Akademisk avhandling

Gene and protein expression in human gliomas

av

Oscar Persson

2008

Som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet
för avläggande av doktorsexamen i medicinsk vetenskap
kommer att offentligen försvaras i Segerfalksalen,
Wallenberg Neurocentrum, Lunds Universitet, Lund

Lördagen den 13:e december kl 9.15

LUND
UNIVERSITY

Avdelningen för Neurokirurgi
Institutionen för kliniska vetenskaper
Medicinska fakulteten
Lunds Universitet

Fakultetsopponent:
Professor John H. Sampson
Division of Neurosurgery
Duke University Medical Center Durham,
North Carolina, USA

Organization
LUND UNIVERSITY
Department of Clinical Sciences
Division of Neurosurgery
Lund University
Lund, Sweden

Document name
DOCTORAL DISSERTATION

Date of issue
December 13, 2008

Sponsoring organization

Author(s)
Oscar Persson

Title and subtitle
Gene and protein expression in human gliomas

Abstract

The human gliomas are the most common primary brain tumours. They are highly aggressive with a rapid fatal progression for the patient. The invasive growth pattern and inherent resistance to radiation and chemotherapy account for a lack of efficient therapies. Several novel therapeutic regimes are being investigated, but they have so far shown limited beneficial effects. The molecular heterogeneity of the gliomas has also made it difficult to define the tumourigenic pathways, and to efficiently subcategorise the tumours according to different molecular origin or therapeutic sensitivity. The aims of this thesis has been to gain novel insights in the genetic and proteomic events underlying tumourigenesis and malignant progression in gliomas, and potentially define specific key events that could be the object of refined or novel therapeutic approaches.
We have utilised gene expression microarrays and 2D differential in-gel electrophoresis (DIGE) with sequential tandem mass spectrometry (MS/MS) protein identification, to investigate the gene and protein expression profiles in a set of gliomas of varying grades, as well as in a number of normal human brain samples. We found that these platforms can efficiently discriminate samples according to their histopathological origin. Both platforms also identified central tumourigenic pathways, deregulated in gliomas. The gene expression analysis further identified several chromosomal regions with deregulated expression. We also identified a number of proteins overexpressed in gliomas as potential objects for targeted therapy. From the combined data we conclude that there is a very weak correlation between mRNA and protein expression.
Furthermore, we investigated the tissue distribution patter and cellular location of the potential tumour associated antigen Ku70/80. We show that the extracellular localisation of this protein complex is specific for tumour cells, and that it can be used as a portal for tumour specific delivery of toxin conjugated antibodies.
Finally, using antibody microarrays we investigated differences in immunoregulatory proteins detected in the plasma of glioblastoma tumour patients and normal controls, as well as in tumour patients undergoing immunotherapy against gliomas. We found that patients can be stratified according to the beneficial effect of the immunotherapy. We also identified a number of key immunoregulatory factors discriminating the different subgroups. These data provide novel insight into tumour immunologic processes, and a step towards refinement of the immunotherapeutic regimes.

Key words:
glioma, targeted therapy, immunotherapy, tumour associated antigen, microarray, proteomics

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language

ISSN and key title:
1652-8220

ISBN
978-91-86059-83-5

Recipient's notes

Number of pages

Price

Security classification

Distribution by (name and address)

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Oscar Persson Date 30/10-08

Academic Dissertation

Gene and protein expression in human gliomas

by

Oscar Persson

2008

LUND
UNIVERSITY

Division of Neurosurgery
Department of Clinical Sciences
Faculty of Medicine
Lund University

It does not take much effort to see the things that are right in front of you,
but a lot of thought to know where to look.

Paper I and II were reprinted with the permission of the publishers.
© Oscar Persson, 2008
Gene and protein expression in human gliomas
Printed by Media-Tryck, Lund 2008

GENE AND PROTEIN EXPRESSION IN HUMAN GLIOMAS

ORIGINAL PAPERS 9

ABBREVIATIONS 11

SUMMARY 13

INTRODUCTION 15

INTRODUCTION TO TUMOUR BIOLOGY 15
GLIOMAS 17
- Aetiology & Epidemiology 17
- Types and grades 17
- Prognostic factors 19
- Genetics 20
- Primary and secondary gliomas 23
- The tumour stem cell model 24

GENE AND PROTEIN EXPRESSION PROFILING IN GLIOMAS 26
- Microarray in gliomas 26
- Proteomics in gliomas 27

CURRENT THERAPEUTIC REGIMES 29
- Surgery 29
- Radiotherapy 29
- Chemotherapy 29

NOVEL AND EXPERIMENTAL THERAPEUTIC REGIMES 31
- Small molecular inhibitors 31
- VEGF and antiangiogenesis 31
- Toxic chimeric proteins and tumour specific antibodies 33
- Immunotherapy 34
- Identification of novel tumour associated antigens 35
- The Ku70/80 protein 36
- Delivery of therapeutic compounds 37

METHODOLOGICAL CONSIDERATIONS 39

- Biological material 39
- Gene expression microarrays 40
- Proteomics and antibody microarrays 40
- Statistical analysis of multivariate data - Unsupervised classification 41
- Statistical analysis of multivariate data - Supervised classifiers 42

AIMS 43

RESULTS AND DISCUSSION 45

DISCRIMINATING TUMOURS BY GENE OR PROTEIN PROFILE (Paper I & II) 45

ABERRATIONS OF CHROMOSOMAL REGIONS (Paper I) 45

DEREGULATION OF SPECIFIC GENES OR PROTEINS (Paper I & II) 46

COMPARISON OF mRNA AND PROTEIN ABUNDANCE (Paper I & II) 47

CHARACTERISATION OF THE TUMOUR ASSOCIATED ANTIGEN Ku70/80 (Paper III) 48

INVESTIGATION OF PERIPHERAL IMMUNOLIGICAL MARKERS AND RESPONSE TO IMMUNOTHERAPY AGAINST GLIOBLASTOMAS (Paper IV) 49

CONCLUSIONS AND FUTURE PERSPECTIVES 53

POPULÄRVETENSKAPLIG SAMMANFATTNING 55

ACKNOWLEDGEMENTS 57

REFERENCES 59

PAPER I - *Microarray analysis of gliomas reveals chromosomal position-associated gene expression patterns and identifies potential immunotherapy targets.* 71

PAPER II - *Proteomic expression analysis and comparison of protein and mRNA expression profiles in human malignant gliomas.* 87

PAPER III - *Distribution, cellular localization and therapeutic potential of the tumor associated antigen Ku70/80 in glioblastoma multiforme.* 103

PAPER IV - *Plasma proteome profiling reveals biomarker pattern associated with prognosis and therapy selection in glioblastoma multiforme patients.* 117

ORIGINAL PAPERS

I. **Oscar Persson***, Morten Krogh*, Lao H Saal*, Elisabeth Englund, Jian Liu, Ramon Parsons, Nils Mandahl, Åke Borg, Bengt Widegren, Leif G Salford.
*These authors share first authorship.
Microarray analysis of gliomas reveals chromosomal position-associated gene expression patterns and identifies potential immunotherapy targets.
Journal of Neurooncology, 2007 Oct;85(1):11-24

II. **Oscar Persson**, Ulrika Brynnel, Fredrik Levander, Bengt Widegren, Leif G Salford*, Morten Krogh*.
*These authors share senior authorship.
Proteomic expression analysis and comparison of protein and mRNA expression profiles in human malignant gliomas.
Proteomics – Clinical Applications, 2009 Jan, In press.

III. **Oscar Persson**, Leif G Salford, Johan Fransson, Bengt Widegren, Carl AK Borrebaeck, Bo Holmqvist.
Distribution, cellular localization and therapeutic potential of the tumor associated antigen Ku70/80 in glioblastoma multiforme.
Submitted Manuscript

IV. Anders Carlsson, **Oscar Persson**, Bengt Widegren, Leif G Salford, Carl AK Borrebaeck, Christer Wingren.
Plasma proteome profiling reveals biomarker pattern associated with prognosis and therapy selection in glioblastoma multiforme patients.
Manuscript

ABBREVIATIONS

2D-DIGE	Two dimensional differential in-gel electrophoresis
ALT	Alternative lengthening of telomeres
AUC	Area under curve
BBB	Blood brain barrier
BRIGTT	Brain Immuno Gene Tumour Therapy
CDK	Cyclin-dependant kinase
CED	Convection enhanced delivery
CSC/TSC	Cancer/tumour stem cell
DC	Dendritic cell
EGFR	Epidermal growth factor receptor
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
hTERT	Human telomerase reverse transcriptase
iDC	Immature dendritic cell
IFN	Interferon
IL	Interleukin
LC	Liquid chromatography
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
MGMT	O6-methylguanine methyltransferase
MHC	Major histocompatibility complex
MMR	Mismatch repair
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NF	Neurofibromatosis
PDGFR-α	Platelet derived growth factor receptor alpha
PE	Pseudomonas exotoxin
PGE_2	Prostaglandin E_2
pI	Isoelectric point
PI3K	Phosphatidylinositol-3-kinase
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
ROC	Receiver operating characteristic
RT	Radiotherapy
RTK	Receptor tyrosine kinase
scFv	Single chain variable fragment of an antibody
SCID	Severe combined immunodeficiency
TAA	Tumour associated antigen
TAM	Tumour associated macrophage
TDSN	Tumour derived supernatant
TGF-β	Transforming growth factor beta
TKI	Tyrosine kinase inhibitor
TMZ	Temozolomide
***TP53*/p53**	Tumour protein 53 (gene/protein)
VEGF	Vascular endothelial growth factor

SUMMARY

The human gliomas are the most common primary brain tumours. They are highly aggressive with a rapid fatal progression for the patient. The invasive growth pattern and inherent resistance to radiation and chemotherapy account for a lack of efficient therapies. Several novel therapeutic regimes are being investigated, but they have so far shown limited beneficial effects. The molecular heterogeneity of the gliomas has also made it difficult to define the tumourigenic pathways, and to efficiently subcategorise the tumours according to different molecular origin or therapeutic sensitivity. The aims of this thesis has been to gain novel insights in the genetic and proteomic events underlying tumourigenesis and malignant progression in gliomas, and potentially define specific key events that could be the object of refined or novel therapeutic approaches.

We have utilised gene expression microarrays and 2D differential in-gel electrophoresis (DIGE) with sequential tandem mass spectrometry (MS/MS) protein identification, to investigate the gene and protein expression profiles in a set of gliomas of varying grades, as well as in a number of normal human brain samples. We found that these platforms can efficiently discriminate samples according to their histopathological origin. Both platforms also identified central tumourigenic pathways, deregulated in gliomas. The gene expression analysis further identified several chromosomal regions with deregulated expression. We also identified a number of proteins overexpressed in gliomas as potential objects for targeted therapy. From the combined data we conclude that there is a very weak correlation between mRNA and protein expression.

Furthermore, we investigated the tissue distribution patter and cellular location of the potential tumour associated antigen Ku70/80. We show that the extracellular localisation of this protein complex is specific for tumour cells, and that it can be used as a portal for tumour specific delivery of toxin conjugated antibodies.

Finally, using antibody microarrays we investigated differences in immunoregulatory proteins detected in the plasma of glioblastoma tumour patients and normal controls, as well as in tumour patients undergoing immunotherapy against gliomas. We found that patients can be stratified according to the beneficial effect of the immunotherapy. We also identified a number of key immunoregulatory factors discriminating the different subgroups. These data provide novel insight into tumour immunologic processes, and a step towards refinement of the immunotherapeutic regimes.

INTRODUCTION

INTRODUCTION TO TUMOUR BIOLOGY

Under normal conditions tissue renewal is strictly regulated via the processes of cell proliferation and cell death. The regulation of these mechanisms protects the organism from an uncontrolled and harmful expansion of any sub-clone of cells. The transformation of normal cells into a tumour phenotype requires the acquisition of a number of features for the cells, overcoming these barriers. Key genetic alterations for tumour initiation and promotion are assembled in the cells through DNA point mutations, deletion, amplification or translocation of chromosomal regions, or epigenetic events such as DNA-methylation, leading to activation of proto-oncogenes or down-regulation of tumour suppressor genes. Although a single genetic alteration is not enough for transformation in human cells, experimental models have indicated that as few as three genetic events might be sufficient for tumour initiation (Hahn et al., 1999). In transgenic mice models, brain tumours can be initiated by alteration of as few as one or two genes (Holland, 2001).

Some common phenotypical features are necessary for malignant transformation and can be found in all tumours. To expand indefinitely, the tumour cells (or at least a sub-clone of cells) have to become *immortalised*, i.e. attain the capability of indefinite cell division. In normal cells a part of the chromosome ends, the telomeres, are lost at each cell division, and after a finite number of divisions the cell enters senescence. To retain the ability of unlimited cell divisions, many tumours are known to express the human telomerase reverse transcriptase (hTERT), which maintains the telomere ends at the replication (Kim et al., 1994). Alternatively, some tumours utilise a recombinant mechanism known as alternative lengthening of telomeres (ALT) for telomere maintenance (Dunham et al., 2000).

Furthermore, tumour cells also need to exhibit *loss of contact inhibition* and to be *self-sustaining for growth- and survival-factors*. Proliferation of untransformed cells is normally inhibited by contact with other cells, and also dependant on paracrine or endocrine addition of mitogenic signals. In tumour cells these regulatory mechanisms are lost and the cells are self-sufficient in mitogenic signals due to autocrine growth-factor secretion, or constitutive activation of the intrinsic signalling pathways. In addition, growth-inhibitory signals can be lost due to mutation of downstream tumour suppressor genes. The growth inhibitory molecule Transforming growth factor (TGF)-β for example exerts some of its growth-inhibitory effect via the retinoblastoma (Rb)-pathway, known to be frequently mutated in gliomas (discussed below) (Laiho et al., 1990).

An additional important feature of tumour cells is *inhibition of apoptosis*. Apoptosis is the mechanism of programmed cell death and can be induced by extrinsic or intrinsic signals. Escape from these signals in tumour cells can, for example, be mediated by overexpression of anti-apoptotic oncogenes such as Bcl-2 (Reed et al., 1996). One of the central orchestrating

proteins in apoptotic signalling is the tumour suppressor p53, which is activated by genomic instability and DNA damage – a common feature in tumours. When p53 is activated it forces the cell into senescence or triggers the apoptotic cascade, and inactivating mutations in the *TP53*-gene is therefore a hallmark of many tumours (Vousden and Lu, 2002). To avoid being recognised by the immune system, tumours also have several ways of *immune evasion*. This can be, for example, downregulation of the antigen presenting major histocompatibility complex (MHC) (Drake et al., 2006) or secretion of immunosuppressive factors (Castelli et al., 1989; Thomas and Massague, 2005). Finally, malignant tumours have the capacity for *invasion* in surrounding tissue. This requires the activation of proteins controlling the adhesive and migratory properties of the cells.

GLIOMAS

Aetiology & Epidemiology

The gliomas are the most common primary intracranial tumours with a yearly incidence of approximately 6 / 100,000 in western countries, with the most malignant form – glioblastoma multiforme – constituting approximately 50% of the cases. Although gliomas can occur throughout the entire life, the low-grade tumours often present within the third or fourth decade of life, while the glioblastomas have their peak incidence in late mid-life. There is a slight overweight of males affected, with reported ratios of approximately 1.5:1 (M:F) for glioblastomas (Kleihues and Cavenee, 2000). Some regional variance in the incidence of brain tumours has been shown with Scandinavian countries having some of the highest incidences in the world (Stewart and Kleihues, 2003). The aetiology of gliomas is largely unknown. Some hereditary syndromes such as Li-Fraumeni (*TP53*), Tuberous sclerosis (*TSC1/TSC2*) and Neurofibromatosis 1/2 (*NF 1/2*) carry a strong predisposition for developing gliomas, and a number of cases has been associated to radiotherapy against the brain, but otherwise no causative factor has been identified (Shapiro et al., 1989; Stewart and Kleihues, 2003).

Types and grades

The first publication trying to classify brain tumours macroscopically was published 1829 by Cruveilier who divided them into categories such as fatty, fleshy or bony tumours. In the second half of the 19th century, Virchow was the first to try to correlate the macroscopic and microscopic features of the brain tumours, and he was also the first one to introduce the term "glioma" (Kaye and Laws, 1995). A complex classification scheme of the gliomas was outlined by Bailey and Cushing, but was replaced by a much simpler scheme put forth by Kernohan in 1949. Kernohan divided the gliomas into five subtypes – astrocytoma, ependymoma, oligodendroglioma, neuro-astrocytoma and medulloblastoma. He was also the first one to attempt a categorisation that would reflect the biological behaviour of the tumours, by introducing a four-step grading scale according to increasing anaplasia and cellularity. However, difficulties using the Kernohan grading-scheme arose due to the subjective classification criteria and interobserver variability. An attempt to introduce a system with more objective definitions of the grades was developed by Daumas-Duport in the 1980s, who proposed a system based on the presence of four morphological characteristics – nuclear atypia, mitoses, endothelial cell proliferation and necrosis. The most commonly used classification scheme today is the WHO-classification, which was also introduced during the 1980s. The most common neuroepithelial tumours in the WHO-system, which are usually considered by the term "gliomas", are the astrocytic, the oligodendroglial and the mixed oligo-astrocytic tumours (Fig 1), and the grading to a large extent depends on the same histopathological features used by Daumas-Duport.

The pilocytic astrocytomas (WHO grade I) are most frequently found in children or sometimes in young adults. They are usually benign, well-circumscribed tumours, and can

	Astrocytic tumours	Oligodendroglial tumours	Mixed tumours
Grade I	Pilocytic (juvenile) astrocytoma		
Grade II	Astrocytoma	Oligodendroglioma	Oligoastrocytoma
Grade III	Anaplastic astrocytoma	Anaplastic oligodendroglioma	Anaplastic oligoastrocytoma
Grade IV	Glioblastoma multiforme		

Figure 1. Outline of the histological types and malignancy grades of the common gliomas according to the WHO classification. The annotation "multiforme" is sometimes left out from the glioblastomas. It has been debated whether the oligodendrogliomas progress to grade IV, but if so, the anaplasticity makes them indistinguishable from other grade IV gliomas.

usually be cured by surgery, and are therefore often concerned with as a distinct group. The malignant gliomas (grade II-IV) are predominantly found within the cerebral hemispheres in adults. They have a diffuse infiltrative growth pattern, expanding into and destroying the surrounding tissue. Invading tumour cells are found migrating along the white matter tracts, the blood vessels and the pia mater, spreading throughout the brain. Tumour cells migrating in this fashion can spread to the contralateral hemisphere via the corpus callosum or anterior commisure, and at the time of diagnosis 50% of glioblastomas have been shown to have infiltrated the contralateral hemisphere or the basal ganglia (Matsukado et al., 1961).

Low-grade diffuse astrocytomas (WHO grade II) histopathologically consist of discreetly increased cellularity and might exhibit a minor degree of nuclear atypia. Radiographically these tumours often appear hypodense, and without any contrast enhancement or surrounding oedema. Approximately 70% of the grade II astrocytomas progress to a grade III/IV tumour within 5-10 years (Furnari et al., 2007). Anaplastic astrocytomas (WHO grade III) often show the addition of focal hypercellularity, and there is an increased anaplasia and mitotic activity of the tumour cells. Glioblastoma multiforme (GBM; WHO grade IV) in addition to a high density of small anaplastic cells and high mitotic activity, also exhibit necrotic areas and neovascularisation. The necrotic areas are often surrounded by pseudopalisading tumour cells. As implied by the name multiforme, these tumours display a high degree of cytopathologic heterogeneity. Radiographically the GBMs are commonly seen as a distinctly contrast-enhanced rim formed by the proliferative tumour front, enclosing a necrotic centre, and which can be surrounded by a prominent vasogenic oedema.

Oligodendrogliomas are categorised into two grades according to the WHO-classification, oligodendrogliomas (grade II) and anaplastic oligodendrogliomas (grade III). The oligodendrogliomas exhibit histopathological morphology distinct from the astrocytomas, with a predominance of oligodendrocyte-like round cells with a clear cytoplasmic space, but the grading criteria are the same as for the astrocytomas. It has been debated whether the anaplastic oligodendrogliomas do progress in malignancy, but if so, the anaplasticity makes them indistinguishable from the rest of the gliomas and they are included in the glioblastoma group. A group of tumours also show a marked mixture of astrocytic and oligodendroglial histopathological morphology. Although these tumours sometimes exhibit distinctly separated juxtaposed astrocytic and oligodendroglial compartments (Kleihues and Cavenee, 2000), identification of shared chromosomal aberrations (LOH 1p/19q) in both compartments indicates a common origin (Kraus et al., 1995).

The classification and grading of the gliomas still remains based upon histological classification criteria. The subjective nature of this classification, and the heterogeneity among the gliomas, have made these tumours prone to misclassification, and studies where samples are examined by several neuropathologists in a blinded fashion often show large interobserver variability in final diagnosis (Kros, 2007). Increased insight into the molecular and genetic underpinnings of these tumours will give rise to improved understanding of the molecular pathogenesis of the gliomas, and may lead not only to more accurate classification and prognosis, but also to new or improved therapies. Microarray-based technologies are powerful tools to survey global gene expression changes in disease, and to relate these changes to phenotype and clinical data. Several studies investigating gene expression in astrocytomas of different grades have been published, and have shown that gene expression profiles can be used to aid conventional histological evaluation in classification of gliomas into biological groups and according to prognosis (Nutt et al., 2003; Freije et al., 2004; Mischel et al., 2004; Phillips et al., 2006). Later on, molecular large-scale investigations have also been extended to proteomic-based platforms, which have been able to successfully separate gliomas from normal tissue, and to find expression profiles related to survival time (Iwadate et al., 2004; Schwartz et al., 2005). Further development of reliable and standardised molecular diagnostic methods is an important task to accomplish successful treatment of glioma patients. The use of stereotactical biopsies, where only small pieces of tissue is retrieved, is always associated with some uncertainties about how representative this sampling is for the entire tumour and could benefit from molecular diagnosties. However, molecular differences have so far been difficult to place in an integrated clinical context, likely due to variations in platforms, study designs, and analytical strategies used.

Prognostic factors

The prognosis for glioma patients is highly dependent on the malignancy grade of the tumour. While low-grade glioma patients frequently have survival times exceeding a decade (CBTRUS, 2008), and reported 5-year survivals between 42-92% (Sanai and Berger, 2008),

the mean survival time of glioblastoma patients remains less than 15 months in spite of state-of-the-art therapy (Stupp et al., 2005), and they have a 5-year survival of less than 4% (CBTRUS, 2008). Within the individual grades, age is the most important prognostic factor (Ohgaki, 2005), and together with Karnofsky performance status the only clinical parameters that repeatedly have shown significant correlation to survival in multivariate analysis. While glioblastoma patients aged 21-44 years have a relative 2-year survival rate of 30%, this decreases to less than 3% in patients older that 65 years (CBTRUS, 2008).

Attempts to find correlation between individual molecular markers have shown variable or no results (Etienne et al., 1998; Newcomb et al., 1998; Balesaria et al., 1999; Kraus et al., 2000; Heimberger et al., 2005). Studies have indicated loss of chromosome 10q to be associated with transition to grade IV and an individual negative prognostic factor (Balesaria et al., 1999; Daido et al., 2004), while loss of 1p/19q is typical for oligodendroglial tumours and has been reported to be prognostically favourable in some materials (Cairncross et al., 1998; McDonald et al., 2005). Nevertheless, so far no single genetic marker has been widely implemented into clinical routine. However, with the development of large-scale gene expression microarrays, and proteome screening methods, expression profiles using multiple mRNAs or proteins have been shown to have the capacity to efficiently categorise tumours according to survival. With the introduction of concomitant temozolomide and radiotherapy, the methylation pattern of the O6-methylguanine methyltransferase (*MGMT*)-gene has also emerged as a new prognostic factor, since the beneficial effect of this regime seems to be limited to patients with promoter methylation (discussed below).

Genetics

The genetic aberrations underlying glioma formation have been extensively studied, and some of the tumourigenic mechanisms are well characterised. However, as pointed out earlier, gliomas are highly heterogeneous tumours and exhibit a high variability to which specific genes or proteins are deregulated in different tumours. By integrative analysis of gene copy number alterations and mutational analysis, a number of core pathways have emerged as critical for tumourigenesis. Examples of these are deregulation of receptor tyrosine kinases (RTKs) or downstream signalling molecules such as phosphatidylinositol-3-kinase (PI3K), and deregulation of the p53 and Rb tumour suppressor pathways (Furnari et al., 2007; Chin et al., 2008),

Epidermal Growth Factor Receptor (EGFR) is an RTK that drives proliferation and cell survival, and one of the most frequently aberrant signalling proteins in glioblastomas. It has been reported to be aberrantly expressed in approximately 60% of the glioblastomas, and in most of those cases (70-90%) the overexpresion is at least partly due to amplification of the *EGFR*-gene (Ohgaki, 2005). The *EGFR*-gene is located on the short arm of chromosome 7, which is often found in trisomy or polysomy in gliomas (Wessels et al., 2002). There are a number of mutated variants of *EGFR* known in glioblastomas, of which the most common is *EGFRvIII* (also known as Δ*EGFR*), which results from an in-frame deletion of exon 2-7 of

the *EGFR*-gene, and produces a truncated extracellular receptor that is constitutively active (Kuan et al., 2001; Pedersen et al., 2001).

Two parallel signalling cascades downstream to the RTKs have been outlined as important for driving proliferation in gliomas (Fig 2). One pathway is the PI3K-signalling cascade, which has been shown to carry frequent mutations in gliomas. These PI3K-complex comprises of one catalytic (PIK3CA) and one regulatory (PIK3R1) subunit, and it has been shown that mutations often locate to the interacting regions of the subunits. The mutations have been suggested to disrupt binding between the subunits and thereby remove the regulatory effect, resulting in a constitutively active PI3K-signaling (Huang et al., 2007; Chin et al., 2008). Another important glioma associated protein that controls the PI3K-pathway is the tumour suppressor *PTEN*, which acts antagonistically to PI3K by dephosphorylation of signalling substrates. The *PTEN* gene is located on the long arm of chromosome 10, which is often deleted in gliomas. *PTEN* is inactivated in approximately 25-40% (Ohgaki et al., 2004; Chin et al., 2008) of the high-grade gliomas via mutations or hypermethylation, resulting in lost control of PI3K-signalling (Vivanco and Sawyers, 2002). In animal models, *PTEN* inactivation has also been associated with increased angiogenesis, which further correlates the loss of expression with transition to high-grade tumours (Xiao et al., 2005). Downstream to PI3K are the Akt and mammalian target of rapamycin (mTOR) proteins, which connects the pathway to cell cycle regulation.

A second important pathway downstream to the RTKs is MAPK-signalling, composed of the Ras/Raf/MEK/Erk-cascade. Constitutively active, mutated forms of Ras are a hallmark of many cancers and are found in approximately 50% of all human tumours. However, although high Ras-activity is often found in gliomas (Guha et al., 1997), few *Ras* mutations, or mutations of any of the other constituents of this pathway, have been identified in astrocytic tumours. This is suggestive of an increased Ras-signalling due to excessive upstream RTK-activation, rather than due to alterations within the MAPK-pathway itself (Furnari et al., 2007).

The Rb-pathway is an important regulator of the G1/S progression in the cell cycle (Takuwa and Takuwa, 2001; Sherr and McCormick, 2002), and a critical target of inactivating mutations in gliomas (Ueki et al., 1996). Mitogenic signalling induces cyclin-D, which associates to cyclin-dependant kinases (CDKs) 4 and 6, and in turn leads to a phosphorylation of Rb. When Rb is phosphorylated it releases the transcription factor E2F, which moves into the nucleus and initiates transcription of the S-phase genes. The most common alteration of the Rb-pathway is deletions of *p16/INK4A*, which is an inhibitor of CDK4/6 (Chin et al., 2008).

A second important cell cycle control signal is exerted by the p53-pathway. Upon recognition of DNA damage, p53-signalling also functions to arrest cells at the G1/S, or to induce apoptosis, and the *TP53*-gene is frequently mutated in a wide variety of cancers (e.g. lung, liver, cervix, colon etc.). Inactivation of p53-signalling can occur either directly as *TP53*-

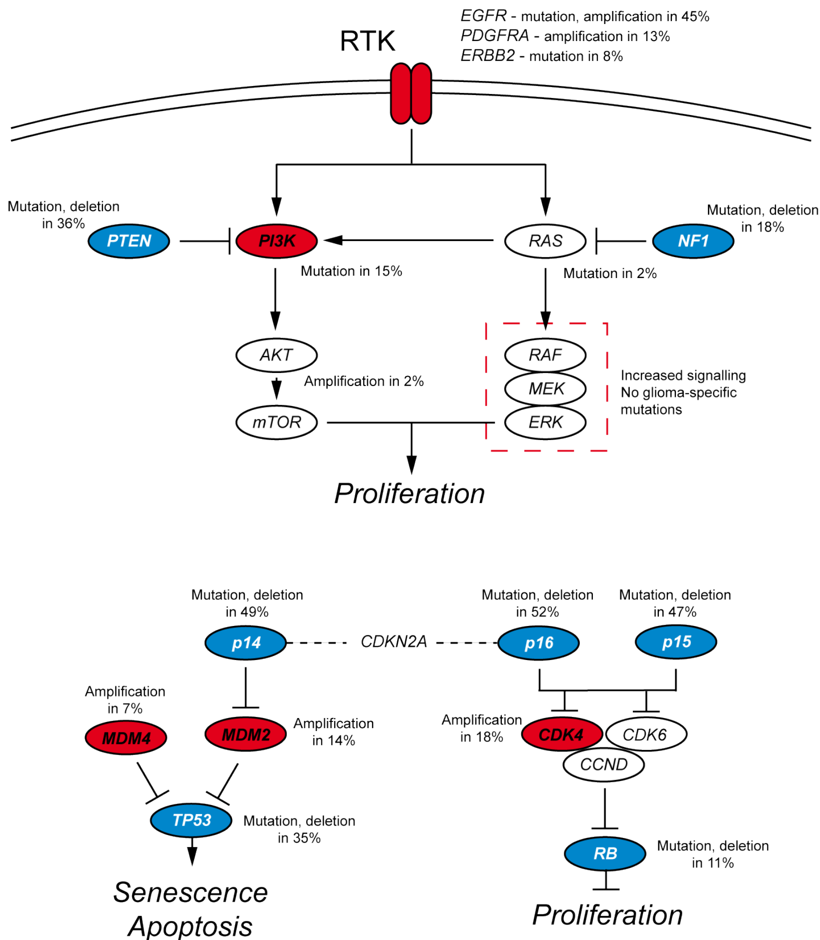

Figure 2. Common alterations in the RTK-, p53-, and Rb-pathways in glioblastomas. Frequently altered oncogenes are shown in red and tumour suppressor genes in blue. Mutations or amplifications in the RAS/RAF/MEK/ERK-pathway are rare in gliomas but an increased activity is often found due to upstream alterations. Both p16 and p14 are transcribed from the *CDKN2A* locus and deletions in this region can therefore target both the p53- and Rb-pathway simultaneously. Frequencies of specific alterations is adopted from Chin et. al. (2008). In total 88% of the tumours were found to have alteration of at least one component in the RTK-pathway, 87% in the p53-pathway, and 78% in the Rb-pathway.

mutations, amplification of the p53-inhibitors *MDM2* or *MDM4*, or by deletion of *p14ARF*. One of the most frequent aberrations in this pathway is homozygous deletion of the *CDKN2A* locus, which encodes both p16 and p14. Deletion of this locus can thus target both the Rb- and p53-pathways simultaneously (Chin et al., 1998; Chin et al., 2008) (Fig 2).

Interestingly, an integrated analysis assessing both copy number alterations and mutation analysis of core tumourigenic pathways in a glioblastoma material, reported a high incidence of concurrent aberrations in all three of the RTK-, p53- and Rb-signalling pathways. This suggests that combined deregulation of all three pathways is a key feature for tumour development (Chin et al., 2008; Zheng et al. 2008). This is an important result since *EGFR* amplification and *TP53* mutation in some earlier studies have been reported to be mutually exclusive, and constitute distinct tumourigenic pathways (Watanabe et al., 1996; Hayashi et al., 1997). Simultaneously, the integrated analysis also revealed that within each pathway, the different aberrations were often mutually exclusive. This is well in line with the hypothesis that mutation or deregulation of one element in a pathway relieves the selective pressure for aberrations in other components of the same pathway (Chin et al., 2008). The key function of these pathways in tumourigenesis in general is emphasised by the study by Hahn et al. (1999), showing that simultaneous disruption of the p53- and Rb-pathways and activation of Ras and hTERT was required and sufficient for transformation.

The most common genetic aberrations in the oligodendrogliomas is the combined loss-of-heterozygosity (LOH) of chromosome 1p and 19q (50-70%). It has been suggested that several different tumour suppressor genes are located within these areas, but the exact tumourigenic mechanism behind this aberration still remains to be elucidated. However, some studies have indicated a better prognosis for oligodendroglial tumours carrying the 1p/19q deletion (Cairncross et al., 1998), proposing that these tumours comprise a special subgroup with a more favourable outcome. Combined loss of 1p/19q has also been proposed to correlate with a proneural gene expression profile (Ducray et al., 2008), which in turn has been show to be prognostically favourable (Phillips et al., 2006). The malignant progression from grade II to anaplastic oligodendroglioma, however, seems to depend on similar pathways as progression of the astrocytomas, with e.g. deletion of the *CDKN2A*-locus as a common feature (Cairncross et al., 1998; Bigner et al., 1999).

In spite of the characterisation of these aberrations, there is still an incomplete understanding of the molecular pathology of the gliomas, and even more is still unknown about their interaction with the surrounding brain.

Primary and secondary gliomas

Attempts to subcategorise gliomas have historically often defined two groups of grade IV tumours based on clinical history. The secondary glioblastomas, which develop through malignant progression from a lower grade tumour, and the primary, or *de novo*, glioblastomas, which arise directly as a grade IV tumour without any evidence of a pre-existing lower grade malignancy. The primary glioblastomas, which constitutes approximately 95% of the cases (Ohgaki and Kleihues, 2007), have been described to occur in older patients and have a more rapid clinical course, while the secondary tumours arise through slow progression in younger patients. It should be noted though, that the discrimination between these groups is conceptual rather than diagnostic. Both groups share a common morphology and are not

possible to distinguish histologically, and no differences in prognosis can be found between them in age-adjusted materials (Dropcho and Soong, 1996; Newcomb et al., 1998; Ohgaki and Kleihues, 2007).

Efforts to distinguish the subtypes on a molecular level have reported the primary GBMs to frequently overexpress EGFR and MDM2, and carry *PTEN* and *p16* mutations, while the secondary glioblastomas are characterised by *TP53* mutation and PDGFR-α overexpression (Kleihues and Ohgaki, 1999). As discussed above, while earlier studies have reported *EGFR*-amplifications and *TP53*-mutations to be mutually exclusive in glioblastomas (Watanabe et al., 1996; Hayashi et al., 1997), supporting the hypothesis of different pathogenic mechanisms, this has been contradicted by later reports showing concurrent aberrations in both pathway (Gil-Benso et al., 2007; Chin et al., 2008; Zheng et al., 2008). Furthermore, in histological examinations, all grade IV tumours, including the primary, contain focal areas with lower malignancy grade, indicating a potential progression from lower grade tumours.

The clinical distinction into primary or secondary does not predict any difference in survival, and attempts to separate the groups according to molecular signature have shown at best limited results (Godard et al., 2003). However, mixed materials containing a natural selection of tumours from both groups can be prognostically subcategorised with high accuracy using molecular classifiers (Petalidis et al., 2008). This underscores that while primary and secondary groups may contain overrepresentation of subgroups of tumours with different molecular signature, there is still a large overlap. For example, the overrepresentation of *EGFR*-amplifications in primary glioblastomas and *TP53*-mutations in secondary glioblastomas, have been found in several studies (Kleihues and Ohgaki, 1999; Ohgaki et al., 2004). However, none of these – or any other genetic marker – is exclusive for their respective group, and a noticeable number of tumours with each genetic aberration is recognised in the opposite clinical group. Potential molecular subclasses of glioblastomas with different temporal pathogenesis are expected to be enriched in either of the primary or secondary group, however, likely with a significant overlap between the groups due to additional clinical factors influencing the time to diagnosis. Hence, the distinctions between primary and secondary likely reflects groups that could be much more efficiently defined on a molecular level – rendering a more clinically useful subcategorisation. This was illustrated by the identification of a group of gliomas carrying mutations in the isocitrate dehydrgenase 1 (IDH1) gene, which was found in a majority of the secondary gliomas but only in a small percentage of the primary. The tumours delineated by this mutation, and especially those defined as primary, showed a significantly better overall survival (Parsons et al., 2008). This demonstrates the superior usefulness of well-defined molecular classification.

The tumour stem cell model

The identification of cancer stem cells (CSC) and the establishment of these as the cancer maintaining population in haematological cancers (Bonnet and Dick, 1997; Hope et al., 2004) sparked an intense search for a CSC also in solid tumours such as gliomas. The classic theory

for glioma growth supposes a wide heterogeneity among the tumour cells, but that all cells have similar proliferation capacity and all share the capability to function as tumour-founding cells (stochastic model). In contrast, the CSC theory (or hierarchical model) proposes a small sub-clone of homogeneous tumour cells with the ability of infinite self-renewal and tumour initiation. These tumour stem cells (TSCs) in turn give rise to a heterogeneous bulk of tumour cells with limited proliferative capability and lacking tumour-initiating capability (Reya et al., 2001; Vescovi et al., 2006). The hierarchical model has won some support by experiments showing that transplantation of tumour cells positive for the cell surface marker CD133 could establish tumours when serially transplanted in SCID-mice, while transplantation of a 1000-fold as many $CD133^-$ cells were not able to induce tumour growth (Singh et al., 2003; Singh et al., 2004). This conceptual revision of how the tumour is propagated could have considerable therapeutical implications. If the hierarchical model holds true, developing therapeutic approaches that specifically kill the TSCs could efficiently deplete the tumour repopulating pool, rendering the rest of the tumour bulk unable to maintain tumour growth and to eventually degenerate (Reya et al., 2001).

The discovery of neural stem/progenitor cells in the adult mammalian and human brain (Reynolds and Weiss, 1992; Eriksson et al., 1998) further inspired the TSC hypothesis. Several conceptual features of the TSCs, and similarities to normal stem cells, make the normal stem cells theoretically conceivable cells of origin. Firstly, normal stem cells already possess the self-renewal machinery necessary in a TSC, which means fewer mutations are required for tumourigenesis compared to reactivating this processes in differentiated cells. Secondly, the longevity of stem cells may account for greater opportunity to accumulate tumourigenic mutations in an individual cell (Reya et al., 2001). Thirdly, the $CD133^+$ potential glioma stem cells share the postulated radiotherapy resistance of normal stem cells (Bao et al., 2006). These notions all point towards a neural stem/progenitor cell as the cell of origin for gliomas, contrary to dedifferentiation of a committed glial cell residing within the parenchyma.

Although often discussed in the same context, it is important to notice that the tumour stem cells theory (concerning tumour propagation) and tumour cell of origin are essentially two distinct and separate issues. Observation of stem cell like properties in glioma cells does not by itself prove neural stem/progenitor cells to be the cell of origin, but could still be the result of abilities required during dedifferentiation (Stiles and Rowitch, 2008). Later studies have also shown results that call for reappraisal of the hierarchical model. Specific genetic alterations have been shown to induce dedifferentiation and tumour maintaining phenotype from committed cells of both haematological and astrocytic lineage (Bachoo et al., 2002; Krivtsov et al., 2006). Furthermore, some $CD133^-$ cells have been shown to also fulfil the functional characteristics of tumour initiating cells (Beier et al., 2007), indicating that potential TSCs cannot be considered one homogenous entity in all gliomas.

GENE AND PROTEIN EXPRESSION PROFILING IN GLIOMAS

Microarray in gliomas

Several investigations have utilised gene-expression microarrays to characterise the transcriptome of gliomas, in order to ultimately try to define differentially expressed genes, distinguish tumour subcategories, and to identify prognostic profiles in these tumours.

Nut et al. (2003) investigated a mixed set of glioblastomas and anaplastic oligodendrogliomas. They identified a prognostic gene-expression profile that could discriminate samples according to survival more efficiently than histopathological diagnosis, and the profile was further validated in a set of tumours showing non-classic histopathologic features. The prognostic classification power of microarrays was also confirmed by Freije et al. (2004), who identified a profile that discriminated glioblastomas and a mixed group of anaplastic astrocytomas/oligodendrogliomas according to survival more efficiently than histopathology. The identified gene-cluster with the best prognosis typically exhibited overexpression of genes involved in neurogenesis, and one of the clusters with poor prognosis exhibited overexpression of mitosis related genes.

Philips et al. (2006) identified three subcategories of high-grade astrocytomas, enriched for overexpression of genes related to proneural, mesenchymal, and proliferative phenotype respectively. They showed a significantly better survival in the proneural group and also correlated this to an intact *EGFR*- and *PTEN*-status, while the opposite was true for the other two groups. The better survival in the proneural group and worse prognosis in the proliferative group are in concordance with the similar discrimination between neurogenesis and mitosis related expression profiles found by Freije et al. The differences in prognosis, and the nature of the genes characterising the three different groups, lead Phillips et al. to propose a model wherein the three groups correspond inversely to the developing stages of the neural stem cell, via neuroblast, to differentiated neuron. Although this reversed correlation to differentiation is conceptually conceivable for explaining a more aggressive nature of some tumours, it still remains to be verified. In contrast to the association with *EGFR*- and *PTEN*-status for the above groups, Rich et al. (2005) found no correlation with survival for expression of the key genes *EGFR*, *TP53*, *p16* or *PTEN*. However, they found that the expression of three migration associated genes (osteonectin, doublecortex and semaphorin3B) correlated negatively to survival.

Several ontological analyses have focused on finding functional groups of genes with diverse expression. Some functional groups frequently found to be differentially expressed in gliomas are associated to angiogenesis, cell migration, extracellular matrix remodelling, cell cycle and DNA maintenance, and signal transduction (Rickman et al., 2001; Petalidis et al., 2008). A number of microarray studies have also tried to identify diverse gene expression patterns in primary and secondary glioblastomas. Tso et al. (2006) found overexpression of a set of mesenchymal stem cell associated genes in a subset of the primary gliomas, compared to

secondary gliomas or normal brain tissue. This result is also in line with the more aggressively behaving mesenchymal group identified by Phillips et al. Some separation between primary and secondary glioblastomas was also found by Godard et al. (2003), who found the secondary tumours to cluster intermediately between low-grade gliomas and primary glioblastomas in a multidimensional scaling model. Hoelzinger et al. (2005) used gene expression microarrays to identify different signatures between migratory cells invading the surrounding parenchyma and the tumour core. Such differences are important to consider in the development of targeted therapies, since these have been focused mainly on the proliferative characteristics of the stationary tumour mass.

The power of gene-expression microarrays to classify gliomas has been further strengthened in later studies (Marko et al., 2008; Petalidis et al., 2008). Although there is often a very a high concordance between the groups found by classification algorithms based on gene expression, and histopathological grade, several studies have shown the gene expression data to have slightly better performance in survival prediction. This strengthens the idea that there are inherent genetic differences in gliomas (heterogeneity), that may define phenotypically different molecular subclasses that are not histologically distinguishable. However, most studies use different platforms and different analytical strategies, and most studies are proof-of-principle rather than providing any common consensus genomic signatures that can be integrated in a clinical context.

Proteomics in gliomas

Although gene expression microarrays are extremely powerful by the large number of genes investigated simultaneously, it is ultimately not the transcriptome but the proteome that governs the phenotype of cells. Many proteins undergo post-translational modifications that can dramatically alter the protein function or activity (Seo and Lee, 2004; Tootle and Rebay, 2005). The amount of protein will also depend not only on abundance of mRNA, but also on stability and breakdown of the protein. Furthermore, microarrays have generally not been able to discriminate different splicing variants of mRNAs. All these factors exemplify processes that can severely alter protein abundance and function, but that will not be recognised by investigations of the transcriptome. Several studies have therefore focused on large-scale proteomic investigations for the identification of tumour specific biomarkers. One of the most well established platforms for such high-throughput proteomic investigations is 2D-gel protein separation with subsequent MS or MS/MS protein identification (see Methodological considerations).

Using this platform, Iwadate et al. (2004) showed that they could efficiently discriminate tumour samples according to histopathologic diagnosis by the proteomic profile, and that tumours with a proteomic profile resembling normal brain had a better prognosis than the rest of the tumours. Further indications of the prognostic power of protein profiling was provided by Schwartz et al. (2005) (using a somewhat different methodological platform), who reported proteomic profile as a significant independent prognostic factor in multivari-

ate analysis. Iwadate et al. also identified a set of proteins differently expressed in gliomas. Several of these proteins belonged either to G-proteins or cytoskeleton regulation associated proteins, processes important for proliferation signalling and cell migration in gliomas.

Chumbalkar et al. (2005) also identified a number of proteins differentially expressed in gliomas, several of which were cytoskeleton associated. Glial fibrillary acidic protein (GFAP) is one of the major intermediate filament proteins in gliomas. It is prominently expressed in lower grade astrocytic tumours, but can disappear with the dedifferentiation in progression to a glioblastoma. Interestingly, Chumbalkar et al. showed a grade specific shift in overexpression of different GFAP isoforms, and suggested a destabilisation of the protein and a decrease of the high molecular weight isoforms with increasing tumour grade.

Proteomic approaches have also been utilised to investigate the induced differences in proteome profile in response to radio- and chemotherapy in glioma cell lines. Trog et al. (2006) showed an upregulation of the extracellular matrix protein vimentin and a downregulation of the cytoskeleton regulatory factor RhoA in response to combined radio/chemotherapy. Both these alterations have earlier been related to more malignant phenotype in gliomas (Dehghani et al., 1998; Forget et al., 2002; Mahesparan et al., 2003), and it is therefore possible that they contribute to the more aggressive phenotype often observed in recurrent tumours after therapy failure. Alterations in the expression of RhoA family members were described also in the studies by Iwadate et al. and Chumbalkar et al.

Additionally, there have been reports trying to discriminate primary and secondary glioblastomas using proteomics. Furuta et al. (2004) identified 11 proteins differentially expressed in primary and secondary gliomas, with EGFR and tenascin-x being overexpressed in the primary tumours. Although this study was performed in a small set of samples, it had the advantage of being performed in a microdissected material, and thus ignoring noise from stromal cells (see Methodological considerations).

A novel platform for high-throughput proteomic research is the antibody microarrays, where proteins can be detected using similar technology as the gene expression microarrays. Our study monitoring immunoregulatory proteins in the plasma of patients undergoing immunisation treatment (Paper IV) is to our knowledge the first time this method is utilised in glioma patients.

CURRENT THERAPEUTIC REGIMES

Surgery

Surgical resection provides the first line therapy against most newly diagnosed gliomas. However, the diffuse infiltrative growth of gliomas, and their propensity to seed microsatellites – or micrometastases – throughout the brain, makes it impossible to achieve a complete surgical removal of the tumours and cure of the patients. Historical cases with a complete surgical removal of a tumour-bearing hemisphere have shown that at the time of diagnosis tumour cells are already spread throughout the brain, with recurrence in the contralateral hemisphere as consequence (Bell and Karnosh, 1949). Although there is often a clear operative indication for alleviating symptoms and obtaining tissue diagnosis, the impact of the extent of resection on patient outcome has been subject of debate. In a meta-analysis covering all studies between 1990 to 2007 in both low- and high-grade gliomas, the correlations between extent of resection and patient outcome was reviewed (Sanai and Berger, 2008). While there seems to be solid data for low-grade tumours, with an overall 50% increase in 5-year survival for gross total resection vs. subtotal resection (90.5% vs. 61.1% respectively), there are still some opposing data concerning the high-grade tumours. Approximately half of the studies concerning high-grade gliomas reported improved overall survival for patients with more extensive surgery, with an approximate 3 month increase in mean survival for patients with gross total resection vs. subtotal resection (14.1 vs. 11.3 months respectively). The beneficial effect of complete resection was further strengthened in another recent study, where a large glioblastoma material was investigated for survival improvement (Stummer et al., 2008).

Radiotherapy

Standard therapy for glioblastomas and other primary malignant gliomas includes radiotherapy (RT) after the performance of most extensive possible surgical resection. Radiotherapy has long been established as the single most beneficial adjuvant therapy for these tumours. Two prospective randomised studies have shown an approximate doubling in overall survival with the addition of RT compared to surgery alone in high-grade gliomas (Walker et al., 1978; Kristiansen et al., 1981). However, the gliomas also display a strong propensity for radioresitance, and in spite of extensive surgery and maximum tolerated radiation dose most patients succumb to local tumour recurrence. Although within the context of the tumour stem cell theory it has been suggested that the slowly dividing TSCs could be responsible for the radioresistance of gliomas, so far little is actually known about the genetic mechanisms behind this resistance.

Chemotherapy

Historically, most chemotherapy trials in gliomas have been focused on different nitrosourea-related compounds. The results of these trials have often been opposing or inconclusive, and positive effects have often been limited in size, at least in astrocytic tumours (Lonardi et al., 2005). A more favourable response has been reported for the oligodendrogliomas and mixed oligoastrocytomas (Cairncross and Macdonald, 1988; Glass et al., 1992; Cairncross et

al., 1998). However, no standard adjuvant chemotherapeutic regime for glioblastomas was established before the beginning of the 21st century. In 2005, Stupp et al. presented a randomised study investigating the effect of RT and concomitant temozolomide (TMZ) administration. This study showed a limited, but significant, increase in median survival in patients treated with RT+TMZ compared to RT alone (14.6 vs. 12.1 months respectively) (Stupp et al., 2005), and this regime soon became standard therapy worldwide. Temozolomide is an alkylating agent and promotes its cytotoxic effect by methylation of DNA guanine-groups, resulting in double-strand breaks and induction of apoptosis. Consecutive studies have shown that the cytotoxic effect of TMZ is highly dependent of the methylation status of the *MGMT*-gene. The beneficial effect of alkylating chemotherapeutics seems to be limited to patients with epigenetic silencing of this gene by promoter methylation, which is found in approximately 45% of GBM patients (Hegi et al., 2005; Hegi et al., 2008). *MGMT* protects the cells by removing methyl-groups from the guanine groups in the DNA. The cytotoxic effect of guanine methylation is believed to be due to cycles of futile mismatch repair (MMR) by the MMR-proteins, which eventually leads to DNA double-strand breaks and induction of apoptosis. Chemotherapy using alkylating agents thus induces a selective pressure on the tumour cells to lose MMR-function. This might in turn lead to induction of a hypermutator phenotype in the remaining tumour cells. Such hypermutating cells might be highly resistant to targeted therapies, such as signalling pathway inhibitors or monoclonal antibodies, but also immunotherapeutic regimes (Chin et al., 2008). This hypothesis therefore calls for caution in combining such therapies with TMZ treatment.

NOVEL AND EXPERIMENTAL THERAPEUTIC REGIMES

Efforts to develop novel targeted therapies against the malignant gliomas have been impeded by the inherent heterogeneity and complexity of the molecular profile of these tumours. Methods to efficiently classify gliomas according to their key molecular events, and potentially also according to inherent resistance to radio- and chemotherapy, might therefore greatly facilitate the development of individualised therapeutic regimes for subgroups of the patients. This development will likely be aided by the progress of genome- and proteome-wide assays in simultaneously screening large numbers of proteins for tumour specific over-expression, and some approaches for assessing readily available cell-surface associated antigens have been developed (Diehn et al., 2000; Stitziel et al., 2004).

Small molecular inhibitors

Several small molecular tyrosine kinase inhibitors (TKI) targeting EGFR have been developed (Fig 3), and two of them evaluated in clinical trials. While some radiographic response was shown for both substances, no clear benefit in terms of survival could be recognised (Sathornsumetee et al., 2007). A monoclonal antibody targeting EGFR, cetuximab, has also shown encouraging results in animal studies (Eller et al., 2005; Yang et al., 2008) and is currently evaluated in an ongoing clinical trial (Combs et al., 2006). Inhibition of intracellular signalling cascades using small molecular inhibitors has also been attempted. Inhibition of the MAPK-pathway using RAS inhibitors has been tested in clinical trials, but showed moderate results (Cloughesy et al., 2006), and trials with RAF inhibitors are ongoing. The PI3K pathway has been targeted using mTOR inhibitors, and clinical trials showed moderate results with some radiographic improvement, but without any noticeable survival benefit (Chang et al., 2005; Galanis et al., 2005). Inhibitors of Akt have also been developed and trials are ongoing (Fig 3).

Overall, the results of the small molecular inhibitors in monotherapy trials have so far failed to show any clear improvement in patient outcome. The absence of success may partly be due to the existence of parallel signalling pathways circumventing an individual target. A recent concept proposing that alkylating chemotherapy might induce a hypermutating state in the tumours (discussed above) also raises concerns regarding combination therapy with alkylating agents and targeted therapies, since such mutations might circumvent the objects of targeted therapies.

VEGF and antiangiogenesis

Gliomas are highly vascularised tumours and neoangiogenic endothelial cell proliferation is one of the hallmarks of transition to high-malignant tumours. Angiogenesis has been indicated to be a vital and rate limiting process for tumour growth in animal models (Folkman, 2007). One of the important factors for angiogenesis is the vascular endothelial growth factor (VEGF). Several TKIs have been developed against the family of VEGF-receptors, expressed on tumour associated endothelial cells, but have so far shown modest effect in clinical trials

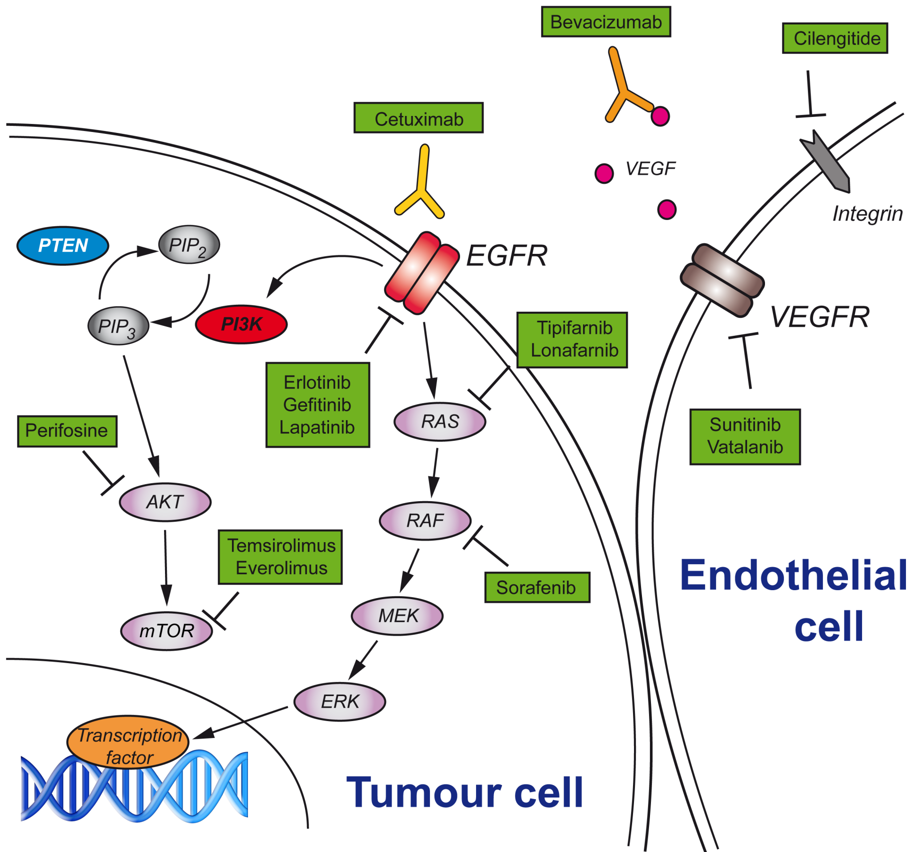

Figure 3. Examples of different targeted therapies investigated in gliomas. Different types of therapeutic compounds are exemplified by common members of the group (green boxes). Proliferation signalling in tumour cells can be blocked by monoclonal antibodies targeting EGFR, intracellular tyrosine kinase inhibitors (TKI), or inhibitors of the downstream signalling cascades. Tumour angiogenesis can be inhibited using monoclonal antibodies against the angiogenic factor VEGF, integrin inhibitors, or TKIs.

(Reardon et al., 2004). One of the most widely investigated of the novel targeted therapeutic agents is the VEGF-antibody bevacizumab (Fig 3). The use of anti-VEGF as monotherapy has also shown small efficacy. However, clinical trials combining bevacizumab and the topoisomerase inhibitor irinotecan have shown encouraging results in recurrent glioblastomas with radiographic response rates of 60-75%, and promising improvements in clinical outcome (Vredenburgh et al., 2007; Ali et al., 2008). One theory about the positive results of the combined therapy is paradoxically an increase in tumour perfusion. The dysfunctional structure characterising proliferating tumour microvessels might compromise the blood flow in the tumour and also increase the interstitial pressure due to vascular permeability. Antiangiogenic

therapy reduces the oedema and might actually normalise the blood flow to the tumour, and thereby increase the delivery of a cytotoxic agent (Pope et al., 2006; Vredenburgh et al., 2007; Kerbel, 2008). However, experimental results indicating that tumour cells might adopt a more invasive phenotype following antiangiogenic therapy (Rubenstein et al., 2000) might call for caution, and further investigations are warranted.

VEGF may also play an instrumental role in tumour immune escape. VEGF acts as a chemoattractant for immature dendritic cells (iDCs) and tumour associated macrophages (TAMs). The iDCs and TAMs are recruited to the tumour site, and can then be recirculated and exert an immunosuppressive function by inhibiting the activation of T-cells (Kim et al., 2006). It has further been shown that tumour-derived supernatant (TDSN) can inhibit interleukin (IL)-12 production and induce IL-10 in DCs in vitro, thus potentially directing the T-cell response towards Th2-type instead of cell mediated Th1 cytotoxicity. However, addition of blocking anti-VEGF antibodies to the supernatant can specifically reverse the inhibitory effect of TDSN on DC maturation (Johnson et al., 2007), and some initial clinical trials of boosting immunotherapy by adding monoclonal anti-VEGF antibodies have been made (Rini et al., 2006), although so far without conclusive results.

Alternative forms of antiangiogenic therapy have emerged with integrin inhibitors, which prevent tumour vessel formation by inhibiting the interaction between endothelial cells and extracellular matrix proteins, such as tenascin and fibronectin, in the tumour. This approach has shown promising results in animal models (Yamada et al., 2006) and is currently undergoing clinical trials.

Toxic chimeric proteins and tumour specific antibodies

An alternative promising strategy to specifically target tumour cells is the use of immunotoxic chimeric ligands or monoclonal antibodies. Immunotoxic antibodies are constructed by conjugating a drug or a toxin to an antibody directed specifically to an extracellular or cell-surface located tumour associated antigen (TAA). Immunotoxic antibodies carry the ability to specifically bind to and internalise in tumour cells, carrying the conjugated toxin into the cell, and thereby selectively killing the tumour cells and minimising collateral damage (Chari, 2008). Alternatively, the antibodies can be conjugated to a radionuclide instead of a toxin (Lambert, 2005; Sharkey and Goldenberg, 2006). Similarly, toxic chimeric ligands are recombinant proteins consisting of a ligand binding to an upregulated or specific receptor on tumour cells, fused with a toxin. The use of toxin-conjugated or radiolabelled antibodies has shown promising results in leukaemia, while the beneficial effect in solid tumours has so far been more modest (Sharkey and Goldenberg, 2006). One of the main concerns in applying these approaches to gliomas is that most TAAs are quantitatively rather than qualitatively altered, which might yield specificity difficulties.

However, a number of different toxic chimeric proteins have been developed against gliomas. Both TGF-α, IL-4 and IL-13 have been conjugated to the Pseudomonas exotoxin

(PE). TGF-α is natural ligand to EGFR and by substituting the native binding domain of PE for TGF-α, the toxin can be specifically targeted to EGFR expressing cells, avoiding unspecific binding. Convection enhanced delivery (see below) of this toxin to recurrent glioma patients has been shown to be safe and show some initial promising results (Sampson et al., 2003; Sampson et al., 2008b). Similarly, the receptors for IL-4 and IL-13 have been shown to be upregulated in gliomas and conjugated PE toxins have been constructed with both these cytokines. They have been shown to be reasonably well tolerated and some promising clinical results have been shown in recurrent glioblastoma patients for IL-13-PE. A phase III clinical study is currently ongoing (Shimamura et al., 2006).

Tenascin-c is an extracellular matrix glycoprotein differentially expressed during embryonic development. It is undetectable in the normal adult brain but is abundantly found in anaplastic astrocytomas and glioblastomas (Bourdon et al., 1983; Leins et al., 2003). Antibodies have been produced targeting alternatively spliced variants of the protein, which confers higher specificity and has made tenascin-c an interesting TAA. Since it is an extracellular matrix protein, anti-tenascin antibodies do not enter the tumour cells and are therefore not suitable for toxin conjugation, but could be efficiently used for delivery of radionuclides that does not rely on intracellular uptake to exert their effect. Injection of radiolabelled anti-tenascin antibodies into the surgical resection cavity of glioma patients has been studied in several clinical trials, and has shown moderate prolongation of survival (Zalutsky, 2005).

Immunotherapy

Due to the blood brain barrier (BBB) and the lack of lymphatic drainage, the brain was long considered to be an immune-privileged organ (Barker and Billingham, 1977). Although this is true to some extent, this view has been revised by later studies showing clear connections between the brain and peripheral immunity (Hickey, 1999; de Vos et al., 2002). The spread of tumour cells within the CNS, making complete surgical removal of the tumour impossible in combination with the delivery difficulties of many local therapies, has turned much interest to regimes actively seeking out tumour cells, e.g. immunotherapy. Gliomas are known to be highly immunosuppressive, and patients often show high levels of immunosuppressive factors such as IL-10, TGF-β, PGE_2 (Kuppner et al., 1989; Hishii et al., 1995) and VEGF (discussed above), T-cell anergy (Brooks et al., 1972) and a shift from Th1 to Th2 cytokine profile (Li et al., 2005).

There are a number of different immunotherapeutic approaches being investigated in glioma therapy. These are based either on the use of autologous tumour cells (Salford et al., 2002; Steiner et al., 2004; Fakhrai et al., 2006), or the use of dendritic cells, loaded with either antigens isolated from whole tumour cells (Yu et al., 2001; Yamanaka et al., 2005) or tumour specific peptides (Heimberger et al., 2006; Sampson et al., 2008b).

The active immunotherapy strategies aim to preferentially initiate a peripheral activation of the cellular adaptive immune response, or T-cell mediated cytotoxicity. After activation,

the T-cells undergo clonal expansion. It has been shown that while naïve T-cells rarely pass the BBB into the parenchyma, activated T-cells can efficiently extravasate into the brain and target remaining tumour cells.

Immunisation with autologous tumour cells with an immuno-stimulatory adjuvans has previously proven to be a feasible and promising method (Steiner et al., 2004; Fakhrai et al., 2006). In the ongoing Brain Immuno Gene Tumour Therapy (BRIGTT) study, patients are immunised using autologous tumour cells transfected with the human interferon-γ gene (Salford et al., 2002; Salford et al., 2005). Interferon-γ (IFN- γ) is one of the main mediators of the Th1-immune response and has an instrumental role in anti-tumour immune-reactivity. It upregulates the peptide presentation on MHC I (Früh and Yang, 1999) and is essential for the activation of cytotoxic T-cells (Janssen et al., 2003). Following resection, tumour cells are cultured *in vitro*. When the cells are regularly karyotyped malignant they are transfected with an adenoviral vector carrying the *IFN*-γ gene. The cells are then irradiated and injected intra-dermaly to the patient. The treatment is repeated every 3 weeks for a total of 8-10 immunisations. Preliminary data from this study shows that the treatment is safe for the patient, and that there is a statistically significant 6.7 months increase in survival in the treatment group.

Identification of novel tumour associated antigens

As discussed above, a promising antigen should be highly specific for the tumour cells to be suitable for targeted therapy, and in the case of toxin-conjugated antibodies also have the ability to internalise in the cells. A further dimension of suitability is also added by the importance of the TAA for the tumour phenotype. In the genome, mutations are often mentioned "driver" mutations if they are required for the phenotype of the tumour cell. However, many tumours also harbour lots of "bystander" or "passenger" mutations that are not essential for tumour proliferation or invasiveness, but have arisen due to the genetic instability in the tumours. Targeting a TAA corresponding to a driver mutation is likely to be more successful than targeting a bystander mutation, since TAAs corresponding to bystander mutations are much more easily lost under selective pressure.

So far, the number of identified and characterised TAAs in gliomas is limited. Due to the heterogeneity of the gliomas and their ability of immune editing, targeting one individual epitope carries a risk of failure, with recurrence of antigen negative tumours. This was illustrated in gliomas in an immunisation study targeting a mutated and constitutively active form of EGFR (EGFRvIII). Although this is thought to be a driver mutation important for tumour proliferation, recurrence of EGFRvIII negative tumours was found in treated patients (Sampson et al., 2008b). This emphasises the potency of immune escape, and the need to target multiple TAAs. This feature is likely to be important not only in immunisation regimes, but also in antibody therapy, and highlights the need for evaluation of a panel of different antibodies targeting the tumour cells. Identification and characterisation of additional TAAs is therefore an important topic if targeted toxins, monoclonal antibodies, or immunotherapy

using tumour specific peptides are going to be successful. It is an intense hope that further development in large-scale screening of the genome, transcriptome and proteome will assist in identifying new potential targets.

The Ku70/80 protein

The Ku70/80 protein complex is a heterodimer comprised of one 70 kDa (Ku70) and one 80 kDa (Ku80) subunit, which was first identified in the sera of autoimmune patients. It is abundantly present in the nuclei of most human cells (Mimori et al., 1981; Francoeur et al., 1986). The Ku70/80 complex binds to double-strand DNA-ends and is a regulatory subunit of the DNA-dependent protein kinase complex, thereby playing an instrumental role in the DNA double-strand break repair (Tuteja and Tuteja, 2000). It has also been shown to play an integral role in the G2/M-phase progression of the cell cycle (Munoz et al., 1998), and to contain an ATPase dependent helicase function (Tuteja and Tuteja, 2000). In addition to DNA maintenance, telomere maintenance, and cell cycle regulation, it has also been purposed to play a role in radioresistance (Chang et al., 2006).

The Ku-protein was first identified to be located on the cell surface of tumour cells in 1990 (Prabhakar et al., 1990), and since then it has been show to be present on the cell surface of a wide range of transformed cell lines. However, in untransformed cells, a cell surface associated localisation has been shown only on macrophages and some endothelial cells (Table 1). Both the Ku70 and Ku80 subunit have been shown to be present at the cell surface and play a role in cell-cell and cell-matrix interaction, and cell migration (Teoh et al., 1998; Lynch et al., 2001). Ku80 has further been shown to functionally interact with matrix metalloproteinase 9 on the cell surface, at least partly explaining the functional role in cell migration (Monferran et al., 2004; Muller et al., 2005).

Recently, a novel recombinant human antibody against Ku70/80 was isolated and shown to rapidly internalise upon binding of the surface antigen. By combining this antibody with a secondary toxin it was shown to exert a potential proliferation inhibiting effect on several tumour cell lines (Fransson and Borrebaeck, 2006). If this effect indeed can be verified to be

Table 1.	**Normal human or tumour cell lines with plasma membrane associated expression of Ku70/80**
Normal human cells	Monocyte derived macorphages (Monferran et al., 2004)
	Umbilical cord endothelial cells (Ginis et al., 1995)
Human tumour cells	Cervix carcinoma (HeLa) (Prabhakar et al., 1990)
	Pancreatic carcinoma (Fransson et al., 2006)
	Rhabdomyosarcoma (Ginis et al., 1995)
	Mammary carcinoma & Neuroblastoma (Ginis et al., 2000)

specific for transformed cells *in vivo*, Ku70/80 could be a novel promising TAA well suited for targeting with monoclonal antibodies.

Delivery of therapeutic compounds

One of the major challenges with targeted therapies is the delivery to the tumour (Muldoon et al., 2007). Therapeutic compounds can be administered either intravasculary or locally. Intravascular delivery requires that the substance can cross the BBB and be accumulated in the tumour. Although the BBB is not fully functional in tumour vessels, there is still a poor uptake of large macromolecules, partly due to the increased interstitial pressure. This also obstructs the diffusion necessary for the substances to reach sparsely vascularised areas of the tumour. Although debulking surgery can relieve interstitial pressure, the size of macromolecules is still a limiting factor for diffusion. In an investigation of radiolabelled anti-tenascin antibodies, Zalutsky et al. (1989 & 2005) showed that upon intravascular administration, only minor amounts of the antibody localised to the tumour, with intolerable toxicity to the liver and spleen when therapeutic doses were reached.

Local infusion in the resection cavity has been shown to be a feasible option in the use of radiolabelled antibodies. Although diffusion of the therapeutic agent is impeded, choosing a radioisotope with appropriate penetration properties in the tissue will guarantee radiation also to tumour cells in the proximity. Unlike the radioisotopes, toxins have to be internalised in the cell to exert its action, and this requires a more uniform distribution within the tumour and peritumoural area. However, once inside the cell, plant and bacteria derived toxins are highly potent, and for some substances one or a few molecules can be enough to kill the cell (Morokoff and Novak, 2004). Convection-enhanced delivery (CED) is the method of choice today for delivery of these substances. With CED, macromolecules are infused into the tumour or peritumoural area using a fluid pressure gradient, which produces a more homogenous regional distribution. However, several issues still remain to be solved to optimise this method and resolve interpatient variability (Muldoon et al., 2007; Sampson et al., 2008a)

Local therapy in itself also does not finally resolve the problem with GBMs, since it is a well-known fact that tumour cells are spread in large areas of the brain. However, with today's therapy, the vast majority of glioblastomas recur locally, making local control an important first step. The importance of local control is further emphasised by results showing that gross total resection to some extent correlates with prolonged survival (discussed above). Furthermore, efficient targeted therapy might also replace aggressive more destructive therapies such as surgery and radiation, and might finally also aid in targeting surgically inaccessible tumours.

An interesting developing field that might prove promising for specific targeting of tumour cells is the use of stem cells. It has been shown that neural stem/progenitor cells and mesenchymal stem cells possess tumour tropism in experimental models. Although the mechanisms for this tropism remain to be elucidated, these stem/progenitor cells have been

indicated to migrate to and infiltrate specifically in solid tumours. This has raised the hope that these cells could be used as delivery vehicles, e.g. for gene therapy or prodrug converting enzymes, specifically to tumour cells. The major advantage of such a system lies in the ability of the stem/progenitor cells to also migrate and trace infiltrating tumour microsatellites (Aboody et al., 2008).

METHODOLOGICAL CONSIDERATIONS

A wide range of important methodological considerations for development of successful research in glioma biology can be defined. Two key methodological issues (as defined by The Brain Tumour Progress Review Group, of the American National Institute of Health), which are especially central to this thesis work are:

• Tissue resource barriers: Existing tumour banks often do not have appropriate, relevant information, such as information on diagnosis, biological characteristics, natural history, and therapeutic outcome. The banks also often do not collect normal tissue or blood.

• Technological barriers: There is an inadequate application to brain tumour biology research of the latest technological advances, such as those in genomics and proteomics, structural biology, chemical biology, and high-throughput screening strategies.

Biological material

High quality clinical information accompanying the biological samples is of vast importance for proper evaluation of gene and protein expression patterns. All biological material used in this thesis work was retrieved from patients operated at the Department of Neurosurgery, Lund University Hospital, and all clinical parameters could be extracted directly from each patient's record, specifically for each study. The close collaboration with the clinical institution ascertains accurate and comprehensive clinical data.

Gene expression analyses are generally performed in three different types of material; primary tumour tissue, microdissected tumour tissue, or tumour cell lines. For the gene expression microarrays and 2D-DIGE proteomic analysis in this thesis, we have used frozen primary tissue samples. While this option is in most circumstances the most feasible, it is important to remember that it also carries some potential disadvantages. Use of primary tissue means that the cells investigated is not a pure tumour cell population. Adding to the cell population is also tumour stroma containing endothelial cells and pericytes from tumour vasculature, reactive astrocytes, and tumour infiltrating lymphocytes. These cells will add to the molecular profile of the tumour, and might either conceal, or induce false, differences between tumour and normal tissue samples. The use of cell lines, in turn, has shown to be a superior alternative for mutational analyses in different tumours (Winter et al., 2006). However, investigations of copy number alterations and gene expression in gliomas have shown cell lines to be poor representatives of *in vivo* gliomas (Li et al., 2008) in these assays. The use of laser microdissection of tissue has the advantages of both circumventing the problem with interfering cell types and attaining proper *in vivo* material, but yields only small amounts of extracted RNA. By steps of RNA amplification it is still possible to achieve sufficient amounts of starting material for gene expression microarrays, but there has been an ongoing debate considering the amount of bias introduced by the amplification procedure (Boelens et al., 2007). The amount of protein extracted from microdissected tissue have earlier been insufficient for proteomic screening methods, and only more recently are techniques emerging, capable of performing proteomic analysis with small amounts of starting material (von Eggeling et al., 2007).

Gene expression microarrays

There are multiple different platforms available for gene expression microarray analysis, and several different methods for protein separation and identification. However, the main focus of this thesis has been the analytical evaluation of the data, rather than the technical methodological issues. In particular, the central concern in the genome- and proteome-wide assays has been the biological analysis, and trying to integrate the results yielded into a glioma biology context. An extensive review of the technical aspects of the large-scale screening methods is therefore out of the scope of this thesis. A brief overview of the principles of the different platforms used is given below.

A cDNA microarray (Paper I) is a high-throughput technology used to simultaneously measure the expression of major parts of the genome. It consists of an arrayed series of specific cDNA sequences that correspond to different mRNAs, spotted onto a glass slide. The arrays used in our study contained approximately 27,000 spots, corresponding to approximately 17,000 unique genes. The mRNA was extracted from the samples to be analysed, and converted to cDNA by reverse transcriptase, and the cDNA was labelled with the Cy3-fluorophore. Simultaneously, a reference sample consisting of pooled mRNA from ten different tumour cell lines was labelled with the Cy5-fluorophore. The sample and the reference were mixed and hybridised to the spotted array under high-stringency conditions, through which each cDNA will hybridise specifically to the corresponding spot on the array. The relative abundance of nucleic acid in the sample compared to the reference was then quantified for each gene by individual scanning for the two fluorophores of all spots on the array. By hybridising all samples investigated together with the same reference the relative abundances between all the samples can be obtained.

Proteomics and antibody microarrays

In 2D-DIGE (Paper II), proteins are separated according to their isoelectric point (pI) and molecular weight. Protein extracts from the samples to be investigated and a reference consisting of pooled protein from all the samples in the study were labelled with different Cy-dyes. The sample and reference were mixed and added to a gel strip containing a pre-formed immobilised pH-gradient. Isoelectric focusing was performed by applying a voltage to the gel strip by which the proteins will migrate to the pH position where their net charge equals zero, i.e. the isoelectric point. The gel strip was then loaded onto a SDS-PAGE gel and the proteins were separated in the second dimension according to molecular weight. The two-dimensional spot pattern was then captured for sample and reference by individual laser scanning of the fluorophores. Approximately 2200 spots were matched between the different gels, and statistical discrimination of samples was performed based on the spot intensities. To identify the proteins in interesting spots, a loading gel with high protein concentration was run, and spots were matched to the main dataset. Selected spots ($n \approx 300$) were then harvested from the gel by a spot-picking robot. The proteins were extracted and analysed using tandem mass spectrometry. The MS/MS-produced peptide fingerprints were used to identify the protein by comparison to protein databases.

Some limitations inherent in the proteomic platform are important to consider in experimental design. The 2D-DIGE is a powerful tool for identifying abundant proteins, but has low sensitivity for highly diluted proteins. This can be an advantage when searching for biomarkers, where highly abundant proteins often are the most suitable, but a disadvantage when searching, e.g., for key tumourigenic proteins, such as transcription factors. The gel separation generally also means that proteins with a pI outside 3-10 or larger than 100 kDa are lost. Furthermore, hydrophobic proteins, such as membrane proteins, are lost in the gel separation.

The use of antibody-arrays is a novel technique for high throughput proteomic investigations. The basic principle is much the same as in gene expression microarrays, but instead of cDNAs, specific antibodies are spotted onto the glass slide. The proteins of patient plasma samples to be investigated were fluorophore-labelled and allowed to bind to the spotted antibodies. The arrays used contained 135 human recombinant single chain variable fragment (scFv) of antibodies directed against 60 different proteins. The arrays were laser scanned and the abundances of the individual proteins quantified by the intensity of the fluorescence for each spot. Although the antibody-arrays today only can investigate a limited number of analytes simultaneously (ranging up to a couple of hundred antibodies), the use of specific antibodies has a great advantage in being able to detect highly diluted proteins. Furthermore, by selecting the antibodies spotted onto the array, this provides the possibility of focusing the arrays for proteins with a specific biological function, e.g. cytokines or other immunoregulatory proteins (Wingren and Borrebaeck, 2008).

Statistical analysis of multivariate data - Unsupervised classification

Unsupervised classification of multidimensional data is often performed using clustering algorithms or any variant of multidimensional scaling algorithms. The standard hierarchical clustering of e.g. a gene expression microarray data set is a matrix, where each column represents one sample, and each row represents one gene. Each position in the matrix is coloured according to the expression intensity of that specific gene in that specific sample. Both samples and genes are then clustered in a dendrogram according to the similarity of the elements (sample or gene), so that the most similar elements are clustered as nearest neighbours. The length of the branches in the dendrogram tree represents the distance between the elements in multidimensional space.

A common mean of visualising high-dimensional data in two or three dimensional images is the use of multidimensional scaling algorithms. These algorithms visualise the samples in three dimensions while trying to preserve as much as possible of the distance between the samples in the original high dimensional space. In the analysis of our microarray data (Paper I) we used a Sammon map, which is very similar to multidimensional scaling but puts more emphasis on preserving the distances between close points.

Hierachical clusterings and Sammon maps are often presented using a filtered set of genes, e.g. with a p-value below some specified cut-off. It is important to note that such

clusterings are not by themselves any proof that this is the most prominent separating profile in the material as a whole, but rather an illustration of the discrimination of those particular samples using that particular set of genes. To generalise the discriminating capability, such a set of genes/proteins have to be verified in independent sets of samples not used for the original selection of the gene/protein set.

Statistical analysis of multivariate data - Supervised classifiers

Supervised classifiers are often trained and used to discriminate groups with different genetic or proteomic profiles in multivariate data. A common measurement of the performance of a classifier is to generate a receiver operating characteristic (ROC) curve, and calculate the area under the curve (AUC, or ROC-area). The discrimination is done pair-wise with two groups at a time, by using a leave-one-out cross validation where each sample is left out once and the classifier trained using the remaining samples. The left out sample is then assigned a numerical value which can be a log odds ratio of belonging to one of the two groups, or a signed distance to the hyperplane between the groups in multidimensional space. The samples are then ordered by their assigned numbers and the ROC-curve is generated by stepwise moving the cut-off between the groups one sample at the time, and plotting the sensitivity vs. 1-specificity curve. A perfect separation between the groups will thus yield an AUC of 1.0, while a total random order of samples yields an AUC of 0.5. The exact mathematics of the classifying algorithms is out of the scope of this thesis.

AIMS

The general aims of this thesis was to gain novel insights in the genetic and proteomic events underlying tumourigenesis and malignant progression in gliomas, and potentially define specific key events that could be object of refined or novel therapeutic approaches. More specific aims were:

- To investigate the potential of genome-wide and proteome-wide assays to discriminate and potentially identify novel subcategories of gliomas.

- To investigate the potential of genome-wide and proteome-wide assays to identify novel tumour specific genetic or proteomic aberrations. Moreover, to investigate the significance of any such aberrations from the perspective of tumour initiation and progression.

- To characterise expression pattern and therapeutic feasibility of specific tumour associated antigens.

- To investigate the immunologic response of patients undergoing immunotherapy against glioblastomas, and potentially identify key immunologic events to optimise this therapeutic regime.

RESULTS AND DISCUSSION

DISCRIMINATING TUMOURS BY GENE OR PROTEIN PROFILE (Paper I & II)

In Paper I we utilised 27k cDNA microarrays to investigate global gene expression profiles primarily in glioblastomas (n=26) and normal human brain (n=4), but also compared to a smaller number of grade II (n=5) and grade III (n=3) gliomas. After initial quality control and filtering, the data set contained approximately 11,500 informative reporters. We show that already before any further filtering of the data set, the global gene expression profiles separate the samples well according to histopathologic diagnosis. A trained supervised classifier was able to separate the samples with perfect or near perfect accuracy. The high-quality performance of the classifier was stable using anywhere between 30 to 1000 genes, above or below which the performance degraded.

The data presented in this paper emphasise the potential of global gene expression analysis for efficient diagnostic classification. In contrast to some earlier published similar studies, we were not able to find any significant correlation to survival (Nutt et al., 2003; Freije et al., 2004; Phillips et al., 2006). This is likely due to two different factors. First, our investigated material might be too small for identification of a survival related gene signature. Second, the investigated material was not selected for a survival analysis and a retrospective overview reveals a potential bias in the survival data since the material also fails to show the expected inverse relation between age and survival.

In Paper II we utilised 2D-DIGE with sequential LC-MS/MS protein identification for large-scale proteomic profiling. A set of glioma and normal brain tissue samples, largely consisting of the same samples analysed for gene expression profile in paper I, was analysed. Approximately 2 200 protein spots were matched between the gels. We found that the proteomic profile was also able to discriminate the samples according to histopathologic diagnosis, although with slightly lower accuracy than the gene expression microarrays (Table 2). This is not unexpected since the microarray data set contains a much larger number of data points.

ABERRATIONS OF CHROMOSOMAL REGIONS (Paper I)

A sliding window model was employed to search for larger chromosomal regions with coordinated up- or downregulation of gene expression. While this approach is often used in comparative genome hybridisations to search for regional amplifications or deletions in the DNA, employing the same method in gene expression data will also allow recognition of alterations due to epigenetic modifications, such as DNA-methylation or histone acetylation. A number of both well known and novel aberrations were identified. Upregulation of large parts of chromosome 7 and downregulation of large areas of chromosome 10 in all tumour

Table 2. **ROC-area (AUC) describing the performance of a supervised classifier based on gene- or protein expression analysis**

	GBM vs. Normal	GBM vs. Grade II	Grade II vs. Normal
Gene expression	1.00	0.95	0.94
Proteomics	0.85	0.94	0.91

groups, and downregulation of parts of chromosome 19q in low-grade tumours are well in line with the literature concerning amplifications and deletions in gliomas. However, overexpression of a smaller region on chromosome 6p constituted a novel and highly interesting finding. This region contains many of the MHC genes responsible for antigen presentation. Since primary tumour tissue was used for the analysis there is a small risk that this upregulation of immunoregulatory genes could reflect infiltration of leukocytes in the tissue. However, the concentration of tumour infiltrating leukocytes is generally not large enough in the proliferating tumour front to be expected to have a significant impact on the gene expression analysis. There is also a possibility that this upregulation originates from reactive astrocytes and microglia in the tumour area. If the overexpression originates from the actual tumour cells, an intriguing theory is provided by the recognition that some of the MHC molecules present within this region actually exert an immunosuppressive function, potentially playing a role in immune evasion (Friese et al., 2004; Varla-Leftherioti, 2004; Wischhusen et al., 2005).

DEREGULATION OF SPECIFIC GENES OR PROTEINS (Paper I & II)

One of the aims of the thesis was to identify tumour specific biomarkers, and potentially also suitable therapeutic targets. Upregulated genes in the tumours (Paper I) were therefore annotated for their cellular location and filtered for extracellular or plasma membrane associated proteins. This yielded a list of 31 significantly overexpressed extracellular proteins in glioblastomas, including both previously well-characterised antigens (e.g. tenascin-c) and novel interesting targets (e.g. PTPRZ1). Three of these proteins were available in a public immunohistochemistry database, and all confirmed our gene expression data.

Our result furthermore underscores the capability of this platform to identify promising TAAs for therapeutic targeting. The large number of identified reporters enables for bioinformatically focusing on proteins with a specific cellular function. A retrospective reanalysis of the data using software for signalling pathway analysis also revealed the Raf/MEK/Erk pathway as one of the top-ranked significantly altered pathways in glioblastomas, indicating the potential also for dissecting the tumourigenic mechanisms underlying tumour formation.

In the proteomic analysis (Paper II), 300 of the matched gel spots were picked for identification using LC-MS/MS. Due to several proteins being present in multiple spots, this yielded a list of approximately 150 unique proteins. A functional analysis of the proteins discriminating gliomas from normal brain revealed cytoskeleton and cell signalling proteins as the top diverging categories. These results are in line with previous proteomic investigations, which have repeatedly reported these categories as the main ontological groups aberrant in gliomas (Iwadate et al., 2004; Chumbalkar et al., 2005). The frequent identification of intermediate filaments and cytoskeleton associated proteins in this platform is no surprising result for two reasons. First, these are highly abundant proteins and therefore easily identified in these proteomic assays. Second, the highly invasive phenotype of gliomas predicts an increased migratory capacity, and would naturally account for aberrations in migration associated or regulatory proteins. A further manual functional analysis revealed altered expression of several proteins associated to the Raf/MEK/Erk pathway, also identified by the reanalysis of the gene expression data from Paper I. However, converting aberrations in ontological groups to specific cellular functions is most often not easily done.

Another intermediate filament protein, GFAP, comprised approximately 50 of the identified spots. This protein showed an overall divergent pattern when all tumours were considered, but when separated according to grade an interesting pattern emerged. We found several high molecular weight isoforms to be specifically upregulated in lower grade tumours, but not in glioblastomas. These data partly supports a theory proposed by Chumbalkar et al. (2005) that GFAP is overexpressed in lower grade tumour but undergoes a destabilisation with progressing tumour grade.

COMPARISON OF mRNA AND PROTEIN ABUNDANCE (Paper I & II)

By using the same biomaterial in both the gene expression analysis (Paper I) and the protein expression analysis (Paper II) we created a highly exclusive combined dataset. This allowed for a paired comparison of gene and protein expression in the same biological samples. We managed to match 216 pairs of mRNA-reporter and identified protein. Because of multiple reporters for some genes and multiple identified spots containing the same protein, the 216 pairs represented approximately 70 distinct proteins. For each matched pair, the Pearson correlation was calculated for the mRNA and protein abundances. For proteins strictly regulated by their mRNA expression, a significant positive correlation would be expected. Interestingly, out of the 216 pairs, only ten showed a positive Pearson correlation with a p-value < 0.05. Five of those ten pairs were different forms of superoxid dismutase 2, which indicates that this protein is tightly regulated by the mRNA abundance, but aside from that, remarkably few pairs were found to correlate. These results bring to light an overall very weak association between the levels of mRNA and protein abundance, which is also in line with two earlier similar comparisons in cancer cell lines (Juan et al., 2002; Chen et al., 2006). These results emphasise the difficulties of directly extrapolating differences in the proteome by investigations

of mRNA. The limited correlation might at first seem surprising, however, there are several processes individually controlling the levels of mRNA and protein. Both mRNA and protein are individually dictated by their own rates of synthesis and turnover, which are in turn controlled by, for example, gene-specific chromatin structure, and binding of accessory proteins. It has also been described that the transcriptome often shows burst-like expression with large temporal differences, and that models measuring expression at several time-points show better correlation than comparisons only considering a single point of time (Dechering, 2005).

The platforms should rather be considered as complementary to each other, since functional analysis of observed differences between samples often shows identification of aberrations within different functional categories. While global gene expression analyses are often considered to provide a more comprehensive overview, the identification of aberrations within distinct groups of proteins, for example, intermediate filament proteins, underscores the complementary value of proteomic large-scale investigations.

CHARACTERISATION OF THE TUMOUR ASSOCIATED ANTIGEN Ku70/80 (Paper III)

The use of radiolabelled or toxin conjugated antibodies for tumour specific therapy requires careful characterisation of potential tumour associated antigens. As discussed above, a potential target should be abundantly expressed on the cell surface of tumour cells, but not, or at least to a significantly lower degree, on normal cells. To be suitable for toxin delivery the antigen should also be able to internalise in the cell, while this is not necessarily obligatory for radiolabelled antibodies.

In Paper III we utilised immunohistochemistry and immunocytochemistry to investigate tissue distribution and cellular localisation of the potential TAA Ku70/80. The Ku70/80 protein complex is ubiquitously present in the nuclei of human cells, where it plays an instrumental role in DNA double strand break repair, but has been shown to be located also on the cell plasma membrane exclusively on tumour cells (discussed above). Analyses were performed using transmission light microscopy, epi-fluorescence microscopy, and confocal laser scanning microscopy. The use of several different parallel methods, and labelling using several antibodies against the same antigen, enabled a thorough evaluation of epitope specificity and performance of the different antibodies under different conditions.

We found that the in-house isolated human anti-Ku70/80 antibody INCA-X binds exclusively to plasma membrane associated Ku70/80 in gliomas. A strong immunoreactivity was observed throughout all tumours investigated (Fig 4). Apart from a very weak cytoplasmic reactivity in a few neurons, no reactivity could be observed in normal human brains. The localisation to the plasma membrane was verified in four out of six primary cell cultures from gliomas. Furthermore, we show indications of an active endosomal internalisation in living glioma cells by granular intracellular co-localisation of INCA-X and the endosome marker

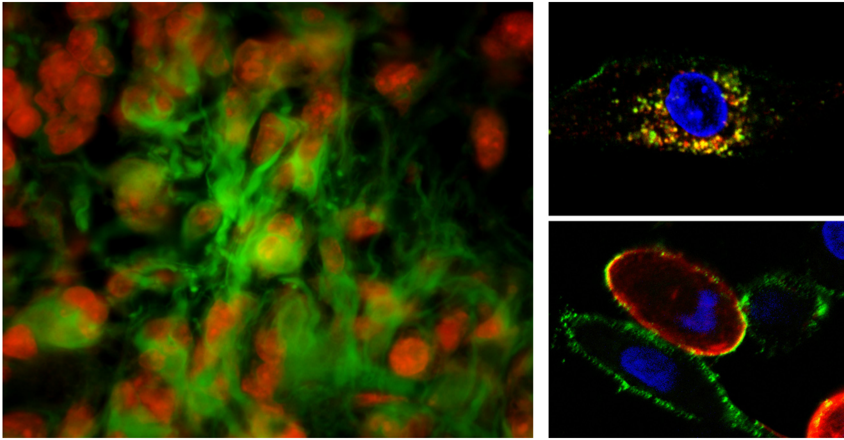

Figure 4. Expression and localisation of the Ku70/80 antigen in glioblastomas. Strong plasma membrane associated immunoreactivity for the Ku70/80 antibody INCA-X (green) was observed in all tumour tissue samples investigated using confocal microscopy (cell nuclei in red; *left*). The plasma membrane associated location of the antigen was verified in tumour cell cultures by co-labelling of the cytoskeleton protein f-actin (red) and cell nuclei (blue) (*bottom right*). Internalisation of the antigen-antibody complex was visualised by co-incubation of live tumour cells with INCA-X and the endosome marker transferrin (red), observed as granular intracellular co-labelling (*top right*).

transferrin (Fig 4). Finally, we show that all cell cultures positive for membrane associated Ku70/80 were sensitive to INCA-X mediated cytotoxicity in an immunotoxicity assay using a secondary toxin conjugated antibody.

The differences in sub-cellular distribution between antibodies targeting the same antigen likely reflect distinct protein epitopes locating to the separate cellular compartments. Optimally, an absorption test of the antibodies should be performed, but unfortunately it was not possible to acquire a full-length protein suitable for this. However, the original isolation of the INCA-X antibody provides solid evidence of the antigen being strongly related to Ku70/80 protein and dependent on the expression of the Ku70 gene. Taken together, these results points to Ku70/80 as a very favourable antigen for tumour specific targeting. The exclusivity of the extracellular localisation in tumours means that therapeutic doses could presumably be administered locally with minimal collateral damage. The potential for this antigen to serve as a glioma specific target should now be evaluated in an *in vivo* model.

INVESTIGATION OF PERIPHERAL IMMUNOLOGIC MARKERS AND RESPONSE TO IMMUNOTHERAPY AGAINST GLIOBLASTOMAS (Paper IV)

In the Brain Immuno Gene Tumour Therapy (BRIGTT) study, patients with glioblastomas are undergoing immunotherapy treatment by the use of autologous, *in vitro* cultured

tumour cells, transduced with the human *IFN*-γ gene. Transfected cells are injected intradermaly every third weak for a total of 8-10 treatments. Inclusion criteria for the study is i) age 50-70 years, ii) diagnosed GBM (WHO grade IV), iii) Karnofsky performance ≥70 and, iv) resection volume ≥80%. Patients fulfilling the inclusion criteria but with insufficient *in vitro* cell growth are used as a control group. The treatment has been shown to be well tolerated by the patients, and preliminary data reveals a 6.7 months prolongation of overall survival in treated patients compared to controls (17.4 vs. 10.7 months respectively). Analysis of the survival data reveals that all patients included benefits from the treatment, but that the positive effect seems to be larger in a subgroup of the patients.

We aimed to investigate if any immunological differences could be observed in plasma samples of patients with glioblastomas compared to healthy controls, in glioblastoma patients during ongoing immunisation treatment, and in the subgroup showing the best effect from treatment compared to the rest of the patients. To this end, plasma samples from immunised tumour patients (n=8), non-immunised tumour patients (n=10), and healthy controls (n=17) were analysed using antibody microarrays, containing 135 antibodies against 60 distinct immunoregulatory proteins. Preoperative samples were analysed from all tumour patients, and from the immunised patients also samples taken at immunisation no. 4 and no. 8.

We found only minor differences between normal controls and the group of tumour patients as a whole. The most prominent upregulated proteins in tumour patients were complement factor 5, VEGF, IL-8 and IL-12. While IL-12 is a Th1 cytokine important for a potential cytotoxic T-cell response, both IL-8 and VEGF are angiogenic factors (Machein and Plate, 2000; Brat et al., 2005), potentially important for neoangiogenesis. VEGF has also been shown to exert immunosuppressive actions in tumours, potentially aiding in tumour immune escape (Kim et al., 2006; Johnson et al., 2007).

When the tumours were subgrouped according to inclusion in the immunisation protocol, and according to long or short survival, a more refined pattern emerged. Interestingly, the immunised patients with long-term survival showed the most distinctly diverging protein profile, both when compared to normal controls and to the other tumour patient groups. This indicates that it is possible to predict which patients will respond best to immunotherapy already in the preoperative sample. Especially, the Th1 directing cytokines IFN-γ and IL-12 were upregulated in this patient group, which points to an ongoing immuneresponse even before immunotherapy initiation. It is therefore possible that the immunisation functions as a boost of this response, rather than inducing an immune reaction. Since all tumour patients in this study fulfilled the inclusion criteria for immunotherapy, and the only factor deciding if they were finally included was the *in vitro* cell growth, it is intriguing that there was also some separation of immunised and not immunised patients pre-operatively. This discrimination might hypothetically indicate some inherent difference influencing the *in vitro* growth capacity of the tumour cells. However, this discrimination was only seen in one of the non-immunised cohorts, and the material is too small to make any conclusions to what this difference represents.

Finally, we investigated whether any difference could be observed over time in patients undergoing immunotherapy. We found clear differences between the immunised long-term survivors (LTS) and short-term survivors (STS). While the LTS showed upregulation of a number of proteins at immunisation no. 4 compared to pre-operatively, no changes could be observed in the STS. Surprisingly, several of the upregulated proteins in LTS were Th2 related cytokines, such as IL-4, IL-10 and IL-13. Whether this represents a genuine Th2 response or a regulatory induction in response to a Th1 reaction is unknown. At immunisation no. 8 there was a decrease in STS of, among others, IFN-γ and IL-12. This might indicate a decreased Th1 reaction. After repeated immunisations, there is a not negligible risk of inducing tolerance instead of an immunereaction, and although speculatively, this could be one hypothesis for the decreasing levels of IFN-γ and IL-12 in STS. Little is so far known about these parameters, and they need to be considered in future optimisation immunotherapeutic regimes.

CONCLUSIONS AND FUTURE PERSPECTIVES

The current thesis has focused on characterising genomic and proteomic aberrations in glioblastomas in comparison to the normal human brain. The main perspectives have been i) identification of discrepancies that could be utilised for refinement of current therapeutic modalities, or development of novel therapeutic regimes, and ii) identification of subgroups of gliomas harbouring clinically relevant differences, based on their gene/protein expression profile.

We conclude that both gene expression and protein expression profiling can efficiently discriminate gliomas from normal brain, and discriminate the tumours according to histopathologic grade. The discriminating performance is somewhat higher in the gene expression microarrays, which can likely be referred to the superior number of reporters. No correlation to survival or identification of novel subgroups could be observed in this material. The molecular heterogeneity of gliomas makes different subgroups hard to discriminate. Future studies striving for identifications of such groups therefore likely need to include a larger number of biological samples to achieve significant results. In the case of gene expression analysis, this should optimally also be combined with sequencing data for mutational analysis.

The general concordance between mRNA and protein abundances as measured by high-throughput platforms is very weak. This can likely be related to individual rates of turnover, and potentially also differences in temporal expression pattern for the mRNAs and proteins. However, while the gene expression microarrays give a very comprehensive overview, proteomic analysis has the advantage of considering post-translational modifications. The platforms also often identify discrepancies within different functional groups of genes/proteins. The platforms should be considered complementary and future extensive investigations of both transcriptome and proteome are important for the understanding of glioma biology.

The Ras/Raf/MEK/Erk signalling pathway can be identified as one of the top significantly deregulated pathways in gene expression analysis, and several proteins associated to this pathway are also identified as deregulated in gliomas. Although few specific mutations in this pathway have been shown in gliomas, these results corroborate this signalling cascade as a central tumourigenic pathway. Furthermore, both the gene expression and the proteomic platforms identified a number of specific proteins, deregulated in gliomas. The implications of these proteins for targeted therapy remain to be evaluated.

The characterisation of the potential TAA Ku70/80 shows highly promising features regarding specific plasma membrane localisation in glioblastomas. Further, the internalising capability and capacity for toxin delivery in glioma cells make this antigen very interesting for targeted therapy using toxic monoclonal antibodies. These results strongly encourage further investigations in *in vivo* models.

Analysis of immunoregulatory proteins in peripheral plasma samples reveals profiles that are indicated to separate patients undergoing immunotherapy into long-term survivor and short-term survivor already pre-operatively. This indicates that there is an inherent difference between patients influencing the beneficial effect of immunisations. Furthermore, the analysis indicates differences in key immunological orchestrating proteins, or proteins associated to tumour progression, between tumour patients and normal controls (e.g. VEGF), and between tumour patients during ongoing immunotherapy (e.g. IFN-γ and IL-10). These differences should be validated in a larger material and by the use of separate methods. A highly interesting future perspective is to analyse immunoregulatory proteins in microdialysate from tumours, in order to directly monitor the local immunologic response.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tumörer i kroppens olika organ uppkommer genom att felaktigheter uppstår i regleringen av cellernas tillväxt och förmåga att infiltrera sin omgivning. Sådana felaktigheter uppstår genom mutationer i gener eller genom att antalet kopior av en gen i cellen förändras, s.k. amplifieringar eller deletioner. Därigenom uppkommer en ökad aktivitet av gener som driver tumörväxten, s.k. onkogener, eller en minskad aktivitet av gener som motverkar tumörutveckling, s.k. tumörsuppressor gener. Detta ger i sin tur upphov till en förändrad aktivitet av de proteiner som dessa gener kodar för.

Glioblastoma multiforme är en tumörtyp som uppstår i centrala nervsystemet, och är en av de mest aggressiva kända tumörformerna. Tumören växer invasivt in den omgivande vävnaden och är resistent både mot strålning och cytostatikabehandling. Detta gör att det idag inte finns någon bra behandling mot denna tumörtyp, och den medför därför en mycket dyster prognos för patienten. Det har även visat sig svårt att exakt kartlägga mekanismerna bakom hur glioblastom uppstår, då de visar en stor molekylär heterogenitet. Syftet med denna avhandling var att kartlägga förändringar i genuttryck och proteinuttryck i tumörer från patienter med glioblastom, samt att försöka identifiera specifika proteiner som skulle kunna användas för att rikta terapi selektivt mot tumörceller. Vidare har vi försökt urskilja mönster i gen- och proteinuttyck, som kan gruppera tumörer på ett mer exakt sätt med avseende på diagnos och optimal terapi, samt eventuellt även prediktera prognos eller nytta av en viss behandling.

Genom att analysera genuttrycket med s.k. microarray – en metod där stora delar av hela genomet kan analyseras simultant – har vi visat att en signatur av vissa gener kan användas för att identifiera och korrekt klassificera tumörpreparat. Dessa analyser visar även att större områden på vissa kromosomer har koordinerade förändringar av genuttryck som kan svara mot amplifieringar eller deletioner av dessa områden, alternativ var nedreglerade genom epigenetiska förändringar. Ett område på kromosom 6 där vi fann uppreglering av flera gener har inte tidigare visats innehålla förändringar i glioblastom. Slutligen har vi identifierat en mindre grupp gener som kodar för protein som skulle kunna var tänkbara att använda för att rikta terapi specifikt mot tumörceller.

En analys av ett stort antal proteiner i tumörerna visar likartad potential för klassificering av tumörerna som analyserna av genexpression. Proteinanalyserna visar även förändringar relaterade till några av de centrala signaleringsvägar som anses viktiga för tumörtillväxt. Vi fann dock att korrelationen mellan genuttrycket – mätt i form av mRNA – och proteinuttrycket var mycket svag. Detta visar på svårigheten att direkt översätta aktiviteten av en gen till aktiviteten av det motsvarande proteinet.

Vi har även genomfört en fördjupad studie av uttrycket och lokalisationen av ett proteinkomplex kallat Ku70/80 i glioblastom. Ku70/80 uttrycks i cellkärnan i nästan alla celler,

men har även visats lokalisera till cellytan specifikt på celler från ett antal tumörformer. Detta skulle göra Ku70/80 till ett möjligt mål för behandling av tumörceller med antikroppar. Då dessa inte kan binda till proteiner inne i celler skulle antikroppar mot Ku70/80 rikta sig specifikt mot tumörceller. Genom att märka in dels odlade tumörceller, dels vävnadssnitt från glioblastom och normal hjärna med fluorescerande antikroppar har vi visat att Ku70/80 finns på ytan även hos glioblastomceller, och att komplexet transporteras in i cellen när antikroppen binder till proteinet. Denna internalisering gör att antikroppar effektivt kan användas för att transportera in ett toxin i tumörcellerna och avdöda dessa. Genom undersökning av vävnadssnitt har vi även visat ett tydligt uttryck av Ku70/80 relaterat till cellytan på celler i glioblastomvävnad, medan detta uttryck saknas helt i normal hjärnvävnad. Detta visar på mycket goda förutsättningar för att antikroppar mot Ku70/80 skulle kunna användas för att leverera toxin till glioblastomceller utan att skada omgivande frisk vävnad.

Slutligen har vi undersökt förändringar i proteiner som styr immunförsvaret i blodprover från patienter som behandlas med immunterapi mot glioblastom. Genom att odla patientens utopererade tumörceller och tillföra dem genen för det immunstimulerande proteinet interferon-γ, och sedan injicera dessa celler i huden på patienten, hoppas man kunna aktivera immunförsvaret att slå ut kvarvarande tumörceller även i hjärnan. Våra resultat från analyser av uttrycket av ca 60 immunrelaterade proteiner indikerar att man redan innan behandlingen påbörjas kan prediktera hur väl patienten kommer att svara på immunterapi. Vi identifierar även ett antal proteiner som skiljer sig åt mellan de patienter som svarar bra, jämfört med dem som svarar mindre bra på behandling. En genomgripande analys av dessa skillnader kan förhoppningsvis ge fördjupad kunskap om de immunologiska mekanismerna vid denna typ av behandling, och bidra till att optimera behandlingen även för de patienter som hittills visat mindre bra effekt av denna.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who in different ways have helped and supported this work, or just made my time as a PhD-student a bit more enjoyable.

My supervisor, *Professor Leif G. Salford*, for his great enthusiasm and tremendous spirits, and for sharing his thorough knowledge and vast experience in neurosurgery and clinical neurooncology. Thank you for taking me in as a PhD-student and creating the possibilities for performing this work. Your attitude that everything should be possible has been an invaluable asset for this thesis work.

My second supervisor, *Professor Bengt Widegren*, for sharing his vast knowledge in tumour biology and immunology. Thank you for guiding me through the genetic "jungle" and for your aid in bridging statistics and tumour biology.

Assoc. Professor Bo Holmqvist, for introducing me to the art of immunofluorescence imaging. Thank you for being the "devils advocate" and for entertaining conversations concerning both science and trivialities during late evenings at the confocal microscope.

Assoc. Professor Morten Krogh, for all his help with the complex statistics in multivariate analysis.

Professor Åke Borg and colleagues at the Department of Oncology, *Professor Peter James* and colleagues at the Department of Protein Technology, and *Professor Carl Borrebaeck* and colleagues at the Department of Immunotechnology, for pleasant and productive collaborations.

Susanne Strömblad and *Catarina Blennow*, for all their help with cell culturing and tissue handling. Thank you for always trying to fix everything in spite of me often calling in the last minute.

Anna Ebbesson, for all the technical support in immunohistochemistry and immunocytochemistry.

Assoc. Professors Peter Siesjö, Gunnar Skagerberg and *Johan Bengzon*, for interesting and insightful discussions about both clinical and scientific aspects of neurosurgery and neurooncology.

All the neurosurgeons and the rest of the personnel at the Department of Neurosurgery for very pleasant collaboration during my clinical practice, and for all the help in collecting the biomaterial necessary for this thesis.

Assoc. Prof. Elisabeth Englund, for sharing her vast knowledge in neuropathology, and always trying to find the time to answer all my questions.

My office roommate and colleague PhD-student throughout the years of this work, *Daniel Bexell*, for numerous interesting and inspiring scientific an academic discussions, and for a very good sense of humour.

My second colleague PhD-student, *Henrietta Nittby*, for her high spirits and for many laughter's during well-needed coffee breaks at EA14.

The Mac-master, *Gunnar Gunnarson*, for always being ever so helpful and just as humoristic.

To *Anna D, Edward, Zhong-Tian* and *Hua*, for always being willing to help me out in lab, and to all the other people in the Rausing laboratory, *Hans-Olov, Xiaolong, Johan R, Anna R, Salina, Sara, Sofia, Anna E, Karin, Shorena, Nivedita, Seema, Man-Li, Dong-Feng* and everybody else, for making it a fantastic place to work.

To *Gunilla Moullin*, for supplying numerous necessary scientific articles.

* * *

To all my fantastic friends, old ones and less old ones, for many joyful times and wonderful experiences throughout the years of this work. All the fun moments have in several ways also contributed to the inspiration for this work.

To my fantastic parents, *Lars* and *Monica Persson*, for always believing in me. Thank you for always having given me the opportunity to do what I find most interesting, and for teaching me the two invaluable qualities of curiosity and persistency.

To *Kajsa Kanebratt*, for being the best sister one could whish for. Thank you for all your concern and for always showing interest, for contributing useful perspectives and for setting the academic standards high.

* * *

This work was supported by grants from The Hans and Märit Rausing Charitable Trust, from The Sten Lexner fund and The Lund University Hospital funds.

REFERENCES

Aboody KS, Najbauer J and Danks MK (2008) Stem and progenitor cell-mediated tumor selective gene therapy. Gene Ther 15:739-752.

Ali SA, McHayleh WM, Ahmad A, Sehgal R, Braffet M, Rahman M, Bejjani G and Friedland DM (2008) Bevacizumab and irinotecan therapy in glioblastoma multiforme: a series of 13 cases. J Neurosurg 109:268-272.

Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, Tang Y, DeFrances J, Stover E, Weissleder R, Rowitch DH, Louis DN and DePinho RA (2002) Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis, in: Cancer Cell, pp 269-277.

Balesaria S, Brock C, Bower M, Clark J, Nicholson SK, Lewis P, de Sanctis S, Evans H, Peterson D, Mendoza N, Glaser MG, Newlands ES and Fisher RA (1999) Loss of chromosome 10 is an independent prognostic factor in high-grade gliomas. Br J Cancer 81:1371-1377.

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD and Rich JN (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756-760.

Barker CF and Billingham RE (1977) Immunologically privileged sites. Adv Immunol 25:1-54.

Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, Aigner L, Brawanski A, Bogdahn U and Beier CP (2007) CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 67:4010-4015.

Bell E, Jr. and Karnosh LJ (1949) Cerebral hemispherectomy; report of a case 10 years after operation. J Neurosurg 6:285-293.

Bigner SH, Rasheed BK, Wiltshire R and McLendon RE (1999) Morphologic and molecular genetic aspects of oligodendroglial neoplasms. Neuro Oncol 1:52-60.

Boelens MC, te Meerman GJ, Gibcus JH, Blokzijl T, Boezen HM, Timens W, Postma DS, Groen HJ and van den Berg A (2007) Microarray amplification bias: loss of 30% differentially expressed genes due to long probe - poly(A)-tail distances. BMC Genomics 8:277.

Bonnet D and Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730-737.

Bourdon MA, Wikstrand CJ, Furthmayr H, Matthews TJ and Bigner DD (1983) Human glioma-mesenchymal extracellular matrix antigen defined by monoclonal antibody. Cancer Res 43:2796-2805.

Brat DJ, Bellail AC and Van Meir EG (2005) The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. Neuro Oncol 7:122-133.

Brooks WH, Netsky MG, Normansell DE and Horwitz DA (1972) Depressed cell-mediated immunity in patients with primary intracranial tumors. Characterization of a humoral immunosuppressive factor. J Exp Med 136:1631-1647.

Cairncross JG and Macdonald DR (1988) Successful chemotherapy for recurrent malignant oligodendroglioma. Ann Neurol 23:360-364.

Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, Silver JS, Stark PC, Macdonald DR, Ino Y, Ramsay DA and Louis DN (1998) Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J Natl Cancer Inst 90:1473-1479.

Castelli MG, Chiabrando C, Fanelli R, Martelli L, Butti G, Gaetani P and Paoletti P (1989) Prostaglandin and thromboxane synthesis by human intracranial tumors. Cancer Res 49:1505-1508.

CBTRUS (2008) Statistical Report: Primary Brain Tumors in the United States, 2000–2004. Central Brain Tumor Registry of the United States.

Chang HW, Kim SY, Yi SL, Son SH, Song do Y, Moon SY, Kim JH, Choi EK, Ahn SD, Shin SS, Lee KK and Lee SW (2006) Expression of Ku80 correlates with sensitivities to radiation in cancer cell lines of the head and neck. Oral Oncol 42:979-986.

Chang SM, Wen P, Cloughesy T, Greenberg H, Schiff D, Conrad C, Fink K, Robins HI, De Angelis L, Raizer J, Hess K, Aldape K, Lamborn KR, Kuhn J, Dancey J and Prados MD (2005) Phase II study of CCI-779 in patients with recurrent glioblastoma multiforme. Invest New Drugs 23:357-361.

Chari RV (2008) Targeted cancer therapy: conferring specificity to cytotoxic drugs. Acc Chem Res 41:98-107.

Chen YR, Juan HF, Huang HC, Huang HH, Lee YJ, Liao MY, Tseng CW, Lin LL, Chen JY, Wang MJ, Chen JH and Chen YJ (2006) Quantitative proteomic and genomic profiling reveals metastasis-related protein expression patterns in gastric cancer cells. J Proteome Res 5:2727-2742.

Chin L, Meyerson M, Aldape K, Bigner D, Mikkelsen T, Vandenberg S, Kahn A, Penny R, Ferguson M, Gerhard D, Getz G, Brennan C, Taylor B, Winckler W, Park P, Ladanyi M, Hoadley K, Verhaak R, Hayes D, Spellman P, Absher D, Weir B, Ding L, Lawrence M, Cibulskis K, Mardis E, Zhang J, Wilson R, Donehower L, Wheeler D, Purdom E, Wallis J, Laird P, Herman J, Weisenberger D, Baylin S, Schultz N, Yao J, Wiedemeyer R, Weinstein J, Sander C, Gibbs R, Gray J, Kucherlapati R, Lander E, Meyer R and Perou C (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061-1068.

Chin L, Pomerantz J and DePinho RA (1998) The INK4a/ARF tumor suppressor: one gene--two products--two pathways. Trends Biochem Sci 23:291-296.

Chumbalkar VC, Subhashini C, Dhople VM, Sundaram CS, Jagannadham MV, Kumar KN, Srinivas PN, Mythili R, Rao MK, Kulkarni MJ, Hegde S, Hegde AS, Samual C, Santosh V, Singh L and Sirdeshmukh R (2005) Differential protein expression in human gliomas and molecular insights. Proteomics 5:1167-1177.

Cloughesy T, Wen PY, Robins HI, Chang SM, Groves MD, Fink KL, Junck L, Schiff D, Abrey L, Gilbert MR, Lieberman F, Kuhn J, DeAngelis LM, Mehta M, Raizer JJ, Yung WK, Aldape K, Wright J, Lamborn KR and Prados MD (2006) Phase II trial of tipifarnib in patients with recurrent malignant glioma either receiving or not receiving enzyme-inducing antiepileptic drugs: a North American Brain Tumor Consortium Study, in: J Clin Oncol, pp 3651-3656.

Combs S, Heeger S, Haselmann R, Edler L, Debus J and Schulz-Ertner D (2006) Treatment of primary glioblastoma multiforme with cetuximab, radiotherapy and temozolomide (GERT)--phase I/II trial: study protocol, in: BMC Cancer, pp 133.

Daido S, Takao S, Tamiya T, Ono Y, Terada K, Ito S, Ouchida M, Date I, Ohmoto T and Shimizu K (2004) Loss of heterozygosity on chromosome 10q associated with malignancy and prognosis in astrocytic tumors, and discovery of novel loss regions. Oncol Rep 12:789-795.

de Vos AF, van Meurs M, Brok HP, Boven LA, Hintzen RQ, van der Valk P, Ravid R, Rensing S, Boon L, t Hart BA and Laman JD (2002) Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. J Immunol 169:5415-5423.

Dechering KJ (2005) The transcriptome's drugable frequenters. Drug Discov Today 10:857-864.

Dehghani F, Schachenmayr W, Laun A and Korf HW (1998) Prognostic implication of histopathological, immunohistochemical and clinical features of oligodendrogliomas: a study of 89 cases. Acta Neuropathol 95:493-504.

Diehn M, Eisen MB, Botstein D and Brown PO (2000) Large-scale identification of secreted and membrane-associated gene products using DNA microarrays. Nat Genet 25:58-62.

Drake CG, Jaffee E and Pardoll DM (2006) Mechanisms of immune evasion by tumors. Adv Immunol 90:51-81.
Dropcho EJ and Soong SJ (1996) The prognostic impact of prior low grade histology in patients with anaplastic gliomas: a case-control study. Neurology 47:684-690.
Ducray F, Idbaih A, de Reynies A, Bieche I, Thillet J, Mokhtari K, Lair S, Marie Y, Paris S, Vidaud M, Hoang-Xuan K, Delattre O, Delattre JY and Sanson M (2008) Anaplastic oligodendrogliomas with 1p19q codeletion have a proneural gene expression profile. Mol Cancer 7:41.
Dunham MA, Neumann AA, Fasching CL and Reddel RR (2000) Telomere maintenance by recombination in human cells. Nat Genet 26:447-450.
Eller JL, Longo SL, Kyle MM, Bassano D, Hicklin DJ and Canute GW (2005) Anti-epidermal growth factor receptor monoclonal antibody cetuximab augments radiation effects in glioblastoma multiforme in vitro and in vivo. Neurosurgery 56:155-162; discussion 162.
Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA and Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313-1317.
Etienne MC, Formento JL, Lebrun-Frenay C, Gioanni J, Chatel M, Paquis P, Bernard C, Courdi A, Bensadoun RJ, Pignol JP, Francoual M, Grellier P, Frenay M and Milano G (1998) Epidermal growth factor receptor and labeling index are independent prognostic factors in glial tumor outcome. Clin Cancer Res 4:2383-2390.
Fakhrai H, Mantil JC, Liu L, Nicholson GL, Murphy-Satter CS, Ruppert J and Shawler DL (2006) Phase I clinical trial of a TGF-Œ≤ antisense-modified tumor cell vaccine in patients with advanced glioma. Cancer gene therapy 13:1052-1052.
Folkman J (2007) Angiogenesis: an organizing principle for drug discovery?, in: Nature reviews Drug discovery, pp 273-286.
Forget MA, Desrosiers RR, Del M, Moumdjian R, Shedid D, Berthelet F and Beliveau R (2002) The expression of rho proteins decreases with human brain tumor progression: potential tumor markers. Clin Exp Metastasis 19:9-15.
Francoeur AM, Peebles CL, Gompper PT and Tan EM (1986) Identification of Ki (Ku, p70/p80) autoantigens and analysis of anti-Ki autoantibody reactivity. J Immunol 136:1648-1653.
Fransson J and Borrebaeck CA (2006) The nuclear DNA repair protein Ku70/80 is a tumor-associated antigen displaying rapid receptor mediated endocytosis. Int J Cancer 119:2492-2496.
Freije WA, Castro-Vargas FE, Fang Z, Horvath S, Cloughesy T, Liau LM, Mischel PS and Nelson SF (2004) Gene expression profiling of gliomas strongly predicts survival. Cancer Res 64:6503-6510.
Friese MA, Steinle A and Weller M (2004) The innate immune response in the central nervous system and its role in glioma immune surveillance. Onkologie 27:487-491.
Früh K and Yang Y (1999) Antigen presentation by MHC class I and its regulation by interferon gamma, in: Curr Opin Immunol, pp 76-81.
Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, DePinho RA and Cavenee WK (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 21:2683-2710.
Furuta M, Weil RJ, Vortmeyer AO, Huang S, Lei J, Huang TN, Lee YS, Bhowmick DA, Lubensky IA, Oldfield EH and Zhuang Z (2004) Protein patterns and proteins that identify subtypes of glioblastoma multiforme. Oncogene 23:6806-6814.
Galanis E, Buckner JC, Maurer MJ, Kreisberg JI, Ballman K, Boni J, Peralba JM, Jenkins RB, Dakhil SR, Morton RF, Jaeckle KA, Scheithauer BW, Dancey J, Hidalgo M, Walsh DJ and Group NCCT (2005) Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a North Central Cancer Treatment Group Study, in: J Clin Oncol, pp 5294-5304.

Gil-Benso R, Lopez-Gines C, Benito R, López-Guerrero JA, Callaghan RC, Pellín A, Roldán P and Cerdá-Nicolas M (2007) Concurrent EGFR amplification and TP-53 mutation in glioblastomas, in: Clin Neuropathol, pp 224-231.

Glass J, Hochberg FH, Gruber ML, Louis DN, Smith D and Rattner B (1992) The treatment of oligodendrogliomas and mixed oligodendroglioma-astrocytomas with PCV chemotherapy. J Neurosurg 76:741-745.

Godard S, Getz G, Delorenzi M, Farmer P, Kobayashi H, Desbaillets I, Nozaki M, Diserens AC, Hamou MF, Dietrich PY, Regli L, Janzer RC, Bucher P, Stupp R, de Tribolet N, Domany E and Hegi ME (2003) Classification of human astrocytic gliomas on the basis of gene expression: a correlated group of genes with angiogenic activity emerges as a strong predictor of subtypes. Cancer Res 63:6613-6625.

Guha A, Feldkamp MM, Lau N, Boss G and Pawson A (1997) Proliferation of human malignant astrocytomas is dependent on Ras activation. Oncogene 15:2755-2765.

Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA (1999) Creation of human tumour cells with defined genetic elements. Nature 400:464-468.

Hayashi Y, Ueki K, Waha A, Wiestler OD, Louis DN and von Deimling A (1997) Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme, in: Brain Pathol, pp 871-875.

Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC and Stupp R (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma, in: N Engl J Med, pp 997-1003.

Hegi ME, Liu L, Herman J, Stupp R, Wick W, Weller M, Mehta MP and Gilbert MR (2008) Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity, in: J Clin Oncol, pp 4189-4199.

Heimberger AB, Hlatky R, Suki D, Yang D, Weinberg J, Gilbert M, Sawaya R and Aldape K (2005) Prognostic effect of epidermal growth factor receptor and EGFRvIII in glioblastoma multiforme patients. Clin Cancer Res 11:1462-1466.

Heimberger AB, Hussain SF, Aldape K, Sawaya R, Archer GA, Friedman H, Reardon D, Friedman A, Bigner D and Sampson JH (2006) Tumor-specific peptide vaccination in newly-diagnosed patients with GBM. Journal of Clinical Oncology 24:2529.

Hickey WF (1999) Leukocyte traffic in the central nervous system: the participants and their roles. Semin Immunol 11:125-137.

Hishii M, Nitta T, Ishida H, Ebato M, Kurosu A, Yagita H, Sato K and Okumura K (1995) Human glioma-derived interleukin-10 inhibits antitumor immune responses in vitro. Neurosurgery 37:1160-1166; discussion 1166-1167.

Hoelzinger DB, Mariani L, Weis J, Woyke T, Berens TJ, McDonough WS, Sloan A, Coons SW and Berens ME (2005) Gene expression profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. Neoplasia 7:7-16.

Holland EC (2001) Brain tumor animal models: importance and progress. Curr Opin Oncol 13:143-147.

Hope KJ, Jin L and Dick JE (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat Immunol 5:738-743.

Huang CH, Mandelker D, Schmidt-Kittler O, Samuels Y, Velculescu VE, Kinzler KW, Vogelstein B, Gabelli SB and Amzel LM (2007) The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. Science 318:1744-1748.

Iwadate Y, Sakaida T, Hiwasa T, Nagai Y, Ishikura H, Takiguchi M and Yamaura A (2004) Molecular classification and survival prediction in human gliomas based on proteome analysis. Cancer Res 64:2496-2501.

Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG and Schoenberger SP (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes, in: Nature, pp 852-856.

Johnson BF, Clay TM, Hobeika AC, Lyerly HK and Morse MA (2007) Vascular endothelial growth factor and immunosuppression in cancer: current knowledge and potential for new therapy. Expert Opin Biol Ther 7:449-460.

Juan HF, Lin JY, Chang WH, Wu CY, Pan TL, Tseng MJ, Khoo KH and Chen ST (2002) Biomic study of human myeloid leukemia cells differentiation to macrophages using DNA array, proteomic, and bioinformatic analytical methods. Electrophoresis 23:2490-2504.

Kaye AH and Laws ER (1995) Brain Tumors. Churchill Livingstone, London.

Kerbel RS (2008) Tumor angiogenesis. N Engl J Med 358:2039-2049.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015.

Kim R, Emi M, Tanabe K and Arihiro K (2006) Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res 66:5527-5536.

Kleihues P and Cavenee W (2000) Tumours of the Nervous System. IARC Press, Lyon.

Kleihues P and Ohgaki H (1999) Primary and secondary glioblastomas: from concept to clinical diagnosis. Neuro Oncol 1:44-51.

Kraus JA, Glesmann N, Beck M, Krex D, Klockgether T, Schackert G and Schlegel U (2000) Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. J Neurooncol 48:89-94.

Kraus JA, Koopmann J, Kaskel P, Maintz D, Brandner S, Schramm J, Louis DN, Wiestler OD and von Deimling A (1995) Shared allelic losses on chromosomes 1p and 19q suggest a common origin of oligodendroglioma and oligoastrocytoma. J Neuropathol Exp Neurol 54:91-95.

Kristiansen K, Hagen S, Kollevold T, Torvik A, Holme I, Nesbakken R, Hatlevoll R, Lindgren M, Brun A, Lindgren S, Notter G, Andersen AP and Elgen K (1981) Combined modality therapy of operated astrocytomas grade III and IV. Confirmation of the value of postoperative irradiation and lack of potentiation of bleomycin on survival time: a prospective multicenter trial of the Scandinavian Glioblastoma Study Group, in: Cancer, pp 649-652.

Krivtsov AV, Twomey D, Feng Z, Stubbs MC, Wang Y, Faber J, Levine JE, Wang J, Hahn WC, Gilliland DG, Golub TR and Armstrong SA (2006) Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature 442:818-822.

Kros JM (2007) From expert opinion to evidence-based: changes in the gold standard of primary brain tumour diagnosis. J Pathol 213:1-3.

Kuan CT, Wikstrand CJ and Bigner DD (2001) EGF mutant receptor vIII as a molecular target in cancer therapy. Endocr Relat Cancer 8:83-96.

Kuppner MC, Hamou MF, Sawamura Y, Bodmer S and de Tribolet N (1989) Inhibition of lymphocyte function by glioblastoma-derived transforming growth factor beta 2. J Neurosurg 71:211-217.

Laiho M, DeCaprio JA, Ludlow JW, Livingston DM and Massague J (1990) Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. Cell 62:175-185.

Lambert JM (2005) Drug-conjugated monoclonal antibodies for the treatment of cancer. Curr Opin Pharmacol 5:543-549.

Leins A, Riva P, Lindstedt R, Davidoff MS, Mehraein P and Weis S (2003) Expression of tenascin-C in various human brain tumors and its relevance for survival in patients with astrocytoma. Cancer 98:2430-2439.

Li A, Walling J, Kotliarov Y, Center A, Steed ME, Ahn SJ, Rosenblum M, Mikkelsen T, Zenklusen JC and Fine HA (2008) Genomic Changes and Gene Expression Profiles Reveal That Established Glioma Cell Lines Are Poorly Representative of Primary Human Gliomas. Mol Cancer Res.

Li G, Hu YS, Li XG, Zhang QL, Wang DH and Gong SF (2005) Expression and switching of TH1/TH2 type cytokines gene in human gliomas. Chin Med Sci J 20:268-272.

Lonardi S, Tosoni A and Brandes AA (2005) Adjuvant chemotherapy in the treatment of high grade gliomas. Cancer Treat Rev 31:79-89.

Lynch EM, Moreland RB, Ginis I, Perrine SP and Faller DV (2001) Hypoxia-activated ligand HAL-1/13 is lupus autoantigen Ku80 and mediates lymphoid cell adhesion in vitro. Am J Physiol Cell Physiol 280:C897-911.

Machein MR and Plate KH (2000) VEGF in brain tumors. J Neurooncol 50:109-120.

Mahesparan R, Read TA, Lund-Johansen M, Skaftnesmo KO, Bjerkvig R and Engebraaten O (2003) Expression of extracellular matrix components in a highly infiltrative in vivo glioma model. Acta Neuropathol 105:49-57.

Marko NF, Toms SA, Barnett GH and Weil R (2008) Genomic expression patterns distinguish long-term from short-term glioblastoma survivors: a preliminary feasibility study. Genomics 91:395-406.

Matsukado Y, Maccarty CS and Kernohan JW (1961) The growth of glioblastoma multiforme (astrocytomas, grades 3 and 4) in neurosurgical practice. J Neurosurg 18:636-644.

McDonald JM, See SJ, Tremont IW, Colman H, Gilbert MR, Groves M, Burger PC, Louis DN, Giannini C, Fuller G, Passe S, Blair H, Jenkins RB, Yang H, Ledoux A, Aaron J, Tipnis U, Zhang W, Hess K and Aldape K (2005) The prognostic impact of histology and 1p/19q status in anaplastic oligodendroglial tumors. Cancer 104:1468-1477.

Mimori T, Akizuki M, Yamagata H, Inada S, Yoshida S and Homma M (1981) Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap. J Clin Invest 68:611-620.

Mischel PS, Cloughesy TF and Nelson SF (2004) DNA-microarray analysis of brain cancer: molecular classification for therapy. Nat Rev Neurosci 5:782-792.

Monferran S, Paupert J, Dauvillier S, Salles B and Muller C (2004) The membrane form of the DNA repair protein Ku interacts at the cell surface with metalloproteinase 9. Embo J 23:3758-3768.

Morokoff AP and Novak U (2004) Targeted therapy for malignant gliomas. J Clin Neurosci 11:807-818.

Muldoon LL, Soussain C, Jahnke K, Johanson C, Siegal T, Smith QR, Hall WA, Hynynen K, Senter PD, Peereboom DM and Neuwelt EA (2007) Chemotherapy delivery issues in central nervous system malignancy: a reality check. J Clin Oncol 25:2295-2305.

Muller C, Paupert J, Monferran S and Salles B (2005) The double life of the Ku protein: facing the DNA breaks and the extracellular environment. Cell Cycle 4:438-441.

Munoz P, Zdzienicka MZ, Blanchard JM and Piette J (1998) Hypersensitivity of Ku-deficient cells toward the DNA topoisomerase II inhibitor ICRF-193 suggests a novel role for Ku antigen during the G2 and M phases of the cell cycle. Mol Cell Biol 18:5797-5808.

Newcomb EW, Cohen H, Lee SR, Bhalla SK, Bloom J, Hayes RL and Miller DC (1998) Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. Brain Pathol 8:655-667.

Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, Pohl U, Hartmann C, McLaughlin ME, Batchelor TT, Black PM, von Deimling A, Pomeroy SL, Golub TR and Louis DN (2003) Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. Cancer Res 63:1602-1607.

Ohgaki H (2005) Genetic pathways to glioblastomas. Neuropathology 25:1-7.

Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schüler D, Probst-Hensch NM, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lütolf UM and Kleihues P (2004) Genetic pathways to glioblastoma: a population-based study, in: Cancer Research, pp 6892-6899.

Ohgaki H and Kleihues P (2007) Genetic pathways to primary and secondary glioblastoma, in: Am J Pathol, pp 1445-1453.

Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, Mclendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE and Kinzler KW (2008) An integrated genomic analysis of human glioblastoma multiforme, in: Science, pp 1807-1812.

Pedersen MW, Meltorn M, Damstrup L and Poulsen HS (2001) The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Ann Oncol 12:745-760.

Petalidis LP, Oulas A, Backlund M, Wayland MT, Liu L, Plant K, Happerfield L, Freeman TC, Poirazi P and Collins VP (2008) Improved grading and survival prediction of human astrocytic brain tumors by artificial neural network analysis of gene expression microarray data. Mol Cancer Ther 7:1013-1024.

Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro JM, Colman H, Soroceanu L, Williams PM, Modrusan Z, Feuerstein BG and Aldape K (2006) Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 9:157-173.

Pope WB, Lai A, Nghiemphu P, Mischel P and Cloughesy TF (2006) MRI in patients with high-grade gliomas treated with bevacizumab and chemotherapy. Neurology 66:1258-1260.

Prabhakar BS, Allaway GP, Srinivasappa J and Notkins AL (1990) Cell surface expression of the 70-kD component of Ku, a DNA-binding nuclear autoantigen. J Clin Invest 86:1301-1305.

Reardon D, Friedman H, Yung WKA, Brada M, Conrad C, Provenzale J, Jackson E, Serajuddin H, B. C and Lauren D (2004) A phase I/II trial of PTK787/ZK 222584 (PTK/ZK), a novel, oral angiogenesis inhibitor, in combination with either temozolomide or lomustine for patients with recurrent glioblastoma multiforme (GBM). Journal of Clinical Oncology 22:1513.

Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S and Hanada M (1996) BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 60:23-32.

Reya T, Morrison SJ, Clarke MF and Weissman IL (2001) Stem cells, cancer, and cancer stem cells, in: Nature, pp 105-111.

Reynolds BA and Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710.

Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BK, Dobra A, Dressman HK, Bigner DD, Nevins JR and West M (2005) Gene expression profiling and genetic markers in glioblastoma survival, in: Cancer Research, pp 4051-4058.

Rickman DS, Bobek MP, Misek DE, Kuick R, Blaivas M, Kurnit DM, Taylor J and Hanash SM (2001) Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. Cancer Res 61:6885-6891.

Rini BI, Weinberg V, Fong L, Conry S, Hershberg RM and Small EJ (2006) Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of prostate cancer after definitive local therapy. Cancer 107:67-74.

Rubenstein JL, Kim J, Ozawa T, Zhang M, Westphal M, Deen DF and Shuman MA (2000) Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption, in: NEO, pp 306-314.

Salford L, Ask E, Siesjo P and al. E (2005) Immunization with autologous glioma cells transfected with IFN-g gene significantly prolongs survival in GBM-patients older than 50 years. Neuro-Oncology 7:370.

Salford LG, Siesjo P, Skagerberg G, Persson BRR, Larsson E-M, Lindvall M, Visse E and Widegren B (2002) Search for effective therapy against glioblastoma multiforme - clinical immunisation with autologous glioma cells transduced with the human interferon-gamma gene. International Congress Series 1247:211-220.

Sampson JH, Akabani G, Archer GE, Berger MS, Coleman RE, Friedman AH, Friedman HS, Greer K, Herndon JE, 2nd, Kunwar S, McLendon RE, Paolino A, Petry NA, Provenzale JM, Reardon DA, Wong TZ, Zalutsky MR, Pastan I and Bigner DD (2008a) Intracerebral infusion of an EGFR-targeted toxin in recurrent malignant brain tumors. Neuro Oncol 10:320-329.

Sampson JH, Akabani G, Archer GE, Bigner DD, Berger MS, Friedman AH, Friedman HS, Herndon JE, 2nd, Kunwar S, Marcus S, McLendon RE, Paolino A, Penne K, Provenzale J, Quinn J, Reardon DA, Rich J, Stenzel T, Tourt-Uhlig S, Wikstrand C, Wong T, Williams R, Yuan F, Zalutsky MR and Pastan I (2003) Progress report of a Phase I study of the intracerebral microinfusion of a recombinant chimeric protein composed of transforming growth factor (TGF)-alpha and a mutated form of the Pseudomonas exotoxin termed PE-38 (TP-38) for the treatment of malignant brain tumors. J Neurooncol 65:27-35.

Sampson JH, Archer GE, Mitchell DA, Heimberger AB and Bigner DD (2008b) Tumor-specific immunotherapy targeting the EGFRvIII mutation in patients with malignant glioma. Semin Immunol.

Sanai N and Berger MS (2008) Glioma extent of resection and its impact on patient outcome, in: Neurosurgery, pp 753-764; discussion 264-756.

Sathornsumetee S, Reardon DA, Desjardins A, Quinn JA, Vredenburgh JJ and Rich JN (2007) Molecularly targeted therapy for malignant glioma. Cancer 110:13-24.

Schwartz SA, Weil R, Thompson RC, Shyr Y, Moore JH, Toms SA, Johnson MD and Caprioli RM (2005) Proteomic-based prognosis of brain tumor patients using direct-tissue matrix-assisted laser desorption ionization mass spectrometry, in: Cancer Research, pp 7674-7681.

Seo J and Lee KJ (2004) Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J Biochem Mol Biol 37:35-44.

Shapiro S, Mealey J, Jr. and Sartorius C (1989) Radiation-induced intracranial malignant gliomas. J Neurosurg 71:77-82.

Sharkey RM and Goldenberg DM (2006) Targeted therapy of cancer: new prospects for antibodies and immunoconjugates. CA Cancer J Clin 56:226-243.

Sherr CJ and McCormick F (2002) The RB and p53 pathways in cancer. Cancer Cell 2:103-112.

Shimamura T, Husain SR and Puri RK (2006) The IL-4 and IL-13 pseudomonas exotoxins: new hope for brain tumor therapy. Neurosurg Focus 20:E11.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J and Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63:5821-5828.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB (2004) Identification of human brain tumour initiating cells. Nature 432:396-401.

Steiner HH, Bonsanto MM, Beckhove P, Brysch M, Geletneky K, Ahmadi R, Schuele-Freyer R, Kremer P, Ranaie G, Matejic D, Bauer H, Kiessling M, Kunze S, Schirrmacher V and Herold-Mende C (2004) Antitumor vaccination of patients with glioblastoma multiforme: a pilot study to assess feasibility, safety, and clinical benefit. J Clin Oncol 22:4272-4281.

Stewart BW and Kleihues P (2003) World Cancer Report. IARC Press, Lyon.

Stiles CD and Rowitch DH (2008) Glioma stem cells: a midterm exam. Neuron 58:832-846.

Stitziel NO, Mar BG, Liang J and Westbrook CA (2004) Membrane-associated and secreted genes in breast cancer. Cancer Res 64:8682-8687.

Stummer W, Reulen HJ, Meinel T, Pichlmeier U, Schumacher W, Tonn JC, Rohde V, Oppel F, Turowski B, Woiciechowsky C, Franz K, Pietsch T and Group A-GS (2008) Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias, in: Neurosurgery, pp 564-576; discussion 564-576.

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987-996.

Takuwa N and Takuwa Y (2001) Regulation of cell cycle molecules by the Ras effector system. Mol Cell Endocrinol 177:25-33.

Teoh G, Urashima M, Greenfield EA, Nguyen KA, Lee JF, Chauhan D, Ogata A, Treon SP and Anderson KC (1998) The 86-kD subunit of Ku autoantigen mediates homotypic and heterotypic adhesion of multiple myeloma cells. J Clin Invest 101:1379-1388.

Thomas DA and Massague J (2005) TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 8:369-380.

Tootle TL and Rebay I (2005) Post-translational modifications influence transcription factor activity: a view from the ETS superfamily. Bioessays 27:285-298.

Trog D, Fountoulakis M, Friedlein A and Golubnitschaja O (2006) Is current therapy of malignant gliomas beneficial for patients? Proteomics evidence of shifts in glioma cells expression patterns under clinically relevant treatment conditions. Proteomics 6:2924-2930.

Tso CL, Freije WA, Day A, Chen Z, Merriman B, Perlina A, Lee Y, Dia EQ, Yoshimoto K, Mischel PS, Liau LM, Cloughesy TF and Nelson SF (2006) Distinct transcription profiles of primary and secondary glioblastoma subgroups. Cancer Res 66:159-167.

Tuteja R and Tuteja N (2000) Ku autoantigen: a multifunctional DNA-binding protein. Crit Rev Biochem Mol Biol 35:1-33.

Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A and Louis DN (1996) CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Res 56:150-153.

Walker MD, Alexander E, Jr., Hunt WE, MacCarty CS, Mahaley MS, Jr., Mealey J, Jr., Norrell HA, Owens G, Ransohoff J, Wilson CB, Gehan EA and Strike TA (1978) Evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. A cooperative clinical trial. J Neurosurg 49:333-343.

Varla-Leftherioti M (2004) Role of a KIR/HLA-C allorecognition system in pregnancy. J Reprod Immunol 62:19-27.

Watanabe K, Tachibana O, Sata K, Yonekawa Y, Kleihues P and Ohgaki H (1996) Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas, in: Brain Pathol, pp 217-223; discussion 223-214.

Vescovi AL, Galli R and Reynolds BA (2006) Brain tumour stem cells. Nat Rev Cancer 6:425-436.

Wessels PH, Twijnstra A, Kessels AG, Krijne-Kubat B, Theunissen PH, Ummelen MI, Ramaekers FC and Hopman AH (2002) Gain of chromosome 7, as detected by in situ hybridization, strongly correlates with shorter survival in astrocytoma grade 2. Genes Chromosomes Cancer 33:279-284.

Wingren C and Borrebaeck CA (2008) Antibody microarray analysis of directly labelled complex proteomes. Curr Opin Biotechnol 19:55-61.

Winter JM, Brody JR and Kern SE (2006) Multiple-criterion evaluation of reported mutations: a proposed scoring system for the intragenic somatic mutation literature, in: Cancer Biol Ther, pp 360-370.

Wischhusen J, Friese MA, Mittelbronn M, Meyermann R and Weller M (2005) HLA-E protects glioma cells from NKG2D-mediated immune responses in vitro: implications for immune escape in vivo. J Neuropathol Exp Neurol 64:523-528.

Vivanco I and Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489-501.

von Eggeling F, Melle C and Ernst G (2007) Microdissecting the proteome. Proteomics 7:2729-2737.

Vousden KH and Lu X (2002) Live or let die: the cell's response to p53. Nat Rev Cancer 2:594-604.

Vredenburgh JJ, Desjardins A, Herndon JE, 2nd, Dowell JM, Reardon DA, Quinn JA, Rich JN, Sathornsumetee S, Gururangan S, Wagner M, Bigner DD, Friedman AH and Friedman HS (2007) Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. Clin Cancer Res 13:1253-1259.

Xiao A, Yin C, Yang C, Di Cristofano A, Pandolfi PP and Van Dyke T (2005) Somatic induction of Pten loss in a preclinical astrocytoma model reveals major roles in disease progression and avenues for target discovery and validation. Cancer Res 65:5172-5180.

Yamada S, Bu XY, Khankaldyyan V, Gonzales-Gomez I, McComb JG and Laug WE (2006) Effect of the angiogenesis inhibitor Cilengitide (EMD 121974) on glioblastoma growth in nude mice. Neurosurgery 59:1304-1312; discussion 1312.

Yamanaka R, Homma J, Yajima N, Tsuchiya N, Sano M, Kobayashi T, Yoshida S, Abe T, Narita M, Takahashi M and Tanaka R (2005) Clinical evaluation of dendritic cell vaccination for patients with recurrent glioma: results of a clinical phase I/II trial, in: Clin Cancer Res, pp 4160-4167.

Yang W, Wu G, Barth RF, Swindall MR, Bandyopadhyaya AK, Tjarks W, Tordoff K, Moeschberger M, Sferra TJ, Binns PJ, Riley KJ, Ciesielski MJ, Fenstermaker RA and Wikstrand CJ (2008) Molecular targeting and treatment of composite EGFR and EGFRvIII-positive gliomas using boronated monoclonal antibodies. Clin Cancer Res 14:883-891.

Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, Yong WH, Incardona F, Thompson RC, Riedinger MS, Zhang W, Prins RM and Black KL (2001) Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration, in: Cancer Research, pp 842-847.

Zalutsky MR (2005) Current status of therapy of solid tumors: brain tumor therapy. J Nucl Med 46 Suppl 1:151S-156S.

Zalutsky MR, Moseley RP, Coakham HB, Coleman RE and Bigner DD (1989) Pharmacokinetics and tumor localization of 131I-labeled anti-tenascin monoclonal antibody 81C6 in patients with gliomas and other intracranial malignancies. Cancer Res 49:2807-2813.

Zheng H, Ying H, Yan H, Kimmelman AC, Hiller DJ, Chen AJ, Perry SR, Tonon G, Chu GC, Ding Z, Stommel JM, Dunn KL, Wiedemeyer R, You MJ, Brennan C, Wang YA, Ligon KL, Wong WH, Chin L and Depinho RA (2008) p53 and Pten control neural and glioma stem/progenitor cell renewal and differentiation. Nature 455:1129-1133.