SALLY JÄRVELÄ

Potential Molecular Targets for Diagnosis and Therapy of Malignant Gliomas

ACADEMIC DISSERTATION
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the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
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To Timo

One Poem

One Dream

To believe in that Dream

One Look

One Seal

To have You, to make that dream Real

Thank you for being here,

Thank you for being You.

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1. Sally Järvelä, Helena Bragge, Niina Paunu, Timo Järvelä, Leo Paljärvi, Hannu Kalimo, Pauli Helén, Vuokko Kinnula, Ylermi Soini, Hannu Haapasalo. Antioxidant enzymes in oligodendroglial brain tumors: Association with proliferation, apoptotic activity and survival. J Neurooncol. 77(2):131-40, 2006

2. S. Järvelä, H. Helin, J. Haapasalo, T. Järvelä, T.T. Junttila, K. Elenius, M. Tanner, H. Haapasalo, J. Isola. Amplification of epidermal growth factor receptor by chromogenic in situ hybridization in astrocytic brain tumors; Association with clinicopathological factors and patient survival. Neuropathol Appl Neurobiol. 32(4):441-50, 2006

3. Sally Järvelä, Seppo Parkkila, Helena Bragge, Marketta Kähkönen, Anna-Kaisa Parkkila, Ylermi Soini, Silvia Pastorekova, Jaromir Pastorek, Hannu Haapasalo. Carbonic anhydrase IX in oligodendroglial brain tumors. BMC Cancer. 8:1, 2008

4. S. Järvelä, I. Rantala, V. Kinnula, Y. Soini, H. Haapasalo. Specific expression profile and prognostic significance of peroxiredoxins in grade II-IV astrocytic tumors. (submitted).

ABBREVIATIONS

AOE	antioxidative enzyme
BBB	blood-brain barrier
CDK4	cyclin-dependent kinase 4
CISH	chromogenic in situ hybridization
CNS	central nervous system
CSF	cerebrospinal fluid
CuZnSOD	copper-zinc superoxide dismutase
DNA	deoxyribonucleic acid
DMBT1/mxi	deleted in malignant brain tumors 1
ECSOD	extracellular superoxide dismutase
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
GBM	glioblastoma multiforme
GBMO	glioblastoma multiforme with oligodendroglial component
GLCL-C	gamma glutamyl cysteinyl synthetase catalytic unit
GLCL-R	gamma glutamyl cysteinyl synthetase regulatory unit
GSH	reduced glutathione
GSSG	oxidized glutathione
HIF	hypoxia-inducible (transcription) factor
INK4a/ARF	cyclin-dependent kinase inhibitor 2A/ alternative reading frame
LOH	loss of heterozygosity
MGMT	O^6-methylguanine DNA methyltransferase
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
NADPH	nicotinamide adenine dinucleotide phosphate
PDGF/R	platelet derived growth factor receptor

Prx	peroxiredoxin
PTEN	phosphatase and tensin homolog
RB	retinoblastoma gene
ROS	reactive oxygen species
RT	room temperature
SOD	superoxide dismutase
TGF	transforming growth factor
Trx	thioredoxin
TrxR	thioredoxin reductase
VEGF	vascular endothelial growth factor
WHO	World Health Organization

ABSTRACT

Malignant gliomas are a challenging entity of tumors. The treatment of gliomas has not proceeded dramatically inspite of advances in the treatment of many other solid tumors. However, research has revealed new approaches for the diagnosis and prognosis and introduced new treatment options. These cornerstones are a basis for breakthroughs in glioma treatment in the future.

In this thesis, new results from molecular biological and clinicopathological topics are introduced: the significance of several antioxidative enzymes in malignant gliomas, a potential therapeutic target molecule, carbonic anhydrase IX, in oligodendrogliomas and an effective way for analyzing gene amplification of epidermal growth factor receptor (EGFR) in astrocytomas.

In study I, the role of antioxidative enzymes manganese superoxide dismutase (MnSOD), thioredoxin (Trx), thioredoxin reductase (TrxR), gamma glutamyl cysteinyl synthetase catalytic unit (GLCL-C) and gamma glutamyl cysteinyl synthetase regulatory unit (GLCL-R) was studied in a series of oligodendroglial tumors. All of the studied antioxidative enzymes (AOEs) except for Trx showed expression in the majority of the tumor samples. However, when tumor cells expressed Trx, it was associated with higher tumor grade, increased proliferation index, increased apoptosis and decreased survival. MnSOD positive tumors were associated with decreasing survival, too. Based on this study it is concluded that at least Trx and MnSOD analysis could be usable in certain cases when more information is needed for prognosis. Antioxidative enzymes are likely to have a role in tumorigenesis, since their enzymatic target, free radicals, are one pathway in the initiation of cancer.

In study II, chromogenic in situ hybridization (CISH) was introduced as a useful diagnostic tool for EGFR amplifications in astrocytomas. EGFR amplification is one of the most common genetic lesions in grade IV astrocytomas, glioblastoma multiformes (GBMs). EGFR analysis is sometimes needed to distinguish primary and secondary GBMs from each other, since EGFR amplification is more characteristic for primary GBMs. EGFR amplification has also been associated with poor prognosis. In this study, a significant association between grade III astrocytomas with EGFR amplification and poor outcome was found, while grade IV astrocytomas did not have such a correlation. However, EGF receptor is a potential target molecule for targeted therapies. In conclusion CISH-analysis was found to be reliable, affordable and easy to put in practice in pathology laboratories.

In study III, carbonic anhydrase (CA) IX was studied in oligodendrogliomas. The majority of the tumor samples had positive expression for CA IX. Furthermore, the proliferation index (MIB-1) was decreased among CA IX positive tumors. Patients with CA IX positive tumors were also associated with decreased survival. CA IX is an attractive molecule for targeted therapies based on its many favorable characteristics. There are multiple studies ongoing for the use of CA targeted inhibitors and CA IX may prove to be usable in the treatment of gliomas, too.

In study IV, peroxiredoxins (Prx) I-VI were studied in a series of astrocytomas. The majority of tumor samples showed expression for Prx I and II, and even more often in recurrent tumors. Higher tumor grade was associated with decreased Prx I or Prx II expression. In a similar manner, Prx I and Prx II positive tumors were associated with decreased apoptotic activity. Patients with these Prx I and Prx II tumors were also

considerably younger and had better prognosis than others. Prxs seemed to have a different profile in gliomas than in normal brain tissue and they certainly represent interesting targets for further studies to evaluate their clinical value.

TIIVISTELMÄ

Aivokasvaimet ovat lisääntyvästä lääketieteellisestä tiedosta ja tekniikasta huolimatta vaikeasti hoidettava kasvainryhmä. Eritoten pahanlaatuiset glioomat ovat haastavia hoitaville lääkäreille ja tutkijoille. Vaikka syöpähoidot ovat kehittyneet monien muiden kiinteiden kasvainten osalta viime vuosikymmenien aikana merkittävästi, ovat glioomat yhä yksi huonoennusteisimmistä kasvaintyypeistä. Tästä huolimatta edistysaskeliakin on tehty. Tutkijat ovat onnistuneet saamaan uutta tietoa, jota käytetään diagnostisoinnissa ja ennusteen arvioinnissa. Myös uusia hoitomenetelmiä on kehitetty. Kipeästi tarvittavat läpimurrot aivokasvainten hoidossa perustuvat toivottavasti näihin tutkimuksiin.

Tässä väitöskirjassa esitellään uusia tuloksia kliinisen molekyylibiologian ja patologian tutkimussaralta. Kirjassa käydään läpi antioksidatiivisten entsyymien esiintymistä pahanlaatuisissa astrosytoomissa ja oligodendroglioomissa, esitellään uusi lupaava täsmähoitoihin soveltuva kohdemolekyyli hiilihappo anhydraasi IX oligodendroglioomissa ja esitellään tehokas tapa epidermaalisen kasvutekijäreseptorin geenin amplifikaation todentamiseen astrosytoomissa.

Ensimmäisessä osatyössä tutkittiin viiden eri antioksidatiivisen entsyymin esiintymistä oligodendroglioomissa: tutkittavat entsyymit olivat MnSOD, Trx, TrxR, GLCL-C ja GLCL-R. Näistä entsyymeistä kaikki paitsi Trx ilmentyivät suurimmassa osassa tutkittuja aivokasvainnäytteitä. Vaikka Trx ei ilmentynytkään yhtä yleisesti kuin muut tutkitut entsyymit, kävi ilmi, että näytteet joissa Trx esiintyi, olivat yleensä pahanlaatuisempia, niissä oli enemmän solujakautumisia, vähemmän apoptoosia ja huonompi ennuste. Myös tuumoreilla, joissa MnSOD ilmentyminen oli selvää, oli yhteys

huonompaan ennusteeseen. Näin ollen vaikuttaa siltä, että Trx:n ja MnSOD:n ilmentyminen voisi antaa lisätietoa etenkin jos ennustetta halutaan tarkentaa. Lisäksi antioksidatiivisilla entsyymeillä lienee vielä selvittämätön osuus kasvaimen kehityksessä, sillä niiden entsymaattisena kohteena olevat vapaat happiradikaalit ovat yksi tärkeä syövän syntyyn altistava tekijä.

Toisessa osatyössä kromogeeninen *in situ* hybridisaatio (CISH) todettiin käyttökelpoiseksi työvälineeksi EGFR geenin monistumisen osoittamiseksi myös astrosytoomissa. EGFR amplifikaatiot ovat yleisimpiä geneettisiä häiriöitä, jotka ovat todennettavissa gradus 4 astrosytoomissa eli glioblastoomissa. EGFR amplifikaatioiden analysointi on eritoten tärkeää, kun halutaan erotella toisistaan primaariset ja sekundaariset glioblastoomat, sillä EGFR amplifikaatio on yleinen primääreissä ja toisaalta hyvin harvinainen sekundaareissa glioblastoomissa. Nämä kaksi alaluokkaa eroavat toisistaan merkittävästi ennusteen ja myös hoitovasteen suhteen. Lisäksi EGF reseptori on osoittautunut mahdolliseksi täsmähoitoihin soveltuvaksi kohdemolekyyliksi. EGFR amplifikaatioiden on jo aiemmissakin tutkimuksissa todettu liittyvän huonoon ennusteeseen. Myös tässä tutkimuksessa etenkin luokan III astrosytoomissa oli nähtävissä yhteys EGFR amplifikaation ja huonon ennusteen välillä. Tärkeimpänä tässä työssä kuitenkin oli osoittaa CISH-analyysin toimivuus. CISH todettiin luotettavaksi, tehokkaaksi ja kustannuksiltaan erittäin kilpailukykyiseksi vaihtoehdoksi EGFR amplifikaatioiden osoittamiseksi myös aivokasvaimissa.

Kolmas osatyö käsitteli hiilihappo anhydraasi IX:n (CA IX) esiintymistä oligodendroglioomissa. Kyseinen entsyymi oli todennettavissa suurimmasta osasta kasvainnäytteitä. Lisäksi CA IX positiivisissa kasvaimissa oli vähemmän

solujakautumisia kuin negatiivisilla verrokeilla ja yllättäen CA IX positiivisten kasvainten ennuste oli huonompi kuin muiden. CA IX on erittäin lupaava kohdemolekyyli täsmähoitoihin. Koska CA IX vaikuttaa olevan lähes yksinomaan syöpäsoluissa esiintyvä entsyymi, sen reseptoriin kohdistuva inhibitio on tulevaisuudessa mahdollisesti myös osa glioomien hoitoa.

Neljännessä osatyössä selvitettiin antioksidatiivisiin entsyymeihin kuuluvien peroksiredoksiinien esiintymistä astrosytoomissa. Prx I ja Prx II ilmentyivät suurimmassa osassa kasvainnäytteitä ja vielä useammin uusiutuneissa kasvaimissa. Pahanlaatuisimmissa kasvaimissa Prx I:n ja Prx II:n esiintyvyys oli vastaavasti vähentynyt ja samalla apoptoosien määrä oli suurempi. Prx I ja Prx II positiivisilla kasvaimilla oli yhteys parempaan ennusteeseen ja potilaan nuorempaan ikään. Muiden peroksiredoksiinien löydökset eivät osoittautuneet yhtä selviksi. Peroksiredoksiinien ilmenemis-profiili on erilainen normaalissa aivokudoksessa kuin aivokasvaimissa. Näin ollen on mahdollista, että niillä on myös vielä löytymätön rooli kasvainten muodostumisessa.

1. INTRODUCTION

During past decades the aim to understand the development of cancer and to find efficient treatments for it has only accelerated. All of that work has already led to remarkable results in the case of some cancers and, for example, the diagnosis and treatment of such common cancers as breast and prostate cancer have improved greatly. However, malignant brain tumors continue to have a reputation as a disease with difficult treatment and a bleak prognosis. Part of this reputation is based on the unchanged, poor prognosis of the most frequent malignant brain tumor, GBM (grade IV astrocytomas). Inspite of progress in anti-cancer drugs, radiation therapy and even surgical techniques, GBMs still are usually lethal within 9-12 months. Other astrocytomas and oligodendrogliomas have a better prognosis than GBMs, but better treatments are still desperately needed. The bony limited space with most sensitive, non-regenerative neural tissue sets limits to all treatments in the head area, not to mention heavy cancer-treatments. Surgical treatments are likely to damage the healthy tissue, too, which in this case may lead to permanent neurological symptoms. The blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier brings challenges to the delivery of anticancer treatment to the tumor site. The first is formed of tight junctions between endothelial cells of capillaries and the latter has tightly bound choroid epithelial cells accompanied with an active organic acid transport system. Furthermore, interstitial pressure in the brain slows down the molecular transfer to cells. Most molecules are not capable of penetrating these boundaries. Generally, molecules that are able to pass the barriers must be electrically neutral, lipid-soluble and small; unfortunately many otherwise potent chemotherapeutic

drugs do not fit in this category. Moreover, even if the cytotoxical agent meets these criteria, it is extremely hard to reach therapeutic drug concentrations and maintain it within the proximity of tumorous tissue. In addition, neural tissue is very sensitive for radiotherapy resulting as cell death of surrounding neural tissue, but also a higher risk for radiation-induced tumors in site. These special limitations make high demands for the use of only carefully planned treatments and only for those tumors that are known to respond.

Considering all of this, it is understood that efficient treatment is based on thorough knowledge on tumor biology. This requires not only accurate diagnosis but also tools to affect tumor behavior. It is emphasized that histopathological examination may not be accurate enough and genetic and molecular pathologic analysis of tumors are being used to complete diagnosis. These analyses are also a base for targeted treatments, attempts to affect only those cells that express certain cancer-cell-specific features, preferably on their surface. This would minimize the damages to surrounding cells and still reach a greater proportion of cancerous cells. However, it has proven difficult to find such targets because no subset of malignant brain tumors, including astrocytomas and oligodendrogliomas, has been shown to be dependant on one oncogene or one pathway in tumorigenesis. Many gene therapies and targeted molecular therapies have their limitations because their target products are resulting from mutation that also makes the treatment unstable, as the genetic data can be varying from cell to cell. In addition, even characteristic features in certain cancer cells can be resulting from changes in very different sites along the molecular pathways. So far it seems that future attempts to develop more effective treatments rely on accurate diagnosis, tools to estimate prognosis

and development of combined therapies; an equation that is more than its several factors alone.

2. REVIEW OF THE LITERATURE

2.1. Malignant brain tumors

Brain tumors are tumors with a grim reputation. Even those cases that are classified as benign are severely interfering with normal brain function, let alone the malignant tumors. In Finland and other Nordic countries as well, central nervous system cancer accounts for approximately 3% of all cancers and the incidence of central nervous system malignancies is 10.6-12.3/100.000 (males-females; Age standardized world incidence per 100.000), incidence tending to increase with age. They account for approximately 3.5% of all cancer-related deaths in Finland (NORDCAN, 2008).

Malignant gliomas are the most common tumors originating in the human nervous system. Traditionally they are divided into subtypes based on their histological features and similarities with differentiated glial cells. A majority of them display morphology resembling either astrocytic or oligodendroglial cells. They are aggressive, invasive and destructive and they have poor prognosis, even among most other malignancies. The most malignant manifestation is World Health Organization (WHO) grade IV GBM, which continues to have a median survival of less than one year, despite all of the progress in surgical techniques, radiation therapy and anticancer-drugs. The grading system of malignant brain tumors is introduced in *Table 1*.

Table 1. Grading system of malignant gliomas by the criteria of WHO.
(eds Louis et al. 2007)

WHO grade	WHO designation (St.Anne/Mayo designation)	Histological criteria
I	Pilocytic astrocytoma	
II	Diffuse astrocytoma (Astrocytoma grade 2)	Nuclear atypia
III	Anaplastic astrocytoma (Astrocytoma grade 3)	Nuclear atypia and mitotic activity
IV	Glioblastoma multiforme (Astrocytoma grade 4)	Nuclear atypia, mitoses, endothelial proliferation and/or necrosis
II	Oligodendroglioma	Nuclear atypia, occasional mitoses
III	Oligoastrocytoma	Nuclear atypia, mitotic activity and prominent microvascular proliferation, occasionally necrosis

2.1.1. Tumorigenesis

Cancer represents an uncontrolled proliferation of cells. According to the first theory attempting to explain gliomas it was thought that central nervous system (CNS) cancer cells arise from glial cells and thus resemble their precursor cells in varying degrees. This resemblance enables specific histopathological diagnosis and also predicts the potential behavior of the neoplasm. This theory suggests that neoplasms of the CNS, as well as other locations, are derived from initially normal cells that maintain or regain a proliferative capacity. Thus, as it is known, mature neurons do not give rise to tumors, but glial cells surrounding them, like astrocytes and oligodendroglial cells that remain proliferative, would be susceptible to tumorigenesis under certain kind of stimulus. However, this stimulus is rarely fully identified and furthermore, tumorigenesis of glial cells is unlikely to result from just one stimulus (eds Louis et al 2007).

Another theory is based on recent findings that suggest that there may actually be cancer stem cells that are responsible for several tumor types, including brain tumors.

These stem cells are portrayed as very primitive cells, resembling embryonic cells and neural stem cells that still are capable of potent mitotic activity and self-renewal. This theory gives a good explanation for the recurrence of tumors and why certain tumors seem to be out of reach for even advanced therapies (Singh et al 2004). Even if the tumor cells are originating from stem cells, they still have features that resemble the cells in the surrounding tissue – in both theories this resemblance is the key factor for prognosis and treatment options.

The molecular background of tumors involves inactivation of normally functioning tumor suppressor genes and activation of normally silent proto-oncogenes. The first specific gene alteration in a human central nervous system tumor was reported in 1985: EGFR gene amplification was found in GBMs (Libermann et al 1985). Since that, many genetic abnormalities and alterations have been revealed and neuro-oncology research has attained a good deal of new information on tumorigenesis of CNS tumors. As is the case for all human cancers, the genes that are altered in CNS tumors can be grouped into oncogenes and tumor suppressors (Klein 1988). Oncogenes, like EGFR, produce proteins that promote cell proliferation and other characteristics important to tumor growth, such as invasion, angiogenesis and resistance to apoptosis. Oncogenes can be activated through different genetic mechanisms, including mutation, amplification and translocation, leading to increased synthesis of their corresponding protein. In CNS tumors oncogene activation is mainly caused by a gene amplification. In a manner opposite to oncogenes, tumor suppressor genes, such as T*P53*, inhibit cell growth. A deletion or a loss of heterozygosity on such a gene leads to disturbance in cell cycle and uncontrolled growth of cell population (Weinberg 2007, eds Louis et al 2007).

Since the knowledge of different triggers for tumorigenesis is constantly increasing, it is extremely important to perceive the logical steps that tumorigenesis has regardless of the means. These steps, or hallmarks, may be reached by different ways, but it is remarkable that whatever countless mechanisms lay behind it, they virtually all lead to six capabilities, *Figure 1*. Hanahan and Weinberg (Hanahan and Weinberg 2000, Weinberg 2007) have described these hallmarks and they are

1) *Self-sufficiency in growth signals. Mitogenic signals are extremely accurately regulated in normal cells. However, tumor cells constantly show reduced dependence on such signals. Many cancer cells seem to be able to synthesize growth signals in a self-supporting way, creating endless positive feedback signals. This can be achieved at least by overexpression of growth factor receptors or by unbalancing the extracellular matrix receptors (integrins) in favor of progrowth signaling. The central cascade that is involved seems to be the Ras-protein that can be affected by various ways. Furthermore, there is evidence that cancerous cells enslave adjacent normal tissue cells, especially inflammatory cells and fibroblasts, enhancing them to produce more growth signals. Activation of oncogenes is a typical genetic change triggering this hallmark.*
2) *Insensitivity to anti-growth signals. Normal cells are constantly regulated by antigrowth signals that will lead them to a quiescent state, waiting to proceed to replication if needed, or a permanently non-proliferative state. A majority, if not all, antigrowth signals are monitored by the retinoblastoma protein (pRb) (and its two near relatives). The pRb activity is affected by means of phosphorylation. In tumors, factors that are responsible of this phosphorylation are disrupted by*

downregulation or mutation of their receptors. These targets for distribution are various, including transforming growth factor β (TGFβ), p15^{INK4B}, p21, cyclin-dependent kinase 4 (CDK4), c-Myc oncoprotein etc. Many tumor suppressor genes are typically inactivated for this hallmark.

3) *Evading apoptosis. Sensors and effectors of the normal cell are monitoring the balance of both extracellular and intracellular environments. They are composed of ligand-receptor pairs. Apoptosis can be triggered by multiple factors, including deoxyribonucleic acid (DNA) damage, oncogene activation, hypoxia, insufficient cell-matrix and cell-cell contact –mediated survival signaling etc. FAS ligand and its receptor, as well as TNFα and its receptor are well-described death-signaling ligand-receptor pairs. They are closely connected to mitochondria released cytochrome C that is a potent catalyst of apoptosis. They both can activate caspases that start the final cascade of cell destruction. In tumorigenesis a very common mutation is the tumor suppressor gene p53. p53 tumor suppressor protein is important because it regulates proteins from the Bcl-2 protein family (including Bax), which in turn are major stimulants for cytochrome C. Another important pathway in escaping apoptosis is the activation of survival signals producing p13 kinase-AKT/PKB pathway, commonly associated with the loss of tumor suppressor gene phosphatase and tensin homolog (PTEN). The capacity to avoid apoptosis often marks the difference between benign and malignant growth.*
4) *Limitless replication. There is evidence that the self-sustained growth signals are not enough for expansive tumor growth. There are many factors that limit cell multiplications and for most cells there are only a limited number of replications*

they are capable of. After that they stop growing, independent of surrounding growth signals. This is associated with the erosion of telomeres, the safety-seals on the end of the chromosomes. After a certain number of doublings, these telomeres are eventually depleted, leaving the chromosomal DNA unprotected and leading to apoptosis. Tumor cells seem to have a capacity to maintain telomeres. At least $p16^{INK4A}$ cell cycle inhibitor is associated with maintaining of the telomere, however there are certainly many factors affecting this machinery. One candidate, Ras-oncogene is commonly activated in tumors.

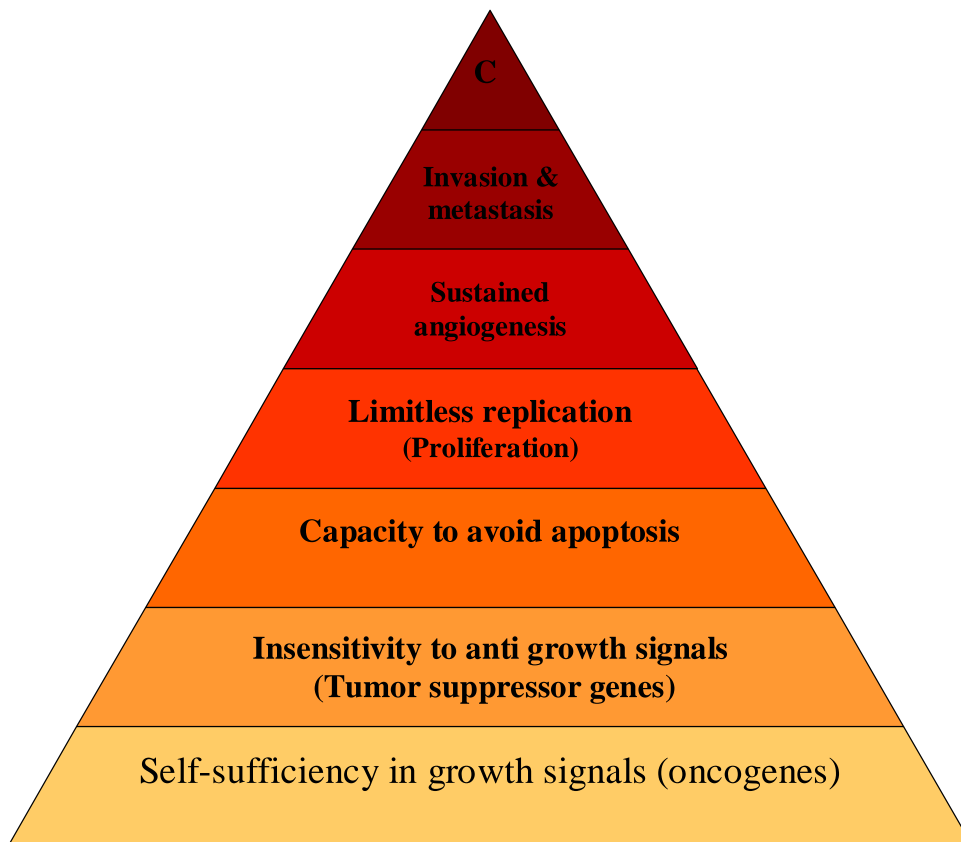
5) *Sufficient angiogenesis. Angiogenesis is crucial for every tissue in the human body and there is strong evidence that without sufficient angiogenesis tumors can not continue to grow very long: in fact, angiogenesis is the one step that is needed not later than in the mid-stage of tumorigenesis, preferably even earlier. Angiogenesis is regulated through a balance of inductors and inhibitors. Signals initiating angiogenesis include vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) 1 and 2. The gene transcription of these factors is often altered in tumor tissue, and the resulting growth factors are expressed in increased amounts. Alternatively, or often simultaneously, the angiogenesis inhibitors are downregulated. Loss of p53 function is associated with downregulated inhibitors.*

6) *Invasion and metastasis. Malignant brain tumors seldom metastasize outside of the CNS, but they are highly invasive. The capacity for invasion and metastasis is the final hallmark. This capacity is the cause of the majority of cancer deaths. Cadherins are molecules that are responsible for cell-cell adhesions, while*

integrins create interaction between cell and extracellular matrix. They both can be targets for changes in tumorigenesis. One common change is the loss of E-cadherin function. It can be inactivated through mutation, its transcription can be disturbed or its extracellular domain can be proteolyzed: several pathways for one goal. Once again, tumor cells can also enslave surrounding normal cells, and stimulate them to secrete the needed proteolyses (Hanahan and Weinberg 2000, Weinberg 2007).

Below is a simplified diagram of these hallmarks. There are a large number of cancer-associated genes and molecules, leading to a limited number of regulatory functions. For intervening with tumorigenesis it is crucial to know the pathways and simultaneously recognize the hallmarks that these pathways are leading to. It may prove to be more applicable to directly affect the hallmarks rather than each and every genetic change present in each tumor, since those changes may vary greatly from tumor to tumor and even within one tumor.

Figure 1. The pyramid of tumorigenesis. Cancer cells share certain acquired capabilities, hallmarks of cancer. There are numerous ways to achieve them since the molecular machinery is sophisticated and versatile. Tumorigenesis is a process with multiple steps, sometimes certain steps can be triggered by one particular genetic lesion, and sometimes a particular step is reached only through the coexistence of different genetic lesions. However, even if there are many traits leading to these essential six hallmarks, and even if the chronology of them may vary, these hallmarks are acquired by virtually all known human malignant cancers. Furthermore, each of these hallmarks represents a feasible target for intervening with tumorigenesis for preventative or therapeutic purposes. C on the top stands for fully functioning cancer.



2.1.2. Astrocytomas

Astrocytomas account for the majority of brain tumors and comprise a wide range of neoplasms that differ in their location within the CNS, histopathological features, biological behavior, growth potential, tendency for progression and clinical course. These

differences are most likely to reflect the type and sequence of genetic alterations during tumorigenesis. Clinically they range from relatively slowly growing diffuse astrocytoma (grade II) to rapidly and destructively growing GBM. They have a tendency for malignant progression, GBMs representing the most malignant phenotype. Pilocytic astrocytoma (grade I) arises mainly in children and differs from diffuse astrocytomas in the manner that it is quite benign and hardly ever undergoes malignant transformation. It too can affect vital neurological functions depending on location. Furthermore, pilocytic astrocytoma often recurs after resection. Nevertheless it is often handled as separate entity, justified by different clinical course and tumor behavior.

Histological criteria for diffuse astrocytoma are nuclear atypia, for anaplastic astrocytoma (grade III) nuclear atypia and mitotic activity and for GBMs the above-mentioned features and endothelial proliferation and/or necrosis, *Table 1*. Diffuse astrocytomas grades II and III are known to invariably progress to more aggressive subtypes, ultimately to GBMs if not fatal before. Mutation of T*P53* tumor suppressor gene is quite common (30-40%) in both grades and alterations in *pRb* are increasing with the tumor grade. Furthermore, loss of *cyclin-dependent kinase inhibitor 2A* (*INK4A*, also known as *p16*) is also frequent in grade III astrocytomas (50%). *INK4a* is closely connected with *pRb*, *Table 2 and Table 3*. They both are part of the antiproliferative machinery of the cell, making the cell insensitive to essential cell cycle signals when disturbed (Hanahan and Weinberg 2000, Kitange et al 2003).

GBMs are divided into two subgroups, primary and secondary glioblastomas. Primary or 'de novo' glioblastomas are considerably more common (>90% of GBMs). They arise after a short clinical history, usually less than 3 months, and the clinical course

is very aggressive. They typically develop in elderly patients (mean 62 years) and the median survival time is less than 5 months. It has been suggested that particularly primary GBMs are arising from the malignant transformation of either a bipotential precursor cell or a neural stem cell, rather than being a result of de-differentiation of mature astrocytes, *Figure 2*. There is some evidence supporting this theory. For example, a small subpopulation of cells with stem cell-like properties has been found from GBM tumors with the capacity for self-renewal as well as tumorigenic behavior. However, there is no direct proof yet presented.

Secondary glioblastomas are less frequent (<10% of GBMs) and they develop through malignant progression from grade II or grade III astrocytoma. A majority of lower grade diffuse astrocytomas transform into a more malignant phenotype within 5-10 years of the first diagnosis, regardless of the prior therapy, and then behave clinically like the higher-grade tumors. Secondary GBMs are more typical of younger patients (mean 45 years). Apparently due to this, the median survival is almost 8 months and thus notably longer than in primary GBMs.

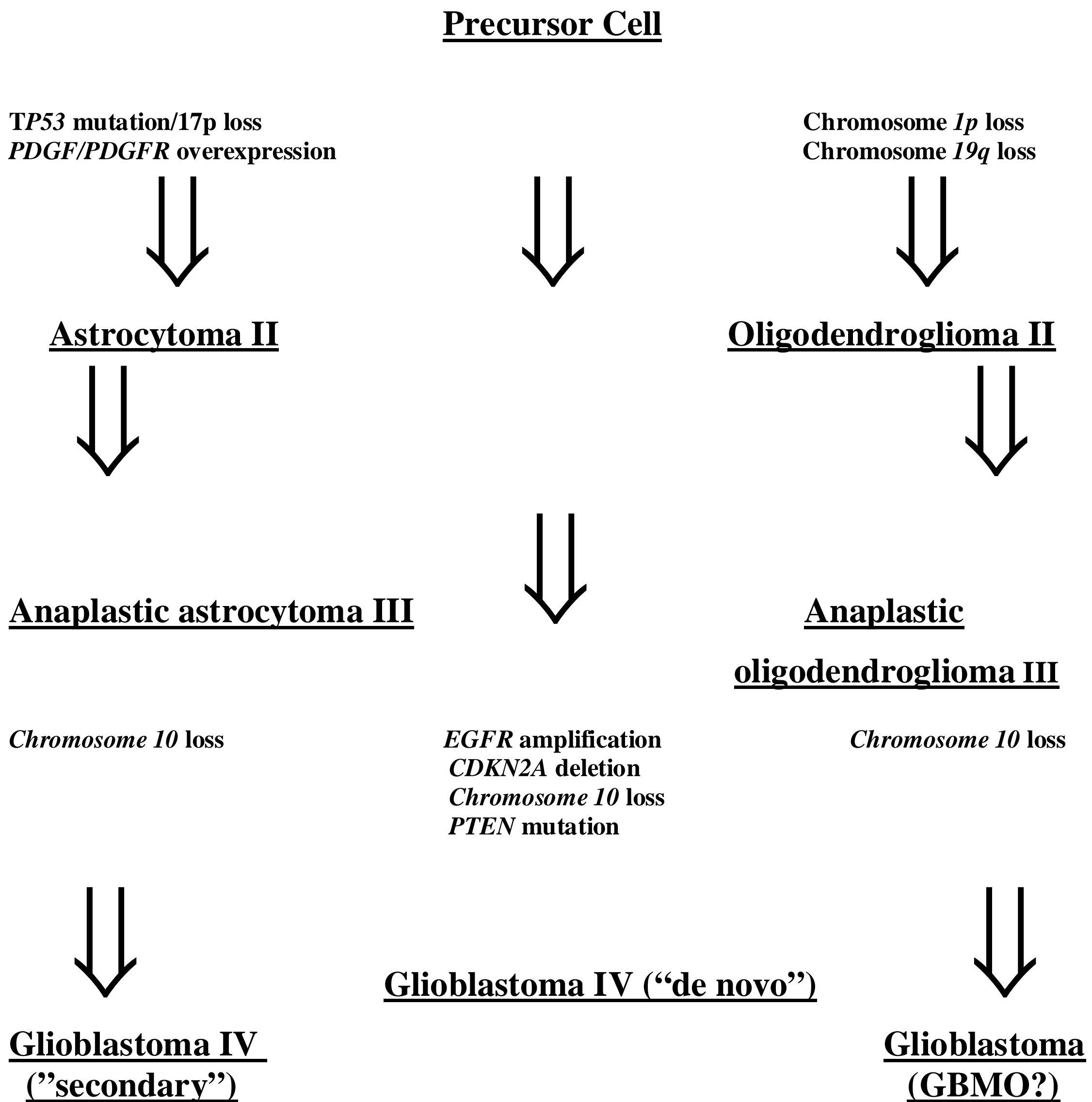
It has been proposed that primary and secondary GBMs should be considered as two distinct clinical entities with two divergent genetic pathways. It is clear that they do have differences in their genetic profiles; for example, a majority of primary GBMs have EGFR amplification (~ 40%) and/or overexpression (~ 60%), while secondary GBMs have EGFR amplification/overexpression rarely. Furthermore, while in primary GBMs T*P53* mutations are rare (~10%), in secondary GBMs T*P53* mutation are quite frequent (>65%). Interestingly, these common genetic changes are mutually exclusive events and hardly ever overlap. Furthermore, another important genetic lesion seen more commonly

in primary GBMs compared to secondary ones is the mutation of the *PTEN* tumor suppressor gene, located on *chromosome 10*. Mutation of *PTEN* ultimately leads to the activation of Akt, a member of antiapoptotic signaling pathway (Hanahan and Weinberg 2000). It is associated with poorer survival, induced angiogenesis, migration and invasion. In addition to the mutation/loss of *PTEN*, genetic losses on other parts of chromosome 10 are the most frequent genetic alteration in primary GBMs. Altogether these genetic lesions in *chromosome 10* add up to 80-90% and it has been debated if loss of heterozygosity (LOH) *10* is not actually an independent prognostic factor, but a requirement for GBM phenotype (Hill et al 2003).

Table 2. Association between gene alterations and malignancy grades in astrocytic brain tumors.

Astrocytoma grade	Grade II	Grade III	Grade IV
Affected gene (frequency)	T*P53* (30-40%)	EGFR (10-15%) CDKN2A (30%) T*P53* (30-40%) p14^{ARF} PTEN (10%) MDM2 (5%) CDK4 (10%)	EGFR (30-40%) CDKN2A (30-40%) T*P53* (20-30%) p14^{ARF} PTEN (25-30%) MDM2 (5-10%) CDK4 (10-15%)

Figure 2. Diagram of genetic alterations common to malignant transformation of glial tumors.



2.1.3. Oligodendrogliomas

Oligodendrogliomas are rather slowly growing tumors of the white matter and cortex. A majority of oligodendrogliomas arise in middle-aged people and in males slightly more frequently than in females. Generally oligodendrogliomas occur locally in the cerebral hemispheres, the frontal lobe being the most frequent place of occurrence, yet they may develop in any location throughout the neuraxis. Although oligodendrogliomas are clinically less aggressive than astrocytomas, they are invasive and are able to traverse into the cerebral spinal fluid (CSF) (eds Louis et al 2007).

Histological examination reveals a moderately cellular tumor with uniformly round, homogenous nuclei. In paraffin sections a typical, although artefactual, swollen and clear cytoplasm can be seen. Sometimes microcalcifications and mucoid/cystic degeneration can be seen and a dense network of capillaries can form a characteristic vascular pattern. Nuclear atypia and occasional mitotic activity are compatible with WHO grade II oligodendroglioma, while more frequent mitosis, prominent microvascular proliferation and necrosis correspond to the WHO grade III anaplastic oligodendrogliomas, *Table 1*.

The differential diagnosis of oligodendrogliomas includes fibrillary astrocytoma and tumors with round nuclei, including clear cell ependymoma and central neurocytoma. Making an accurate diagnosis is not only a challenge but also essential, since the clinical course and therapeutic approach to oligodendrogliomas differs from that of other malignant brain tumors, mainly because oligodendrogliomas are relatively chemosensitive. A useful diagnostic and prognostic tool is the analysis of chromosomes *1p* and *19q*. Loss of heterozygosity (LOH) on *1p* and *19q* are the most common genetic

alterations in oligodendroglial tumors, *Table 3*. It occurs in 50-70% of both low-grade and anaplastic oligodendrogliomas suggesting that it represents an early change in tumorigenesis of oligodendroglioma (eds Louis et al 2007). *1p* and *19q* loss makes a good signature for oligodendrogliomas, because they are tightly associated with one another, and at the same time they are a quite rare alteration combination in other tumors (Cairncross et al 1998, Ino et al 2001). Furthermore, combined loss of *1p* and *19q* or isolated loss of *1p* are shown to be significantly associated with both chemosensitivity and longer recurrence-free survival after chemotherapy and genetic testing is clinically widely used in diagnostic and prognostic purposes (Cairncross et al 1998, Smith et al 2000).

2.1.4. Oligoastrocytomas

Oligoastrocytomas are tumors composed of neoplastic cells morphologically resembling the tumor cells in oligodendroglioma and diffuse astrocytoma. When nuclear atypia and occasional mitoses are present, the tumor corresponds histologically to WHO grade II. If increased cellularity, pleomorphism and frequent mitoses are present, as well as other features of increased malignancy (prominent angiogenesis, necrosis) the tumor is graded as grade III. The diagnosis of oligoastrocytomas is based on recognition of two different neoplastic glial components. In the literature, there are no specific percentages of the proportion of these two components to qualify the tumor as an oligoastrocytoma, but it is suggested that there should be less than 50% of astrocytic cells in the tumor (eds Louis et al 2007).

The chromosomal changes found in oligoastrocytomas are often a combination of common alterations in both oligodendroglial and astrocytic tumors, *Table 3*. LOH on *1p* and *19q* is commonly found, and like in oligodendrogliomas, has a favorable effect on chemosensitivity and prognosis. *P53* mutation is frequently found in low-grade astrocytomas and oligoastrocytomas. Furthermore, similarly to higher grade astrocytomas, the loss of *INK4A* is often observed (40%). However, the molecular genetic basis of oligoastrocytomas remains less well defined, mainly because the diagnosis is, after all, subjective (eds Louis et al 2007).

In the literature there is an additional mention of malignant astrocytoma with small cell phenotype, histologically resembling closely anaplastic oligodendroglioma or oligoastrocytoma. However, clinically it resembles GBMs and the wrong diagnosis can greatly mislead therapeutic decisions and prognosis. It can be distinguished from a more favorable diagnosis of oligodendroglioma, since it lacks the loss of *1p* and *19q* and in addition it frequently displays the loss of *chromosome 10q* (Fuller and Perry 2005). However, a rare minority of GBMs contains areas showing oligodendroglioma-like tumor cell differentiation and loss of *1p* and *19q*. Although these tumors are not considered as a distinct disease entity, they are not of importance. These tumors have been described as glioblastoma with oligodendroglial component (GBMO). GBMO may represent the most malignant form in the oligodendroglial lineage, *Figure 2*. The oligodendroglial component and combined loss of chromosomal arm *1p* and *19q* in GBM seems to indicate better prognosis via increased chemosensitivity and radiosensitivity (Vordermark et al 2006).

Table 3. Relationship between median survival, histological features and major genetic changes associated with each tumor.

	Low-grade Oligodendroglioma/ oligoastrocytoma	Anaplastic oligodendroglioma/ oligoastrocytoma	Low-grade Astrocytoma	Anaplastic astrocytoma	Glioblastoma
Survival (years)	**5-10**	**3-5**	**5-10**	**2-3**	**<1**
Proliferation	+/-	+ +	+/-	+ +	+ + +
Invasion	+	+ +	+ +	+ +	+ + +
Angiogenesis	-	+/-	-	-	+ + +
Necrosis	-	+/-	-	-	+ + +
Genetic lesions	***-1p***[d] ***-19q***[d]	***-1p***[d] ***-19q***[d]	***-p53***[m] **-PDGF/R over-expression**	**-RB**[m] **-CDK4**[a] **-INK4a/ ARF**[d] **-PTEN**[d] **-DMBT1/ mxi**[d] ***-19q***[d] ***-11p***[d]	**-EGFR**[a,m] **-INK4a/ ARF**[d] **-PTEN**[d] **-RB**[m]

Type of gene alterations: [a] = amplification, [d] = deletion, [m] = mutation
- = not present; +/- = if present, only very weakly; + = weakly present; ++ = present moderately; +++= present strongly

2.1.5. Histopathological diagnosis and genetic analysis

Histological diagnosis and additional genetic analyses can be thought of as a reflection of the true biological behavior of a tumor. Most of the treatment options have side effects that limit their use to tumor entities that are known to be sensitive for the

selected treatment. Thus factors that can reliably help to choose optimal therapies as well as predict the course of illness are needed. The basis of good treatment is to identify the illness (histological diagnosis, tumor grade), recognize the factors influencing its phenotype (protein overexpression, genetic changes) and be aware of characteristics that might have an effect on the outcome (specific proteins, genotype, for instance *1p* and *19q* deletions in oligodendroglial brain tumors).

Histopathological analysis will certainly remain as a cornerstone of the diagnosis of malignant gliomas. However, it is also evident that efficient and cost-effective tools for further analysis are needed. As treatment options develop there will be an increasing need to predict more accurately the clinical behavior of individual tumors. In recent years microarrays and fluorescence in situ hybridization tissue arrays have been offering new opportunities to obtain genetic information with reasonable efforts and costs. Other testing techniques include Western, Southern and Northern blots, reverse transcriptase polymerase chain reaction, PCR, DNA sequencing, microsatellite analysis, comparative genomic hybridization, etc (Fuller and Perry 2005). It is clear that there is a growing need for molecular analysis in the future. A good example is the analysis of chromosome *1p* and *19q* deletions, since the results affect greatly not only diagnosis, but more importantly the therapeutic decisions.

Genetic changes behind malignant transformation are multiple and, furthermore, it seems that tumors that result from one specific change are in vast rarity. However, some changes are present in different frequencies in different subtypes of gliomas, and moreover, the frequencies of some genetic alterations are changing along with the tumor grades. EGFR amplification or mutation is present in approximately 10-15% of grade III

astrocytomas, whereas in grade IV GBMs up to 40% of tumors have EGFR gene alteration. Similarly, gene alteration of *PTEN* is present in 10% of grade III, but the frequency at least doubles in GBMs. Respectively, up to 40% of grade II and III astrocytomas have T*P53* mutation, while it is present in only approximately 20% of primary GBMs.

2.1.6. Prognostic factors

Tumor grade. Like in most of the solid tumors, WHO grade in malignant gliomas is composed of multiple factors that together give a rough estimation of the outcome of tumor subtypes. These factors may vary between each tumor entity, but it typically contains assessment of proliferation, differentiation and general morphology of tumor cells. The more specific prognosis that is wanted, the more information about other factors, genetic and clinical findings, is needed. However, the tumor grade is quite reliable on its own too; patients with WHO grade II tumors are likely to survive more than five years while on the opposite extreme are patients with grade IV malignant gliomas that seldom have a life expectancy for more than one year (eds Louis et al 2007).

Age. Patient's age at the time of diagnosis seems to be a rather good prognostic marker. Younger age has been associated with a more favorable clinical course in low-grade astrocytomas, GBMs and oligodendrogliomas, as well as oligoastrocytomas (eds Louis et al 2007).

Tumor size and gross total resection. Large tumor size is a negative predictor. The attempt to reach gross total resection as precise as possible is justified by

unequivocal association with longer survival in malignant gliomas. Infiltrative growth to the adjacent normal brain tissue is very typical for gliomas, thus the macroscopic estimation of tumor size and gross total resection are very difficult (eds Louis et al 2007).

Proliferation. Increased proliferation index (>5% MIB-1 positive cells) has a distinct correlation with clinical outcome in low-grade astrocytomas and oligodendrogliomas predicting shorter survival (eds Louis et al 2007).

Necrosis. Extent of necrosis is associated with shorter survival in all tumors. The presence of necrosis is also taken into account while grading tumors and it is associated with grade III oligodendrogliomas and IV GBMs. Especially large necrotic areas resulting from insufficient blood supply, i.e. ischemic necroses, are typical for primary GBMs and correlate with poor clinical outcome (Tohma et al 1998, Louis et al 2007).

EGFR amplification. Epidermal growth factor receptor amplification has been connected with shorter survival in anaplastic astrocytomas (eds Louis et al 2007).

T*P53* mutation. The prognostic value of T*P53* mutation remains contradictory. In some studies the presence of T*P53* mutation has been suggested to be a favorable prognostic factor in GBMs, while in low-grade astrocytomas it has been suggested to accelerate progression of the tumor even if not affecting outcome. Several large studies have not been able to find any association at all. However, *p53* is known to be one of the G1 checkpoint regulating tumor suppressor genes and also an apoptotic factor (eds Louis et al 2007).

LOH *10*. Loss of heterozygosity of *chromosome 10* is the most common genetic alteration in GBMs and is suggested to predict shorter survival. Furthermore, several candidates for tumor suppressor genes have been mapped to *10q*, especially *PTEN*. LOH

10q seems to be exclusive of LOH *1p* and *19q*, since they extremely seldom are present in the same tumor (Zhou et al 1999, Louis et al 2007).

LOH *1p* and *19q*. Loss of chromosome *1p* or combined loss of chromosomes *1p* and *19q* are suggested to appear in particularly slowly growing oligodendrogliomas and thus making the prognosis significantly better. However, *1p* and *19q* loss is associated with better prognosis also in oligoastrocytomas and anaplastic oligodendrogliomas, even though it is associated most often with typical histological features of oligodendrogliomas. Moreover, loss of *1p* and *19q* has been associated with better response to chemotherapy and longer recurrence-free survival in all subtypes of gliomas, even in rare case of GBMs expressing this particular loss (Hill et al 2003, Louis et al 2007).

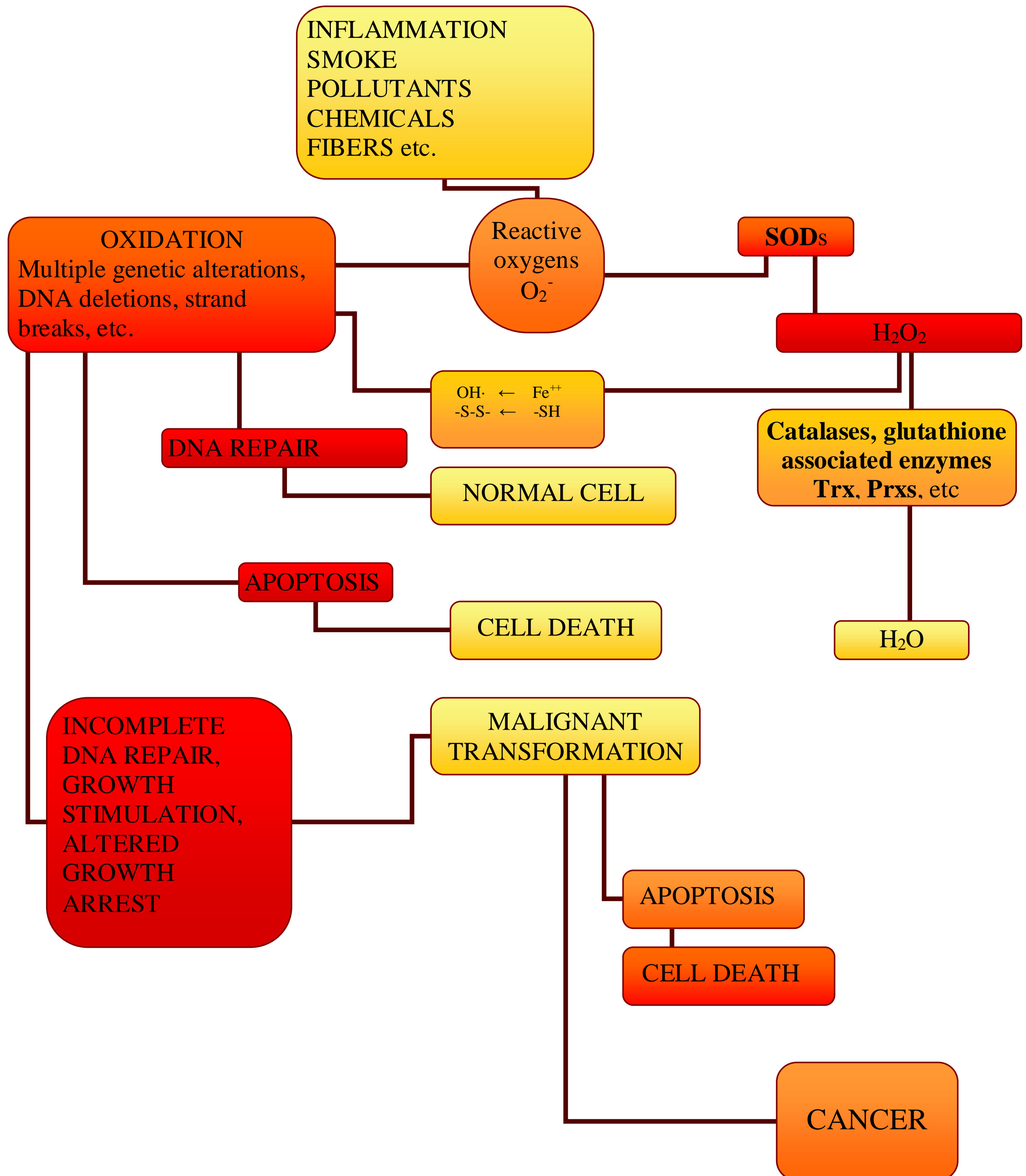
2.2. Antioxidative enzymes

Reactive oxygen species (ROS) are extremely reactive and unstable molecules, comprised of free radical oxygen, which has two unpaired electrons (Halliwell 1991). These unpaired electrons react eagerly and form compounds that are classified as ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and peroxynitrite. ROS are strongly oxidizing compounds and they are produced by various human cell enzyme systems. In particular mitochondrial oxygen metabolism is a major source of ROS. Furthermore, there are many factors including environmental pollutants, carcinogenic chemicals, chronic inflammation and smoking that expose cells to ROS. Under normoxic conditions the balance between oxidizing and reducing species within the cell is maintained carefully (Kinnula et Crapo 2004). Although they are part of normal cell

physiology, ROS are thought to play a role in initiation of carcinogenesis through DNA damage (Valko et al 2006). When these DNA damages lead to alterations in oncogenes or tumor suppressor genes, malignant conversion is inevitable. Furthermore, ROS have an effect on proliferation, transformation, apoptosis, angiogenesis and invasion. The boundaries of normal redox balance are very narrow and exposure to reactive oxygen species above those boundaries leads to a situation where DNA repair cannot be accomplished as fast as DNA damages are formed. If this exposure continues, proliferation accelerates at the expense of normal apoptosis due to ROS stimulation of TGFβ production and by the induction of tumor suppressor gene PTEN. This leads to dysplastic lesions and finally malignant cells. In addition, exposure to ROS stabilizes hypoxia-inducible (transcription) factor (HIF) and via this means causes an induction of many genes effecting angiogenesis, including VEGF. The synthesis of matrix metalloproteinase (MMP) is also stimulated, accelerating invasion of cancerous cells. Chronic and excess levels of ROS are toxic to even cancer cells, finally leading to apoptosis (Fruehauf et Meyskens 2007), and necrotic areas within the tumor. To date, the role of ROS in the pathogenesis of several malignancies is well established.

AOEs are vital for cells capacity to convert ROS to nontoxic compounds. Superoxide dismutases (SODs) are the only enzymes capable of transmuting ROS to hydrogen peroxide that is then further converted to water by various enzymes including catalases, thioredoxins (and thioredoxin reductases), peroxiredoxins, glutathione associated enzymes, etc, *Figure 3*. Metal-binding proteins (for example ferritin) and vitamins C and E are also part of these important antioxidative mechanisms of the cell.

Figure 3. Antioxidative enzymes in mammalian cells. This simplified diagram shows the vitally important role that antioxidative enzymes have to all mammalian cells. They convert hydrogen peroxide to water. If cell's capacity to convert ROS to nontoxic compounds is exceeded, it leads to apoptosis or in worst scenario initiates malignant transformation.

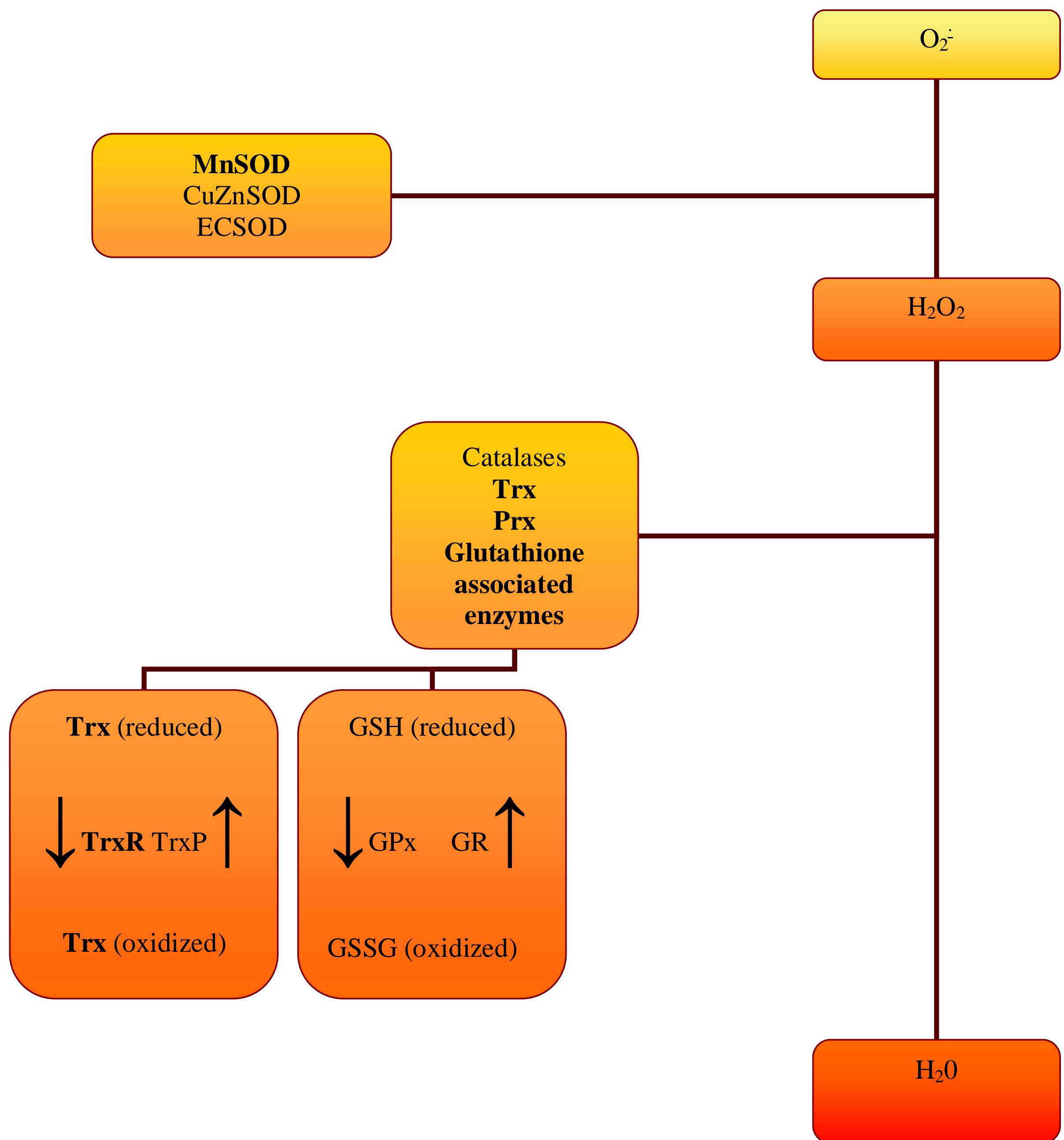


2.2.1. General aspects

AOEs main function is to neutralize toxic oxidants, such as ROS. This is achieved by detailed enzymatic reactions: Superoxide dismutases (SOD) catalyze the conversion of superoxide to hydrogen peroxide, which is further converted to water by catalase or reduced glutathione (GSH) peroxidase coupled with glutathione reductase. Trx coupled with TrxR as well as peroxiredoxins scavenge H_2O_2 and alky peroxides, using GSH as a substrate, *Figure 4*. As GSH plays a central role in maintaining redox balance, the ratio between GSH and oxidized glutathione is a good estimate of the redox buffering capacity of the cell (Schafer et Buettner 2001). This balance is particularly important because of its effects on regulation of signaling pathways and gene expression, the previous through changes in kinase and phosphatase activity and the latter through modulation of transcription factor function (Thannickal et Fanburg 2000, Biswas et al 2006).

The enzymes that are included in the group of AOEs have other functions besides antioxidation. Some of them are connected to changes in chemosensitivity and resistance to radiation therapy, others seem to have versatile roles including as growth factors or angiogenic factors. These properties are discussed below in a more detailed way.

Figure 4. The neutralizing of toxic oxygen radical to water by antioxidative enzymes. Elements in the multiple pathways scavenging superoxide and hydrogen peroxide include MnSOD, CuZnSOD, ECSOD, Trx, TrxR, Prxs, glutathione-associated enzymes like GLCL-C, and GLCL-R. GSH is reduced glutathione and GSSG is oxidized glutathione, GPx is glutathione peroxidase and GR is glutathione reductase. The enzymes that are debated on more closely in this review are on bold figures in the diagram.



$O_2^{\cdot-}$
MnSOD
CuZnSOD
ECSOD
H_2O_2
Catalases
Trx
Prx
Glutathione associated enzymes
Trx (reduced)
TrxR TrxP
Trx (oxidized)
GSH (reduced)
GPx GR
GSSG (oxidized)
H_20

2.2.2. Manganese superoxide dismutase

Superoxide dismutases are an important family of antioxidative enzymes that are the only mammalian enzymes that are capable of converting O_2^- to H_2O_2. These enzymes include cytosolic copper-zinc SOD (CuZnSOD), extracellular SOD (ECSOD) and mitochondrial manganese superoxide dismutase (MnSOD). As mitochondrial oxygen metabolism continuously produces O_2^-, it is obvious that this mitochondrial superoxide dismutase is essential to the vitality of all mammalian cells (Kinnula et Crapo 2003). This is also proved by different studies and, for example, MnSOD knockout mice die at the age of 10-21 days with cardiovascular and neuronal problems (Li et al 1995, Carlsson et al 1995). These results underline MnSODs' essential biological role in the brain and when the characteristics of neural cells are taken in the consideration, it seems evident that the neural cells are particularly vulnerable to reactive oxygen species.

MnSOD is an 88kDA homotetramer, encoded by a gene located at *6q25.3* (Church et al 1992), *Table 4*. It is notable that the promoter area of MnSOD contains binding sites for transcription factors such as AP-1, AP-2, SP-1 and NF-κB (Yeh et al 1998). This, at least for some parts, explains why MnSOD is induced by several stress factors, such as TNF-alpha, radiation, hyperoxia, interleukin-1, lipopolysaccharides and interferon-γ (Kinnula et Crapo 2003, Marklund et al 1982). Cytotoxic drugs also induce it, and this may reflect an important pathway for the development of drug resistance in tumor cells (Akashi et al 1998). Increased MnSOD expression has been reported in various malignancies, such as breast, esophageal, gastric, colon, lung and astrocytic tumors and mesotheliomas (Soini et al 2001, Cobbs et al 2003, Kinnula et al 1996, Kahlos et al 1998, Kahlos et al 2000a, Kahlos et al 2000b, Haapasalo et al 2003), and in some of them it has been correlated with poor prognosis.

2.2.3. Thioredoxin

Thioredoxins (Trxs) are a family of redox proteins with various properties. First of all, Trx is one of the major redox regulators. After reactive oxygen species are converted to hydrogen peroxide by superoxide dismutases like MnSOD, thioredoxins participate in the important cycle where H_2O_2 is further converted to H_2O. The two main thioredoxins are thioredoxin-1, a cytosolic and nuclear form, and less studied thioredoxin-2 that is located in the mitochondria. The different subcellular locations suggest that Trx may have different roles within cells: in addition to its important role as an antioxidative enzyme, it acts as a growth factor, inducer of cell proliferation, inhibitor of apoptosis and a cofactor for ribonucleotide reductase (Powis et Montfort 2001). Some of these roles include thioredoxin reductase (TrxR) in order to function properly. The necessity of Trx has been proven in several studies; mice with targeted disturbance of the Trx-1 gene die shortly after implantation when homozygous, while heterozygous mice seem to do fine. In the early stage of development Trx knockout is obviously lethal (Powis et Montfort 2001).

Thioredoxin is a compact globular protein, with a molecular weight of 12kDA. The gene for Trx is located on chromosome *9q32*, *Table 4*. Its activation is induced by oxidative stress, hypoxia, lipopolysaccharides, viral infections, X-radiation and UV irradiation.

When the versatile biological activities of Trx are viewed more closely, it seems quite clear that Trx and its expression may have some impact not only on normal cell functions but on malignant transformation, too. It is known to stimulate angiogenesis by increasing the secretion of VEGF as much as 41-79%. This phenomenon can be seen in normoxic conditions, and even more importantly in hypoxic conditions that are usually

the case in solid tumors (Welsh et al, 2002). Trx also stimulates cell proliferation. The exact mechanism of this is still unclear, but it appears to depend on the increased production of cytokines and by changes in growth factor activity (Powis et Montfort 2001). Furthermore, via redox balance, it influences several transcription factors, such as *p53*, NF-κB and AP-1 and AP-2 by influencing their binding to DNA (Nishinaka et al 2001). Trx itself binds to several cellular proteins and thus regulates their biological activity. When coupled with TrxR it affects DNA synthesis by having a critical role in the generation of deoxyribonucleotides, where Trx provides the electrons needed in the synthesis (Powis et Montforth 2001). With TrxR it also protects the cell against apoptosis with a so far unknown mechanism (Powis et al 1998). In tumors, Trx overexpression increases the expression of HIF-1α both in normoxic and hypoxic conditions. This seems to give the tumor cells an advantage in survival, as HIF-1α induces hexokinase and thus the metabolism of the tumor cells is provided with glycolysis as a predominant energy source (Nakamura et al 2000, Yasuda et al 2004). In addition, there is evidence with different tumor-cell lines that increased Trx expression increases the resistance of cancer cells to cytotoxic drugs (Powis et Montfort 2001). No wonder that several tumors, including mesotheliomas, lung, colorectal, cervical, hepatic, and pancreatic cancer, have been shown to express high levels of Trx and/or TrxR. Increased expression of these enzymes have been associated with poor prognosis (Soini et al 2001, Powis et al 1998, Powis et Montfort 2001, Welsh et al 2002, Raffel et al 2003, Kim et al 2003, Lincoln et al 2003, Han et al 2002, Hedley et al 2004, Choi et al 2002, Kakolyris et al 2001).

2.2.4. Thioredoxin reductase

The above-mentioned numerous functions of Trx cannot be sufficiently discussed without consideration of the functions of TrxR. TrxRs are selenocysteine-containing flavoenzymes, the only known enzymes capable of reducing the active site of Trx (Powis et al 1994, Holmgren et al 1995, Sun et al 1999, Mustacich et Powis 2000, Powis et al 2001). By itself this makes the role of TrxR indispensable for mammalian cells, and it has been suggested that TrxR could serve as a novel redox target for cancer therapy (Biaglow et Miller 2005, Hashemy et al 2006). Like Trx, also TrxR is known to have at least two isoforms, thioredoxin reductase-1 and -2. TrxR-1 is mainly cytosolic 54.4kDa homodimer, while TrxR-2 is 56.2kDa homodimer in mitochondria, *Table 4*. TrxR is able to undergo reversible oxidation-reduction in a similar manner to Trx (Powis et Montfort 2001).

2.2.5. Glutamate cysteine ligase, catalytic and regulatory unit

Glutathione is a tripeptide that, with several other AOEs, participates in the scavenging of H_2O_2 and transport of toxic metabolites through the cell membrane. Glutathione is responsible for a great part of oxygen metabolism and cell defense; it scavenges hydrogen peroxide both in cytosol and mitochondria. Glutathione peroxidase uses GSH as a co-factor in a reaction where two molecules of oxidized glutathione (GSSG) are produced for every molecule of H_2O_2. Then GSSG is re-reduced to GSH by glutathione reductase, with reduced nicotinamide adenine dinucleotide phosphatase (NADPH) as a co-factor.

The synthesis of glutathione is regulated by glutathione synthetase and γ-glutamylcysteine synthetase (i.e. glutamate cysteine ligase) (GLCL). GLCL is the rate-limiting enzyme in glutathione synthesis (Tew 1994). It consists of a heavy or catalytic (GLCL-C) and a light or regulatory (GLCL-R) unit. The genes for these heterodimers are located on chromosomes *6* (catalytic unit) and chromosome *1* (regulatory unit), *Table 4*. GLCL-C has a molecular weight of 73kDA, while GLCL-R weighs 30kDA. It has been suggested that under oxidizing stress not only are they both needed for enzymatically active compound, but also they are capable of conformation between subunits to change this enzymatic activity (Huang et al 1993). Via effects on glutathione synthesis GLCL-C and GLCL-R influence a variety of cellular events along with antioxidative and detoxification reactions, including signal transduction, regulation of cell proliferation, apoptosis, mitochondrial respiration, remodeling of extra-cellular matrix and regulation of the cysteine reservoirs of the cell (Rahman et MacNee 2000). GLCL-C knockout mice die soon after birth and therefore it can be assumed that GLCL-C is vitally important for the enzymatic function of the cell (Dalton et al 2000). Expression of GLCL has been scarcely studied in human tumors but its overexpression has been shown in lung cancer and mesotheliomas (Soini et al 2001, Järvinen et al 2002). Glutathione-associated enzymes have been shown to be also associated with the development of anticancer drug resistance (Tew 1994, Zhang et al 1998).

2.2.6. Peroxiredoxins

Peroxiredoxins (Prx) are a family of thiol-specific antioxidant enzymes that also have an influence in the signal transduction of mammalian cells by controlling cytokine-

induced peroxide levels (Wong et al 2000, Kang et al 1998, Wood et al 2003). Prxs are also thought to be associated with cell proliferation, apoptosis, differentiation, gene expression and resistance to radiation or chemotherapy (Chae et al 1999, Kim et al 2000, Sasagawa et al 2001, Park et al 2000, Chung et al 2001). So far six subclasses of peroxiredoxins from mammals, peroxiredoxin I-VI, have been introduced, *Table 4*. These are further divided into three classes according to the amount of redox-active cysteines the protein contains. PrxI, PrxII, PrxIII and PrxIV are so called 'typical' 2-Cys Prxs, containing two cysteines and equivalent structure and enzymatic mechanism; PrxV contains also two cysteines but diverges structurally from the aforementioned Prxs and is therefore known as 'atypical' 2-Cys Prx. PrxVI has only one cysteine and is often referred as 1-Cys Prx.

Prxs are produced at high levels in cells. In mammalian cells they compose 0.1-0.8% of the soluble proteins, excluding erythrocytes where they are even more abundantly produced (Chae et al 1999, Wood et al 2003). In the normal mammalian brain, immunoreactivity for different subtypes of Prxs in different types of neural cells is varying conspicuously. The localization of Prx I immunoreactivity is predominantly concentrated in the nuclei of oligodendrocytes. Concurrently PrxII has been found principally in the cytosol of most neurons of grey matter (Jin et al 2005, Sarafian et al 1999, Lee et al 2003). PrxII has also been found in the nuclei of medial habenular neurons (Jin et al 2005). These are involved in many important and even vital biological functions, and the nuclear localisation of PrxII indicates that it may have a role that goes beyond that of an antioxidative protein in normal neural tissue. Furthermore, PrxII seems to have several important independent functions in various other human cells. For example, lack of expression is known to damage severely the normal function of erythrocytes (Lee et al 2003).

In addition, the expression of Prx II is known to be aberrant in the pathogenesis of neurodegenerative disorders, including Alzheimer's disease, Pick's disease and Down syndrome (Krapfenbauer et al 2003). Elevated Prx II expression has also been reported at least in lung carcinoma and pleural mesothelioma (Lehtonen et al 2004, Kinnula et al 2002), and it is known to affect radiation sensitivity and drug resistance (Park et al 2000, Chung et al 2001).

In normal brain tissue Prx III immunoreactivity is concentrated in neurons especially in the hippocampal area, where it has a protective role against excitotoxic injuries (Jin et al 2005, Hattori et al 2003). The uneven concentration of Prx III immunoreactivity in the hippocampus results not only from its function, but derives from its subcellular localisation in mitochondria. It has been suggested that Prx III is a critical regulator of apoptotic signalling by virtue of its regulatory effect on the abundance of mitochondrial H_2O_2 (Chang et al 2004).

Moderate immunoreactivity for Prx IV in normal mammalian brain tissue has been reported in the cytoplasm of neurons while strong, mainly nuclear positivity is seen in oligodendrocytes (Jin et al 2005). This could mean that Prx IV has at least partially different roles in these cells. This is also the case with Prx V, which is found in normal neural tissue in the mouse brain and is known to have a protective role against excitotoxic brain lesions in newborn mice (Jin et al 2005, Plaisant et al 2003). Furthermore, Prx V is endowed with peroxynitrite reductase activity. This is highly significant to mammalian cells, since superoxide O_2^- is often accompanied by simultaneous production of nitric oxide NO, and when both are present, they rapidly form extremely toxic peroxynitrite $ONOO^-$. Its toxicity is based on further conversion to two potent radical species HO and NO_2 via haemolytic decomposition or to CO_3^- through a reaction with CO_2. So far Prx V and Prx

VI are shown to have peroxynitrite reductase activity; it is likely that other mammalian Prxs share this capability at least to some extent (Dubuisson et al 2004).

Prx VI immunoreactivity has been previously found in normal mouse astrocytes and oligodendrocytes. Interestingly, the Prx VI protein is expressed in the nuclei of astrocytes and oligodendrocytes, although Prx VI is known to be cytosolic in other mammalian cells (e.g. Jin et al 2005, Wood et al 2003b). This could mean that Prx VI carries out a different function in the neural system than in other organs, such as the lungs and kidneys, where it is expressed in the cytosol (Fujii et al 2001).

It is quite fascinating to notice that there is a distinct divergence in peroxiredoxin expression in different neural cell types, indicating that they are likely to have very specific and different functions albeit they represent the same family of antioxidative enzymes. It is also clear, that alterations in this fine balance can cause severe problems for the cell. It is also possible that Prx proteins have a direct influence on tumorigenesis. Studies on human carcinomas of the thyroid gland, pleural mesotheliomas, oral and lung cancer, prostate cancer and breast carcinoma suggest that Prxs may at least have a role both in tumor progression and in drug resistance (Yanagawa et al 1999, Yanagawa et al 2000, Noh et al 2001, Lehtonen et al 2004, Kinnula et al 2002, Shen et al 2002, Karihtala et al 2003).

Table 4. Summary of antioxidative enzymes; molecular size, chromosomal location and cellular location

	Polypeptide length (amino acids)	Human chromosomal location	Cellular location
MnSOD	183	6q25.3	Mitochondrial
Trx	104	9q31.3	Cytoplasm, nucleus, mitochondria, extracellular
TrxR (-1)	497	12q23-q24.1	Cytoplasm, mitochondria
Glutamate-cysteine ligase, also known as gamma-glutamylcysteine synthetase	637 GLCL-C 637 GLCL-R 274	 6p12 1p22.1	Mitochondrial, cytosol
PrxI	199	1p34.1	Cytosol, nucleus
PrxII	198	19p13.2	Cytosol, membrane
PrxIII	256	10q25-q26	Mitochondria
PrxIV	271	10p22.13	Cytosol, Golgi, secreted
PrxV	214	11q13	Mitochondria, peroxisome, cytosol
PrxVI	224	1q25.1	Cytosol

2.3. Carbonic anhydrases

Carbonic anhydrases (CAs) are physiologically important zinc metalloenzymes; CAs catalyze the reversible interconversion between CO_2 and HCO_3^-, one of the most fundamental chemical reactions in cells. This reaction is essential for organisms as it influences respiration, pH regulation and homeostasis, exchange of electrolytes and several metabolic biosynthetic pathways (Sly et al 1995, Harris et al 2002, Wykoff et al 2000, Pastorekova 2004b). Aberrant changes in this fine machinery are implicated in many diseases, including cancer.

CAs are present in broad range of organisms, from bacteria to plants and animals, including human. However, during the last decade it was understood that these enzymes belong to five genetically unrelated families (alpha, beta, gamma, delta, and zeta CAs) (Zimmerman and Ferry, 2008). Out of 16 mammalian alpha CA isoforms described so far, 13 are enzymatically active (CA I-IV, VA, VB, VI, VII, IX, XII-XV) and of these, only CA XV is not expressed in humans (Hilvo et al 2005). Enzymatically active CAs contain four important histidine residues: three residues for the coordination of a zinc ion in the active site and one for a proton shuttle (Supuran et al 2003, Pastorekova et al 2004). Three known enzymatically inactive isoforms (CA VIII, X and XI) lack one of the three histidines and therefore lose the capability for CO_2 hydration. In contrast to acatalytic isoforms, high activity isoforms are ranked among the most efficient enzymes because of extremely fast turnover values. Each CA isozyme has characteristic subcellular localization. CA I, II, III, VII, and XIII are cytosolic enzymes, CA IV, IX, XII, XIV, and XV are associated with the plasma membrane, CA VA and VB are present in mitochondria, and CA VI is the only secretory form (Pastorekova et al 2006).

2.3.1. General aspects

The expression of CA isoforms in the human body varies from those widely expressed throughout the body (CA II, IV, XII, XIII) to the others which are restricted to only few tissues (e.g. CA III in muscles and adipocytes; CA VA in liver, CA VI in salivary and mammary glands). Furthermore, tissues such as brain, lung, gut, liver, kidney, testis and red blood cells have abundant CA expression involving various

isoforms which contribute to different physiological reactions. Although some of the CA functions are still poorly understood, it is widely accepted that CAs play important roles in several processes. CA VA and VB are involved in metabolic processes including gluconeogenesis and ureagenesis. Some CAs can improve the transmembrane movement of bicarbonate by physically interacting with bicarbonate transporters, thus increasing the local concentration of bicarbonate and thereby accelerating its flux through the plasma membrane. This protein kinase C regulated mechanism modulates pH at the both sides of the membrane, and is fundamental for the physiological functioning of normal, metabolically active cell (Pastorekova et al 2004b, Pastorekova et al 2006).

Abnormal CA activity is implicated in several diseases. Since the functions and cellular locations of different isoforms show great versatility, there are also various clinical manifestations associated with CA deficiency and overexpression. CA deficiency of one isoform can cause a severe syndrome with multiple health problems (e.g. hereditary CA II deficiency). On the other hand, increased CA activity may contribute to various neurological and neuromuscular disorders, glaucoma, epilepsy, obstructive pulmonary disease, etc. (Sly et al 1995, Pastorekova et al 2006).

It would be easy to assume that in different cancers CA expression is systematically increased since cancer cells would benefit from excessive extracellular acidification. Several studies report, however, that CA (isoforms CA I, II, III, XIII) activity is reduced and/or their expression is decreased in colorectal, hepatocellular and lung tumors when compared to the corresponding normal tissues (Kummola et al 2005, Kuo et al 2003, Chiang et al 2002, Kivelä et al 2001). Furthermore, the expression of these isoforms is usually lost in poorly differentiated tumors, suggesting that they are mainly connected with

processes other than contributing to tumorigenesis (Kuo et al 2003, Kivelä et al 2001). On the other hand, there is evidence that the expression of the inactive isoform (CA VIII) is increased at least in lung and colorectal carcinomas, although it is not accurately known what its role is in these tumors and tumor progression. Out of thirteen catalytically active isoforms only two have been associated with different types of tumors: CA XII is over expressed in some tumor types, including breast carcinoma, while CA IX is almost exclusively expressed in different tumors, making it an attractive molecule not only for cancer research but also for targeted cancer therapy (Pastorekova et al 2006).

2.3.2. Carbonic anhydrase IX

CA IX was originally identified from mammary tumor-derived MaTu cells and grown in a mixed culture with human cervical carcinoma cells HeLa. This new protein was initially called MN, and only later it was found to contain a well-conserved CA domain and to act like other members of the alpha CA-family (Pastorek et al 1994, Opavsky et al 1996, Pastorekova et al 2006). It is mainly localized on the cell surface and contains extracellular proteoglycan and CA domains, a transmembrane part, and an intracellular C-terminal tail (Pastorek et al 1994, Opavsky et al 1996, Hilvo et al 2008). The antibody for CA IX, M75, binds to the extracellular proteoglycan domain. Another interesting feature is its capacity to oligomerize. The recombinant human CA IX enzyme tends to form dimers, and in the excess of certain metal ions shows the highest catalytic activity ever measured for any CA (Hilvo et al 2008). The gene for CA IX was initially mapped to *17q21.2* by fluorescence in situ hybridization; however, radiation hybrid mapping localized it to *9p13-p12* (National Center for Biotechnology Information, National Institutes of Health).

CA IX has a special role among human CA isoenzymes because it can be found in very few normal tissues, but it is abundant in several tumors, such as colorectal, bladder, cervical, lung and breast carcinomas (Chrastina et al 2003, Ivanov et al 2001, Zavada et al 1993). Even though the expression of CA IX in these carcinomas is evident, the tissues from which the carcinomas are originally derived are known to be CA IX-negative, or they show only low enzyme expression. Furthermore, the few normal tissues or cell types that express CA IX, such as gastrointestinal and gallbladder epithelial cells, have been reported to lose most of the CA IX expression during carcinogenesis (Karhumaa et al 2001, Pastorekova et al 1997, Saarnio et al 1998, Saarnio et al 2001). This quite exceptional phenomenon makes CA IX an interesting tumor-associated protein.

CA IX expression is strongly induced by hypoxia. This transcriptional activation is accomplished via the HIF-1α transcription factor, which accumulates in tissue under the hypoxic conditions that are often present in growing tumors. These conditions, in turn, are an outcome of poorly organized and insufficient vasculature in uncontrollably growing malignant tissue. The HIF-1 transcription factor is a trigger for several hypoxia-regulated genes linked to cell survival, proliferation, apoptosis, angiogenesis and metabolism in tumor cells. The activation of these genes helps the cell to adapt to the stress caused by low oxygen level in a particular tissue. Earlier studies have shown that CA IX expression is induced as early as two hours after HIF activation and persists for several days, even if HIF-1 expression has ceased. This means that CA IX reflects both previous and present hypoxia in cells (Rafajova et al 2004, Sobhanifar et 2005, Vordermark et al 2005). In addition to the important correlation between hypoxia and CA IX expression, it has been found that CA IX expression correlates with poor prognosis in various tumors (Chia et al 2001,

Giatromanolaki et al 2001, Haapasalo et al 2006, Loncaster et al 2001). It has also been suggested that CA IX has a direct role in tumor progression and the regulation of pH balance during tumorigenesis. CA IX also affects cell adhesion, and has been suggested to play a role in tumor invasion through weakening of cell-cell adhesion as E-cadherin is competing for β-catenin (Svastova et al 2003). However, the effect of CA IX on tumor cell invasion is still under debate as some recent results have suggested that there is no evidence of such a correlation (Robertson et al 2004). Even though we do not know the exact mechanisms of CA IX in cancer development, it is undoubtedly a promising target for anticancer treatments. It is also known that CA IX is strongly inhibited by aromatic and heterocyclic sulfonamides. Some of these compounds act even at sub-nanomolar concentrations and work better with CA IX than with other CAs. Up to date, several potent inhibitors for CAs have already been designed, although their systemic clinical use is still in its infancy except for treatments designed for glaucoma and epilepsy (Vullo et al 2003, Ilies et al 2003, Franchi et al 2003, Vullo et al 2004, Pastorekova et al 2004a, Abbate et al 2004).

2.4. Epidermal growth factor receptor

Epidermal growth factor, EGF, is a small polypeptide that belongs to the family of HER/Erbb-kinases, which includes EGFR (ErbB1), HER2 (ErbB2, c-Neu), ErbB3 (HER3) and ErbB4 (HER4). All these proteins have been under considerable interest during the last ten years, since agents targeting them (monoclonal antibodies or tyrosine kinase inhibitors) have shown rather potent therapeutic effects and have been introduced into clinical use as well. A good example is the use of trastuzumab for the treatment of HER-2 positive breast cancer since the end of 1990s. After that the EGFR targeted agents

were approved into clinical use for the treatment of metastatic colorectal cancer, non-small- cell lung cancer, metastatic pancreatic cancer and certain head and neck carcinomas (Rocha-Lima et al 2007).

EGF gene is located on chromosome 7p12. EGF is present in most mammalian cell types, but is most abundant on epithelial cells. It binds to a glycosylated transmembrane receptor of the cell and via that activates proliferation of the cell by first activating tyrosine kinase in the cytoplasmic end of the membrane, thereby phosphorylating intracellular proteins and the receptor itself. Since EGF has this capacity of generating potent mitogenic signals, it is also a potential oncogene and when activated overrides the normally carefully controlled cell cycle pathways.

2.4.1. Epidermal growth factor receptor in the gliomas

In astrocytic gliomas the most frequent oncogenic activation is amplification of the gene for EGFR, which results in overexpression of its protein product, a transmembrane tyrosine kinase receptor. Amplification of the EGFR gene is present in 30- 70% of astrocytomas, and it confers advantages in regards to growth and radio- and chemoresistance (Libermann et al 1988, Wong et al 1987, Carpenter et al 1990, Ekstrand et al 1991, Chaffanet et al 1992, Fuller et al 1992, Schlegel et al 1994, Schrober et al 1995, Barker et al 2001, Krishnan et al 2003). In addition to amplification, a variety of mutations in the EGFR gene have also been described (Libermann et al 1984, Sugawa et al 1990, Wong et al 1992, Fredrick et al 2000). The most common EGFR mutation is EGFRvIII, which leads to a protein with a truncated ligand-binding domain and that is constitutively phosphorylated. Several investigators have shown that EGFR amplification

and vIII mutation can coincide in the same tumors, typically in GBMs (Shinojima et al 2003, Aldape et al 2004). Amplification and overexpression of the EGFR gene are generally associated with poor prognosis, but it is less clear whether the prognostic correlation is independent or whether it is the result of close association with the grade of malignancy (Hurtt et al 1992, Watanabe et al 1996, Etienne et al 1998, Huncharek et al 2000, Simmon et al 2001, Smith et al 2001, Shinojima et al 2003, Quan et al 2005).

3. AIMS OF THE RESEARCH

The main purpose of this study was to investigate possible target molecules in gliomas with special emphasis on the following:

1. Study the expression of antioxidative enzymes both in oligodendroglial and astrocytic brain tumors
2. Evaluate the molecular pathology of carbonic anhydrase IX in oligodendrogliomas
3. Validate a new method for EGFR-gene amplification analysis in glial tumors

4. MATERIALS AND METHODS

4.1. Patients

For analysis that included **oligodendroglial brain tumors**, we analyzed tumor samples altogether from 86 patients who underwent surgery during 1980-2004 at Tampere University Hospital, Turku University Hospital and Kuopio University Hospital, Finland. For AOE study 85 patients were included and by the time of CA IX study, one newly diagnosed recurrent tumor sample was included, too. Of these tumors 55 were grade II and 31 were grade III. Forty-eight tumors were pure oligodendrogliomas and 38 were oligodendrogliomas with an additional astrocytic component (mixed oligoastrocytomas). 71 samples were from primary tumors and 15 from recurrent ones. All of the patients underwent neurosurgical operation with the intention of gross radical tumor resection. Post-operative treatment varied according to the changing standards in oncology during the time period. Information on radiation therapy was available for 35 patients (patients who were treated at Tampere University Hospital), 27 (77%) of which had been given the treatment. Information on usage of anticancer drugs was available for 30 individuals, of which 11 (37%) had been treated. The regimens most often used were single-CCNU (lomustine) or PCV (a combination of lomustine, vincristine and procarbazine), in a few cases temozolomide or other types of anticancer drugs were given. The median age of the patients at the time of operation was 40.0 years (mean 41.5, SD +14.2). The median follow-up time was 5.4 years (mean 6.1, SD 4.9) of the patients alive after the follow-up.

Histological samples of **astrocytic tumors** were obtained from 383 patients who underwent surgery at Tampere University Hospital, Tampere, Finland in 1983-1997. 299 samples were from primary tumors and 84 from recurrent ones. 62 of the tumors were grade II, 54 grade III and 267 GBMs. All of the patients underwent neurosurgical operation with the intention of gross radical tumor resection. The patients with primary tumors that were included in this study did not receive any anticancer medication prior to the operation. The postoperative treatment was not studied here. The median age of the patients at the time of operation was 48.9 years (mean 48.8, SD 15.0) and the male-female ratio was 1.3:1. On average, patients with recurrent tumors were nine years younger than those with primary tumors. When assessing the role of malignancy grade, grade II and III astrocytomas were considered as one group and compared to GBMs (grade IV astrocytomas). The median follow-up time was 5.7 years (mean 5.3, SD 2.9) of the patients alive after the follow-up.

4.2. Histological tumor samples

Both oligodendroglial and astrocytic tumor samples were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin. Histopathological typing and grading were carried out according to WHO criteria (eds Louis et al 2000). A neuropathologist selected histologically representative tumor regions and the samples from these areas were applicated in tissue microarray blocks using a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The diameter of the tissue cores in the microarray blocks was 600 µm. All the blocks

contained 30-100 samples and were stained at the same time. We used only 1 core for block, but this core was representative as described before.

4.3. Immunohistochemistry: antioxidative enzymes

Expression of antioxidative enzymes was studied as follows. Four-micrometer thick sections were cut from representative tissue microarray paraffin blocks. First they were deparaffinized in xylene and rehydrated in descending ethanol series. Then the sections were incubated in 10mM citrate buffer (pH 6.0), boiled in a microwave oven for 2 min at 850W, and after that 8 min in 350W for the purpose of enhancing immunoreactivity. Endogenous peroxidase activity was eliminated by incubation in 0.1% hydrogen peroxidase in absolute methanol for 10 min. The antibodies used in the study were as follows. A polyclonal rabbit antibody to human MnSOD (dilution 1:1000) (gift from prof. James Crapo, Jewish Medical and Research Center, Denver, CO, USA). Rabbit polyclonal antibodies to human GLCL-C and GLCL-R peptides (dilution 1:1000 for both) (gifts from T. Kavanagh, University of Washington, Seattle, WA, USA). An affinity purified polyclonal goat antibody to human Trx (dilution 1:200) (American Diagnostica, Greenwich, CT, USA). The antibody to TrxR was the gammaglobulin fraction of a polyclonal rabbit anti-rat antibody directed against cytosolic TrxR in rat liver (dilution 1:1000) (gift from prof. A Holmgren, Karolinska Institutet, Stockholm, Sweden). The bound MnSOD, GLCL-C, GLCL-R and TrxR antibodies were visualized using the Histostain-Plus Kit (Zymed Laboratories Inc. San Francisco, CA, USA). The primary antibodies were substituted with phosphate-buffered saline (PBS) or non-

immune rabbit serum in negative controls. For Trx, a biotinylated secondary anti-goat antibody was applied (dilution 1:400) followed by an avidin-biotin-peroxidase complex (all from Dakopatts, Glostrup, Denmark). The color was developed using 3,3`-diaminobenzidine, and the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Replacement of the primary antibody by PBS at pH 7.2 and goat IgG immunoglobulin isotype (Zymed Laboratories Inc.) were used as a negative control. For positive controls a validated sample from non-small cell lung carcinoma and mesothelioma were used (Kahlos 2000a, Kahlos 2000b, Soini 2001, Järvinen 2002). The procedure for peroxiredoxins was the same as for other antioxidative enzymes and the polyclonal anti-Prx-antibodies were a gift from Dr Kang (Center for Cell Signalling Research and Division of Molecular Sciences, Ewha Womans University, Seoul, Korea). The dilution for the primary antibodies was 1:1500 for Prx I, 1:1000 for Prx II, 1:500 for Prx III, 1:1000 for Prx IV and 1:2000 for Prxs V and VI. For positive controls a validated sample from breast carcinoma and mesothelioma were used (Kinnula 2002, Karihtala 2003).

4.4. Immunohistochemistry: carbonic anhydrase IX

For CA IX the immunohistochemical procedure was the following. The monoclonal antibody M75, which recognizes the N-terminal proteoglycan domain of human CA IX was used (Pastorekova et al 1992). Automated immunostaining, which was performed using Power Vision+™ Poly-HRP IHC Kit (ImmunoVision Technologies, Co.) reagents, included the following steps: (a) rinsing in wash buffer; (b) treatment in 3% H_2O_2 in ddH_2O for 5 min and rinsing in wash buffer; (c) blocking with Universal IHC

Blocking/Diluent for 30 min and rinsing in wash buffer; (d) incubation with the primary antibody (M75 for CA IX) or normal rabbit serum (NRS) diluted 1:200 (M75) or 1:2000 (NRS) in Universal IHC Blocking/Diluent for 30 min; (e) rinsing in wash buffer for 3 x 5 min; (f) incubation in poly-HRP-conjugated anti-rabbit or anti-mouse IgG for 30 min and rinsing in wash buffer for 3 x 5 min; (g) incubation in DAB (3,3' –diaminobenzidine tetrahydrochloride) solution (one drop of DAB solution A and one drop of DAB solution B in 1 ml) ddH_2O for 6 min; (h) rinsing with ddH_2O ; (i) $CuSO_4$ treatment for 5 min to enhance the signal; and (j) rinsing with ddH_2O. All procedures were carried out at room temperature. The sections were mounted in Entellan Neu (Merck; Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 x objective microscope (Carl Zeiss; Göttingen, Germany).

4.5. Scoring of immunohistochemical stainings

The immunohistochemical staining results were evaluated for each immunohistochemical target during one session on a multiheaded microscope by three observers semiquantitatively by dividing the AOE, PRX or CA IX staining reaction into four categories based on the reactivity of the staining taking into account the intensity and extent of the staining: 0 = no immunoreactivity present; 1 = weak immunoreactivity; 2 = moderate immunoreactivity; 3 = strong immunoreactivity present. On these studies the evaluation was subjective, but there was strong consensus between the three observers. For the analysis, the four categories were divided into two groups: the AOE(-), PRX(-) and CA IX(-) group contained negatively and weakly stained tumors. The AOE(+),

PRX(+) and CA IX(+) group contained tumors showing moderate or strong immunoreactivity.

4.6. Cell proliferation and apoptotic rate

For the analysis of cell proliferation, a mouse monoclonal antibody MIB-1 recognizing the Ki-67 antigen was used (Immunotech, S.A. Marseille, France) (dilution 1:40). After immunostaining, the tissue sections were counterstained with methyl green. Proliferative activity was reported as a percentage of nuclei with positive immunoreaction. Analysis of MIB-1 positive tumor cells was done with a carefully standardized image analysis system (CAS-200™ Software, Becton Dickinson & Co., USA) as described previously (Sallinen et al 1994a, Sallinen et al 1994b).

Apoptosis activity was determined by TUNEL–labeling. The deparaffinized tumor microarray tissue sections were first digested with proteinase K (20µg/ml) for 15 min. Apoptotic cells were demonstrated using ApopTag In Situ Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. In the terminal deoxynucleotidyl transferase nick end labeling method, the recommended concentration was reduced eightfold. Direct immunoperoxidase detection of digoxigenin labeled dUTP was followed by counterstaining in methyl green (Miettinen et al 2001). Staining was analyzed by counting all the tumor cells in the core tissue with an image analysis system (CAS-200™). The obtained scores were reported as a percentage of nuclei with positive immunoreaction.

4.7. Epidermal growth factor receptor and chromogenic *in situ* hybridization

Expression of EGFR wild type protein and variant (vIII) protein was studied immunohistochemically using mouse monoclonal antibodies to epidermal growth factor receptor (NCL-EGFR) and truncated epidermal growth factor receptor (NCL-EGFRT) at a concentration of 1 μg/ml (Novocastra Laboratories, Newcastle, UK) and high temperature antigen retrieval. Immunoreactivity present on the plasma membrane or in the cytoplasm of the tumor cells was scored as negative (-), positive (+) or strongly positive (++). Amplification of the EGFR gene in astrocytic brain tumors was assessed by means of CISH, using a slight modification of the method described in a previous study (Tanner et al 2001). In brief, the slides were de-paraffinized and heat-treated in Tris-HCl (pH 7.0) in a temperature-controlled microwave oven (92 °C for 10 min). After pepsin digestion (10 min at room temperature, using Digest-All III, Zymed Laboratories, South San Francisco, CA), the slides were washed with phosphate-buffered saline (PBS) and dehydrated in graded ethanol solutions. A ready-to-use digoxigenin-labelled EGFR DNA probe (Zymed) was applied to the slides, coverslips were placed on and the sections denatured on a thermal plate (94°C for 3 min). After overnight hybridization at 37 °C, the slides were washed with 0.5 x SSC (standard saline citrate; 5 min at 75 °C), followed by three washes in PBS (at room temperature). The EGFR probe was detected by means of sequential incubations with mouse anti-digoxigenin (diluted 1:300; Roche Biochemicals, Mannheim, Germany), and Powervision+ immunoperoxidase kit reagents (Immunovision Inc., Daly City, CA). Diaminobenzidine was used as chromogen. The sections were counterstained with haematoxylin and embedded. Positive and negative controls were

included in every hybridization batch. These controls were samples from previously well-defined tumorous tissues (Tanner 2001). The sections were evaluated using a ×40 dry objective. A non-amplified gene copy number was defined as 1 to 5 signals per nucleus. Amplification was defined as 6 or more signals per nucleus in over 50% of cancer cells, or when a large gene copy cluster was seen.

4.8. Analysis of *p53*

Immunohistochemical analysis for *p53* status was carried out as follows. Sections (thickness 5 μm) routinely processed tumor microarray paraffin blocks were cut and mounted on SuperFrost Plus- or poly-L-lysine-coated slides and dried overnight at 37 °C. They were then dewaxed and rehydrated. Antigen retrieval was carried out by heating the sections in a microwave oven for 2 × 7 minutes in 0.01 M citrate buffer (pH 6.0). The sections were then incubated with primary antibodies for 25 minutes at room temperature. The antibody used for *p53* was clone DO-7 (dilution 1:40) (Novocastra Laboratories, Newcastle, UK). The bound antibodies were visualized by using a streptavidin-biotin-peroxidase kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as chromogen. Methyl green was used for counterstaining. Status was evaluated as described previously (Haapasalo et al 1993).

4.9. Analysis of *1p* and *19q*

Fluorescence in situ hybridization (FISH) analysis was performed on tumor cell nuclei isolated from paraffin embedded sections. The sections were first deparaffinized with xylene, followed by an ethanol row with decreasing concentrations. After transfer into distilled water, the deparaffinized sections were incubated with 0.1% protease in 0.1 M Tris, 0.07 M NaCl at 370C for 30 minutes and occasionally agitated. To check cell density, 5 µl of cell suspension was put on a slide and examined under the microscope. After preparing the slides using 10 µl of cell suspension, the slides were air-dried overnight at room temperature and fixed with 4% buffered formaldehyde for 10 minutes. The slides were used immediately or stored in sealed boxes at -70°C. FISH analysis for *1p* and *19q* were performed with *LSI 1p36/LSI 1q25* and *LSI19q13/LSI 19p13* probes (Vysis). Hybridization was done according to the protocols of Vysis included in the probe package insert.

4.10. Statistical analysis

The statistical tests were all performed using part of SPSS 11.0 (SPSS Inc., Chicago, Illinois) for Windows software. χ^2-test, t-test, Mann-Whitney test and Wilcoxon test were used, as well as multivariate analysis of variance (ANOVA). The log-rank test and Cox multivariate analysis were used for the analysis of prognostic factors. The χ^2 test was used to test for associations between factors. Life tables were calculated according to the Kaplan-Meier method. Survival curves were compared with the log-rank test and hazard ratios (HR) were calculated using Cox regression. In survival analysis overall

survival was defined from the day of surgery until the death of the patient. The death of patient was considered as an event in the analysis. Coding of variables in Cox regression model is discussed in more detail in the results section. The significance level was set at $p < 0.05$ and all p values are two-tailed.

4.11. Ethics

The study designs for all the original publications were approved by the Ethics committee of Tampere University Hospital and the National Authority for Medicolegal Affairs.

5. RESULTS

5.1. Expression

AOEs. Antioxidative enzymes were studied in 71 primary and 14 recurrent oligodendroglial tumors (MnSod, Trx, TrxR, GLCL-C, GLCL-R) and in 299 primary and 84 recurrent grade II-IV astrocytic tumors (Prx I-VI).

Immunoreactivity for Trx and MnSOD were seen in the cytoplasmic compartment of the positive oligodendroglial cells, for MnSOD in a granular pattern. Trx could also be detected in the nuclear compartment of the cell. TrxR had cytoplasmic perinuclear staining positivity possibly indicating Golgi associated staining. For GLCL-C diffuse cytoplasmic immunoreactivity was seen. GLCL-R was also stained diffusely in the cytoplasm and occasional strong staining was concentrated in perikaryon. The distribution of immunopositivity for AOEs is also shown in *Table 5*. MnSOD positivity was seen in 89% of tumors, 31% showing strong, 35% moderate and 23% weak positivity. Twenty-nine percent of the oligodendrogliomas expressed Trx; 9% were moderately and 20% weakly positive. There were no cases showing strong staining for Trx. In the case of TrxR, 76% were positive, 9% strongly, 28% moderately and 39% weakly. GLCL-C showed cytoplasmic immunoreactivity in 70% of tumor cells and GLCL-R in 68%. Strong GLCL-C positivity was found in 20%, moderate in 27% and weak in 23% of tumors. The corresponding values for GLCL-R were 2%, 14% and 52%. When primary and recurrent tumors were compared, Trx, TrxR and GLCL-C were more often moderately or strongly stained in recurrent tumors than in their primary

counterparts (p=0.036, p=0.028 and p<0.001, χ^2-tests). Of primary tumors, 5% showed moderate staining for Trx, whereas in recurrent tumors 27% were moderately stained (there was no strong staining for Trx at all). TrxR was strongly or moderately stained in 34% of primary tumors and in 50% of recurrent tumors, GLCL-C correspondingly in 37% of primary tumors and in 90% of recurrent tumors. When AOE expression was compared between pure oligodendrogliomas and mixed oligo-astrocytomas, MnSOD was found to be significantly stronger in pure oligodendroglial tumors (p=0.021, χ^2-test). When tumor grade was also considered, only Trx showed significantly increased expression along with the malignancy grade (p=0.016, χ^2-test).

Table 5. Expression of antioxidative enzymes and carbonic anhydrase IX in malignant glial tumors

Intensity of expression	negative	Weak	moderate	strong	Tumors with positive staining
Oligodendroglial tumors					
MnSod	**11%**	**23%**	**35%**	**31%**	**89%**
Trx	**71%**	**9%**	**20%**	**-**	**29%**
TrxR	**24%**	**39%**	**28%**	**9%**	**76%**
GLCL-C	**30%**	**23%**	**27%**	**20%**	**70%**
GLCL-R	**32%**	**52%**	**14%**	**2%**	**68%**
CA IX	**20%**	**62%**	**10%**	**8%**	**80%**
Astrocytic tumors					
Prx I	**32%**	**47%**	**18%**	**3%**	**68%**
Prx II	**16%**	**32%**	**30%**	**22%**	**84%**
Prx III	**10%**	**31%**	**39%**	**20%**	**90%**
Prx IV	**95%**	**5%**	**<1%**	**-**	**5%**
Prx V	**96%**	**4%**	**-**	**-**	**4%**
Prx VI	**53%**	**36%**	**10%**	**1%**	**47%**

Prxs. In astrocytomas, positive expression for Prx I, Prx II and Prx VI was seen as staining in the nucleus and in the cytoplasm for Prx III. Prx IV and Prx V stainings were mainly negative in this material. Prx I was positively stained in 68% of the astrocytomas, 47% weakly, 18% moderately and 3% strongly. 84% of the same tumors were positive for Prx II, 32% weakly, 30% moderately and 22% strongly. For Prx III already 90% of the tumor samples were stained, 31% weakly, 39% moderately and 20% strongly. A majority of the tumors were negative for Prx IV and Prx V, only 5% were weakly stained for Prx IV and 4% as well weakly stained for Prx V. Prx VI showed more stained tumors again, since 47% of the tumors were stained, 36% weakly, 10% moderately, 1% strongly. The distribution of immunopositivity for Prxs is also shown in *Table 5***.** Prx I was positive in 66% of primary tumors and in 75% of recurrent tumors (p=0.008, χ^2-test). Similarly, Prx VI was positive in 45% of primary tumors and in 54% recurrent tumors (p=0.01, χ^2-test). Higher-grade tumors showed a significant decrease in immunoreactivity for Prx I and Prx II (p<0.001 and p<0.001, χ^2-tests). Prx IV, on the other hand, was associated with an increasing malignancy grade (p=0.014, χ^2-test). We also studied the intercorrelations of Prxs. Prx I positivity correlated significantly with Prx II and Prx III positivity (p<0.001 and p=0.014, χ^2-tests), and Prx II positivity correlated significantly with Prx III and Prx VI positivity (p=0.005 and p=0.031, χ^2-tests): these Prx(+) tumors increased considerably when the other Prxs expressed positivity.

The correlation between Prxs and other AOEs was studied as well. The results are summarized in the *Table 6*. In brief, Prx I(+) tumors were significantly more often also GLCL-R positive than their negative counterparts (p<0.001, χ^2-test). Prx II(+) positive tumors were statistically more often MnSOD, GLCL-C and GLCL-R positive

than Prx II(-) tumors (p=0.008, p<0.001 and p=0.037, x^2-tests). In addition, Prx III(+) tumors were on average more often TrxR(+) than Prx III(-) tumors (p=0.013, x^2-test).

Table 6.
The association of peroxiredoxin expression with antioxidative enzymes MnSOD, GLCL-C, GLCL-R, TRX and TRXR in astrocytic brain tumors

Characteristic	PrxI +/-	PrxII +/-	PrxIII +/-	PrxIV +/-	PrxV +/-	PrxVI +/-
MnSOD						
Negative	24/ 92	49/ 64	61/ 50	- / 109	#/ 110	8/ 104
Positive	38/ 142	107/ 76	109/ 71	1/ 178	#/ 179	25/ 153
	n.s.	p=0.008*	n.s.	n.s.		n.s.
GLCL-R						
Negative	14/ 135	63/ 87	88/ 62	- / 148	#/ 148	16/ 131
Positive	49/ 102	94/ 56	88/ 58	1/ 146	#/ 147	17/ 131
	p<0.001*	p<0.001*	n.s.	n.s.		n.s.
GLCL-C						
Negative	25/ 120	69/ 78	82/ 64	1/ 142	#/ 143	16/ 128
Positive	38/ 116	88/ 64	92/ 56	- / 150	#/ 150	17/ 133
	n.s.	p=0.037*	n.s.	n.s.		n.s.
TRX						
Negative	50/ 196	123/ 124	138/ 105	- / 241	#/ 240	24/ 216
Positive	13/ 42	33/ 21	37/ 17	1/ 53	#/ 55	9/ 46
	n.s.	n.s.	n.s.	n.s.		n.s.
TRXR						
Negative	38/ 163	98/ 101	106/ 90	- / 195	#/ 195	21/ 176
Positive	25/ 74	58/ 43	67/ 31	1/97	#/ 98	12/ 86
	n.s.	n.s.	p=0.013*	n.s.		n.s.

*X^2-test
#No cases in prx-positive group, analysis of significance not possible

CA IX. CA IX expression was analyzed in 71 primary and 15 recurrent oligodendroglial tumors. The distribution of CA IX expressive tumors is shown in *Table 5*. There was no significant difference in CA IX expression between grade II and grade III tumors or between primary and recurrent tumors or between pure oligodendroglial tumors and mixed oligoastrocytomas. CA IX expression was significantly higher in

MnSOD(+) tumors (p=0.008, χ^2-test). On the other hand, CA IX expression was decreased in GLCL-R(+) tumors (p=0.044, χ^2-test). The other AOEs did not reach any statistical association with CA IX.

EGFR. Amplification of EGFR was easily distinguishable as an abundance of gene copies in the tumor cell nucleus. In the total material of 338 astrocytic tumors, including both primary and recurrent tumors, two of the 48 (4%) grade II tumors and 9 of the 43 (21%) grade III tumors showed EGFR gene amplification. In grade IV GBMs 96 of 247 tumors (39%) showed EGFR gene amplification (p<0.001 χ^2-test). Thus, gene amplification correlated strongly with malignancy grade and furthermore, with both primary and recurrent tumors (p<0.001 and p=0.034, respectively, χ^2-test). The incidence of EGFR gene amplification was higher in primary than in recurrent astrocytic tumors (33% vs. 25%), but the difference was not statistically significant (p=0.21, χ^2-test). Primary GBMs were significantly more often EGFR gene-amplified than secondary GBMs, as 41% of primary and 16% of secondary GBMs had EGFR gene amplification (p=0.033, χ^2-test). In 29 tumors (26 primaries) EGFR protein status was investigated immunohistochemically for the expression of wild-type and variant (vIII) protein. Wild-type and variant type EGFR positivity was found to correlate with gene amplification status. All but one of the EGFR gene-amplified tumors overexpressed wild-type protein as detected by immunohistochemistry. In non-amplified tumors, 8 of 15 showed overexpression (p=0.035, χ^2-test). Variant-type EGFR protein (vIII) was found in half of the gene-amplified tumors, but only in 1 of 15 tumors without amplification (p=0.014, χ^2-test). Quantitative assessment of EGFR mRNA was successful in 10 tumors. The levels

of EGFR mRNA were on average 6 times higher in gene-amplified tumors than in tumors without amplification (*p*=0.028, Mann-Whitney test).

5.2. Proliferation and apoptosis

In proliferation index, apoptosis index and *p53* immunohistochemistry analyses, comparison were made between two categories: negative and faint staining was considered as the negative (-) expression group and moderate and strong staining as the positive (+) expression group. This categorization was used with AOEs, including Prxs and also with CA IX. For EGFR the categories were formed by results of genetic analysis; the EGRF negative group did not have EGFR gene amplification, while the EGFR positive group had.

AOEs. In pure oligodendrogliomas, proliferation differed significantly between Trx categories, as Trx positive tumors had higher MIB-1 index (p=0.001, Mann-Whitney test) than Trx negative tumors. In mixed oligoastrocytomas GLCL-C positive tumors showed significantly higher proliferation (p=0.007, Mann-Whitney test) than their negative counterparts. Apoptotic rate was significantly higher in Trx positive oligodendroglial tumors (p=0.003, Mann-Whitney test).

Prxs. In the total material of the astrocytic tumors, the mean MIB-1 proliferation index was 16.0 (SD 20.88) and median 9.7%. Proliferation activity was compared between Prx(+) and Prx(-) groups. Prx I(+) and Prx VI(+) tumors had a significantly lower proliferation rate than their IHC negative counterparts (p=0.019 and p=0.033, Mann-Whitney test). In contrast, Prx III(+) tumors had a marginally higher proliferation

rate than Prx III(-) tumors (p=0.056, Mann-Whitney test). The mean for the TUNEL labeling index in the total material was 9.6 (SD 15.2) and median 3.6. The tumor apoptotic rate was decreased in Prx I(+) and Prx II(+) tumors (p<0.001 and p=0.007, Mann-Whitney tests).

When the malignancy grades were divided into two groups (grades II and III vs. grade IV) and analyzed separately, the proliferation index differed significantly in Prx III(+) and Prx VI(+) lower grade (II and III) astrocytomas compared to their Prx III(-) and Prx VI(-) counterparts. The mean for Prx III(+) tumors was 14.7 (median 4.1, SD 26.2); in Prx(-) counterparts it was 8.4 (median 3.0, SD 17.8) (p=0.043, Mann-Whitney test). The mean proliferation rate in Prx VI(+) cases was 1.6 (median 2.2, SD 1.6) and in Prx VI(-) counterparts 12.1 (median 4.1, SD 22.4) (p=0.016, Mann-Whitney test). There was no statistically significant difference in the proliferation index when only GBMs were included in the analysis, or in the case of other Prxs. The apoptotic rate differed significantly only in the case of Prx I and in GBMs, when the lower grade astrocytomas and GBMs were analyzed separately. The mean apoptotic rate for Prx I(+) GBMs was 8.8 (median 3.6, SD 17.6) and for Prx I(-) GBMs 12.8 (median 7.1, SD 17.8) (p=0.009, Mann-Whitney test).

CA IX. When oligodendroglial tumors were further analyzed and CA IX expression was considered, it was found that cell proliferation index (MIB-1) was decreased among CA IX(+) tumors. In Ca IX(+) tumors median was 2.9, mean 3.3, SD± 4.8 vs. median 5.8, mean 11.9 and SD± 13.9 in CA IX(-) tumors (p=0.015, Mann-Whitney test). No significant difference was seen in apoptotic activity in CA IX(-) vs. CA IX(+) tumors.

EGFR. Amplification of the EGFR gene was not associated with proliferation as measured by Ki-67 expression, but apoptosis was significantly more common in tumors with EGFR amplification ($p<0.001$, Mann-Whitney test). Mean apoptotic rate in EGFR gene amplified tumors was 13.2, median 7.1 and SD $\pm$19.0 while in no EGFR amplified tumors they were mean 7.7, median 3.6 and SD $\pm$12.4.

5.3. *1p* and *19q* and *p53*

Analysis of *1p* and *19q* loss was done by FISH for the 30 most recent oligodendroglial tumor samples. In 24 samples there were *1p* and *19q* deletions, 1 had only *1p* deletion, 1 only *19q* deletion, and 4 had no deletions. We investigated the association between AOEs, CA IX and *1p* and *19q* status. We found that there was no correlation between AOE or CA IX and *1p* and *19q* status in this material.

Both astrocytic and oligodendroglial tumor samples were also analyzed for *p53* mutations. A significant correlation was found in oligodendroglial tumors between *p53* status (*p53* negative and *p53* positive tumors) and Trx status: Trx positive tumors were less frequently *p53* positive than Trx negative tumors (p=0.043, χ^2-test). The other AOEs had no significant correlation with *p53*. An inverse association was found between EGFR gene amplification and *p53* overexpression in astrocytomas. Gene amplification was found in 20% of *p53*-positive tumors, in contrast to 37% of *p53*-negative tumors ($p=0.048$, χ^2-test).

5.4. Other clinicopathological features

Several clinicopathological features were included in our analysis, both in oligodendrogliomas and astrocytomas. These features include patient dependent features such as age and sex, as well as tumor dependent features such as histological phenomenon like calcifications, necrosis etc. We found that patients with Prx I(+) and Prx II(+) primary astrocytic tumors were significantly younger, in average 7 years younger than their Prx(-) counterparts (p=0.014 and p=0.005, t-test). The age of the patients at the time of the operation did not differ significantly between the AOE or CA IX(-) and AOE (+) / CA IX(+) groups in oligodendrogliomas.

EGFR gene amplification correlated significantly with age of the patients. The incidence of amplification was 48% in patients aged over 50 years, compared with 13% in the age group 50 years or less ($p<0.001$, χ^2-test) in astrocytomas.

5.5. Survival

AOEs. For survival analysis only the primary cases of **oligodendrogliomas** were used (45% alive after the mean follow-up of 5.2 years). When the patients were divided into four groups according to the original AOE-immunoreactivity grouping (0,1,2 or 3) only MnSOD seemed to have prognostic value (p=0.035, log-rank test); patients with high MnSOD expressing oligodendroglial tumors had the most unfavorable survival. Four-graded comparison was not possible by Trx, because there were no cases with strong expression. Furthermore, because only 5% of primary tumors had moderate immunoreactivity for Trx, the sample size in survival analysis for cases with moderate expression is small. However when AOE-positive and AOE-negative tumors were

compared, Trx-positivity was associated with decreased survival (p=0.034, log-rank test). In Cox multivariate analysis Trx seemed to have a trend for being an independent prognostic factor (p=0.018) along with age [age in categories as below: HR= 6.187, 95% CI for HR 1.944-19.689 (p=0.002)], but the small sample size in tumors with moderate expression sets a limit for interpreting this result. The other AOEs, tumor malignancy grade or histological typing (pure oligodendroglioma or mixed oligoastrocytoma) had no independent prognostic value. For this analysis age was graded for three categories: ≤35years, 36-54 years and ≥55years. The criteria for cut-off values were approximately equal patient groups.

Prxs. When Prx(+) and Prx(-) **astrocytic tumors** were compared, patients with Prx I(+)(p=0.0052, HR=0.623, 95% CI 0.356-1.090, log-rank test) and Prx II(+) tumors showed a significantly better survival rate than their negative counterparts (p=0.0002, HR=0.636, 95% CI 0.427-0.946, log-rank test). Other peroxiredoxins did not reach statistical significance. A further survival analysis was carried out for the patients with GBMs (N=216) or lower grade (grade II and III) astrocytomas (N=83). The mean follow-up for GBMs was 1.2 years (16.2% alive after mean follow-up) and for lower grade astrocytomas 4.5 years (30.1% alive after mean follow-up). In this comparison, Prx I had a favourable effect on prognosis in lower grade astrocytomas (p=0.0072). In GBMs no corresponding statistical significance was found in patient survival. Proliferation index, patient age, tumor grade, sex and Prxs were included in the Cox multivariate analysis in these astrocytomas. Only patient age and tumor grade had independent prognostic significance (p<0.001, HR=2.207 and p<0.001, HR =1.874). For this analysis age was graded for three categories: <45years, 45-60 years and >60years. The criteria for cut-off

values were approximately equal patient groups. Proliferation index (MIB-1) was also divided into three categories: low, moderate and high proliferation index, cut-off made for equal size groups.

CA IX. Univariate survival analysis was carried out by comparing all the four CA IX categories. In multivariate survival analysis the tumors that had no CA IX immunoreactivity were compared to those that had weak, moderate or strong staining. This was done to reach the best prognostic cut-off possible.

When the patients with **oligodendroglial tumors** that had no immunoreactivity for CA IX were compared in univariate survival analysis with those who had positive immunoreactivity for CA IX, the negative tumors tended to associate with a better survival rate than their positive counterparts. However, this difference did not reach statistical significance. In the same material, Cox multivariate analysis showed that the expression of CA IX ($p=0.009$, $HR=7.370$), patient age ($p=0.003$, $HR=3.422$) and histological component (mixed oligoastrocytoma vs. pure oligodendroglioma) ($p=0.022$, $HR= 0.351$) showed independent prognostic significance. CA IX positivity, older age and astrocytic component predicted poorer outcome. Other clinicopathological features, including proliferation status and *1p 19q* status, did not reach statistical significance in association to the prognosis. For this analysis age was graded for three categories: ≤35years, 36-54 years and ≥55years. The criteria for cut-off values were approximately equal patient groups. Proliferation index (MIB-1) was also divided into three categories: low, moderate and high proliferation index, cut-off made for equal size groups.

EGFR. For EGFR-analysis in **astrocytic tumors**, the survival analysis was carried out in grades III and IV separately and in the total material as a whole. Patients

with EGFR gene-amplified grade III tumors had a significantly poorer prognosis than those with no amplification (p=0.03, log-rank test), whereas no such correlation was found in grade IV tumors (p=0.5, log-rank test). In the total material, EGFR gene amplification was significantly associated with poorer prognosis, (p=0.009, log-rank test). There were only two EGFR gene-amplified grade II astrocytic tumors, precluding survival analysis in this group. EGFR gene amplification status was not found to be an independent prognostic factor, unlike malignancy grade HR=2.033 and age HR=1.945) (p<0.001, Cox multivariate analysis). For this analysis age was graded for three categories: <45years, 45-60 years and >60years. The criteria for cut-off values were approximately equal patient groups.

Table 7. Summary of impact on prognosis.

Target molecule	Impact on prognosis	
Oligodendrogliomas		
MnSOD +	↓	
Trx +	↓	
CA IX +	↓	
Astrocytomas		
EGFR +	↓	**Especially in grade III tumors**
Prx I +	↑	**Especially in grade II and III tumors**
Prx II +	↑	

↑= better prognosis; ↓= worse prognosis; compared to target molecule- negative counterparts

6. DISCUSSION

6.1. Present prospects

Oxidative stress caused by several factors, including environmental pollutants, carcinogenic chemicals, chronic inflammation and smoking, just to mention a few, has been known to enable either programmed cell death, i.e. apoptosis or to lead to cancer after several events. The machinery protecting cells from reactive oxygen species is complex. There are several components taking part of this machinery. Some components are irreplaceable and essential, others may have tasks overlapping with others, but none the less they are fundamental to the normal function of the cell. Here it was decided to study the expression of different types of antioxidative enzymes both in astrocytic and oligodendroglial tumors. They represent different phases of antioxidation, each of which could potentially lead to malignant transformation of the cell in the case of dysfunction.

MnSOD. MnSOD is understandably essential to mammalian cells, as it is responsible for conversion of O_2^- to H_2O_2 with two other members of the superoxide dismutase family. There are no substitutive enzymes for this reaction and the detoxifying of reactive oxygen species cannot proceed without it. The role of MnSOD in malignant transformation is not yet understood. However, chromosome 6q, where the genetic information for MnSOD is located, is altered in many cancers. Furthermore, this chromosome area is frequently lost in familial gliomas, the rare exception of more homogenous malignant brain tumors (Paunu et al 2000). The majority of oligodendrogliomas

studied here were positive for MnSOD; expression was slightly more intense in grade III oligodendrogliomas compared to grade II oligodendrogliomas and significantly stronger in pure oligodendrogliomas compared to mixed oligoastrocytomas. When previous results concerning astrocytomas are considered it can be estimated that the role of MnSOD in mixed oligoastrocytomas and astrocytomas is very similar, while pure oligodendroglial tumors are distinguished with more intense immunoreactivity for MnSOD possibly reflecting an alternative role (Haapasalo et al 2003). These two studies with astrocytoma samples investigated before and oligodendrogliomas investigated now are comparable because the immunostainings were done at the same time with the same antibody and similar antibody concentrations. So far it is not entirely clear whether the expression of MnSOD in cancer cells reflects merely the redox state of the cell or if it is a mark of more versatile acivity within the cell. In vitro studies have suggested that MnSOD is actually a tumor suppressor gene. There is evidence from breast cancer and melanoma cells, that when the MnSOD gene is transfected it significantly suppresses the malignant phenotype (Church et al 1993, Zhang et al 2002). Furthermore, glioma cells that are overexpressing MnSOD show decreased cell growth (Zhang et al 2002).

MnSOD and CAIX. MnSOD also showed interesting correlation aside from histopathological component. Oligodendroglial tumors with MnSOD positive expression more frequently showed CA IX expression, as well. Although factors that induce MnSOD expression are numerous, hyperoxia is among these factors (Marklund et al 1982, Kinnula et al 2003). Furthermore, the basic reaction of MnSOD where O_2- is converted to H_2O_2 is known to affect HIF-1α, by letting it accumulate in the cytosol. Conversely, it is known

that CA IX is induced by hypoxia and HIF-1α (Pastorekova et al 2004b). These states represent extremes and still seem to be present in the same cancerous tissue. It may be that the factors inducing MnSOD are so complex that this association has nothing to do with the hyperoxia-hypoxia axis. Another explanation is that they indeed reflect phenomena that take place during malignant transformation. As explained earlier, MnSOD has been suggested to be a tumor suppressor gene with an effect on the phenotype of the tumor. Increased MnSOD could thus reflect the attempts of the cell to control tumor environment during tumorigenesis. However, when the growth of poorly organized and improperly proliferated cancer cells continues, the environment becomes more and more hostile for surrounding normal cells. Rapidly growing cancer tissue leads to low pH and insufficient vasculature thus suppressing normal cells, while malignant cells have features favoring their growth in this imbalanced environment. This further activates elements in response to hypoxia, inducing genes that are involved with adaptation of low oxygen, including CA9 gene. In conclusion of this peculiar association of MnSOD and CA IX it may thus represent overlapping phases of malignant transformation during which both these proteins are still present in the cancerous tissue. When the transformation proceeds, it may be that the expression of one or another diminishes, e.g. when a lower grade tumor proceeds towards more a malignant phenotype. HIF-1α is likely to play a major part in this exciting and complicated continuum.

MnSOD and Prx II. Consistent with previous findings are the further findings from astrocytomas, that MnSOD is also associated with Prxs, in particular with Prx II. In this astrocytic tumor material, Prx II+ tumors showed MnSOD positivity more often than

their negative counterparts. Prx II itself was associated with younger age and less malignant grade in astrocytomas, and therefore it also seemed to have a favorable effect on prognosis for patients with astrocytoma. In a previous study by Haapasalo et al., using the same concentration and same antibody for MnSOD in astrocytic tumors, it was shown that a lack of MnSOD predicted poor prognosis in diffuse astrocytomas (Haapasalo et al 2003). However, here it was shown that in oligodendrogliomas the situation is the reverse, since strongly MnSOD positive tumors had the worst prognosis. This emphasizes that subtle differences between different subtypes of malignant gliomas have an impact on expression of redox regulative enzymes in the cancer cells, and furthermore, impacts on cell proliferation and even prognosis.

GLCL-C and GLCL-R. The synthesis of GSH is crucial for every normal cell and also for malignant cells. It involves not only antioxidative functions but also takes part in basic detoxification reactions and maintains the viable microenvironment inside the cell. The level of glutathione has also been associated with drug resistance of malignant tumors (Zhang et al 1998). Thus it is obvious that GLCL-C and GLCL-R, which are the major regulators of GSH synthesis, should have a role in malignant transformation or at least show disturbed expression during it. In oligodendrogliomas GLCL-C was found to associate with increased proliferation and with tumor recurrence. However, GLCL-C and GLCL-R seemed not to have any clear associations with prognosis or tumor grade. However, in astrocytic tumor material, an association with Prxs was found. Prx I+ tumors were more often GLCL-R positive than their negative counterparts and similar phenomenon was found with Prx II+ tumors, since they were more often both GLCL-C

and GLCL-R positive than Prx- tumors. Once again, this could reflect the redox state of the disturbed cell, or more probably the complex machinery that intertwines the antioxidative enzymes together.

Trx and TrxR. Thioredoxin and thioredoxin reductase seem to have effects on multiple cell functions. The major limitation of the study is the small sample size. In addition to the limited statistical power, comparison of subgroups of patients and individual prognostic markers is compromised due to the small sample size. In practise, the reported results of possible new prognostic markers are not based on high-level evidence. Therefore, our results are inconclusive and should be interpreted cautiously. However, based on results presented in this study, it may be cautiously suggested that Trx expression has a trend for poor prognosis in oligodendrocytic tumors. Interestingly, immunoreactivity for Trx was more often seen not only in higher grade tumors but also in recurrent tumors, suggesting a role in more aggressive tumors. In contrast to MnSOD, Trx has low expression in oligodendrogliomas, mixed oligoastrocytomas and astrocytomas. There was also a similarity between astrocytomas and oligodendrogliomas, as Trx positive tumors were associated with higher proliferation activity as well as correlation with worse prognosis (Haapasalo et al 2003). However, Trx or TrxR did not have correlations with CA IX like MnSOD, even though it would be tempting to logically assume that the same factors induce all antioxidative enzymes in a similar manner, and thus there could have been some kind of trend between them. It seems that the versatility of Trx and TrxR is far too extensive to make other assumptions that they are a good example of the so-called butterfly-effect, merely microscopically.

Prxs. Both the role and the function of peroxiredoxins are tightly bound to that of thiol-based antioxidative enzymes (including Trx and TrxR) and glutathione-based antioxidative enzymes (including GLCL-C and GLCL-R). Prxs are shown to utilize thioredoxins and glutaredoxins as the electron donor in plants, and they are likely to have similar connections in mammalian cells (Rhee et al 2005). Prxs are not the most potent antioxidative enzymes. However, they were found to have interesting correlations with astrocytic tumor cells in this study.

Prx I, II and III were present in a majority of the astrocytic tumor samples, while lower, if any, expression in corresponding samples was found for Prx IV, V and VI. Based on previous studies concerning normal mammalian brain tissue, the expectation was to find only weak or at most moderate expression in astrocytic tissue, and mainly for Prx VI (Jin et al 2005) or for Prx I (Sarafian et al 1999). It is remarkable that in this study it was observed that the pattern for Prx expression is strikingly different in the tumor tissue compared to normal mammalian brain tissue. It is obvious that the previous studies on normal mammalian brain tissue and on this study of astrocytomas cannot be compared straightforwardly, since the methods (antibodies, concentrations, etc) may differ in some extent. However, they can be discussed in general level. Only neurons or oligodendroglial cells are found to express Prxs considerably in the normal mouse brain, the former expressing Prx II (Sarafian et al 1999) and latter mainly Prx I and Prx IV (Jin et al 2005). In addition to increased overall expression in astrocytic tumor cells compared to previous findings in normal brain, Prx I and II expression was found to be associated with malignancy grade. The expression of these two peroxiredoxins was more common in grade II and III astrocytic tumors than in GBMs. In lower grade tumors there are often

signs of logical reactions of the cell to cellular stress, caused by the malignant growth itself. One of these signs may be the increased Prx production. However, it seems that Prx production is lost in grade IV tumor cells, at least it seems to be no longer indispensable for malignant cell growth in GBMs. This may result from the very badly organized cells of GBM. Since the ultimate goal of cancerous cells is to proliferate as effectively as possible, it sacrifices all mimicking of normal cell functions that does not lead to inescapable apoptosis. Interestingly, Prx I expression was also found far more often in recurrent tumors than primary ones. The primary tumor is often originally lower grade and when malignant transformation proceeds to invasion and metastasis, recurrent tumors occur. However, usually they still maintain similarities with the original tumor tissue. Thus, those lower grade tumors that have increased Prx expression already in the first place may continue to have that characteristic in their outcome as recurrent tumors. It is remarkable that Prx I and II were shown to have this association, while other Prxs lacked it. It can be debated that the role of Prx I and II within the cell may resemble each other more than the rest of the Prxs, even though they all belong to the same family by their structural base.

As discussed above, particularly Prx I and II seem to have an association with cellular events in malignant astrocytic cells. They also were associated with clinical prognosis, since patients with Prx I and Prx II positive tumors consistent with the lower grade tumors lived longer than their negative counterparts. Since at the same time the patients with Prx positive tumors were also younger, it is not clear whether the effect on prognosis is purely associated with the younger age or histologic grade, or if Prx positivity has straightforward correlations with more favorable outcome for the patients.

Furthermore, as Prxs are part of very complex antioxidative systems, with close connections with, for example, protein kinase activity, NF-κB activation, cell proliferation and growth control, differentiation, immune responses etc. (Hofmann et al 2002, Bryk et al 2000, Wood et al 2003, Veal et al 2004, Hirotsu et al 1999, Krapfenbauer et al 2003, Chen et al 2002, Nonn et al 2003), it would be extremely hard to prove that kind of straightforward association. Prx I had still further associations. Prx I positive tumors showed both lower proliferation rate and decreased apoptotic activity, when compared to their negative counterparts. Since these same Prx I(+) tumors were also associated with better prognosis, decreased apoptotic activity is seemingly contradictory to this finding. However, with closer analysis, Prx I positivity loses the statistically significant correlation with prognosis in grade IV GBMs, perhaps because of too few survivors, and findings on apoptotic rate were focused particularly on grade IV tumors. Thus these two findings, better prognosis and decreased apoptotic activity, represent tumors in different phases of malignant transformation.

Prx II was associated with tumor apoptotic rate in a corresponding way, but without clear association with grade IV. This may suggest that Prx II has a more prominent effect on apoptosis in different grade astrocytic malignant cells. Remarkable, however, is that in the previous literature Prx II has not been found to be expressed in astrocytes at all in the normal brain. It is peculiar that Prx II expression was observed in the majority of the tumor samples in this study. Thus it may have a particular role in malignant transformation (either as part of a cascade or as a by-product) or otherwise to exist in the cells that originate the malignant transformation.

Prx III has previously been shown to have its highest immunoreactivity in hippocampal area of normal brain, where it is suggested to have role against excitoxic

injuries (Hattori et al 2003). In this study it was shown that it was expressed in almost all astrocytic brain tumor samples. It correlated with higher proliferation rate in lower grade (II-III) tumors, but despite its abundant expression, it lacks any further correlations in this material. Prx IV and V expression were rarely present in this tumor material, and any implications of their role in astrocytic brain tumors could not thus be examined. However, in the literature they both have been found to be expressed in neurons and Prx IV even in abundant amounts in oligodendrocytes in normal mammalian brains (Jin et al 2005). This suggests that even though no straightforward conclusions can be made based on this study, they are likely to have at least partially different roles in normal and malignant CNS cells.

Prx VI it was expected to be expressed in this material, since it is commonly found in both astrocytes and oligodendrocytes of normal mammalian brain. Prx VI is interesting enzyme, because it has been reported to be expressed in the nuclei of glial cells, while it is cytosolic in other mammalian cells (Wood et al 2003). The localization of its expression in the present study was in the line with the previous literature. It was found to be particularly common in recurrent tumors. In addition, Prx VI had a correlation with proliferation rate, too, similarly to Prx I.

CA IX. Carbonic anhydrase IX has been of interest in cancer research due to its cancerous cell specific expression and other unique qualities that make it a candidate for targeted cancer therapies. This was an encouragement to study its expression in a series of 86 oligodendroglial brain tumors. The most important finding, in addition to its abundant expression in oligodendroglial brain tumors, was its independent prognostic

value, since CA IX positive tumors had poorer survival than their negative counterparts. In previous studies CA IX has shown to be a good marker indicating hypoxia, and furthermore, is also associated with the presence of necrosis (Ivanov et al 2001, Beasley et al 2001, Preusser et al 2005). This suggests that the same severe disturbances in the cell function (including hypoxia and insufficient microvasculature) that as an endpoint cause necrosis are also enhancing CA IX expression. There is already good evidence for this suggestion, since perinecrotic increase of expression has been reported, associated with microvessel immunostaining based on microvascular proliferation, an attempt of the cell to retain sufficient vasculature in severely hypoxic oligodendroglial tissue (Preusser et al 2005). In the present study, perinecrotic accentuation was seen in a majority of those oligodendroglioma cases that presented necrotic areas, too.

Hypoxia is known to be present in several tumors, particularly in those that grow very rapidly. In addition, the identification of hypoxic tumors gives an option for hyperbaric oxygenation preceding radiotherapy, which has to be allocated to tumors with true tissue hypoxia. There is a study that reports that CA IX expression is often accompanied with the expression of hypoxia-inducible factor 1α (HIF-1α) (Birner et al 2005). In that study it was concluded that when expressed together, they represent a true tissue hypoxia and not just oncogenic activation, which might otherwise be the case. Since HIF-1α also has been shown to have independent prognostic value in oligodendrogliomas (Preusser et al 2001), using a combination of these two hypoxia markers would not only give an estimation of prognosis, but would also give support for decision making when designing individual therapies.

CA IX expression has several clinical applications as discussed above that alone make CA IX a very useful tool in limited cases of tumors, when further information is needed. However, there are several other prognostic markers for oligodendroglial tumors, many of them already routinely used (i.e. *1p* and *19q* analysis), and even though association with CA IX and prognosis is peculiar, there are several other, even more attractive reasons to be interested in CA IX expression in oligodendrogliomas. Increasing knowledge of malignant transformation and phenomena that take place in cancerous cells has led to the realization that when the neoplasia is caused by several mutations and alterations in more than one oncogene or tumor suppressor gene, the targeted therapies are also not likely to be solved by just one target molecule. There is an extensive spectrum of cells expressing different, sometimes even opposite typse of molecules and enzymes, not only between different histopathological categories, but also within the same tumor. Thus the criteria for a very good target molecule is not just a monochromic list of characteristics needed, rather it is a combination of qualities that make it usable, preferably in combination with other treatments. CA IX is extremely promising as such a target molecule.

EGFR. For many years, much hope has been raised towards EGFR as a therapeutic target molecule, and agents targeting EGF receptor, usually combined with other treatments, have already shown some applicable clinical results. It fulfills the criteria for an ideal target molecule in several ways; one very important aspect is that it can be used as a target either by using monoclonal antibodies or by using tyrosine kinase inhibitors. Furthermore, it is already in common use in, for example, breast, colorectal,

pancreatic and other cancers, and our knowledge of its practical features are getting more and more familiar (Rocha-Lima et al 2007). The role of EGFR is particularly interesting, since a current study by Soeda et al (2008) suggests that the maintenance of human brain tumor stem cells requires EGF (Soeda et al 2008). Malignant stem cells are at the present thought be responsible for gliomagenesis and to be responsible for recurrences. Stem cells are a reservoir for cancer and even if treatments are effective for the offspring of these progenitors, a single surviving stem cell is enough for the cancer to proceed. Furthermore, if the stem cells are destroyed completely, the offspring cells are not likely to carry on proliferation endlessly and they are more vulnerable to apoptosis, too. Thus treatments that are especially targeted for stem cells are particularly desired. Soeda lists EGF, fibroblast growth factor-2, VEGF, platelet derived growth factor (PDGF) and hepatocyte growth factor to be growth factors that are necessary for normal neural stem cells. They found that EGF is absolutely needed, at least in vitro, for malignant stem cells survival (Soeda et al 2008). This makes the role of EGFR targeted therapies even more intriguing.

As always, great hopes can lead to huge disappointments, too. A recent article by Brandes et al (2008) summarizes the results of ongoing trials using EGFR based molecules as targets. They conclude that so far the results are very controversial and that more studies must be done before common clinical use (Brandes et al 2008). It is not only a matter of knowing your target on the molecular level, but good patient selection, including sufficient knowledge of many other genetic and clinicopathological features, that is the base for better treatments. However, clinical assessment of EGFR status in gliomas gives valuable information even when we are not using new therapies. It is quite clear that the

frequency of EGFR amplifications and mutations are increasing in a straightforward manner with increasing tumor grade. EGFR is also a landmark for one of the major pathways of carcinogenesis of glial tumors. This argument is backed up by the findings that in GBMs the EGFR alterations are common in “de novo” tumors, while secondary GBMs often have mutation of *p53*. More interestingly, these two alterations seldom overlap, as observed in this study, as well. Furthermore, many studies suggest that analyzing of EGFR gives valuable information for the prognosis, resistance to therapies etc. As a method, CISH offers a sensitive, novel way to analyze EGFR status in glial tumors with reasonable costs and reasonable equipments. A major part of the technique needed for CISH is already present in the average pathological laboratory that handles brain tumor specimens.

We believe that these reported new markers and methods are applicable in glioma research, and may benefit clinical diagnostics and decision making, after they have been confirmed in larger studies. Therefore, to overcome the methodological compromises (small sample size in some subgroups etc.), large clinical trials with patients receiving standardized glioma treatments are required.

6.2. Future prospects

Targeted therapies. Target molecules, proteins that serve as a candidate target for cytotoxins or other kind of therapeutical agents, have been under great interest. The designing of a target molecule starts with identification of a target candidate. Its function

within the cell must be characterized, its expression and specificity for the cancer cell must be screened and the gene must be able to be cloned. A target molecule's structure has to be modeled and the active site has to be defined. High affinity ligands must be identified. Blood brain barrier permeability has to be tested, as well as metabolic and other pharmacodynamical features, too. The entire process before the first clinical trials often takes several years itself, and to find good candidates for targeted therapies is also quite challenging. A target molecule should be easily detected, specific to cancer cells, have a high antibody affinity and preferably at least partially locate in the cell surface to make it more reachable. Some potential targets for malignant brain tumor therapeutics have been suggested, and some already have clinical applications. *P53*, p10 and p16 are quite common gene alterations in glial tumors, and as tumor suppressor genes, they represent an early phase of the malignant transformation pathway. O^6-methylguanine DNA methyltransferase (MGMT) targeted therapies have also been studied in malignant gliomas. MGMT is a repair enzyme that also affects drug-resistance, especially for temozolomide. When MGMT is silenced via gene promoter methylation, it decreases DNA repair activity and the cell is more vulnerable to apoptosis caused by cancer drugs (Hegi et al 2005). The challenge with targeted therapies is that most potential targets have coactivation of multiple enzymes and redundant pathways. Thus, single agents for any treatment, are an unlikely option. Antiangiogenic agents are under interest for adjuvant therapies, since malignant gliomas are extremely vascular tumors. Glioma stem cells are thought to produce VEGF and be in most part responsible for angiogenesis in the glioma microenvironment (Wen et Kesari 2008). This suggests that glioma stem cells are vulnerable to

decreased angiogenesis and antiangiogenetic agents may be an effective means to target stem cells.

CA IX and EGFR represent two molecules that already have ongoing clinical studies or even applications for clinical use as a target molecule for specially designed treatments of certain malignancies. Antioxidative enzymes, especially otherwise interesting MnSOD, Trx, Prx I and Prx II, may not be the key for targeted therapies, but nevertheless they have importance in prognosis and they can provide intriguing information regarding imbalances and incidents in the tumorous tissue. The hunt for the Holy Grail of the perfect target molecules continues. By providing further knowledge of the basic steps of malignant transformation and taking advantage of already identified molecules leads to better results already today, both for basic diagnostic and prognostic use, but also for clinical results, for the patients.

7. CONCLUSIONS

1. Altogether eleven different antioxidative enzymes were studied, five in oligodendroglial brain tumors and six in astrocytic tumors. **In oligodendrogliomas,** MnSOD, TrxR, GLCL-C and GLCL-R were expressed in a majority of the tumors. The expression of MnSOD was concentrated in pure oligodendroglial tumors with no astrocytic component. Increased Trx expression was associated with higher tumor grade, cell proliferation and apoptosis, and Trx seemed to have a trend for poorer prognosis. Recurrent tumors showed a clear increase in Trx, TrxR and GLCL-C expression when compared to primary ones. The results suggest that the biology of these five AOEs in oligodendrogliomas is extremely complex, and at least some of them seem to play a role that makes it worthwhile to study them more in larger clinical trials to gain further information of patient prognosis but perhaps also in the selection of therapies. Despite the small number of patients, which is partly due to the low population-based incidence of oligodendrogliomas in Finland, our results were statistically significant as well as biologically relevant.

   In astrocytomas Prx I, II and III were found to be expressed in a majority of the tumor samples and Prx VI in many. Prx I and Prx II had several associatiosn with both clinical and histopathological characteristics; when they were expressed in the tumor they both were associated with younger patient age, decreased

malignancy grade, lower apoptosis rate and finally, with better survival. Prx I showed an association also with proliferation rate. These findings suggest that Prxs, in this case particularly Prx I and Prx II, have a role that is yet to be fully identified in the malignant transformation of astrocytic brain tumors.

2. CA IX was expressed in a majority of oligodendroglial tumors and it had an independent prognostic value in oligodendrogliomas. CA IX expression is induced by hypoxia, and analyzing CA IX status may give additional information needed for choosing optimal treatment options. CA IX has many qualities that make it an attractive target for molecular therapies.

3. Chromogenic in situ hybridization (CISH) proved to meet expectations to be effective, straightforward and readily applicable to pathology laboratories for detecting e.g. EGFR gene amplification in astrocytic brain tumors. EGFR amplifications have been previously shown to be commonly present in astrocytic tumors and the gene status is also often needed in clinical use. In this study EGFR amplification was found in almost 40% of GBMs and more rarely in lower grade astrocytomas. However, in grade III astrocytomas, EGFR amplification was associated with poor survival and thus to have clinical relevance. Furthermore, EGFR based targeted therapies are already being tested, some of them used. The need for its reliable analysis is likely to increase in the future.

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