medicine in Rostock between August 2009 and October 2012. Patient data were anonymized by assigning the prefix HRO (for the city of Rostock), G (for glioma tumor) and a consecutive number. The median age of the HROG patient cohort at surgery was 59 years and ranged from 13 to 80 years; 34 patients were male (56%) and 27 patients were female (44%), the male to female ratio was 1.3 to 1. The majority of patients included were diagnosed with GBM grade IV tumors (42/61; 69%) by the Institute of Pathology. The median age here was 62 years with a range from 44 to 80 years; 22 (52%) patients were male and 20 (48%) patients were female with a male to female ratio of 1.1 to 1. Of the GBM grade IV tumors, 39 (93%) were primary and 3 (7%) were secondary GBM; hereof 26 (62%) were newly diagnosed primary tumors, 2 (5%) newly diagnosed secondary tumors, 13 (31%) relapses and 1 (2%) relapsed secondary tumor. Patient characteristics are summarized in Table 1.

Sample ID	Sex	Age	Diagnosis	Localization	Survival
HROG02	M	68	GBM (IV)	R; parietooccipital	† 7
HROG03	M	50	anaplastic Oligodendroglioma (III)	R; parietal	? 9
HROG04	F	53	relapsed GBM (IV)	R; frontal	† 13
HROG05	F	60	relapsed GBM (IV)	L; temporal	† 3
HROG06	M	53	GBM (IV)	L; frontal	† 8
HROG07	M	55	relapsed GBM (IV)	R; temporoparietal	† 6
HROG08	M	47	relapsed GBM (IV)	R; frontal	? 29
HROG09	M	66	anaplastic Astrocytoma (II-III)	L; temporal	**33**
HROG10	M	74	GBM (IV)	R; temporal	† 7
HROG11	F	54	GBM (IV)	L; frontal	**30**
HROG12	M	64	GBM (IV)	R; frontoparietal	† 5
HROG13	F	77	GBM (IV)	L; temporal	† 8
HROG14	F	81	Subependymoma (I)	IV. ventricle	† 3
HROG15	M	56	GBM (IV)	R; parietal	**23**
HROG16	M	53	GBM (IV)	R; parietal	† 26
HROG17	M	70	relapsed GBM (IV)	L; parietooccipital	† 3

HROG18	M	71	relapsed Oligoastrocytoma (II)	cerebrum	? 7
HROG19	M	69	GBM (IV)	L; temporoparietal	† 15
HROG20	M	34	diffuse Astrocytoma (II)	L; temporal	**24**
HROG21	M	44	secondary GBM (IV)	R; parietal	**21**
HROG22	M	66	relapsed GBM (IV)	L; temporal	† 4
HROG23	F	60	relapsed GBM (IV)	L; parietal	**20**
HROG24	F	73	GBM (IV)	L; occipital	† 10
HROG25	F	77	relapsed GBM (IV)	L; temporal	† 3
HROG26	M	63	relapsed Astrocytoma (II)	R; parietal	† 8
HROG27	M	76	Meningioma (I)	cerebrum	**23**
HROG28	F	76	Meningioma (I)	cerebrum	? 4
HROG29	M	39	diffuse Oligoastrocytoma (II)	cerebrum	**19**
HROG30	M	67	Meningioma (I)	frontal	? 3
HROG31	F	59	GBM (IV)	R; occipitotemporal	**21**
HROG32	F	76	GBM (IV)	R; temporal	**22**
HROG33	F	46	GBM (IV)	L; occipitotemporal	† 13
HROG34	F	69	GBM (IV)	L; frontal	† 5
HROG35	M	64	relapsed GBM (IV)	R; occipital	† 6
HROG36	F	80	GBM (IV)	R; parietal	† 5
HROG37	F	20	pilocytic Astrocytoma (I)	L; occipital	? 2
HROG38	F	49	GBM (IV)	R; parietooccipital	**19**
HROG39	F	59	Meningioma (I)	cerebrum	**18**
HROG41	M	71	secondary GBM (IV)	L; frontal	† 2
HROG42	F	70	GBM (IV)	L; frontal	**16**
HROG43	M	55	Meningioma (I)	L; frontal	? 8
HROG44	M	69	Meningioma (I)	L; frontal	? 8
HROG45	M	61	relapsed Astrocytoma (II)	L; parietal	**13**
HROG46	F	69	GBM (IV)	R; parietotemporal	**15**
HROG47	M	59	GBM (IV)	R; temporal	† 16
HROG48	M	13	pilocytic Astrocytoma (I)	L; occipital	**13**
HROG49	M	45	relapsed secondary GBM (IV)	R; parietooccipital	? 6
HROG50	F	33	diffuse Oligoastrocytoma (II)	L; frontal	**14**
HROG52	M	47	GBM (IV)	L; temporobasal	**13**
HROG53	F	50	anaplastic Astrocytoma (III)	cerebrum	? 4
HROG54	M	58	GBM (IV)	R; parietal	**8**
HROG55	F	74	GBM (IV)	R; parietal	? 1
HROG56	F	76	GBM (IV)	trigonum	? 5
HORG57	F	60	relapsed GBM (IV)	R; parietal	**8**
HROG58	F	57	GBM (IV)	R; frontal	**7**
HROG59	M	60	relapsed GBM (IV)	R; temporal	† 8
HROG60	M	51	relapsed GBM (IV)	R; temporal	? 1
HROG61	F	50	diffuses Astrocytoma (II)	L, frontal	**6**
HROG62	M	71	GBM (IV)	R; temporoparietal	**4**
HROG63	M	48	relapsed GBM (IV)	L; temporal	**3**

HROG64	F	57	GBM (IV)	R; temporal	**1**

Table 1: Patient characteristics
The table summarizes patient information on sex (F = female; M = male), age at time point of resection in years, diagnosis by the department of pathology including WHO grading in parentheses, tumor localization (L = left hemisphere; R = right hemisphere) and survival († = patient died; **bold** = patients still alive on January 25th 2013; ? = no information available for > 6 months) in months after resection.

Attachment and outgrowth rates of the 42 consecutive WHO grade IV GBM tumor samples *in vitro* were assessed. Under standard *in vitro* conditions in 37/42 (88%) cells of the tumors attached; 25/28 (89%) newly diagnosed tumors and 12/14 (86%) relapses. Establishment of outgrowing cell lines was successful in 25/42 (60%) cases, hereof 17/28 (61%) were derived from newly diagnosed tumors and 8/14 (57%) from relapses. Twelve (8 from newly diagnosed and 4 from relapsed tumors) of the cell lines divide rapidly and stable. These cell lines could be passaged over 40 times, which implies far more than 50 cell divisions and thus exceeds the Hayflick limit (number of cell divisions a normal, healthy cell can undergo) and proves immortality[139]. Consequently, they were characterized in detail and subsequently considered as permanent cell lines. No differences in attachment (p=1.000), outgrowth (p=1.000) or cell line establishment (p=1.000) rates between newly diagnosed and relapsed tumors were observed.

The prognosis for GBM patients is poor; it is even more devastating for patients diagnosed with relapsed tumors compared to those with newly diagnosed tumors (median survival is 15 months and 5 - 7 months respectively). The survival time of patients with GBM grade IV tumors after surgery correlated with the diagnosis (Figure 1); patients suffering from newly diagnosed tumors survived significantly longer than patients diagnosed with a relapse (p=0.029). The cohort of patients for whom cell line establishment was successful, was termed “cell line patients”; here the survival benefit of patients with newly diagnosed tumors might also be reached. However, no level of significance could be reached for the “cell line patients”; most likely since the cohort was too small.

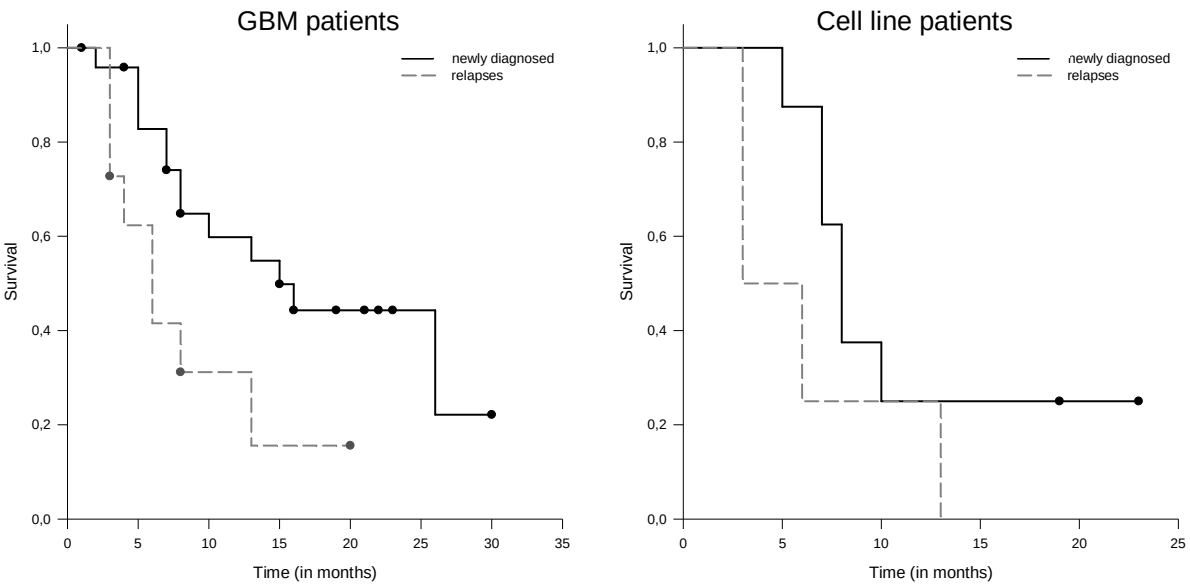


Figure 1: Survival of patients with newly diagnosed and relapsed GBM tumors

The Kaplan-Meier survival curves plot the survival interval (in months) of patients after the surgery of the GBM tumor, comparing survival intervals of patients with newly diagnosed and relapsed tumors. The graph on the left side (GBM patients) indicates the survival interval of the Rostock GBM patients (n=42); the graph on the right side ("cell line patients") indicates the survival interval of the "cell line patients" (n=12).

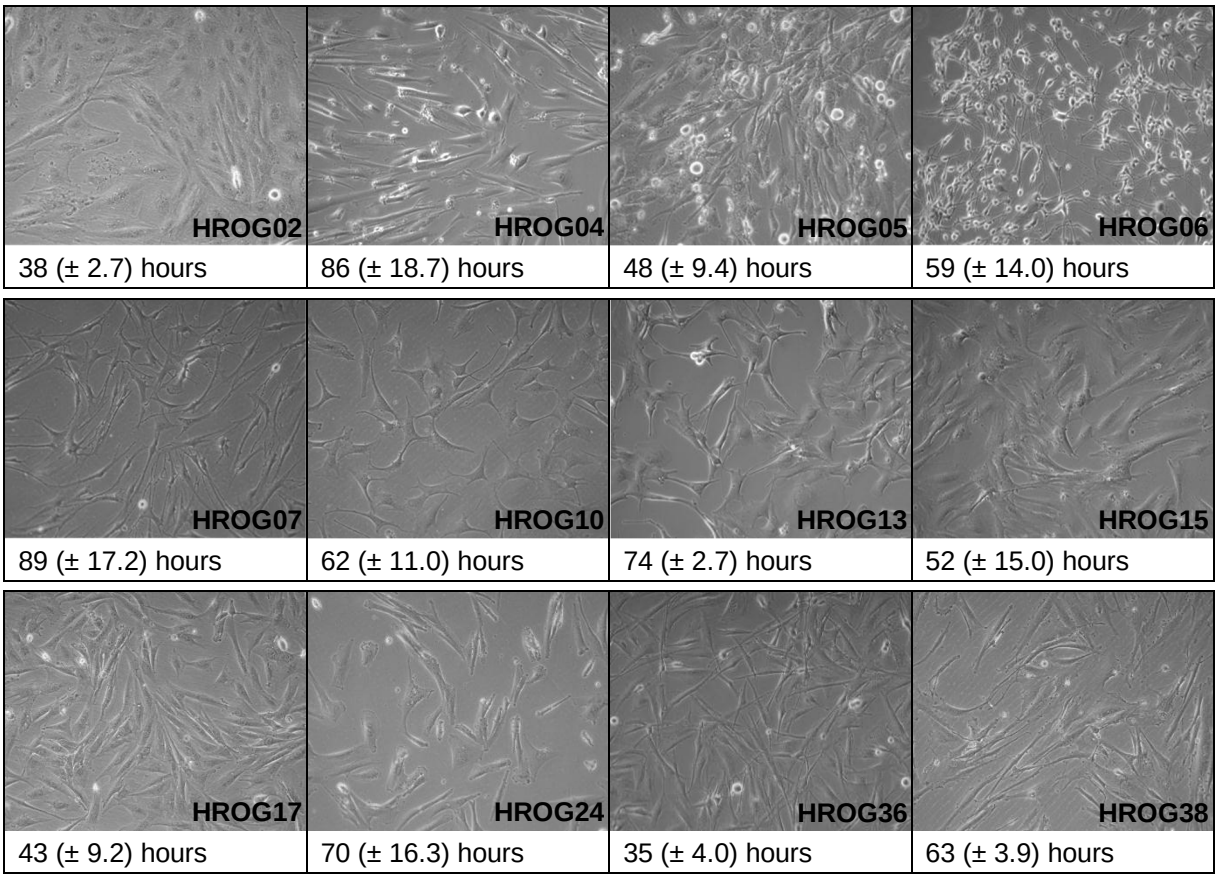
Engraftment of tumor pieces into immunodeficient mice was successful (tumor outgrowth) in 8/36 (22%) cases. Four (4/25; 16%) xenografts were derived from newly diagnosed tumors and four (4/11; 36%) from relapsed tumors. Engraftment was successful in 2/10 (20%) cases when tumor material was implanted fresh directly after patient surgery and six times (6/36; 17%) when the tumor material was vitally frozen before engraftment. Passaging of outgrowing tumors in immunodeficient mice was successful in the two cases assessed. No level of significance was reached since the sample size was too little. However, a study on a direct comparison of the rate of success between fresh and frozen GBM samples is ongoing. Small tumor pieces (3 x 3 x 3 mm) were implanted fresh (directly after neurosurgery) into immunodeficient mice and of the same sample tumor tissue was implanted into immunodeficient mice after vital cryo-preservation (1 - 12 months in nitrogen).

4.2 Model characterization

Morphology and doubling time

In most cases, the morphology of monolayer GBM cell lines showed fibroblast-like or epithelial-like appearances, while polygonal or spindle-like cells were less frequent.

In a first step, the cell lines were micro-photographed to compare their morphology (Figure 2); all cell lines show a fibroblast-like phenotype but differences between the individual cell lines are obvious, HROG10 with a larger cell body and shorter extensions compared to HROG06 having a smaller cell body and long extensions or HROG17 encompassed of long skinny cells. Furthermore, doubling times of the cell lines were assessed. Doubling times range from 35 hours for HROG36 to 89 hours for HROG07. The average doubling time is 60 hours.



38 (± 2.7) hours	86 (± 18.7) hours	48 (± 9.4) hours	59 (± 14.0) hours
89 (± 17.2) hours	62 (± 11.0) hours	74 (± 2.7) hours	52 (± 15.0) hours
43 (± 9.2) hours	70 (± 16.3) hours	35 (± 4.0) hours	63 (± 3.9) hours

Figure 2: Cell line morphology and doubling time
Depicted in this figure are micro-photographed pictures (100x enlarged) and doubling times in hours (± standard deviation) of the cell lines.

Molecular pathology

In the era of targeted and individualized therapy unraveling molecular characteristics – in common or individually, for an entity and for patients – must be a prime aim; this obviously enables defining (novel) target structures. These targets however, may not only have therapeutic impact but may also serve as prognostic as well as predictive markers.

GBM relevant molecular features such as the methylation status of the MGMT promoter, the amplification rate of the EGFR, as well as mutation status of the genes IDH 1 and 2, TP53, K-Ras, B-Raf and PTEN were assessed in comparison to the original tumor material (Table 2). The methylation status of the MGMT promoter was consistent between original tumor and cell lines. Methylation of the promoter occurred in 11/42 (26%) tumors and was maintained in the cell lines HROG02, HROG05, HROG13, HROG15 and HROG17. This coincided with no or only marginal (<0.001) cDNA expression. No methylation of the promoter was detectable in 27/42 (64%) tumors; for four tumor samples the status could not be assessed. The tumors HROG24 and HROG36 were scored unmethylated after bisulfide sequencing; however cDNA expression analyses revealed only marginal expression of MGMT cDNA (< 0.001) for both cell lines. Further the cell lines HROG04, HROG06, HROG07, HROG10 and HROG38, which were also scored unmethylated after bisulfide sequencing, did express detectable levels of MGMT cDNA (Table 2).

All cell lines expressed detectable levels of PTEN cDNA; however, mutations in the gene were very frequently (8/13; 62%) detected (HROG04, HROG05, HROG06, HROG15, HROG17, HROG24, HROG36 and HROG38). No mutations were detected in cell lines HROG02, HROG07, HROG10, HROG13 and HROG59.

A genomic amplification of the EGFR was present in 22/42 (52%) tumors; 13 (31%) of these tumors had a high amplification (>10x). No amplification was detectable in 15/42 (36%) tumors and five samples could not be analyzed. The amplification rate of the EGFR differed in seven (HROG02, HROG04, HROG05, HROG06, HROG07, HROG10, HROG17 and HROG24) out of the twelve cases when comparing the status of the original tumor to the one of the cell line (Table 2). Loss of the genomic amplification is a frequently described phenomenon in literature and explained by extra-chromosomal EGFR amplification (in form of mini-chromosomes) which is gradually lost in cell culture due to absence of selective pressure[118-120].

Of note, all mutations of the original tumors were maintained in the cell lines except those affecting IHD1. HROG02, HROG06 and HROG24 show a mutation in the TP53 gene; HROG05 has a mutation in the K-Ras gene. One mutation of B-Raf was detected in the tumor HROG23 (no successful culture). Mutations in the gene IDH1 were present in tumors HROG21 (still in culture – but very slowly growing) and HROG41; the mutation, however, was not maintained in the cell line HROG41. The

MGMT gene was completely deleted in HROG36 and only one allele was left in HROG24. No mutations in the analyzed genes were detected in tumors and cell lines HROG04, HROG07, HROG10, HROG13, HROG15, HROG17 or HROG38 (Table 2).

Sample ID		MGMT promoter status	MGMT cDNA expression	PTEN	EGFR [x fold]	Mutations
HROG02	tumor	M			3	TP53 R248Q
	cell line		<0.001	3.68	1	
HROG04	tumor	U			36	PTEN W274L
	cell line		1.02	12.73	1	
HROG05	tumor	M			82	K-Ras G12D PTEN P169S / del 212-229
	cell line		<0.001	1.01	1	
HROG06	tumor	U			82	TP53 R273H / R306* PTEN (+1 at 126)
	cell line		0.07	2.31	1	
HROG07	tumor	U			12	wt
	cell line		0.34	14.92	1	
HROG10	tumor	U			2	wt
	cell line		0.27	3.73	1	
HROG11	tumor	U			3	wt
	cell line		n.a.	n.a.	1	
HROG13	tumor	M			1	wt
	cell line		<0.001	2.88	1	
HROG15	cell line	M	<0.001	3.70	1	TP53 R273H PTEN S170N
HROG17	tumor	M			4	PTEN R130*
	cell line		<0.001	0.55	1	
HROG24	tumor	U			43	TP53 R273C MGMT CN=1 PTEN exon 3 del / spliced
	cell line		<0.001	2.21	1	
HROG33	tumor	U			31	wt
	cell line		n.a.	n.a.	1	
HROG36	tumor	U			1	MGMT CN=0 PTEN I5S
	cell line		<0.001	3.72	1	
HROG38	tumor	U			1	PTEN I224M / R234W
	cell line		0.23	0.02	1	
HROG41	tumor	M			1	IDH1 R132H
	cell line		n.a.	n.a.	1	
HROG59	tumor	U			16	wt / n.a.
	cell line		0.73	8.55	1	

Table 2: Molecular comparison of tumors and cell lines

This table summarizes molecular characteristics of tumors in comparison to the corresponding cell line. Listed are the methylation status of the MGMT promoter (M = methylated; U = unmethylated), the relative cDNA expression of the MGMT gene compared to the housekeeping gene TBP, the relative cDNA expression of the PTEN gene compared to the housekeeping gene TBP, the genomic amplification rate of the EGFR compared to the normal

diploid status (1 = 2n) and detected mutations of the genes TP53, PTEN, IDH 1 and 2, K-Ras and B-Raf (wt = wild type, if no mutations were detected; mutations are indicated by the position with the wt amino acid in front and the amino acid resulting from the mutation behind or * in case of a stop codon; n.a. = not assessed; wt / n.a. = wild type for IDH genes, B-Raf and PTEN; TP53 and K-Ras were not assessed; CN = copy number 1, when one copy of the gene was lost and 0 if both copies of the gene were lost; del = deletion of amino acids; spliced = alternatively spliced; +1 = insertion of a base leading to a frame shift).

Tumor samples of patients from whom cell line establishment was not successful were still molecularly analyzed (see Table 3).

Sample ID	MGMT promoter status	EGFR [x fold]	Mutations
HROG12	U	37	wt
HROG16	U	1	wt
HROG19	U	9	wt
HROG21	U	21	IDH1 R132H
HROG22	M	1	wt / n.a.
HROG23	U	2	B-Raf V600E
HROG25	U	1	wt
HROG31	U	2	wt
HROG32	U	44	wt
HROG34	U	97	wt
HROG42	U	1	wt
HROG46	M	125	wt
HROG47	U	70	wt
HROG49	U	1	wt
HROG54	M	1	wt / n.a.
HROG55	M	1	wt / n.a.
HROG56	U	1	wt / n.a.
HROG57	U	1	wt / n.a.
HROG58	U	1	wt / n.a.
HROG60	U	2	wt / n.a.
HROG63	U	19	wt / n.a.
HROG64	M	1	wt / n.a.

Table 3: Molecular details of tumor samples
This table summarizes molecular characteristics of the tumors. Listed are the methylation status of the MGMT promoter (M = methylated; U = unmethylated), the genomic amplification rate of the EGFR compared to the normal diploid status (1 = 2n) and mutations of the genes TP53, IDH 1 and 2, K-Ras and B-Raf (wt = wild type, if no mutations were detected; mutations are indicated by the position with the wt amino acid in front and the amino acid resulting from the mutation behind; wt / na = wild type for IDH genes and B-Raf, TP53 and K-Ras were not assessed).

The methylation status of the MGMT promoter still is the only well-established prognostic factor for patients diagnosed with a GBM tumor. Hence, the survival interval of patients with methylated MGMT promoter compared to patients with no

such methylation was assessed. In the Rostock patient cohort survival did not correlate with the methylation status of the MGMT promoter, neither for all patients nor for the cell line patients (Figure 3).

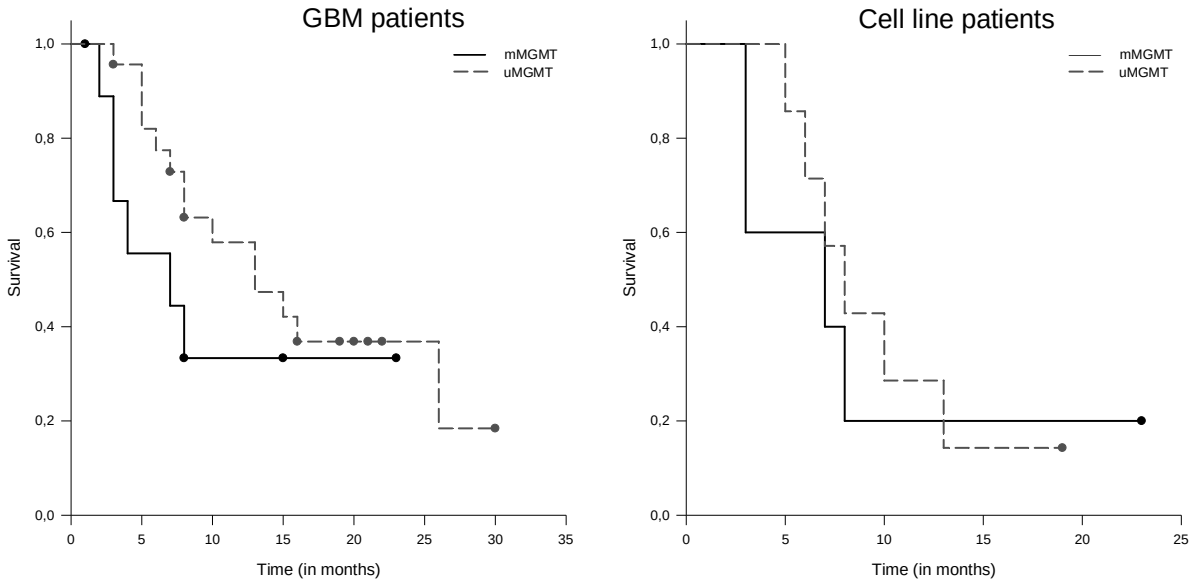


Figure 3: Survival of patients with methylated or unmethylated MGMT promoter
The Kaplan-Meier survival curves plot the survival interval (in months) of patients after the surgery of the GBM tumor, comparing survival intervals of patients with MGMT promoter methylated tumors (mGMGT) and without methylation (uMGMT). The graph on the left side (GBM patients) indicates the survival interval of the Rostock GBM patients (n=42); the graph on the right side ("cell line patients") indicates the survival interval of the "cell line patient" (n=12).

An amplification of the EGFR is a prevalent feature of GBM tumors; it equips these tumors with a growth benefit by promoting cell division and invasion as well as playing a role in therapy resistance[140,141]. However, controversial aspects on a prognostic value are reported ranging from positive over neutral to negative prognosis[142]. The survival interval of patients with a high amplification of the EGFR (>10x) was assessed in comparison to that of patients with no or only low EGFR amplification (<10x). In the Rostock patient cohort survival did not correlate with the amplification of the EGFR, neither for all patients nor for the cell line patients (Figure 4).

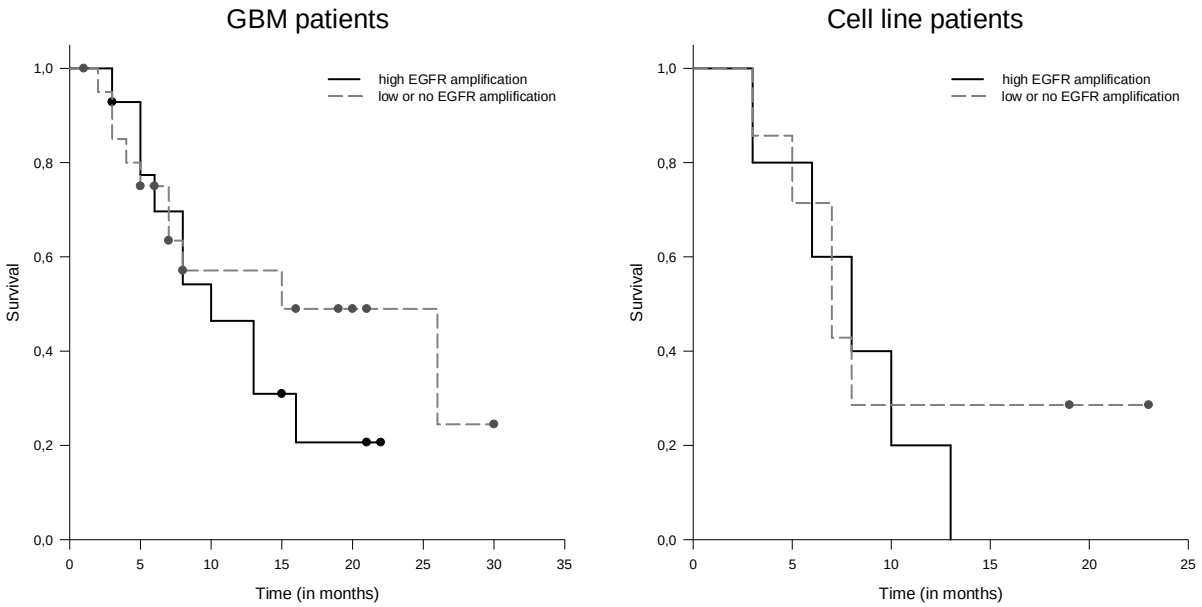


Figure 4: Survival of patients in regard to EGFR amplification

The Kaplan-Meier survival curves plot the survival interval (in months) of patients after the surgery of the GBM tumor, comparing survival intervals of patients with highly amplified EGFR in the tumors (>10x) to tumors with no or only low amplification (<10x). The graph on the left side (GBM patients) indicates the survival interval of the Rostock GBM patients (n=42); the graph on the right side ("cell line patients") indicates the survival interval of the "cell line patient" (n=12).

CGH arrays

A variety of chromosomal abnormalities are described for GBM (see above). For a detailed analysis addressing this issue in the GBM cell line collection (except for HROG38), a genomic analysis with very high resolution taking advantage of the SNP Array 6.0 from Affymetrix was performed. All, except for one cell line (HROG07) showed almost complete loss of at least one copy of chromosome 10. Chromosome 13q was deleted in 4/11 (HROG02, HROG05, HROG24 and HROG36). The most frequent amplification was for chromosome 7; in 8/11 cases an amplification was present (HROG04, HROG05, HROG06, HROG13, HROG15, HROG17, HROG24 and HROG36). The long arm of chromosome 9 (9q) was amplified in cell lines HROG02, HROG04, HROG05, HROG07, HROG17 and HROG36. Merely cell line HROG13 had a deletion at 1p and HROG36 at 19q; no co-deletions of the loci were detected (for a detailed view see supplementary material).

Molecular sub-typing

According to the molecular data and pieces of information obtained by the CGH arrays an attempt at sub-classifying the cell lines into the proneural, neural, mesenchymal and classical GBM types was undertaken (see Table 4). All but three

cell lines could easily be assigned to one specific sub-type. The cell lines HROG05, HROG17, HROG36 and HROG38 could not be categorized definitely. Despite the fact that HROG05, HROG17 and HROG36 lacked the loss of 17q11.2 and for HROG38 no CGH data was available, they were assigned into the mesenchymal subtype. This is based on the fact that all four cell lines had mutated PTEN genes and the mesenchymal sub-type is the most common one described for GBM cell lines[57].

All in all, 5/12 (42%) cell lines were categorized as mesenchymal, 4/12 (33%) as proneural and 3/12 (25%) as classical sub-type. None of the cell lines was classified as neuronal sub-type, which is also due to the lack of robust markers here for.

Sample ID	GBM sub-type	molecular characteristics
HROG02	proneural	TP53 mutated; 4q12 (PDGFRA) amplified
HROG04	classical	EGFR amplified; 9p21.3 (CDKN2A) deleted
HROG05	mesenchymal*	PTEN mutated
HROG06	proneural	TP53 mutated; 4q12 (PDGFRA) amplified
HROG07	classical	EGFR amplified; 9p21.3 (CDKN2A) deleted
HROG10	proneural	4q12 (PDGFRA) amplified
HROG13	classical	Chr. 7 amplified; chr. 10 lost; 9p21.3 (CDKN2A) deleted
HROG15	mesenchymal	PTEN mutated; 17q11.2 (NF) deleted
HROG17	mesenchymal*	PTEN mutated
HROG24	proneural	TP53 mutated; 4q12 (PDGFRA) amplified
HROG36	mesenchymal*	PTEN mutated
HROG38	mesenchymal*	PTEN mutated

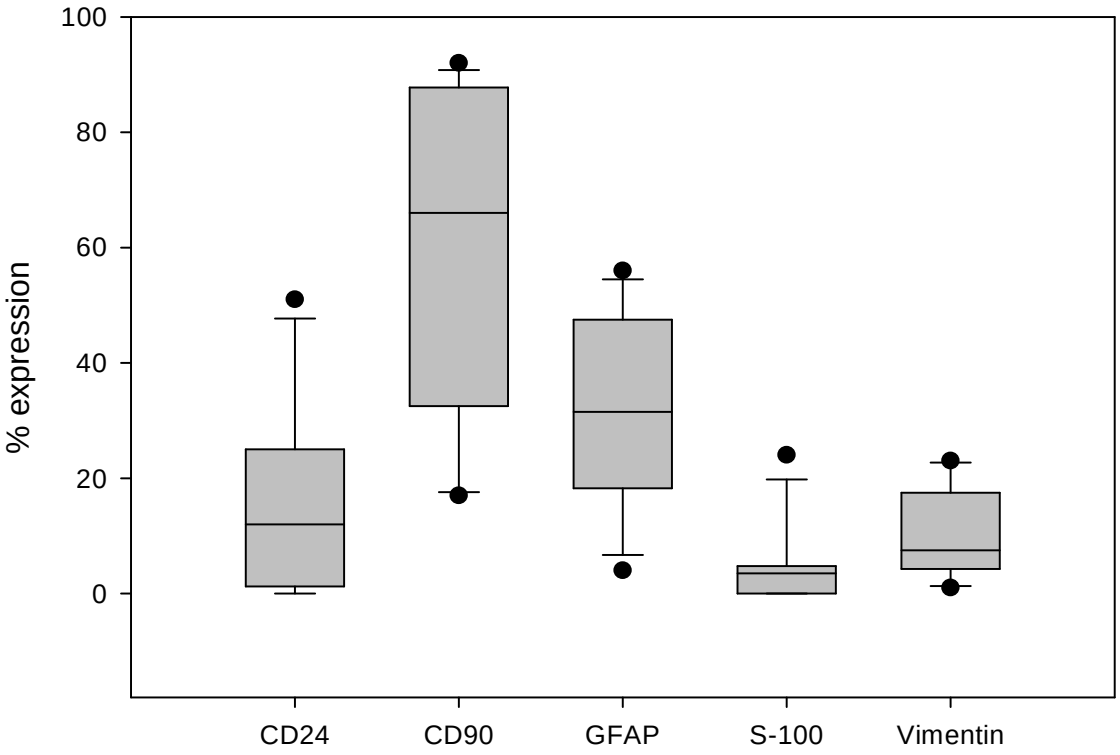
Table 4: Molecular sub-classification of cell lines

This table gives an overview on the sub-classification of the cell lines according to the characteristics described by Verhaak et al., 2010. Classifications for which only partial correlation with the molecular characteristics is fulfilled are marked with an asterisk. The respective molecular characteristics for each sub-type present in the cell line (or tumors of the patients for EGFR amplification) are indicated in the right column (chr. = chromosome).

Expression of neuronal and GBM markers

Proving neuronal origin, the expression of GBM (associated) cell surface markers such as CD24 and CD90 as well as neuronal markers glial fibrillary acidic protein (GFAP), S-100 and Vimentin was analyzed by flow cytometry (see Figure 5). In all twelve cell lines a high level of GBM (associated) markers, in especially CD90, was detectable. The expression of neuronal markers varied between the cell lines but was detectable in all cases (Figure 5). A population staining positive for GFAP (of at least 10%; except for HROG13 with only 5%) and Vimentin was present in all cell lines; Vimentin was expressed in less than 10% of cells for cell lines HROG02, HROG04,

HROG07, HROG13, HROG15, HROG17 and HROG38 and cell lines HROG05; HROG06, HROG10, HROG24 and HROG36 stained positive for more than 10% of cells. No expression of S-100 was observed in cell lines HROG02, HROG07, HROG17 and HROG38; cell lines HROG04, HROG05, HROG06, HROG10, HROG13, HROG15 and HROG24 had less than 10% positively stained cells; merely HROG36 had a population composed of more than 20% of cells. No differences in expression were observed between cell lines of newly diagnosed and relapsed



tumors.

Figure 5: Expression of GBM and neuronal markers

The percentage of cells expressing cell surface GBM markers CD24 and CD90 as well as intracellular expression of neuronal proteins GFAP, S-100 and Vimentin is depicted in the boxplot graphic. The grey box represents middle 50% of values; the line in the box is the median expression; whiskers indicate the range of the data set; outliers are plotted as dots.

Expression of BTSC markers

CSC are said to be responsible for sustaining the tumor and play a substantial role in therapy resistance, relapse and the metastasizing process.

The proportion of BTSC-like cells in the twelve cultures was accessed by flow cytometry (see Figure 6). In all cell lines at least 80% of cells expressed CD44; not only a BTSC marker but also a relevant factor for EMT and characteristic for the

mesenchymal subtype[57]. The degree of expressed BTSC markers (CD15, CD34, CD133 and Nestin) varied from cell line to cell line, but a small positive population was always present. Cell lines HROG02, HROG04, HROG05, HROG10, HROG13, HROG15 and HROG24 had only a small population (<10%) of CD15 expressing cells; HROG06, HROG07, HROG17 and HROG36 had more than 10% CD15 positive cells; only HROG38 did not stain positive for CD15. Only cell lines HROG04, HROG13 and HROG38 had less than 10% CD34 expressing cells; for the other nine cell lines more than 10% of cells stained positive for CD34. In half of the cell lines (HROG06, HROG10, HROG15, HROG17, HROG24 and HROG38) the population of CD133 positive cells was smaller than 10%. For the marker Nestin only HROG07 and HROG13 had less than 10% positive cells.

No differences concerning BTSC-like populations were detected between the cell lines from newly diagnosed and relapsed tumors.

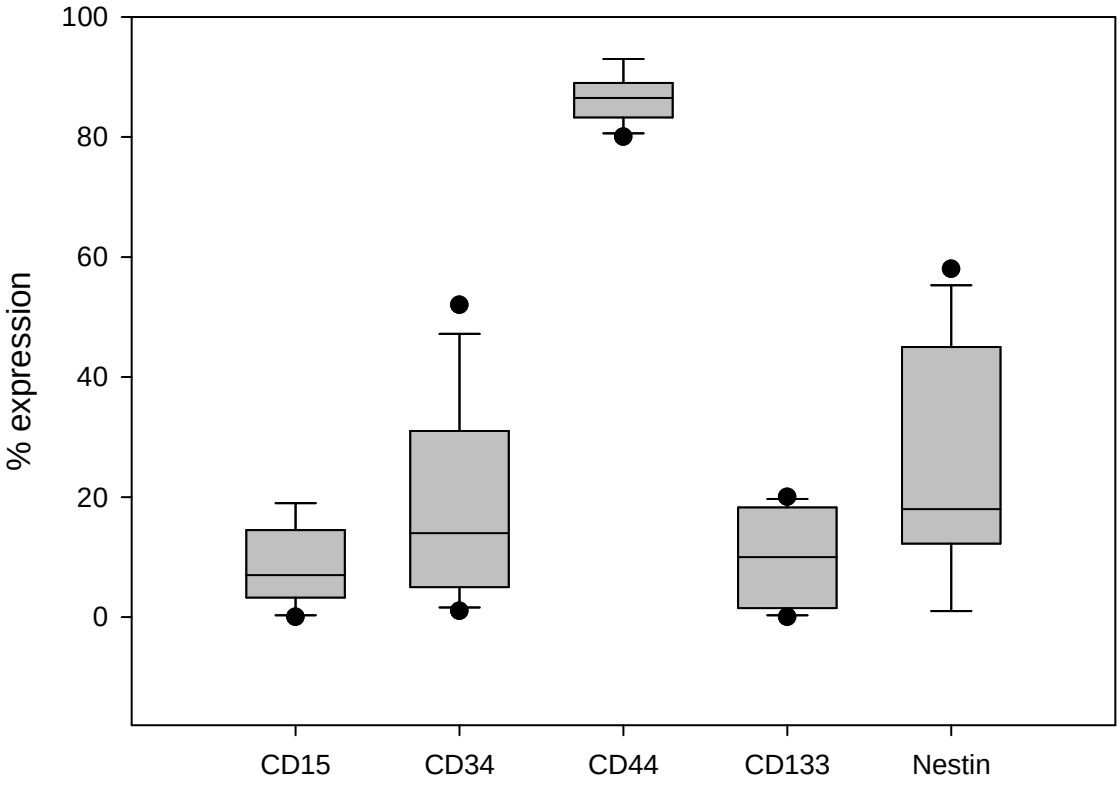


Figure 6: Analyses of BTSC populations

The percentage of cells expressing BTSC markers CD15, CD34, CD44, CD133 and Nestin is depicted in the boxplot graphic. The grey box represents middle 50% of values; the line in the box is the median expression; whiskers indicate the range of the data set; outliers are plotted as dots.

Expression of tumor associated antigens

Tumor specific antigens (TSA) or tumor associated antigens (TAA) qualify as prime target structures in therapeutic interventions. The expression (level) of TAA is of particular interest for immunotherapeutic interventions. Thus the presence of CEA, a TAA expressed by a variety of tumor entities such as melanoma, lung cancer, colon and gastric carcinoma[143-146] and of the GBM associated / specific TAA IL-13 receptor alpha (IL-13Rα), TGF-β and huntingtin interacting protein 1 (HIP1) was analyzed by flow cytometry (see Figure 7). Surprisingly, rather high levels of CEA were detectable in all cell lines. No differences were observed between cell lines form newly diagnosed and relapsed tumors. In contrast, the degree of GBM TAA varied less from cell line to cell line; generally only few cells stained positive for GBM TAA, yet a small positive population was always present (Figure 7). Again, no differences between cell lines from newly diagnosed and relapsed tumors were observed.

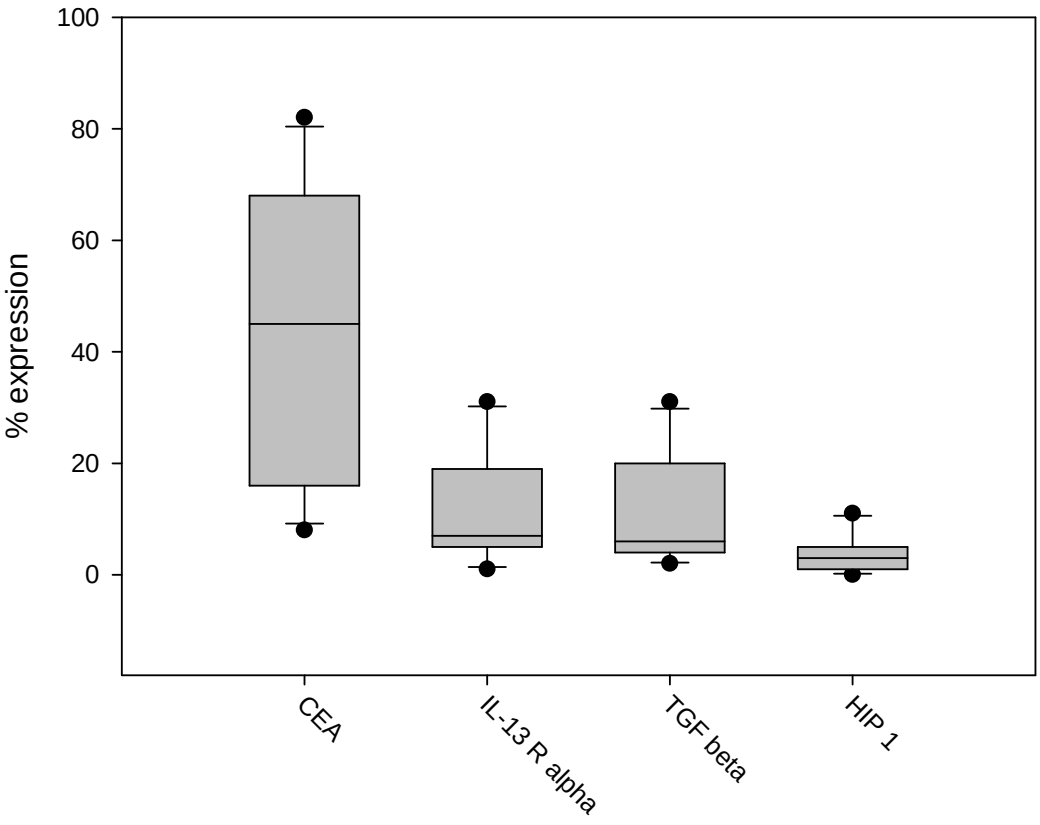


Figure 7: Expression of TAA

The percentage of cells expressing general and GBM specific TAA as CEA, IL-13Rα, TGF-β and HIP1 is depicted in the boxplot graphic. The grey box represents middle 50% of values; the line in the box is the median expression; whiskers indicate the range of the data set; outliers are plotted as dots.

HLA typing

With regard to experimental immunological analyses and for future development of immunotherapeutic strategies a two-digits encompassing HLA typing of the HLA loci HLA-A, -B, -C and HLA-DR and -DQ was performed. Of the assessed cell lines 10/12 (83%) were HLA-A2 positive and two of those even homozygous (see Table 5). The average distribution in the Caucasian race is about half the population is HLA-A2 positive[147]. However, since most studies, aiming at the identification of immunogenic epitopes from novel candidate antigens, are performed in an HLA-A2 restricted manner, this finding is of great academic interest.

Sample ID	HLA class I						HLA class II			
	A		B		C		DRB1		DQB1	
HROG02	*01	*02	*08	*13	*06	*07	*03	*07	*02	-
HROG04	*01	*02	*08	*51	*07	*15	*03	*11	*02	*03
HROG05	*02	-	*07	*40	*03	*07	*12	*13	*03	*06
HROG06	*01	*03	*08	*35	*04	*07	*01	*13	*05	*06
HROG07	*02	*26	*15	*27	*03	*07	*08	*15	*06	-
HROG10	*02	*23	*15	*44	*01	*04	*07	*09	*02	*03
HROG13	*02	-	*15	*44	*03	*05	*03	*06	*04	*13
HROG15	*02	*03	*15	*35	*03	*04	*03	*13	*02	*06
HROG17	*11	*66	*14	*40	*01	*08	*01	*12	*03	*05
HROG24	*02	-	*40	*44	*02	*05	*07	*13	*02	*06
HROG36	*02	*25	*40	*55	*03	-	*04	*14	*03	*05
HROG38	*02	*11	*13	*51	*03	*06	*04	*09	*03	-

Table 5: HLA typing results

The results of a 2-digits encompassing HLA typing for the loci HLA-A, -B and -C as wells as HLA-DR and -DP are listed. Information on both alleles is provided; in case of homozygosity the “second” allele is marked by -.

Tumor cell secreted cytokines

One frequent event in immune escape of tumors is establishing an immunosuppressive environment by attracting regulatory immune cells or secreting immunosuppressive cytokines. Tumor cells also can “communicate” with the surrounding tissue (the micromilieu) by cytokines.

The secretion levels of several cytokines with immunosuppressive and/or tumor relevant functions were assessed (see Table 6). All but one (HROG38) GBM cell line secreted high levels of IL-8. High secretion of IL-6 was detectable in 6/12 (50%) cell lines: HROG06, HROG10, HROG15, HROG24 and HROG36. Little IL-6 was present

in the supernatant of HROG05; and cell lines HROG02, HROG04, HROG07, HROG13 and HROG38 secreted no IL-6. Merely the cell line HROG04 secreted TGF-β. None of the cell lines secreted CEA or TNFα (results not shown).

Sample ID	IL-6 [pg/ml]	IL-8 [pg/ml]	TGF-β [pg/ml]
HROG02	0.0	482.0 / 483.0	0.0
HROG04	0.0	240.0 / 316.0	131.0 / 70.0
HROG05	0.0 / 13.3	498.0 / 477.0	0.0
HROG06	197.5 / 204.4	213.0 / 264.0	0.0
HROG07	0.0	347.0 / 281.0	0.0
HROG10	794.6 / 0.0	544.0 / 304.0	0.0
HROG13	0.0	559.0 / 394.0	0.0
HROG15	612.8 / 625.5	514.0 / 495.0	0.0
HROG17	658.8 / 656.0	519.0 / 520.0	0.0
HROG24	413.6 / 493.3	395.0 / 418.0	0.0
HROG36	478.8 / 708.0	502.0 / 526.0	0.0
HROG38	0.0	0.0 /11.0	0.0

Table 6: Cytokine secretion
The amount of cytokines secreted after 72 hours (value before the slash) and 120 hours (value after the slash) of cell culture are listed.

Drug response

A first step towards individualized therapy, response and resistance prediction may be establishing drug-response profiles for a variety of molecularly and phenotypically different patients – or more feasible from patient individual ultra-low passage cell lines.

Therefore, response of the GBM cell lines to increasing doses of drugs was assessed. The concentrations tested were oriented according to realistically achievable plasma levels in treated patients. Classical chemotherapeutics such as alkylating agents (BCNU, CCNU, Procarbazine and TMZ), anti-metabolites (Cytarabine and Methotrexate), topoisomerase inhibitors (Irinotecan and Topotecan) and other common chemotherapeutic agents (Cisplatin and Vincristine) were analyzed as well as substances ascribed to the rapidly growing class of targeted therapeutics. These included small molecules (Celecoxib, Imatinib, Nilotinib and Rapamycin) and therapeutic antibodies (Bevacizumab and Cetuximab). Further, Thalidomide, developed as a sedative (infamous due to the Contergan scandal in the 1950s) with detrimental consequences for the unborn of pregnant women, was found

to have anti-tumoral effects by inhibiting angiogenesis, was tested (IC_{50} values; Table 7a, b).

Responses to an agent varied between cell lines and sensitivity of a cell line to various agents differed as well. Response to CCNU, Cisplatin, Cytarabine and Topotecan correlated with the methylation status of the MGMT promoter or with cDNA expression levels. *In vitro* sensitivity was significantly higher in hypermethylated (cDNA expression <0.001) cell lines; with p=0.033 for CCNU, p=0.002 for Cisplatin, p=0.016 for Cytarabine and p=0.024 for Topotecan. In the case of the remaining alkylating substances BCNU, Procarbazine and TMZ however, no correlation of sensitivity towards the agents and the methylation status of the MGMT promoter could be observed. A general strong *in vitro* response to Vincristine was detected, yet the greatest variance between the different cell lines was observed for this substance (IC_{50} values ranged from 0.3nM to 244nM). Interestingly, in the five most sensitive cell lines (HROG02, HROG06, HROG15, HROG17, HROG24) to Irinotecan all four cell lines with mutated TP53 (HROG02, HROG06, HROG15 and HROG24) were found. In terms of serum level achievable amounts of Methotrexate had no influence on cell viability *in vitro*.

Sample ID	BCNU [µM]	CCNU [µM]	Procarbazine [µM]	Temozolomide [mM]	Cisplatin [µM]	Cytarabine [µM]	Methotrexate [mM]	Irinotecan [µM]	Topotecan [µM]	Vincristine [nM]
HROG02	68.0	20.0	20.0	2.0	1.0	1.0	>1.0	0.07	0.02	2.0
HROG04	209.0	241.0	33.3	3.5	>30	>500	>1.0	2.0	3.0	4.0
HROG05	110.0	137.0	35.7	1.2	4.0	0.5	>1.0	0.6	0.02	0.3
HROG06	111.8	98.3	37.2	0.5	10.0	500	>1.0	0.07	0.1	18.0
HROG07	279.0	359.0	38.7	2.0	>30	>500	>1.0	28.0	1.5	244
HROG10	158.0	101.0	25.0	1.5	16.3	>500	>1.0	65.5	1.2	244
HROG13	312.0	198.0	34.4	2.0	12.0	>500	>1.0	58.8	1.2	200
HROG15	52.0	101.0	35.7	0.8	7.0	3.7	>1.0	0.07	0.02	3.6
HROG17	21.3	61.0	3.8	0.05	3.2	0.1	>1.0	0.07	0.01	1.4
HROG24	28.3	21.8	30.1	0.2	1.3	3.8	>1.0	0.03	0.02	9.0
HROG36	46.0	27.5	31.6	1.2	3.2	0.5	>1.0	0.1	0.01	1.8
HROG38	136.6	237.4	32.6	1.0	17.0	32.0	>1.0	0.8	1.0	1.8

Table 7a: Response to chemotherapeutic agents

Calculated IC_{50} values (from three independent assessments in triplicates) for 144 hour incubation periods with the therapeutic agents are provided for all cell lines.

None of the three cell lines most sensitive to Thalidomide had detectable MGMT cDNA expression (>0.001), the cell lines HROG05 and HROG17 were methylated and in HROG36 the MGMT gene was completely deleted. The range for the IC_{50} values for Celecoxib was surprisingly narrow (48µM – 171µM). One cell line (HROG17) was sensitive in its response towards Imatinib (IC_{50} = 0.06µM) as well as to Nilotinib (IC_{50} = 0.02µM); otherwise the responses were rather heterogeneous. The cell lines most sensitive to Rapamycine were HROG17 and HROG24, both of which did not express MGMT cDNA due to methylation of the promoter in HROG17 and loss of one MGMT copy in HROG24. In contrast, the least sensitive cell line HROG38 expressed MGMT cDNA, as did the three next sensitive cell lines (HROG06, HROG07 and HROG10). No (cytotoxic) effects of the therapeutic antibodies could be observed *in vitro*.

Sample ID	Thalidomide [nM]	Celecoxib [µM]	Imatinib [µM]	Nilotinib [µM]	Rapamycin [µM]	Bevacizumab [mg/ml]	Cetuximab [µg/ml]
HROG02	>40	68	3.9	5.1	1.0	>2.5	>20
HROG04	>40	48	21.3	0.3	1.0	>2.5	>20
HROG05	40	51	149.6	5.3	1.5	>2.5	>20
HROG06	>40	126	3.9	6.3	3.0	>2.5	>20
HROG07	>40	171	39.1	7.5	6.0	>2.5	>20
HROG10	>40	74	0.06	8.9	6.0	>2.5	>20
HROG13	>40	60	39.6	9.8	2.7	>2.5	>20
HROG15	>40	67	15.6	6.3	3.0	>2.5	>20
HROG17	0.62	58	0.06	0.02	0.4	>2.5	>20
HROG24	>40	68	25.3	4.4	0.9	>2.5	>20
HROG36	10.0	60	4.2	>10.0	2.4	>2.5	>20
HROG38	>40	68	15.3	3.6	31.0	>2.5	>20

Table 7b: Response to small molecules and experimental drugs

Calculated IC_{50} values (after three independent assessments in triplicates) for 144 hour incubation with the therapeutic agents are provided for all cell lines.

4.3 Technical optimization of Model Generation

Since a feasible methodology and expedient protocols could be established, the next logic step seemed improving or rather optimizing these procedures with regard to higher efficacy and breaking down the logistics.

Success rates

We assessed attachment and outgrowth rates of 26 consecutive WHO grade IV GBM tumor samples and one relapsed astrocytoma, when prepared fresh directly after resection (culture #1) or after vital storage for varying periods of time in liquid nitrogen (culture #2). After fresh preparation, cells attached in 85% (24/27) of the cases and after vital freezing before preparation, attachment of cells occurred in 78% (21/27). Vital cryo-storage had no significant influence on attachment (p=1.000). Establishment of stable outgrowing cell lines was successful in 63% (17/27) of freshly prepared material and in 59% (16/27) after transient cryo-conservation. Again, cryo-storage had no significant influence on outgrowth of cells (p=1.000). The comparison of fresh and vitally frozen material prior to preparation is summarized in Table 8. The six most rapidly and stable outgrowing pairs of cell cultures (could be passaged >40 times) were subsequently characterized in detail. Cell lines derived from fresh material were marked with the suffix #1 and cell lines from vitally frozen material with the suffix #2.

Sample ID	Outgrowth		Sample ID	outgrowth		Sample ID	outgrowth	
	#1	#2		#1	#2		#1	#2
HROG02	✓	✓	HROG15	✓	✓	HROG26		
HROG04	✓	✓	HROG16			HROG31		
HROG05	✓	✓	HROG17	✓	✓	HROG32		✓
HROG06	✓	✓	HROG19			HROG33	✓	✓
HROG07	✓	✓	HROG21	✓	✓	HROG34		✓
HROG10	✓	✓	HROG22		✓	HROG36	✓	✓
HROG11	✓	✓	HROG23	✓		HROG38	✓	
HROG12			HROG24	✓		HROG41	✓	
HROG13	✓	✓	HROG25			HROG42		

Table 8: Outgrowth of *in vitro* models

A comparative overview on the success of cell line establishment from the fresh and vitally frozen tumor material; successful cell line establishment is indicated by a check mark.

Morphology and growth kinetics

In a first step, the cell lines were micro-photographed to compare the morphology of the cell line pairs. In Figure 8, the morphology of the newly established tumor cell lines is depicted, showing the pairs side by side for a direct comparison. Furthermore, doubling times of the cell lines were assessed and are presented pairwise in Figure 8. In all cases the pairs showed high similarity in regard to their morphology and doubling times but differences between the different cell lines were obvious. Morphologically all cell lines show a fibroblast-like phenotype, and no differences between cell lines established fresh and from previously frozen tumors became apparent. Doubling times ranged from 35/40 hours (#1/#2) for HROG36 to 74/65 hours for HROG13. HROG17 having doubling times of 43/32 hours, followed by HROG02 with 36/54 hours, HROG05 with 48/44 and finally HROG06 with 59/57 hours.

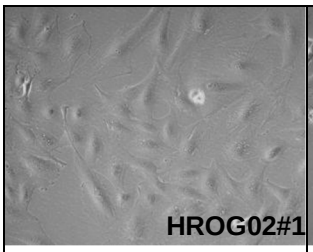
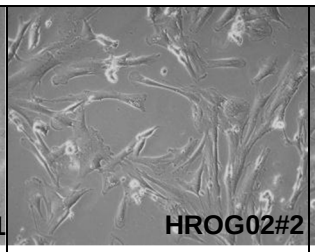
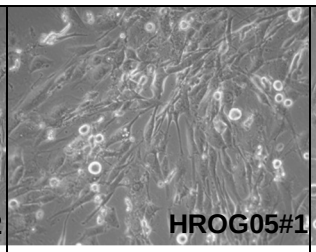
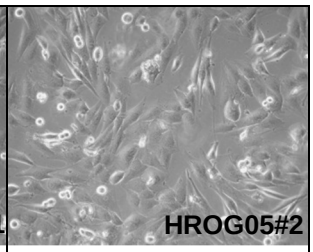
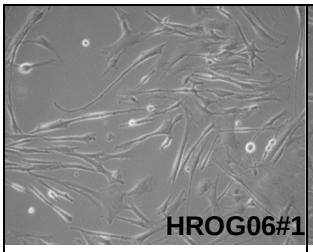
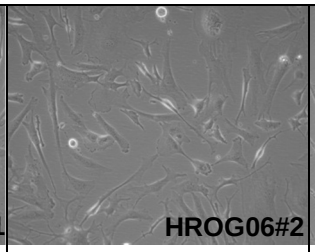
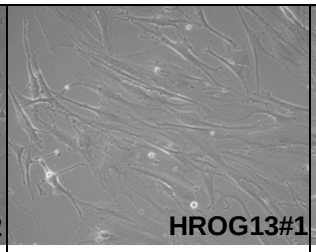
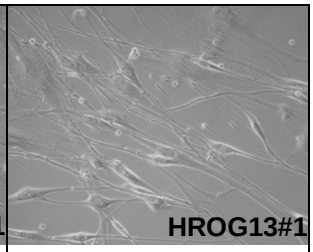
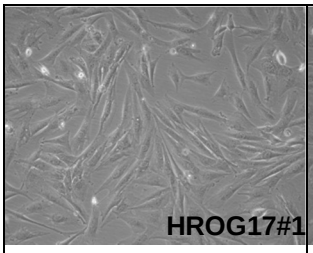
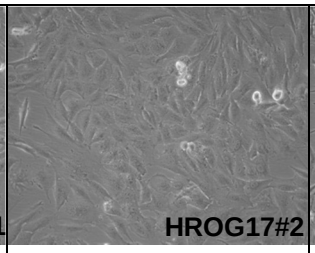
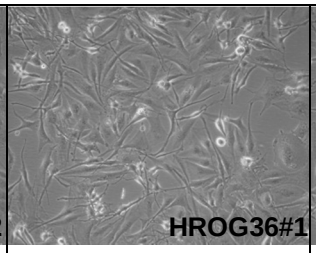
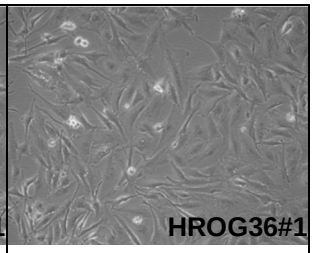
 HROG02#1	 HROG02#2	 HROG05#1	 HROG05#2
38 (± 2.7) hours	48 (± 3.4) hours	48 (± 9.4) hours	44 (± 9.4) hours
 HROG06#1	 HROG06#2	 HROG13#1	 HROG13#1
59 (± 14) hours	57 (± 5.6) hours	74 (± 2.7) hours	65 (± 4.3) hours
 HROG17#1	 HROG17#2	 HROG36#1	 HROG36#1
43 (± 9.2) hours	32 (± 5.0) hours	35 (± 4.0) hours	40 (± 8.0) hours

Figure 8: Cell line pair morphology and doubling time

Phenotypes of the cell lines captured by micro-photography (100x enlarged) are displayed pairwise and doubling times (in hours) of the cell lines are given.

Molecular data

Molecular markers relevant for GBM such as the methylation status of the MGMT promoter, the amplification rate of EGFR, as well as mutation status of the genes IDH 1 and 2, TP53, K-Ras and B-Raf were assessed for the cell line pairs in comparison to the original tumor material (Table 9). The methylation status of the MGMT promoter was always consistent between original tumor and cell line pairs.

The amplification rate of the EGFR differed in 4 (HROG02, HROG05, HROG06 and HROG17) out of 6 cases when comparing the status of the original tumor to the cell lines. No differences were, however, observed between the cell line pairs (Table 9).

Of note, all mutations detected in the original tumors were maintained in the cell lines. HROG02 and HROG06 show a mutation in the TP53 gene and HROG05 has a mutation in the K-Ras gene. No mutations were detected in HROG13, HROG17 or HROG36 and similarly, we did not observe any hot spot mutations in the genes IDH1 and 2 or B-Raf (see above Table 2).

Sample ID		MGMT promoter PMR	MGMT promoter status	EGFR [x fold]
HROG02	tumor	25	M	3
	#1	39		1
	#2	47		2
HROG05	tumor	35	M	82
	#1	13		2
	#2	58		2
HROG06	tumor	0	U	82
	#1	0		3
	#2	0		2
HROG13	tumor	4	M	1
	#1	22		1
	#2	19		1
HROG17	tumor	13	M	4
	#1	6		1
	#2	4		1
HROG36	tumor	0	U	1
	#1	0		1
	#2	0		1

Table 9: Molecular comparison

This table summarizes molecular characteristics of tumors in comparison to the corresponding cell line pairs. Listed are the PMR values and thereby scored methylation status of the MGMT promoter (M = methylated; U = unmethylated) and the genomic amplification rate of the EGFR compared to the normal diploid status (1 = 2n).

Drug response

For functional comparison of the cell line pairs, the sensitivity of each cell line (pair) towards a panel of therapeutic agents commonly used for GBM treatment was assessed (summarized in Table 10). As expected, the response to different drugs varied within a given cell line. Similarly, the response to one agent varied between the different cell lines. Notably, no severe differences in regard to sensitivity to one agent were observed when comparing the cell lines in matched pairs (TMZ p=0.551; BCNU p=0.431; Vincristine p=0.259; Imatinib p=0.247). There was only one exception from this rule. For HROG36 minor dissimilarities were observed with regard to the substance Vincristine (p=1.000). The IC_{50} values of HROG36#1 and HROG36#2 are 79nM and 42nM respectively; but HROG36#1 plateaus at about 50% of dead cells.

Sample ID	TMZ [µM]		BCNU [µM]		Vincristine [µM]		Imatinib [µM]	
	#1	#2	#1	#2	#1	#2	#1	#2
HROG02	2,010	2,004	88	48	105	97	218	105
HROG05	1,205	1,245	23	66	0.2	0.9	144	86
HROG06	490	575	88	95	3	1	88	88
HROG17	39	15	85	57	0.9	0.4	151	133
HROG36	1,201	1,235	223	159	0.8	0.4	159	184

Table 10: Comparison of drug responses

This table summarizes calculated IC_{50} values (after three independent assessments in triplicates) for 144 hour incubation with the therapeutic agents for all cell lines.

In summary, no obvious discrepancies in drug sensitivity of the cell line pairs were observed. Thus, functional drug response measurements of tumor samples obtained from individual GBM patients are not influenced by a transient cryo-preservation step before the start of culture; this may be of special interest for future clinical studies.

4.4 Model application for translational Research

Recent research in GBM focuses on novel targeted compounds, in addition to standard chemotherapy. One of the emerging compounds is CGT, which by binding to integrins (i.e. αvβ3 and αvβ5) may inhibit angiogenesis and also is directly cytotoxic to tumor cells by interfering with intracellular signaling pathways. At the present an interventional study on the combination of CGT and metronomic TMZ in children and adolescents with relapsed or refractory high grade gliomas or diffuse intrinsic pontine gliomas is recruiting patients (HGG-CilMetro; NTC01517776). The

Children´s Hospital of the University Medicine Rostock is part of this multicenter clinical trial, thus an accompanying laboratory investigation on the effects of a combination therapy was performed.

Integrin expression

The expression of integrins targeted by CGT was analyzed by flow cytometry. In all cell lines a high general expression of av integrins was detectable (Figure 9 and Table 11). The degree of avβ3 and avβ5 expression varied between the cell lines but was positive in all cases (Figure 9). Strong staining for avβ3 and avβ5 integrins was detected in the cell lines HROG02, HROG15 and HROG17. An intermediate staining was seen in HROG05, HROG10 and HROG36, followed by relatively weak staining in HROG04, HROG06, HROG13 and HROG38.

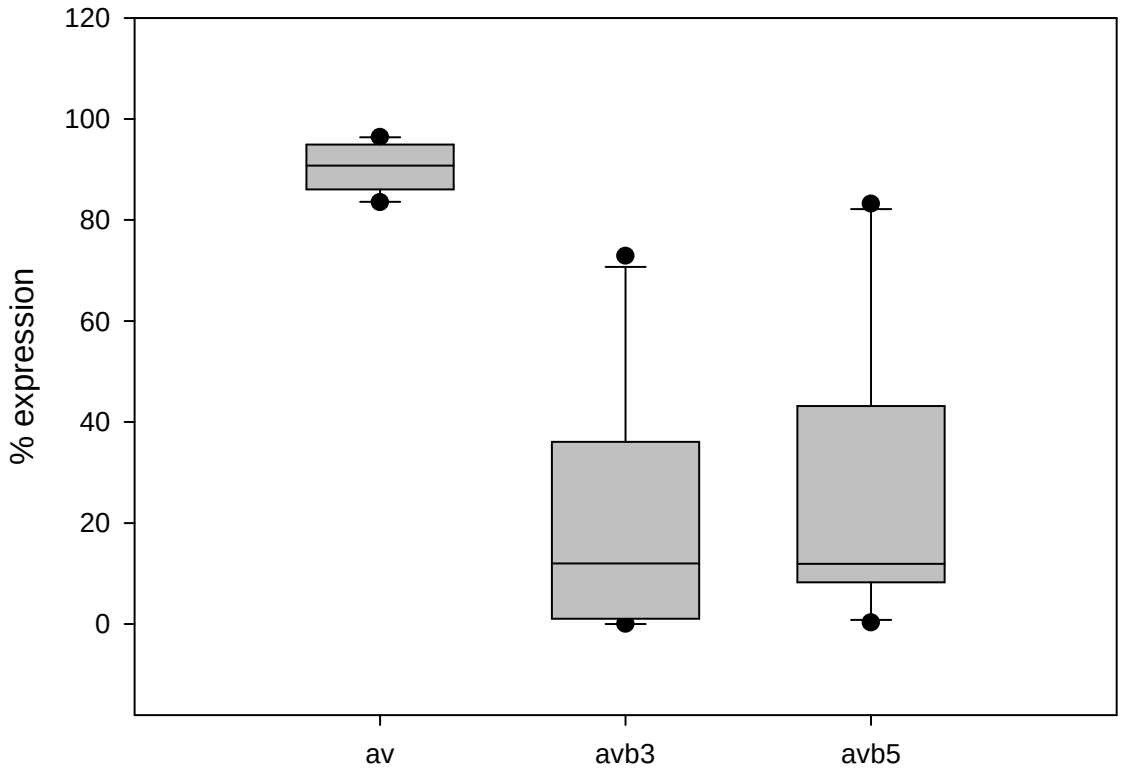


Figure 9: Integrin expression

The percentage of cell lines expressing pan-av, avβ3 and avβ5 integrins, as assessed by flow cytometry, is illustrated in a box plot diagram. The grey box represents middle 50% of values; the line in the box is the median expression; whiskers indicate the range of the data set; outliers are plotted as dots.

Determination of IC_{50} values for TMZ and CGT

In a first step, response of the GBM cell lines to increasing doses of TMZ and CGT was assessed (IC_{50} values; Table 11). Response to TMZ correlated with the methylation status of the MGMT promoter and was significantly higher in hypermethylated cell lines (p=0.016). In addition, we observed strong responses to CGT; IC_{50} values did not exceed 20µM in any of the assays (Table 11), while plasma levels in patients receiving CGT peak at 40 – 50µM[85]. Responses to CGT also seemed to correlate with the methylation status of the MGMT promoter. Contrary to TMZ, cell lines with unmethylated promoter strongly tended to respond better to CGT (p=0.066).

Sample ID	IC_{50} values		Integrin expression [% / MFI]		
	CGT [µM]	TMZ [mM]	pan αv	αvβ3	αvβ5
HROG02	7.0	0.5	88.7 / 5.8	51.0 / 3.6	72.8 / 6.2
HROG04	5.4	3.5	96.4 / 2.9	3.0 / 0.9	10.8 / 1.2
HROG05	6.0	0.5	90.1 / 2.8	14.8 / 1.4	23.8 / 2.8
HROG06	8.0	1.5	96.3 / 6.7	1.4 / 0.1	5.5 / 1.3
HROG10	5.4	1.5	83.5 / 3.6	10.6 / 0.6	9.2 / 1.4
HROG13	2.0	2.0	84.4 / 0.7	0.1 / 0.2	0.3 / 0.0
HROG15	10.0	0.8	94.5 / 9.1	31.1 / 2.2	83.2 / 7.2
HROG17	5.0	0.1	86.6 / 4.5	72.9 / 3.9	33.3 / 2.5
HROG36	20.0	0.8	94.2 / 4.5	13.4 / 1.6	12.6 / 1.8
HROG38	0.8	1.0	91.4 / 0.2	0.0 / 0.2	11.3 / 4.0

Table 11: IC_{50} values and integrin expression

Calculated IC_{50} values (mean of three independent assessments in triplicates) for TMZ and CGT are provided for the ten cell lines. The expression of integrins was assessed by flow cytometry and is given as % expressing cells and as MFI [= (fluorescence intensity of sample – fluorescence intensity of control) / fluorescence intensity of control].

Combination treatment

Next, we studied potential additive or synergistic effects of combined CGT and TMZ treatment. A functional *in vitro* test regimen was performed by combining three CGT concentrations with three TMZ concentrations. We decided on the following three TMZ doses for subsequent analysis: a low concentration (5µM), comparable to that used in metronomic treatment[148], an intermediate concentration (50µM) consistent with plasma levels in patients receiving standard treatment[149]; and a very high concentration (500µM) to study maximum effects. In case of CGT we also chose three doses: a low concentration (1.4µM), comparable to the IC_{50} value of the sensitive cell lines; an intermediate concentration (7µM), which is close to the IC_{50}

value of the majority of tested cell lines; and a high concentration (10µM), representing the average IC_{90} value.

In all cases CGT monotherapy was more effective than TMZ monotherapy. In cell lines harboring a methylated MGMT promoter addition of TMZ had a beneficial effect (Figures 10). The effects of the combined *in vitro* treatment regimen ranged from almost additive (HROG17 and HROG36) to synergistic (HROG02 and HROG15) (Figure 10). In the unmethylated promoter setting, CGT monotherapy had even greater effects on cell viability than in the methylated setting; but the addition of TMZ showed no further benefit (Figure 10).

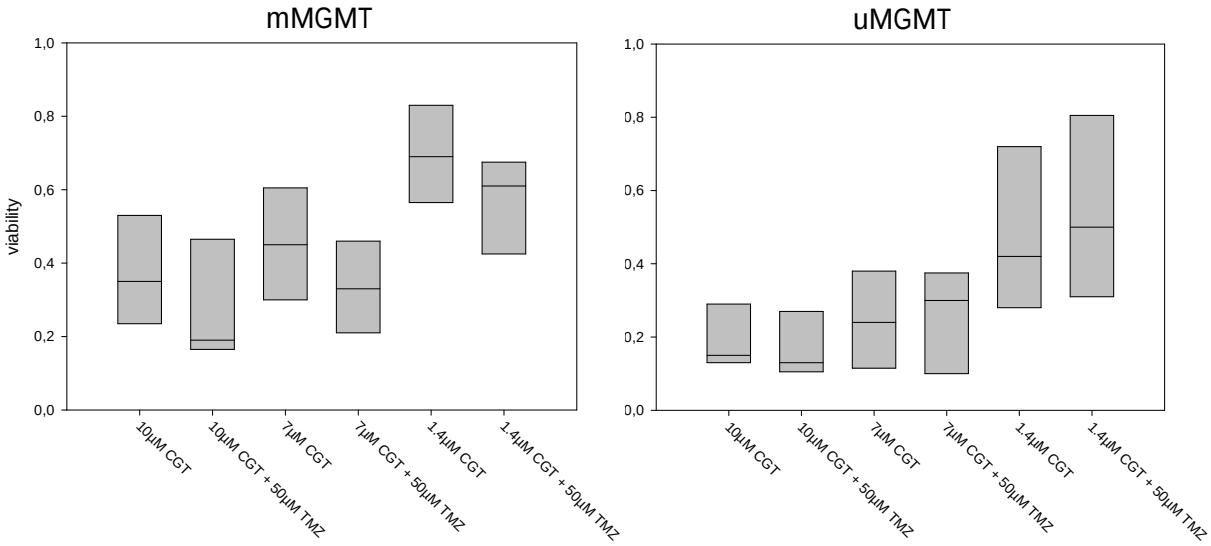


Figure 10: Combination treatment

Viability of cells treated with decreasing amounts of CGT (10µM, 7µM and 1.4µM) alone and in combination with 50µM TMZ is depicted. The fluorescence intensity is normalized (1 = untreated cells; 0 = alcohol treated/dead cells). On the left hand side (mMGMT) MGMT promoter hypermethylated cell lines, on the right hand side (uMGMT) MGMT promoter unmethylated cell lines.

5. Discussion

Over a decade ago, Prof. Stefaan van Gool initiated the HGG-IMMUNO group and corresponding meeting with the aim of uniting forces of physicians and scientist, and promoting the idea and concepts of an autologous DC vaccine for patients with brain tumors. The ultimate goal remains providing all patients suffering from (GBM) tumors with access to such an immunotherapeutic intervention. Patients with relapsed GBM were first vaccinated in the university hospital in Leuven (UZ Leuven) in 2000[64]. As source of antigens for presentation, the DC are loaded with tumor lysate and after repeated subcutaneous applications are capable of inducing a tumor specific immune reaction. The great success of this treatment strategy not only led to the extension of the Leuven treatment setting and research but also inspired many partners to follow their example. One of the limiting factors in this process is the amount of accessible tumor material; in fact “no tumor material” is an exclusion criterion. As part of the consortium, the AG Hirntumorvakzine supports the concept with basic and translational research. A simple yet elegant solution for the antigen source / tumor material may be the establishment of permanent cell lines out of a small piece of the resected tumor. Nearly unlimited amounts of tumor lysate could be produced *in vitro*. At the same time these cell lines would also provide an opportunity to address a multitude of questions concerning the biology, immunology and genetic composition of these tumors. Finally, individualized response prediction for other treatments in conjunction with DC could be ideally tested.

In consequence, the aim of this work was to generate a collection of GBM models reflecting the entire clinical appearance of GBM grade IV cases. Successful and feasible protocols for model generation could be established. The clinical characteristics of the HROG patient cohort and thus of the analyzed cell line collection matched fairly well with published data of “typical” GBM patient cohorts. The median age at surgery in the Rostock patients was 62 years; when stratifying the patients by diagnosis with primary GBM tumors and secondary, 93% of the patients had been diagnosed with a primary and only 7% with a secondary GBM. Such a predominance of primary GBM (about 90%) versus secondary (about 10%) is well established in literature[4]. The median age for primary GBM was 63 years and 53 years for secondary GBM. This correlates with data from the literature with 64 years in median for primary GBM and 45 years for secondary[6,7]. Since there were only

three secondary GBM tumors represented (age 44, 71 and 45 years) one outliner largely raised the median age. The overall male to female ratio was 1.3 to 1 and 1.1 to 1 for the grade IV tumors. In literature preponderance for the male sex is described with male to female ratios ranging from 1.3 – 1.5 to 1[1,8]. Survival of patients correlated with the WHO grading of the tumors (worst for patients with WHO grade IV tumors) and patients with newly diagnosed GBM survived significantly longer than those suffering of a relapse (p=0.029) as is described in literature[150,151]. Patients with a methylated MGMT promoter tended to survive longer than patients showing no such methylation, the correlation of MGMT promoter methylation and prolonged survival is common knowledge[15]. However, in the Rostock cohort, no significance was reached. This may be due to the still low number but should be followed in future clinical data analysis in order to exclude any therapeutic bias.

The analyzed patients from Rostock reflected age and gender distribution fairly well and the cohort size seemed to be representative.

The distribution of all patients between newly diagnosed and relapsed GBM were 28/42 (67%) newly diagnosed tumors and 14/42 (33%) relapses. Of note, the distribution in the “cell line patients” was 8/12 (67%) newly diagnosed GBM and 4/12 (33%) relapses, too.

In vitro attachment rates to the culture dishes were above 85% when cells were prepared freshly directly after surgery. No differences were observed between attachment of newly diagnosed and relapsed GBM (p=1.000). These attached cells would already recommend themselves as a starting point for decision making in an individualized therapy manner (response prediction for single agent treatment or combinations) or at least for predicting development of resistance in a first simple screen.

In vitro outgrowth of cells was successful in 60% (61% for newly diagnosed and 57% for relapsed GBM). Again no differences between newly diagnosed and relapsed GBM were observed (p=1.000). The HROG cell line establishment rates are for the most part superior to what is described in literature. Take rates range from 3% for pediatric brain tumors[152] over 10%[153] to 21%[154] for adult GBM tumors. However, most authors do not comment on any statistics at all. The most stable outgrowing and subsequently characterized twelve cell lines included eight newly diagnosed and four relapsed tumors, meaning successful stable outgrowth in 29% of cases for both

newly diagnosed and relapsed tumors. Obviously, there was no difference between the two types (p=1.000).

In contrast, i*n vivo* engraftment of tumor pieces into immunodeficient mice led to outgrowth in 22% of tumors. Separating newly diagnosed tumors from relapses, the success was 16% and 36%, respectively. Take rates from the literature vary largely depend on sample size and mouse strains used as hosts for engraftment: for subcutaneous engraftment into the flanks of mice, about one third successful engraftment into Nude-Foxn1 nu mice[136] is comparable to the observed rates for the HROG tumors. However, successful engraftment in half of cases into NMRI nu/nu mice[154] and nearly 60% (but mouse strain not mentioned)[119] are also described in the literature. Orthotopic implantation into the brain of NSG mice was successful in three quarters of cases[137].

Although differences observed between newly diagnosed and relapsed tumors were apparent, no level of significance was reached – most likely due to the limited sample size. The question whether vital cryo-storage of tumor pieces has an influence on outgrowth in immunodeficient mice or not is currently being addressed in a direct comparative study. Here, pieces of a tumor are engrafted immediately after surgery and after vital storage in nitrogen. This has not been performed so far and thus represents a valuable twist to xenografting, since it allows engraftment of many tumors at the same time and pre-selection according to pathological diagnosis and grading.

Establishment of cell lines was more successful than *in vivo* engraftment. This is in sharp contrast to success rates for colorectal carcinomas in our group[135] but very much in line with data from the literature on GBM[114]. Thus, any handling artifacts are very unlikely but it rather underlines differences between entities and modeling systems. However, success rates both *in vitro* and *in vivo* may be improved. Although the rate of *in vitro* attachment is satisfyingly high this may even be augmented further by applying different coating substances beside collagen, e.g. fibronectin or laminin[125,155] and supplementing the media with further additives such as EGF, VEGF and bFGF[125,156]. Different cell culture options such as spheroid culture, stem cell conditions or different additives are possible and might have positive impact on success. In this manner success rates of nearly 100% are described[125]. Growth factor supplementation such as EGF and bFGF is thought to enhance stemness and

promote expansion of BTSC (-like) cell populations[24,125,126]. However, this is ongoing work and shall be analyzed in detail in the near future.

For the *in vivo* system, different immunodeficient mouse strains are available as touched on above and may be associated with differences in engraftment rates. The NMRI nu/nu mice used in the HROG study have deficient T cells but fully functional B and NK cells. NOD SCID or SCID Beige mice may be more suitable, since these mouse strains have deficient T and B cells as well as impaired NK cells. NSG mice additionally lack functional NK cells and are considered best for achieving high engraftment rates (Dr. I. Fichtner, EPO GmbH, Berlin, personal communication). Implanting the tumor (pieces) in an orthotopic manner may further augment the success rate since GBM tumors are strongly dependent on the microenvironment[157]. Further means may include co-implanting matrigel (provides a matrix for cell attachment and contains growth factors) as well as tumor-supporting fibroblasts or even glia cells[157]. On the pro side of *in vitro* models are the fast and easily feasible method and high success rates[114] thus cell cultures provide a good model for a first drug screen on response and resistance development. Subsequent testing and verification may then more selectively be performed using *in vivo* model(s) – preferably established in parallel to the cell lines.

The morphology of the established HROG cell lines was a fibroblast-like appearance of the cells with some polygonal variations. The most common GBM cell morphology is a fibroblast-like or epithelial-like one [ATCC and DSMZ]. Cells rarely changed their morphology during the culturing process.

The doubling times ranged from 35 to 89 hours, which is in accordance with what was described by others for GBM cell lines[158,159]. Of note, the doubling times for all cell lines were assessed for passage 20 and lower; the doubling time tends to increase with culturing time[160,161].

One major drawback of tumor models in general is a trend towards genetic drift (in comparison to the original tumor material)[162]. Thus, the GBM models generated in this work were compared to the primary GBM tumor tissue presented to the department of pathology for routine diagnosis.

High preservation of the GBM tumor´s molecular features was achieved in the cell cultures. Particularly the methylation status of the MGMT promoter and mutations in

the genes TP53, K-Ras and B-Raf were maintained in the cell lines. There was one exception from this rule concerning the mutation status of the IDH 1 gene, which was not maintained in the *in vitro* model (HROG41). Another exception is the gradual loss of EGFR genomic amplification under standard *in vitro* culture conditions. However, this is no drawback of the present work since both phenomena are well described in literature[118-121].

More and more extensive molecular pathological analyses are being performed on GBM tumors. This is no longer restricted to immunohistochemistry (hematoxylin and eosin staining, Ki67, GFAP, Nestin) and methylation analyses (of the MGMT promoter) but meanwhile includes a broad spectrum of mutation and gene amplification analyses. An antibody directed against the most common mutation (R132H) of the IDH 1 gene is used to distinguish between primary and secondary GBM[163] and has simplified the pathologist's decision making in this regard substantially (personal information of Prof. Prall, Institute for Pathology, University Medicine Rostock). In rare cases, fluorescent *in situ* hybridization analyses may also be part of the repertoire, e.g. for EGFR amplification detection[164].

The extensive molecular pathological analyses are not only required for detailed diagnosis but also have clinical relevance. In breast carcinoma patients the human epidermal growth factor receptor 2 (HER2)/neu is relevant for therapy with the monoclonal antibody Trastuzumab; only patients with an amplification of the HER2/neu receptor profit of this therapy[165]. For GBM patients not responding to the first line therapy (radio-chemotherapy with TMZ) a variety of alternatives including targeted therapeutics are available. Patients with amplification of the EGFR may receive Cetuximab, a monoclonal antibody directed against this. Alternatively, Panitumumab, a further monoclonal antibody directed against EGFR might be used[166]. However, effectiveness of both antibodies seems to be restricted to the expression of the wild type form of the receptor[167]. Bevacizumab is an antibody directed against the growth factor VEGF and thus could inhibit tumor vascularization and (neo-) angiogenesis[168].

Many recent studies, i.e. clinical testing of targeted therapeutics, have not led to the expected results but rather fell short of the high expectations[169].

This is to a big part attributable to the fact that GBM consist of very heterogeneous tumors[169] and thus comes as no big surprise. One line of argumentation is that given the great heterogeneity, and thus not adequately considering this fact when recruiting

for clinical studies, it may obscure beneficial effects for individual GBM sub-types. Taking this argumentation further one could even dare to state that this may imply that GBM is not one disease but rather a common heading of different glioma tumors. So these “individual” diseases should be treated as such and patient cohorts for clinical trials should be carefully defined. In line with this argumentation is the initiative to sub-classify GBM tumors into the categories, e.g. in proneural, neural, mesenchymal and classical by designating specific molecular characteristics to these sub-groups[57]. Duarte and coworkers used this approach for a correlation of GBM sub-types and prognosis. They described a gene signature in the proneural subtype of GBM which may be responsible for the poor prognosis by chemotherapy and/or radiation resistance in those cells[170]. Their results might have strong implications both for better prediction models for survival and improved understanding of the underlying subtype-specific molecular mechanisms for GBM tumor progression and treatment response.

A key feature of cancer development is the progressive accumulation of genomic changes resulting in the loss of tumor suppressor functions, the activation of oncogenes and/or the generation of fusion genes with oncogenic potential[171]. Such complex structural and numerical alterations in the genome leading to changes in the DNA copy number are characteristic also of GBM tumors[172].

In essence, amplifications recommend themselves as (proto-) oncogenes and deleted sequences as tumor suppressor candidates. An in-depth analysis of a large number of GBM samples and cell lines will provide the most accurate information on the amplifications and deletions most frequent in GBM. This might further advance sub-classification and shed light into this heterogeneous disease.

In this regard, a genomic analysis with very high resolution taking advantage of the SNP Array 6.0 was performed. This technique allows comparative genomic analyses at a very high resolution[173,174]. The most frequent alteration was a complete or at least partial loss (minimum one allele) of chromosome 10. It was observed in 10/11 cell lines (91%) and this loss has been associated with a poor prognosis[53]. However, numerous more unique amplifications and deletions were present in all GBM cell lines. Some of these alternations serve as markers for a GBM sub-classification. Amplification of chromosome 7 and loss of chromosome 10 are common in the

classical, deletion of 17q11.2 in the mesenchymal and amplification of 4q12 in the proneural sub-type[57].

In the context of the present work, these data were not further analyzed concerning candidate tumor suppressor or oncogenes. A provisional analysis performed together with Dr. B. Schneider from the Institute of Pathology revealed a number of promising candidate genes which shall be validated in the near future.

I would like to hint towards another point that can be brought forward in favor of cell lines: any contamination with normal cells can be excluded here and thus the data obtained from array CGH analyses are less likely to contain misleading results. However, even then, one cannot guarantee to see all differences which may be present in sub-clonal populations of these cell lines.

The neuronal origin of the cell lines was proven by staining the cells with different neuronal and GBM (specific) markers and detection was performed by flow cytometry. As expected, all cell lines were of neuronal origin (highly positive for CD90). CD90 plays a primal role in BTSC formation and $CD90^+$ cells localize around vascular structures[175]. The expression of CD90 is significantly higher in WHO grade III and IV tumors compared to grade I and II; thus CD90 is a marker for high grade gliomas[175]. Moreover, a higher expression is associated with an undifferentiated GBM cell type[176].

In contrast to CD90 the other markers (CD24, GFAP, Nestin and S-100) were expressed to a much lower and also more varying degree in the HROG cell lines.

The two major intermediate filament proteins of astrocytes are Vimentin and GFAP. Early in development, radial glia and immature astrocytes express mainly Vimentin[177]. Expression of Vimentin in GBM cells is dependent on the density; in low density cell cultures the expression is highest[178]. The expression is associated with a more aggressive tumor behavior, with a metastatic phenotype and thus a poor prognosis[179]. Finally, Vimentin is also a marker for EMT[179].

Towards the end of gestation, a switch occurs whereby Vimentin is progressively replaced by GFAP in differentiated astroglial cells. GFAP has been widely recognized as an astrocyte differentiation marker[177]. In the adult stage, GFAP is induced upon brain damage, during CNS degradation and in the aged brain in general[180]. Decreased GFAP expression has been associated with glioma growth, especially in

high grade gliomas (grade III and IV)[181]. Lastly, GFAP has proven as a valuable diagnostic marker for GBM[182].

CD24 enhances metastatic potential of malignant cells by functioning as a ligand for P-selectin[183]. CD24 is overexpressed in GBM tumors; however a clear association with clinicopathological parameters has only recently been established by Deng and colleagues who could correlate CD24 overexpression with a poor prognosis in GBM[184].

The S-100 protein is specific for the nervous system and is expressed at high levels in the brain, primarily by astrocytes and astrocytic tumors[185]. Its expression increases with the level of malignancy[186]. Some S-100 proteins are associated with angiogenesis[186]. Thus, the S-100 protein is likely to be involved in the progression of GBM malignancy[186].

In contrast to CD90, the other markers (CD24, GFAP, Nestin and S-100) were expressed to a much lower and also more varying degree in the HROG cell lines.

The CSC hypothesis was initiated in the 1990s by Dick and colleagues[187]. Their report on Leukemia initiating cells became the paradigm for later studies proposing CSC to be at the top of a hierarchical pyramid[188]. Since then a broad spectrum of tumor entities encompassing CSC populations have been described[23,24,26-29]. These CSC maintain hallmarks of normal stem cells such as the ability to self-renewal and to differentiate into all cell types of the tissue they originate from[189]. However, CSC are not only responsible for tumor maintenance but are also thought to be the key players in recurrence and therapy resistance[3,20,22,30].

The lack of robust markers allowing the identification of BTSC is an obstacle in the development of specific treatments. Nevertheless, frequently proposed cell surface markers (alone or in combination) are CD133, CD15, Nestin, CD34 and CD44[22,30-32].

Since BTSC seem to be a part of GBM development, maintenance and therapy resistance, the presence of BTSC-like populations was assessed in the HROG cell lines. The markers CD15, CD34, CD133 and Nestin were expressed in a percentage of cells in the range of what might be expected for CSC. However, CD44 was present on about 90% of cells in the cultures and thus highly improbable of representing only CSC. In fact, CD44 is a marker for EMT and thus also of the mesenchymal sub-type of GBM tumors[190]. Of note the mesenchymal sub-type is the most commonly represented in cell lines[57].

A number of strategies have been developed and tested to treat GBM tumors by specifically targeting the BTSC or BTSC-like cells. Yang and coworkers used a CSC specific miRNA (miR145) to target $CD133^+$ cells and showed that this approach significantly inhibited their tumorigenic and BTSC-like abilities and facilitated their differentiation into non-BTSC. Furthermore, the expression of drug-resistance and anti-apoptotic genes was suppressed and cells were rendered sensitive to radiation and TMZ treatment[191]. Wang et al. studied the efficacy of photothermolysis challenging cells with single-walled carbon nanotubes conjugated with an anti-CD133 monoclonal antibody and then irradiated with near-infrared laser light. This resulted in an eradication of BTSC-like cells and blocked the tumorigenic and self-renewal capability[192]. Also immunological targeting of BTSC was tested. The group around Xu isolated BTSC from tumor specimen of GBM patients and lethally irradiated the cells to load autologous monocyte derived DC with the apoptotic BTSC. These loaded DC were capable of eliciting a “BTSC-specific” Th1 immune response. Finally, this DC vaccination achieved a robust antitumor T cell immunity and led to a significant survival benefit for rats challenged with BTSC neurospheres[193]. As a possible exploitation of the HROG cell line collection, it would be promising to validate this principle approach by isolating BTSC from HROG lines and testing their immunological potential in comparison to non-BTSC and untouched HROG cells.

The brain is an immunologically privileged organ and under normal physiological conditions only minimal entry of immune cells takes place for purpose of immune surveillance[194]. The normal brain does not possess a lymphatic system and furthermore brain cells do not express HLA molecules which are necessary for the initiation of an immune response[195]. However, there is increased awareness of and appreciation for the complex interplay between the nervous system and the immune system in the setting of many disease states, including neoplasms[196].

Maintaining a healthy steady state is the role of the immune system. Tumor cell recognition and elimination by the immune system does not simply rely on the ability to differentiate between “host” and “non-host” cells but on a more subtle differentiation between “host” and “transformed”[197]. To fulfill this function the immune system must be in constant interaction with the cells in an immunosurveillance and immunoediting manner[197]. The immune system not only controls tumor quantity (tumor size) but also tumor quality (immunogenicity). In this regard three distinct

phases are important, which have magnificently been described by Schreiber and coworkers. (I) In the elimination phase the innate and adaptive immune system work together to detect and destroy transformed cells before a cancer becomes clinically apparent. (II) In the equilibrium phase transformed cells that survived elimination persist. The adoptive immune system however prevents tumor cell growth and thus sculpts the immunogenicity of these cells and residual tumor cells are maintained in a state of dormancy. (III) In the final escape phase tumor cells have acquired the ability to circumvent immune recognition and destruction and thus tumor progression and visible growth proceed[198].

So, differentiation between tumor cells and non-transformed counter parts is achieved by antigens expressed specifically by the tumor cells. These antigens include: (I) differentiation antigens (e.g. melanocyte differentiation antigens), (II) mutational antigens (e.g. mutated p53), (III) overexpressed cellular antigens (e.g. EGFR, HER2/neu), (IV) viral antigens (e.g. CMV) and (V) cancer/testis antigens, which are normally only expressed in germ cells of testis and ovary (e.g. NY-ESO-1)[198]. Based on these hypotheses different immunotherapeutic strategies have been developed and these are roughly divided into active (induction of host immune response) and passive (supporting the host immune response) as well as specific (tumor/antigen specific interventions) and unspecific (induction of a general immune response e.g. by microbes)[197].

In the process of malignant transformation, astrocytoma cells display a number of surface proteins not expressed by their normal adult counterparts. These include TAA as well as HLA class I and II. The concomitant expression of TAA and HLA molecules together with the disruption of the blood brain barrier often occurring in tumor patients may elicit an acquired (humoral or cell mediated) immune response[195]. TSA or TAA qualify as prime target structures for therapeutic interventions, since they are highly (and rather exquisitely) expressed on the tumor cells. The expression (level) of TAA and HLA molecules on GBM cells is thus of particular interest for immunotherapeutic interventions.

The most frequent HLA-A allele is the *A2; 50% of people of the Caucasian race harbor this allele[147]. Most of the initial studies aiming at the identification or validation of novel T cell epitopes of TAA are thus designed in an HLA-A2 restricted manner. In this regard it is of special interest that ten out of the twelve HROG cell lines carry this

allele; they recommend themselves as tools for studying the immunogenicity of candidate TAA.

HIP 1 is predominantly expressed in neuronal cells of the CNS[199]. In yeast the protein is involved in organization and polarization of the cytoskeleton[199]. Its oncogenic potential is ascribed to the fact that it is part of the oncogenic HIP 1/platelet-derived growth factor beta receptor (PDGFβR) fusion protein[200]. HIP 1 is overexpressed in cancers of the prostate, colon and breast as well as in primary brain tumors[200]. The oncogenic function may arise from an altered biology of brain cells by overexpression of the protein and thus inducing malignant transformation[200]. An interesting observation is that the overexpression of HIP 1 correlates with the overexpression of EGFR and PDGFβR in GBM tumors[200]. However, on the HROG cell lines it is expressed rather marginally. Shedding some doubt on the latter finding.

The IL-13Rα, a cancer associated receptor for IL-13[201], is a promising target for immunotherapy since it is abundantly expressed on GBM cells but not on host CNS cells[202,203]. IL-13 is a pro-inflammatory cytokine inducing apoptosis[204,205]. Despite the presence of high levels of IL-13 in GBM tumors, often only a low level of apoptosis is observed[206]. IL-13Rα may bind IL-13 with an extremely high affinity and is internalized as well but the signal is not transduced[205]. Thus, the IL-13Rα is termed a decoy receptor given its inability to mediate downstream signaling[207,208] and thus may protect GBM cells of IL-13 induced apoptosis. The receptor was expressed in all HROG cell lines but as HIP 1 only with a low(er) intensity.

Okano and coworkers could show that a novel epitope of IL-13Rα stimulates $CD8^+$ T cells, which secreted interferon gamma (IFNγ) and were capable of lysing IL-13Rα-expressing GBM cells *in vitro*. In line with the argumentation to the relevance of HLA-A2, they showed this for the $HLA\text{-}A2^+$ situation[209]. An *in vivo* targeting of IL-13Rα was performed by tagging IL-13 with a mutated form of the pseudomonas exotoxin[202,205,210]. The fusion protein (IL-13-PE38QQR), termed Cintredekin besudotox, injected intracranially led to both tumor regression and prolonged survival in an animal model[211].

While only few cells staining positive for the more GBM-specific antigens, all the HROG cell lines expressed the general TAA CEA; most HROG lines even high levels. CEA was first described by Gold and Freedman in 1965 in human colon cancer tissue[212]. Twenty years later, immunohistochemical evidence of CEA expression in GBM tumors was presented[213]. In the late 1970s and early 1980s CEA

became a favored target antigen[214]. Since then, numerous therapeutic strategies targeting CEA have been developed such as vaccines, antibodies and recombinant retroviruses[215,216]. Today, CEA is not only a therapeutic target but also an important diagnostic marker in a variety of tumor entities[217-219]. Analysis of the amino acid sequence of CEA and of the other members of the CEA family revealed that they belong to the immunoglobulin superfamily[214]. CEA plays a role in tumor progression[220,221] and metastasis[220,222].

GBM tumors are said to be exquisitely good in surrounding themselves with an immunosuppressive environment; they secret cytokines – mostly IL-6 and IL-8, attract regulatory cells and regulate HLA expression as well as antigen presentation[223,224]. Thus, by rendering a very immunosuppressive milieu, immunological recognition through the patients´ immune system is prevented. A challenge to overcome in advancing GBM therapy is the complexity of the GBM microenvironment[225]. One of the emerging strategies to counteract GBM tumors is activating the immune system and thus overcoming this GBM-induced immunotolerance. Although lymphocyte infiltration into the tumor is observed in GBM tissue[226] this is not sufficient to induce tumor rejection. The main players hereby are $CD4^+$ $CD56^+$ T cells[226]. It is becoming clearer that it is not sufficient to stimulate the immune system in order to elicit a (cytotoxic) immune response. Overcoming GBM resistance to traditional therapies requires consideration not only of the tumor cells intrinsic properties but also to analyze how these cells interact with neural precursor cells, vascular endothelial cells, stromal cells, astrocytes, microglia, lymphocytes, extracellular matrix proteins, and cytokines; in short the tumor micromilieu[227]. This dynamic interplay of diverse cell populations, cytokines, and extracellular matrix proteins is what enables GBM tumorigenesis, progression, and invasion. In essence, effective therapies not only have to be directly cytotoxic to a molecularly diverse population of tumor cells, but must also overcome the pro-tumorigenic properties of the microenvironment[227].

It is established that this hierarchy is also maintained by the cytokine milieu[228]. Immunosuppressive cytokines such as IL-10, TGF-β and prostaglandin E2, in the GBM microenvironment suppress the antitumor immune response in concert with other factors such as indoleamine-2,3-dioxygenase (IDO) and Galectin-1[229-231]. The sources of these molecules and the details of their interactions are yet to be fully

unraveled. Although the expression of IDO is associated with a poor prognosis and tends to predominate in high grade gliomas, it is essential whether IDO is tumor-derived (increased regulatory T cell (T reg) recruitment and decreased survival) or is peripheral (non-tumor derived IDO has no effect on T cells and overall survival of patients)[230]. Galectin-1 expression correlates with a high grade of malignancy in astrocytomas and poor overall survival. The immunosuppressive functions concern both arms of the immune system; it counteracts anti-tumor immune response of the innate arm by inducing tolerogenic DC and macrophages and induces accumulation of T reg cells and impairs T cell function in the adaptive arm[231].

Since GBM cell lines have long been known to secret high levels of immunosuppressive cytokines[232], it is necessary to fully understand the relationship between distinct cytokines and the variety of cell populations composing the GBM microenvironment. In order to fully comprehend the interaction, a detailed sub-classification might be necessary[227].

Cytokines are factors known to regulate cell proliferation and differentiation. They can be divided into several groups such as growth factors, interleukins, interferons and tumor necrosis factors[233].

Physiologically, IL-6 is produced by T cells, involved in B cell maturation and secreted in acute chronic inflammation[224]. However, IL-6 release is commonly attributed to several tumor entities and especially to GBM cells[234]. High levels of the cytokine are thought to be involved in (tumor) cell growth and angiogenesis, e.g. by induction of VEGF[235]. GBM invasion and migration is promoted by IL-6 induced activation of signal transducer and activator of transcription 3[236]. It is a key player in immunosuppression by inhibiting DC differentiation[235]. IL-6 also plays a role in therapy resistance and it counteracts p53 induced apoptosis[235,237-239]. IL-6 is more frequently and to higher levels expressed in the most malignant gliomas (GBM) and inversely correlates with patient survival[235]. Thus, it comes as no surprise that more than half of the HROG cell lines secreted (high) levels of the cytokine.

Under normal conditions IL-8 is undetectable in the brain but aberrant expression leads to pathogenesis[240]. The cytokine is highly secreted by GBM cell lines and stem cells[234]. The pathophysiological role is mainly that of an angiogenic factor[241]. IL-8 secretion also correlates with increased proliferation, invasiveness and tumor growth[36,242]. These functions are mostly mediated by the two receptors chemokine (C-X-C motif) receptor 1 and chemokine (C-X-C motif) receptor 2[241,243,244]. Lastly, the

level of IL-8 correlates with the histopathological grade[245]. All but one HROG cell line (HROG38) secreted substantial amounts of the cytokine into the supernatant.

Many different immune cells secrete TNFα, primarily monocytes, macrophages, activated natural killer cells and T cells[246]. In the normal, healthy brain TNFα is responsible for DC maturation[246]. But in a tumor environment the expression correlates positively with the tumor grade[247]. As a member of the Th1 pro-inflammatory cytokines it can elicit cell mediated immune responses and thus exert anti-tumoral functions[246,248]. However, once produced by astrocytes and microglial cells, TNFα can induce the transcription of IL-6, IL-1 and IL-8[241,249]. These pleiotropic effects are dependent on the dosage and range from tumor suppression (high dosage) to promotion of tumorigenesis (low doses)[224,250]. On the side of the pro-tumorigenic capacity is the promotion of glioma formation and progression by angiogenesis[251]. Vascularization is promoted through IL-8 and VEGF induction[251]. However, none of the analyzed HROG cell lines produced detectable amounts of TNFα.

Among the multiple pathways associated with GBM, the TGF-β pathway plays a very crucial role in regulating the behavior of the tumor cells[252]. So it comes as no surprise that TGF-β is one of the best characterized cytokines in GBM tumors[224]. At the cellular level TGF-β affects processes such as cell growth, cell survival, differentiation, migration and immune cell activation in a cell type-dependent manner and depending on the cellular context[99]. In the normal brain TGF-β is not produced: however, in GBM tumors it is often over-expressed[224]. In cancer it has a dual role and thus can exhibit both promoting and suppressive functions[253,254] which depends on tumor stage and entity[255,256]. This phenomenon has been termed TGF-β paradox[257,258]. As a tumor suppressor it is a potent inhibitor of astrocytes, epithelial and immune cells. Some tumors can escape the TGF-β influence by acquiring mutations in the pathway; other tumors including GBM selectively abolish the capacity of TGF-β to inhibit proliferation while maintaining beneficial aspects (to the tumors) of the pathway[259]. In such tumors, TGF-β can activate proliferation, cell survival and maintain stemness, induce (neo-) angiogenesis, promote invasion and migration, boost therapy resistance, induce EMT and help create immune suppression[99,100,260]. Inspired by the preclinical data, it is clear that TGF-β-targeted therapy may be of great value for the treatment of gliomas. Therefore, a variety of therapeutics targeting TGF-β or the pathway has been or is currently being studied in GBM patients. This

encompasses a TGF-β specific antisense oligo-deoxynucleotide (Trabedersen), neutralizing antibodies, small molecule inhibitors and specific kinase inhibitors (NCT00761280; NCT01472731; NCT01220271)[101,261].

By regulating gene expression via transcription factors of the SMAD family pro-angiogenic factors such as VEGF are induced[262], or molecules associated with invasion and migration (such as MMP) are up-regulated in GBM[263].

TGF-β also directly influences the immune system. As part of the Th3 immune response it exerts an exquisitely strong immunosuppressive function on tumor infiltrating lymphocytes[248]. Especially reduction of T, B and NK cell proliferation[264,265], inhibition of T cell activation and suppression of NK cell activity as well as promotion of T reg activity[232,266] lead to immunosuppression. But also interference with DC maturation[267] and down regulation of HLA class II[99].

Irradiation leads to increased levels of TGF-β[268,269]. And finally elevated levels of TGF-β correlate with tumor grade, an advanced stage and poor prognosis[100,252,270,271].

Secretion of TGF-β was only detected in one cell line, HROG04, at high levels. The cell line was established of a patient with a relapsed GBM tumor. However, none of the other cell lines established from relapsed tumors secreted detectable levels of the cytokine. This may be explained – in addition to the lack of CEA secretion – by changes in immunological phenotype and cytokine secretion of GBM cells after *in vitro* passaging[272].

Beside defining and uncovering tumor initiating, propagating and metastasizing processes, and identifying new (molecular) target structures, high throughput screening of drugs is the main purpose of tumor models, especially in cell lines for the latter. Cell cultures are broadly used since cell lines are easy to handle, and manageable in high quantities; they represent a relatively low-cost approach and are ethically preferable to other methods; i.e. experimental animals[273]. Thus they have somewhat become the pharmaceutical industries favorite “pet”. One big obstacle remains. Cell lines established in the 1970s and 1980s when there was a big hype for GBM cell lines[114], have been passaged uncountable many times and certainly have “acquired” culturing artifacts. Many changes over long term *in vitro* culturing are well described.

Doubling time increases over culturing periods with the number of passages[160,161] and this may have an influence on drug sensitivity, since many chemotherapeutic agents

aim at rapidly proliferating cells. Extensive *in vitro* passaging may lead to a hypermethylated phenotype. In this respect, Danam and colleagues demonstrated that with increasing cell culture passage, methylation progressively increased and revealed a concomitant trend to a completely MGMT-silenced phenotype[274]. In contrast, elimination or loss of amplification of the EGFR during GBM cell culturing is a well-known phenomenon yet causes remain enigmatic[118-120]. Also IDH mutations, present rather frequently in GBM tumors, are an obstacle to be maintained in culturing of GBM cells[121]. Acquisition of "new" mutations and chromosomal aberrations are further described for highly passaged cell lines[114], also the above mentioned changes in immunological phenotype and cytokine secretion after passaging *in vitro* are possible[272]. These (very) long term cultures have little in common with the original situation in the patients and thus only have limited model capability and drug testing potential.

The gold standard chemotherapeutic agent for GBM tumors is since 2005 TMZ[14]. All novel therapeutics must measure up to it and prove significant benefit for GBM patients or lower toxicity towards normal tissue, i.e. have fewer side effects.

Responsiveness of the patient-derived low passage Rostock GBM cell line collection was assessed for conventional chemotherapeutic agents as the alkylating agents (BCNU, CCNU, TMZ and Procarbazine) and other conventional therapeutics (Cisplatin, Cytarabine, Irinotecan, Methotrexate, Topotecan and Vincristine). The sensitivity to CCNU, Cisplatin, Cytarabine and Topotecan correlated with the methylation status of the MGMT promoter or MGMT cDNA expression and was significantly higher in hypermethylated (cDNA expression <0.001) cell lines ($p=0.033$; $p=0.002$; $p=0.016$ and $p=0.024$ respectively). In contrast, no correlation of the methylation status of the MGMT promoter could be detected for the agents BCNU, TMZ and Procarbazine. This is somewhat in contrast to the positive correlation described for methylated MGMT promoter and response to alkylating agents[275]. However, the presence of MGMT cDNA tended to correlate with a better response in methylated cell lines; a final conclusion only would be possible when directly correlating the protein expression to drug response. Consequently, such a staining protocol will be established in the near future. This emphasizes the value of these patient-derived low passage cell lines since detailed characterization revealed for example a deletion of the entire MGMT sequence in HROG36 which was scored unmethylated but did not even harbor the sequence. Same holds true for HROG24

with only one copy of the MGMT gene. Methylation scoring for such cases may have to be reconsidered.

Responses to Irinotecan and Topotecan strongly tended to be strongest for cell lines with mutated TP53. This finding goes well with the fact that GBM cells treated with the DNA topoisomerase inhibitor SN-38 only underwent cell cycle arrest and even re-proliferated after withdrawal of the inhibitor in an wild type p53 setting, whereas in cells with mutations in the tumor suppressor treatment caused apoptosis[276].

A general strong response to Vincristine was observed. In contrast, Methotrexate had no influence on cell viability *in vitro*.

Further, *in vitro* response to several so called targeted therapeutics (Imatinib, Nilotinib, Bevacizumab, Cetuximab, Rapamycin, and Celecoxib) were assessed. The therapeutic antibodies Bevacizumab and Cetuximab had absolutely no influence on GBM cell viability; although binding of the antibody to the cell lines could be demonstrated by flow cytometry. A general response to the tyrosin kinase inhibitors could be observed with a strong tendency towards a better response to Imatinib compared to Nilotinib. Gleevec (Imatinib) failed in large scale studies to prove significant benefit for GBM patients[277]. However, repeating the trails or re-evaluation might produce sub-groups which might very well profit from the drug[278].

The cell lines most sensitive to Rapamycin (HROG17 and HROG24) did not express MGMT cDNA. In contrast, the least sensitive cell line HROG38 expressed MGMT cDNA, as did the three next most sensitive cell lines (HROG06, HROG07 and HROG10). Such a correlation has not been described so far and this finding should be evaluated *in vivo* using the xenografts models. The mTOR inhibitor Rapamycin not only has direct cytotoxic effects on the tumor cells but also modulates the immune response[279].

Cyclooxigenase inhibition had surprisingly strong effects on cell viability and all cell lines tested were rather sensitive to Celecoxib with a narrow range.

Thalidomide, initially applied as a sedative, has proven anti-cancer efficacy[280,281]. Three cell lines: HROG05, HROG17 and HROG36 were highly responsive to the agent. Currently Lenalidomide (derived from Thalidomide; CC-5013) is under clinical investigation for treatment of advanced cancers and GBM in particular. Four clinical trials are listed currently on clinicaltrials.gov. Two, which have been completed: (1) Study of Lenalidomide and radiotherapy in patients with newly diagnosed GBM (NCT00165447) and (2) CC-5013 in treating patients with recurrent glioma

(NCT00036894), one active but not recruiting: Irinotecan plus Lenalidomide in adult patients with recurrent GBM (NCT00671801) and one currently recruiting: Lenalidomide in combination with Bevacizumab, Sorafenib, Temsirolimus, or 5-Fluorouracil, Leucovorin, Oxaliplatin (FOLFOX) in patients with advanced cancers (NCT01183663).

Current efforts of improving GBM treatment include the addition of novel targeted agents to the standard of care regimen. Thus, analyses regarding the addition of CGT to the standard chemotherapeutic agent TMZ were performed. Possible beneficial effects as published previously[85,86] were assessed *in vitro*. Further, defining responses to CGT alone or in combination with TMZ in correlation with molecular characteristics of the tumor cells were of particular interest. Somewhat unexpected, the amount of surface integrin expression, by which CGT is thought to inhibit angiogenesis and induce cytotoxicity, did not correlate with the response to CGT. The observed strong *in vitro* reaction to CGT monotherapy is in accordance with the positive *in vivo* response of the randomized phase II study published by Reardon and colleagues[94]. Further, a positive correlation of MGMT promoter methylation with the response towards TMZ was observed as described earlier[282,283]. Contrary to that, a strong trend indicating a better response to CGT (monotherapy) in non-promoter-methylated cells was found. This finding is somehow conflicting with data of Maurer et al. who demonstrated complete lack of influence of MGMT expression on response towards CGT[284]. However, this shall be clarified in the near future since several clinical studies are currently addressing this question, i.e. the CORE study only recruiting GBM patients with an unmethylated MGMT promoter (NCT00813943), the CENTRIC study, including exclusively patients with a methylated promoter (NCT00689221), and the pediatric HGG-CilMetro study (NCT01517776), including both. Recently, two studies revealed a significant benefit of a combination therapy with TMZ and CGT for patients with a methylated MGMT promoter[85,86]. I would like to stress the fact that this effect was also observed with the ultra-low passage HROG cell line collection. The response analysis on the ultra-low passage GBM cell lines to treatment with TMZ and CGT allows the following conclusions: (I) There is a clear positive correlation between the MGMT promoter methylation status and response to TMZ. (II) Addition of CGT resulted in an at least additive effect, suggesting that patients with MGMT promoter methylated GBM will most likely benefit from the

addition of CGT to the standard treatment with TMZ. (III) In contrast, patients suffering from GBM with unmethylated MGMT promoter may benefit most from CGT monotherapy.

The results on drug response further strengthen the hypothesis that patient-derived (ultra-) low passage cell lines represent a model system with ideal features for response and resistance prediction and are a good step towards individualized therapy and screening in drug development, particularly when molecularly and clinically well characterized.

Since the establishment of cell lines was crowned by such high success rates, further focus was laid on optimizing the methodology and improving the logistics. Therefore practicability of cryo-preservation prior to model establishment was assessed. Tumor tissue from GBM surgical resection specimens could be vitally stored and successful *in vitro* culture establishment remained feasible. As a technically very simple method, cryo-preservation of GBM tumor tissue prior to model establishment may be quite appealing to both clinical and basic researchers alike for the following reasons:
(1) The methodology is easy and as successful as for the cell cultures established from fresh tumor material. Even though success rates tend to be lower after vital freezing than with tumor tissue fresh from surgery, this difference did not reach statistical significance (p=1.000). This finding is in line with similar analyses of gastrointestinal tumors[135,285] as well as for established GBM xenografts and cancer-initiating cells[286,287]. In seven cases no successful tumor outgrowth was possible. In four of these cases, the tissue was captured from surgery for recurrent glioblastoma. Besides the comparable little tumor mass in these patients, the tumors were heavily pretreated according to standard therapy often causing necrotic tissue as previously described.
(2) There were no clear-cut differences observed neither in morphology and growth kinetic nor in the sensitivity towards the tested drugs. Moreover, the mutational patterns of the original tumors were maintained in the cell line pairs. The latter may quite possibly not withstand whole genome sequence analysis. It is, however, very likely that slight differences will also be observed when analyzing several micro samples originating from the same GBM case as has been shown for methylation patterns and the levels of receptor amplification in different sub-clones[288,289].

(3) Finally and most important, the cryo-method will allow pre-selection of interesting cases before model establishment according to molecular data of the original tumor, clinical course, therapy response or development of resistance.
Taken together, the simple cryo-step does not interfere with successful establishment of ultra-low passage GBM cell lines or primary cultures. This comparison has been performed under the best possible conditions imaginable including an in-depth molecular characterization and functional tests. Ultra-low passage primary cell cultures are considered superior to continuous cell lines in brain tumor research[152]. However, their availability can be limiting to scientific progress. Thus, separating the pure collection of clinical GBM specimens from the more complex logistics of model establishment will simplify the successful generation of individual GBM models.

Not only pre-selection of interesting cases prior to laborious cell line establishing processes is possible, but the technique also enables repetitive establishment procedures and therefore allows going “back” to primary cultures. Of course, this will depend on the amount of GBM tissue stored immediately after operation. One simple problem is the cost for generating many cryo-aliquots of a tissue sample. This problem is obvious for academics but most likely not so in the context of clinical studies. Also, the procedure does not necessarily interfere with pathological analysis of the operated GBM, since cryopreserved tissue pieces can be substitute of fresh material; at least for diagnostic pathological procedures.

5.1 Conclusion

Patient derived individual model establishment is feasible and could be performed with very acceptable success rates (in especially for the *in vitro* system), even from vitally frozen material. Noteworthy, the latter has never been performed before; neither for cell culturing nor for xenografting. A large collection of patient-individual models could be established by these means. Subsequently, these models could be well characterized and preserved in the established biobank in (ultra-) low passages. Molecular analyses revealed that the cell lines exhibit only few alterations when compared to the original patients' tumors. Finally, proof-of-concept studies allow the conclusion that these (ultra-) low passage patient-derived models can readily be applied for drug sensitivity testing. Thus, these cultures may very well be suitable for clinical response prediction and represent a valuable step towards true individualized therapy.

5.2 Future perspectives

The next steps for this research work beyond the analyses described here would be the testing of neurosphere cultures as well as stem cell conditions for the *in vitro* systems. Concerning the *in vivo* setting, establishing an orthotopic xenograft model will be the next step.

The extensive molecular analyses, including the CGH array, should be further extended and candidate tumor suppressor genes can be functionally validated with the – ongoing – large model collection.

From a more clinical aspect of view, the actual clinical outcome of the therapeutic intervention should be compared to *in vitro* drug sensitivity and validated starting in a retrospective manner but ultimately attempting prospective predictions.

Finally, further functional and in especially immunological analyses will be performed by taking advantage of the biobanked patient material (lymphocytes and sera).

6 Summary

6.1 English

The aim of this thesis on glioblastomas, the most common form of brain tumor with a devastating prognosis, was to establish individual patient-derived tumor models both *in vitro* (cell lines) and *in vivo* (xenografts). Generating models was successful from fresh and vitally frozen GBM patient material – the latter representing a completely novel technique. These models were subject to subsequent detailed characterization in direct comparison to the patients´ tumors. Generally, molecular characteristics such as mutations, gene amplifications and epigenetic alterations were maintained in the models. Immortality, neuronal origin and stem cell characteristics of the cell lines could be demonstrated. Extensive drug sensitivity screens were performed. These well-defined patient-individual models are ideal for establishment of individualized therapy approaches and enable testing of immunological strategies.

6.2 German

Ziel dieser Arbeit zu Glioblastomen, der häufigsten und aggressivsten Form von Hirntumoren, war die Etablierung von Patienten-individuellen Tumormodellen *in vitro* (Zelllinie) und *in vivo* (Xenograft). Eine neue Technik zur vitalen Kryo-Asservierung von Patientenmaterial konnte etabliert werden. Die Modelle wurden detailliert im Vergleich zu den Primärtumoren charakterisiert. Grundlegende molekulare Eigenschaften wie Mutationen, Amplifikationen und epigenetische Veränderungen blieben in der Regel in den Modellen erhalten. Der neuronale und maligne Ursprung der Zelllinien konnte nachgewiesen werden. Das Ansprechen der Zelllinien auf ein umfangreiches Panel an Therapeutika wurde getestet. Diese umfassend charakterisierten Modelle eignen sich hervorragend als Ausgangspunkt zur Etablierung individualisierter Therapieansätze und zur Testung immunologischer Strategien.

7. References

1.Louis, D. N. Molecular pathology of malignant gliomas. *Annual review of pathology* **1**, 97-117 (2006).

2.Louis, D. N. *et al.* The 2007 WHO classification of tumours of the central nervous system. *Acta neuropathologica* **114**, 97-109 (2007).

3.Alves, T. R. *et al.* Glioblastoma cells: a heterogeneous and fatal tumor interacting with the parenchyma. *Life sciences* **89**, 532-9 (2011).

4.Ohgaki, H. & Kleihues, P. The definition of primary and secondary glioblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 764-72 (2013).

5.Preusser, M. *et al.* Current concepts and management of glioblastoma. *Annals of neurology* **70**, 9-21 (2011).

6.Dolecek, T. A., Propp, J. M., Stroup, N. E. & Kruchko, C. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. *Neuro-oncology* **14 Suppl 5**, v1-49 (2012).

7.Ohgaki, H. *et al.* Genetic pathways to glioblastoma: a population-based study. *Cancer research* **64**, 6892-9 (2004).

8.Iacob, G. & Dinca, E. B. Current data and strategy in glioblastoma multiforme. *Journal of medicine and life* **2**, 386-93 (2009).

9.Ohgaki, H. & Kleihues, P. Epidemiology and etiology of gliomas. *Acta neuropathologica* **109**, 93-108 (2005).

10.Wrensch, M. *et al.* Variants in the CDKN2B and RTEL1 regions are associated with high-grade glioma susceptibility. *Nature genetics* **41**, 905-8 (2009).

11.Shete, S. *et al.* Genome-wide association study identifies five susceptibility loci for glioma. *Nature genetics* **41**, 899-904 (2009).

12.Inskip, P. D. *et al.* Cellular-telephone use and brain tumors. *The New England journal of medicine* **344**, 79-86 (2001).

13.Henriksson, R., Asklund, T. & Poulsen, H. S. Impact of therapy on quality of life, neurocognitive function and their correlates in glioblastoma multiforme: a review. *Journal of neuro-oncology* **104**, 639-46 (2011).

14.Stupp, R. *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine* **352**, 987-96 (2005).

15.Hegi, M. E. *et al.* MGMT gene silencing and benefit from temozolomide in glioblastoma. *The New England journal of medicine* **352**, 997-1003 (2005).

16.Muñoz, D. M. & Guha, A. Mouse models to interrogate the implications of the differentiation status in the ontogeny of gliomas. *Oncotarget* **2**, 590-8 (2011).

17.Rankin, S. L., Zhu, G. & Baker, S. J. Review: insights gained from modelling high-grade glioma in the mouse. *Neuropathology and applied neurobiology* **38**, 254-70 (2012).

18.Liu, C. *et al.* Mosaic analysis with double markers reveals tumor cell of origin in glioma. *Cell* **146**, 209-21 (2011).

19.Al-Hajj, M., Becker, M. W., Wicha, M., Weissman, I. & Clarke, M. F. Therapeutic implications of cancer stem cells. *Current opinion in genetics & development* **14**, 43-7 (2004).

20.Lima, F. R. *et al.* Glioblastoma: therapeutic challenges, what lies ahead. *Biochimica et biophysica acta* **1826**, 338-49 (2012).

21.Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-74 (2011).

22.Denysenko, T. *et al.* Glioblastoma cancer stem cells: heterogeneity, microenvironment and related therapeutic strategies. *Cell biochemistry and function* **28**, 343-51 (2010).

23.Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine* **3**, 730-7 (1997).

24.Singh, S. K. *et al.* Identification of human brain tumour initiating cells. *Nature* **432**, 396-401 (2004).

25.Fang, J. *et al.* [Isolation and identification of brain tumor stem cells from human brain neuroepithelial tumors]. *Zhonghua yi xue za zhi* **87**, 298-303 (2007).

26.Prince, M. E. *et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 973-8 (2007).

27.Sadri-Ardekani, H. *et al.* Propagation of human spermatogonial stem cells in vitro. *JAMA : the journal of the American Medical Association* **302**, 2127-34 (2009).

28.Ricci-Vitiani, L. *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**, 111-5 (2007).

29.Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 3983-8 (2003).

30.Dimov, I., Tasić-Dimov, D., Conić, I. & Stefanovic, V. Glioblastoma multiforme stem cells. *TheScientificWorldJournal* **11**, 930-58 (2011).

31.Brescia, P., Richichi, C. & Pelicci, G. Current strategies for identification of glioma stem cells: adequate or unsatisfactory? *Journal of oncology* **2012**, 376894 (2012).

32.Gilbert, C. A. & Ross, A. H. Cancer stem cells: cell culture, markers, and targets for new therapies. *Journal of cellular biochemistry* **108**, 1031-8 (2009).

33.Brat, D. J. & van Meir, E. G. Vaso-occlusive and prothrombotic mechanisms associated with tumor hypoxia, necrosis, and accelerated growth in glioblastoma. *Laboratory investigation; a journal of technical methods and pathology* **84**, 397-405 (2004).

34.Furnari, F. B. *et al.* Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes & development* **21**, 2683-710 (2007).

35.Jensen, R. L. Brain tumor hypoxia: tumorigenesis, angiogenesis, imaging, pseudoprogression, and as a therapeutic target. *Journal of neuro-oncology* **92**, 317-35 (2009).

36.Bonavia, R., Inda, M., Cavenee, W. K. & Furnari, F. B. Heterogeneity maintenance in glioblastoma: a social network. *Cancer research* **71**, 4055-60 (2011).

37.Armstrong, T. S. *et al.* A case of soft tissue metastasis from glioblastoma and review of the literature. *Journal of neuro-oncology* **103**, 167-72 (2011).

38.Kraus, J. A., Felsberg, J., Tonn, J. C., Reifenberger, G. & Pietsch, T. Molecular genetic analysis of the TP53, PTEN, CDKN2A, EGFR, CDK4 and MDM2 tumour-associated genes in supratentorial primitive neuroectodermal tumours and glioblastomas of childhood. *Neuropathology and applied neurobiology* **28**, 325-33 (2002).

39.Masui, K., Cloughesy, T. F. & Mischel, P. S. Review: molecular pathology in adult high-grade gliomas: from molecular diagnostics to target therapies. *Neuropathology and applied neurobiology* **38**, 271-91 (2012).

40.Dunn, G. P. *et al.* Emerging insights into the molecular and cellular basis of glioblastoma. *Genes & development* **26**, 756-84 (2012).

41.Belden, C. J. *et al.* Genetics of glioblastoma: a window into its imaging and histopathologic variability. *Radiographics : a review publication of the Radiological Society of North America, Inc* **31**, 1717-40 (2011).

42.Chow, L. M. & Baker, S. J. Capturing the molecular and biological diversity of high-grade astrocytoma in genetically engineered mouse models. *Oncotarget* **3**, 67-77 (2012).

43.Bleeker, F. E., Molenaar, R. J. & Leenstra, S. Recent advances in the molecular understanding of glioblastoma. *Journal of neuro-oncology* **108**, 11-27 (2012).

44.Nagane, M., Lin, H., Cavenee, W. K. & Huang, H. J. Aberrant receptor signaling in human malignant gliomas: mechanisms and therapeutic implications. *Cancer letters* **162 Suppl**, S17-S21 (2001).

45.Hatanpaa, K. J., Burma, S., Zhao, D. & Habib, A. A. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia (New York, N.Y.)* **12**, 675-84 (2010).

46.Gan, H. K., Kaye, A. H. & Luwor, R. B. The EGFRvIII variant in glioblastoma multiforme. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* **16**, 748-54 (2009).

47.Montano, N. *et al.* Expression of EGFRvIII in glioblastoma: prognostic significance revisited. *Neoplasia (New York, N.Y.)* **13**, 1113-21 (2011).

48.Bakhoum, S. F. & Compton, D. A. Chromosomal instability and cancer: a complex relationship with therapeutic potential. *The Journal of clinical investigation* **122**, 1138-43 (2012).

49.Martinez, R. *et al.* Molecular mechanisms associated with chromosomal and microsatellite instability in sporadic glioblastoma multiforme. *Oncology* **66**, 395-403 (2004).

50.Martinez, R. *et al.* Low-level microsatellite instability phenotype in sporadic glioblastoma multiforme. *Journal of cancer research and clinical oncology* **131**, 87-93 (2005).

51.Vaish, M. & Mittal, B. DNA mismatch repair, microsatellite instability and cancer. *Indian journal of experimental biology* **40**, 989-94 (2002).

52.Zheng, S., Chheda, M. G. & Verhaak, R. G. Studying a complex tumor: potential and pitfalls. *Cancer journal (Sudbury, Mass.)* **18**, 107-14 (2012).

53.Hill, C., Hunter, S. B. & Brat, D. J. Genetic markers in glioblastoma: prognostic significance and future therapeutic implications. *Advances in anatomic pathology* **10**, 212-7 (2003).

54.Martinez, R. *et al.* A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics : official journal of the DNA Methylation Society* **4**, 255-64 (2009).

55.Laffaire, J. *et al.* Methylation profiling identifies 2 groups of gliomas according to their tumorigenesis. *Neuro-oncology* **13**, 84-98 (2011).

56.Shinawi, T. *et al.* DNA methylation profiles of long- and short-term glioblastoma survivors. *Epigenetics : official journal of the DNA Methylation Society* **8**, (2013).

57.Verhaak, R. G. *et al.* Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer cell* **17**, 98-110 (2010).

58.Paccapelo, A. *et al.* A retrospective pooled analysis of response patterns and risk factors in recurrent malignant glioma patients receiving a nitrosourea-based chemotherapy. *Journal of translational medicine* **10**, 90 (2012).

59.Scoccianti, S. *et al.* Second-line chemotherapy with fotemustine in temozolomide-pretreated patients with relapsing glioblastoma: a single institution experience. *Anti-cancer drugs* **19**, 613-20 (2008).

60.Glas, M. *et al.* Nimustine (ACNU) plus teniposide (VM26) in recurrent glioblastoma. *Oncology* **76**, 184-9 (2009).

61.Schreiber, R. D. Cancer vaccines 2004 opening address: the molecular and cellular basis of cancer immunosurveillance and immunoediting. *Cancer immunity* **5 Suppl 1**, 1 (2005).

62.Gilboa, E. DC-based cancer vaccines. *The Journal of clinical investigation* **117**, 1195-203 (2007).

63.Ardon, H. *et al.* Integration of autologous dendritic cell-based immunotherapy in the standard of care treatment for patients with newly diagnosed glioblastoma: results of the HGG-2006 phase I/II trial. *Cancer immunology, immunotherapy : CII* **61**, 2033-44 (2012).

64.de Vleeschouwer, S. *et al.* Postoperative adjuvant dendritic cell-based immunotherapy in patients with relapsed glioblastoma multiforme. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 3098-104 (2008).

65.Fadul, C. E. *et al.* Immune response in patients with newly diagnosed glioblastoma multiforme treated with intranodal autologous tumor lysate-dendritic cell vaccination after radiation chemotherapy. *Journal of immunotherapy (Hagerstown, Md. : 1997)* **34**, 382-9 (2011).

66.Caruso, D. A. *et al.* Results of a phase 1 study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children and young adults with brain cancer. *Neuro-oncology* **6**, 236-46 (2004).

67.Wu, A. *et al.* Identification of EGFRvIII-derived CTL epitopes restricted by HLA A0201 for dendritic cell based immunotherapy of gliomas. *Journal of neuro-oncology* **76**, 23-30 (2006).

68.Phuphanich, S. *et al.* Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. *Cancer immunology, immunotherapy : CII* **62**, 125-35 (2013).

69.Söderberg-Nauclér, C. & Johnsen, J. I. Cytomegalovirus infection in brain tumors: A potential new target for therapy? *Oncoimmunology* **1**, 739-740 (2012).

70.Dziurzynski, K. *et al.* Glioma-associated cytomegalovirus mediates subversion of the monocyte lineage to a tumor propagating phenotype. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 4642-9 (2011).

71.Ghazi, A. *et al.* Generation of polyclonal CMV-specific T cells for the adoptive immunotherapy of glioblastoma. *Journal of immunotherapy (Hagerstown, Md. : 1997)* **35**, 159-68 (2012).

72.Crough, T. *et al.* Ex vivo functional analysis, expansion and adoptive transfer of cytomegalovirus-specific T-cells in patients with glioblastoma multiforme. *Immunology and cell biology* **90**, 872-80 (2012).

73.Harris, M. Monoclonal antibodies as therapeutic agents for cancer. *The lancet oncology* **5**, 292-302 (2004).

74.Bulgaru, A. M., Mani, S., Goel, S. & Perez-Soler, R. Erlotinib (Tarceva): a promising drug targeting epidermal growth factor receptor tyrosine kinase. *Expert review of anticancer therapy* **3**, 269-79 (2003).

75.Culy, C. R. & Faulds, D. Gefitinib. *Drugs* **62**, 2237-48; discussion 2249-50 (2002).

76.Sahade, M., Caparelli, F. & Hoff, P. M. Cediranib: a VEGF receptor tyrosine kinase inhibitor. *Future oncology (London, England)* **8**, 775-81 (2012).

77.Reardon, D. A. *et al.* Phase I pharmacokinetic study of the vascular endothelial growth factor receptor tyrosine kinase inhibitor vatalanib (PTK787) plus imatinib and hydroxyurea for malignant glioma. *Cancer* **115**, 2188-98 (2009).

78.Reardon, D. A. *et al.* Phase I study of sunitinib and irinotecan for patients with recurrent malignant glioma. *Journal of neuro-oncology* **105**, 621-7 (2011).

79.Ma, S. & Rosen, S. T. Enzastaurin. *Current opinion in oncology* **19**, 590-5 (2007).

80.van Maanen, J. M., Retèl, J., de Vries, J. & Pinedo, H. M. Mechanism of action of antitumor drug etoposide: a review. *Journal of the National Cancer Institute* **80**, 1526-33 (1988).

81.Seeliger, H., Guba, M., Kleespies, A., Jauch, K. & Bruns, C. J. Role of mTOR in solid tumor systems: a therapeutical target against primary tumor growth, metastases, and angiogenesis. *Cancer metastasis reviews* **26**, 611-21 (2007).

82.Kang, K. B. *et al.* Enhancement of glioblastoma radioresponse by a selective COX-2 inhibitor celecoxib: inhibition of tumor angiogenesis with extensive tumor necrosis. *International journal of radiation oncology, biology, physics* **67**, 888-96 (2007).

83.Mikkelsen, T. *et al.* Radiation sensitization of glioblastoma by cilengitide has unanticipated schedule-dependency. *International journal of cancer. Journal international du cancer* **124**, 2719-27 (2009).

84.Nabors, L. B. *et al.* Phase I and correlative biology study of cilengitide in patients with recurrent malignant glioma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **25**, 1651-7 (2007).

85.Stupp, R. *et al.* Phase I/IIa study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 2712-8 (2010).

86.Nabors, L. B. *et al.* A safety run-in and randomized phase 2 study of cilengitide combined with chemoradiation for newly diagnosed glioblastoma (NABTT 0306). *Cancer* **118**, 5601-7 (2012).

87.Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-87 (2002).

88.Humphries, J. D., Byron, A. & Humphries, M. J. Integrin ligands at a glance. *Journal of cell science* **119**, 3901-3 (2006).

89.Jin, H. & Varner, J. Integrins: roles in cancer development and as treatment targets. *British journal of cancer* **90**, 561-5 (2004).

90.Weis, S. M. & Cheresh, D. A. αv Integrins in Angiogenesis and Cancer. *Cold Spring Harbor perspectives in medicine* **1**, a006478 (2011).

91.Dechantsreiter, M. A. *et al.* N-Methylated cyclic RGD peptides as highly active and selective alpha(V)beta(3) integrin antagonists. *Journal of medicinal chemistry* **42**, 3033-40 (1999).

92.Nisato, R. E., Tille, J., Jonczyk, A., Goodman, S. L. & Pepper, M. S. alphav beta 3 and alphav beta 5 integrin antagonists inhibit angiogenesis in vitro. *Angiogenesis* **6**, 105-19 (2003).

93.MacDonald, T. J. *et al.* Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. *Neurosurgery* **48**, 151-7 (2001).

94.Reardon, D. A. *et al.* Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **26**, 5610-7 (2008).

95.Westphal, M. *et al.* A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. *Neuro-oncology* **5**, 79-88 (2003).

96.Puduvalli, V. K. *et al.* Phase II trial of irinotecan and thalidomide in adults with recurrent glioblastoma multiforme. *Neuro-oncology* **10**, 216-22 (2008).

97.Triscott, J. *et al.* Disulfiram, a drug widely used to control alcoholism, suppresses the self-renewal of glioblastoma and over-rides resistance to temozolomide. *Oncotarget* **3**, 1112-23 (2012).

98.Ikushima, H. *et al.* Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. *Cell stem cell* **5**, 504-14 (2009).

99.Joseph, J. V., Balasubramaniyan, V., Walenkamp, A. & Kruyt, F. A. TGF-β as a therapeutic target in high grade gliomas - Promises and challenges. *Biochemical pharmacology* **85**, 478-85 (2013).

100.Platten, M. *et al.* N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) inhibits transforming growth factor-beta relesase and reduces migration and invasiveness of human malignant glioma cells. *International journal of cancer. Journal international du cancer* **93**, 53-61 (2001).

101.Bogdahn, U. *et al.* Targeted therapy for high-grade glioma with the TGF-β2 inhibitor trabedersen: results of a randomized and controlled phase IIb study. *Neuro-oncology* **13**, 132-42 (2011).

102.Haseley, A., Alvarez-Breckenridge, C., Chaudhury, A. R. & Kaur, B. Advances in oncolytic virus therapy for glioma. *Recent patents on CNS drug discovery* **4**, 1-13 (2009).

103.Fulci, G. & Chiocca, E. A. Oncolytic viruses for the therapy of brain tumors and other solid malignancies: a review. *Frontiers in bioscience : a journal and virtual library* **8**, e346-60 (2003).

104.Geletneky, K. *et al.* Phase I/IIa study of intratumoral/intracerebral or intravenous/intracerebral administration of Parvovirus H-1 (ParvOryx) in patients with progressive primary or recurrent glioblastoma multiforme: ParvOryx01 protocol. *BMC cancer* **12**, 99 (2012).

105.Gilbert, M. R. Recurrent glioblastoma: a fresh look at current therapies and emerging novel approaches. *Seminars in oncology* **38 Suppl 4**, S21-33 (2011).

106.Bai, R., Staedtke, V. & Riggins, G. J. Molecular targeting of glioblastoma: Drug discovery and therapies. *Trends in molecular medicine* **17**, 301-12 (2011).

107.Farias-Eisner, G. *et al.* Glioblastoma biomarkers from bench to bedside: advances and challenges. *British journal of neurosurgery* **26**, 189-94 (2012).

108.Schuster, J. M., Friedman, H. S. & Bigner, D. D. Therapeutic analysis of in vitro and in vivo brain tumor models. *Neurologic clinics* **9**, 375-82 (1991).

109.Garson, K., Shaw, T. J., Clark, K. V., Yao, D. & Vanderhyden, B. C. Models of ovarian cancer--are we there yet? *Molecular and cellular endocrinology* **239**, 15-26 (2005).

110.Reyes, G. *et al.* Orthotopic xenografts of human pancreatic carcinomas acquire genetic aberrations during dissemination in nude mice. *Cancer research* **56**, 5713-9 (1996).

111.Vogel, C. L., Reddy, J. C. & Reyno, L. M. Efficacy of trastuzumab. *Cancer research* **65**, 2044 (2005).

112.Voskoglou-Nomikos, T., Pater, J. L. & Seymour, L. Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 4227-39 (2003).

113.Fiebig, H. H., Maier, A. & Burger, A. M. Clonogenic assay with established human tumour xenografts: correlation of in vitro to in vivo activity as a basis for anticancer drug discovery. *European journal of cancer (Oxford, England : 1990)* **40**, 802-20 (2004).

114.Huszthy, P. C. *et al.* In vivo models of primary brain tumors: pitfalls and perspectives. *Neuro-oncology* **14**, 979-93 (2012).

115.Fischer, H., Schwechheimer, K., Heider, M., Bernhardt, S. & Zang, K. D. Establishment and characterization of a human glioblastoma cell line with a stable karyotype and nullisomy 13. *Cancer genetics and cytogenetics* **17**, 257-68 (1985).

116.Bakir, A. *et al.* Establishment and characterization of a human glioblastoma multiforme cell line. *Cancer genetics and cytogenetics* **103**, 46-51 (1998).

117.Romaguera-Ros, M. *et al.* Cancer-initiating enriched cell lines from human glioblastoma: preparing for drug discovery assays. *Stem cell reviews* **8**, 288-98 (2012).

118.Bigner, S. H. *et al.* Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. *Cancer research* **50**, 8017-22 (1990).

119.Pandita, A., Aldape, K. D., Zadeh, G., Guha, A. & James, C. D. Contrasting in vivo and in vitro fates of glioblastoma cell subpopulations with amplified EGFR. *Genes, chromosomes & cancer* **39**, 29-36 (2004).

120.Witusik-Perkowska, M. *et al.* Glioblastoma-derived spheroid cultures as an experimental model for analysis of EGFR anomalies. *Journal of neuro-oncology* **102**, 395-407 (2011).

121.Piaskowski, S. *et al.* Glioma cells showing IDH1 mutation cannot be propagated in standard cell culture conditions. *British journal of cancer* **104**, 968-70 (2011).

122.Brewer, G. J. & Torricelli, J. R. Isolation and culture of adult neurons and neurospheres. *Nature protocols* **2**, 1490-8 (2007).

123.Janka, M. *et al.* Comparative amplification analysis of human glioma tissue and glioma derived fragment spheroids using reverse chromosome painting (RCP). *Anticancer research* **16**, 2601-6 (1996).

124.Ignatova, T. N. *et al.* Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* **39**, 193-206 (2002).

125.Pollard, S. M. *et al.* Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell stem cell* **4**, 568-80 (2009).

126.Galli, R. *et al.* Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer research* **64**, 7011-21 (2004).

127.Mohr, U. *et al.* Pathology of tumours in laboratory animals. Tumours of the lower respiratory tract and pleura in the rat. *IARC scientific publications* 275-99 (1990).at <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=2093651&retmode=ref&cmd=prlinks>

128.Schmidek, H. H., Nielsen, S. L., Schiller, A. L. & Messer, J. Morphological studies of rat brain tumors induced by N-nitrosomethylurea. *Journal of neurosurgery* **34**, 335-40 (1971).

129.DRUCKREY, H., IVANKOVIC, S. & PREUSSMANN, R. [SELECTIVE INDUCTION OF MALIGNANT TUMORS IN THE BRAIN AND SPINAL CORD OF RATS BY N-METHYL-N-NITROSOUREA]. *Zeitschrift für Krebsforschung* **66**, 389-408 (1965).

130.Macleod, K. F. & Jacks, T. Insights into cancer from transgenic mouse models. *The Journal of pathology* **187**, 43-60 (1999).

131.Talmadge, J. E., Donkor, M. & Scholar, E. Inflammatory cell infiltration of tumors: Jekyll or Hyde. *Cancer metastasis reviews* **26**, 373-400 (2007).

132.Talmadge, J. E. Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 5243-8 (2007).

133.Woolfenden, S., Zhu, H. & Charest, al A Cre/LoxP conditional luciferase reporter transgenic mouse for bioluminescence monitoring of tumorigenesis. *Genesis (New York, N.Y. : 2000)* **47**, 659-66 (2009).

134.Senner, V., Sturm, A., Hoess, N., Wassmann, H. & Paulus, W. In vivo glioma model enabling regulated gene expression. *Acta neuropathologica* **99**, 603-8 (2000).

135.Linnebacher, M. *et al.* Cryopreservation of human colorectal carcinomas prior to xenografting. *BMC cancer* **10**, 362 (2010).

136.Carlson, B. L., Pokorny, J. L., Schroeder, M. A. & Sarkaria, J. N. Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery. *Current protocols in pharmacology / editorial board, S.J. Enna (editor-in-chief) ... [et al.]* **Chapter 14**, Unit 14.16 (2011).

137.Joo, K. M. *et al.* Patient-specific orthotopic glioblastoma xenograft models recapitulate the histopathology and biology of human glioblastomas in situ. *Cell reports* **3**, 260-73 (2013).

138.Ogino, S. *et al.* Epigenetic profiling of synchronous colorectal neoplasias by quantitative DNA methylation analysis. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **19**, 1083-90 (2006).

139.Hayflick, L. Recent advances in the cell biology of aging. *Mechanisms of ageing and development* **14**, 59-79 (1980).

140.Chakravarti, A., Chakladar, A., Delaney, M. A., Latham, D. E. & Loeffler, J. S. The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. *Cancer research* **62**, 4307-15 (2002).

141.Mazzoleni, S. *et al.* Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. *Cancer research* **70**, 7500-13 (2010).

142.Hobbs, J. *et al.* Paradoxical relationship between the degree of EGFR amplification and outcome in glioblastomas. *The American journal of surgical pathology* **36**, 1186-93 (2012).

143.Gambichler, T., Grothe, S., Rotterdam, S., Altmeyer, P. & Kreuter, A. Protein expression of carcinoembryonic antigen cell adhesion molecules in benign and malignant melanocytic skin lesions. *American journal of clinical pathology* **131**, 782-7 (2009).

144.Dango, S. *et al.* Elevated expression of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) is associated with increased angiogenic potential in non-small-cell lung cancer. *Lung cancer (Amsterdam, Netherlands)* **60**, 426-33 (2008).

145.Ieda, J. *et al.* Re-expression of CEACAM1 long cytoplasmic domain isoform is associated with invasion and migration of colorectal cancer. *International journal of cancer. Journal international du cancer* **129**, 1351-61 (2011).

146.Zhou, C. *et al.* The different expression of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) and possible roles in gastric carcinomas. *Pathology, research and practice* **205**, 483-9 (2009).

147.Ellis, J. M. *et al.* Frequencies of HLA-A2 alleles in five U.S. population groups. Predominance Of A*02011 and identification of HLA-A*0231. *Human immunology* **61**, 334-40 (2000).

148.Kurzen, H., Schmitt, S., Näher, H. & Möhler, T. Inhibition of angiogenesis by non-toxic doses of temozolomide. *Anti-cancer drugs* **14**, 515-22 (2003).

149.Agarwala, S. S. & Kirkwood, J. M. Temozolomide, a novel alkylating agent with activity in the central nervous system, may improve the treatment of advanced metastatic melanoma. *The oncologist* **5**, 144-51 (2000).

150.Nieder, C., Grosu, A. L. & Molls, M. A comparison of treatment results for recurrent malignant gliomas. *Cancer treatment reviews* **26**, 397-409 (2000).

151.Brada, M. *et al.* Multicenter phase II trial of temozolomide in patients with glioblastoma multiforme at first relapse. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **12**, 259-66 (2001).

152.Xu, J. *et al.* Novel cell lines established from pediatric brain tumors. *Journal of neuro-oncology* **107**, 269-80 (2012).

153.Maunoury, R. Establishment and characterization of 5 human cell lines derived from a series of 50 primary intracranial tumors. *Acta neuropathologica* **39**, 33-41 (1977).

154.Stockhausen, M. *et al.* Maintenance of EGFR and EGFRvIII expressions in an in vivo and in vitro model of human glioblastoma multiforme. *Experimental cell research* **317**, 1513-26 (2011).

155.Lathia, J. D. *et al.* Laminin alpha 2 enables glioblastoma stem cell growth. *Annals of neurology* **72**, 766-78 (2012).

156.Ferla, R., Bonomi, M., Otvos, L., Jr & Surmacz, E. Glioblastoma-derived leptin induces tube formation and growth of endothelial cells: comparison with VEGF effects. *BMC cancer* **11**, 303 (2011).

157.Gilbertson, R. J. & Rich, J. N. Making a tumour's bed: glioblastoma stem cells and the vascular niche. *Nature reviews. Cancer* **7**, 733-6 (2007).

158.Ishiwata, I. *et al.* Establishment and characterization of human glioblastoma cell line (HUBT-n). *Human cell* **18**, 59-65 (2005).

159.Wang, J. *et al.* Establishment of a new human glioblastoma multiforme cell line (WJ1) and its partial characterization. *Cellular and molecular neurobiology* **27**, 831-43 (2007).

160.Bin Park, Y. *et al.* Alterations of proliferative and differentiation potentials of human embryonic stem cells during long-term culture. *Experimental & molecular medicine* **40**, 98-108 (2008).

161.Chang-Liu, C. M. & Woloschak, G. E. Effect of passage number on cellular response to DNA-damaging agents: cell survival and gene expression. *Cancer letters* **113**, 77-86 (1997).

162.Capes-Davis, A. *et al.* Match criteria for human cell line authentication: Where do we draw the line? *International journal of cancer. Journal international du cancer* (2012).doi:10.1002/ijc.27931

163.Joseph, N. M. *et al.* Diagnostic implications of IDH1-R132H and OLIG2 expression patterns in rare and challenging glioblastoma variants. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* (2012).doi:10.1038/modpathol.2012.173

164.Kim, B. *et al.* The clinicopathologic values of the molecules associated with the main pathogenesis of the glioblastoma. *Journal of the neurological sciences* **294**, 112-8 (2010).

165.Montserrat, M., Leveque, D., Barthelemy, P. & Bergerat, J. P. Duration of adjuvant trastuzumab treatment in routine practice. *Anticancer research* **32**, 4585-8 (2012).

166.Berezowska, S. & Schlegel, J. Targeting ErbB receptors in high-grade glioma. *Current pharmaceutical design* **17**, 2468-87 (2011).

167.Gajadhar, A. S., Bogdanovic, E., Muñoz, D. M. & Guha, A. In situ analysis of mutant EGFRs prevalent in glioblastoma multiforme reveals aberrant dimerization, activation, and

differential response to anti-EGFR targeted therapy. *Molecular cancer research : MCR* **10**, 428-40 (2012).

168.Laviolette, P. S. *et al.* Vascular change measured with independent component analysis of dynamic susceptibility contrast MRI predicts bevacizumab response in high-grade glioma. *Neuro-oncology* (2013).doi:10.1093/neuonc/nos323

169.Wang, Y. & Jiang, T. Understanding high grade glioma: Molecular mechanism, therapy and comprehensive management. *Cancer letters* (2013).doi:10.1016/j.canlet.2012.12.024

170.Duarte, C. W. *et al.* Expression signature of IFN/STAT1 signaling genes predicts poor survival outcome in glioblastoma multiforme in a subtype-specific manner. *PloS one* **7**, e29653 (2012).

171.Forment, J. V., Kaidi, A. & Jackson, S. P. Chromothripsis and cancer: causes and consequences of chromosome shattering. *Nature reviews. Cancer* **12**, 663-70 (2012).

172.Ruano, Y. *et al.* Identification of survival-related genes of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma multiforme. *Cancer* **112**, 1575-84 (2008).

173.Maletzki, C. *et al.* Establishment, characterization and chemosensitivity of three mismatch repair deficient cell lines from sporadic and inherited colorectal carcinomas. *PloS one* **7**, e52485 (2012).

174.Linnebacher, M. *et al.* Single nucleotide polymorphism array analysis of microsatellite-stable, diploid/near-diploid colorectal carcinomas without the CpG island methylator phenotype. *Oncology letters* **5**, 173-178 (2013).

175.He, J. *et al.* CD90 is identified as a candidate marker for cancer stem cells in primary high-grade gliomas using tissue microarrays. *Molecular & cellular proteomics : MCP* **11**, M111.010744 (2012).

176.Parry, P. V. & Engh, J. A. CD90 is identified as a marker for cancer stem cells in high-grade gliomas using tissue microarrays. *Neurosurgery* **70**, N23-4 (2012).

177.Bramanti, V., Tomassoni, D., Avitabile, M., Amenta, F. & Avola, R. Biomarkers of glial cell proliferation and differentiation in culture. *Frontiers in bioscience (Scholar edition)* **2**, 558-70 (2010).

178.Trog, D., Yeghiazaryan, K., Schild, H. H. & Golubnitschaja, O. Up-regulation of vimentin expression in low-density malignant glioma cells as immediate and late effects under irradiation and temozolomide treatment. *Amino acids* **34**, 539-45 (2008).

179.Satelli, A. & Li, S. Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cellular and molecular life sciences : CMLS* **68**, 3033-46 (2011).

180.Middeldorp, J. & Hol, E. M. GFAP in health and disease. *Progress in neurobiology* **93**, 421-43 (2011).

181.Chumbalkar, V. C. *et al.* Differential protein expression in human gliomas and molecular insights. *Proteomics* **5**, 1167-77 (2005).

182.Jung, C. S. *et al.* Serum GFAP is a diagnostic marker for glioblastoma multiforme. *Brain : a journal of neurology* **130**, 3336-41 (2007).

183.Taniuchi, K., Nishimori, I. & Hollingsworth, M. A. Intracellular CD24 inhibits cell invasion by posttranscriptional regulation of BART through interaction with G3BP. *Cancer research* **71**, 895-905 (2011).

184.Deng, J. *et al.* CD24 expression as a marker for predicting clinical outcome in human gliomas. *Journal of biomedicine & biotechnology* **2012**, 517172 (2012).

185.Reeves, R. H. *et al.* Astrocytosis and axonal proliferation in the hippocampus of S100b transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 5359-63 (1994).

186.Camby, I. *et al.* Differential expression of S100 calcium-binding proteins characterizes distinct clinical entities in both WHO grade II and III astrocytic tumours. *Neuropathology and applied neurobiology* **26**, 76-90 (2000).

187.Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-8 (1994).

188.Rosen, J. M. & Jordan, C. T. The increasing complexity of the cancer stem cell paradigm. *Science (New York, N.Y.)* **324**, 1670-3 (2009).

189.Tan, C. & Scotting, P. J. Stem cell research points the way to the cell of origin for intracranial germ cell tumours. *The Journal of pathology* **229**, 4-11 (2013).

190.Thiery, J. P. Epithelial-mesenchymal transitions in tumour progression. *Nature reviews. Cancer* **2**, 442-54 (2002).

191.Yang, Y. *et al.* Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI. *Biomaterials* **33**, 1462-76 (2012).

192.Wang, C. *et al.* Photothermolysis of glioblastoma stem-like cells targeted by carbon nanotubes conjugated with CD133 monoclonal antibody. *Nanomedicine : nanotechnology, biology, and medicine* **7**, 69-79 (2011).

193.Xu, Q. *et al.* Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. *Stem cells (Dayton, Ohio)* **27**, 1734-40 (2009).

194.Prendergast, C. T. & Anderton, S. M. Immune cell entry to central nervous system--current understanding and prospective therapeutic targets. *Endocrine, metabolic & immune disorders drug targets* **9**, 315-27 (2009).

195.Sawamura, Y. & de Tribolet, N. Immunotherapy of brain tumors. *Journal of neurosurgical sciences* **34**, 265-78 (1990).

196.Sunit, das, Srikanth, M. & Kessler, J. A. Cancer stem cells and glioma. *Nature clinical practice. Neurology* **4**, 427-35 (2008).

197.Halama, N., Zoernig, I. & Jäger, D. [Immunotherapy for cancer--modern immunologic strategies in oncology]. *Deutsche medizinische Wochenschrift (1946)* **133**, 2105-8 (2008).

198.Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science (New York, N.Y.)* **331**, 1565-70 (2011).

199.Hackam, A. S. *et al.* Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *The Journal of biological chemistry* **275**, 41299-308 (2000).

200.Bradley, S. V. *et al.* Huntingtin interacting protein 1 is a novel brain tumor marker that associates with epidermal growth factor receptor. *Cancer research* **67**, 3609-15 (2007).

201.Wykosky, J., Gibo, D. M., Stanton, C. & Debinski, W. Interleukin-13 receptor alpha 2, EphA2, and Fos-related antigen 1 as molecular denominators of high-grade astrocytomas and specific targets for combinatorial therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 199-208 (2008).

202.Debinski, W., Gibo, D. M., Slagle, B., Powers, S. K. & Gillespie, G. Y. Receptor for interleukin 13 is abundantly and specifically over-expressed in patients with glioblastoma multiforme. *International journal of oncology* **15**, 481-6 (1999).

203.Debinski, W., Slagle, B., Gibo, D. M., Powers, S. K. & Gillespie, G. Y. Expression of a restrictive receptor for interleukin 13 is associated with glial transformation. *Journal of neuro-oncology* **48**, 103-11 (2000).

204.Nishimura, Y., Nitto, T., Inoue, T. & Node, K. STAT6 mediates apoptosis of human coronary arterial endothelial cells by interleukin-13. *Hypertension research : official journal of the Japanese Society of Hypertension* **31**, 535-41 (2008).

205.Kawakami, M., Leland, P., Kawakami, K. & Puri, R. K. Mutation and functional analysis of IL-13 receptors in human malignant glioma cells. *Oncology research* **12**, 459-67 (2001).

206.Hsi, L. C. *et al.* Silencing IL-13Rα2 promotes glioblastoma cell death via endogenous signaling. *Molecular cancer therapeutics* **10**, 1149-60 (2011).

207.Rahaman, S. O. *et al.* IL-13R(alpha)2, a decoy receptor for IL-13 acts as an inhibitor of IL-4-dependent signal transduction in glioblastoma cells. *Cancer research* **62**, 1103-9 (2002).

208.Chiaramonte, M. G. *et al.* Regulation and function of the interleukin 13 receptor alpha 2 during a T helper cell type 2-dominant immune response. *The Journal of experimental medicine* **197**, 687-701 (2003).

209.Okano, F., Storkus, W. J., Chambers, W. H., Pollack, I. F. & Okada, H. Identification of a novel HLA-A*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8**, 2851-5 (2002).

210.Husain, S. R., Joshi, B. H. & Puri, R. K. Interleukin-13 receptor as a unique target for anti-glioblastoma therapy. *International journal of cancer. Journal international du cancer* **92**, 168-75 (2001).

211.Kawakami, K., Kioi, M., Liu, Q., Kawakami, M. & Puri, R. K. Evidence that IL-13R alpha2 chain in human glioma cells is responsible for the antitumor activity mediated by receptor-directed cytotoxin therapy. *Journal of immunotherapy (Hagerstown, Md. : 1997)* **28**, 193-202 (2005).

212.Gold, P. & Freedman, S. O. Specific carcinoembryonic antigens of the human digestive system. *The Journal of experimental medicine* **122**, 467-81 (1965).

213.d'Aquino, S. *et al.* [Immunohistochemical study on the presence of CEA in primary brain tumors. Preliminary note]. *Minerva medica* **76**, 1587-91 (1985).

214.Hammarström, S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Seminars in cancer biology* **9**, 67-81 (1999).

215.Turriziani, M. *et al.* Carcinoembryonic antigen (CEA)-based cancer vaccines: recent patents and antitumor effects from experimental models to clinical trials. *Recent patents on anti-cancer drug discovery* **7**, 265-96 (2012).

216.Chester, K. A. *et al.* Recombinant anti-carcinoembryonic antigen antibodies for targeting cancer. *Cancer chemotherapy and pharmacology* **46 Suppl**, S8-12 (2000).

217.Bolocan, A., Ion, D., Ciocan, D. N. & Paduraru, D. N. Prognostic and predictive factors in colorectal cancer. *Chirurgia (Bucharest, Romania : 1990)* **107**, 555-63 (2012).

218.Garud, S. S. & Willingham, F. F. Molecular analysis of cyst fluid aspiration in the diagnosis and risk assessment of cystic lesions of the pancreas. *Clinical and translational science* **5**, 102-7 (2012).

219.Tamura, K. [Tumor markers for thyroid malignant disease]. *Nihon rinsho. Japanese journal of clinical medicine* **69 Suppl 2**, 298-303 (2011).

220.Fiori, V., Magnani, M. & Cianfriglia, M. The expression and modulation of CEACAM1 and tumor cell transformation. *Annali dell'Istituto superiore di sanità* **48**, 161-71 (2012).

221.Nouvion, A. & Beauchemin, N. [CEACAM1 as a central modulator of metabolism, tumor progression, angiogenesis and immunity]. *Médecine sciences : M/S* **25**, 247-52 (2009).

222.Thomas, P., Forse, R. A. & Bajenova, O. Carcinoembryonic antigen (CEA) and its receptor hnRNP M are mediators of metastasis and the inflammatory response in the liver. *Clinical & experimental metastasis* **28**, 923-32 (2011).

223.Pellegatta, S., Cuppini, L. & Finocchiaro, G. Brain cancer immunoediting: novel examples provided by immunotherapy of malignant gliomas. *Expert review of anticancer therapy* **11**, 1759-74 (2011).

224.Zhu, V. F., Yang, J., Lebrun, D. G. & Li, M. Understanding the role of cytokines in Glioblastoma Multiforme pathogenesis. *Cancer letters* **316**, 139-50 (2012).

225.Charles, N. A., Holland, E. C., Gilbertson, R., Glass, R. & Kettenmann, H. The brain tumor microenvironment. *Glia* **59**, 1169-80 (2011).

226.Waziri, A. *et al.* Preferential in situ CD4+CD56+ T cell activation and expansion within human glioblastoma. *Journal of immunology (Baltimore, Md. : 1950)* **180**, 7673-80 (2008).

227.Jackson, A. The changing face of brain tumours. Preface. *The British journal of radiology* **84 Spec No 2**, S79-81 (2011).

228.Harling-Berg, C. J., Park, T. J. & Knopf, P. M. Role of the cervical lymphatics in the Th2-type hierarchy of CNS immune regulation. *Journal of neuroimmunology* **101**, 111-27 (1999).

229.Rodrigues, J. C. *et al.* Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. *Neuro-oncology* **12**, 351-65 (2010).

230.Wainwright, D. A. *et al.* IDO expression in brain tumors increases the recruitment of regulatory T cells and negatively impacts survival. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 6110-21 (2012).

231.Verschuere, T., de Vleeschouwer, S., Lefranc, F., Kiss, R. & van Gool, S. W. Galectin-1 and immunotherapy for brain cancer. *Expert review of neurotherapeutics* **11**, 533-43 (2011).

232.Wrann, M. *et al.* T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-beta. *The EMBO journal* **6**, 1633-6 (1987).

233.Veldhuis, G. J., Willemse, P. H., Mulder, N. H., Limburg, P. C. & de Vries, E. G. Potential use of recombinant human interleukin-6 in clinical oncology. *Leukemia & lymphoma* **20**, 373-9 (1996).

234.Yeung, Y., McDonald, K., Grewal, T. & Munoz, L. Interleukins in glioblastoma pathophysiology: implications for therapy. *British journal of pharmacology* **168**, 591-606 (2013).

235.Rolhion, C. *et al.* Interleukin-6 overexpression as a marker of malignancy in human gliomas. *Journal of neurosurgery* **94**, 97-101 (2001).

236.Liu, Q. *et al.* IL-6 promotion of glioblastoma cell invasion and angiogenesis in U251 and T98G cell lines. *Journal of neuro-oncology* **100**, 165-76 (2010).

237.Weissenberger, J. *et al.* IL-6 is required for glioma development in a mouse model. *Oncogene* **23**, 3308-16 (2004).

238.Saidi, A. *et al.* Combined targeting of interleukin-6 and vascular endothelial growth factor potently inhibits glioma growth and invasiveness. *International journal of cancer. Journal international du cancer* **125**, 1054-64 (2009).

239.Goswami, S., Gupta, A. & Sharma, S. K. Interleukin-6-mediated autocrine growth promotion in human glioblastoma multiforme cell line U87MG. *Journal of neurochemistry* **71**, 1837-45 (1998).

240.Xie, K. Interleukin-8 and human cancer biology. *Cytokine & growth factor reviews* **12**, 375-91 (2001).

241.Brat, D. J., Bellail, A. C. & van Meir, E. G. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-oncology* **7**, 122-33 (2005).

242.La Iglesia, de, N. *et al.* Deregulation of a STAT3-interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 5870-8 (2008).

243.Holmes, W. E., Lee, J., Kuang, W. J., Rice, G. C. & Wood, W. I. Structure and functional expression of a human interleukin-8 receptor. *Science (New York, N.Y.)* **253**, 1278-80 (1991).

244.Murphy, P. M. & Tiffany, H. L. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science (New York, N.Y.)* **253**, 1280-3 (1991).

245.Carlsson, A. *et al.* Plasma proteome profiling reveals biomarker patterns associated with prognosis and therapy selection in glioblastoma multiforme patients. *Proteomics. Clinical applications* **4**, 591-602 (2010).

246.Morse, M. A. *et al.* Immunotherapy of surgical malignancies. *Current problems in surgery* **41**, 15-132 (2004).

247.Maruno, M., Kovach, J. S., Kelly, P. J. & Yanagihara, T. Distribution of endogenous tumour necrosis factor alpha in gliomas. *Journal of clinical pathology* **50**, 559-62 (1997).

248.Hao, C. *et al.* Cytokine and cytokine receptor mRNA expression in human glioblastomas: evidence of Th1, Th2 and Th3 cytokine dysregulation. *Acta neuropathologica* **103**, 171-8 (2002).

249.Hong, J. *et al.* Suppressing IL-32 in monocytes impairs the induction of the proinflammatory cytokines TNFalpha and IL-1beta. *Cytokine* **49**, 171-6 (2010).

250.Fajardo, L. F., Kwan, H. H., Kowalski, J., Prionas, S. D. & Allison, A. C. Dual role of tumor necrosis factor-alpha in angiogenesis. *The American journal of pathology* **140**, 539-44 (1992).

251.Yoshida, S. *et al.* Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Molecular and cellular biology* **17**, 4015-23 (1997).

252.Rich, J. N. The role of transforming growth factor-beta in primary brain tumors. *Frontiers in bioscience : a journal and virtual library* **8**, e245-60 (2003).

253.Curtin, J. F. *et al.* Combining cytotoxic and immune-mediated gene therapy to treat brain tumors. *Current topics in medicinal chemistry* **5**, 1151-70 (2005).

254.Rolle, C. E., Sengupta, S. & Lesniak, M. S. Challenges in clinical design of immunotherapy trials for malignant glioma. *Neurosurgery clinics of North America* **21**, 201-14 (2010).

255.Derynck, R., Akhurst, R. J. & Balmain, A. TGF-beta signaling in tumor suppression and cancer progression. *Nature genetics* **29**, 117-29 (2001).

256.Siegel, P. M. & Massagué, J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nature reviews. Cancer* **3**, 807-21 (2003).

257.Rahimi, R. A. & Leof, E. B. TGF-beta signaling: a tale of two responses. *Journal of cellular biochemistry* **102**, 593-608 (2007).

258.Massagué, J. & Gomis, R. R. The logic of TGFbeta signaling. *FEBS letters* **580**, 2811-20 (2006).

259.Seoane, J. Escaping from the TGFbeta anti-proliferative control. *Carcinogenesis* **27**, 2148-56 (2006).

260.Peñuelas, S. *et al.* TGF-beta increases glioma-initiating cell self-renewal through the induction of LIF in human glioblastoma. *Cancer cell* **15**, 315-27 (2009).

261.Hau, P. *et al.* Inhibition of TGF-beta2 with AP 12009 in recurrent malignant gliomas: from preclinical to phase I/II studies. *Oligonucleotides* **17**, 201-12 (2007).

262.Di Chunhui, Mattox, A. K., Harward, S. & Adamson, C. Emerging therapeutic targets and agents for glioblastoma migrating cells. *Anti-cancer agents in medicinal chemistry* **10**, 543-55 (2010).

263.Li, A., Dubey, S., Varney, M. L., Dave, B. J. & Singh, R. K. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *Journal of immunology (Baltimore, Md. : 1950)* **170**, 3369-76 (2003).

264.Llopiz, D. *et al.* Peptide inhibitors of transforming growth factor-beta enhance the efficacy of antitumor immunotherapy. *International journal of cancer. Journal international du cancer* **125**, 2614-23 (2009).

265.Okada, H. *et al.* Immunotherapeutic approaches for glioma. *Critical reviews in immunology* **29**, 1-42 (2009).

266.de Martin, R. *et al.* Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-beta gene family. *The EMBO journal* **6**, 3673-7 (1987).

267.Grauer, O., Pöschl, P., Lohmeier, A., Adema, G. J. & Bogdahn, U. Toll-like receptor triggered dendritic cell maturation and IL-12 secretion are necessary to overcome T-cell inhibition by glioma-associated TGF-beta2. *Journal of neuro-oncology* **82**, 151-61 (2007).

268.Tabatabai, G., Frank, B., Möhle, R., Weller, M. & Wick, W. Irradiation and hypoxia promote homing of haematopoietic progenitor cells towards gliomas by TGF-beta-dependent HIF-1alpha-mediated induction of CXCL12. *Brain : a journal of neurology* **129**, 2426-35 (2006).

269.Ehrhart, E. J., Segarini, P., Tsang, M. L., Carroll, A. G. & Barcellos-Hoff, M. H. Latent transforming growth factor beta1 activation in situ: quantitative and functional evidence after low-dose gamma-irradiation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **11**, 991-1002 (1997).

270.Sasaki, A. *et al.* Secretion of transforming growth factor-beta 1 and -beta 2 by malignant glioma cells. *Neurologia medico-chirurgica* **35**, 423-30 (1995).

271.Gold, L. I. The role for transforming growth factor-beta (TGF-beta) in human cancer. *Critical reviews in oncogenesis* **10**, 303-60 (1999).

272.Anderson, R. C. *et al.* Changes in the immunologic phenotype of human malignant glioma cells after passaging in vitro. *Clinical immunology (Orlando, Fla.)* **102**, 84-95 (2002).

273.Matarese, G., Cava, A. L. & Horvath, T. L. In vivo veritas, in vitro artificia. *Trends in molecular medicine* **18**, 439-42 (2012).

274.Danam, R. P., Howell, S. R., Remack, J. S. & Brent, T. P. Heterogeneous methylation of the O(6)-methylguanine-DNA methyltransferase promoter in immortalized IMR90 cell lines. *International journal of oncology* **18**, 1187-93 (2001).

275.Zhang, K., Wang, X., Bin Zhou & Zhang, L. The prognostic value of MGMT promoter methylation in Glioblastoma multiforme: a meta-analysis. *Familial cancer* (2013).doi:10.1007/s10689-013-9607-1

276.Wang, Y., Zhu, S., Cloughesy, T. F., Liau, L. M. & Mischel, P. S. p53 disruption profoundly alters the response of human glioblastoma cells to DNA topoisomerase I inhibition. *Oncogene* **23**, 1283-90 (2004).

277.Wen, P. Y. *et al.* Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 4899-907 (2006).

278.Desjardins, A. *et al.* Phase II study of imatinib mesylate and hydroxyurea for recurrent grade III malignant gliomas. *Journal of neuro-oncology* **83**, 53-60 (2007).

279.Li, J. *et al.* [Inhibitory effects of rapamycin on proliferation of chronic myelogenous leukemia cells and its mechanism]. *Zhonghua xue ye xue za zhi = Zhonghua xueyexue zazhi* **33**, 843-6 (2012).

280.Kumar, S., Witzig, T. E. & Rajkumar, S. V. Thalidomide as an anti-cancer agent. *Journal of cellular and molecular medicine* **6**, 160-74 (2002).

281.Thomas, D. A. & Kantarjian, H. M. Current role of thalidomide in cancer treatment. *Current opinion in oncology* **12**, 564-73 (2000).

282.Martinez, R. & Esteller, M. The DNA methylome of glioblastoma multiforme. *Neurobiology of disease* **39**, 40-6 (2010).

283.Palanichamy, K., Erkkinen, M. & Chakravarti, A. Predictive and prognostic markers in human glioblastomas. *Current treatment options in oncology* **7**, 490-504 (2006).

284.Maurer, G. D. *et al.* Cilengitide modulates attachment and viability of human glioma cells, but not sensitivity to irradiation or temozolomide in vitro. *Neuro-oncology* **11**, 747-56 (2009).

285.Sorio, C. *et al.* Successful xenografting of cryopreserved primary pancreatic cancers. *Virchows Archiv : an international journal of pathology* **438**, 154-8 (2001).

286.Goike, H. M. *et al.* Cryopreservation of viable human glioblastoma xenografts. *Neuropathology and applied neurobiology* **26**, 172-6 (2000).

287.Foong, C. S. *et al.* Cryopreservation of cancer-initiating cells derived from glioblastoma. *Frontiers in bioscience (Scholar edition)* **3**, 698-708 (2011).

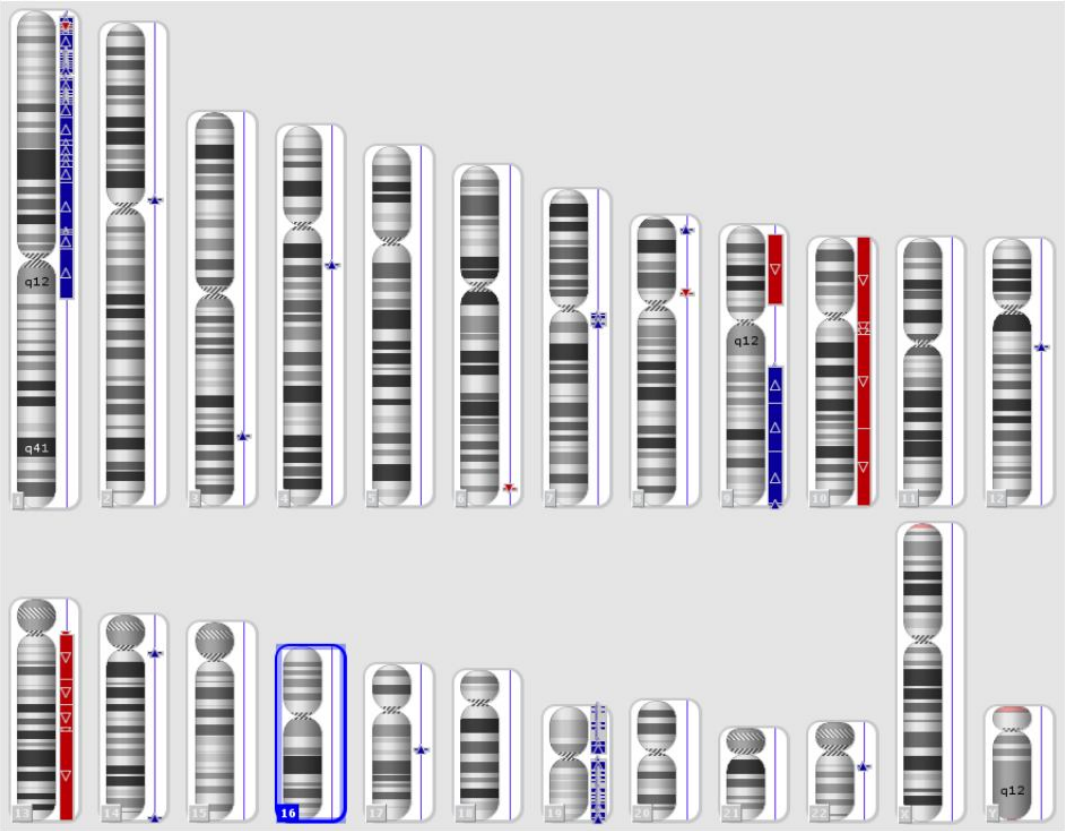
288.Szerlip, N. J. *et al.* Intratumoral heterogeneity of receptor tyrosine kinases EGFR and PDGFRA amplification in glioblastoma defines subpopulations with distinct growth factor response. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3041-6 (2012).

289.Hamilton, M. G. *et al.* Determination of the methylation status of MGMT in different regions within glioblastoma multiforme. *Journal of neuro-oncology* **102**, 255-60 (2011).

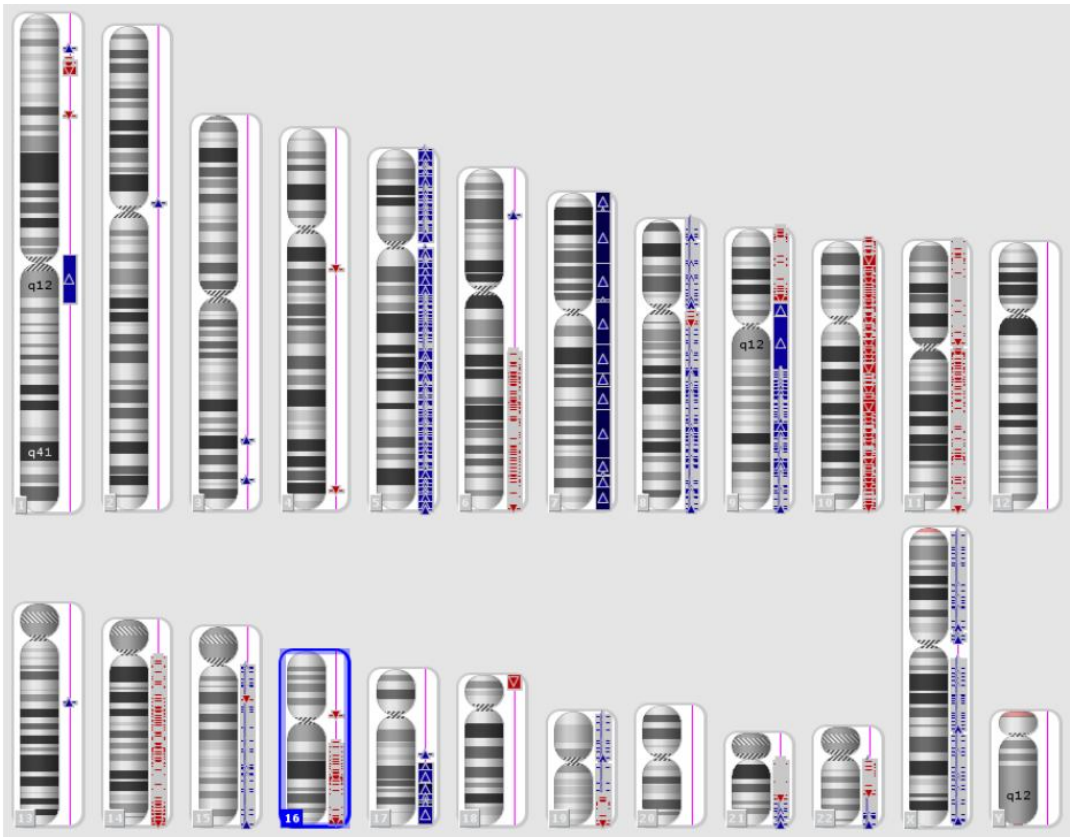
8. Supplementary Material

CGH array data: Pseudo-karyograms of the HROG cell lines obtained from the Affymetrix Genotyping Console 4.1.2 software generated from hybridizations of cell lines gDNA on Affymetrix SNP 6.0 chips. Given is the cell line name followed by the karyogram. Blue indicates genomic gains (i.e. amplifications), whereas red indicates genomic losses (i.e. deletions) in comparison to a reference genome (human reference 103 from Axiom). Detailed information on how this technology is to be used is available at: www.affymetrix.com.

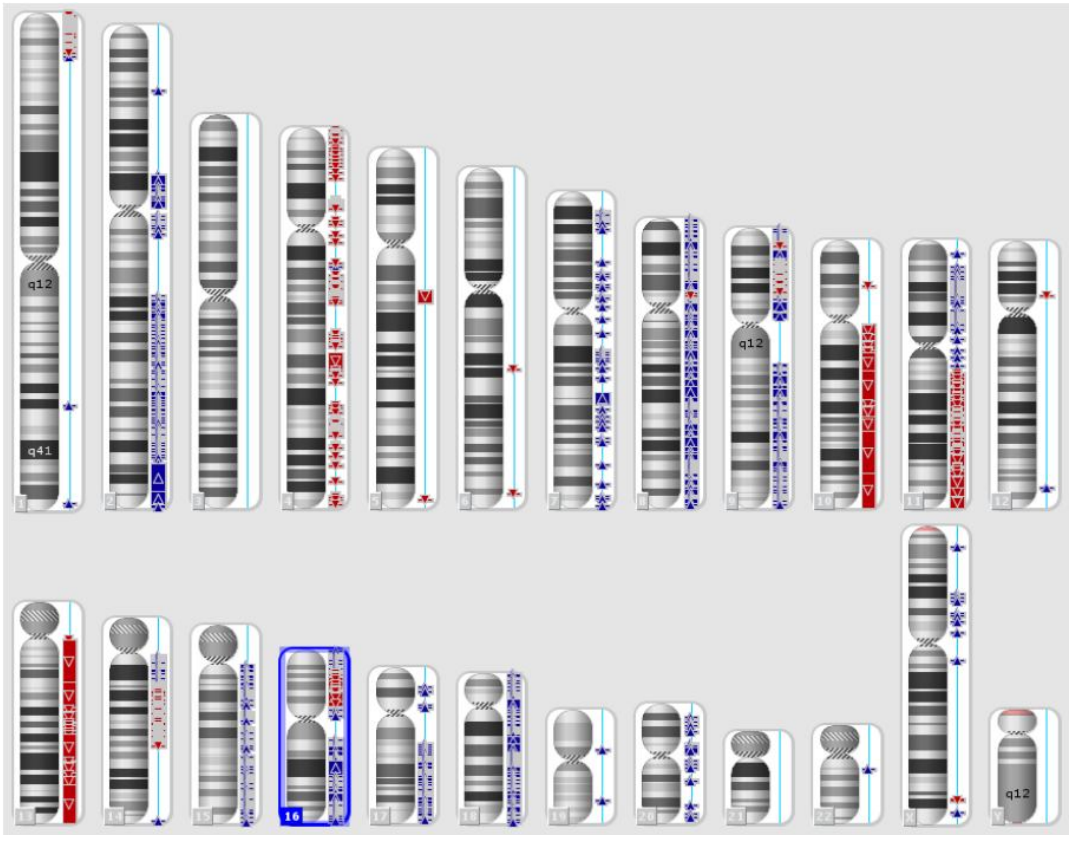
HROG02



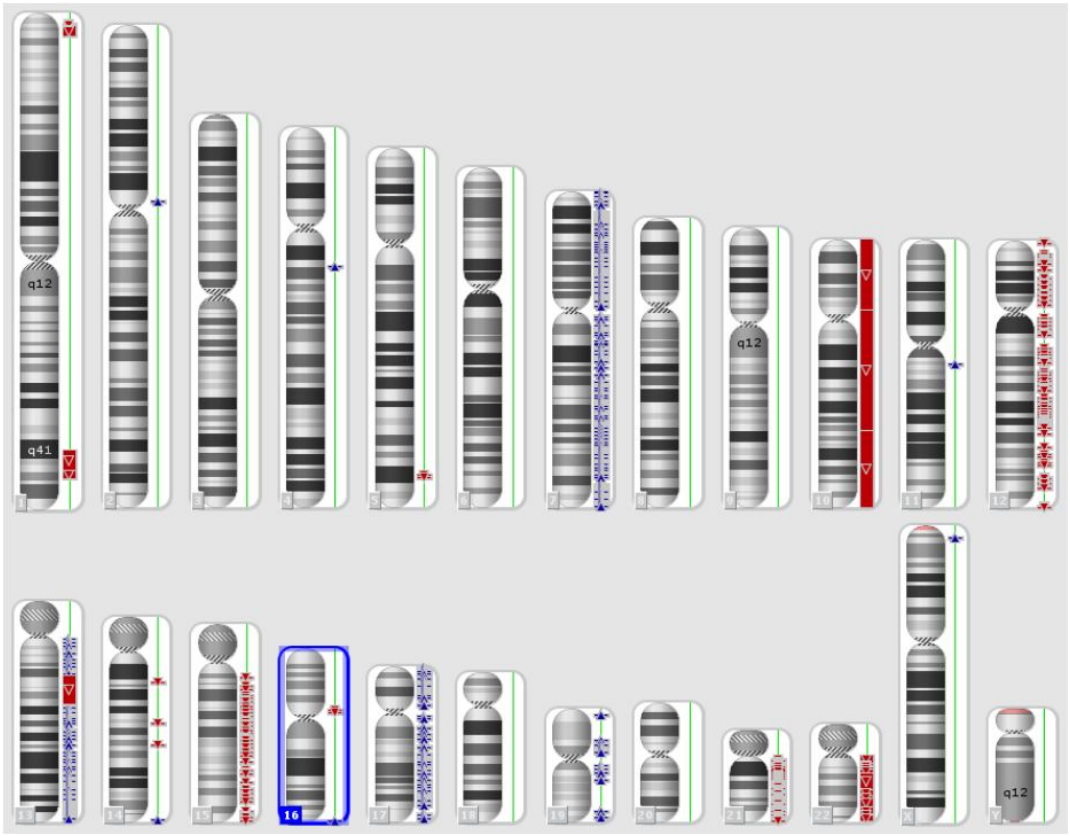
HROG04



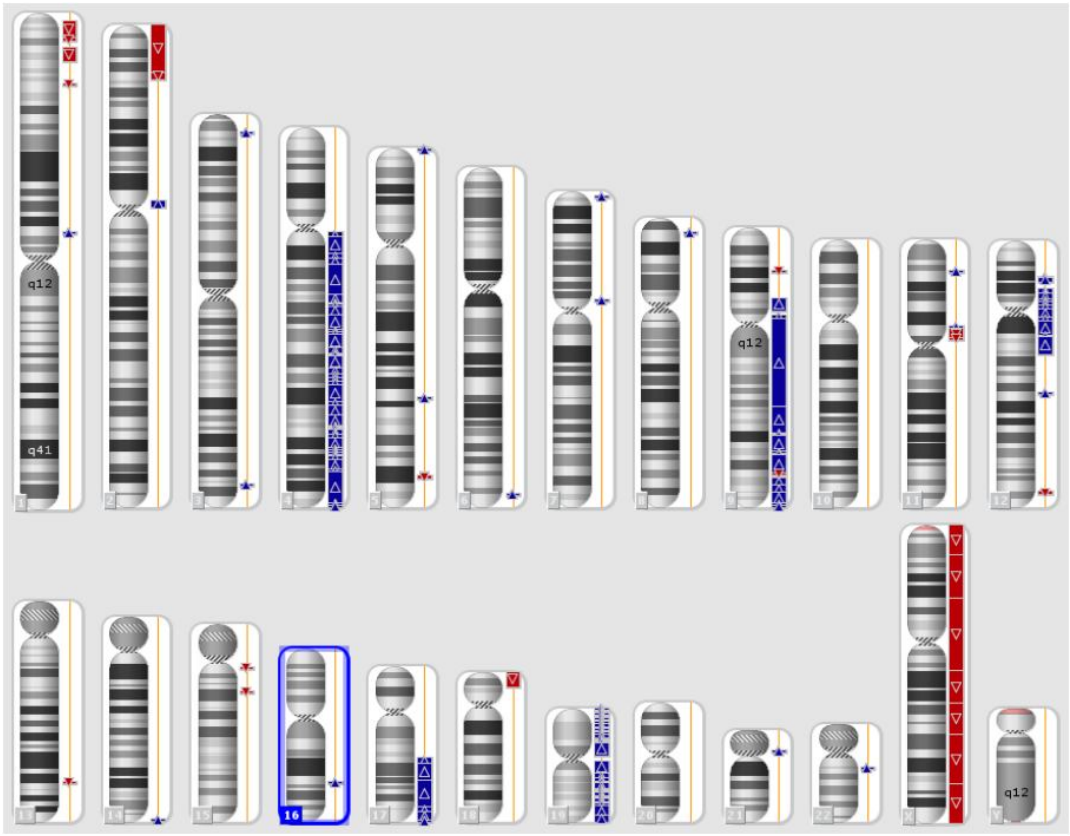
HROG05



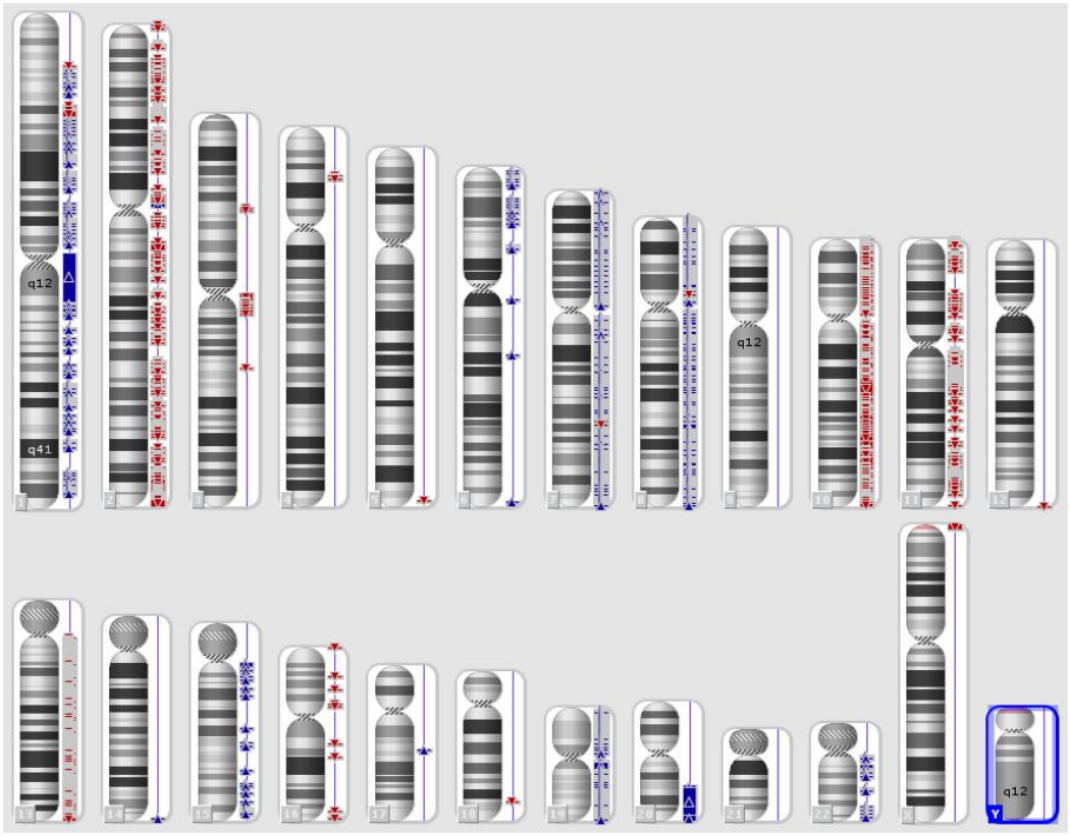
HROG06



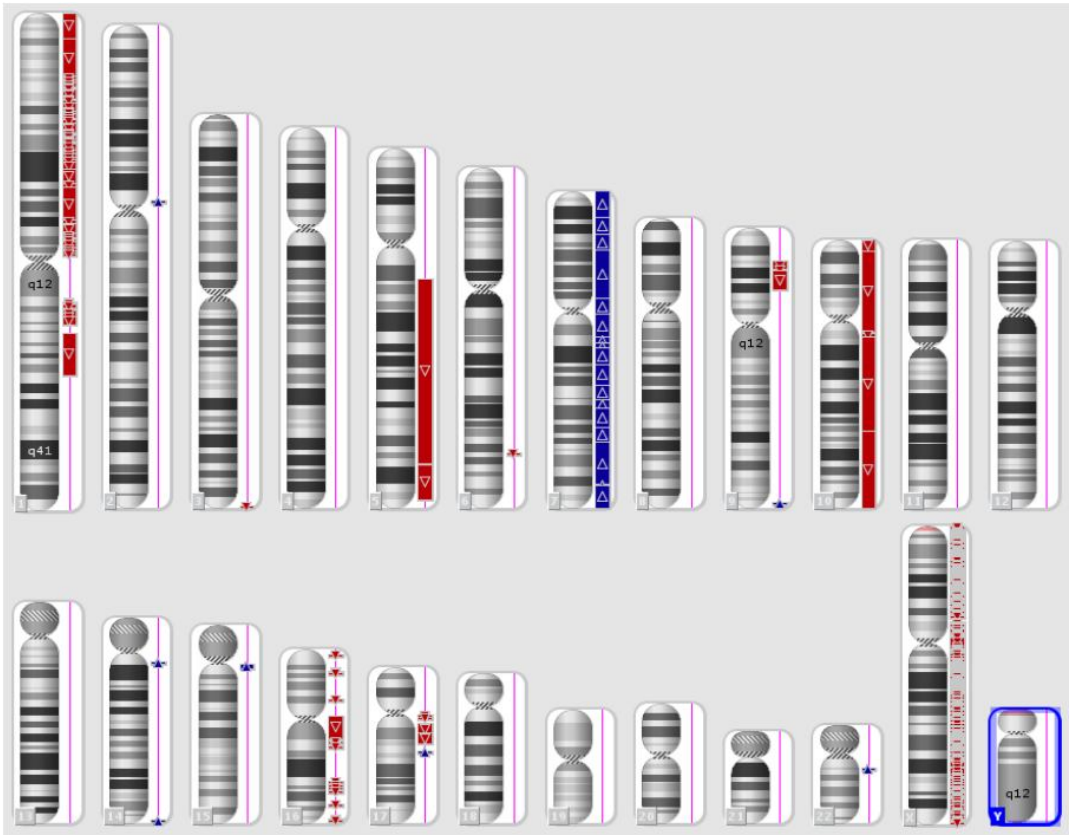
HROG07



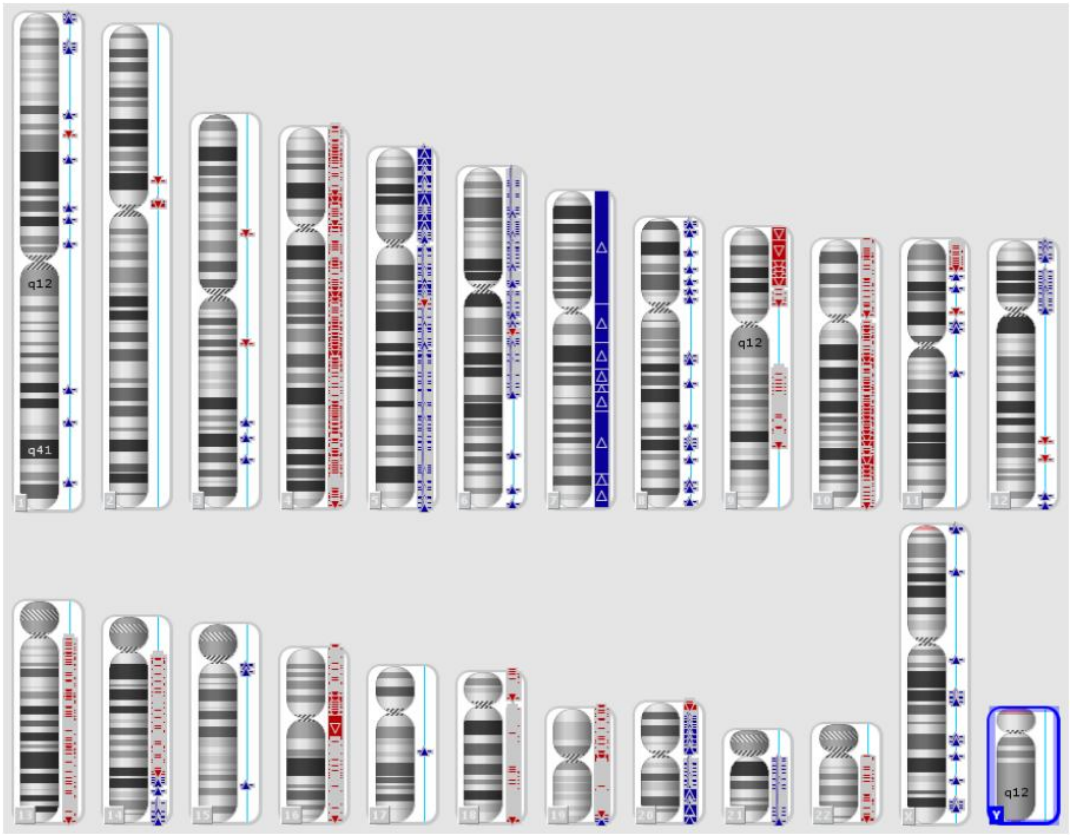
HROG10



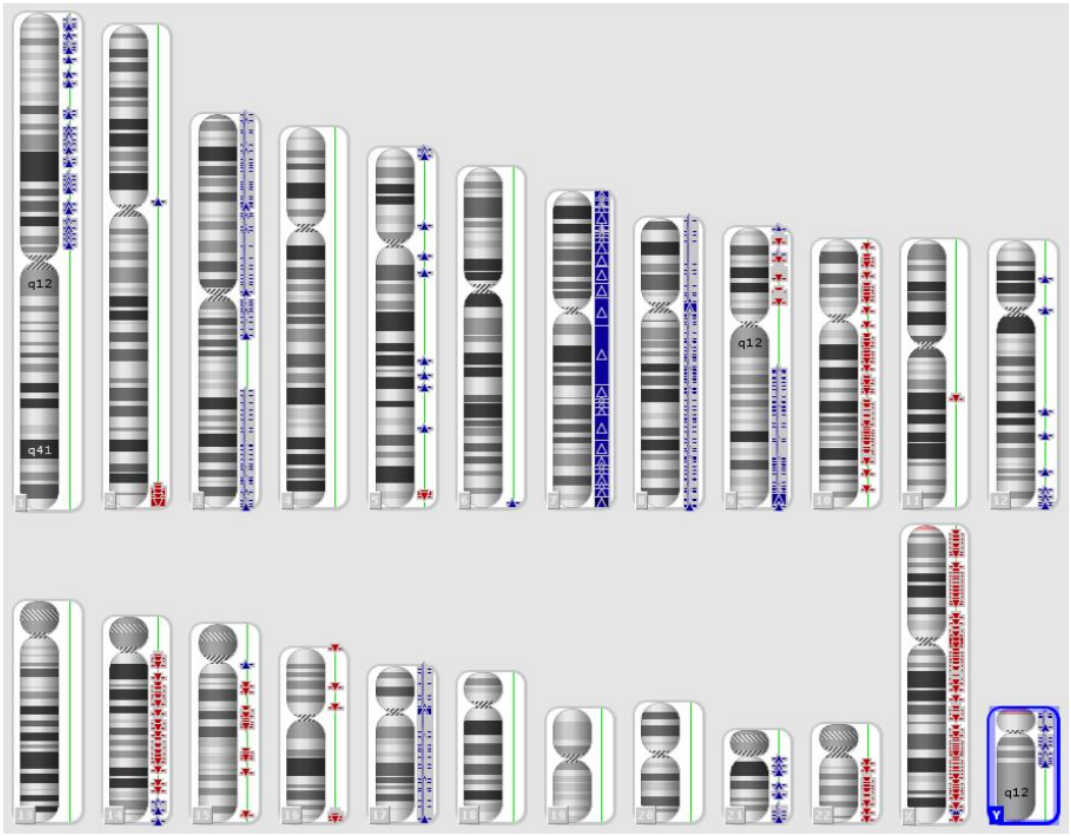
HROG13



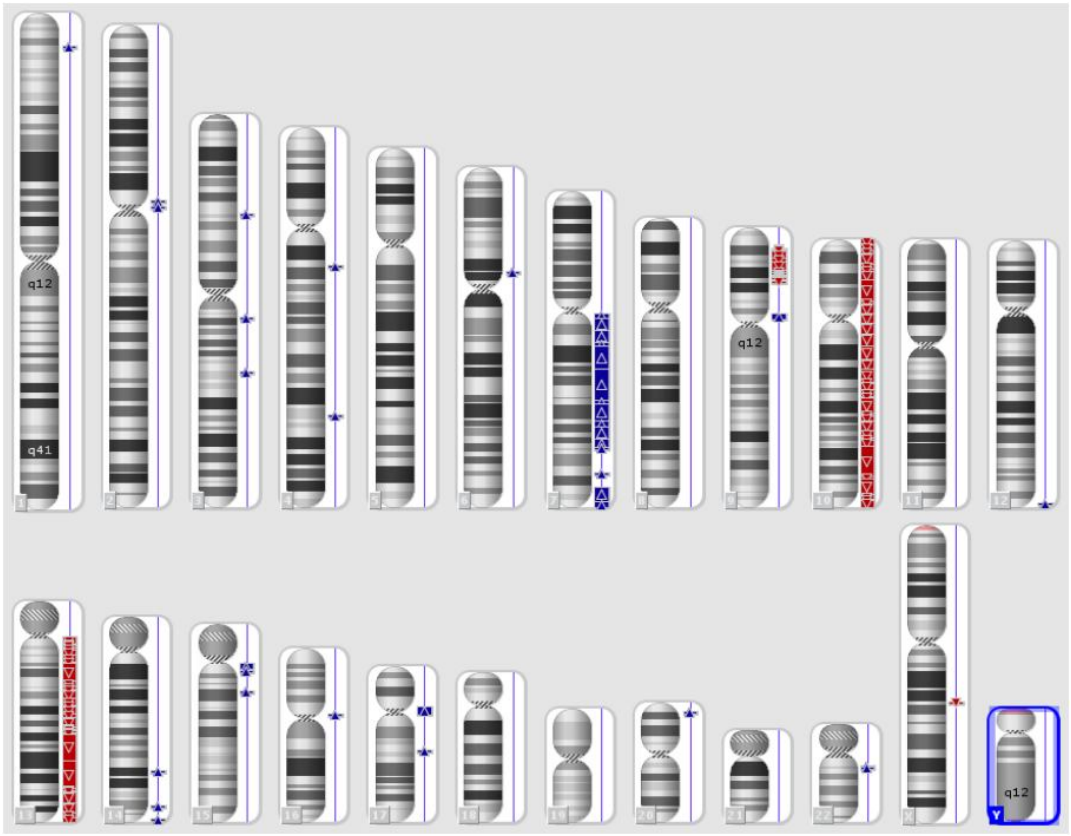
HROG15



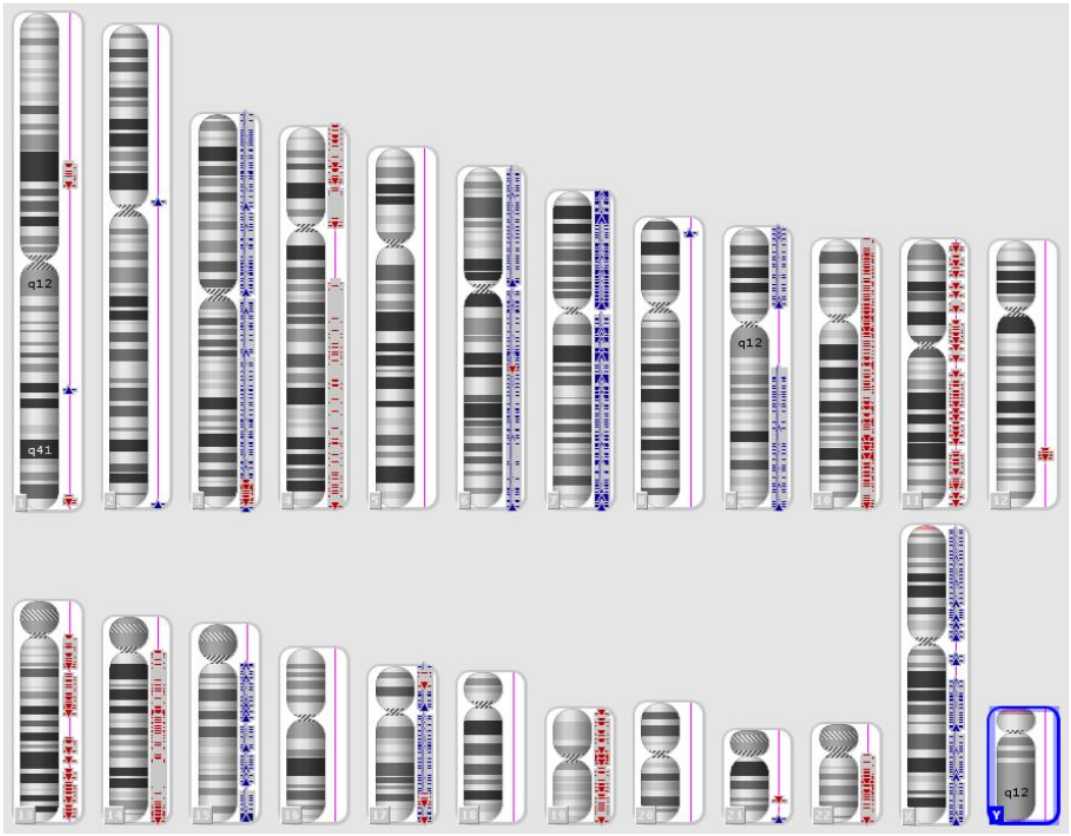
HROG17



HROG24



HROG36



9. Curriculum Vitae

Personal information

Surname:	Mullins
First Name:	Christina
Middle Name:	Susanne
Date of Birth:	21.08.1984
Place of Birth:	Tübingen
Nationality:	German

Scholastic Education

Since 08/2009:	PhD student at the University Rostock and member of research staff in the laboratory of PD Dr. Linnebacher (AG MOI) and PD Dr. Classen (AG Hirntumorvakzine)
10/2004 – 07/2009:	Universität Hohenheim, Stuttgart Advanced studies in Biology Diploma thesis: "**Analysen zur Proteinkinase D beeinflussten Zellmigration in *Drosophila melanogaster***"
10/2006 – 09/2007:	Student research assistant at the Department of Zoology
04/2007 – 03/2008:	Student research assistant at the Department of Genetics
1995 – 2004:	Johann – Sebastian – Bach Gymansium, Mannheim
08/2001 – 12/2001:	Stay abroad at the Lycée Jean Perrin, Lyon, France
1993 – 1995:	Almenhof Grundschule, Mannheim
1991 – 1993:	Grundschule in Tamm, Tamm-Hohenstange

Fellowship and awards

10/2010 – 03/2013:	Landesgraduiertenförderung des Landes Mecklenburg-Vorpommern
10/20/2010:	Prize: Alexander-Karl-Preis 2010 der Stiftung Tumorforschung Kopf-Hals
2011:	Grant by Forschungsförderung der Medizinischen Fakultät der Universität Rostock (FORUN)
2012 – 2013:	Grant by the Wilhelm Vaillant Stiftung

10. List of Publications

1. Mullins CS, Eisold S, Klar E, Linnebacher M. Multidrug-resistance proteins are weak tumor associated antigens for colorectal carcinoma. BMC Immunol. 2011 Jul 10;12:38. doi: 10.1186/1471-2172-12-38.

2. Mullins CS, Linnebacher M. Endogenous retrovirus sequences as a novel class of tumor-specific antigens: an example of HERV-H env encoding strong CTL epitopes. Cancer Immunol Immunother. 2012 Jul;61(7):1093-100. doi: 10.1007/s00262-011-1183-3.

3. Mullins CS, Linnebacher M. Human endogenous retroviruses and cancer: causality and therapeutic possibilities. World J Gastroenterol. 2012 Nov 14;18(42):6027-35. doi: 10.3748/wjg.v18.i42.6027.

4. Mullins CS, Schubert J, Schneider B, Linnebacher M and Classen CF. Cilengitide response in ultra-low passage glioblastoma cell lines: relation to molecular markers. Under review in the Journal of Neuro-oncology.

11. List of Presentations

11.1 List of oral presentations

Mullins CS, Stockhammer F, Linnebacher M and Classen CF: In vitro Modelle zur Optimierung einer dendritischen Zellvakzine für GBM. 5. Rostocker Symposium für Tumorimmunologie, Rostock 19.02.**2011**.

Mullins CS, Schubert J, Linnebacher M and Classen CF: Establishment and characterization of primary GBM cell lines. 20. Arbeitstagung "Experimentelle Neuroonkologie", Minden 29.04.**2011**.

Mullins CS, Schneider B, Stockhammer F, Linnebacher M and Classen CF: Patient-individual models of glioblastoma multiforme – pros, cons and tasks. HGG-IMMUNO meeting, Leuven (Belgium) 23.10.**2011**.

Mullins CS, Klar E and Linnebacher M: Endogenous retrovirus sequences as a novel class of tumor-specific antigens: an example of HERV-H *env* encofing strong CTL epitopes. 129. Kongresses der Deutschen Gesellschaft für Chirurgie, Berlin 25.04.**2012**.

Mullins CS, Schneider B, Stockhammer F, Classen CF and Linnebacher M: Establishment and characterization of primary GBM cell lines from fresh and frozen material: a detailed comparison. 16. Chirurgische Forschungstage, Regensburg 04.10.**2012**.

Mullins CS, Schubert J, Schneider B, Stockhammer F, Linnebacher M and Classen CF: Glioblastoma models: will they lead the way to individualized therapy? HGG-IMMUNO meeting, Leuven (Belgium) 06.12.**2012**.

Mullins CS, Wegner T and Linnebacher M: Optimizing the nucleofection process for professional antigen presenting cells. 7. Rostocker Symposium für Tumorimmunologie, Rostock 15.02.**2013**.

11.2 List of poster presentations

Mullins CS, Eisold S, Klar E and Linnebacher M: Multidrug-resistance proteins are weak tumor associated antigens for colorectal carcinoma. 5. Mildred Scheel Cancer Conference, Königswinter 14.07.**2011**.

Mullins CS, Eisold S, Klar E and Linnebacher M: Multidrug-resistance proteins are weak tumor associated antigens for colorectal carcinoma. 15. Chirurgische Forschungstage, Dresden 23.09.**2011**.

12. Eidesstattliche Erklärung

Hiermit erkläre ich eidesstattlich, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzen Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Christina Susanne Mullins Rostock, Februar 2013

13. Acknowledgments

“An allem Unfug, der passiert, sind nicht nur die schuld, die ihn tun, sondern auch die, die ihn nicht verhindern.“ [Erich Kästner]

I want to sincerely thank my mentors PD Dr. Kalle Classen and PD Dr. Michel Linnebacher not only for giving me the opportunity to perform my PhD project in their laboratories but also for always making sure I neither lose sight of the clinical nor the scientific aspects. I want to thank Prof. Dr. Schröder for his interest in and supervision of this translational project.

I thank the wonderful technicians Mathias Krohn and Anne Lehmann for their technical support and brightening my lab hours. I thank the students Alexander Walter, Julia Schubert, Robert Luck and Tabea Wegner for accepting, loving and at times “hating” me as their co-mentor. Julia is awarded “student of the year” for asking and following (at least most of the times) my advice and help with the Cilengitide project. Tabea wins the “toughness award” for her endurance with the nucleofection project. And I thank all members of the lab team for making this a memorable experience.

A warm thank you goes to the Department of Neurosurgery, in especially to PD Dr. Stockhammer and Dr. Mann, for providing me with the resection specimens and to the always friendly surgery team.

I want to extend a special thank you to the Department of Pathology, in especially Prof. Prall for fruitful conversations on glioma and insights into their histopathology and Dr. Björn Schneider for his major help with the molecular pathological analyses.

Last but not least, I want to thank my family for supporting me during this time!