HELENA E. MIETTINEN

Evaluation of Prognostic Factors in Glial Tumors

University of Tampere
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ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi,
Lenkkeilijänkatu 6, Tampere, on September 16th, 2000, at 12 o'clock.

University of Tampere
Tampere 2000

To my family

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-IV.

I Miettinen H, Kononen J, Haapasalo H, Helen P, Sallinen P, Harjuntausta T, Helin H, Alho H. Expression of peripheral-type benzodiazepine receptor and diazepam binding inhibitor in human astrocytomas: relationship to cell proliferation. Cancer Res 1995; 55:2691-5.

II Miettinen H, Kononen J, Sallinen P, Alho H, Helen P, Helin H, Kalimo H, Paljarvi L, Isola J, Haapasalo H. CDKN2/p16 predicts survival in oligodendrogliomas: comparison with astrocytomas. J Neurooncol 1999; 41: 205-11.

III Miettinen H.E, Paunu N, Rantala I, Kalimo H, Paljärvi L, Helin H, Haapasalo H. Cell cycle regulators (p53, p21 and pRb) in oligodendrocytic tumors: A study by novel tumor microarray technique. Submitted.

IV Miettinen H.E, Järvinen T.A.H, Kauraniemi P, Kellner U, Parwaresch R, Rantala I, Kalimo H, Paljärvi L, Isola J, Haapasalo H. High Topoisomerase IIα expression associates with High Proliferation Rate, p53 and Retinoblastoma status and poor prognosis in oligodendrogliomas. Neuropathology & Applied Neurobiology, in press.

ABBREVIATIONS

BCNU	bischloroethyl-nitrosourea
BrdU	bromodeoxyuridine
cAMP	cyclin adenosine monophosphate
CCNU	chloroethylcyclohesyl nitrosurea
CDK	cyclin-dependent kinase
CNS	central nervous system
Cpm	counts per minute
CT	computed tomography
DAB	diaminobenzidine
dATP	deoxyadenosinetriphosphate
DBI	diazepam binding inhibitor
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GFAP	glial fibrillary acidic protein
Ki-67/MIB-1	MIB-1 antibody directed against the Ki-67 antigen
LI	labelling index
LOH	loss of heterozygozity
MMAC1	mutated in multiple advanced cancers
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NF	neurofibromatosis
O2A	oligodendrocyte-Type 2 astrocyte
PET	positron emission tomography
PBR	peripheral-type benzodiazepine receptors
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCV	procarbazine, lomustine (CCNU) and vincristine sulfate
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
ROC	receiver operating characteristics
SPF	s-phase fraction
T1A	type 1 astrocyte
TEP-1	TGF-β-regulated and epithelial cell-enriched phosphatase
TGF	tumor growth factor
topoIIα	topoisomerase IIα
WHO	the World Health Organization
wt	wild-type

ABSTRACT

Gliomas, are the most common type of intracranial tumors. Diffuse astrocytomas are the most frequent gliomas followed by oligodendrogliomas. Oligoastrocytomas, consisting of a mixture of oligodendroglioma and astrocytoma cells, are the most common type of mixed gliomas. The classification of gliomas is difficult and controversial especially in case of oligodendrocytic tumors. It has been suggested that oligodendrogliomas are underdiagnosed and that they may actually represent 25-33% of all glial tumors in contrast to the reported 6%. The exact histopathological diagnosis is of crucial importance because glioma types respond differently to treatments. Oligodendrogliomas are among the most chemosensitive solid malignancies whereas most astrocytic tumors are resistant to chemotherapy.

This study focused on finding new potential molecular biological markers to help prognostication, differential diagnosis and selection of treatment. The amount of peripheral benzodiazepine receptor (PBR) immunopositivity associated significantly with histological grade and proliferation rate of the studied astrocytic tumors. Diazepam binding inhibitor (DBI) was observed to localize in the same cells with PBR. Intense PBR expression predicted poor survival but however, did not reach significance in multivariate analysis.

CDKN2/p16 immunoreactivity decreased with increasing malignancy grade in both oligodendrocytic and astrocytic tumors. Lack of CDKN2/p16 immunoreactivity was closely associated with poor patient survival in both astrocytoma and oligodendroglioma patients. The expression of tumor supressors p53 and p21 increased with malignancy in oligodendrocytic tumors. The amount of pRb expression was found have significant effect on cell proliferation rate, whereas p21 immunopositivity had independent prognostic value in multivariate analysis suggesting use for p21 immunostaining in routine neuropathology.

Topoisomerase IIα immunoreactivity increased with proliferation and malignancy grade in oligodendrocytic tumors. Multivariate analysis showed that topoIIα had an independent prognostic value for patient survival high expression predicting poor outcome. TopoIIα, being a target for multiple cytotoxic drugs, may offer a tool for selecting therapy for patients with oligodendrocytic tumors. These two qualities make it a potential tool for clinical use.

INTRODUCTION

Neoplasms arising from glial tissue are the most common type of intracranial tumors. Diffuse astrocytomas are the most common gliomas accounting for approximately 30% of all primary brain tumors (Central Brain Tumor Registry Of The United States 1996). Oligodendrogliomas are the second largest group of adult gliomas comprising 6 % of them and 3% of all primary intracranial tumors (Central Brain Tumor Registry of The United States 1996). Oligoastrocytomas, consisting of a mixture of oligodendroglioma and astrocytoma cells, are the most frequent type of mixed gliomas. The classification of gliomas is difficult and controversial. It has been suggested that oligodendrogliomas are underdiagnosed and that they may actually represent 25-33% of all glial tumors (rev by Fortin *et al*. 1999). A correct histopathological diagnosis is of crucial importance because glioma types respond differently to treatments and the patient prognosis varies by subtypes (figure 1). Abnormal proliferation and inability of cells to die result from accumulation of genetic aberrations, which are often typical of a specific malignancy. Better understanding of biology and pathogenesis provides means for predicting tumor behaviour and affecting it. Recognizable molecular biological differences between different glioma groups and subgroups would be most useful in aiding the demanding work of a neurosurgeon, pathologists and oncologists.

In order to pass on their genetic material the cells must divide. Prior to this the cells must replicate their DNA and segregate their chromosomes into two identical sets. The described events are finely regulated in cell cycle consisting of two fundamental parts. Interphase occupies the majority of cell cycle and mitosis, which lasts about 30 minutes. Continuous processes occur throughout the interphase and are referred to collectively as growth. Stepwise processes occur once per cell cycle. DNA synthesis and chromosome replication are restricted to S phase. S phase occurs in the middle of interphase preceded by a gap called G1 and followed by a gap called G2. The replicated chromosomes divide into two daughter cells during mitosis.

The percentages of cells in different phases in a cell population gives an idea about the proliferative activity. Identified cell cycle regulators and their amounts may provide even more precise information about the cell cycle regulation and possible malfunction. All this information may be of use in clinical practise predicting tumor growth and aggressiveness.

In addition to surgery, affecting cell cycle or its regulation and dysfunction is the only way to affect tumor growth. New targets for more effective therapies and methods are investigated intensively. In this thesis, basic cell cycle regulation and its malfunction in human gliomas were studied in order to find new prognostic factors and possible targets for therapy.

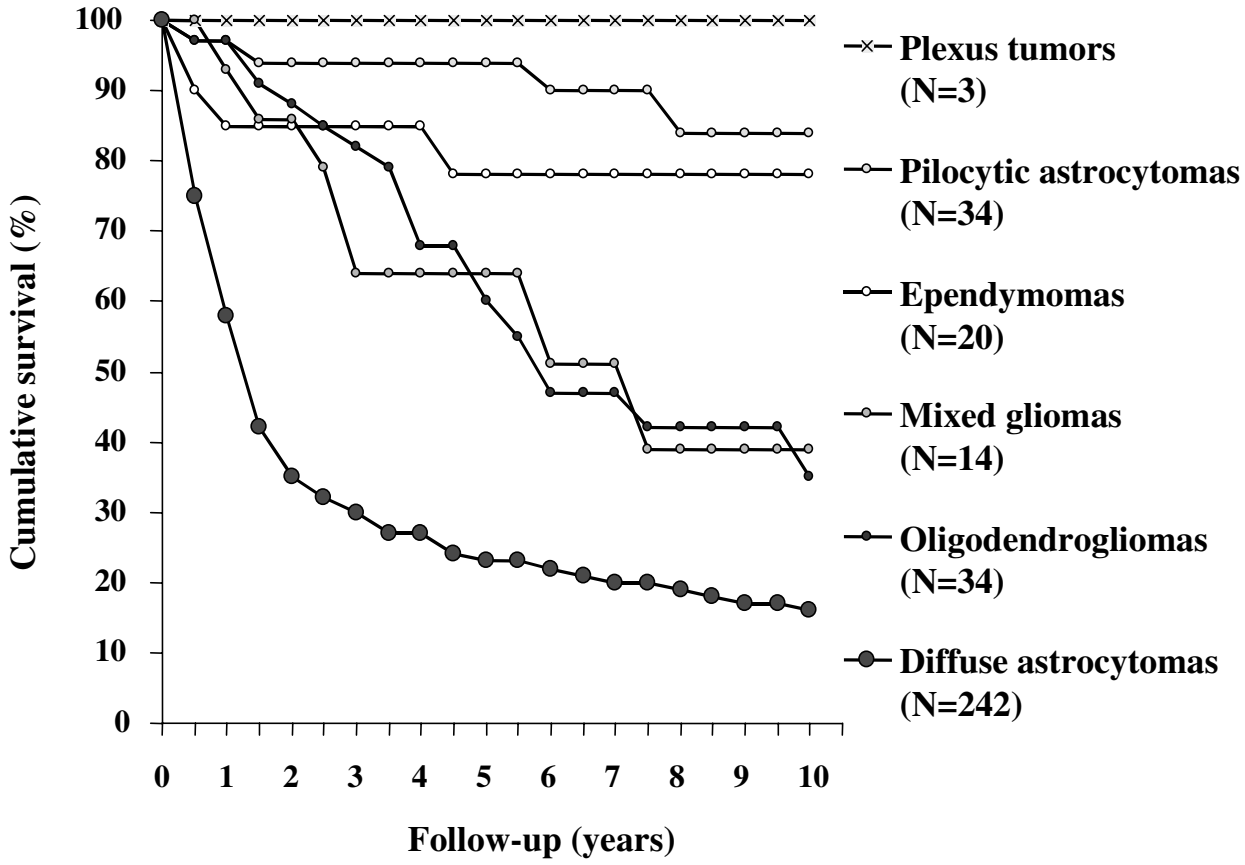


Figure 1. The glioma patients operated at Tampere University Hospital between 1983-1994 and their survival by diagnosis ($p<0.0001$). The group of diffuse astrocytomas includes grades II-IV.

REVIEW OF THE LITERATURE

1. Glial cells

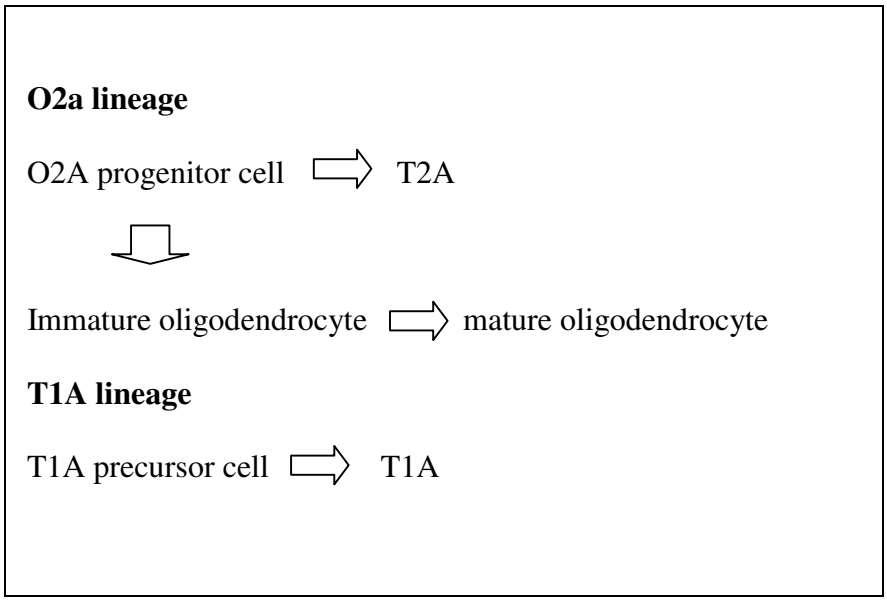
All CNS cells arise originally from neuroectoderm and thus all different glial cells as well as neurons must share a common origin at some point in development.
Oligodendrocyte differentiation involves three physiological stages (Raible *et al*. 1989). The first stage involves exiting from the cell cycle, which occurs in an immature oligodendrocyte (Raff *et al*. 1983a, Raff *et al*.1985). The second stage is the synthesis of myelin components, and the third stage is the actual assembly of myelin and the maintenance of extended cell membranes. Cells in culture develop only to the second physiological stage because structural myelin is not produced in the absence of neurons (Raible and McMorris 1989). As oligodendrocytes progress through the stages of differentiation, they go from expressing the embryonic form of the neural adhesion molecule (oligodendrocyte-Type 2 astrocyte, O2A progenitor cell) to expressing predominantly the adult neural cell adhesion molecule (immature oligodendrocyte). As they develop to oligodendroblasts, they express exclusively the adult form of the neural cell adhesion molecule (mature oligodendrocyte) (rev by Linskey and Gilbert 1995).

Type 1 astrocyte (T1A) cells are formed directly from T1A precursor cells. Both O2A progenitor cells and immature oligodendrocytes can be induced to differentiate into Type 2 astrocyte (T2A), but mature oligodendrocytes cannot. The differentiation of T2As from O2A progenitor cells seems to be an induced developmental pathway requiring an environmental stimulus to occur (rev by Linskey and Gilbert 1995).

Morphologically, T1As appear fibroblast-like whereas T2As appear neuron-like in cell cultures. Increased cyclin adenosine monophosphate (cAMP) can cause T1As to change shape and look like T2As without affecting other characteristics that distinguish the two cell types. T1As are stimulated to divide by extracts of bovine pituitary and epidermal growth factor (EGF) whereas T2As are not (Raff *et al.* 1983b). Biochemically T2As exhibit γ-aminobutyric acid accumulation via high affinity transport system, have excitatory amino acid receptors (e.g., glutamate) coupled to amino acid neurotransmitter release, and synthesize chondroitin sulfate, whereas T1As do not . T1As and T2As also differ in the type of ion channels present in their cell membranes (rev by Linskey and Gilbert 1995).

As far as the function of the cells of these two lineages is concerned, both oligodendrocytes and T2As provide myelin insulation for axons. The T2As are mostly located in white matter tracts which may contribute to the structure of nodes of Ranvier and which have high-affinity neurotransmitter uptake mechanisms. These observations suggest that O2A lineage cells may be specialized for functional role in axonal conduction whereas T1As assume a more structural role in the CNS (rev by Linskey and Gilbert 1995).

Figure 2. All glial cells develop from one of the two lineages (rev by Linskey and Gilbert 1995).



2. Histology of oligodendrocytic and astrocytic gliomas

The histological typing of gliomas by the WHO (2000) names five different glioma types; astrocytic tumors, oligodendroglial tumors, ependymal tumors, mixed gliomas and choroid plexus tumors (table 1). Each group has variants and grades of their own but the features determining malignancy, e.g, nuclear atypia, mitotic activity, cellularity, vascular proliferation and necrosis, are mainly common to all groups. Grading is based on the areas showing the highest degree of anaplasia because the most anaplastic and genetically abnormal cells most likely determine the course of disease (WHO Classification of Tumours 2000).

2.1 Oligodendroglial tumors

2.1.1 Oligodendroglioma (WHO grade II)

Oligodendroglioma is a diffusely growing, moderately cellular tumor composed predominantly of neoplastic oligodendrocytes. A typical honey-comb appearance featuring clear cytoplasm around central spherical nuclei is seen in routinely formalin-fixed and paraffin embedded material but not in frozen sections. This phenomenon aiding diagnosis is due to acute swelling of cells during fixation process. Mitotic activity in oligodendrogliomas is generally low. Microcalcifications and branching capillaries resembling chicken-wire are typical hallmarks. Glial fibrillary acidic protein (GFAP) positive microgemistocytes may be present. GFAP immunoreactivity can be seen also in typical oligodendroglial tumor cells which have been designated as gliofibrillary oligodendrocytes.

Biological behaviour: Oligodendrogliomas generally recur locally. The period of neurological symptoms preceding oligodendroglioma diagnosis is usually longer than in case of astrocytic tumors. Malignant progression is not uncommon but it is less frequent than in diffuse astrocytomas. The five year survival is 57.8% (Central Brain Tumor Registry Of The United States 1996).

2.1.2 Anaplastic oligodendroglioma (WHO grade III)

Anaplastic oligodendrogliomas are cellular gliomas with the same hallmarks as oligodendrogliomas but with increased focal or diffuse anaplasia. The majority of tumor cells show morphological features of oligodendroglial cells but occasional tumors demonstrate marked cellular pleomorphism with multinucleated giant cells. Mitotic activity is present. Vascular proliferation and necroses are possible. Gliofibrillary oligodendrocytes and minigemistocytic elements are frequent.

Biological behaviour: These neoplasms may progress to a highly anaplastic tumor that may be morphologically indistinguishable from glioblastoma multiforme (grade IV). Most patients die of local recurrence. Occasional patients may develop

metastases. A rare complication is spread to leptomeninges. The reported survival rate for five years is 32.7% (Central Brain Tumor Registry Of The United States 1996).

2.2 Mixed Gliomas

2.2.1 Oligoastrocytoma (WHO grade II)

A tumor consisting of a mixture of neoplastic oligodendrocytes and astrocytes, either diffusely intermingled or separated into distinct areas. Cellularity is moderate and mitotic activity is low. Microcalcifications as well as microcystic degeneration may be present. Necrosis and vascular proliferation are absent. GFAP and vimentin positivity vary.

Biological behaviour: Slowly growing, although due to incomplete surgical resection tumors may reoccur. The reported survival rate for five years is 53.4% (Central Brain Tumor Registry Of The United States 1996).

2.2.2 Anaplastic oligoastrocytoma (WHO grade III)

A mixed diffusely infiltrating tumor showing signs of anaplasia, e.g. increased cellularity, nuclear atypia and high mitotic activity. Vascular proliferation and focal necrosis may be present. In most tumors the signs of anaplasia are evident in both components but there are individual tumors in which the anaplasia is restricted to only one of the components.

Biological behaviour: Strong growth potential. The reported survival rate for five years is 36% (Shaw *et al.* 1994).

2.3 Astrocytic tumors

2.3.1 Pilocytic astrocytoma (WHO grade I)

A circumscribed astrocytoma composed, at least in part, of bipolar fusiform cells with dense fibrillation. Microcystic formation apparently coalescing to larger cysts is often seen. GFAP immunoreaction is always positive at least to some degree. Eosinophilic, club-shaped structures called Rosenthal fibers and eosinophilic intracytoplasmic protein droplets called granular bodies are histological hallmarks of this tumor type. The criteria for pilocytic astrocytoma do not allow significant mitotic activity but endothelial proliferation is possible. Calcification may also be seen but is usually inconspicuous. Local invasion of the subarachnoid space can be seen and may be accompanied by desmoplastic leptomeningeal reaction but it is not indicative of malignancy.

***Biological behaviour*:** This tumor grows slowly and malignant transformation occurs very rarely. The five year survival is 85.7% (Central Brain Tumor Registry Of The United States 1996).

2.3.2 Astrocytoma (WHO grade II)

Cell density is low to moderate. Astrocytic nuclei are hyperchromatic, oval to irregular and may show occasional mitoses. Phenotypically, neoplastic astrocytes may vary considerably with respect to size, prominence and disposition of cell processes, and the abundance of cytoplasmic glial filaments. Microcyst formation is seen occasionally. Diffusely infiltrating astrocytomas (grades II-IV) tend to infiltrate the surrounding brain diffusely and pre-existing cell types, e.g. neurons, are often entrapped. GFAP immunostaining is scant.

***Biological behaviour*:** These well differentiated diffusely infiltrating astrocytomas grow slowly. However, due to incomplete surgical resection, they almost always recur. There is an inherent tendency for progression to anaplastic astrocytoma, and eventually, to glioblastoma. The reported five year survival rate is 46.8 % (Central Brain Tumor Registry Of The United States 1996).

2.3.3 Anaplastic astrocytoma (WHO grade III)

Diffusely infiltrating astrocytoma with focal or dispersed anaplasia, e.g. increased cellularity, pleomorphism, nuclear atypia. Displays mitotic activity. Necrotic areas are absent. Immunoreactivity for GFAP is not consistent in all tumor cells but the expression of S-100 protein, vimentin and αB-crystallin is.

***Biological behaviour*:** Anaplastic astrocytomas show inherent and often rapid tendency to progress, ultimately transitioning to glioblastomas. The five year survival rate years is 32.7% (Central Brain Tumor Registry Of The United States 1996).

2.3.4 Glioblastoma (WHO grade IV)

Highly cellular astrocytic tumor consisting of poorly differentiated pleomorphic neoplastic astrocytes and occasional multinucleated giant cells. Areas of vascular proliferation and/or necrosis are essential for diagnosis. Pseudopalisating around areas of tumor necrosis is often present. Mitotic activity and occurrence of atypical mitoses as well as expression of GFAP vary considerably.

Biological behaviour: Highly malignant diffusely infiltrating glioma. The reported survival rate for five years is 3% (Central Brain Tumor Registry Of The United States 1996).

Table 1. WHO classification (2000) of gliomas.

Astrocytic tumors
Diffuse astrocytomas
- Fibrillary astrocytoma
- Protoplasmic astrocytoma
- Gemistocytic astrocytoma

Anaplastic astrocytoma
Glioblastoma
- Giant cell glioblastoma
- Gliosarcoma

Pilocytic astrocytoma
Pleomorphic xanthoastrocytoma
Subependymal giant cell astrocytoma

Oligodendroglial tumors
Oligodendroglioma
Anaplastic oligodendroglioma

Mixed gliomas
Oligoastrocytoma
Anaplastic oligoastrocytoma

Ependymal tumors
Ependymoma
- Cellular
- Papillary
- Clear cell

Tanycytic
Anaplastic ependymoma
Myxopapillary ependymoma
Subependymoma

Choroid plexux tumors
Choroid plexus papilloma
Choroid plexus carcinoma

Glial tumors of uncertain origin
Astroblastoma
Gliomatosis cerebri
Chordoid glioma of the 3rd ventricle

3. Tumorigenesis

3.1 Molecular genetics

3.1.1 Oligodendrogliomas

The most frequent genetic alteration in oligodendroglial tumors as determined by loss of heterozygozity (LOH) analysis, is LOH on the on the long arm of chromosome 19. The incidence of LOH on 19q varies from 50% to more than 80% of studied tumors (Bello *et al*. 1995, Kraus *et al*. 1995, Reifenberger *et al*. 1994a, von Deimling *et al*. 1992a). The oligodendroglioma-associated tumor suppressor gene suspected on 19q remains unidentified. The second most common genetic alteration in oligodendroglial neoplasms is LOH on the short arm of chromosome 1. The reported incidences of LOH on 1p range from 40% up to 92% (Reifenberger *et al*. 1994a, Kraus *et al*. 1995, Bello *et al*. 1994). Recent studies suggest that several tumor suppressor genes from 1p may be involved in the pathogenesis of oligodendrogliomas, one of them being CDKN2C (p18 ^{INK4C}) at 1p32 (Husemann *et al*. 1999, Pohl *et al*. 1999). Almost all oligodendrogliomas, which have lost alleles on 1p, have lost alleles on 19q as well (Reifenberger *et al*. 1994a, Kraus *et al*. 1995). In addition to losses on 19q and 1p, grade II oligodendrogliomas have been shown to contain abnormalities on chromosomes 4, 6, 11p, 14 and 22q (Reifenberger *et al*. 1994a, Weber *et al.* 1996, Zhu *et al*. 1998, Bigner *at al.* 1999, Kross *et al* 1999).

Anaplastic oligodendrogliomas show the same genetic losses as grade II oligodendrogliomas but in addition display frequent losses on 9p and 10q. These two genetic alterations are also common progression associated changes in malignant astrocytomas (Reifenberger *et al.* 1994a, Kross *et al* 1999). The CDKN2A tumor suppressor gene at 9p21 has been shown to be homozygously deleted in about 25% of anaplastic oligodendrogliomas, including tumors with and without losses on 1p and/or 19q (Cairncross *et al*. 1998, Bigner *et al*.1999). The incidence of CDKN2A deletions in anaplastic oligodendrogliomas and astrocytomas is similar (Ichimura et al. 1996). At 10q23.3 there is a tumor suppressor gene candidate PTEN (phosphatase and tensin homology) also known as MMAC1 (mutated in multiple advanced cancers) and TEP-1 (TGF-β-regulated and epithelial cell-enriched phosphatase) which is commonly mutated in glioblastomas (Li *et al*. 1997, Steck *et al*. 1997) . However, PTEN mutations have been found only in a small fraction of anaplastic oligodendrogliomas (Duerr *et al.* 1998). In addition to losses on 9p and 10, anaplastic oligodendrogliomas show an increased frequency for multiple deletions on chromosomes 4, 6, 11, 15 and 18 supporting the hypothesis that malignant progression is associated with augmentation of genetic aberrations (Reifenberger *et al.* 1994a, Kross *et al* 1999, Zhu *et al*. 1998, Bigner *et al*. 1999).

Unlike in astrocytic tumors, loss of 17p harboring p53 gene is infrequent, being limited to under 10% of oligodendroglial tumors (Maintz *et al* 1997, Reifenberger *et al.* 1994a, Bigner *et al* 1999). Mutations in the TP53 gene have been shown in 10-15% of reported oligodendrogliomas (Maintz *et al* 1997, Ohgaki *et al*. 1991, Reifenberger *et al.* 1996a).

Less than 10% of anaplastic oligodendrogliomas show amplification of proto-oncogenes, including EGFR, MYC, MYCN, CDK4, SAS, GLI and the renin gene REN (rev by Reifenberger *et al.* 1996b).

3.1.2 Mixed tumors

Loss of heterozygocity on 19q and 1p occurs in about 30-50% of oligoastrocytomas (Kraus *et al.* 1995, Maintz *et a*l.1997, Reifenberger *et al*. 1994a) whereas about 30% of oligoastrocytomas display the genetic aberrations commonly found in astrocytomas, i.e. mutations of the TP gene and/or LOH on 17p. The tumors with TP53 mutations and/or 17p loss do not have LOH in 1p and 19q, and vice versa (Maintz *et al.*1997, Reifenberger *et al*. 1994a). The predominant component and therefore the histological appearance of the tumor seems to depend on the genetic alterations. Microdissection of three oligoastrocytomas studying e.g. LOH of alleles 1p and 19q found common genetic alterations in both components independent of oligodendrocytic or astrocytic appearance (Kraus *et al.* 1995).

3.1.3 Astrocytomas

Allelic loss of chromosome 17p and TP53 mutations are present in approximately one-third of astrocytic tumors (Fults *et al*. 1992, Sidransky *et al*. 1992, von Deimling *et al*. 1992b) excluding pilocytic astrocytomas in which it does not seem to play any role (Ohgaki *et al*. 1993). The important consequence of TP53 mutation is increased genomic instability and therefore a tendency to augmentation of genetic aberrations, which leads to more and more malignant neoplasms. In case of gliomas, the lethal result of this gradual malignant formation is secondary glioblastoma. If there is not any evidence of pre-existing less malignant neoplasm, the glioblastoma is called primary. In these tumors TP53 mutations are limited to 10% suggesting that glioblastomas can evolve at least in two distinct pathways (Watanabe *et al*. 1996, Watanabe *et al*. 1997, Ohgaki *et al*. 1999). One mechanism to escape the TP53-regulated control of cell proliferation is through aberrations in genes affecting the function of TP53. MDM2 gene, which is located to 12q, encodes at least five proteins, two of which interact with TP53. The MDM2 protein binds to both mutant and wild-type (wt) TP53 proteins thereby inhibiting their function (Momand *et al*. 1992, Olson *et al*. 1993, Haines *et al*. 1994). The transcription of the MDM2 gene is induced by wt TP53, and MDM2 promotes the degradation of TP53 (Barak *et al*. 1994, Zauberman *et al*. 1995). This autoregulatory loop is unbalanced at least in the 8% of primary glioblastomas with MDM2 amplification but no TP53 mutation (Reifenberger *et al*. 1993) . Wild-type TP53 upregulates the expression of p21(WAF1/CIP1) which is needed for p53 mediated growth arrest (El Deiry *et al*. 1993). No mutations in gliomas have been found in the p21 encoding CDKN1A gene on chromosome 6p (Jung *et al*. 1995, Koopmann *et al*. 1995, Li *et al*. 1998).

The aberrations in chromosome 9p are frequent in high-grade gliomas. The genes encoding tumor supressors p16 and p15, CDKN2A and CDKN2B, respectively, are located on chromosome 9p21 (James *et al*. 1991, Olopade *et al.* 1992). The percentage CDKN2A deletions has been shown to increase with malignancy in

gliomas being 56% in high-grade astrocytomas (Walker *et al*. 1995). In addition to deletions the regulatory function of p16 can be overcome by transcriptional silencing. *De novo* methylation of 5'CpG island of CDKN2A gene has been found in approximately 20% of different neoplasms but not in normal tissues (Merlo *et al*. 1995). This very mechanism for inactivation has been shown for the CDKN2B gene in gliomas (Herman *et al*. 1996).

The overexpression of CDK4 or CDK6 might override the normal function of p16 and p15. The gene encoding CDK4 resides at 12q and the gene encoding CDK6 maps to chromosome 7q. Both proteins have catalytic kinase activities promoting proliferation. The CDK4 gene is amplified in nearly 15% of high-grade gliomas, especially in the ones without CDKN2A/CDKN2B alterations (Nishikawa et al. 1995, Reifenberger et al. 1994b).

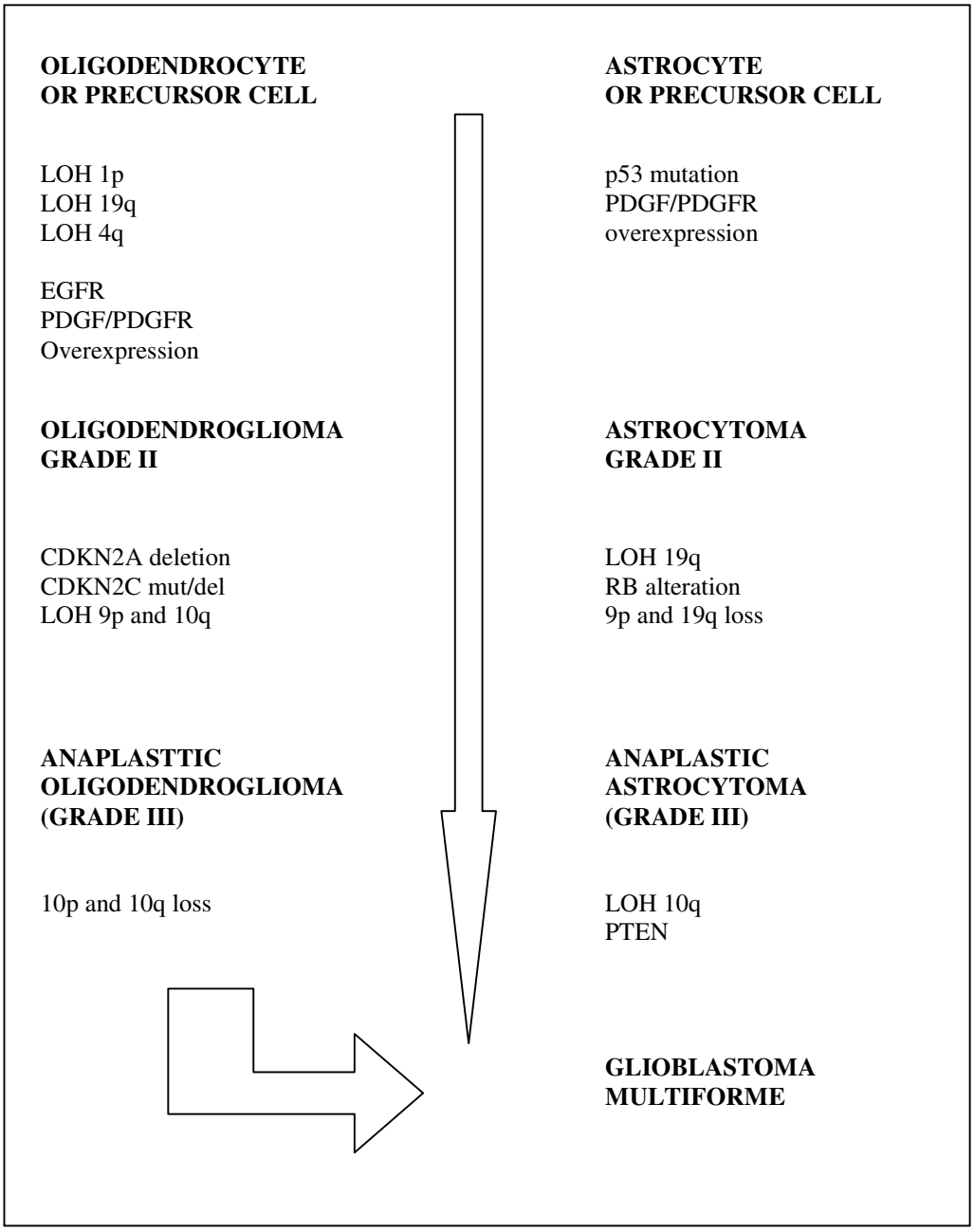
The Retinoblastoma gene (RB1) is located on chromosome 13q14, which is altered in about one-third of high-grade astrocytic tumors (James et al. 1988, Henson *et al*. 1994). This protein is the ultimate target of kinase activities controlling cell proliferation and the amplification of RB1 has the same growth promoting consequences as CDK4 and CDK6 amplifications (Serrano *et al*. 1993).

The epidermal growth factor receptor (EGFR) gene, residing at chromosome 7, is the most frequently amplified gene in astrocytic tumors (rev by Fuller and Bigner 1992). It is amplified and overexpressed in about one third of glioblastomas and in a few anaplastic astrocytomas (Wong *et al.* 1987). Interestingly, more than 60% of primary glioblastomas, whereas only 10% of secondary glioblastomas show upregulated EGFR expression (Watanabe *et al*. 1996). Therefore EGFR amplification and overexpression could be regarded as a hallmark of primary glioblastoma.

The frequency of loss of heterozygozity on chromosome 10 increases with malignancy in astrocytic tumors being the most frequent genetic alteration in glioblastomas (James *et al*. 1988, von Deimling *et al*. 1993, rev by Bigner and Vogelstein 1990). At 10q23.3 there is a tumor suppressor gene candidate PTEN, which has been shown to be altered in 30-44% of high-grade gliomas (Wang *et al*. 1997).

Loss of heterozygocity of chromosome 19q occurs in 21-44% of high-grade astrocytic tumors (von Deimling *et al.* 1992a, von Deimling *et al.* 1994). Chomosome 22q has lost one of its' alleles in 20-30% of gliomas of all grades (Huang *et al*. 1996). The possible tumor suppressors in these regions remain unidentified.

Figure 3. Genetic changes in oligodendroglial and astrocytic tumorigenesis.



3.2 Aetiology and genetic suspectibility

The only proven environmental cause of gliomas is therapeutic X-irradiation (rev by Huang et al. 1987, Lantos et al. 1996, Brat et al. 1999).There is increasing evidence that a significant fraction of human neoplasms including gliomas contain sequences from SV40 virus. The occurrence of SV40 DNA in gliomas has been shown to be up to 50% in astrocytic tumors and 25% of oligodendrogliomas (Martini et al. 1996, Huang et al. 1999). However, the role of viral infections in aetiology of human malignancies remains unclear at present.

Only occasional cases of familial clustering have been reported among oligodendrogliomas (Roosen *et al*. 1984, Roelvink *et al*. 1986) whereas among astrocytic tumors familial clustering is not as uncommon. Astrocytomas are associated with inherited tumor syndromes including Li-Fraumeni syndrome, Turcot syndrome, tuberous sclerosis, neurofibromatosis (NF1) and multiple enchondromatosis (Maffucci/Ollier disease) syndrome (Mellon *et al*. 1988, rev by Frappaz *et al.* 1999) .

4. Clinical factors

4.1 Patient age

Patient age has been shown to be a prognostic factor of major significance in several studies of both oligodendrocytic and astrocytic tumors. This has been suggested to be due to the occurrence of more malignant gliomas at older age and by decreased host resistance with age (Cohadon *et al*. 1985, Burger *et al*. 1991). Patient age has, however, been shown to be an independent prognostic factor in multivariate analyses and to even have a role within the tumor malignancy categories (Burger *et al*. 1985, Burger and Green 1987, Schiffer *et al*. 1997a, Shaw *et al*. 1992). Therefore Burger and Green (1987) concluded that the effect of age must be caused by factors that are not detected on morphology. The mean patient ages for different gliomas are shown in table 1.

Table 2. Age and gender in glial neoplasms. The figures are adapted from Düsseldorf series (reviewed by Kleihues and Cavenee (eds. 2000)) except for pilocytic astrocytomas (Haapasalo et al. 1999).

Tumor type	Mean age	Gender Male:female
Oligodendroglioma grade II	43	1.1 : 1
Oligodendroglioma grade III	49	1.5 : 1
Oligoastrocytoma grade II	45	1.2 : 1
Oligoastrocytoma grade III	45	1.3 : 1
Pilocytic astrocytoma (grade I)	14	1 : 1.2
Diffuse astrocytoma (grade II)	34	1.2 : 1
Anaplastic astrocytoma (grade III)	41	1.2 : 1
Glioblastoma multiforme (grade IV)	53	1.5 : 1

4.2 Tumor location and surgery

Both oligodendrocytic and astrocytic tumors arise preferentially in the cortex and white matter of the cerebral hemispheres. Of oligodendrogliomas, 50-65% reside in the frontal lobe. The temporal, parietal and occipital lobes are affected with decreasing frequencies (Kros *et al*. 1994, Shaw et al. 1992, Nijjar *et al*.1993). Diffuse astrocytomas involve most commonly frontal and temporal lobes as well, one third of the cases arising from each site (Kleihues and Cavenee (eds.) 2000). Frontal location is considered favourable for prognosis of oligodendroglioma patients whereas no such lobe specific correlation has been shown with astrocytomas (Kros *et al*. 1994).

Macroscopically complete surgical removal (Shaw *et al.* 1992, Celli *et al*. 1994) and irradiation after partial resection have been shown to favour a better outcome of oligodendroglioma patients (Gannett *et al*. 1994). Gross total resection is considered predictive of delayed recurrence and progression also among patients with astrocytic tumors (Winger *et al.* 1989*,* Peraud *et al*.1998). Because of the diffusely infiltrating nature of diffuse astrocytomas and oligodendrogliomas, the gross total resection is unfortunately not possible with the exception of cerebellar astrocytomas which tend to demarcate well from surrounding white matter.

4.3 Patient's performance status

Karnofsky's ten-tiered scale is used to evaluate the patient's ability to manage every-day life and work. Karnofsky score has been suggested to be a significant prognostic factor for both oligodendroglioma and astrocytoma patients (Chandler *et al.* 1993, Philippon *et al*. 1993, Schiffer *et al*. 1997a). In clinical trials, Karnofsky score has been largely replaced by a five tiered WHO scale which is easier to use and provides sufficient information on patient's overall performance.

5. Treatment of gliomas

Surgery is needed to establish an accurate pathological diagnosis of the tumor. Therefore it is essential for treatment even if gross total resection can not be accomplished. Surgery is also necessary for tumor mass reduction and relieving possible compression and neurological deficit. It can be assumed that by removing as much as possible of the neoplastic tissue, the pool of acquired genetic abnormalities in the tumor would be decreased, and according to the theory of the genetic cascade of gliomas, this might slow down dedifferentation and delay recurrence or progression. Secondly, by removing the bulk of the lesion and the most central portion which is the less well vascularized and oxygenated part of the tumor, the delivery and effectiveness of complementary therapy is maximized. With the reduced genetic abnormalities the chance of developing resistance to chemotherapy would be reduced as well (rev by Fortin *et al*. 1999). In cases of pilocytic astrocytomas surgery may be curative.

Oligodendrogliomas are among the most chemosensitive of all human solid malignancies. PCV regimen, a combination of procarbazine, lomustine (CCNU), and vincristine sulfate, is the most commonly used chemotherapy but oligodendrogliomas have been shown to be sensitive to a variety of cytotoxic drugs including melphalan, thiotepa, temozolomide, paclitaxel (Taxol) and platinum-based regimens. Responses have been shown with patients treated upfront with PCV chemotherapy before radiotherapy with both pure and mixed oligodendroglial tumors of both low and high grades. About 75% of patients with recurrent anaplastic oligodendroglioma treated with PCV chemotherapy gain at least 50% reduction in area of enhancing tumor and many of these responses are complete (Rev by Perry JR *et al.* 1999).

The response to cytotoxic chemotherapy in astrocytic tumors seems to be inferior to that of oligodendrogliomas. Of agents with confirmed activity against astrocytic tumors, intravenous carmustine (BCNU, bischloroethyl-nitrosourea) is often the first choice. Locally implanted BCNU-wafers have been tried with variable results. Subach *et al*. (1999) did not find the treatment beneficial for the patients whereas Valtonen *et al.* (1997) were able to achieve a considerable increase in the life span of treated high grade glioma patients. CCNU alone as well as metotrexate are also used to treat astrocytoma patients. Interestingly, some patients with grade III astrocytomas have been reported to respond to PVC regimen better than carmustine treatment (Levin *et al*. 1990). Promising results from temozolomide-treated patients with anaplastic astrocytoma and anaplastic oligoastrocytoma at first relapse have been recently reported (Yung *et al*. 1999)

Radiation therapy and its life prolonging effect are well established for patients with malignant astrocytomas (Kirby *et al*. 1995, Kirby *et al*. 1996). Although radiotherapy appears to delay recurrence of low-grade gliomas its effect on survival is controversial (Knisely et al. 1997). The use of irradiation has been formerly shaded by severe neurobehavioral deficits such as memory impairment, progressive global dementia, apathy and personality changes (Taphoorn *et al*. 1992). Abnormalities in gait, balance and coordination have also been reported (Crossen et al. 1994). Currently conformal and stereotactic techniques which effectively spare normal brain from unwanted

irradiation are increasingly being used to overcome long-term side effects of radiotherapy (Joensuu and Tenhunen 1999). However, a recent review based on several studies on factors affecting patients' outcome (Fortin et al. 1999) suggests that radiotherapy should be used to treat patients with oligodendrogliomas and oligoastrocytomas only if chemotherapy has failed.

There are ongoing studies about the efficacy and usefulness of gene therapy (Puumalainen *et al.* 1998), boron neutron capture therapy (rev by Diaz *et al*. 2000) anti-angiogenic (rev by Lund et al. 1998) and immunotherapy (rev by Lillehei et al. 1999) for gliomas but none of them are in routine use yet.

6. Potential prognostic factors

6.1 Proliferation

6.1.1 Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdU) is a thymidine analogue which is incorporated into cells actively synthesizing DNA. BrdU can be detected by specific antibodies immunohistochemically in tissue sections or by flow cytometry. BrdU labelling indices have been suggested to be of prognostic significance in gliomas (Hoshino *et al.* 1993, Wacker *et al.* 1994, Struikmans *et al.* 1999). However, this method requiring fresh tissue is not in widespread use and it is replaced by other immunohistochemical proliferation markers, e.g. by MIB-1 staining, which has also been shown to have more prognostic power (McKeever *et al*. 1997).

6.1.2 Proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is a nuclear polypeptide, which has an important role in the initiation of cell proliferation (Baserga 1991). There are two populations of PCNA accumulating in cells during the S-phase of the cell cycle: one nucleoplasmic as in quiescent cells and extracted by detergent, and another that is associated with DNA replication sites and not extractable with organic solvents (Bravo and Macdonald-Bravo 1987). High immunohistochemical PCNA labelling indices have been suggested to be indicative of a poor prognosis in both oligodendrocytic and astrocytic tumors (Ehrmann *et al*.1997, Kirkegaard *et al*. 1998, Schiffer *et al*. 1997). In comparison, the prognostic value of MIB-1 labelling has been shown to be more significant in gliomas (Sallinen *et al.* 1994, McKeever *et al*. 1997).

6.1.3 MIB-1/Ki-67

MIB-1/Ki-67 is a nuclear antigen, which is expressed throughout the cell cycle from late G1 to mitosis but not in quiescent cells suggesting that Ki-67 could be used as a marker of mitogenic stimulation (Gerdes *et al*. 1983, Gerdes *et al*.1984). MIB-1 labelling index has been shown to be a significant prognostic factor in gliomas, some studies reaching significance independent of histological grade, patient age and tumor site in both oligodendroglial and astrocytic tumors (Jaros *et al*. 1992, Sallinen *et al*. 1994, Heegaard *et al*. 1995, Kros *et al.* 1996, Coons *et al*.1997, McKeever *et al*. 1997, Schiffer *et al*. 1997). Several different cut-off values for dividing the patients into different prognostic subgroups have been suggested. A study of 89 oligodendroglioma patients reported 83% as the 5-year survival rate for the patients whose tumors showed MIB-1 labelling indices under 5% but only 24% for patients with tumors displaying MIB-1 scores higher than 5%. (Dehghani *et al*. 1998) Similarly, a study of 133 astrocytic tumors reported survival rates decreasing with increasing MIB-1 scores. The five year survival was 70% for the patients with tumors disclosing MIB-1 indices lower than 10%. As the index grew from 10 to 20% the

survival dropped to 45%. None of the patients with tumors expressing MIB-1 indices over 20% survived the follow-up (Sallinen et al. 2000).

6.1.4 Mitoses

Mitotic count can be expressed as the number of mitoses per ten microscopic high-power fields. Another estimate, mitotic index, is the percentage of mitoses of all nuclei counted. Volume-corrected mitotic index gives the mitotic count per square millimeter of neoplastic tissue and therefore eliminates some of the variation factors related to mitotic counts (Haapasalo *et al*. 1989). High mitotic activity has been shown to be indicative of a worse prognosis in both oligodendrocytic and astrocytic tumors (Coons *et al*. 1998, Kros *et al.* 1992, Shiffer *et al*. 1997, Struikmans *et al*. 1998, Struikmans *et al*. 1999, Sallinen *et al*. 2000).

6.1.5 Flow cytometric proliferation analysis (S-phase fraction)

The phases of the cell cycle can be detected by DNA flow cytometry based on the nuclear DNA content. Flow cytometric S-phase fraction (SPF) has been shown to be higher in more malignant gliomas and to have prognostic significance in both oligodendrocytic and astrocytic tumors (Struikmans *et al.* 1998, Coons *et al.* 1994a and b).

6.2 Cell cycle regulators

Cell cycle is regulated by factors promoting or inhibiting proliferation (Fig 4). Malignancies result from imbalance in control caused by increased effect of proliferation promoting factors, i.e. oncogenes or decrease in inhibition, namely tumor suppressor genes.

6.2.1 p53

The tumor supressor gene TP53 is located on the short arm of chromosome 17p13.1. It encodes a protein which is called the wild-type (wt) p53 protein in its' normal variant. It regulates gene transcription (Ginsberg *et al*. 1991) and participates in the induction of a G1 arrest and inhibition of DNA synthesis that follows DNA damage (Kastan *et al*. 1991, Kuerbitz *et al*. 1992). p53 has a role in apoptosis and control of the G2/M checkpoint of the cell cycle (rev. by Liebermann *et al*. 1995). p53 also participates in DNA repair by exhibiting 3'-to-5' exonuclease activity (Mummenbrauer *et al*. 1996). A significant correlation has been found for p53 protein accumulation and tumor grade in gliomas but the significance of TP53 gene mutations is circumstantial (Pavelic *et al*, 1994, Hagel *et al*. 1996, Hilton *et al*. 1998). The p53 immunoreactivity has also been shown to be indicative of poor prognosis in both oligodendrocytic and astrocytic tumors (Hagel *et al* 1999, Kros *et al*. 1993, Bouvier-Labit *et al*.1998).

6.2.2 p21

The expression of p21/WAF1/CIP1 gene, which is located on chromosome 6p21.2 (El-Deiry *et al*. 1993, Harper *et al.* 1993), is transcriptionally regulated by the wt p53 protein. A normal p21 gene is necessary for the p53 mediated cell cycle arrest at G1 (Brugarolas *et al*. 1995, Waldman *et al*. 1995), although p21 can also be activated independently of p53 (Michieli *et al*. 1994, Parker *et al*. 1995). The expression of p21 has been shown to be low in normal and reactive brain but elevated p21 levels have been shown in gliomas (Arvanitis *et al.* 1991, Jung *et al*. 1995, Korkopoulou *et al.* 1998). In mutation studies, no association has been found between any polymorphism of the gene encoding p21, p53 mutations and histopathological tumor type (Koopman *et al.* 1995). The expression of p21 has been shown to increase with malignancy in studies pooling astrocytic and oligodendrocytic gliomas (Arvanitis *et al.* 1991, Jung *et al*. 1995, Korkopoulou *et al.* 1998). It has been suggested that p53-dependent p21 expression is tumor grade specific (Jung *et al*. 1995).

6.2.3 pRb

Retinoblastoma gene, located to chromosome 13q14, was the first tumor suppressor gene to be discovered (Friend *et al*. 1986). At the G1/S checkpoint pRb is the downstream target of many cell cycle regulators. Levels of pRb expression do not normally vary during the cell cycle, but the pRb phosphorylation status and the consequent activity does (rev by Weinberg 1995). In addition to retinoblastomas, mutations of pRb gene occur commonly in sporadic osteosarcomas (Toguchida *et al*. 1989), lung (Harbour *et al* 1988) and breast carcinomas (Lee *et al*. 1988). Loss of heterozygosity at 13q harboring Rb gene has been show in 32- 54% of malignant gliomas (Ueki et al. 1996, Lee et al. 1995). Elevated pRb expression has been shown to correlate with malignancy in astrocytic tumors but only five oligodendrogliomas have been investigated so far (Henson *et al.* 1994, Korkopoulou *et al.* 1998).

6.2.4 CDKN2A/p16

The p16 tumor suppressor inhibits cyclin-dependent kinases CDK4 and CDK6 reducing their capacity to phosphorylate the retinoblastoma protein thereby controlling G1/S phase transition of cell cycle (Weinberg 1993, Serrano *et al*. 1993). Aberrations in CDKN2/p16 gene, located at chromosomal region 9p21, have been characterized in several malignant tumors, including gliomas (Nobori *et al*. 1994, Nishikawa *et al*. 1995, Ono *et al.* 1996). The association of the CDKN2/p16 with astrocytomas has been well established, some studies including oligodendrogliomas and oligoastrocytomas with similar results (Nobori *et al*. 1994, Nishikawa *et al*. 1995, Ono *et al.* 1996, Cairncross *et al*. 1998, James et al. 1999). Walker *et al*. (1995) reported homozygous deletions of the CDKN2/p16 gene in 56% of the 71 high grade astrocytomas, and Kyritsis *et al*. (1996) found multiple mutations in five of 35 gliomas examined.

6.3 Chromosomal changes

6.3.1 1p and 19q

A study including 39 patients with anaplastic or aggressive oligodendrogliomas treated with alkylating chemotherapy showed that allelic loss of 1p was a significant predictor of chemosensitivity; all the tumors with 1p loss were chemosensitive. Loss of chromosome 19q alone was not indicative of response, whereas combined loss involving chromosomes 1p and 19q associates with both chemosensitivity and longer recurrence-free survival after chemotherapy (Cairncross *et al*. 1998). The loss of 19q has been shown to be strongly associated with oligodendrocytic tumor type in comparison to diffuse astrocytomas, which are rather associated to retention of 19q and loss of 19p (Ritland *et al*. 1995).

6.3.2 Aberrations in chromosomes 7 and 10

A study of 207 patients with gliomas (153 diffusely infiltrating astrocytic tumors, 23 pilocytic astrocytomas and 31 oligodendrogliomas) showed that aberrations in chromosome 7 were indicative of a worse prognosis as well as aberrations in chromosome 10 (Ganju *et al*. 1994). The loss of heterozygozity on chromosome 10 is typical in diffuse astrocytic tumors the frequency increasing with malignancy (James *et al*. 1988, von Deimling *et al*. 1993, rev by Bigner and Vogelstein 1990). Aberrations at chromosome 7 have been suggested to be an early event in diffuse astrocytomas (Schrock *et al*. 1996).

6.4 Peripheral-type benzodiazepine receptor and diazepam binding inhibitor

Peripheral-type benzodiazepine receptors (PBR) are found in most tissues. In brain they seem to be localized mainly in glial cells (Benavides *et al.* 1983, Anholt *et al*. 1984, Gehlert *et al*. 1985). They are often located subcellularly into mitochondria and therefore also referred to as mitochondrial benzodiazepine receptors (Anholt *et al*. 1986). However, other subcellular locations have been reported (Oke *et al*. 1992). The functional significance of PBRs and their effector mechanisms is not yet clear but they seem to be involved at least in mitochondrial cholesterol transport, mitochondrial and cell proliferation, immune response and modulation of voltage-dependent calcium channels (Papadopoulos 1993, Alho *et al.* 1994).

Diazepam binding inhibitor (DBI) is an endogenous polypeptide capable of displacing benzodiazepine binding from both central-type and peripheral-type receptors (rev by Costa and Guidotti 1991). DBI is involved in multiple biological processes such as stimulation of cell growth, stimulation of steroidogenesis and inhibition of glucose-induced insulin secretion from pancreas (rev by Papadopoulos 1993, Costa and Guidotti 1991). The polypeptide has also been purified by its ability to bind long chain acyl-CoA esters and accordingly entitled to acyl-CoA-binding protein (Knudsen *et al*. 1993). In central nervous system the highest levels of DBI immunoreactivity have been detected in ependymal and glial cells, although also neurons contain DBI.

High concentration of DBI is present in circumventricular organs and cerebellum, but not in the normal cerebral cortex (Alho *et al.* 1985). Several studies have shown increased binding of benzodiazepine ligands in different brain tumors (Ferrarese *et al*. 1989, Black *et al*. 1990, Cornu *et al*. 1992). In cell line studies the highest PBR/DBI immunoreactivity has been shown in dividing cells (Alho *et al.* 1994).

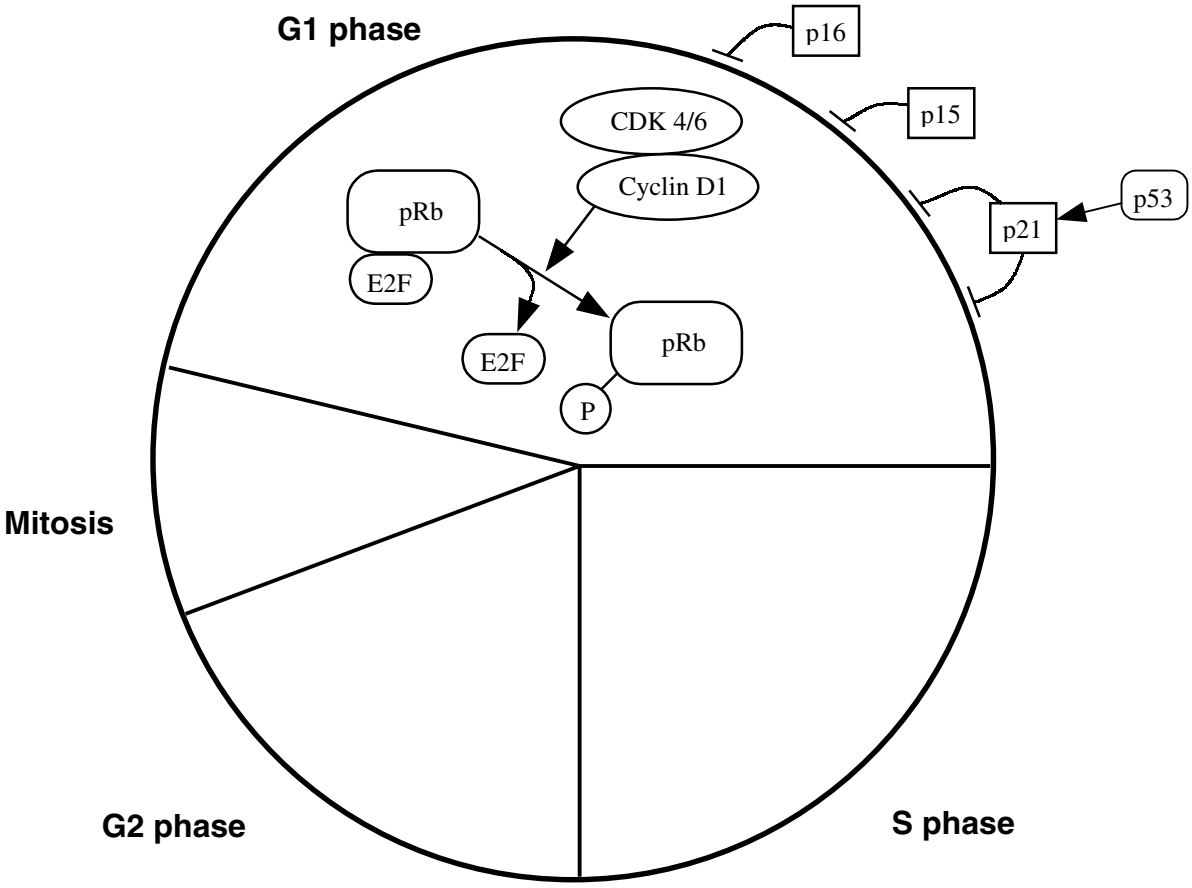
6.5 Topoisomerase IIα

Topoisomerase IIα (topoIIα) is a 170-kD enzyme which generates and reseals double-stranded DNA breaks. It is encoded by a gene residing at chromosome 17q21-q22 (rev by Watt and Hickson 1994, Watt 1996). Because of its function it is a logical molecular target for anticancer therapy. Among the commonly used cytotoxic agents, which act by inhibiting topoIIα, there are such important anticancer drugs as anthracyclines, epipodophyllotoxins, actinomycin and mitoxantrone. The chemosensitivity of cancer cells to topoII-inhibitors, in turn, correlates with the expression level of topoIIα (Isaacs *et al*. 1998). There are two previous studies including altogether 52 astrocytic tumors reporting immunopositivity increasing with malignancy and proliferation as determined by MIB-1. Both studies also showed that the amount of topoIIα staining was a significant prognostic factor (Holden and Townsend 1999, Taniguchi *et al*. 1999). There are no reports concerning oligodendrogliomas.

6.6 Apoptosis

Apoptosis, in other words programmed cell death, provides a means to eliminate cells with damaged DNA. In neoplastic cells the regulatory mechanisms are often dysfunctional and the cells may become immortal. Some studies have shown the amount of apoptosis increasing with malignancy in gliomas and even suggested a prognostic significance (Schiffer *et al*. 1997a, Sipos *et al*. 1998), whereas other studies have not been able to confirm the results (Shiffer et al. 1997b, Wharton et al. 1998).

Figure 4. The G1 phase of the cell cycle as a concentrated target for the mutations in glioma. The proteins required for cell cycle to proceed are shown within the phase. Those in circles are checkpoint accelerators or regulators. The proteins in squares are inhibitors. At the G1/S checkpoint pRb is the downstream target of many cell cycle regulators. Levels of pRb expression do not normally vary during the cell cycle, but the pRb phosphorylation status and the consequent activity does. Cyclin D1 is a cell cycle regulatory protein which activates the cyclin-dependent kinases 4 and 6 to phosphorylate the retinoblastoma protein during G1 phase. This leads to the activation of the transcription factor E2F and thus the cell cycle to proceed from phase G1 to S (rew. by Weinberg 1995). This is inhibited by tumor suppressors shown (Weinberg 1993, Serrano *et al*. 1993).



AIMS OF THE STUDY

1. To investigate the expression of PRB and DBI in astrocytic tumors and their possible correlation to cell proliferation, tumor malignancy and patient survival.

2. To study the expression of cell cycle regulators (p16/CDKN2A , p53, pRb, p21) in gliomas and to evaluate their role in tumor biology and clinical relevance.

3. To characterize the expression of topoisomerase IIα (topo IIα) in oligodendrocytic tumors. To correlate the topo IIα expression with cell proliferation and patient prognosis.

MATERIALS AND METHODS

1. Patients and Tumors

For study I, representative samples from eighty-six primary astrocytic tumors were obtained from surgeries performed at Tampere University Hospital between February 1988 and February 1992. The material included 9 pilocytic astrocytomas (grade I) and 77 diffuse (grade II-IV) astrocytomas (table 2). There were 21 female and 65 male patients. Their age varied from 5 to 77, median age was 47 years (mean 44 years). The follow-up period was 24 months.

The 78 primary gliomas included in the study II are described in the table 2. There were 15 male and 27 female oligodendroglioma patients (median age was 35, range 5-68). The median age of the 26 male and 10 female astrocytoma patients was 43 years (range 6-68). The oligodendroglioma patients were followed-up for at least 63 months and the astrocytoma patients for at least 40 months, or until death. The patients were operated at Tampere University Hospital, Tampere, Finland, Turku University Hospital, Turku, Finland and Kuopio University Hospital, Kuopio, Finland between 1983 and 1994.

The 88 patients with oligodendrocytic tumors included in the study IV were operated at the same hospitals between 1980 and 1999 (table 2). Altogether, there were 48 male and 40 female patients (median age was 40, range 8-76). The 80 patients with primary tumours available (40 male and 40 female patients, median age 40, range 8-76) were followed-up for 5 years (n=62, of whom alive 46, dead 16) or until November 1999 (n=18, all alive).

Of the 88 patients evaluated in the study IV 84 patients with representative samples for further analyses were included in the study III (table 2). Altogether, there were 44 male and 40 female patients (median age was 40.5, range 8-76). Patients included 37 males and 39 females, median age 40.0, range 8-76. The patients were followed-up for 5 years (n=61, of whom alive 45 , dead 16) or until November 1999 (n=15, all alive).

When possible, patients were treated by gross radical resection of the tumor. After resection of the tumor, adjuvant radiation and/or chemotherapy were combined to the treatment in cases of high grade malignancy or recurrence. Frequent clinical check-ups included brain imaging with computed tomography (CT) or magnetic resonance imaging (MRI).

Table 3. Histopathological characteristics of patients with glioma

Study	Grade	Oligodendrogliomas	Astrocytomas	Mixed glioma	Target
I	I II III IV Total number		9 19 21 37 86		PBR DBI
II	I II III IV Total number	33 9 42	7 10 9 10 38		CDKN2/p16
III	II III Total number	42 16 58		16 10 26	P53, pRb,p21
IV	II III Total number	43 16 59		17 12 29	Topo II α

2. Methods

2.1 Immunohistochemistry

2.1.1 PBR and DBI

The tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. In addition to HE-stained sections for routine histology, five µm sections were cut and taken onto poly-L-lysine coated slides. The slides were dried at 37ºC overnight. Dewaxed sections were treated with solution containing methanol and 1.5% hydrogen peroxide and hydrated. To reduce nonspecific immunostaining, the sections were incubated in PBS (pH 7.4) containing 10% BSA for 30 min. For visualization of PBR, the samples were incubated for 16 to 22 h either with a polyclonal (1:500) or a monoclonal PBR antibody (donated by Dr. Krueger, Alho *et al.* 1994) at 4ºC. For demonstration of DBI, a polyclonal rabbit DBI antibody was used at dilution 1:4000 (Alho *et al*. 1985). The slides were then incubated with biotinylated goat anti-rabbit antibody (1:400, Vector Laboratories, Burlingame, CA) and avidin-biotin complex for 30 min each. The immunoreaction

was visualized by incubating the sections with 0.025% diaminobenzidine-0.01% hydrogen peroxide for 5 min, or for fluorescence double labeling either with rabbit or mouse FITC- or rhodamin-conjugated antisera. All the antibodies were diluted in PBS containing 1% BSA and 0.3% Triton-X 100. Aquamount mountaint was used for embedding the slides.

2.1.2 p16/CDKN2A

Representative five µm sections from archival formalin-fixed and paraffin-embedded tissues were cut and taken onto poly-L-lysine coated slides, and dried. For improved antigen retrieval, dewaxed sections were immersed in 10mM EDTA (ph 8.0) at 80°C and incubated at 120°C, (pressure 1.05 bar) for 10 minutes in an ordinary autoclave, washed at room temperature and processed for immunostaining. The samples were incubated for 12-18 h with a polyclonal (1:300) CDKN2/p16-antibody (Pharmingen, San Diego, CA) at 4ºC. The slides were then incubated with biotinylated goat-anti-mouse antibody (1:400; Vector Laboratories, Burlingame, CA) and ABC-complex for 30 minutes each. The immunoreaction was visualized by incubating the sections with 0.025 % diaminobenzidine in 0.01% hydrogen peroxide for 4 minutes. Alternatively, diaminobenzidine peroxidase reaction was intensified by incubating the slides in silver methenamine solution for 4 minutes, at 60ºC. All the antibodies were diluted in PBS containing 1% BSA and 0.3% Triton-X 100. After staining the sections were embedded in Pertex (Histolab Products, Västra Frölunda, Sweden).

2.1.3 MIB-1, topoisomerase IIα, p53, pRb and p21

For immunohistochemical analysis 5 µm sections from routinely processed tumor microarray paraffin blocks, or in case of MIB-1 staining also from regular tumor blocks, were cut and mounted on SuperFrost, SuperFrost Plus or poly-L-lysine coated slides and dried overnight at 37°C. The sections were then dewaxed and rehydrated. Antigen retrieval was done by heating the sections in microwave oven for 2 x 7 minutes in 0.1 mol/l citrate buffer (pH 6.0). The sections were then incubated with primary antibodies for 25 minutes at RT except for anti-p21 in which the sections were incubated overnight at +4°C. The following antibodies were used for proteins studied: clone MIB-1 for Ki-67 (Immunotech S.A. Marseilles, France, dilution 1:40), Ki-S1 (Boehringer Mannheim, dilution 1:1000) and Ki-S4 (generous gift from Dr. Udo Kellner, Univ. of Kiel, Germany, dilution 1:1000) for topoisomerase IIα, clone DO-7 for p53 (Novocastra Laboratories, Newcastle, UK, dilution 1:40), clone IF8 for Rb (NeoMarkers, Union City, CA, dilution 1:100), and goat polyclonal antiserum for p21 (Santa Cruz Biothechnology, Santa Cruz, CA, USA, dilution 1:150). The bound antibodies were visualized using appropriate streptavidin-biotin-peroxidase kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as chromogen. For counterstaining methyl green was used with the exception of p21 for which hematoxylin was used.

2.2 Apoptosis

Determination of apoptosis was done by TUNEL-labelling. The deparaffinized tumor tissue sections as well as microarray sections were first digested with proteinase K (20 µg/ml) for 15 minutes. Apoptotic cells were demonstrated using ApopTag^{TM} In Situ Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. In the terminal deoxynucleotidyl transferase (TdT) nick end labelling method, the recommended TdT concentration was reduced by 8-fold. Direct immunoperoxidase detection of digoxigenin labelled dUTP was followed by counterstaining in 0.03 % methyl green in 0.1 mol/l acetate buffer (pH 5.2) for 15 min.

2.3 mRNA in situ hybridization

2.3.1 MBR

Fresh frozen tissue sections were cut onto Superfrost Plus (Menzel, Germany) slides. An oligonucleotide probe directed against PBR mRNA (GenBank accession number M36035) was labelled at the 3-end with ^{35}S-dATP (DuPont-N England Nuclear Research Products, Boston, USA). The sections were hybridized at 42 °C for 18 hours with $1\times 7\ 10^7$ cpm/ml of the probe, washed 4 times for 15 minutes each in 1 X SSC at 55°C, and while in the final rinse they were left to cool to room temperature (approximately 1 h). After exposure for 3 weeks the autoradiograph films (Amersham β-max, Amersham Int.) were developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY, USA). Alternatively, the slides were dipped in Kodak NTB2 nuclear track emulsion and exposed for 3 weeks. The dipped sections were counterstained with hematoxylin.

2.3.2 p16/CDKN2A

For the p16/CDKN2A study half of the cases were randomly chosen for mRNA in situ hybridization. 7 µm sections were cut and mounted on Superfrost Plus (Menzel, Germany) slides. 45mer oligonucleotide probe with 55% GC content directed against CDKN2/p16 mRNA (GenBank accession number L27211) was labelled at the 3-end with ^{33}P-dATP (DuPont-NEN Research Products, Boston, USA) using terminal deoxynucleotidyltransferase (Amersham Int., Buckinghamshire, UK). The sections were hybridized at 42 °C for 18 hours with $1\times 7\ 10^7$ cpm/ml of the probe (approximately 0.5 ng/slide), washed 4 times for 15 minutes each in 1 X SSC at 55°C, and while in the final rinse they were left to cool to room temperature (approximately 1 h).

Control sections were hybridized with the presence of 100-fold excess of unlabeled probe, which reduced the hybridization signal considerably. Other control sections were treated with RNase before hybridization, which resulted in complete absence of hybridization signal. After exposure for 6-8 weeks the autoradiograph films (Amersham β-max, Amersham Int.) were developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY, USA). The specificity of the probe was tested with Northern hybridization of RNAs extracted from brain and testes, which showed a single band hybridization signal.

2.4 Tumor array

Histologically the most representative tumor areas were selected and marked to hematoxylin-eosin stained sections of each tumor. Thereafter, tissue cylinders (diameter 0.6 mm, height 3-4mm) from corresponding areas of each individual paraffin-embedded tumor blocks were taken and applicated into a new recipient paraffin block using a custom-built arrayer (Beecher Instruments, Silver Spring, MD). From mixed gliomas a sample from both oligodendroglioma and astrocytoma component was obtained separately.

2.5 Quantitation

The PBR, DBI and CDKN2/p16 immunostainings were evaluated semiquantitavely under a light microscope.

The percentage of MIB-1, p53, pRB, p21 and p27 immunoreactive and apoptotic cells in tissues were evaluated quantitavely using a computer-assisted image analysis system (CAS-200 TM Software, Becton Dickinson & Co., USA. Only neoplastic cells were included in the analysis. Necrotic and hemorrhagic areas were omitted.

2.6 Statistical methods

Mann-Whitney test, chi-square test, Wilcoxon test and Pearson correlation coefficient (r) were used for the statistical evaluation as described in studies I-IV. To compare the effect of cell cycle regulators (immunopositive vs. immunonegative) to proliferation rate in study III, multivariate variance analysis was used. For variance analysis log-transformed MIB-index was used, because the Kolmogorov-Smirnov test showed that the distribution of the MIB indices was normal only after log-transformation. The best prognostic cut-off point for study IV was determined by the receiver operating characteristics (ROC) curve. Log-rank test was used for comparison of survival of patients in the primary presentation phase. Cox's proportional hazards model was used for multivariate analysis of prognostic factors. All statistical analyses were performed using SPSS for Windows software (SPSS Inc., Chigaco, IL).

RESULTS

The studies included 77 patients with primary oligodendrocytic tumors. Eight out of 51 (16%) patients with grade II tumors died whereas eight of 26 (31%) patients with grade III tumors died during the five year follow up (p=0.11). Median age (40 years) was a significant prognostic factor (p=0.009). There was no significant difference in survival when the patients were divided into two groups by median of proliferation as determined by MIB-1 (p=0.11) nor when the patients were divided into groups by histological subtype (pure oligodendroglioma vs. mixed glioma: p=0.36).

1. PBR and DBI

The peripheral benzodiazepine receptor (PBR) immunostaining was granular and localized around nucleus and cytoplasm in astrocytic tumors. The amount of PBR immunoreaction was significantly associated with the histological grade of the tumor (p=0.0036, χ^2 test) of the glioblastomas (grade IV) disclosing the strongest immunopositivity. The amount of PBR expression increased independently of cell density. The in situ hybridization confirmed immunostaining results. High PBR expression was significantly associated with high proliferation rate (p=0.0039, Mann-Whitney test) and poor survival (p=0.024, log-rank test). Of patients with tumors expressing high amounts of PBR only 35% survived, whereas 65% of the patients with tumors disclosing low or no immunopositivity survived the two year follow-up period. In multivariate survival analysis, the predictive power of PBR immunopositivity was lower than the predictiveness of the histological grade, patient age, and MIB-1 immunopositivity, which all were independent prognosticators.

DBI immunopositivity was observed to be higher in malignant astrocytomas than in normal brain or low-grade astrocytomas. The majority of cells disclosing DBI were found to express PBR whereas there were numerous PBR immunoreactive cells lacking DBI expression.

2. CDKN2A/p16

Twenty-five of the 42 oligodendroglioma specimens expressed nuclear CDKN2/p16. Eleven of the seventeen patients with CDKN2/p16 immunonegative tumors died during the follow-up period, whereas 18 of the 25 patients with tumors disclosing CDKN2/p16 survived (p=0.0045).

In pilocytic and grade II astrocytomas there were numerous heterogeneously distributed cells with nuclear CDKN2/p16 immunoreactivity, in contrast to the high grade astrocytomas. Five out of 19 high grade astrocytomas versus 14 out of 17 low grade astrocytomas were CDKN2/p16 immunopositive (p=0.002, χ^2 test). Although high grade astrocytomas were almost completely devoid of mRNA in situ hybridization signal, low mRNA expression levels could be observed in areas with nuclear CDKN2/p16 immunopositivity. In comparison to immunopositive astrocytomas, CDKN2/p16 immunonegative tumors showed significantly increased

cell proliferation activity as determined by MIB-1 (median 9.6% vs. 25.6%, p=0.004, Mann-Whitney test) and mitotic index (median 0.12% vs. 0.70%, p=0.007, Mann-Whitney test). Furthermore, lack of CDKN2/p16 immunoreactivity was closely associated with poor patient survival in astrocytomas (p=0.025, log-rank test).

3. p53, pRb and p21

All the stainings disclosed strong nuclear immunopositivity with no background reactivity. The percentage of p53 immunopositive cells associated with the grade of the tumor (p=0.01, Mann-Whitney test). The p53-immunopositivity associated with high pRb expression (p=0.015 Mann Whitney test) and showed a trend towards higher rate of proliferation (p=0.073, Mann Whitney test). Sixteen (94%) of the 17 patients with p53 negative primary tumors survived whereas only 44 (75%) of the 59 patients with p53 positive primary tumors survived the five year follow up (p=0.13, log-rank test).

The p21 score associated strongly with grade (p<0.001, Mann-Whitney test, Fig 2). The immunopositive tumors had a higher rate of proliferation (p=0.021 Mann-Whitney test). The p21 immunopositivity associated strongly with p53 immunopositivity: of the 33 p21 immunopositive tumors 30 were p53 immunopositive and three immunonegative (p=0.017, chi-square test). Of the 46 patients with immunonegative primary tumors 42 (91%) survived whereas only 18 (60%) of the 30 patients with immunopositive primary tumors survived the five year follow-up (p=0.0017, log-rank test).

The pRb immunopositive tumors had clearly higher proliferation than immunonegative tumors (p= 0.002, Mann-Whitney test) but there was no correlation with survival.

Multivariate variance analysis was used to study the combined effect of the studied cell cycle regulators to cell proliferation rate. Log-transformed MIB-index was used as a dependent variable in the analysis. Only the amount of pRb expression reached statistical significance (p=0.004) but p21 and p53 showed a trend (p=0.068 and 0.071).

The expression of p53 and pRb did not differ between pure oligodendrogliomas and oligoastrocytomas or between the oligodendroglioma and astrocytoma components of of oligoastrocytomas. There were ten oligoastrocytomas with representative p21 samples stained for both components. The oligodendroglial component was observed to express p21 even when the astrocytic component did not (p= 0.046, Wilcoxon test).

Cox regression analysis was used to test the independent prognostic effect of the studied proteins for survival. Of all the studied cell cycle regulators (p53, p21, pRb) and pathobiological factors analyzed (grade of the tumor, histology: oligodendroglioma vs. mixed glioma, tumor proliferation rate, apoptosis), p21 was the only one that reached statistical significance (p=0.01, exp(B)=5.5).

4. Topoisomerase IIα

Positive topoIIα immunostaining was restricted to the nuclei of neoplastic cells in 88 glioma samples whereas the stroma and non-malignant cells remained always negative. There were 22 oligoastrocytomas which had representative oligodendroglioma and astrocytoma tissue cores for the analysis. These histologically different components of oligoastrocytomas were analyzed separately for their topoIIα expression. The topoIIα scores were higher in the oligodendroglioma than in the astrocytoma component of the tumour (5.37% ± 5.58% vs. 1.89% ± 2.49%, p=0.018, Wilcoxon test). The difference in the topoIIα scores between the oligodendroglioma and the astrocytoma components was significant without significant difference in MIB-1 indices between the components (p>0.05, Wilcoxon test).

TopoIIα scores were significantly higher in poorly differentiated, grade III tumours than in grade II gliomas (p<0.0001, Mann-Whitney test). The patients with primary tumours were divided into two groups with low and high topoIIα expression, respectively. There was a strong association between the high topoIIα expression and high tumour proliferation rate (p<0.0001, Mann-Whitney test, r=0.663, Pearson correlation), high expression of tumour suppressor protein, Rb (p=0.023, Mann-Whitney test) and p53 overexpression (p=0.01, Mann Whitney test).

Significantly higher proportion of patients with low topoIIα scores (37 of 42, 88%) were alive than of patients with high topoIIα scores (24 of 35, 69%) at the end of the five year follow-up (p=0.03, log-rank test). Cox regression analysis was used to demonstrate that the topoIIα had an independent prognostic value for survival (exp(B)=2.98, p=0.034) among the other pathobiological factors analyzed. None of the other factors included in the analysis reached statistical significance in multivariate analysis.

DISCUSSION

The classification of gliomas, especially that of oligodendrogliomas and mixed gliomas, is difficult and controversial. It has been suggested that oligodendrogliomas are underdiagnosed and that they may actually represent 25-33% of all glial tumors (rev by Fortin *et al*. 1999). Oligodendrocytic tumors have been shown to respond well to chemotherapy unlike diffuse astrocytomas (rev by Perry JR *et al.* 1999) and therefore definite diagnosis is of crucial importance. The underlying mechanism explaining the difference in response might provide means to improve the treatment of patients with astrocytic tumors. Histological grade does not provide definite prognostic information in oligodendrocytic tumors, which makes it difficult to evaluate the aggressiveness of the tumor and to decide about the therapy. Factors aiding to find differently progressing subgroups within histological grades would be useful for the astrocytic tumor group as well.

1. PBR and DBI

Peripheral benzodiazepine has been shown to stimulate DNA synthesis and cell proliferation in rat glioma cells (Ikezaki and Black 1990). Accordingly, the highest peripheral benzodiazepine receptor (PBR) immunoreactivity has been shown in dividing cells (Alho *et al.* 1994). However, the biological function in human brain tumors has remained unclear. In study I, PBR expression was observed to increase with tumor malignancy and proliferation in astrocytic tumors. The amount of diazepam binding inhibitor (DBI) immunopositivity, the suggested endogenous ligand of PBR, increased with PBR expression and was localized into the same cells. Our result is supported by a later study that showed a significantly higher density of PBR sites in both low and high-grade gliomas when compared with a normal brain using autoradiographic and liquid scintillation measurements. A significant difference was found between low and high grades as well. The authors suggested that it might be possible to differentiate high and low-grade gliomas by in vivo imaging with 11C-labelled PK-11195 using positron emission tomography (PET) (Miyazawa et al. 1998). Based on our study, clinical use of PBR staining is still arguable, because multivariate analysis did not show independent statistical significance.

The colocalization of PRB and DBI supports the suggested autocrine function of DBI (Ferrarese *et al.* 1989). The parallel increase in expression of PBR and DBI with proliferation could be due to increased metabolism caused by mitotic activity. This hypothesis is supported also by Ferrarese *et al*. (1994) who showed that PBR density and glucose utilization increase in parallel with malignancy in untreated tumors.

2. CDKN2/p16

Cyclin dependent-kinase 4 (CDK4)-cyclin D complexes inactivate the retinoblastoma protein (pRb) by phosphorylation, thereby promoting proliferation. The catalytic activity of CDK4-cyclin D complexes is inhibited by CDKN2/p16. The CDKN2/p16-CDK4-cyclin D1-pRb pathway has been frequently altered in various malignancies (Serrano *et al.* 1993, Weinberg 1995).

A clear correlation between decreased CDKN2/p16 expression and poor survival of both oligodendroglioma and astrocytoma patients was seen in study II. However, CDKN2/p16 was statistically associated with proliferation activity only in astrocytomas. Decreased CDKN2/p16 expression was present in 17 of 42 oligodendrogliomas and in 14 of 19 high-grade astrocytomas. The prognostic significance of the staining was better than that of histological grade but worse than that of proliferation index as determined by MIB-1 staining. Therefore the staining may not provide any further information for clinical use.

The lowered CDKN2/p16 mRNA and protein levels could be due to deletions or mutations of the CDKN2/p16 gene. This is supported by Bigner *et al*. (1999) who suggested that CDKN2 deletions are associated with anaplastic progression in oligodendrogliomas. However, glioma cells may escape CDKN2/p16 cell cycle checkpoint also by transcriptionally silencing its expression (Merlo *et al.* 1995, Costello et al. 1996). The number of glioblastomas and malignant astrocytomas exhibiting lowered protein levels is slightly greater than what would be expected on the basis of previous deletion and alteration assays, supporting the possibility of transcriptional regulation of the CDKN2/p16 gene. Methylation of 5'CpG island is one possible mechanism for transcriptional silencing of the CDKN2/p16 gene, and such de novo methylation of the 5´CpG island of CDKN2/p16 has been found in approximately 20% of different primary neoplasms, but not in normal tissues (Merlo *et al*. 1995, Fueyo *et al*. 1996). In addition, there may be an alternative mechanism for abrogating the CDKN2/p16 growth-regulatory pathway: the target gene CDK4 may be amplified (He *et al.* 1994, Schmidt *et al*. 1994). The existence of multiple mechanisms to avoid the regulation of the pathway is also suppported by a study of 120 glioblastomas of which 40% had no wild-type CDKN2/p16 gene and 12% had CDK4 gene amplication. The ultimate target of these regulatory proteins, wild-type retinoblastoma gene, was lacking in 14% of the studied tumors. With two exceptions, each tumor had only one of these abnormalities (Ichimura *et al*. 1996).

3. p53, pRb and p21

Tumor suppressor proteins p15, p16, p21 and p27 inhibit the activities of cyclin D-CDK4 and cyclin E-CDK2 complexes and, furthermore, the activity of p21 is regulated by p53. Normally cyclin D and cyclin E form complexes with CDK4 and CDK2. The activated complexes phosphorylate pRb and the transcription factor E2F are released triggering entry into the S phase.

Results of study III indicate intact function of p53 and p21 in oligodendrocytic tumors. Their expression levels rise with malignancy as in response to DNA damage, but their attempt to halt cell proliferation seems not to be sufficient. It is also possible that they are overrun by other factors promoting tumorigenesis.

Correlation of p53 immunoreactivity with malignancy confirms the similar result of a previous study of oligodendrogliomas (Pavelic *et al.* 1994). The patients with p53 immunopositive tumors had a tendency to poor prognosis in our study but statistical significance was not reached.

Expression of p21 correlated with both grade and proliferation and it was a significant independent prognostic factor. In agreement with previous studies describing cell cycle regulation in astrocytic tumors, results of study III also indicate that p21 is expressed almost exclusively only when p53 is immunohistochemically detected. To our knowledge, three earlier glioma studies report on p21 expression in altogether 7 oligodendrogliomas pooled with astrocytomas (Arvanitis *et al.* 1991, Jung *et al.* 1995, Korkolopoulou *et al*. 1998). Two of these studies show p21 expression to increase with malignancy in a pooled series of astrocytomas and oligodendrogliomas. The study by Jung *et al*. (1995) concludes that p53-dependent p21 expression seems to be tumor grade specific. The mutation studies have shown that p21 mutations are infrequent in gliomas (Jung *et al*. 1995, Koopman *et al.* 1995), and p53 mutations are rare in oligodendrogliomas (Ohgaki *et al*. 1991, Maintz *et al* 1997). This, together with the simultanous expression of p21 and p53, supports the hypothesis that the immunohistochemically detectable p53 could be wild type in oligodendrogliomas. This might indicate that p21 expression is a consequence of the elevated expression of wt p53 which is in turn due to DNA damage and can also be regarded as a sign of pathological cell cycle regulation.

The expression of pRb correlated with positive p53 status and high cell proliferation. Earlier studies with astrocytic and several other tumors have shown that elevated pRb correlates with malignancy but only five oligodendrogliomas have been investigated so far (Korkolopoulou *et al.* 1998). According to multivariate analysis with 84 oligodendrogliomas in this study, pRb status seems to correlate with cell proliferation better than any of the other studied regulators.

Oligodendrogliomas and mixed oligoastrocytomas did not differ in expression from any of the studied factors in their oligodendroglioma component. The oligodendroglioma and astrocytoma components were observed to differ only by their p21 expression which was significantly more frequent in the oligodendroglial component of mixed gliomas. Because of the small number of samples the issue needs further studies but the result may suggest a different expression profile of p53-p21 pathway of different components of mixed gliomas.

In multivariate survival analysis of the studied regulatory proteins (p53, p21 and pRb) and pathobiological factors analysed (histological grade of the tumor, oligodendroglioma vs. mixed glioma, tumor proliferation rate and apoptosis rate) p21 was the only one that reached statistical significance ($p=0.01$, $\exp(B)=5.5$). This strongly suggests clinical relevance for p21 staining in routine neuropathological practice.

4. Topoisomerase IIα

Topoisomerase IIα is a key enzyme in DNA metabolism, first generating and then resealing double-stranded DNA breaks, and thus a target for numerous cytotoxic drugs (rev by Watt and Hickson 1994). Results of study IV indicate that immunohistochemically demonstrable high topoIIα expression characterizes oligodendrogliomas which have high cellular proliferation rate and poor overall prognosis.

The expression pattern of topoIIα is known to increase in parallel with proliferation (Isaacs *et al*. 1998) whereas tumor suppressor gene p53 is capable of repressing topoIIα expression (Sandri *et al*. 1996, Wang Q *et al.* 1997). Supporting this, tumors with aberrant p53 expression were found to express higher topoIIα scores. Interactions, possibly linked to regulation of the expression of topoIIα, have been reported with pRb and accordingly we observed a positive correlation. (Bhat *et al.* 1999).

It has been shown *in vitro* that sensitivity to topoII-inhibitors correlates with the expression level of topoIIα in malignant cells (Isaacs *et al*. 1998). Therefore, the analysis of topoIIα expression may prove to be a key determinant in the selection of the appropriate chemotherapy for oligodendroglioma patients. TopoIIα expression does not have only prognostic significance, but it at the same time may define the target of optimal chemotherapy and thus it may also improve the prognosis.

Oligodendroglioma components of mixed gliomas were shown to disclose significantly higher topoIIα scores than the astrocytoma components independently of difference in proliferation. This may indicate effect of an additional factor specific for oligodendrogliomas.

Peripheral benzodiazepine receptor (PBR) and diazepam binding inhibitor expression were shown to increase with malignancy in astrocytic tumors. However, statistical significance was not reached in multivariate analysis, and clinical use of the staining can not be suggested on the bases of this study. Potentially interesting application would be in vivo imaging of PBR density in gliomas with 11C-labelled PK-11195 and PET for grading of malignancy and evaluation of novel therapeutic approaches.
CDKN2/p16 expression increased in both astrocytic and oligodendrocytic tumors with malignancy. The prognostic significance was better than that of histological grade but worse than that of proliferation index as determined by MIB-1 staining. Clinical utility of CDKN2/p16 staining is, therefore, questionable.
The results of this study suggest that topoisomerase IIα and p21 expression are useful in differential diagnosis of astrocytic and oligodendrocytic tumors. Both factors were also significant prognosticators in both uni- and multivariate analyses whereas the histopathological grade did not reach statistical significance in either one.
The clinical use of both stainings can therefore be well recommended. In addition to prognostication, evaluation of topo IIα reveals the possible existence of a target of cytotoxic drugs. High levels of topo IIα in many oligodendrocytic tumors suggest a rationale for clinical trials of novel topoisomerase IIα inhibitors for patients with oligodendroglial tumors.

CONCLUSIONS

1. High peripheral benzodiazepine receptor (PBR) labeling index is associated with high histological grade and proliferation rate in astrocytic tumors. Diazepam binding inhibitor (DBI) was observed to localize in the same cells with PBR. Intense PBR expression predicted poor survival but did not, however, reach significance in multivariate analysis.

2. CDKN2/p16 immunoreactivity decreased with increasing malignancy grade in both oligodendrocytic and astrocytic tumors. Lack of CDKN2/p16 immunoreactivity was closely associated with poor patient survival in both astrocytoma and oligodendroglioma patients. The expression of tumor suppressor genes p53 and p21 increased with malignancy in oligodendrocytic tumors. The amount of pRb expression was found to have a significant effect on cell proliferation rate, whereas p21 immunopositivity had independent prognostic value in multivariate analysis suggesting clinical applicability of p21 immunostaining.

3. Topoisomerase IIα immunoreactivity increased with proliferation and malignancy grade in oligodendrocytic tumors. Multivariate analysis showed that a high expression of topoIIα was an independent predictor of poor outcome. TopoIIα, being a target for multiple cytotoxic drugs, may offer a tool for selecting therapy for patients with oligodendrocytic tumors. These two features make it an attractive tool for further clinical evaluation.

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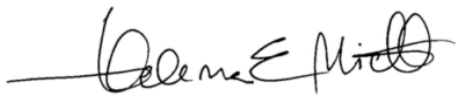

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