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MOLECULAR DETERMINANTS OF GLIOMA SUBSETS WITH DISTINCT HISTOLOGY OR SENSITIVITY TO SIGNAL TRANSDUCTION INHIBITORS

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Stockholm 2007

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7357-208-8

ABSTRACT

Gliomas are brain tumors that currently are treated with surgery, radiation and chemotherapy. Less than 7.5% of patients with GBM survive more than 2 years after diagnosis. The conclusion, from several decades of intense research on glioma biology, is that gliomas are tumors with high genetic, morphological and clinical variation, which makes them hard to diagnose and treat. Predicative markers are therefore highly warranted. In this thesis we identified new molecular characteristics of glioma subsets with distinct histology or sensitivity to signal transduction inhibitors.

Grade II gliomas are histologically diverse and are commonly divided into astrocytomas, oligodendrogliomas and oligoastrocytomas. We have identified rPTPβ/ζ as a novel marker for grade II oligodendrogliomas, which is the type with better survival and response to chemotherapy.

Glioblastomas are currently subdivided into primary and secondary glioblastomas, which follow different biological as well as clinical routes. Current knowledge about glioma genetics suggests the need for a refined sub-categorization. We have defined a high grade glioma subtype which is sensitive to PDGFR inhibitor (imatinib, Glivec). This subtype expresses high levels of PDGFR-α and CXCL12. Potentially this subtype constitutes a group of tumors which would best respond to imatinib treatment.

IGF-1R signaling has been shown to be important for glioma growth. NVP-AEW541 is a novel small-molecule inhibitor of IGF-1R. Analysis of the effects of NVP-AEW541 on a panel of high grade glioma cell cultures revealed differences in sensitivity. PIK3CA, PTEN and AKT status was analyzed to investigate their effect on NVP-AEW541 sensitivity. 3/4 high-sensitive cultures demonstrated serum-dependent AKT phosphorylation, whereas AKT phosphorylation was only affected in 1/5 cultures with low sensitivity. Exon 9 mutations in PIK3CA were found in 2/2 sensitive and in 3/5 low sensitive. PTEN expression varied more than ten-fold between cultures, in a manner not associated with drug sensitivity. Interestingly down-regulation of PTEN decreased sensitivity to NVP-AEW541.

CD133-expressing tumor cells with stem cell characteristics have been identified and isolated from several malignancies including glioblastomas. These are suggested to be responsible for the tumor formation. Two novel $CD133^+$ glioma cell subsets were identified. These subsets differed with regard to marker gene expression, ability to grow as neurospheres and in sensitivity to tyrosine kinase inhibitors. Furthermore, genes defining these two subsets were found to be co-expressed in glioblastoma tissue.

These studies have thus generated a series of novel observations relevant for the continued rational development of new treatment strategies, and improved diagnosis, of different types of gliomas. Furthermore, the identification of two novel types of tentative brain cancer stem cells should possibly aid in the ultimate identification of the cell types that undergo the initial transformation in glioma formation.

LIST OF PUBLICATIONS

I. **Hägerstrand D**, Smits A, Eriksson A, Sigurdadottir S, Olofsson T, Hartman M, Nistér M, Kalimo H and Östman A.
Gene expression analyses of grade II gliomas and identification of rPTPβ/ζ as a candidate oligodendroglioma marker
Neuro-Oncolog, 2007 *(in press)*

II. **Hägerstrand D**, Hesselager G, Achterberg S, Wickenberg Bolin U, Kowanetz M, Kastemar M, Heldin C-H, Isaksson A, Nistér M and Östman A.
Characterization of an imatinib-sensitive subset of high-grade human glioma cultures
Oncogene, 2006 Aug 10;25(35):4913-22

III. **Hägerstrand D**, Bradic M, Garcia-Echeverria C, Nistér M, Hofmann F and Östman A.
Analyses of roles of gene expression and PTEN-PI3K-AKT pathway in determination of sensitivity to the IGF-1 receptor inhibitor NVP-AEW541 in high grade glioma cell cultures
Submitted

IV. **Hägerstrand D***, He X*, Hoefs S, Bradic M, Hesselager G, Östman A, Nistér M. (* These authors contributed equally)
Identification of novel subsets of CD133 positive high grade glioma cells with distinct differences in sensitivity to tyrosine kinase inhibitors
Submitted

CONTENTS

LIST OF ABBREVIATIONS

ASCL1	achaete-scute complex homolog 1
AML	acute myeloid leukemia
$\alpha_2\beta_1$	alfa-2 beta-1 integrin
ALL	acute lymphoblastic leukemia
ARF	cyclin-dependent kinase inhibitor 2A
ATP	adenosine triphosphate
BCRP1	ATP binding cassette (ABC) transporter G2 (ABCG2)
BCR-ABL	breakpoint cluster region – Abelson
bFGF	basic fibroblast growth factor
Bmi1	B lymphoma Mo-MLV insertion region 1
BMP	bone morphogenetic protein
CGH	comparative genomic hybridization
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CML	chronic myeloid leukemia
COX2	cyclooxygenase 2
CTGF	connective tissue growth factor
DFSP	dermatofibrosarcoma protuberans
DLBCL	diffuse large B-cell lymphoma
DLL3	delta like 3
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Emx2	empty spiracle homeobox 2
ESA	epidermal surface antigen
FABP7	fatty acid binding protein 7
FDA	food and drug administration
FIP1L1	factor interacting with PAP 1-like 1
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GIST	gastro intestinal stromal tumor
HDAC	histone deacetylase
HES	hypereosinophilic syndrome
HSC	haematopoietic stem cell
HSP90	heat shock protein 90
IAP	inhibitor of apoptosis
IGFBP2	insulin-like growth factor-binding protein 2
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor receptor 1
KRAS	kirsten rat sarcoma viral oncogene homolog
LOH	loss of heterozygosity
MCL1	myeloid cell leukemia sequence 1
MGMT	O-6-methylguanine-DNA-methyltransferase
MMP1	matrix metalloproteinase 1

MMP2	matrix metalloproteinase 2
NSCLC	non-small cell lung cancer
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDGFR-α	platelet derived growth factor receptor α
PDGFR-β	platelet derived growth factor receptor β
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	phosphatase and tensin homolog
RCC	renal cell carcinoma
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
Shh	sonic hedge hog
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SOX2	SRY-box containing gene 2
SPARC	secreted protein acidic and rich in cysteine
VEGF	vascular endothelial growth factor
WHO	world health organization

1 INTRODUCTION

1.1 GLIOMAS

1.1.1 Classification of gliomas

Glial tumors are according to WHO standards graded into four grades (Kleihues et al., 2002). Grading is based on histological criteria such as nuclear atypia, mitotic activity, vascular thrombosis, microvascular proliferation and necrosis. Grade II tumors are generally divided into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. Grade III is divided into anaplastic astrocytomas and anaplastic oligodendrogliomas. Grade IV, the most malignant form, is commonly known as glioblastoma multiforme (GBM).

1.1.2 Low grade gliomas

Grade II gliomas are well differentiated gliomas (Kleihues et al., 2002). Grade II astrocytomas are defined by immunoreactivity for GFAP, Vimentin and S-100 protein. The nucleus are often elongated, hyper chromatic and irregular (Cavaliere et al., 2005). Astrocytomas can further be subdivided into fibrillary, protoplasmic or gemistocytic astrocytomas (Kleihues et al., 2002). Oligodendrogliomas display a distinct histology with a homogenous population of cells with a round nucleus. Artificial clearing of the cytoplasm gives a characteristic “fried-egg” appearance. They also have a distinct vascular pattern referred to as “chicken-wire” (Cavaliere et al., 2005).

Oligodendrogliomas have lately received a lot of attention since they are successfully treated with chemotherapy (Cairncross et al., 1994). Oligodendrogliomas with loss of chromosomes 1p and 19q display longer survival and better response to chemotherapy (Cairncross et al., 1998). The third type, oligoastrocytomas, possesses a mix of characteristics from both oligodendroglial and astrocytic tumors as the name suggests. Histological definition of oligoastrocytomas is difficult since the mixed pattern can be biphasic with distinct areas or the two components are intermingled (Cavaliere et al., 2005). Unfavorable prognostic factors for patients with low grade gliomas are age over 40, a tumor over 6 cm in diameter, tumor crossing the midline, astrocytic tumor and presence of neurological deficit before surgery (Pignatti et al., 2002).

1.1.3 Glioblastomas

GBM is a brain tumor for which a successful treatment does not exist. The yearly incidence of GBMs is around 2-3/100000 (Ohgaki et al., 2004). GBM is a heterogeneous tumor type both on the genetic and histological level. The best prognostic factor for GBM is age (Ohgaki et al., 2004). Median survival is 10 months, with fewer than 5% surviving longer than 5 years. Main treatment is by surgical removal, followed by radiation treatment and chemotherapy (Gaya et al., 2002).

In a meta-analysis based on 12 randomized clinical trials, it was calculated that combined radiotherapy and chemotherapy gave 46% 1-year overall survival as compared to 40% with only radiation (Stewart, 2002). In a recent study with 573 GBM patients, temozolomide in combination with radiation gave a significant benefit and minimal toxicity as compared to radiation alone (Stupp et al., 2005). Markers for

response of temozolomide treatment have been described. The MGMT gene is epigenetically silenced by methylation in about 45% of GBMs. Patients with methylated MGMT promoter benefits more from temozolomide treatment (Hegi et al., 2005). MGMT is involved in DNA repair and patients with no MGMT methylation express more of the protein and therefore have better DNA repair, and consequently are less sensitive to DNA alkylating reagents, like temozolomide. Recently it has been shown that MGMT is under regulation of p53, which commonly is mutated in GBMs (Blough et al., 2007). These results indicate that analysis of MGMT regulation and DNA reparation should improve usage of temozolomide.

GBMs may appear *de novo* as primary tumors or progress from lower grade gliomas and are then called secondary glioblastomas. On a histological level these are indistinguishable. In two population based studies the relationship between primary and secondary GBMs were found to be approximately 95% primary and 5% secondary GBMs (Ohgaki et al., 2004, Dropcho et al., 1996). However, there might be an underestimation of secondary glioblastomas due to rapidly progressing secondary tumors, which mistakenly are considered, as primary GBMs. Primary GBMs are more common in older patients whereas secondary GBMs frequently occur in middle-aged individuals. In survival analysis, primary GBM patients were shown to have a shorter survival but when corrected for age this difference disappeared. On a genetic level primary GBMs are characterized by mutation or deletion of INK4a, EGFR mutation or amplification, loss of PTEN and MDM2 amplification. Secondary GBMs usually have perturbed p53 signaling (Watanabe et al., 1996, Ohgaki et al., 2004). The platelet derived growth factor receptor (PDGF) is also thought to be important in the development of secondary glioblastomas (Hermansson et al., 1988).

GBMs are characterized by a highly unstable genome and harbor several different genetic aberrations. The most common defect is LOH 10q. It has been seen that LOH 10q typically occurs in combination with at least one of the other known genetic aberrations (Ohgaki et al., 2004). Other common mutation are activating mutations of PIK3CA which are found in about 30% of GBMs (Samuels et al., 2004). PTEN, a negative regulator of the same signaling pathway as PIKCA, has been described to be mutated or lost in about 24% of GBMs (Ohgaki et al., 2004). If PTEN and PIK3CA mutations are mutually exclusive, still remain to be determined. Amplification and mutations of the EGFR receptor has been well characterized and occurs in about 40% of primary GBMs (Tohma et al., 1998, Ohgaki et al., 2004). Genes involved in regulation of cell cycle and apoptosis are also commonly mutated. Inactivating mutations of INK4a are found in about 30-40% of primary GBMs (Ohgaki et al., 2004, Ichimura et al., 1994, Schmidt et al., 1994) and mutations of p53 in about 30% of GBMs (Ohgaki et al., 2004).

1.2 RECEPTOR TYROSINE KINASES AND GLIOBLASTOMAS

1.2.1 The human kinome

Roughly 520 protein kinases have been identified in the human genome (Manning et al., 2002). Of these, 58 are receptor tyrosine kinases, belonging to the family of 90 tyrosine kinases. Tyrosine kinases transfer phosphate groups from ATP to tyrosine residues in proteins. This leads to changes in conformation, localization or binding

capacity and is extensively used in eukaryotic cell signaling. The negative regulators, that remove the phosphate group, are called protein phosphatases (Alonso et al., 2004). Tyrosine kinases are highly involved in human malignancies. Development of targeted therapies against these have been the most successful story of cancer drug development during the last decade (Baselga, 2006).

1.2.2 Receptor tyrosine kinases in cancer

RTKs control cellular processes, e.g. proliferation and apoptosis (Schlessinger, 2000). The receptors span the cellular membrane and relay extra-cellular ligand mediated signals into the cell. Ligand binding stabilizes complex-formation between receptors, usually in the form of dimers, which allows them to become activated by phosphorylation in trans. Activation of the receptor leads to signaling via binding and activation of intracellular signal transduction molecules and subsequent generation of second messengers. These signals are integrated, typically into the nucleus, leading to specific cell responses such as cell cycle progression, proliferation, apoptosis and migration.

RTK-signaling contributes to different aspects of cancer growth (Blume-Jensen et al., 2001). Activation of RTK signaling can arise from point-mutations of the receptor affecting the kinase activity, from amplifications resulting in over-expression, from chromosomal translocations giving rise to dysfunctional RTKs or dysregulated growth factor production. Tumor angiogenesis is also driven by RTKs, *e.g.* by the VEGF-receptors (Carmeliet, 2005).

1.2.2.1 PDGF signaling and cancer

The platelet-derived growth factor α- and β-receptors (PDGFR-α or -β) are RTKs, encoded by two separate genes. The PDGF ligands are expressed as homo- or hetero-dimers of A-, B-, C- or D-chains kept together by intermolecular cysteine-bonds. Because of the ligands different affinity for the two receptor types, they cause either homo- or hetero-dimerization of the receptors leading to distinct signaling activities (Heldin et al., 2002).

PDGF receptor signaling contributes to various tumor-associated phenomena (Ostman et al., 2007, Pietras et al., 2003). A PDGF autocrine loop is a driving force in some tumors like DFSP and GBM. Translocations between PDGFR-β and TEL or PDGFR-α and FIP1L1 has been described in CMML and HES, respectively (Golub et al., 1994, Cools et al., 2003). PDGF may also contribute to the angiogenesis of tumors, through the recruitment and differentiation of pericytes (Furuhashi et al., 2004, Benjamin et al., 1998, Guo et al., 2003), and also via induction of other growth factors, such as VEGF (Dong et al., 2004, Brogi et al., 1994). Tumor stroma development and function is also controlled by the PDGF receptor tyrosine kinase (Forsberg et al., 1993, Pietras et al., 2001, Pietras et al., 2002). In tumors with PDGF receptor expression in tumor stroma cells, PDGF antagonists have been shown to lower the interstitial fluid pressure and increase uptake and efficacy of cytotoxic drugs and radioimmunotherapy (Baranowska-Kortylewicz et al., 2005, Pietras et al., 2003, Pietras et al., 2002, Pietras et al., 2001)

1.2.3 Receptor tyrosine kinase inhibitors

1.2.3.1 Receptor tyrosine kinase inhibiting antibodies

Ways to inhibit cancerous RTK-signals has been subject of intensive research, which has given rise to novel cancer drugs (Shawver et al., 2002)**.** Trastuzumab (Herceptin) is a HER-2 targeting antibody, which is used against advanced breast cancer with HER-2 amplification (Arteaga et al., 2002). Three additional FDA approved RTK-blocking antibodies are the EGFR-targeting antibodies cetuximab and panitumumab, and the VEGF-directed antibody bevacizumab.

1.2.3.2 Tyrosine kinase inhibiting small molecules

Imatinib mesylate (STI571, Glivec, Gleevec) is a low-molecular weight compound, which bind to the catalytic, ATP binding pocket of the BCR-ABL, PDGFR and c-kit tyrosine kinases (Capdeville et al., 2002). Imatinib was first approved by the FDA for treatment of CML and GIST where it acts by blocking BCR-ABL and the c-kit receptor, respectively (Druker et al., 2001, van Oosterom et al., 2001). Imatinib is also active in PDGFR-dependent malignancies such as CMML, DFSP and HES (Apperley et al., 2002, McArthur et al., 2005, Cools et al., 2003). Erlotinib is also an ATP-competitive inhibitor with specificity for the EGFR, and has been approved for second- and third line treatment of metastatic NSCLC (Shepherd et al., 2005). Another EGFR targeting molecule is lapatinib, which was recently approved for second line treatment of trastuzumab resistant HER-2^+ breast cancer (Geyer et al., 2006).

NVP-AEW541 is a novel pre-clinical inhibitor with high specificity for IGF-1R (Garcia-Echeverria et al., 2004). The high similarity between IGF-1R and insulin receptor is a big hurdle in developing drugs against IGF-1R, since inhibition of the insulin receptor will cause diabetes. NVP-AEW541, which in cellular assays preferentially blocks IGF-1R, has been successful in inhibiting tumor growth in several different experimental malignancies.

1.2.3.3 Response to receptor tyrosine kinase inhibitors

It was early recognised that not all cancer patients with similar diagnosis will respond to treatment with tyrosine kinase inhibitors. Erlotinib, an EGFR inhibitor was approved by the FDA as a treatment for NSCLC in 2004. This approval was based on results from a study where treatment increased median overall survival from 4.7 to 6.7 months (Shepherd et al., 2005). In parallel, it was found that about 10% of NSCLC patients had a mutated EGFR and that better response to EGFR inhibitor occurred in this subgroup (Paez et al., 2004, Lynch et al., 2004). KRAS status has also been found to be important in predicting response to EGFR inhibition and is suggested to be used in combination with EGFR analyses in treatment decision for NSCLC patients (Pao et al., 2005).

1.2.3.4 Resistance to receptor tyrosine kinase inhibitors

Patients treated with targeted therapies can initially be resistant to treatment, or will develop this later even when they initially were responsive. One example is GIST patients receiving imatinib. About 90% and 5% of GIST patient have activating mutations in the c-kit receptor or the PDGFR-α, respectively (Heinrich et al., 2003). Molecular studies have demonstrated that different types of mutations are differently

sensitive to imatinib (Heinrich et al., 2003). Some patients lacking mutation in c-kit were found to have activating mutations in the PDGFR-α gene. Many of the PDGFR mutation variants are insensitive to imatinib (Corless et al., 2005).

Resistance to imatinib treatment can develop through secondary mutations in the c-kit gene (Heinrich et al., 2006). This has been solved by using another inhibitor effective on these variants. In a study on patients who were resistant or intolerant to treatment with imatinib it was shown that these patients benefited from treatment with sunitinib (Demetri et al., 2006).

1.2.3.5 Multi kinase inhibitors

Drugs hitting multiple targets could be advantageous from several points of view. Initially it was thought that drugs hitting several targets would have many side effects. On the other hand, multi-kinase inhibitors that impact on multiple targets at the same time can better control tumors dependent on several pathways for growth. These could also affect several cell types at the same time and should be less prone to drug resistance. Two successful drugs with these abilities are sorafinib and sunitinib who target both VEGFR and PDGFR. These have been approved and are effective in treatment of RCC where the main effect is thought to be anti-angiogenic (Escudier et al., 2007, Motzer et al., 2007).

1.2.3.6 Oncogene addiction

Many pathways have been described to promote cancer cell proliferation. Still little is known about how these pathways differ between normal and tumor cells. Oncogene addiction explains why some targeted therapies have such a selective killing effect on cancer cells. Oncogene addiction means that cancer cells are dependent on a certain oncogene for growth and survival, while other cells can lose this gene without any obvious consequences. Turning of the oncogene will result in apoptosis, cell cycle block or differentiation. This addiction appears for example when actively mutated RAS is expressed in normal lung cells. These cells will start to grow in an uncontrolled manner, but later when RAS subsequently is turned off the cells do not return to their original state but die from apoptosis (Fisher et al., 2001). RAS has reprogrammed the cell to be dependent on its signalling.

Several observations have been made on oncogene addiction, but no real mechanistic explanation has been given. Signalling events were analysed when BCR-ABL was turned of in BCR-ABL addicted cells (Sharma et al., 2006). Kinetic differences in pathways for survival and apoptosis were observed. In the normal steady-state signalling situation survival is higher than apoptosis. BCR-ABL signalling raises the level of both states, but survival domintes. Upon removal of BCR-ABL the survival state decreases faster that the apoptosis state and apoptotic signalling take over and kills the cell (Sharma et al., 2006).

1.2.4 Receptor tyrosine kinases and glioblastomas

1.2.4.1 PDGFR signaling in glioblastoma

Several studies have indicated the importance of PDGF and EGF signaling in GBMs. The expression patterns of the PDGF ligand and receptor in GBM suggests the presence of both paracrine and autocrine signaling. By *in situ* hybridization and immunohistochemistry it was shown that PDGF receptors and their ligands were over expressed in GBMs as compared to normal brain tissue (Hermansson et al., 1988).

The potential role of PDGF as an initiator of the formation of GBMs has also been shown. Injection of a murine retrovirus coding for PDGF-B into mouse brain induced formation of GBM-like tumors (Uhrbom et al., 1998). In a comparison of PDGF ligand-induced tumors in mouse strains having a wild type or mutated Ink4a-Arf, it was shown that PDGF ligand was able to induce GBMs in both types, but in the mutated mice the tumors had a more malignant phenotype (Dai et al., 2001).

By turning off PDGF signaling via introduction of a truncated PDGF receptor into C6 rat glioma cells it was possible to decrease cell proliferation (Strawn et al., 1994). Also, with PDGF receptor inhibitors like SU101 and imatinib, inhibition of glioma cell growth, in tissue culture and intracranial tumors grown in nude mice, has been demonstrated (Kilic et al., 2000, Shawver et al., 1997).

Results from initial clinical studies with imatinib GBM treatment have been modest. Imatinib treatment on 50 patients with recurrent high grade gliomas had minor effects. Only 3% had 6-months progression free survival in the GBM group and 10% in the anaplastic astrocytomas group (Wen et al., 2006).

More promising results have been achieved in studies using a combination of imatinib and hydroxyurea. In the initial study, a 32% 6-month progression free survival was achieved in 30 patients (Dresemann, 2005). Similar results were observed in a separate phase II trial with a 6-month progression free survival of 27% measured on 33 patients (Reardon et al., 2005). Subsequently this treatment was tested on 39 patients diagnosed with grade III gliomas. Here 24% of the patients had 6-month progression free survival (Desjardins et al., 2007).

1.2.4.2 EGFR signaling in glioblastoma

Another tyrosine kinase receptor, which has been shown to be involved in the GBM formation, is the EGFR. Autocrine signaling occurs as a consequence of co-expression of EGF receptor and its ligands TGF-α and EGF (Ekstrand et al., 1991). Over-active signaling of the EGFR in GBMs can also be caused by amplification and over-expression of wild-type or mutated EGFR (Wong et al., 1992). Introduction of constitutively active EGFR into glioma cells, having low basal EGFR expression, did not increase proliferation rate in cell culture, but enhanced the tumorigenic capacity in nude mice (Nishikawa et al., 1994). By using AG1478, an EGFR inhibitor, it was possible to inhibit the growth of U87 tumors grown intracranially in mice. It has also been possible to inhibit U87 tumor growth with the mono clonal antibody mAb806, which specifically targets the truncated EGFR form (Mishima et al., 2001, Luwor et al.,

2001). Clinical benefit has been observed after treatment of GBM with the EGFR inhibitor gefitinib (Rich et al., 2004).

In two studies, increased knowledge was added concerning the mechanisms predicting response to EGFR inhibitors in GBM (Mellinghoff et al., 2005, Haas-Kogan et al., 2005). Co-expression of EGFRvIII and PTEN, and high levels of EGFR and low levels of phosphorylated Akt, were associated with response to EGFR inhibitor (Mellinghoff et al., 2005, Haas-Kogan et al., 2005). This was experimentally validated by over-expression of EGFRvIII in PTEN-lacking cells, which became sensitive to EGFR inhibitor. Restoration of PTEN decreased sensitivity, showing the importance of PTEN status (Mellinghoff et al., 2005).

1.2.4.3 IGF-1R signaling in glioblastoma

IGF-1R is a critical regulator of cell proliferation and protection of apoptosis. Overexpression of both IGF-1 and IGF-2 mRNA has been found in glioma tissue (Sandberg et al., 1988). Both are ligands to IGF-1R, which suggests autocrine or paracrine signaling. Furthermore, in experimental studies of C6 glioma cells grown intracranially in rat, growth was abolished when cells were stably transfected with antisense RNA against IGF-1R (Resnicoff et al., 1994).

1.3 GENE EXPRESSION ANALYSIS OF GLIOBLASTOMAS

1.3.1 Unsupervised analyses

Methods for studying class relationships in tumor microarray experiments can be divided into two main categories, unsupervised and supervised class discovery methods. In unsupervised analysis the gene expression pattern is used to identify previously unrecognized subsets with distinct properties.

In a pioneering study of this kind, Alizadeh et al. were able to show the presence of two molecularly distinct classes of diffuse large B-cell lymphomas (DLBCL). One of the classes had a gene expression profile more similar to germinal centre B cells, while the other was more similar to *in vitro* activated peripheral blood B cells. The patients with DLBCLs more similar to the germinal centre B cells had a better overall survival than those with activated B-like DLBCL (Alizadeh et al., 2000).

Subsequently similar approaches have been used for many tumor types, including breast cancer. Tumor types defined by gene expression patterns showed significant differences in clinical outcome. The basal-like subtype of the tumors had worse prognosis than the other groups (Sorlie et al., 2003).

1.3.2 Supervised analyses

Supervised classification methods in tumor microarray experiments are mainly used for discovery of markers of a desired trait, e.g. response to treatment or clinical course. This approach was first used to define a gene set which could distinguish acute myeloid leukemia (AML) and acute lymphoblast leukemia (ALL) (Golub et al., 1999). In a land-mark paper van't Veer et al. studied the gene expression in 98 breast cancer tumors, with known clinical course, and used this information to establish a gene list

which could predict whether tumors would develop metastases or not. The gene-list based classification outperformed old methods of classification (van 't Veer et al., 2002). Results were subsequently confirmed in a larger study including 295 patients (van de Vijver et al., 2002).

1.3.3 Explorative analyses

1.3.3.1 Metastasis profiles

Microarray methods have also been used as a tool in explorative tumor biology studies. Two of the most interesting studies of this kind have aimed at a better understanding of the mechanisms of tumor metastasis (Clark et al., 2000, Kang et al., 2003). In the study of Clark et al. a cell line with poor metastatic ability was injected into mice. From the few pulmonary metastases found, cells were isolated and put into cell culture or back into the mouse. Re-injected cells had a higher metastatic ability. The cycle was repeated three times. Thereafter gene expression was compared between cells from the different cycles, and genes that changed in relative expression level were considered as candidates responsible for the metastatic process. RhoC expression was increased in the metastatic cell line and was selected for further validation. Over-expression of RhoC in the initial cell line conferred a higher metastatic ability, indicating that this gene was one of the true effectors (Clark et al., 2000). Furthermore, over-expression of a dominant-negative RhoC inhibited the metastatic ability.

Bone metastases are a common cause of morbidity and sometimes mortality in breast cancer patients. By using a similar selection method as described above it was possible to find genes responsible for bone metastases in a model of breast cancer bone metastasis (Kang et al., 2003). Among the metastasis-promoting genes were genes encoding secreted and cell surface proteins like interleukin-11, CTGF and MMP1, which could possibly serve as targets for novel therapies.

The metastatic program is thought to be a multi step progress, which is dependent on several factors. To target several of these is desirable. This was tested with a combination of drugs targeting different pathways thought to be involved in the metastatic program (Gupta et al., 2007). Factors that were targeted and thereby shown to be involved were the EGFR ligand epiregulin, COX2, MMP1 and MMP2.

Also, human tumors have been analyzed to identify metastatic profiles. 128 genes were found to differ between 64 primary adenocarcinomas and 12 metastatic adenocarcinomas (Ramaswamy et al., 2003). When this gene list was used to cluster 62 primary lung adenocarcinomas two groups were formed, which differed significantly with regard to survival. The 128 gene list was distilled to 17 genes and a significant difference was still observed. The 17-genes long list was also able to significantly distinguish two groups of breast cancer with different time to metastasis, overall survival in medulloblastomas and time to PSA recurrence in prostate cancer.

1.3.3.2 Wound-healing profile

It has been noted that tumors resemble wound tissue. To further investigate this, a wound healing gene expression signature was derived from fibroblast cell cultures

stimulated with serum (Chang et al., 2004). The wound profile was consequently tested on tumor tissue gene expression data. A signature match was detected in some early tumors and more frequently in progressed tumors. The signature effectively clustered patients into two subsets with either a quiescent or activated profile. These two groups significantly differed in survival and metastasis. The tumor with activated profile had less favorable prognosis.

This study nicely illustrate how a gene expression profile can be build from experimental imitation of a physiological event in a controlled setting and then be used to interpret gene expression profiles of complex clinical samples (Chang et al., 2004).

1.3.3.3 The Connectivity map

The Connectivity map was presented in a publication, which was followed by two other where its usability was demonstrated (Lamb et al., 2006, Hieronymus et al., 2006, Wei et al., 2006). The Connectivity map is a database containing 453 gene expression profiles. The profiles are derived from the cancer epithelial cell line MCF7 which has been treated with 164 different small-molecule compounds. To test the capacity of this data set, profiles from drugs with similar mechanisms were compared. Several HDAC inhibitor profiles were studied and similarity was found.

The Connectivity map data base can thus be queried with expression profiles generated by compounds with unknown function, and relationship to compounds with known mechanism of action can be retrieved (Lamb et al., 2006).

In the first application a high-throughput screen for small-molecules that activate the androgen receptor in prostate cancer cells was analyzed (Hieronymus et al., 2006). One hit was the natural product gedunin, purified from Cedrela sinensis (*Meliaceae*). The gedunin gene expression profile was then queried with the Connectivity map. The profile was similar to those of three known HSP90 inhibitors. It could thereby be shown that gedunin regulates the androgen receptor by inhibition of HSP90.

In the second study, a drug with capability of reversing glucocorticoid resistance in ALL was sought (Wei et al., 2006). A gene expression signature associated with glucocorticoid resistance was first generated by comparing expression data from sensitive and resistant patients. The Connectivity database was then queried with the profile to find drug signatures with similarity to this profile. The best matched signatures were from the mTOR inhibitor rapamycin. Rapamycin was then tested in glucocorticoid resistant cells and was found to change the resistance profile. Changes of expression of apoptosis regulatory genes was then studied after rapamycin treatment and the anti-apoptotic regulator MCL1 was found to be down-regulated. This was found to be functionally significant since overexpression of MLC1 made primary lymphocytes resistant to glucocorticoids.

1.3.3.4 Prediction of oncogene activity

In another series of experiments, gene expression profiling was used to predict oncogene activation in tumors. Gene expression profiles were generated from Ras, E2F or Myc transformed embryonic mouse fibroblasts (Huang et al., 2003). These profiles

properly predicted the oncogene activity in *in vivo* tumor models with deregulation of these components (Huang et al., 2003). A similar study was also performed on human clinical material (Bild et al., 2006). The signatures were able to identify adenocarcinomas with Ras mutations. Hierarchical clustering was performed of lung and ovarian tumor samples according to the total oncogene profile and subsets were found with different prognosis. This shows that oncogene activation can be measured by gene expression profiling and that tumor types with different clinical outcome can be identified by these.

1.3.4 Gene expression analyses of glioblastomas

1.3.4.1 Gene expression profiles classifying histological grades of gliomas

The first microarray papers concerning brain tumors dealt with the issue of finding genes which differ between tumor and normal brain. Huang et al were able to identify 23 genes which were differently expressed in grade II astrocytomas as compared to normal brain (Huang et al., 2000). Other studies have also found genes regulated in higher grade gliomas. IGFBP2 and Vimentin were found to be highly up-regulated in grade II, grade III and grade IV gliomas. This was confirmed with tissue micro arrays containing 418 brain tumors. It was found that high expression of IGFBP2 is associated with poor progression in diffuse astrocytomas (Sallinen et al., 2000). Oligodendrogliomas, with or without loss of heterozygosity of 1p have also been compared and were found in a non-supervised analysis to form two distinct subgroups (Mukasa et al., 2002, Tanwar et al., 2002). To find classifier genes which can distinguish gliomas of different grades, a study was performed where a 170 genes long gene list was made which could distinguish between different tumor grades (Shai et al., 2003). Hierarchical clustering of different glioma stages was also found to be able to cluster the different histological subtypes in a similar study (Fuller et al., 2002). Serum markers for glioma progression have been identified by gene expression analysis. YKL-40 was found to be one of the most over-expressed genes in GBMs. Serum levels of YKL-40 measured by ELISA correlated with tumor grade and tumor burden in GBM patients (Tanwar et al., 2002). In a later study these results were confirmed in a larger patient group (Hormigo et al., 2006).

1.3.4.2 Glioblastoma gene expression profiles for prognosis

Histological diagnosis strongly affects therapeutic decisions in today's treatment of GBMs. Histological grading is sometime very challenging and also does not reflect the underlying molecular cause of the disease. With the arrival of targeted therapies there is a dramatically increased need to define these. Several studies have been performed trying to distinguish different subtypes of GBMs by gene expression analysis.

Classical GBMs and anaplastic oligodendrogliomas follow markedly different clinical courses but are challenging to diagnose. For expression-based classification of these, a 20-feature classifier was built from 21 tumors. The classifier was able to correctly classify 18 of the 21 tumors in a leave-one-out validation. When the classifier was applied on a separate set of tumors, not having classical histology, the prediction of survival by the gene-expression-based classifier out-performed the prediction based on histological criteria (Nutt et al., 2003). Another study used 85 diffuse infiltrating

gliomas to generate a predictive list of 44 genes. This classifier list subsequently divided 50 additional gliomas into groups with different survival. (Freije et al., 2004). Liang et al. were also able to find 70 genes that were highly expressed in rapidly progressing tumors. This list divided GBM patients into two groups which differed 4-fold in median survival. FABP7 was selected from the list and was further tested in a unrelated cohort of 105 patients, where it also was found to be associated with survival (Liang et al., 2005). Other genes, isolated by gene expression analysis, which have been suggested to predict survival in GBMs are SPARC, doublecortex and Semaphorin3B (Rich et al., 2005).

Efforts have also been made to explore the differences between primary and secondary GBMs by gene expression and CGH analysis. Gene lists were generated which could distinguish these two types. Genes associated with secondary GBMs included mitotic cell cycle components, suggesting the loss of proper cell cycle regulation, whereas genes associated with primary GBMs were typically involved in stromal response, suggesting the importance of extracellular signaling in these (Tso et al., 2006). The same group also went on producing a second publication where they describe primary glioblastomas to possess similarities in gene expression with mesenchymal stem cells (Tso et al., 2006).

The notion that one subset of glioblastomas posses mesenchymal characteristics has been described in other studies as well. Philips et al. nicely described that high-grade astrocytomas can be divided into three categories according to gene expression analysis; one proneural, one proliferative and one mesenchymal. The two latter display a shorter survival. They also suggested a two-gene prognostic model using PTEN and DLL3 expression, indicating that Akt and Notch signaling are hallmarks for poor prognosis in GBMs (Phillips et al., 2006).

The inhibition of Notch signaling may be an important event in the development of grade II gliomas and the subsequent progression. ASCL1, a protein involved in inhibiting Notch signaling, has also been described to be up regulated during glioma progression and is also suggested to be a marker which can distinguish secondary from primary GBMs (Somasundaram et al., 2005).

Differences between primary and secondary GBMs have also been studied on a genetic level by CGH (Maher et al., 2006). Primary GBMs were defined by gains of chromosome 7, 19, 20 and a small region from 12 and loss of chromosome 10, 9 and 11. These results were compared to the Tso study and showed that 55 genes over-expressed in primary glioblastomas fell within regions that were selectively amplified in primary GBM. In the same study chromosomal features were found for secondary GBMs which divided these into two novel subclasses. One group was characterized by chromosomal loss of 6, 9, 10, 13, 18 and 19 whereas the other had regional gains on chromosomes 4, 8 and 12 and focal gains on 7 and 11.

Three novel subsets of high grade gliomas have also been found by unsupervised gene expression analysis in combination with protein expression data. These were characterized by either high expression of EGFR receptor, high expression of EGFR in

combination with over-expression of genes from chromosome 12q13-15 or by none of these features (Mischel et al., 2003).

1.4 TUMOR STEM CELLS AND GLIOBLASTOMAS

1.4.1 The cancer stem cell hypothesis

The cancer stem cell hypothesis postulates the existence of tumor cells, which possess self renewal potential and are capable of forming tumors in immune-deficient mice that recapitulate the heterogeneity of the original tumor. Cancer stem cells also possess other characteristics similar to normal stem cells like the capacity of multi-lineage differentiation and high potential in *in vivo* growth assays (Polyak et al., 2006, Tan et al., 2006).

The difference between a normal and a cancer stem cell is that the later possesses genetic disturbances giving a transformed phenotype. Existence of transformed cells with stem cell similarities can be explained in at least two ways. Either an adult stem cell have become genetically altered to give rise to a transformed cancer stem cell, thus it has inherited the stem cell phenotype. This idea is supported by the multi-step model of carcinogenesis were a long-lived cell is required for multiple genetic hits to occur (Vogelstein et al., 1988). Alternatively the transformation process, through genetic and epigenetic alterations, in itself produces stem cell similarities (Passegue et al., 2003).

Some studies have suggested prognostic significance of cancer stem cells. Overall survival was poorer in AML patients with a high percentage of $CD34^{+}CD38^{-}$ leukemia cells (van Rhenen et al., 2005).

The behavior of cancer stem cells suggests that total removal of this population is required to achieve complete cure. Therefore efforts in developing therapies directed against tumor stem cells have received a lot of attention. However the similarities between normal and cancer stem cells have also raised the question of whether therapies targeting the cancer stem population, but sparing the normal stem cells, can be developed. This was addressed in a recent study were deletion of PTEN in adult haematopoietic cells gave rise to myeloproliferative disease within days and transplantable leukemia cells within weeks (Yilmaz et al., 2006). PTEN deletion also promoted HSC proliferation. Treatment with the mTOR inhibitor rapamycin prevented the leukemia and normalized the non-leukemic $PTEN^{-}$ HSC. This illustrates the possibility to specifically target the cancer. It also shows that PTEN is an important regulator of HSC homeostasis.

1.4.2 Cancer stem cells in solid tumors

During the last years tentative cancer stem cells have been isolated from many types of solid tumors including e.g. breast cancer, colorectal cancer, prostate cancer, pancreatic cancer, medulloblastomas and GBMs (Singh et al., 2003, Singh et al., 2004, Ricci-Vitiani et al., 2007, Al-Hajj et al., 2003, Collins et al., 2005, Li et al., 2007). However, in most cases the molecular characteristics of cancer stem cells are still poorly defined. The cancer stem cells have mainly been identified based on their expression of certain previously characterized stem cell markers.

Breast cancer stem cells were isolated by collecting CD44/$CD24^{-/low}$/ESA^{+} cells (Al-Hajj et al., 2003). CD44 is an adhesion protein which binds to hyaluronic acid. It has been shown to be up-regulated in certain malignancies and to be directly involved in promoting the proliferation of melanomas (Naor et al., 1997, Ahrens et al., 2001). CD24 is a small, heavily glycosylated, mucin-like surface protein, which directly has been shown to predict poor prognosis in colon cancer and other malignancies (Weichert et al., 2005, Kristiansen et al., 2004). ESA is a glycoprotein expressed on most epithelial cells (Litvinov et al., 1994).

Prostate cancer stem cells were isolated guided by their $CD44^{+}/\alpha_2ß_1^{hi}/CD133^{+}$ profile. Among these α2-integrin has previously been shown to be a marker of normal prostate stem cells (Collins et al., 2001). CD133 (prominin-1) was initially found to be expressed in epithelial, including neuro-epithelial, cells and haematopoietic stem cells (Weigmann et al., 1997, Yin et al., 1997). Pancreatic cancer stem cells were isolated, with the expression profile of CD44/$CD24^{+}$/ESA^{+} (Li et al., 2007).

Together these findings suggest that several markers are shared between cancer stem cells but some are tumor-type-specific.

1.4.3 Neural stem cells

Research on neural stem cells is very intense due to the interest to understand and to use these for treatment of neurodegenerative diseases and after different neural damages. Neural stem cells can give rise to glial and neuronal cell lineages. Such cells have been directly isolated from the central nervous system based on their expression of CD133 (Uchida et al., 2000).

Most neural stem cell studies have focused on identifying cells from the sub ventricular zone, olfactory bulb and hippocampal dentate gyros (Alvarez-Buylla et al., 2001). Lately brain stem cells have also been isolated from the cerebellum (Lee et al., 2005). Markers for neural stem cells are nestin (Lendahl et al., 1990), Musashi-1 (Sakakibara et al., 1996) and CD133 (Uchida et al., 2000). The role of these genes, and their expression in the developmental hierarchy, in neural biology is still poorly understood, but under intense research.

Several growth factors have been reported to be important for the regulation of neural stem cells. EGF and bFGF were early detected as factors important for maintenance of neural stem cells. They are also the factors used to grow neural stem cells in the neurosphere assay (Gritti et al., 1996, Reynolds et al., 1992). PDGFR been shown to be an early inducer of partial differentiation of neuronal stem cells (Erlandsson et al., 2006). Other growth factor systems which in general are important during development and later in adult stem cell regulation are members of the Notch family. It has been shown that Notch1 signaling is important for the maintenance of neural stem cells (Hitoshi et al., 2002).

Shh is a developmental factor, known to orchestrate tissue pattering during embryogenesis, and has been shown to regulate and maintain adult neural stem cells. In

a study where Shh signaling was blocked with cyklopamine it was seen *in vivo* that it had profound inhibitory effects on $GFAP^+$ sub ventricular neural stem cells (Palma et al., 2005). Shh is also a key mediator in the formation of medulloblastomas. Forced over expression of Shh by retroviral transduction of Shh in mice induces the formation of medulloblastomas (Weiner et al., 2002).

Bmi1, a member of the Polycomb transcription factors group, was identified as an oncogene collaborating with c-myc in lymphomagenesis (Haupt et al., 1993). Bmi1 has also been shown to regulate normal haematopoietic and neural stem cells as well as tumor stem cells in these two tissues (Lessard et al., 2003, Molofsky et al., 2003). There is a clear connection between Bmi1 regulation and Shh signaling, since Bmi1 and Gli1 are up regulated after stimulation by Shh (Leung et al., 2004). The Bmi1 regulation of neural stem cells has been shown to be mediated through $p16^{Ink4a}$ and $p14^{Arf}$, which are negatively regulated by Bmi1. In Bmi1 knock out animals, the hypo-proliferative effects on cerebellum development can be completely rescued by crossing with $Arf^{-/-}$ mice, and partially by crossing with $Ink4a^{-/-}$ mice (Bruggeman et al., 2005).

Other transcription factors involved in the maintenance of neural stem cells are Sox2 and Emx2. Sox2 is expressed in embryonic early neural precursors of the ventricular zone and when knocked out gives abnormalities in neurons, and diminish the GFAP/nestin positive population of neural stem cells in the hippocampus (Ferri et al., 2004). Emx2 is a homeobox transcription factor, which has been reported to be expressed in cells in the periventricular zone. Adult neural stem cells isolated from the perivascular region express Emx2, but expression is lost when they differentiate into neurons or glia. When Emx2 expression is abolished, the symmetric divisions that generate two stem cells increase, whereas they decrease when Emx2 expression is enhanced (Galli et al., 2002).

1.4.4 Cancer stem cells in brain tumors

1.4.4.1 Isolation of glioblastoma stem cells

Based on previous knowledge about neural stem cells and stem cells in other malignancies, the existence of cancer stem cells in GBMs has been investigated in several studies (Singh et al., 2003, Singh et al., 2004, Galli et al., 2004). These cells can be isolated based on their expression of CD133, a marker previously used for isolating normal brain stem cells (Uchida et al., 2000). The brain tumor stem cells display a high capacity for self renewal, high proliferation, multi-lineage differentiation and a disturbed karyotype. CD133 isolated brain tumor stem cells implanted into mice also show a high capacity to phenocopy the original tumor (Singh et al., 2004, Galli et al., 2004, Tunici et al., 2004).

1.4.4.2 Characteristics of brain tumor stem cells

In an attempt to find factors that differ between normal neural stem cells and GBM stem cells, Calabrese and colleges found that the $CD133^+nestin^+$ GBM stem cells were associated with the tumor tissue vessels and dependent on growth stimulating factors released from endothelial cells (Calabrese et al., 2007). Bevacizumab treatment, which depleted blood vessels, resulted in tumor growth arrest. In connection to these findings

it has also been shown that VEGF enhance survival of neural stem cells (Wada et al., 2006), and that endothelial cells stimulate self-renewal and expansion of normal neural stem cells (Shen et al., 2004). This opens up possibilities for new therapeutic strategies (Yang et al., 2007). A phase II clinical trial combining bevacizumab and irinotecan, a topoisomerase I inhibitor has already been performed where the treatment was active and associated with acceptable toxicity (Vredenburgh et al., 2007).

GBM tumor stem cells have also been shown to be insensitive to many GBM treatment regimens (Bao et al., 2006, Liu et al., 2006). The high frequency of recurrence of GBMs, which have been treated by operation, radiation or chemotherapy, could possibly be explained by re-growth of the stem cell population. This was indicated in a study where the amount of $CD133^+$ cells was compared in paired primary and recurrent GBMs, which showed that that the fraction of $CD133^+$ cells had increased in the recurrences (Liu et al., 2006). In this study the $CD133^+$ GBM cells grown in culture were shown to be insensitive to chemotherapeutic treatment with temozolomide, carboplatin, etoposide and taxol. The drug resistance of the $CD133^+$ population was suggested to be caused by higher expression of BCRP1, MGMT and IAPs.

Ionizing radiation is commonly used for treatment for GBMs, but gliomas are in general quite radio-resistant. Bao et al. have presented results which indicate that this is mainly due to lack of sensitivity of the $CD133^+$ GBM stem cells (Bao et al., 2006). It was observed that the fraction of $CD133^+$ tumor cells increased after radiation both in patients and in cell culture. The $CD133^+$ cells were more efficient than the $CD133^-$ in repairing the DNA. This was shown to be mediated through a CHK1/CHK2 dependent mechanism, by the treatment of debromohymenialsine, a CHK1/CHK2 inhibitor, which sensitized the $CD133^+$ tumor stem cells to radiation.

1.4.4.3 Manipulation of signaling pathways in brain tumor stem cells

Several studies have tried to develop strategies to target glioma stem cells. Piccirillo and colleagues have utilized the fact that $CD133^+$ GBM stem cells express receptors for BMPs (Piccirillo et al., 2006). These factors are involved in the normal development of the brain (Panchision et al., 2002). Transient exposure of BMP4 to transplanted $CD133^+$ GBM cells abolishes the capacity of these to establish intra cerebral tumors. Also, *in vivo* delivery of BMP4 effectively blocks the tumor growth and associated mortality that occurs after intra cerebral grafting of human GBM stem cells (Piccirillo et al., 2006).

Inhibition of the Notch pathway depletes and blocks engraftment of medulloblastoma cell lines in embryonic brain tumors. The fraction of $CD133^+$ cells was decreased after inhibition, and apoptosis in $nestin^+$ cells was 10-fold higher, as compared to the negative cells, indicating that these cells are dependent on Notch signaling (Fan et al., 2006). Conversely it has been shown that Notch signaling enhances nestin expression in GBMs, suggesting that Notch indeed contributes to the stem cell characteristics of brain tumor stem cells (Shih et al., 2006).

1.5 GLIOBLASTOMA ANIMAL MODELS

Many different experimental systems can be used for studying glioblastoma biology and they all have their advantages and disadvantages depending on which question is addressed. Some of the most common systems are introduced and briefly discussed below.

1.5.1 Cell cultures

Glioma cell cultures have extensively been used to study glioblastoma biology. These can be primary cultures or established cell lines. Primary cell cultures are thought to resemble the original brain tumor cell better than established cell lines. On the other hand, primary cultures quickly go through in vitro adaptations, selection and finally become cell lines.

Glioma tumor cells can also be grown as tumor neurospheres in serum free medium supplemented with EGF and bFGF. A recent study compared the difference between growing cells in neurosphere conditions or in 10% serum (Lee et al., 2006). On gene expression level, cells grown under neurosphere-conditions were more similar to the mother tumor and to normal neural stem cells. Genetic characteristics from the original tumor were also better preserved during neurosphere conditions as measured by SNP, Giemsa banding and SKY. Serum-grown cells had genomic rearrangements as early as passage 10, whereas cells grown in neurosphere conditions maintained parental tumor genotype after more than 70 passages. This study indicates that cell culture systems in the future should rely on neurosphere growth conditions. However, more studies should be performed since only two cell cultures were used.

One general drawback with cell systems is the absence of other cell types, which are present in the real tumor situation. Still, cell systems will remain as tumor models, mostly because they are easily subjected to experimental manipulations.

1.5.2 Xenografts

Xenografts can be grown either intracranially or subcutaneous and the tumor growth recapitulates the tumor microenvironment better than cell cultures. Intracranial grafting is preferable, but growth is difficult to monitor. In this setting however luciferase expressing cells can be used to monitor intracranial tumor growth (Uhrbom et al., 2004). The xenografts can be derived either from cell cultures or from intact brain tumor tissue. Additionally, subpopulations of tumor cells can be isolated from tumor material and used for xenograft tumor growth.

1.5.3 Genetic models

Several genetically engineered GBM mouse models exist. Genetically engineered mice are advantageous since they well mimic the tumor-host interactions in normal tumor development. Some problems with genetic brain tumor models are that they usually have bad reproducibility and low penetrance. Also clonal tumor growth is not considered since the oncogene is expressed in all cells.

One system which circumvents some of these problems is the RCAS/TVA model (Holland et al., 1998). TVA, a virus receptor, is expressed under a tissue or cell type specific promoter. The gene of interest is then inserted in adult stage into cells expressing the receptor by RCAS-virus infection.

In addition, other viral systems have been used to insert genes of interest to induce brain tumors. An example is the Moloney murine leukemia virus which was used to express the PDGFB-chain in mice leading to formation of brain tumors (Uhrbom et al., 1998). This model has further been used to search for new genes involved in glioma formation by insertional deletion (Johansson et al., 2005, Johansson et al., 2004). Retroviral insertion sites were cloned and the flanking genes identified. Several loci known to be involved in glioma formation were tagged like Ddr1, Trp53, Fancc, Rad51l1, Eef1a1, Gli and Fos.

2 AIM OF THE THESIS

The aim of the thesis was to combine immuno-histochemical, biochemical, and gene-expression-based analyses of glioma tumor tissue, and cultured glioma cells, to identify novel biologically and therapeutically relevant subsets of gliomas.

More specifically the studies aimed at

- an improved characterization of differences and similarities among the three major types of grade II gliomas; astrocytomas, oligodendrogliomas and oligodendrogliomas

- an identification of characteristics associated with, or causally coupled to, sensitivity to different glioma-relevant tyrosine kinase inhibitors

- an investigation of the possible existence of biologically distinct types of brain cancer stem cells

3 RESULTS

3.1 PAPER I

Gene expression analyses of grade II gliomas and identification of rPTPβ/ζ as a candidate oligodendroglioma marker

Grade II gliomas are morphologically heterogeneous. Histopathological typing remains the major tool for clinical classification. The major histological subtypes are astrocytomas, oligodendrogliomas and oligoastrocytomas. If these constitute true biological entities is incompletely understood. Morphological classification is not trivial. Novel markers for the subtypes would thus make diagnosis easier and more robust.

Following gene-expression analyses of twenty-three grade II gliomas, hierarchical clustering was performed. All six oligodendrogliomas were grouped together in one of the two major clusters. Histopathological subtype and gene expression was thus found to be correlated. To identify novel candidate markers for oligodendrogliomas, a supervised gene expression analyses was performed. The phosphatase rPTPβ/ζ was found among the most differentially expressed genes. Subsequent immunohistochemistry analyses, of an independent set of grade II gliomas, revealed positive staining in 11/11 oligodendrogliomas. Neoplastic astrocytes were mostly negative for rPTPβ/ζ staining.

A correlation between gene expression pattern and histological subtype in grade II gliomas was thus demonstrated, suggesting that the histologically defined subgroups represent valid biological entities. The results indicating oligodendroglioma-selective expression of rPTPβ/ζ should stimulate to continued evaluation of the usefulness of this protein as a novel marker.

3.2 PAPER II

Characterization of an imatinib-sensitive subset of high-grade human glioma cultures

Improved treatment is urgently needed for high-grade gliomas, including glioblastomas. Studies on the biology of these tumors suggest large heterogeneity and indicate that distinct subsets exist. Platelet-derived growth factor (PDGF) receptor signaling is a candidate target for novel therapies. In this study, the sensitivity of human high-grade glioma primary cultures to exposure to the PDGF receptor inhibitor imatinib/Glivec/Gleevec/STI571 was analyzed. Among 15 cultures, 6 displayed more than 40% growth inhibition after imatinib treatment, whereas seven cultures showed less than 20% growth inhibition. In the more sensitive cultures, apoptosis were found to contribute to the growth inhibition. A search for differences between high- and low-sensitive cultured demonstrated that high levels of platelet-derived growth factor receptor expression and phosphorylation was associated with high sensitivity. Micro-array-analyses, supported by real-time PCR analyses identified high levels of the chemokine CXCL12/SDF-1 (stromal cell-derived factor 1) as a predictor of imatinib

sensitivity. A functional link was suggested by the observation that exogenous addition of CXCL12 conferred some imatinib sensitivity to imatinib-insensitive cultures. In addition, analyses of gene-expression data from glioblastoma biopsies demonstrated co-regulation of CXCL12 and PDGF alpha-receptor in human tumor tissue.

In summary, the characteristics of a novel imatinib-sensitive subset of glioma cultures were defined, including high expression of PDGF receptors and CXCL12. Searches for sensitivity-determinants in samples from ongoing clinical trials with PDGF receptor inhibitors should be assisted by these novel observations.

3.3 PAPER III

Analyses of roles of gene expression and PI3K-PTEN-AKT pathway in determination of sensitivity to the IGF-1 receptor inhibitor NVP-AEW541 in high-grade glioma cell cultures

NVP-AEW541 is a novel IGF-1-recpetor inhibitor. The sensitivity to this inhibitor of fifteen high-grade glioma cultures was analyzed. The response varied extensively, with the most and least sensitive culture showing 340% and 20% growth inhibition, respectively. Growth-inhibition occurred through apoptosis induction and reduction of proliferation. Un-supervised gene-expression-based clustering of cultures did not reveal any significant correlations between global gene expression and drug sensitivity.

It was hypothesized that status of the PI3K/PTEN/AKT-pathway was involved in determining sensitivity. Nine cultures were selected to test this. Interestingly, 3/4 high-sensitive cultures demonstrated serum-dependent AKT phosphorylation, whereas AKT phosphorylation was only affected in 1/5 cultures with low sensitivity. However, sequence analyses of PI3K, and analyses of PTEN expression, failed to yield results strongly associating these read-outs with either sensitivity to drug-treatment or “wild-type” behavior of Akt phosphorylation following serum depletion.

To more directly investigate the functional role of PTEN expression levels in determining sensitivity to NVP-AEW541, the effects of siRNA-mediated down-regulation of PTEN was analyzed. PTEN down-regulation increased AKT phosphorylation. Interestingly, the growth inhibitory response to NVP-AEW541 was also reduced following PTEN down-regulation. This was most evident at lower drug concentrations.

The reduced sensitivity following PTEN down-regulation, together with the association between high sensitivity and serum-dependent Akt phosphorylation, thus suggest a causal relationship between these signaling pathways and sensitivity. However, the absence of strong correlations between results from the analyses of PTEN expression and PI3-kinase mutations, and drug sensitivity, indicate that analyses restricted to these individual components is insufficient to correctly predict sensitivity to NVP-AEW541 sensitivity.

3.4 PAPER IV

Two subsets of CD133$^+$ glioma cells differ in gene expression, neurosphere formation and tyrosine kinase inhibitor sensitivity

GBM growth has been suggested to be dependent on CD133$^+$ cancer stem cells. This study identified two subsets - designated type I and type II cultures – of CD133$^+$ brain cancer stem cells. The two subsets were originally suggested by differences in global gene expression. Real time PCR analyses of a set of stem cell and lineage marker genes revealed that type I cultures were characterized by higher expression of CXCR4, SOX2, EAAT1 and GFAP, and lower expression of CNP, PDGFRB, CXCL12 and extra cellular matrix proteins. Type I and II cultures also differed with regard to neurosphere formation, and sensitivity to mono-treatment with PDGF- and IGF-1-receptor inhibitors. Type I cultures showed better ability to form neurospheres and a reduced sensitivity to the growth factor inhibitors. Clinical significance of these subsets was suggested by analyses of gene-expression patterns in human GBM samples. The genes defining the two subsets were also differentially expressed in different tumors.

The description of these novel subsets of CD133$^+$ glioma cells should possibly help in identifying the cells subjected to transformation during brain tumor development. Also, these findings suggest novel possibilities for rational selection of therapeutic strategies for different types of tumors.

4 DISCUSSION AND FUTURE PERSPECTIVES

4.1 PAPER I

This study identified rPTPβ/ζ as a candidate marker for oligodendroglioma. For a better understanding of the potential of rPTPβ/ζ as a novel marker for oligodendrogliomas, further tests on larger clinical materials, including different glioma grades and histological subtypes, are required. rPTPβ/ζ has also been suggested as important for glioma cell invasion (Muller et al., 2003, Wu et al., 2006) and is considered as target in new therapies against glioma (Foehr et al., 2006, Ulbricht et al., 2006). Our studies suggest that oligodendrogliomas might be particularly sensitive to drugs targeting rPTPβ/ζ.

4.2 PAPER II

The existence of a novel imatinib-sensitive glioma subset expressing PDGFR-α and CXCL12 is intriguing. Tissue material from clinical trials with imatinib used in combination with hudroxyurea has been made available and tests of the significance of these proteins as markers predicting response is ongoing. Furthermore the role of CXCL12 in glioma biology needs to be explored. The observation that imatinib non-sensitive glioma cultures can be sensitized by CXCL12 treatment also requires further exploration.

4.3 PAPER III

The analysis of determinants of sensitivity to NVP-AEW541 strongly suggested that the status of the PTEN pathway is important. However, the inconclusive results from the analyses of two individual components of the pathway – PTEN and PI3-kinase – also illustrate the importance of developing assay that provides information of the functionality of the pathway. Ideally, these assays should be possible to use on patient-derived material. Along these lines it would be highly interesting to monitor pAkt status in tumor pre- and post-treatment and see how this correlates with later clinical response. It is also noted that data is still incomplete concerning the effects of the different PI3K mutations on activity. Furthermore, sequence analyses should be performed on non-tumor DNA to investigate if these are somatic mutations. Finally, the lack of concordance between PTEN measurements and drug sensitivity could of course be caused by differenced in activity status of PTEN in the different cultures.

4.4 PAPER IV

To test if cells of the two $CD133^+$ subsets exist in clinical material who are similar has high priority. Another important issue which still needs to be addressed is how normal neural stem cells and glioma tumor stem cells differ. Among the type I of markers are already proteins suggested to be important for glioma growth, like CXCR4. Interestingly, experimental glioblastoma growth has been shown to be inhibited by AMD 3100, a CXCR4 inhibitor (Rubin et al., 2003). The effects of this drug on type I cultures should be explored. Furthermore, the mechanisms underlying the striking combinatorial effect of the two inhibitors should be investigated.

5 ACKNOWLEDGEMENTS

The work underlying this thesis started in Uppsala at the Ludwig Institute for Cancer Research and has continued in Stockholm at the Karolinska Institute.

I would like to thank everyone who I have meet during my PhD years and made it an enjoyable time. Special thanks go to:

My supervisor **Arne** for all the time you have spent helping me with all kind of things. I have learned a lot from you and I hope you learnt something in return. The scientific projects have been very exciting and I am very satisfied. Ok, they have not exactly been straight-out-of-the-box, but I never asked for something like that. When I think what I will remember the best and have most use for it must, funny enough, be the "candy-rule". Creates a nice atmosphere and brings people together.

Many thanks also goes to the present lab group, I will always remember you **Plupp-Christina, Janna-panna, Jeroen, Jörn, Maja, Martin, Paparazzi-Markus, Kakbakar-Åsa**. Have almost too many memories of all the crazy things we have done together. But for your own sake I won't mention them here. Just have to keep on pumping those experiments and one day you will also be defending…snarf, snarf!

Former short/long time members/visitors of the lab group. **Tobias, Camilla, Kristian, Jill, Carina, Olli, Maria, Sefanja, Kai, Masao, Irene, Jens, Frank & Annette, Jan, Mitchan, Patrick**

All the people working on the CCK who always are fun to talk to. **Jacob**, for taking me, Lars and Dan on that crazy long bike ride…"just for lunch"! I still have nightmares from that day. I have enjoyed all the time on the spinning with "grodlåren", and the push-up competitions, as well. **Mira** for always having time for some lunch, and for open-mindedly sharing your ideas and experiences. **Salle** for all the good and bad pubs organized for the other colleges. Sometime it goes up, some times it goes down. Just keep on going! **CC, running man**. Thanks for all the late nights out for relaxing, and all the bad movies, which I still enjoyed because they were so bad.

The crowd on the fifth floor, who strangely enough always is having "fika" when I come by to ask something scientific!? Thanks for great scientific collaborations and other things as well. **Monica, Xiobing, Ulrika, Rona, Inga, Tomadher, Crister, Anna, Saskia, Ulrika, Wessen, Salah, Markus, Jian and Juan**

Marcus, a.k.a. Schwein. Yea what can I say, you know! I owe you quite a few for all the too late nights and too much pearls. Guess you are one of the few Stockholm-tourists who knows more about Stockholm than the Stockholmers them self.

Alexander, for all the football championship watching. Brave to sit with all the Swedes and watch the Germany game. Thanks also teaching me the art of Panini-sticker-swapping, or buying when not so many to swap with. Childish, but I felt sorry for you when your girlfriend gave all the stickers away to a bunch of kids.

All the people from MF that have come and gone. The water hole after a long hard week. Always a good way to relax a bit on your way home or somewhere else in the night.

All the people on our floor who make life so much easier and things run smoothly. **Sören, Joe, Eva-Lena, Marie and Ann-Gitt**.

Also all the other people on CCK who help with the every day work. **Elisabeth, Emily, Elle, Anders and Evi**

Although it seems like a long time ago I still remember all the fun time in Uppsala and on the Ludwig Institute and would like to thank **all of you** for many, many, many nice times with all the fika-breaks, lunch-excursions, crazy parties and ski-trips.

Calle for managing the institute in such efficient manner and for having time with every each and one.

Pathologic Micke it was a great time in Uppsala with Schlager, car racing, marathon running and football training.

Håkan Hellström, if you ever need entertainment for your birthday party just call. Otherwise enjoy your time cross-dressing like a chicken and crossing chickens.

Christer W for fulfilling my dream and passing on to me the very honorable task of being Santa at the annual Ludwig Christmas party. It took a very long time before anyone realized it was me. Also thank for you great interest in the colored spots I was sitting with all day.

Rainer, I had a real blast with all the adrenalin rushes from the bike riding in the wild Uppsala countryside.

All the spex groups, the most strange and fun thing during the whole PhD time.

Also a great thank to all the people from Rudbeck, Uppsala, who have somehow been involved in my projects. **Anja, Anders I, Guran, Hannu, Marianne K, Sunna, Tommie and Ulrika W-B**

Kissemissen for beeing patient, supportive and understand that I need some time to do this.

My warmest thanks go to my family **Bengt, Else-Marie and Joel** for always being there and for all the great holidays on the countryside where you really can relax with fresh air, good food and beautiful nature.

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