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PROGRESSION OF DIFFUSE GLIOMAS

FROM THE FIRST DIAGNOSIS TO RECURRENCE

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ACADEMIC DISSERTATION

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To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I   Tynninen O, Aronen HJ, Ruhala M, Paetau A, von Boguslawski K, Salonen O, Jääskeläinen J, Paavonen T. MRI enhancement and microvascular density in gliomas. Correlation with tumor cell proliferation. Invest Radiol 34:427-434, 1999

II   Tynninen O, Carpén O, Jääskeläinen J, Paavonen T, Paetau A. Ezrin expression in tissue microarray of primary and recurrent gliomas. Neuropathol Appl Neurobiol 30:472-477, 2004

III   Puputti M, Tynninen O, Sihto H, Blom T, Mäenpää H, Isola J, Paetau A, Joensuu H, Nupponen NN. Amplification of KIT, PDGFRA, VEGFR2, and EGFR in gliomas. Mol Cancer Res 4:927-934, 2006

IV   Tynninen O, Paetau A, Haglund C, Ristimäki A. HuR is a marker of reduced survival in glioma patients and associates with cyclooxygenase-2 expression and tumour grade. Manuscript

The publications are referred to in the text by their roman numerals. The publications are reprinted with permission from the copyright holders. Publication III has appeared in the thesis of Marjut Puputti.

ABBREVIATIONS

2HG   2-hydroxyglutarate
aCGH   array-based CGH
BAC   bacterial artificial chromosome
BCR-ABL   breakpoint cluster region-Abelson
CBV   cerebral blood volume
CCNU   1-(2-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea
CDKN2A   cyclin-dependent kinase inhibitor 2A
CEP   centromere evaluation probe
CGH   comparative genomic hybridisation
CISH   chromogenic in situ hybridisation
CNS   central nervous system
COX   cyclooxygenase
CpG   cytosine-guanine dinucleotide
CT   computed tomography
DAPI   4',6-diamidino-2-phenylindole
DHPLC   denaturing high-performance liquid chromatography
DIG   digoxigenin
DNA   deoxyribonucleic acid
DTPA   diethylene triamine pentaacetic acid
ECL   enhanced chemiluminescence
ECM   extracellular matrix
EGF   epidermal growth factor
EGFR   epidermal growth factor receptor
EGFRvIII   epidermal growth factor receptor variant III
ErbB   erythroblastic leukemia viral oncogene homolog B
ERM   ezrin-radixin-moesin
FDG   [^{18}F]fluorodeoxyglucose
FISH   fluorescence in situ hybridisation
Flk-1   fetal liver kinase-1
FVIII   factor VIII
GFAP   glial fibrillary acidic protein
Gy   gray
H&E   hematoxylin and eosin
HIF-1   hypoxia-inducible factor 1
HRP   horseradish peroxidase
HuR   human antigen R
IDH1   isocitrate dehydrogenase
IF   intermediate filament
K+   potassium ion
kD   kilodalton
KDR   kinase-insert domain receptor

MET   [^{11}C]methionine
MGMT   O^6 methylguanine-DNA methyltransferase
MLPA   multiplex ligation-dependent probe amplification
MMP   matrix metalloproteinase
MRI   magnetic resonance imaging
mRNA   messenger ribonucleic acid
MRS   magnetic resonance spectroscopy
n.a.   not applicable
NeuN   neuronal nuclear antigen
NSAID   non-steroidal anti-inflammatory drug
OLIG2   oligodendrocyte transcription factor 2
PCR   polymerase chain reaction
PCV   procarbazine, CCNU and vincristine
PDGF   platelet-derived growth factor
PDGFRA   platelet-derived growth factor receptor
PET   positron emission tomography
PlGF   placenta growth factor
Rb   retinoblastoma
RNA   ribonucleic acid
SD   standard deviation
SDS-PAGE   sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPECT   single-photon emission computed tomography
SVZ   subventricular zone
TGF-α   transforming growth factor α
uPA   urokinase type plasminogen activator
VEGF   vascular endothelial growth factor
VEGFR   vascular endothelial growth factor receptor
WHO   World Health Organization

ABSTRACT

Gliomas are the most frequent primary brain tumours. The cardinal features of gliomas are infiltrative growth pattern and progression from low-grade tumours to a more malignant phenotype. These features of gliomas generally prevent their complete surgical excision and cause their inherent tendency to recur after initial treatment and lead to poor long-term prognosis.

Increasing knowledge about the molecular biology of gliomas has produced new markers that supplement histopathological diagnostics. Molecular markers are also used to evaluate the prognosis and predict therapeutic response. The purpose of this thesis is to study molecular events involved in the malignant progression of gliomas.

Gliomas are highly vascularised tumours. Contrast enhancement in magnetic resonance imaging (MRI) reflects a disrupted blood-brain barrier and is often seen in malignant gliomas. In this thesis, 62 astrocytomas, oligodendrogliomas and oligoastrocytomas were studied by MRI and immunohistochemistry. Contrast enhancement in preoperative MRI was associated with angiogenesis, tumour cell proliferation and histological grade of gliomas.

Activation of oncogenes by gene amplification is a common genetic aberration in gliomas. EGFR amplification on chromosome 7p12 occurs in 30-40% of glioblastomas. PDGFRA, KIT and VEGFR2 are receptor tyrosine kinase genes located on chromosome 4q12. Amplification of these genes was studied using in situ hybridisation in the primary and recurrent astrocytomas, oligodendrogliomas and oligoastrocytomas of 87 patients. PDGFRA, KIT or VEGFR2 amplification was found in 22% of primary tumours and 36% of recurrent tumours including low-grade and malignant gliomas. The most frequent aberration was KIT amplification, which occurred in 10% of primary tumours and in 27% of recurrent tumours.

The expression of ezrin, cyclooxygenase 2 (COX-2) and HuR was studied immunohistochemically in a series of primary and recurrent gliomas of 113 patients. Ezrin is a cell membrane-cytoskeleton linking-protein involved in the migration of glioma cells. The COX-2 enzyme is implicated in the carcinogenesis of epithelial neoplasms and is overexpressed in gliomas. HuR is an RNA-stabilising protein, which regulates the expression of several proteins including COX-2. Ezrin, COX-2 and HuR were associated with histological grade and the overall survival of glioma patients. However, in multivariate analysis they were not independent prognostic factors.

In conclusion, these results suggest that contrast enhancement in MRI can be used as a surrogate marker for the proliferative and angiogenic potential of gliomas. Aberrations of PDGFRA, KIT and VEGFR2 genes, as well as the dysregulated expression of ezrin, COX-2 and HuR proteins, are linked to the progression of gliomas.

INTRODUCTION

Gliomas are the most frequent primary brain tumours. They account for one third of all intracranial tumours (Central Brain Tumor Registry of the United States, 2011). Gliomas are neoplasms of the supporting cells of the CNS (central nervous system). Their main subtypes are astrocytomas, oligodendrogliomas, mixed oligoastrocytomas and ependymomas. The clinical behaviour of gliomas varies greatly from benign to extremely malignant. Half of the gliomas belong to the most malignant category, i.e. glioblastomas with a median survival time of 1 year.

Infiltrating growth pattern and progression from a low-grade tumour to a more malignant phenotype are characteristic of gliomas. These features generally prevent their complete surgical removal and curative treatment. Tumour recurrences are common and ultimately fatal to the patient.

The histological transformation from low-grade neoplasia to secondary malignant glioma and the accumulation of genetic alterations is best documented in astrocytomas but is also feature of oligodendroglial tumours. The two main types of genetic abnormalities involved in glioma progression are activation of oncogenes and inactivation of tumour suppressor genes. Oncogenes promote cell proliferation and invasion as well as inhibit apoptosis. Inactivation of tumour suppressor genes leads to impaired regulation of cell growth.

Management of gliomas has progressed substantially during the past 20 years. Advances in surgery, radiation and chemotherapy have improved the survival of glioma patients. Developments in neuroradiology and neuropathology have refined the diagnostics of gliomas. Understanding of the molecular biology of brain tumours has increased rapidly. This knowledge has been assimilated into clinical neuropathology to complete the histopathological diagnosis. The molecular profiles of gliomas are also used in clinical neuro-oncology to the estimate prognosis for patients (prognostic markers) and to predict responses to chemotherapy or biological therapy (predictive markers).

The objective of this thesis is to study molecular markers related to the progression and recurrence of low-grade gliomas.

REVIEW OF THE LITERATURE

Pathobiology of glial cells

The main cell types that comprise the CNS are neurons and neuroglial cells. Rudolf Virchow, in 1856, first described neuroglia as a connective substance where the nervous elements are embedded (Virchow, 1856 cited in Somjen, 1988). Neurons are electrochemically active cells responsible for nerve impulse conduction. The three types of neuroglial cells are astrocytes, oligodendrocytes and ependymal cells, together known as macroglial cells. They are supporting cells, which have important functions in maintaining the homeostasis in the CNS. Neuroglial cells comprise approximately half of the total brain volume (Noback et al., 2005). Neurons and neuroglial cells are developmentally derived from neural tube ectoderm. The fourth type of supporting cells in the CNS is the microglial cells, which are brain equivalent for macrophages capable of obtaining antigen-presenting and phagocytic properties (Graeber and Streit, 2010). The name microglia is a misnomer, however. Microglial cells are not true neuroglial cells since they are of mesodermal origin. Precursor cells of microglia migrate to the brain parenchyma during early embryonic development (Monier et al., 2006).

Astrocytes

Astrocytes are the main supporting cells of the CNS; they constitute 20-50% of brain volume depending on the anatomical region (Squire et al., 2008). Astrocytes provide physical support for neurons and other cells of the CNS. Historically, the supportive properties of astrocytes were considered their main function. In recent years, however, their role in interactions with neurons and CNS function has been established (Sofroniew and Vinters, 2010).

Astrocytes are further subclassified in fibrous and protoplasmic astrocytes on the basis of their morphology and anatomical distribution. Fibrous astrocytes with long processes are found in cerebral white matter. Protoplasmic astrocytes have extensively branching processes and are located in grey matter (Kiernan, 1998).

Processes of astrocytes ensheath synapses and even participate in synaptic function through the release of regulating molecules called gliotransmitters (Halassa et al., 2007). Astrocytic processes express transport molecules that clear the synaptic space by facilitating the re-uptake of neurotransmitters such as glutamate (Sofroniew and Vinters, 2010). Astrocytes maintain the fluid and ion homeostasis in the CNS through the aquaporin 4 water channel and K+ transporters (Seifert et al., 2006). Astrocytes have numerous contacts

with blood vessels and they are involved in controlling the cerebral microcirculation (Iadecola and Nedergaard, 2007).

During the CNS development, the precursor cells of astrocytes are called radial glia, which give rise to astrocytes later in development (Vinters and Kleinschmidt-DeMasters, 2008; Kriegstein and Alvarez-Buylla, 2009). Radial glia establish guiding fibers for neuroblasts when they migrate to the cerebral cortex. In fact, the radial glia are also progenitor cells which give rise to cortical pyramidal neurons as well (Kriegstein and Alvarez-Buylla, 2009).

Astrocytes have a crucial role in the reaction to any tissue damage in the CNS. Tissue injury, such as ischaemia, trauma, radiation injury, infection or neoplastic invasion, can evoke an astrocytic reaction termed gliosis, the CNS equivalent to scarring elsewhere in the body. Astrocytic hypertrophy and proliferation are seen in both acute and chronic injuries to brain tissue. In a normal resting brain, the cytoplasm of astrocytes is barely visible in H&E staining. In reactive gliosis, the morphology of astrocytes is transformed and astrocytes with abundant cytoplasm, known as gemistocytic astrocytes, are formed.

Astrocytic scarring may form borders along the region of tissue damage caused by ischaemia, infection or neoplasms. Glial scars are chronic and may exist in the brain tissue for a long period after the original insult has resolved (Sofroniew and Vinters, 2010). In clinical neuropathology, florid astrocytic gliosis is seen as a reaction to non-glial brain neoplasms and metastases. Benign slowly growing gliomas such as pilocytic astrocytomas may evoke reactive gliosis in the tumour boundaries as well.

Oligodendrocytes

Oligodendrocytes are responsible for producing and maintaining the myelin sheaths, which surround axons in the CNS (Kiernan, 1998). In the grey matter, oligodendrocytes are also clustered perineuronally as satellite cells. In the white matter, interfascicular oligodendrocytes are arranged in rows along myelinated axons. Oligodendrocytes are smaller and have fewer processes than astrocytes. In H&E-stained paraffin sections the cytoplasm of oligodendrocytes is not visible, but instead an artefactual perinuclear halo is often present (Vinters and Kleinschmidt-DeMasters, 2008). Oligodendrocytes share a common precursor cell with neurons and astrocytes. They are also derived from radial glia through intermediate progenitor cells (Kriegstein and Alvarez-Buylla, 2009).

Oligodendrocyte responses to tissue injury are limited. They are vulnerable to oxidative damage, which contributes to oligodendrocyte loss in many disorders including multiple sclerosis and ischaemia (Bradl and Lassmann, 2010). In demyelinating diseases, such as multiple sclerosis, oligodendrocyte proliferation can be seen as a reparative response to injury (Vinters and Kleinschmidt-DeMasters, 2008).

Ependyma

Ependymal cells line ventricular surfaces of the brain and the central canal of the spinal cord. Morphologically ependymal cells share similarities with columnar and cuboidal epithelial cells, but unlike epithelium, ependyma lacks basal lamina. The apical surface of ependymal cells is covered by microvilli and cilia (Del Bigio, 2010). Ependymal cells contribute to fluid homeostasis between brain parenchyma and cerebrospinal fluid.

Gliomas

Origin of gliomas

The origin of gliomas has excited neuropathologists and neuro-oncologists for decades and is still enigmatic. The neoplastic transformation of mature glial cells has been introduced as an explanation for gliomagenesis. Since the first publication on the classification of gliomas by Bailey and Cushing (Bailey and Cushing, 1926 cited in Martin-Villalba et al., 2008), the basis of classifications has been that the morphology of low-grade astrocytomas resembles astrocytes and that oligodendrogliomas share common features with differentiated oligodendrocytes. Therefore, it seems reasonable that gliomas are derived from their mature counterpart cells. However, evidence supporting this hypothesis is lacking. The formation of gliomas through dedifferentiation of mature glial cells raises an interesting question. Occasionally, gliomas contain several morphological components; e.g. oligoastrocytomas are composed of two types of glial cells and glioneuronal tumours contain glial and neuronal cells. How are these gliomas formed if the cell of origin is a mature glial cell?

Glial cell turnover in the adult brain is low. Glial cells, especially astrocytes, are able to proliferate as a response to injury after trauma or demyelination. The cells could acquire mutations and other genetic changes when DNA duplicates in mitosis during reactive proliferation. However, epidemiological studies do not show convincing evidence of increased glioma incidence after trauma (Ohgaki and Kleihues, 2005a).

In diffuse gliomas, precursor lesions are not identified and thus, the first morphologically recognisable step in gliomagenesis is a low-grade neoplasm, e.g. grade II astrocytoma. In epithelial cancer such as colon carcinoma, the neoplastic lesion develops in continuously dividing epithelium. Furthermore, a continuum of the progression from a premalignant adenoma to a malignant invasive carcinoma can be identified in colon carcinoma (Fearon, 2010). In contrast to carcinomas, gliomas lack premalignant lesions equivalent to epithelial dysplasia; therefore, understanding of molecular events prior to low-grade glioma is still limited.

The identification of neural stem cells and glial progenitor cells in adult brain has brought new perspectives to gliomagenesis. Neural stem cells are multipotent cells capable of generating multiple cell types and they are self-renewing; i.e. they can remain undifferentiated after cell

division (Yadirgi and Marino, 2009). Progenitor cells are precursors capable of producing cells that are of either neuronal or glial lineage, but not both (Sanai et al., 2005). The largest region containing neural stem cells in the human brain is the subventricular zone (SVZ) in the lateral wall of the lateral ventricle (Sanai et al., 2004). The SVZ contains a population of astrocytes that behave as multipotent neural stem cells (Sanai et al., 2004).

Glial progenitor cells have been identified in the human brain (Armstrong et al., 1992; Roy et al., 1999). During development, these glial precursors have three possible fates: 1) they can differentiate into mature glia, 2) they could die during early postnatal development or 3) a small subset remain immature and cycling through adult life (Canoll and Goldman, 2008). It has been estimated that glial precursor cells account for up to 4% of adult white matter cells (Canoll and Goldman, 2008). Hypothetically, if these progenitor cells undergo transformation they could give rise to tumours that could differentiate into astrocytomas or oligodendrogliomas. In fact, the same signalling pathways that regulate self-renewal of normal stem cells are active in the transformation of tumour cells (Sanai et al., 2005). Tumour cells that are multipotent and capable of self-renewal are called cancer stem cells or tumour-initiating cells (Jordan et al., 2006; Hadjipanayis and Van Meir, 2009). Cancer stem cells constitute only a small subpopulation of the tumour bulk but they are essential to tumour growth. Cancer stem cells show in-creased resistance to radio- and chemotherapy. Therefore, it has been suggested that failure in cancer treatment reflects incomplete elimination of the cancer stem cells resulting in the recurrence of tumours (Prestegarden and Enger, 2010).

Tumour-initiating cells have been isolated from human gliomas and transplanted into mouse brains where they produce tumours that histologically mimic the original gliomas (Galli et al., 2004; Singh et al., 2004).

Epidemiology

Tumours of the CNS account for 1.7% of all new cancers worldwide (Parkin et al., 2005). Gliomas represent 31% of the primary CNS tumours (Central Brain Tumor Registry of the United States, 2011). In Finland, the incidence of gliomas was 4.7 per 100000 in a recent population-based study (Larjavaara et al., 2007). An increase in the incidence of gliomas during the 1970s and early 1980s has been reported in Finland (Kallio, 1993) and other Nordic countries (Lönn et al., 2004). This increase coincides with the introduction of computed tomography (CT) into clinical practise and may reflect improved detection of gliomas (Lönn et al., 2004).

The peak incidence of gliomas is between the ages of 45 and 70, which is mainly due to glioblastomas, the most malignant type of gliomas (Louis et al., 2008). In children, the benign pilocytic astrocytoma forms a lower incidence peak. Low-grade diffuse gliomas are most common in the 35-44 age group

(Okamoto et al., 2004; Central Brain Tumor Registry of the United States, 2011). Males are more commonly affected; the male/female ratio of gliomas is 1.26 (Ohgaki and Kleihues, 2005b).

Therapeutic high-dose radiation is the only environmental risk factor that is clearly associated with an increased risk of gliomas. Children who have received prophylactic CNS irradiation for acute lymphoblastic leukemia have an increased risk for developing brain tumors such as gliomas and primitive neuroectodermal tumors (Ohgaki and Kleihues, 2005a). The rapid increase in mobile phone use during the last 20 years has raised concerns about the possible link between radiofrequency electromagnetic radiation and brain tumors. A recent international case-control study of mobile phone users concludes that they have no increased risk for glioma or meningioma (INTERPHONE Study Group, 2010).

Several hereditary tumour syndromes are characterised by predisposition to gliomas among other tumours (Ohgaki and Kleihues, 2005a). Neurofibromatosis 1 is associated with pilocytic astrocytomas of the optic nerve and less frequently diffuse astrocytomas or glioblastomas (Rodriguez et al., 2008). Neurofibromatosis 2 patients have an increased risk for spinal ependymomas. Patients with Li-Fraumeni syndrome carry germline mutations of the p53 gene and have increased incidence of astrocytic gliomas (Louis et al., 2007).

Classification

The first attempt at classification of gliomas on their histogenetic basis was made by Bailey and Cushing in 1926 (Bailey and Cushing, 1926 cited in Martin-Villalba et al., 2008). Their classification showed that the histopathology of the tumour is can give valuable information about the patient outcome. Predicting the behaviour of the tumour by means of histological grading is the principle of modern glioma classification as well.

The World Health Organization (WHO) classification of gliomas edited by Zülch was first published in 1979 (Zülch, 1979). The second edition, edited by Kleihues, appeared in 1993 (Kleihues et al., 1993). In 1997, the International Agency for Research of Cancer in collaboration with the International Society of Neuropathology, published a reference book that combined pathology and the genetics of brain tumours in one book (Kleihues and Cavenee, 1997). Thereafter, the WHO classification of brain tumours has included genetic alterations in the descriptions of tumour entities (Kleihues and Cavenee, 2000; Louis et al., 2007). The current WHO classification of gliomas published in 2007 is still based on the histopathological morphology of the tumours (Louis et al., 2007).

Grading

Histological grading of gliomas gives a powerful estimation of the biological behaviour of the tumour and forms the basis for planning adju-

vant therapies after surgical resection.

WHO grade I is applied to tumours, which are clearly circumscribed, benign in their behaviour and can be cured following total surgical resection. WHO grade II tumours are slowly progressing, low-grade malignant tumours that exhibit cellular atypia without extensive proliferation or anaplasia. Typical survival for grade II neoplasms is over 5 years (Figure 1). WHO grade III tumours show increased cellularity, anaplasia and mitotic figures. Survival for grade III tumour is 2-3 years. In grade IV tumours the histological hallmarks are necrosis and vascular proliferation in addition to grade III features. Together grade III-IV gliomas are called malignant gliomas. In a population-based study, the median survival for grade IV gliomas i.e. glioblastomas was less than 1 year (Ohgaki et al., 2004). In recent trials, there has been a promising tendency of improved prognosis when glioblastomas are treated with combined radiotherapy and chemotherapy (Glas et al., 2009; Stupp et al., 2009). Currently, there is considerable interobserver variability in the grading of gliomas, and new grading schemes are needed, in which histological criteria are unequivocally defined and scored (Kros, 2011). The main difference between grade I and grade II-IV gliomas is their growth pattern. Benign grade I gliomas e.g. pilocytic astrocytomas are typically discrete while grade II-IV tumours show
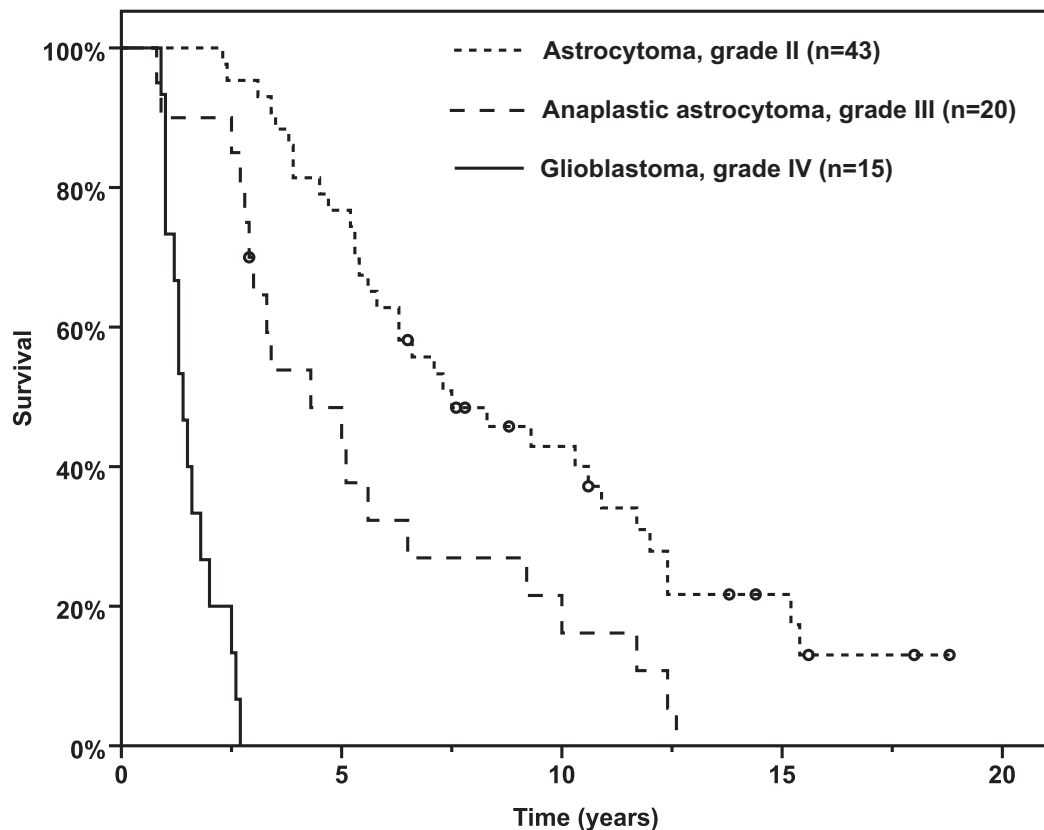
Figure 1 Survival of 78 patients with astrocytic gliomas WHO grade II-IV (study IV).

diffuse infiltration of neoplastic cells into the surrounding brain. The emphasis of this thesis is on diffuse gliomas, i.e. astrocytomas and oligodendrogliomas, grade II-IV.

Histopathology of diffuse gliomas

Diffuse astrocytoma

The peak incidence of grade II diffuse astrocytomas is between ages 30 and 40. There is a slight male predominance 1.18:1 (Louis et al., 2007). Cerebral hemispheres are mainly affected in adults. Brain stem and thalamus are more commonly affected in children.

Diffuse astrocytomas WHO grade II show increased cellularity (2-3 times) compared to normal white matter. Due to their infiltrative nature, the margins of diffuse astrocytomas cannot be clearly defined. Neoplastic cells intermingle with normal structures and cells; they also follow axons and tracts. Astrocytoma cells infiltrate the cortex and deep gray matter and surround neurons (perineuronal satellitosis). Microcystic change of tumour tissue is a common feature.

The two main histological subtypes are fibrillary and gemistocytic astrocytoma. In fibrillary astrocytoma, tumour cells have scant cytoplasm with numerous processes forming a fibrillary background. Nuclei are enlarged, hyperchromatic and irregular in shape. Gemistocytic astrocytomas are characterised by the plump eosinophilic cytoplasm and eccentric nuclei of the neoplastic cells. The rare variant protoplasmic astrocytoma consists of small cells with few cytoplasmic processes and mucoid or microcystic matrix. Some studies suggest that gemistocytic morphology is an unfavourable prognostic feature in astrocytomas (Shaw et al., 1989; Krouwer et al., 1991; Okamoto et al., 2004). Gemistocytic astrocytomas are prone to undergo malignant progression more rapidly than fibrillary astrocytomas (Louis et al., 2007).

There is no strict cut-off value for mitotic figures in grade II astrocytomas but generally occasional mitoses are accepted especially in large tumour samples. However, in small stereotactic biopsies, even single mitotic figure may allow grading to anaplastic astrocytoma if other cellular features are consistent with anaplasia.

Anaplastic astrocytoma

Anaplastic astrocytomas share the main histopathological features with diffuse astrocytomas with signs of focal or diffuse anaplasia. Anaplastic astrocytomas are hypercellular with marked mitotic activity and nuclear pleomorphism. The shape of nuclei is more angular and chromatin structure coarse compared to grade II astrocytomas. Anaplastic astrocytomas occur in the older age group compared to the grade II astrocytomas. Their peak incidence is approximately 45 years (Louis et al., 2007).

Glioblastoma

Glioblastomas are the most common primary brain tumours. They

account for 12-15% of all intracranial neoplasms (Louis et al., 2007). Glioblastomas occur preferentially in adults; their peak incidence is between 40 and 70 years.

Glioblastomas are the most malignant gliomas. The morphology of glioblastomas may be extremely heterogeneous as indicated in their previous name glioblastoma multiforme. Both cellular and nuclear pleomorphism is a prominent feature. Mitotic figures and atypical mitoses are frequent. Cellular composition varies from small fusiform cells to multinucleated giant cells.

The key features distinguishing glioblastomas from lower-grade astrocytomas are necrosis and microvascular proliferation. Typically, glioblastomas have large central necroses surrounded by vital tumour tissue as seen in MRI imaging. The histologically characteristic form of necrosis consists of irregular necrotic foci surrounded by a radially oriented pseudopalisading zone of glioma cells. Thrombosed vessels are frequently seen in necrotic tumour areas. Brat et al. have postulated that glioma cells form pseudopalisades when migrating away from the hypoxic focus, which is often caused by vessel thrombosis (Brat et al., 2004).

Microvascular proliferation appears as glomeruloid vascular structures, which consist of hyperplastic endothelial and smooth muscle cells (Wesseling et al., 1995). Glomeruloid vessels are often seen near necrotic foci and less frequently at the infiltrating edge of glioblastomas.

Glioblastomas are divided into two subtypes that are histologically indistinguishable. Primary glioblastomas (de novo glioblastomas) develop without a previous clinical history of glioma. Secondary glioblastomas arise through malignant progression from pre-existing lower-grade astrocytomas (Louis et al., 2007).

The vast majority (over 90%) of glioblastomas are primary. They usually develop at old age (mean 62 years) with short clinical history of symptoms (mean 6 months) (Ohgaki et al., 2004; Ohgaki and Kleihues, 2005b). Secondary glioblastomas are rare tumours comprising less than 10% of all glioblastomas. They develop at younger age (mean 45 years) and more frequently in women (male/female ratio 0.65) (Ohgaki and Kleihues, 2005b). Progression from grade II glioma to glioblastoma takes an average for 5.3 years and from grade III glioma for 1.4 years (Ohgaki and Kleihues, 2005b). Approximately 70% of grade II gliomas progress into grade III/IV (Furnari et al., 2007).

Several morphological variants of glioblastoma are recognised. Occasionally glioblastomas contain oligodendroglioma-like areas. According to the WHO 2007 classification, these tumours should be called *glioblastomas with oligodendroglial component*, and they may have better prognosis than ordinary glioblastomas (Louis et al., 2007). *Small cell glioblastomas* are composed of a dense infiltrate of small monomorphic tumour cells with round or slightly elongated nuclei. These tumours should be distinguished from poorly

differentiated anaplastic oligodendrogliomas. *Gliosarcoma* is a biphasic variant of glioblastoma consisting of a malignant glial and sarcomatous mesenchymal component. *Giant cell glioblastoma* consists of bizarre, multinucleated giant cells. This variant may exhibit a less infiltrative growth pattern and slightly favourable prognosis (Louis et al., 2007).

Oligodendroglioma

Oligodendrogliomas account for 5% of all gliomas Oligodendrogliomas arise primarily in the cerebral hemispheres of adults with a peak incidence between 40 and 45 years of age (Louis et al., 2007). In children, oligodendroglioma is rare. The cardinal histological features of oligodendrogliomas are round nuclei and perinuclear halo artefact in paraffin sections, producing a typical morphology resembling fried egg. Oligodendrogliomas typically infiltrate to cerebral cortex where neoplastic cells gather around neurons (perineuronal satellitosis). Migrating tumour cells often accumulate at the subpial surface of the cortex. These growth patterns are called secondary structures of Scherer (Scherer, 1938 cited in Claes et al., 2007). Microcysts and calcifications are also features of oligodendrogliomas, although they are non-specific.

Occasionally oligodendrogliomas contain minigemistocytes resembling gemistocytic astrocytes. Reactive astrocytes are usually scattered evenly throughout the oligodendroglioma. The branching capillary network of oligodendrogliomas resembles chicken wire pattern. Occasional

mitoses are compatible with the diagnosis of grade II oligodendroglioma (Louis et al., 2007).

Anaplastic oligodendroglioma

Anaplastic oligodendrogliomas account for 1.3% of gliomas. They manifest in the slightly older age group compared to low-grade oligodendrogliomas, with a peak incidence between 45 and 50 years (Louis et al., 2007).

Morphologically anaplastic oligodendrogliomas show high cellularity. There is marked nuclear atypia and mitotic figures are frequent. In addition to characteristic branching capillaries of oligodendrogliomas, microvascular proliferation may be present. Foci of necrosis are present in one third of anaplastic oligodendrogliomas (Miller et al., 2006).

Oligoastrocytoma and anaplastic oligoastrocytoma

Oligoastrocytomas are defined as tumours with two cell components resembling astrocytoma and oligodendroglioma. The two cellular components are usually intermingled. In rare cases, the tumour is biphasic and can be divided into astrocytic and oligodendrocytic areas. Currently, there is considerable interobserver variability in the diagnostics of oligoastrocytic tumours due to lack of definite histological criteria (Kros et al., 2007).

Grade II oligoastrocytomas show moderate cellularity and low mitotic activity. Microcystic change and calcification may be present. In anaplastic oligoastrocytomas, diag-

nostic features are high cellularity, obvious mitotic activity, cellular pleomorphism and microvascular proliferation. Anaplastic oligoastrocytomas with necrosis should be classified as glioblastomas with an oligodendroglial component, because necrosis is marker of poor prognosis in these tumours (Miller et al., 2006; Louis et al., 2007).

Histological types and survival

Median survival times by histological glioma type from population-based studies are shown in Table 1. Oligodendroglial tumours generally have a better prognosis than astrocytomas of the corresponding WHO grade. Clinical trials usually show better outcomes than population-based studies due to bias towards recruitment of younger patients and patients with better performance status (Louis et al., 2007). The glioblastoma patients presented in Table 1 were diagnosed and treated before the era of modern chemoradiotherapy (see later).

Molecular pathogenesis of gliomas

Natural course of diffuse gliomas

Recurrence after treatment and progression towards a more malignant phenotype are inherent characteristics of diffuse gliomas. Most low-grade gliomas develop a recurrent tumour after variable periods of time. Often the recurrent tumour shows histologically increased cellularity, nuclear atypia and mitoses as features of morphological progression (Figure 2). Molecular pathogenesis of gliomas is a stepwise process where genetic alterations accumulate in glial cells causing initiation and progression of the tumour. Therefore, the number of genetic changes is greatest in glioblastomas. Single molecular events can be dated to certain steps of tumour evolution and correlated with histological phenotype. The path of progression at both the histological and molecular level has

Table 1   Mean age and median survival of gliomas in a population-based material from Switzerland, diagnosed in 1980-1994.

Tumour histology	WHO grade	n	Mean age	Median survival (years)
Astrocytoma	II	52	41.0	5.6
Oligoastrocytoma	II	20	41.1	6.6
Oligodendroglioma	II	50	40.9	11.6
Anaplastic astrocytoma	III	47	45.5	1.6
Anaplastic oligoastrocytoma	III	11	48.2	–
Anaplastic oligodendroglioma	III	13	50.4	3.5
Glioblastoma	IV	680	62.2	0.4

Source: (Okamoto et al., 2004; Ohgaki and Kleihues, 2005b).

been best characterised in astrocytomas. Hanahan and Weinberg have suggested that cancer cells share six critical alterations, the hallmarks of cancer, that enable their malignant behaviour: 1) self-sufficiency in growth signals, 2) insensitivity to growth-inhibiting signals, 3) resistance to apoptosis, 4) limitless replication, 5) angiogenesis and 6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). In their recent review, the authors have updated the cancer hallmarks and added two emerging characteristics of cancer cells: 1) metabolic reprogramming (aerobic glycolysis) and 2) capability of avoiding destruction by the immune system (Hanahan and Weinberg, 2011). Most of these characteristics are common for different types of cancers including gliomas and will be reviewed in the following sections.

Oncogenes

EGFR

Oncogenes are altered genes that promote the neoplastic transforma-
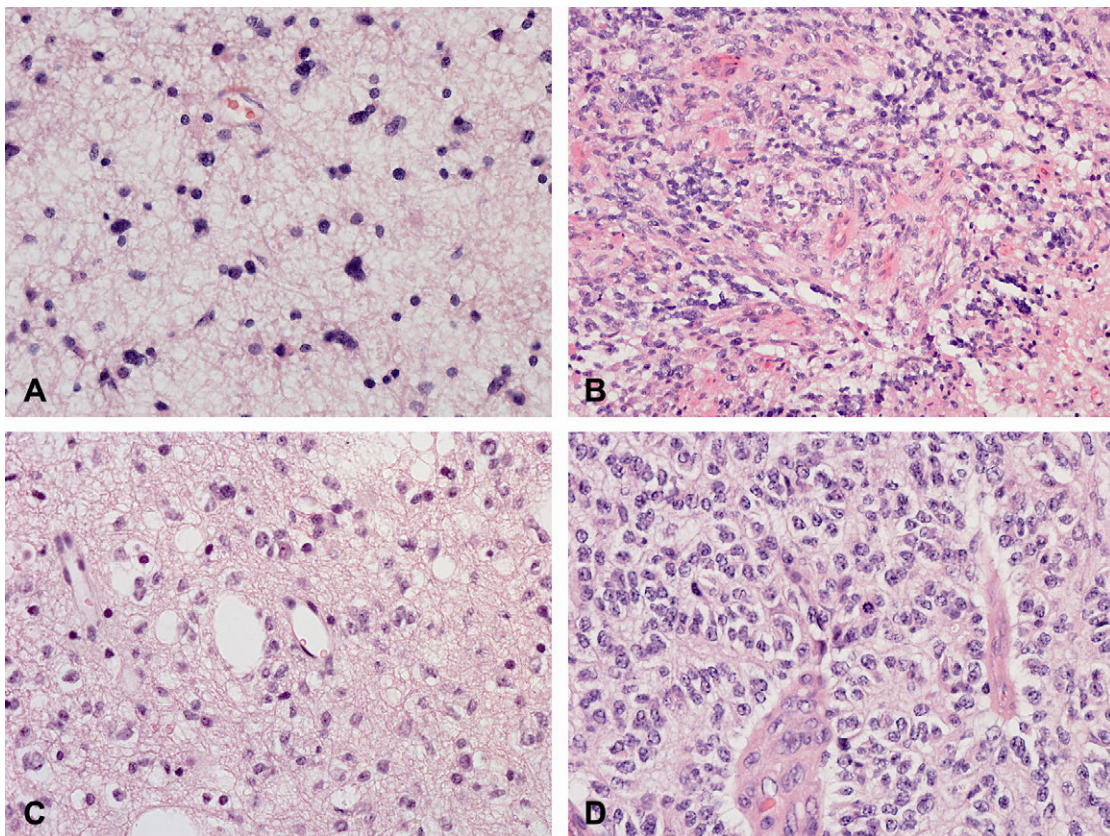
Figure 2 Histological progression of gliomas. **A**, low-grade astrocytoma showing low cellularity and nuclear atypia. **B**, 5 years later the tumour in fig. A has progressed to glioblastoma with high cellularity, vascular proliferation and necrosis. **C**, low-grade oligodendroglioma shows round nuclei and prinuclear halos. **D**, recurrence of the tumour in fig. C 10.5 years later with increased cellularity and vascular proliferation.

tion of cells. Activation of onco-genes occurs by mutation or amplifi-cation of their normal cellular counterparts proto-oncogenes.

Epidermal growth factor receptor (EGFR) is the most frequently am-plified gene in glioblastomas. EGFR is a member of the ErbB family of tyrosine kinase receptors, which are often dysregulated in cancer. EGFR amplification and overexpression are reported in over 30% of all glioblas-tomas (including primary and secon-dary) (Louis, 2006). The EGFR gene is located at chromosome 7p12. It encodes a 170 kD transmembrane tyrosine kinase receptor protein. EGF and TGF-α are ligands for the EGFR. Activation of wild-type EGFR by its ligand triggers signal-ling cascade, which enhances cell proliferation and migration (Yarden and Sliwkowski, 2001). Amplifica-tion of the EGFR gene leads to overexpression at the mRNA and protein level and is often associated with structural alterations of the gene. Amplified EGFR genes are usually extrachromosomal double minute fragments. EGFR amplifica-tion is typical genetic alteration in primary glioblastomas, present in ca. 40% of cases (Ohgaki and Kleihues, 2007). In secondary glioblastomas, EGFR amplification is rare (Ohgaki et al., 2004).

The most common mutant of EGFR gene is EGFRvIII, which occurs in 50-60% of tumours with EGFR amplification (Furnari et al., 2007). The mutant EGFRvIII results from deletions of exons 2-7 and lead to truncated form of EGFR protein lacking part of extracellular domain. EGFRvIII is constitutively active

resulting in increased proliferation and reduced apoptosis (Furnari et al., 2007).

KIT, PDGFRA and VEGFR2

KIT, PDGFRA and VEGFR2 (KDR) are receptor tyrosine kinase genes that are clustered on chromo-somal segment 4q12. Under physio-logical conditions, KIT is activated by the binding of its ligand stem cell factor (Antonescu, 2011). Activation of KIT triggers signalling cascades that result in cell proliferation and inhibition of apoptosis (Kitamura and Hirotab, 2004). Oncogenic mu-tations of KIT are found in the ma-jority of gastrointestinal stromal tumours (Hirota et al., 1998) as well as in a subset of melanomas (Curtin et al., 2006) and seminomas (Tian et al., 1999) but they are uncommon in other types of solid cancer (Sihto et al., 2005). High-level KIT amplifica-tion is present in 33% of glioblas-tomas but its overexpression at protein level is rare (Joensuu et al., 2005).

PDGFRA and its ligand PDGF are overexpressed in malignant gliomas (Fleming et al., 1992; Guha et al., 1995; Lokker et al., 2002; Joensuu et al., 2005; Paulsson et al., 2011), which suggests the presence of an autocrine signalling loop. Am-plification of the PDGFRA gene has been found in a subset of glioblas-tomas (Fleming et al., 1992; Her-manson et al., 1996; Joensuu et al., 2005; Verhaak et al., 2010). In an experimental cell culture and mouse model, overexpression of PDGF in neural progenitor cells by gene trans-fer has been shown to induce tu-

mours identical to human oligodendrogliomas (Dai et al., 2001).

VEGFR2 is predominantly expressed in endothelial cells of glioblastomas but may be present in tumour cells as well (Hatva et al., 1995; Steiner et al., 2004). The VEGFR2 gene is amplified in 22% of glioblastomas (Joensuu et al., 2005). KIT, PDGFRA, and VEGFR2 genes are concurrently amplified in up to 21% glioblastomas (Joensuu et al., 2005). The role of PDGFRA and VEGFR2 in the angiogenesis of gliomas will be discussed later.

Tumour suppressor genes

p53

Tumour suppressor genes are growth-controlling genes of the cells. Their inactivation by deletions, mutations or epigenetic mechanisms leads to dysregulation of the cell cycle and is a common mechanism in the development of cancer.

p53 is a tumour suppressor gene located at chromosome 17p13. Inactivation of p53 by mutations leads to the accumulation of non-functioning protein in the cell. Dysfunction of the p53 pathway is one of the most common molecular alterations in human malignancies. Intact p53 responds to DNA damage or oncogene activation by blocking the cell cycle in the G1 phase or by inducing apoptosis, preventing possible neoplastic transformation of the cell (Furnari et al., 2007). The p53 mutation is a hallmark of diffuse astrocytomas (Louis et al., 2007). p53

mutation is an early event in the progression of astrocytomas (Figure 3); over half of grade II astrocytomas carry the p53 mutation (Watanabe et al., 1997; Okamoto et al., 2004). Therefore, the p53 mutation is frequent in secondary glioblastomas, which develop from lower-grade gliomas (Ohgaki and Kleihues, 2007). p53 protein accumulation is frequent in astrocytomas, but elevated protein expression is not always due to mutation. Immunohistochemical p53 staining is useful in differentiating between astrocytomas and oligodendrogliomas in neuropathological diagnostics. Strong nuclear staining for p53 often reflects mutation of the gene and is more frequent in astrocytomas than in oligodendrogliomas (Gupta et al., 2005). Mutations of the p53 gene can be heterogeneous, and one glioma may contain tumour cells with different p53 mutations (Ren et al., 2007).

PTEN and Rb1

The PTEN gene, located at 10q23.3, is a tumour suppressor gene that is inactivated by deletion or mutation in up to 40% of glioblastomas (Knobbe et al., 2002; Cancer Genome Atlas Research Network, 2008). PTEN is part of the PI3K/PTEN/AKT pathway, and its inactivation eventually leads to cell proliferation (Knobbe et al., 2002).

The retinoblastoma gene (Rb1) encodes Rb protein that regulates transition from G1 phase to S phase in the cell cycle (Harbour and Dean, 2000). Rb1 is inactivated by deletion in one third of high-grade astrocy-
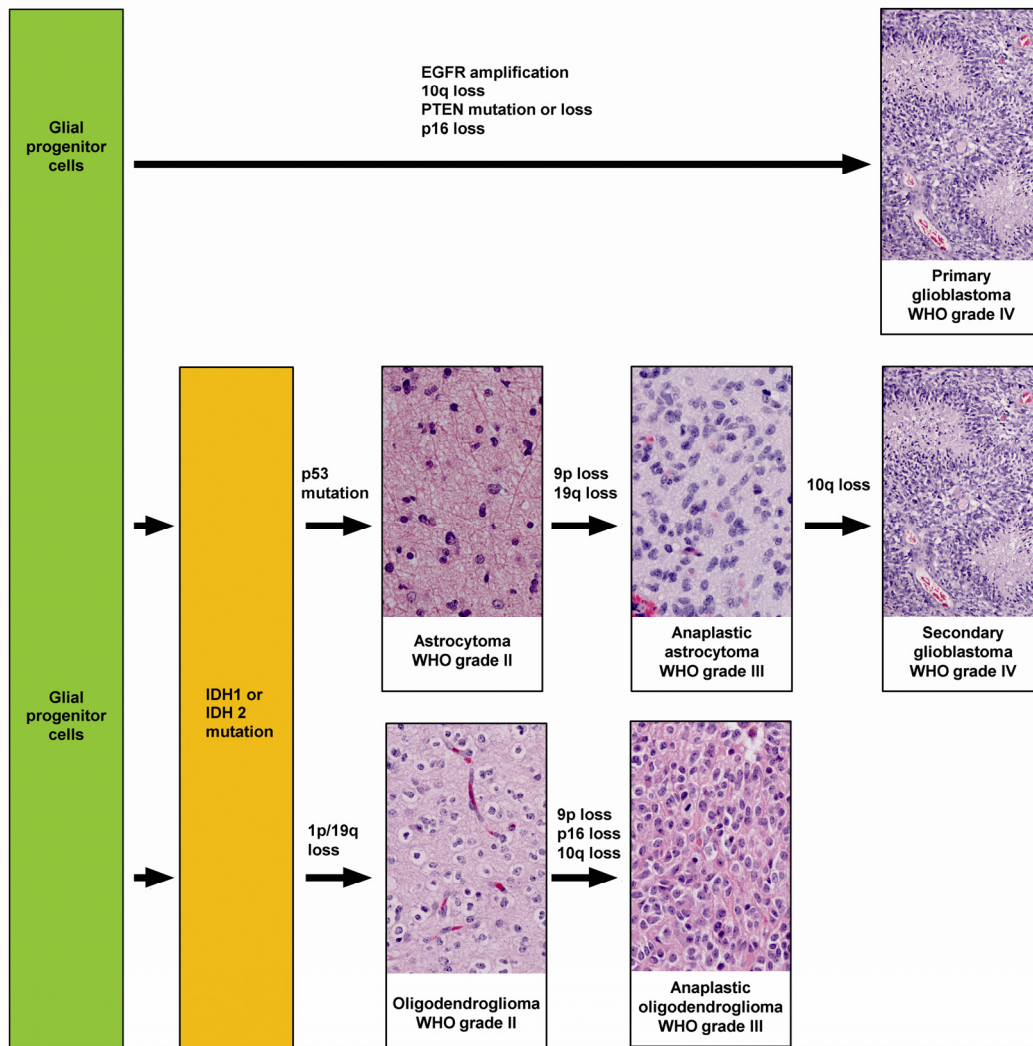
Figure 3 Summary of the most frequent genetic alterations during the progression of gliomas (Louis et al., 2007; Riemenschneider et al., 2010).

tomas (Henson et al., 1994; Ichimura et al., 1996).

1p and 19q deletions

Deletions of chromosome arms 1p and 19q are characteristic of oligodendroglial tumours (von Deimling et al., 1992; Reifenberger et al., 1994; Cairncross et al., 1998). Co-deletion of 1p and 19q is present in 44-80% of oligodendrogliomas (Smith et al., 2000; Watanabe et al., 2002; Okamoto et al., 2004; Jeuken et al., 2010) and in 20-30% of oligoastrocytomas (Smith et al., 2000; Jeuken et al., 2001; Jeuken et al., 2010). 1p/19q deletion frequently involves the chromosome arms in their entirety. Loss of heterozygosity (LOH) of 1p and 19q is mediated by unbalanced translocation t(1;19)(q10;p10), where derivative chromosome 1p-19q is lost (Griffin et al., 2006; Jenkins et al., 2006). So

far, the search for tumour suppressor genes in these chromosome arms has been unsuccessful (Jansen et al., 2010).

Oligodendrogliomas that have classic histological features such as uniform round nuclei with perinuclear halo and a vascular pattern resembling chicken wire show strong correlation with 1p/19 co-deletion (Giannini et al., 2008; Scheie et al., 2008).

1p/19q deletion is a powerful prognostic and predictive marker for oligodendroglial tumours. Loss of 1p/19q was reported to associate with better response to chemotherapy and longer overall survival in anaplastic oligodendrogliomas in 1998 (Cairncross et al., 1998). The prognostic value of 1p/19q loss in anaplastic oligodendrogliomas and oligoastrocytomas has been confirmed in clinical trials (Cairncross et al., 2006; van den Bent et al., 2006). Deletion of 1p/19q predicts a chemotherapy response and better overall survival in low-grade gliomas as well (Kaloshi et al., 2007). However, the effect of 1p/19q deletion on patient outcome may be smaller in low-grade gliomas and its role in the management of patients is still controversial (Jansen et al., 2010; Tabatabai et al., 2010).

Loss of 1p/19q seems to have prognostic importance only in patients treated with either radiotherapy or combined radiotherapy and chemotherapy. 1p/19q loss was not prognostic marker for progression-free survival in patients who underwent surgical resection for their oligodendroglial tumour without

further treatment (Weller et al., 2007).

MGMT

MGMT (O^6 methylguanine-DNA methyltransferase) is an enzyme that repairs DNA damage caused by alkylating chemotherapeutic agents used in the treatment of gliomas (Esteller et al., 2000). Therefore, functioning MGMT enzyme causes resistance to alkylating drugs such as temozolomide. The MGMT gene is located at chromosome 10q26. Its transcription is inactivated by methylation of CpG islands in the gene promoter area in several human cancers including gliomas (Esteller et al., 1999). MGMT gene is methylated in 41-45% of glioblastomas (Esteller et al., 1999; Hegi et al., 2005).

MGMT promoter methylation is associated with a response to temozolomide treatment in glioblastoma patients and has therefore been introduced as a predictive marker for chemotherapy (Hegi et al., 2005). However, MGMT methylation is a favourable marker for survival even in glioblastoma patients who have received radiation treatment, but not chemotherapy (Rivera et al., 2010). Thus, MGMT methylation may not be just a positive predictive marker for chemotherapy, but indicates in general a favourable prognostic phenotype in glioblastoma (Jansen et al., 2010). MGMT methylation is correlated with 1p/19q deletion and prognosis in anaplastic oligodendrogliomas as well (van den Bent et al., 2009).

IDH1

The IDH1 gene in chromosome 2q33 encodes cytoplasmic isocitrate dehydrogenase-1 enzyme, which participates in the citric acid cycle. Mutations of the IDH1 gene are a novel finding in gliomas. IDH1 gene mutations were reported in 12% of 22 glioblastomas in a comprehensive genomic analysis of over 20000 protein-coding genes. In this genome-wide analysis, the investigators searched for mutations in genes that were previously not known to be altered in glioblastomas (Parsons et al., 2008). In a larger series of 321 gliomas, IDH1 was frequently mutated in low-grade astrocytomas (88%) and oligodendrogliomas (79%) (Watanabe et al., 2009). IDH2 is a mitochondrial counterpart of IDH1, and it is less frequently mutated in gliomas. Three percent of diffuse gliomas carry the IDH2 mutation (Hartmann et al., 2009).

IDH1 mutation is a very early event in gliomagenesis (Figure 3). In multiple biopsies of gliomas, IDH1 mutation is present before p53 mutations or 1p/19q loss (Watanabe et al., 2009). This suggests that IDH1 mutation takes place in glial precursor cells, and subsequent acquisition of the p53 mutation may lead to astrocytic differentiation while loss of 1p/19q leads to an oligodendrocytic phenotype (Watanabe et al., 2009). IDH1 mutation is present in 82% of secondary glioblastomas and rarely in primary glioblastomas (5%) suggesting different origins of these glioblastoma subtypes (Watanabe et al., 2009).

IDH1 mutation is a favourable prognostic factor in all grades of diffuse gliomas (Dubbink et al., 2009; Sanson et al., 2009; Weller et al., 2009; Wick et al., 2009; van den Bent et al., 2010). Furthermore, anaplastic astrocytomas without the IDH1 mutation have an even worse outcome than glioblastomas with mutated IDH1 (Hartmann et al., 2010). IDH1 mutation is also associated with 1p/19q co-deletion and MGMT promoter methylation in gliomas (Sanson et al., 2009; Labussiere et al., 2010; van den Bent et al., 2010).

Mutations of the IDH1 and IDH2 genes can be detected by DNA sequencing (Parsons et al., 2008). Over 90% of IDH1 mutations result in substitution the of amino acid arginine by histidine at codon 132 (p.R132H) (Hartmann et al., 2009). Monoclonal antibodies against mutant p.R132H IDH1 protein have been recently developed (Capper et al., 2009; Kato et al., 2009). These antibodies are suitable for paraffin-embedded tissues and allow inexpensive immunohistochemical detection of the most common IDH1 mutation in routine clinical neuropathology.

Currently, there are different hypotheses on the oncogenic mechanism of IDH1 mutations. The heterozygous mutation of IDH1 leads to reduced formation of its product α-ketoglutarate, which increases the levels of HIF-1α in cultured glioblastoma cells (Zhao et al., 2009). HIF-1 is a transcription factor that regulates genes implicated in tumour angiogenesis and cell proliferation (Semenza, 2010). These results suggest that IDH1 could act as a tumour suppressor gene (Zhao et

al., 2009). However, the direct relationship between IDH1 mutation and HIF-1α upregulation has been questioned. Williams and co-workers analysed 120 gliomas by IDH1 and HIF-1α immunohistochemistry (Williams et al., 2011). In their publication, the link between the expression of mutant IDH1 and HIF-1α was not evident, suggesting that activation of the HIF-1α pathway is not primarily regulated by IDH1 mutation.

Another mechanism for IDH1-related tumourigenesis has been suggested by Dang et al. They have shown that mutated IDH1 gains a novel enzymatic activity and is able convert α-ketoglutarate to 2-hydroxyglutarate (2HG) (Dang et al., 2009). Accumulation of 2HG could be a tumourigenic event in the brain, suggesting an oncogenic gain of function of mutated IDH1 (Jansen et al., 2010).

Markers of tumour cell differentiation and phenotype

Intermediate filaments

Identification of the origin and differentiation of tumour cells by studying their protein expression is a basic method in modern pathology. Immunohistochemistry is used to investigate cytoplasmic, nuclear and membranotic antigens in the classification of tumours.

Intermediate filaments (IF) are constituents of the cell cytoskeleton. The IF proteins are subclassified by their sequence homology into six classes: I-II) cytokeratins, III)

vimentin, desmin and glial fibrillary acidic protein (GFAP), IV) neurofilament proteins and α-internexin, V) nuclear proteins lamins, VI) nestin (Dahlstrand et al., 1992; Herrmann and Aebi, 2000).

GFAP is expressed in normal, reactive and neoplastic astrocytic cells. GFAP immunohistochemistry is a useful tool in clinical neuropathology differentiating glial tumours from meningeal neoplasms and metastases. Although GFAP expression tends to decrease during the course of glioma progression (Louis et al., 2008), focal GFAP reactivity is usually still seen even in poorly differentiated glioblastomas. GFAP expression is found in 80% of oligodendroglial tumours; therefore, its use in differentiating oligodendrogliomas from astrocytomas is limited (Ikota et al., 2006). In oligodendrogliomas, expression of α-internexin associates with 1p/19q deletions (Ducray et al., 2009) and the proneural gene expression profile, which is related to good prognosis (Ducray et al., 2008).

Vimentin is expressed in both astrocytic and oligodendroglial tumours (Dehghani et al., 1998; Koperek et al., 2004; Ikota et al., 2006). Nestin is a class VI intermediate filament that is expressed in neural stem cells and in high-grade astrocytomas and oligodendrogliomas (Lendahl et al., 1990; Dahlstrand et al., 1992; Ikota et al., 2006). Poorly differentiated and metaplastic glioblastomas rarely express cytokeratins (Oh and Prayson, 1999). Therefore, cytokeratin immunohistochemistry combined with GFAP can be used in differentiating glio-

mas from metastatic carcinomas. Cytokeratin antibody CAM 5.2 is useful in this setting. However, cytokeratin antibody AE1/AE3 should be avoided because it is frequently positive in glioblastomas, probably due to non-specific cross-reactivity with other intermediate filaments (Oh and Prayson, 1999).

Other markers of differentiation

OLIG2 is a recently identified transcription factor that regulates oligodendroglial development (Yokoo et al., 2004). OLIG2 expression is high in diffuse gliomas but is also found in other primary CNS malignancies (Ligon et al., 2004). Immunohistochemical OLIG2 staining can be used in differentiating between primary CNS tumours and metastasis (Ligon et al., 2004).

Histological classification of gliomas includes specific entities of mixed glioneuronal tumours. However, neuronal differentiation at the molecular level is present in a subset of diffuse gliomas even without histological features of neuronal morphology. Neurofilament expression has been found immunohistochemically in astrocytomas and in oligodendrogliomas (Wharton et al., 1998; Wharton et al., 2002). Other neuronal antigens such as synaptophysin, neuronal nuclear antigen (NeuN) and chromogranin are expressed in glioblastomas, especially in the giant cell subtype (Donev et al., 2010).

Molecules linked to glioma progression

Actin and ERM proteins

Actin microfilaments are a major constituent of the cell cytoskeleton. Actin has a key role in cell motility and migration. In brain physiology, actin cytoskeleton is involved in axon formation of neurons. Actin filaments are in closely involved with the cell membrane. Important mediators of this interaction are the members of the ERM (ezrin-radixin-moesin) protein family (Tsukita and Yonemura, 1997; Vaheri et al., 1997).

Astrocytes are capable of migrating to the site of tissue damage in the CNS. Ezrin and radixin are localised in peripheral astrocytic processes in rat cell cultures suggesting their possible role in astrocyte motility (Derouiche and Frotscher, 2001). Ezrin expression has been confirmed immunohistochemically in human astrocytes as well (Geiger et al., 2000; Grönholm et al., 2005).

Ezrin has been implicated in the progression of several human cancers. Ezrin participates in motility and invasion of pancreatic cancer cells (Meng et al., 2010) as well as in osteosarcoma metastasis (Khanna et al., 2004). Furthermore, ezrin expression is associated with the invasion of cutaneous melanoma (Ilmonen et al., 2005). Ezrin is an unfavourable prognostic factor in uveal melanoma and colorectal carcinoma (Mäkitie et al., 2001; Elzagheid et al., 2008).

In neuroepithelial tumours, ezrin expression has been detected in as-

trocytomas (Geiger et al., 2000), ependymomas (Böhling et al., 1996; Snuderl et al., 2008), gangliogliomas (Majores et al., 2005) and medulloblastomas (Osawa et al., 2009). Overexpression of ezrin is correlated with increasing malignancy grade in astrocytomas (Geiger et al., 2000). In medulloblastomas, ezrin promotes tumour cell invasion (Osawa et al., 2009). Merlin (schwannomin), another member of the **m**oesin-**e**zrin-**r**adixin (i.e. ERM) family is the tumour suppressor gene involved in neurofibromatosis 2 syndrome (Asthagiri et al., 2009).

COX-2

Cyclooxygenase (COX) is the key enzyme in prostaglandin synthesis. The two isoforms COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandins (Dubois et al., 1998). COX-1 is constitutively expressed in several tissues including brain (Menter et al., 2010). COX-2 is an inducible enzyme, which is normally not expressed in most tissues, but is upregulated in inflammation (Wang and Dubois, 2010a).

COX-2 has been implicated in carcinogenesis and it is dysregulated in many cancers such as carcinomas of the colon (Eberhart et al., 1994), stomach (Ristimäki et al., 1997) and breast (Hwang et al., 1998). Epidemiological studies have indicated that prolonged use of non-steroidal anti-inflammatory drugs (NSAID) reduces the risk of developing colorectal cancer (Thun et al., 2002). It has been hypothesized that antitumour effects of NSAIDs are medi-

ated mainly by inhibition of COX-2 and subsequent reduction of prostaglandin synthesis (Wang and Dubois, 2010b).

COX-2 is expressed in neurons of normal human brain tissue (Yasojima et al., 1999; Joki et al., 2000). Overexpression of COX-2 has been reported in astrocytomas (Deininger et al., 1999; Joki et al., 2000; Shono et al., 2001) and in oligodendrogliomas (Castilla et al., 2003). Elevated COX-2 is correlated with high histological grade (Joki et al., 2000) and poor prognosis (Shono et al., 2001; Castilla et al., 2003) in gliomas.

HuR

Hu proteins are a family of four RNA-binding proteins that participate in post-transcriptional regulation of RNA. HuB, HuC and HuD are primarily expressed in neurons and have important functions in neuronal development and memory (Hinman and Lou, 2008). HuR is ubiquitously expressed in human tissues and is predominantly present in the nucleus (Lopez de Silanes et al., 2005). HuR is able to shuttle between the nucleus and cytoplasm, which is considered a main mechanism for its RNA-stabilising function (Doller et al., 2008). HuR overexpression can increase the half-life of its target RNAs such as RNA of TNFα (Dean et al., 2001). HuR also binds to mRNA of VEGF and COX-2 and regulates their expression (Levy et al., 1998; Sengupta et al., 2003; Mrena et al., 2005; Young et al., 2009). Cytoplasmic expression of HuR is associated with high COX-2 expression in carcinomas of

the colon, ovary and stomach (Erkinheimo et al., 2003; Mrena et al., 2005; Denkert et al., 2006). Furthermore, cytoplasmic HuR expression is an independent prognostic factor in cancers of the breast (Heinonen et al., 2005) and ovary (Denkert et al., 2004a). Expression of HuR has been found in brain tumours such as gliomas and medulloblastomas (Nabors et al., 2001; Ido et al., 2008).

Mechanisms of glioma invasion

Gliomas have an inherent tendency to infiltrate the surrounding brain. This growth pattern usually prevents their complete neurosurgical resection. Infiltrative growth is histologically seen already in low-grade gliomas. Unlike in many organs, basal membrane does not inhibit tumour cell invasion in brain parenchyma. In brain, well-defined basal membrane is limited to subpial and perivascular locations. Mechanisms involved in glioma cell invasion to surrounding brain include detachment of the cell from its original site, attachment to the extracellular matrix (ECM) and proteolytic degradation of ECM (Nakada et al., 2007). These alterations facilitate the subsequent cell migration by modifications of the actin cytoskeleton.

Typical invasion routes of malignant glioma cells are along white matter tracts and basal laminas of blood vessels (Furnari et al., 2007). Haematogenous spread and metastasis of malignant gliomas is extremely rare. There is experimental evidence that glioma cell lack the ability to invade intact blood vessels (Bern-stein and Woodard, 1995). However, the molecular basis of this feature of malignant gliomas is not known.

Proteases including matrix metalloproteinases (MMP2, MMP9), urokinase type plasminogen activator (uPA) and cathepsin B are expressed in gliomas by grade dependent manner and they have a potential role in glioma cell invasion (Rao, 2003).

Tumour cell proliferation

Mitotic activity

Mitotic activity is one of the four major histopathological grading criteria of gliomas, with the others being atypia, microvascular proliferation and necrosis. The presence of mitotic activity separates grade III-IV tumours from grade II. In the current WHO classification, the numbers of mitotic figures for different grades are not defined. Occasional mitoses in grade II gliomas are accepted (Louis et al., 2007). In the St. Anne/Mayo grading scheme for astrocytomas even single mitotic figure is a criterion for grade III. However, the clinical behaviour of astrocytomas with a solitary mitosis is more like that of grade II tumours supporting tolerance for occasional mitoses in clinical practice (Giannini et al., 1999).

KI-67 / MIB-1 labelling index

In clinical neuropathology glioma grading can be problematic due to small histological samples or the absence of mitotic figures in an other-

wise anaplastic tumour. Immuno-histochemical labelling of proliferating cells with monoclonal antibody MIB-1 has become an invaluable aid in glioma diagnostics. MIB-1 antibody recognizes a nuclear Ki-67 antigen, which is expressed in the cell cycle except the resting phase G0 (Cattoretti et al., 1992).

The tumour proliferation index as measured by the fraction of MIB-1-positive tumour cells correlates with histological grade and survival in astrocytomas (Sallinen et al., 1994; Wakimoto et al., 1996; Giannini et al., 1999). Prognostic significance of MIB-1 index has also been shown in oligodendrogliomas (Heegaard et al., 1995; Kros et al., 1996; Dehghani et al., 1998).

Apoptosis and apoptotic index

Apoptosis or programmed cell death is a physiological characteristic of cells during embryogenesis and adult life enabling normal turnover of tissues (Holcik et al., 2005). Apoptosis can be found in malignant tumours where it determines tumour net growth together with cell proliferation. Tumour cell apoptosis can be spontaneous or it can be induced by irradiation or chemotherapeutic agents (Kerr et al., 1994). Apoptotic cells can be quantified in tissue sections to calculate the apoptotic index of the tumour (Gavrieli et al., 1992). In gliomas, the apoptotic rate does not correlate with prognosis (Schiffer et al., 1995; Heesters et al., 1999).

Angiogenesis and microvascular proliferation

Angiogenesis in gliomas is a complex process involving tumour cells, endothelial cells, growth factors and their receptors as well as extracellular matrix. Cerebral capillaries are formed by endothelial cells and their basal lamina, which are enveloped by pericytes and astrocytic foot processes (Ballabh et al., 2004). These cells form the blood-brain barrier, which inhibits the exchange of molecules between the bloodstream and brain. Tight junctions (zonula occludens) between endothelial cells are the main anatomical component of the blood-brain barrier.

Angiogenesis plays an important role in glioma growth and progression. Neovascularisation in gliomas means an increase in vascular density and formation of abnormal vascular structures (Figure 4). Microvessel density correlates with tumour grade in astrocytomas (Leon et al., 1996). Vascular density is also an independent prognostic factor in adult astrocytomas (Leon et al., 1996; Abdulrauf et al., 1998; Birlik et al., 2006). The blood-brain barrier of brain tumour vessels is defective because the endothelial cells lack tight junctions, and the vessels are enveloped by only a few pericytes or astrocytic foot processes. Consequently, these defects lead to the accumulation of fluid and plasma proteins in the extracellular space and peritumoural vasogenic oedema (Furnari et al., 2007). The brain lacks lymphatic vessels through which the extravasated fluid is transported back into circulation in other organs. Thus, in the brain the extravasated

fluid must move within the narrow extracellular space, which further enhances the oedema.

Microvascular proliferation is a hallmark of glioblastomas, but it can also be found in anaplastic oligodendrogliomas. Microvascular proliferation is not a categorical sign of malignancy, however. It may be present in benign pilocytic astrocytomas without any prognostic significance. A dramatic shift in tumour angiogenesis is seen in the transition from low-grade and anaplastic astrocytomas to glioblastomas. The most common feature of microvascular proliferation is capillary tufts resembling glomeruli in the kidney (Figure 4). Previously, these glomeruloid

vessel structures were called endothelial cell proliferation. However, because they consist of both multilayered endothelial cells and pericytes/smooth muscle cells (Wesseling et al., 1995), they were renamed microvascular proliferation.

Molecular mechanisms of angiogenesis

The molecular basis of glioma angiogenesis is complex and several mechanisms have been introduced. Angiogenesis in gliomas is driven by hypoxia and it is mediated by hypoxia inducible factor (HIF-1), which is a transcription factor se-
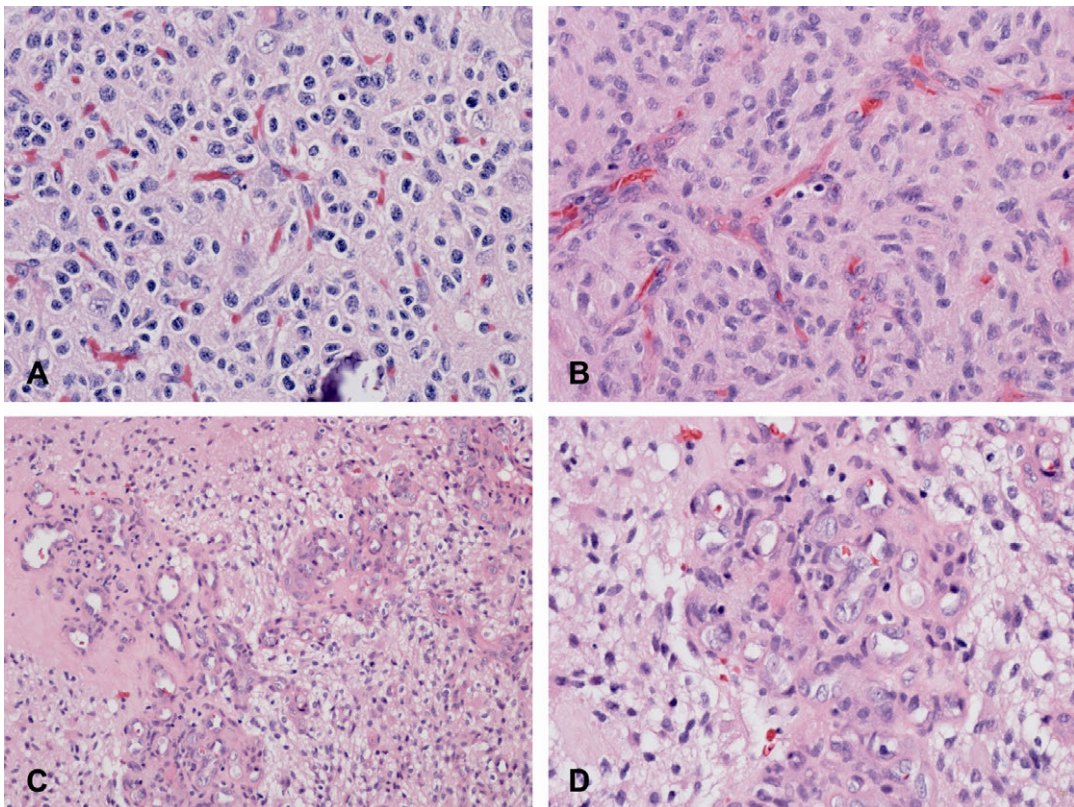
Figure 4 Vascular patterns of gliomas. **A**, dense network of capillaries in oligodendroglioma. **B**, highly vascular glioblastoma with plump, multilayered endothelial cells. **C** and **D**, microvascular proliferation of glioblastoma with glomeruloid structures.

creted by pseudopalisading tumour cells of glioblastoma (Fischer et al., 2005). HIF-1 consists of two subunits HIF-1α and HIF-1β. HIF-1 regulates the transcription of several genes that are involved in angiogenesis, tumour cell growth and invasion (Semenza, 2010). Vascular endothelial growth factor (VEGF) expression of tumour cells is activated by HIF-1 (Semenza, 2010).

VEGF and platelet-derived growth factor (PDGF) are key angiogenic mediators in glioblastomas. VEGF (or VEGF-A) belongs to the growth factor family with four other members: placenta growth factor (PlGF), VEGF-B, VEGF-C and VEGF-D (Lohela et al., 2009). VEGF and PDGF are produced by perinecrotic tumour cells and induce angiogenesis (Hermanson et al., 1992; Plate et al., 1992). VEGF increases vascular permeability of both existing and newly formed vessels (Fischer et al., 2005). VEGF is highly expressed in malignant gliomas (Plate et al., 1992; Hatva et al., 1995) and correlates with microvessel density of the tumours (Schmidt et al., 1999).

VEGFR 1 and 2 are tyrosine kinase receptors of VEGF, which are expressed in endothelial cells of high-grade gliomas, but not in normal brain endothelium (Plate et al., 1994). VEGF secreted by tumour cells binds to VEGFR2 on the endothelial cells, resulting in the paracrine signalling loop that stimulates endothelial cell growth and division (Norden et al., 2009).

The PDGF signalling pathway is involved in tumourigenesis and in the progression of gliomas by promoting cell proliferation and angiogenesis. The PDGF family consists of four ligands PDGFA-D and their receptors PDGFRA and PDGFRB (Shih and Holland, 2006). PDGFB and its receptor PDGFRB, are expressed in hyperplastic endothelial cells in glioblastoma (Hermansson et al., 1988; Hermanson et al., 1992). The angiogenic effect of PDGFB is at least partly mediated by the upregulation of VEGF expression (Guo et al., 2003).

Alternative mechanisms for vascularisation have been found in a subset of glioblastomas. In the experimental glioma model, the tumour may co-opt existing host vessels. The co-opted vasculature supports the tumour until the neoangiogenesis starts (Holash et al., 1999). In vasculogenic mimicry, glioblastoma cells presenting stem cell properties may express endothelium-associated genes and form blood vessels de novo (El Hallani et al., 2010). Recent studies have shown that endothelial cells of glioblastomas harbour the same genetic alterations with tumour cells such as EGFR amplification (Ricci-Vitiani et al., 2010; Wang et al., 2010). These results suggest that a subpopulation of endothelial cells of glioblastoma originate from tumour stem-like cells.

Diagnostics and treatment of gliomas

Magnetic resonance imaging

Magnetic resonance imaging is currently the method of choice in brain

tumour imaging. MRI is an invaluable tool in preoperative diagnostics and anatomical localization of gliomas as well as in the planning of the surgical treatment. Postoperatively MRI is utilized in planning radiotherapy, assessing response to treatment and tumour progression (Henson et al., 2005).

MRI with contrast medium is the most sensitive non-invasive diagnostic method in neuro-oncology (Jenkinson et al., 2007). Administration of intravenous paramagnetic gadolinium-DTPA improves contrast in MR imaging and increases sensitivity in brain tumour diagnostics (Felix et al., 1985; Brant-Zawadzki et al., 1986). Contrast-enhanced MRI is useful in differentiating tumours from other pathological processes of the CNS (Essig et al., 2006).

The intact blood-brain-barrier is impermeable to water-soluble gadolinium chelates, and it prevents the access of contrast medium into brain tissue (Neuwelt, 2004). Therefore, contrast enhancement in MRI is a sign of a disrupted blood-brain-barrier reflecting the tumour-induced abnormal neovascularisation (Essig et al., 2006).

MRI characteristics correlate with the WHO grade of diffuse gliomas (Henson et al., 2005; Jenkinson et al., 2007). In low-grade gliomas, MRI usually shows a mildly expansive lesion without contrast enhancement or substantial oedema. In anaplastic grade III tumours, there is characteristically more oedema and expansion into the surrounding brain with patchy areas of gadolinium enhancement. The typical MRI appearance of glioblastoma shows irregular or ring-like contrast enhancement with a central non-enhancing area corresponding to necrosis. Glioblastomas produce a pronounced mass effect and vasogenic oedema as well. Although gadolinium enhancement is associated with anaplastic features in gliomas, up to one third of the non-enhancing gliomas are histologically malignant (Barker et al., 1997; Scott et al., 2002). Furthermore, benign pilocytic astrocytomas often show intense contrast enhancement reflecting the microvascular proliferation that is an exceptional feature in a benign astrocytoma (Zimmerman and Bilaniuk, 2009).

Functional MRI techniques can be utilised preoperatively to identify eloquent brain areas (Vlieger et al., 2004). The functional information can be applied for planning neurosurgical operations in order to minimize damage to eloquent brain regions.

The association between MRI characteristics and molecular profiles of gliomas have been reported recently. Oligodendrogliomas that harbour the 1p/19q deletion are infrequently located in temporal lobes and show indistinct borders in MRI (Zlatescu et al., 2001; Megyesi et al., 2004; Jenkinson et al., 2006; Kim et al., 2011). Secondly, fuzzy tumour borders in the MRI of glioblastomas may predict EGFR amplification at the molecular level (Aghi et al., 2005). Furthermore, the volume ratio of contrast enhancement and tumour necrosis in MRI correlates with EGFR overexpression in glioblastomas (Diehn et al., 2008).

Positron emission tomography

Positron emission tomography (PET) is an imaging method that can be used to measure brain metabolism. PET detects radionuclide-labelled tracer molecules such as [18F]fluorodeoxyglucose (FDG) and [11C]methionine (MET), which reflect glucose and amino acid uptake of the brain tissue, respectively (Minn, 2005). The advantage of PET in tumour imaging is based on enhanced energy metabolism of cancer cells compared to normal tissue. However, the basal level of glucose metabolism in brain is high, resulting in a low signal-background ratio in FDG-PET. Generally, high-grade gliomas show increased glucose metabolism in PET, while low-grade gliomas are hypometabolic (Di Chiro, 1987; Padma et al., 2003).

Background uptake of amino acid tracers in normal brain tissue is low, which allows high contrast for tumour uptake (Jager et al., 2001). MET uptake in PET is associated with glioma grade and survival (Ogawa et al., 1993; Nuutinen et al., 2000; De Witte et al., 2001). FDG and MET uptake in brain tumours is independent of blood-brain-barrier disruption, which is advantageous in tumour imaging (Minn, 2005). A recent study suggests that PET can be used to detect glioma cell invasion into white matter tracts, which were visualised by diffusion tensor MRI (Stadlbauer et al., 2009).

Currently, the main indications for PET in the imaging of gliomas are detecting tumour recurrence and guiding diagnostic biopsy site. PET using FDG or MET is more sensitive than standard MRI to detect glioma recurrence (Minn, 2005). However, specificity of PET in differentiating between radiation injury and true recurrence is 75% (Dhermain et al., 2010). PET can be used to direct biopsy to hypermetabolic tumour tissue and avoid reactive changes on the tumour edge (Klasner et al., 2010).

Molecular diagnostics

Genetic alterations are important steps in gliomagenesis as well as biomarkers in diagnostics. Traditionally, genetics of gliomas have been studied by basic cytogenetic methods like G band karyotyping and fluorescence in situ hybridisation (FISH). Modern techniques for studying genetic aberrations include whole genome karyotyping by comparative genomic hybridisation (CGH) and array-based CGH (aCGH) (Kallioniemi et al., 1992; Pinkel et al., 1998).

Currently, testing of 1p/19q deletions by either FISH or aCGH is recommended for oligodendroglial tumours to complement histopathological diagnosis and to add prognostic information of the tumour (Yip et al., 2008; Jansen et al., 2010). The EGFR amplification assay is a useful diagnostic aid in small tumour biopsies with only few infiltrating neoplastic cells, where histopathological criteria alone do not allow diagnosis of malignant glioma. Identification of tumour cells with EGFR amplification by FISH (Mott et al., 2008) or by chromogenic in situ hybridisation (CISH) (Järvelä et al., 2006) can be used as a surrogate marker for high-grade astrocytoma.

The methylation status of the MGMT promoter provides prognostic information of glioblastomas. However, the lack of standardised methods has inhibited the wide use of MGMT testing in clinical neuropathology (von Deimling et al., 2011). Furthermore, it is not yet known which CpG sites are relevant for silencing of MGMT transcription (Riemenschneider et al., 2010).

An immunohistochemical assay of the p.R132H IDH1 mutation may be helpful in differential diagnostics of diffuse gliomas. Positive IDH1 immunostaining can rule out pilocytic astrocytomas and ependymomas, which lack IDH1 mutations (Capper et al., 2010). Moreover, IDH1 immunohistochemistry helps in the discrimination of oligodendrogliomas from tumours with similar morphology, such as central neurocytomas and dysembryoplastic neuroepithelial tumours (Capper et al., 2011). Assessment of IDH1 and p53 immunohistochemistry is useful in differentiating reactive gliosis from astrocytoma, which is a common diagnostic problem in surgical pathology (Camelo-Piragua et al., 2011). However, negative staining with IDH1 antibody to mutant p.R132H does not rule out other IDH1 mutations that are present in less than 10% of mutated cases or rare IDH2 mutation.

Treatment of gliomas

Surgery

The current treatment options for diffuse gliomas include surgical resection, radiotherapy and chemotherapy (Tabatabai et al., 2010). The goals for surgical resection are decreasing the tumour mass, lowering intracranial pressure and confirming the diagnosis. Surgical treatment is not curative because of the infiltrative nature of diffuse gliomas. The impact of the extent of surgical resection on patient survival has been controversial (Sanai and Berger, 2008). A recent meta-analysis indicates that cytoreductive resection of malignat glioma is associated with better survival as compared to biopsy (Tsitlakidis et al., 2010). However, in elderly patients the benefit from open surgery seems to be limited (Vuorinen et al., 2003). If surgical resection is not feasible because of tumour location, a stereotactic biopsy should be performed to obtain tissue for histopathological diagnostics and molecular studies (Wen and Kesari, 2008; Soffietti et al., 2010).

Radiotherapy

Radiotherapy is an effective treatment of malignant gliomas and prolongs the survival of the patients (Walker et al., 1978). The standard therapy for anaplastic gliomas (WHO grade III) consists of adjuvant radiotherapy after surgery (Stupp et al., 2010). The total dose of focal radiotherapy for malignant gliomas is 50-60 Gy (Laperriere et al., 2002; Wen and Kesari, 2008). In low-grade gliomas, radiotherapy extends the patients' progression-free period, but does not affect their overall survival (van den Bent et al., 2005). Adjuvant radiotherapy is indi-

cated for low-grade glioma patients with unfavourable prognostic factors such as incomplete surgical resection or advanced age (Soffietti et al., 2010). Radiotherapy may induce long-term adverse reactions including cognitive impairment and leukoencephalopathy (Surma-aho et al., 2001).

Chemotherapy

The blood-brain barrier restricts the entry of many chemotherapeutic agents into the CNS. In high-grade gliomas, the blood-brain-barrier is often partially disrupted due to neoangiogenesis of the tumour, which may facilitate drug delivery to the tumour (Muldoon et al., 2007).

The main chemotherapeutic agents currently used in treatment of gliomas are alkylating drugs such as temozolomide, lomustine (CCNU 1-(2-Chloroethyl)-3-Cyclohexyl-1-Nitrosourea) and procarbazine, which are able to cross the blood-brain-barrier (Rao and Buckner, 2004). Combined treatment with procarbazine, CCNU and vincristine (PCV chemotherapy) is particularly effective in anaplastic oligodendrogliomas with 1p/19q deletion (Cairncross et al., 1998).

In low-grade gliomas, chemotherapy is mainly used for tumour recurrences after surgical resection and radiotherapy (Soffietti et al., 2010). Adjuvant PCV chemotherapy does not prolong survival of patients with anaplastic oligodendroglial tumour compared to radiotherapy alone (Cairncross et al., 2006; van den Bent et al., 2006). Concomitant chemotherapy and radiation (chemo-radiotherapy) are currently the treatments of choice for glioblastoma. Patients who receive concomitant temozolomide and radiotherapy have significantly longer survival compared to patients with radiotherapy alone (median survival 14.6 vs. 12.1 months) (Stupp et al., 2009).

AIMS OF THE STUDY

The purpose of this thesis was to study molecular alterations involved in glioma progression. Therefore, we collected a retrospective series of gliomas from patients who had been operated on for glioma and one or more recurrences.

Specifically, we studied:

1. the correlation of tumour contrast enhancement in MRI with microvessel density and cell proliferation in gliomas (study I)

2. the genetic alterations and proteins related to recurrence and progression of gliomas (studies II-IV)

3. the molecular changes in the longitudinal course of individual gliomas (studies II-IV)

MATERIALS AND METHODS

Tissue samples

This retrospective study included diffuse gliomas diagnosed at the Department of Pathology, University of Helsinki and HUSLAB, Helsinki, Finland between 1979 and 2000. All patients were operated on at the Department of Neurosurgery, Helsinki University Central Hospital, Helsinki, Finland. Formalin-fixed and paraffin-embedded tumour samples were retrieved from the archive, and the histopathological diagnoses were reviewed by the author and experienced neuropathologist (A.P.). All tumours were diagnosed according to the latest WHO classification at the time of the study (Kleihues et al., 1993; Kleihues and Cavenee, 2000; Louis et al., 2007).

Primary supratentorial astrocytomas, oligodendrogliomas and oligoastrocytomas, grade II-IV, from 62 patients were included in study I. Studies II and IV included 229 primary and recurrent astrocytomas, oligodendrogliomas and oligoastrocytomas, grade II-IV, from 113 patients. Study III included primary and recurrent astrocytomas, oligodendrogliomas and oligoastrocytomas, grade II-IV, from 87 patients.

Magnetic resonance imaging (study I)

Magnetic resonance imaging of the 62 patients in study I was performed preoperatively using a Magnetom 42

SP 1.0 Tesla imager (Siemens, Erlangen, Germany). Sagittal T1-weighted localizing images, unenhanced T1-, T2- and proton-weighted spin echo images were obtained. The contrast agent gadopentetate dimeglumine (Magnevist, Schering AG, Berlin, Germany) was injected intravenously 0.1 mmol/kg, and axial T1-weighted spin echo images were repeated after 5-10 minutes. Contrast enhancement was studied in T1-weighted post-contrast images by two radiologists. Any detectable contrast enhancement of the tumour was considered positive.

Immunohistochemistry

Tissue microarray (TMA) blocks were prepared for studies II-IV with a manual tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA). A representative area of the tumour was selected in H&E-stained sections, and 1-3 tissue cylinders with a 0.6 mm diameter were obtained from each tumour. Four-micrometer sections (5 μm in study III) were cut from TMA blocks and conventional paraffin blocks for immunohistochemistry.

The antibodies used in immunohistochemical studies are summarized in Table 2. In study I, capillary endothelium was highlighted by polyclonal antibody against factor VIII-related antigen (FVIII, Dako, Glostrup, Denmark, dilution 1:2000). The slides were pretreated

Table 2   Antibodies used in immunohistochemical studies.

Antibody	Clonality	Host	Dilution	Pretreatment	Study
Factor VIII	Polyclonal	Rabbit	1:2000	Pepsin	I
MIB-1 (Ki-67)	Monoclonal	Mouse	1:50	Heating	I
Ezrin	Monoclonal	Mouse	1:2000	Heating	II
KIT (CD117)	Polyclonal	Rabbit	1:300	Heating	III
VEGFR2 (Flk-1)	Polyclonal	Rabbit	n.a.	Heating	III
EGFR	Monoclonal	Mouse	1:150	Heating	III
p53	Monoclonal	Mouse	1:500	Heating	III
Nestin	Monoclonal	Mouse	1:500	Heating	III
Prominin 1 (CD133)	Monoclonal	Mouse	1:10	Heating	III
COX-2	Monoclonal	Mouse	1:100	Heating	IV
HuR	Monoclonal	Mouse	1:20000	Heating	IV

with pepsin and the primary antibody was detected using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Tumour cell proliferation was detected by monoclonal antibody MIB-1/Ki-67 (Immunotech S.A., Marseille, France, dilution 1:50). Antigen retrieval was performed in a microwave oven (15 min. in 10mM citrate buffer). A histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA) was used to visualize the primary antibody.

In study II, monoclonal antibody against human ezrin was used (clone 3C12, dilution 1:2000) (Böhling et al., 1996). The monoclonal antibody, raised against the carboxyterminal part of ezrin (amino acids 362 to 585), detects a single 80 kDa band compatible with ezrin in immunoblotting. Antigen retrieval was performed in a microwave oven (10 min. in 10mM citrate buffer). The slides were stained using a LabVision Autostainer automatic immunostaining device and UltraVision detection kit (Lab Vision Inc., Fremont, CA).

In study III, the following antibodies were used: polyclonal KIT/CD117 antibody (A 4502, Dako, Glostrup, Denmark, dilution 1:300), monoclonal EGFR antibody (NCL-EGFR, Novocastra Laboratories Ltd., Newcastle, United Kingdom, dilution 1:150), monoclonal p53 antibody (NCL-p53-D07, Novocastra Laboratories Ltd., Newcastle, United Kingdom, dilution 1:500) and polyclonal VEGFR2 antibody (Flk-1/ VEGFR2 Ab-1, NeoMarkers, LabVision Corp., Fremont, CA). Antigen retrieval for KIT staining was performed in a microwave oven (7 min and 3 × 5 min in Tris-EDTA buffer, pH 9.0). Autoclave heating was used as pretreatment for EGFR and p53 antibodies (120ºC, 2 min in 1× Reveal buffer; Biocare Medical, Walnut Creek, CA) and for VEGFR2 (120ºC, 2 min in a10 mM citrate buffer, pH 6.0). The binding of KIT antibody was detected with a Dako EnVision detection kit (Peroxidase/DAB, rabbit/mouse, Dako). The EGFR, p53 and VEGFR2 antibodies were visualized with Powervi-

sion+ Poly-HRP detection kit (DPVB+110DAB, ImmunoVision Technologies Co., Daly City, CA).

In study IV, monoclonal antibodies against COX-2 (dilution 1:100) and HuR (dilution 1:20000, clone 19F12) were used. For antigen retrieval, the sections were pretreated in for 20 min at 85°C in Tris-HCl buffer (pH 8.5). Immunostainings were performed with a Lab Vision Autostainer 480 and Dako REAL EnVision detection kit. All tissue sections were counterstained with hematoxylin.

Scoring of immunohistochemical stainings

In study I, microvessel density was measured from the most vascularised area of the tumour. The number of FVIII positive vessels was counted from three microscopic fields (1.3 mm² each). The average of three counts was considered as the vascular density of the tumour. In cases of the multiluminal hyperplastic vessel, each lumen was considered as one capillary. The cell proliferation rate was measured from the most proliferative area of the tumour. The cell proliferation index was defined as a percentage of MIB-1-positive tumour cells. At least 500 tumour cells were analysed in each tumour.

In study II, two observers (the author and O.C.) evaluated ezrin expression independently. Immunostaining was graded from 0 to 3 using these ranges: 0%, 1-30%, 31-70% and >70% positive tumour cells respectively. Grading of the two observers was averaged.

In study III, the KIT, EGFR, p53, VEGFR2, nestin and prominin-1 immunostainings were graded semiquantitatively as either negative (-), faintly positive (+), moderately positive (++) or strongly positive (+++).

In study IV, granular cytoplasmic staining with COX-2 antibody was considered positive and scored as low (0-20% of tumour cells) or high (>20% of tumour cells). HuR staining was scored as negative (only nuclear staining in tumour cells) or positive (cytoplasmic staining in tumour cells).

Immunoblotting (study II)

Ezrin expression was confirmed by an immunoblotting assay of eight gliomas. Fresh tumour samples were snap frozen and stored in liquid nitrogen. Tumour samples were homogenized in RIPA buffer and protein concentration was measured. Equal amounts of protein (30 µg) were separated with SDS-PAGE electrophoresis in 10% polyacrylamide gel and blotted on nitrocellulose sheet. The filter was blocked by incubating overnight in non-fat milk protein and incubated with monoclonal ezrin antibody (clone 3C12, dilution 1:1000) for 1 h. Anti-actin antibody was used to control the loaded protein in the gel. The primary antibody was detected using an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

Chromogenic in situ hybridisation and fluorescence in situ hybridisation (study III)

In study III, bacterial artificial chromosome (BAC) probes were used to study gene copy numbers of KIT (clone RP11-586A2), PDGFRA (RP11-231C18), VEGFR2 (RP11-662M13) and EGFR (RP11-815K24) (Invitrogen Ltd, Paisley, UK). The BAC DNA was isolated and labelled with a DIG-Nick translation mix (Roche, Mannheim, Germany). Pretreatment for in situ hybridisation was performed in a microwave oven (92°C, 10 min in 0.1 M Tris-HCl buffer, pH 7.0) and by enzymatic digestion (Digest-All 3 solution, Zymed, Inc., South San Francisco, CA). The probes were applied onto the slides, sections were denatured and hybridisation was done overnight at 37°C. The probes were detected with mouse anti-digoxigenin antibody (dilution 1:300; Roche Biochemicals, Mannheim, Germany) and a Powervision+ kit (ImmunoVision Technologies). The tissue sections were counterstained with hematoxylin.

Chromogenic in situ hybridisation showed weak or absent KIT, PDGFRA or VEGFR2 signals in 13 tumours. Therefore, fluorescence in situ hybridisation was performed to study gene copy numbers in those tumours. The BAC DNA probes described earlier were labelled with a DIG-Nick and BIOTIN-Nick translation mix (Roche, Mannheim, Germany). Centromeric probes for chromosomes 4 and 7 (CEP 4 and CEP 7, Vysis Inc., Downers Drive,

IL) were used as controls. After hybridisation, the probes were detected immunochemically with avidin-fluorescein isothiocyanate and anti-digoxigenin rhodamine. Slides were counterstained with DAPI in antifade solution and viewed under a fluorescence microscope.

Gene amplification was defined as 6 or more hybridisation signals per nucleus. Tumours with 3-5 signals were considered aneuploid.

Screening of p53 gene mutations (study III)

Polymerase chain reaction (PCR), denaturing high-performance liquid chromatography (DHPLC) and DNA-sequencing methods were used for studying p53 mutations. These methods are described in detail in the original publication.

Statistical analysis

Associations between categorical variables were studied using the χ^2 test and Fischer's exact test. Differences of non-normally distributed variables between two or more groups were compared with the non-parametric Mann-Whitney U test and Kruskall-Wallis test. Paired observations of primary and recurrent gliomas were studied using the Wilcoxon signed rank test. Level of interobserver agreement was assessed by kappa statistics. The relationship between continuous variables was studied using simple linear regression and Spearman rank correlation. Survival analysis was performed us-

ing the Kaplan-Meier method and survival curves were compared using the log-rank test. Multivariate survival analysis was performed using Cox proportional hazards model. The statistical significance level was set at 5%. Statistical analysis was performed using Statview (SAS Institute Inc., San Francisco, CA) and SPSS (SPSS Inc., Chicago, IL) statistical software.

Approvals

This study was approved by the Ethical Committee of the Hospital District of Helsinki and Uusimaa and the National Supervisory Authority for Welfare and Health.

RESULTS AND DISCUSSION

MRI enhancement and histological features of gliomas (study I)

We studied the correlation between MRI contrast enhancement and histological characteristics of 62 diffuse gliomas (Table 3). Contrast enhancement in MRI was present in 45 of 62 (73%) gliomas. Enhancement was seen in 23 of 38 (61%) low-grade gliomas, in 11 of 13 (85%) anaplastic gliomas and in all 11 glioblastomas. Contrast enhancement in MRI was significantly associated with higher tumour grade in the whole tumour group (p=0.005) and in the astrocytoma subgroup (n=51, p=0.006), but not in the oligodendroglial tumours.

Grading of astrocytomas by MRI characteristics has been previously studied by Asari et al. (Asari et al., 1994). They developed MRI scoring that was based on nine criteria: 1) tumour heterogeneity, 2) cyst formation or necrosis, 3) haemorrhage, 4) tumour crossing the midline, 5) oedema and/or mass effect, 6) definition of border, 7) flow void, 8) degree of contrast enhancement and 9) heterogeneity of contrast enhancement. Using this MRI scoring they could differentiate low-grade and high-grade astrocytomas. Degree and heterogeneity of contrast enhancement were related to histological grade by multiple regression analysis (Asari et al., 1994). The MRI scoring developed by Asari et al. was later used in non-invasive grading of gliomas in two other studies (Rie-

Table 3 Tumour histology, WHO grade and contrast enhancement of 62 gliomas (study I).

Tumour histology and grade	N	Contrast enhancement	
		Yes	No
Astrocytoma			
Grade II	31	19	12
Grade III	9	8	1
Grade IV	11	11	0
Oligodendroglioma			
Grade II	4	3	1
Grade III	3	2	1
Oligoastrocytoma			
Grade II	3	1	2
Grade III	1	1	0
Total	62	45	17

mann et al., 2002; Kumar et al., 2006). In these studies, there was a significant difference in MRI score between low-grade and high-grade gliomas. Furthermore, the degree of contrast enhancement was one of the criteria that were significantly associated with tumour grade (Riemann et al., 2002; Kumar et al., 2006). Our results are in congruence with an earlier study by Aronen et al., where contrast enhancement in MRI imaging was associated with histological grade of diffuse gliomas (Aronen et al., 1994). One must keep in mind, though, that even benign pilocytic astrocytomas commonly show contrast enhancement in MRI (Zimmerman and Bilaniuk, 2009). However, pilocytic astrocytomas are usually sharply demarcated, whereas diffuse gliomas are ill-defined in MRI.

Nonenhancing tumours should not be considered as low-grade merely based on their enhancement pattern. In our series, 2 of 17 (12%) nonenhancing gliomas were malignant (i.e. grade III-IV). In other studies, 9-40% of nonenhancing tumours were histologically malignant (Barker et al., 1997; Ginsberg et al., 1998; Scott et al., 2002).

Next, we studied the correlation of MRI contrast enhancement with endothelial proliferation, which is a hallmark of glioblastomas and characteristic of anaplastic oligodendrogliomas as well. Tumour endothelial proliferation was significantly associated with contrast enhancement in MRI (p=0.003). All tumours (n=16) with endothelial proliferation in histological sections showed contrast enhancement. This finding has been

confirmed by Reiche et al., who studied oligodendrogliomas and found a significant association between contrast enhancement and tumour grade as well as endothelial proliferation (Reiche et al., 2002). Endothelial proliferation is not a *sine qua non* of contrast enhancement, however. We detected contrast enhancement in 63% of gliomas that did not have endothelial proliferation in histology.

Correlation of MRI enhancement, tumour cell proliferation, vascular density and tumour grade (study I)

In the present study, the mean vascular density (i.e. the number of capillaries per 1.3 mm² microscopic field) was 53.2 in grade II, 98.9 in grade III and 149.8 in grade IV gliomas (p=0.001). The correlation between vascular density and tumour grade was found also in the subgroup (n=45) that showed contrast enhancement in MRI (p=0.0017).

Angiogenesis is necessary for tumour growth. Without the formation of new vessels and establishment of perfusion, a tumour could not exceed a diameter of 2-3 mm (Folkman, 1971). Intra-tumoural microvessel density is a reliable method for measuring angiogenic activity of solid tumours (Hasan et al., 2002). Immunohistochemically determined microvessel density is an independent prognostic factor in breast cancer (Weidner et al., 1992) and in several other epithelial can-

cers as reviewed by Hasan and co-workers (Hasan et al., 2002).

Our results are in line with previous studies that found an association between microvessel density and tumour grade in astrocytomas (Leon et al., 1996; Wesseling et al., 1998). This association has been confirmed in later studies as well (Korkolopoulou et al., 2002; Quon et al., 2010). Furthermore, microvessel density is an independent prognostic factor in astrocytomas (Leon et al., 1996; Abdulrauf et al., 1998; Birlik et al., 2006).

In our study, the immunohistochemically assessed microvessel density was significantly higher in the tumours that showed MRI contrast enhancement compared to tumours with no enhancement (mean 47.9 vs. 92.0, p=0.01). This finding most likely reflects a defective blood-brain-barrier in newly formed blood vessels. Contrast enhancement in MRI is caused by the accumulation of intravenously administered gadolinium-containing contrast material in the interstitial space (Jayaraman and Boxerman, 2009). In a normal brain, the blood-brain-barrier of the capillaries prevents the entrance of water-soluble contrast material in the interstitium. The current understanding is that contrast enhancement in MRI is due to defective capillaries rather than the active destruction of the blood-brain-barrier of the existing vessels (Jayaraman and Boxerman, 2009). Neoangiogenesis of gliomas causes the formation of capillaries that have fenestrated blood-brain-barrier, which subsequently leads to the interstitial accumulation of contrast material in MRI.

In our study, there was a significant difference in the immunohistochemically measured cell proliferation index between the nonenhancing and enhancing gliomas (mean 2.0% vs. 8.1%, p<0.001). Association between tumour enhancement and proliferation index may simply reflect that both variables are associated with the histological grade of the tumour. However, this result suggests that preoperative conventional MRI provides information on proliferation activity of the tumour. The fact that MRI contrast enhancement may be used as a non-invasive predictor of cell proliferation is clinically interesting, because the immunohistochemically measured Ki-67 index is a widely used ancillary diagnostic method in approximating the clinical behaviour of gliomas. Previously, Aronen and co-workers have studied proliferative activity and MRI enhancement in diffuse gliomas. They found that mitotic activity of gliomas was associated with contrast enhancement in MRI (Aronen et al., 1994).

In their recent review Alexiou et al. have evaluated non-invasive methods for assessment of glioma proliferation (Alexiou et al., 2010). The imaging modalities that have been used for studying glioma proliferation in vivo include MRI, PET and single-photon emission computed tomography (SPECT). Alexiou et al. conclude that proton magnetic resonance spectroscopy (MRS), which provides information on the metabolic status of tumour tissue, is a promising tool for the

non-invasive assessment of glioma proliferation.

In the present study, vascular density correlated positively with tumour cell proliferation of gliomas (r=0.51, p<0.0001). This association is not surprising because the cell proliferation index, as measured by Ki-67 (MIB-1) immunohistochemistry, correlates positively with glioma grade (Montine et al., 1994; Sallinen et al., 1994; Giannini et al., 1999; Sallinen et al., 2000). It has been suggested that the Ki-67 index could be included in the grading criteria of gliomas in the future (Kros, 2011).

Functional MRI techniques can be used to characterise brain tumours more accurately than conventional contrast-enhanced MRI. Cerebral blood volume (CBV) can be measured in vivo by functional MRI techniques (Belliveau et al., 1991). CBV studied by MR imaging can provide information on angiogenesis and proliferation of gliomas as well. In high-grade gliomas CBV is significantly elevated compared to low-grade gliomas (Maia et al., 2005). Furthermore, CBV correlates with tumour vascularity and mitotic activity of gliomas (Aronen et al., 1994) as well as with metabolic activity and angiogenesis of gliomas (Aronen et al., 2000). Callot et al. found an association between CBV and endothelial hyperplasia in low-grade gliomas (Callot et al., 2007).

Ezrin is expressed in astrocytomas and oligodendrogliomas (study II)

We studied ezrin expression by immunohistochemistry in 229 primary and recurrent gliomas of 113 patients. Characteristics of the studied tumours are presented in Table 4. Ezrin expression was observed in both astrocytic and oligodendroglial tumours by immunohistochemistry and immunoblotting. The mean ezrin immunoreactivity score was 2.5 in astrocytomas, 2.2 in oligoastrocytomas and 2.1 in oligodendrogliomas (p=0.006). Oligodendrogliomas showed moderate staining in tumour cells and more intensive reactivity in astrocytic cells within the tumour. Ezrin immunoreactivity was associated with WHO grade in astrocytomas (mean 2.4 in grade II vs. 2.4 in grade III vs. 2.6 in grade IV, p=0.04) but not in oligodendrogliomas or oligoastrocytomas. In pairwise comparison, ezrin expression was significantly higher in glioblastomas than in grade II astrocytomas (p=0.04, Mann-Whiney test with Bonferroni correction). Differences between grade II vs. III and grade III vs. IV were not significant.

Ezrin expression was further confirmed by immunoblotting of frozen tumour samples in a subset of 8 gliomas (2 astrocytomas, 2 glioblastomas and 4 oligodendrogliomas). Immunoblotting for ezrin was positive in 3 oligodendrogliomas and in 1 glioblastoma. One low-grade astrocytoma and one anaplastic oligodendroglioma with negative immunoblotting were weakly positive for ezrin by immunohistochemistry. The other two tumours with negative immunoblotting were anaplastic astrocytoma and glioblastoma. These tumours showed moderate or high positivity by immunohistochemistry. Negative

Table 4   Tumour histology, WHO grade and ezrin immunoreactivity (study II).

Tumour histology and grade	First diagnosis		Recurrence	
	N	Ezrin score mean (SD)	N	Ezrin score mean (SD)
Astrocytoma				
Grade II	42	2.4 (0.5)	16	2.5 (0.6)
Grade III	20	2.2 (0.7)	22	2.6 (0.5)
Glioblastoma, primary	14	2.6 (0.5)	13	2.8 (0.4)
Glioblastoma, secondary	n.a.	n.a.	24	2.6 (0.5)
Oligodendroglioma				
Grade II	16	2.2 (0.6)	5	2.0 (0.4)
Grade III	8	2.1 (0.8)	15	2.0 (0.7)
Oligoastrocytoma				
Grade II	6	2.0 (0.5)	6	2.3 (0.4)
Grade III	4	2.0 (0.8)	18	2.4 (0.6)
Total	110		119	

SD standard deviation, n.a. not applicable

immunoblotting result may be due to tissue sampling, i.e. intratumoural heterogeneity and/or focal necrosis.

Our results are in line with Geiger et al., who found a positive correlation between ezrin expression and WHO grade in 74 diffuse astrocytomas grade II-IV (Geiger et al., 2000). However, 27 oligodendrogliomas were almost completely negative in ezrin immunohistochemistry in the series by Geiger et al. They found only a few positive astrocytic cells in the ezrin staining of oligodendrogliomas. The difference in the staining pattern in oligodendroglial tumours between the studies may be due to different antigen retrieval methods. We used heat-induced antigen retrieval, which improves the reactivity of the 3C12 antibody in paraffin sections. Our

results suggest that ezrin is linked to the malignant progression of astrocytomas. However, ezrin immuno-histochemistry may not be directly applicable to the grading of gliomas because of considerable overlapping in ezrin scores between low-grade and high-grade gliomas.

In a recent proteome-wide profiling of low-grade oligoastrocytomas, ezrin was one of the proteins differentially expressed in tumours with intact vs. deleted chromosome arms 1p and 19q (Grzendowski et al., 2010). Grzendowski et al. found that ezrin expression was significantly higher in tumours with intact chromosomes 1p and 19q. Furthermore, they studied the hypermethylation of CpG islands of the ezrin gene and found that hypermethylation was associated with 1p/19q deletion.

However, hypermethylation of CpG islands did not decrease ezrin expression at the mRNA level to a statistically significant degree (Grzendowski et al., 2010). Grzendowski et al. did not include survival analysis in their study of oligoastrocytomas. Nevertheless, it is interesting that epigenetic silencing by hypermethylation of the ezrin gene is associated with 1p/19q deletion, which is a marker of favourable prognosis in oligodendroglial tumours (Grzendowski et al., 2010).

Ezrin and patient outcome in gliomas (study II)

In the present study, ezrin expression was significantly higher in tumour recurrences (mean ezrin score 2.5 vs. 2.3, p=0.02), suggesting that ezrin is involved in the progression of gliomas. High ezrin expression of primary tumours was associated with shorter recurrence-free time (median 3.6 vs. 4.1 vs. 2.5 in low, intermediate and high ezrin score respectively, p<0.05) and poor overall survival (median 9.2 vs. 7.7. vs. 4.5 years, p<0.05) in the whole tumour material. The non-linear relationship between ezrin score and recurrence-free time may be partly explained by the small number of cases in the low ezrin group (n=9). High ezrin expression was also a negative prognostic factor for overall survival in the astrocytoma subgroup (p=0.03) but not in oligodendroglial tumours. However, in multivariate analysis, only WHO grade, histological tumour type and patient age were in-

dependent prognostic factors. Ezrin expression was not independently associated with patient prognosis probably due to its correlation with tumour grade.

Invasion and migration in brain tissue are characteristic of glioma cells. Destruction of brain structures and deterioration of neurological function due to infiltration of glioma cells are eventually fatal to the patient. Tumour cells of diffuse gliomas typically accumulate in subpial areas and perineuronally, and the cells preferentially invade along white matter tracts (secondary structures of Scherer). There is experimental evidence that ezrin is linked to glioma cell migration in the brain. Wick and co-workers transfected mutant ezrin plasmids into four glioma cell lines that originally expressed wild-type ezrin (Wick et al., 2001). Expression of mutant ezrin acted in a dominant negative manner in malignant glioma cells and inhibited their migration in vitro. Furthermore, when human glioma cells expressing a dominant-negative ezrin were xenografted in mouse brain they produced smaller tumours with longer survival time than controls (Wick et al., 2001). Similar results of the role of ezrin in tumour cell motility and invasion have been reported in medulloblastoma cells (Osawa et al., 2009).

EGFR amplification in gliomas (study III)

In the present study, the EGFR amplification rate in secondary glioblastomas was 14% in the 14

informative cases (Table 5). Our results are well in line with Järvelä and co-workers, who studied EGFR amplification in 338 grade II-IV astrocytomas by CISH (Järvelä et al., 2006). In their material, EGFR amplification was present in 16% of secondary glioblastomas.

EGFR amplification is a genetic hallmark of primary glioblastomas. In a previous study, we found EGFR amplification in half of primary glioblastomas (Joensuu et al., 2005). In a population-based study, the EGFR amplification frequency of glioblastomas was a little lower (36%) and secondary glioblastomas carried EGFR amplification in less than 10% of tumours (Ohgaki et al., 2004).

In the present study, the frequency of EGFR amplification in anaplastic astrocytomas was 12% in primary tumours and 6% in recurrent tumours. We also detected EGFR amplification in two recurrent low-grade astrocytomas that did not show amplification at first diagnosis (Figure 5, cases 33 and 70). However, these two astrocytomas did not progress at histological level; both primary and recurrent tumours were WHO grade II. Järvelä et al. found EGFR amplification in 4% of grade II and in 21% of grade III astrocytomas (Järvelä et al., 2006).

In our series, we found EGFR amplification in one (n=4) primary anaplastic oligoastrocytoma and in one (n=4) recurrent low-grade oligodendroglioma. EGFR amplification is rare in low-grade oligodendroglial tumours. Fallon et al. studied 138 oligodendrogliomas and oligoastrocytomas by FISH and found only one EGFR amplification in an anaplastic oligoastrocytoma (Fallon et al., 2004). In a series of 33 oligodendrogliomas, Reifenberger et al. found EGFR amplification by Southern blotting in one case of anaplastic oligodendroglioma (Reifenberger et al., 1996). Our results are in agreement with the previous studies, although the number of tumours in these subgroups is small. Anaplastic oligodendroglial tumours carry EGFR amplification at a significantly higher rate. In a prospective chemotherapy study, EGFR amplification was present in 18% of anaplastic oligodendrogliomas (Kouwenhoven et al., 2009).

Amplification of genes on chromosome 4q12 (study III)

PDGFRA

In the present study PDGFRA amplification was found mainly in high-grade astrocytomas. PDGFRA was amplified in one primary (n=35) and one recurrent (n=12) low-grade astrocytoma. Anaplastic astrocytomas carried PDGFRA amplification in 33% at first diagnosis and in 12% in recurrent tumours. In secondary glioblastomas, PDGFRA amplification was found in 31% of cases. PDGFRA amplification emerged in two tumours during progression from low-grade astrocytoma to glioblastoma (Figure 5, cases 13 and 39). These results are well in line with other studies, where PDGFRA amplification has been found in 8-21% of glioblastomas (Fleming et al.,

1992; Joensuu et al., 2005; Verhaak et al., 2010; Nobusawa et al., 2011). Fleming and co-workers found PDGFRA amplification in one of seven anaplastic astrocytomas by Southern blotting (Fleming et al., 1992). In a recent array-CGH study, PDGFRA amplification was detected in 13% of anaplastic astrocytomas (Toedt et al., 2010). In low-grade astrocytomas, PDGFRA amplification has been reported in 14-50% of tumours by real-time quantitative PCR analysis (Arjona et al., 2005; Martinho et al., 2009).

In the present study, oligodendroglial tumours did not exhibit PDGFRA amplification at the time of the diagnosis. However, at recurrence one anaplastic oligodendroglioma (n=11) and four anaplastic oligoastrocytomas (n=10) had acquired PDGFRA amplification (Table 5, Figure 5). Our results are in agreement with Martinho and co-workers who found PDGFRA amplification in 1 out of 10 low-grade oligodendrogliomas and in 3 out of 15 anaplastic oligodendroglial tumours (Martinho et al., 2009). Smith et al. reported PDGFRA amplification in 4 out of 21 anaplastic oligodendrogliomas and in 1 out of 11 anaplastic oligoastrocytomas (Smith et al., 2000).

In glioblastomas, PDGFRA amplification accumulates in a subtype that has a proneural gene expression profile (Verhaak et al., 2010). The proneural subtype of glioblastomas is characterised by genes associated with the progress of neurogenesis (Phillips et al., 2006), and it is associated with younger patient age and better outcome (Phillips et al., 2006;

Verhaak et al., 2010). According to a recent study, activating rearrangements of the PDGFRA gene are present in 40% of glioblastomas with amplified PDGFRA (Ozawa et al., 2010). Furthermore, Ozawa et al. have reported a novel gene fusion between PDGFRA and VEGFR2 in glioblastomas (Ozawa et al., 2010).

Imatinib is a selective tyrosine kinase inhibitor of KIT, PDGFRs and BCR-ABL fusion protein, which is the oncogenic protein in chronic myelogenous leukaemia (Capdeville et al., 2002). Imatinib has revolutionized the therapy of patients with chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor (GIST), which is characterised by activating mutations of KIT or PDGFRA (Antonescu, 2011). Imatinib inhibits tumour cell growth in primary cultures of high-grade gliomas (Hagerstrand et al., 2006). However, in a clinical trial imatinib therapy showed only limited efficacy in patients with recurrent gliomas (Raymond et al., 2008).

KIT

In the present study, KIT amplification was found more frequently in recurrent tumours than in primary gliomas (27% vs. 10%, p<0.01). We found KIT amplification most frequently in malignant astrocytic tumours. KIT amplification was present in 28% of primary, in 50% of recurrent anaplastic astrocytomas and in 36% of secondary glioblastomas as well. At first presentation, low-grade astrocytomas and oligodendroglial tumours carried KIT amplification in significantly lower

Table 5   Histological diagnoses and gene amplifications in primary gliomas and their recurrences (study III).

Tumour histology and grade			n	Amplification at first diagnosis n (n of informative tumours)				Amplification at recurrence n (n of informative tumours)			
First diagnosis		Recurrence		PDGFRA	KIT	VEGFR2	EGFR	PDGFRA	KIT	VEGFR2	EGFR
A2	→	A2	12	0 (11)	0 (12)	2 (11)	0 (11)	1 (8)	2 (9)	1 (9)	2 (9)
A2	→	A3	13	1 (13)	0 (11)	2 (11)	0 (11)	1 (12)	5 (11)	1 (12)	0 (11)
A2	→	GBM	7	0 (6)	1 (7)	1 (7)	0 (6)	2 (7)	1 (7)	0 (7)	0 (7)
A3	→	A3	6	1 (5)	2 (6)	1 (6)	1 (6)	1 (4)	2 (4)	1 (4)	1 (4)
A3	→	GBM	8	3 (8)	3 (8)	2 (8)	1 (7)	3 (8)	4 (6)	2 (8)	2 (7)
A2	→	OA3	5	0 (4)	1 (5)	0 (5)	0 (5)	1 (4)	0 (4)	1 (5)	0 (5)
A3	→	A2	3	1 (2)	0 (3)	0 (3)	0 (3)	0 (2)	0 (2)	0 (3)	0 (2)
A3	→	O3	1	n.a.	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
A2	→	O3	1	0 (1)	0 (1)	0 (1)	n.a.	0 (1)	0 (1)	0 (1)	0 (1)
O2	→	O2	4	0 (3)	0 (3)	0 (3)	0 (4)	0 (3)	0 (3)	0 (3)	1 (3)
O2	→	O3	3	0 (3)	0 (3)	0 (2)	0 (2)	0 (3)	0 (3)	0 (3)	0 (3)
O3	→	O3	5	0 (5)	0 (4)	0 (5)	0 (5)	1 (5)	1 (5)	1 (5)	0 (5)
O2	→	OA3	2	0 (2)	0 (2)	0 (2)	0 (2)	0 (1)	0 (1)	0 (1)	0 (1)
O2	→	OA2	2	0 (2)	0 (2)	1 (2)	0 (2)	0 (2)	0 (2)	1 (2)	0 (2)
O3	→	OA3	1	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	n.a.	0 (1)
O3	→	O2	1	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
O2	→	A3	1	0 (1)	0 (1)	0 (1)	0 (1)	n.a.	n.a.	n.a.	n.a.
O3	→	A3	1	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)	1 (1)	0 (1)	0 (1)
OA2	→	OA2	1	0 (1)	0 (1)	0 (1)	n.a.	0 (1)	1 (1)	0 (1)	0 (1)
OA2	→	OA3	4	0 (3)	1 (3)	0 (3)	0 (3)	3 (4)	2 (4)	2 (3)	0 (4)
OA3	→	OA3	1	0 (1)	0 (1)	0 (1)	0 (1)	n.a.	n.a.	n.a.	n.a.
OA3	→	GBM	1	n.a.	n.a.	1 (1)	1 (1)	0 (1)	0 (1)	0 (1)	n.a.
OA2	→	A2	2	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)
OA3	→	O3	2	0 (2)	0 (2)	0 (2)	0 (2)	0 (1)	0 (1)	0 (1)	0 (1)
Total			87	6 (78)	8 (81)	10 (81)	3 (78)	13 (73)	19 (71)	10 (74)	6 (72)

A astrocytoma, GBM glioblastoma O oligodendroglioma, OA oligoastrocytoma, WHO grade by Arabic numerals, n.a. not available

53
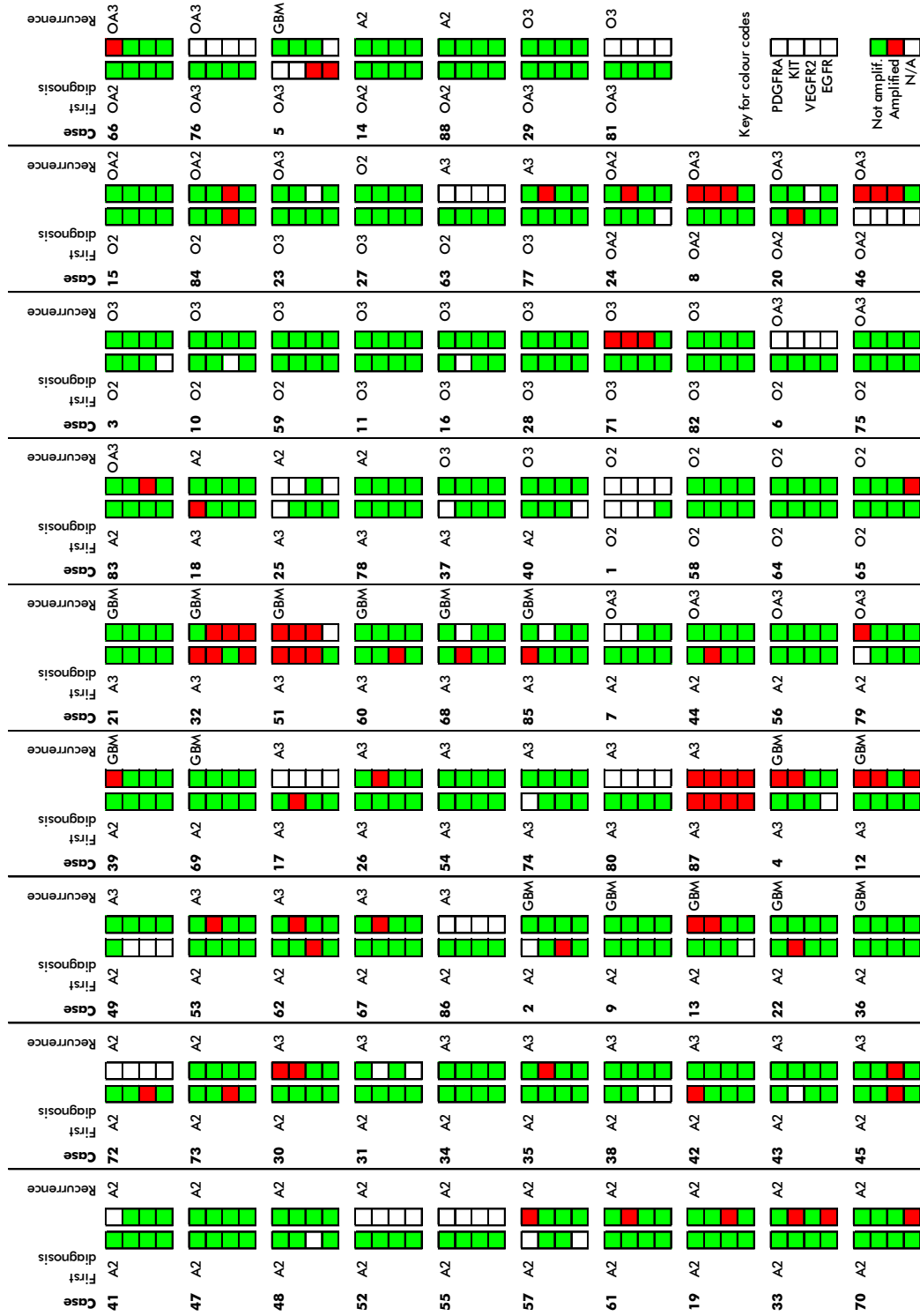
Figure 5 Amplification status of PDGFRA, KIT, VEGFR2 and EGFR genes in gliomas at first diagnosis and subsequent recurrence are indicated by green (not amplified) and red (amplified) boxes. A astrocytoma, O oligodendroglioma, OA oligoastrocytoma, GBM glioblastoma, WHO grade by Arabic numerals, N/A not available. Courtesy of M. Puputti.

frequency than anaplastic astrocytomas (5% vs. 28%, p=0.01).

The frequency of KIT amplification in a recent population-based study of glioblastomas was 4.4%, as studied by differential PCR (Nobusawa et al., 2011). Holtkamp et al. studied 87 gliomas using the multiplex ligation-dependent probe amplification (MLPA) method and found KIT amplification in 5% of glioblastomas but not in any anaplastic astrocytomas or oligodendrogliomas (Holtkamp et al., 2007). The differences in KIT amplification frequency between the studies may be due to different detection methods and patient material.

Nobusawa and co-workers did not detect a significant difference in KIT amplification frequency between primary and secondary glioblastomas (Nobusawa et al., 2011). This result suggests that KIT amplification may be involved in the pathogenesis of both primary and secondary glioblastomas. Notably, in our material, 5 of 13 astrocytomas acquired KIT amplification during progression from grade II to III, suggesting that KIT may play a role in the progression of low-grade astrocytomas as well.

Mutations of KIT have not been reported in gliomas. Joensuu and co-workers did not detect any KIT mutations in a series of 47 glioblastomas (Joensuu et al., 2005). At protein level, KIT expression was rare in our material; moderate or strong immunoreactivity was present in only two anaplastic astrocytomas and two secondary glioblastomas. All four tumours that expressed KIT by immunohistochemistry harboured KIT amplification.

VEGFR2

In the present study, VEGFR2 amplification was found in 5 (14%) low-grade astrocytomas, in 3 (17%) anaplastic astrocytomas and in 2 (11%) oligodendroglial tumours at first diagnosis. There was no significant difference in the VEGFR2 amplification rate between primary and recurrent gliomas (12% vs. 14%). Two (12%) secondary glioblastomas harboured VEGFR2 amplification. In their population-based study of glioblastomas, Nobusawa et al. detected VEGFR2 amplification in 3.3% of tumours (Nobusawa et al., 2011). Holtkamp and co-workers did not find VEGFR2 amplifications in a series of 10 anaplastic astrocytomas and 12 oligodendrogliomas (Holtkamp et al., 2007).

We found immunohistochemical VEGFR2 expression in 28 of 51 (47%) of astrocytic tumours at first diagnosis. Oligodendroglial tumours were negative by VEGFR2 immunohistochemistry at first diagnosis. In our material, VEGFR2 expression did not correlate with VEGFR2 amplification. Steiner and co-workers have demonstrated coexpression of VEGF and its receptor VEGFR2 in glioblastomas, suggesting a presence of an autocrine signalling loop (Steiner et al., 2004). VEGFR2 expression has been detected in glioblastoma cell lines as well (Holtkamp et al., 2007). VEGF upregulation and simultaneous amplification of its receptor VEGFR2 in gliomas cells may facilitate tumour growth.

Co-amplification of KIT, PDGFRA and VEGFR2

We found co-amplification of KIT, PDGFRA and VEGFR2, which are located at 4q12, in 2 primary anaplastic astrocytomas (3% of informative primary tumours, Figure 5). At recurrence, co-amplification of the three genes was present in 5 (7%) tumours (1 astrocytoma grade III, 1 glioblastoma, 1 oligodendroglioma grade III and 2 oligoastrocytomas grade III). In a recent series of glioblastomas, co-amplification of the three genes was present in 3.1% of tumours (Nobusawa et al., 2011).

Prognostic value of KIT, PDGFRA and EGFR amplifications

In our study, PDGFRA (p=0.047), KIT (p=0.015) and EGFR (p=0.0003) amplifications were prognostic factors for overall survival in univariate analysis. However, in multivariate analysis that included tumour histology and grade, the gene amplifications were not associated with prognosis. This result is probably due to the fact that amplification of PDGFRA (p=0.014) and EGFR (p=0.048) were associated with tumour grade in primary tumours. Järvelä and co-workers reported a negative association between EGFR amplification and survival in anaplastic astrocytomas (Järvelä et al., 2006) but two other studies found no such association (Smith et al., 2001; Liu et al., 2005). In glioblastomas, EGFR amplification is not an independent prognostic factor (Liu et al., 2005; Järvelä et

al., 2006) with an exception of patients over 60 years of age (Smith et al., 2001).

Mutations of p53 gene

We detected p53 gene mutations at first diagnosis in 11 (29%) low-grade astrocytomas, in 5 (28%) anaplastic astrocytomas and in 7 (22%) oligodendroglial tumours. There was no significant difference in the frequency of p53 mutations between first diagnosis and recurrence, indicating that the p53 mutation is an early event in the progression of gliomas. In a population-based study of low-grade gliomas, p53 mutations were present in 59% of astrocytomas and in 23% of oligodendroglial tumours (Okamoto et al., 2004). The lower frequency of p53 mutations detected in our material may be due to different patient population and detection methods. p53 mutation is a genetic hallmark of secondary glioblastomas (Ohgaki and Kleihues, 2007). In our material, 6 (38%) secondary glioblastomas harboured p53 mutation.

Expression of COX-2 and HuR in gliomas (study IV)

COX-2

We studied COX-2 expression in primary and recurrent gliomas of 113 patients. Histological diagnoses and WHO grade of the tumours are shown in Table 6. We found a high level of COX-2 expression in 65 (28%) of gliomas (Table 6). COX-2

expression was observed in all subtypes of diffuse gliomas. There was no significant difference in COX-2 immunostaining between astrocytomas, oligodendrogliomas and oligoastrocytomas. Upregulated COX-2 expression was associated with higher WHO grade ($p<0.001$) in the whole tumour group and in subgroups of astrocytomas ($p<0.001$) and oligodendrogliomas ($p<0.001$). Our results are in accordance with previous studies, where COX-2 immunoexpression correlated positively with tumour grade in astrocytomas (Joki et al., 2000; Shono et al., 2001; Hara and Okayasu, 2004). Similarly, Castilla and co-workers found elevated COX-2 expression in high-grade oligodendrogliomas (Castilla et al., 2003).

HuR

We detected nuclear expression of HuR in all studied tumours. The nuclei of neurons that were inside the infiltrating glioma tissue were positive by immunohistochemistry as well. Cytoplasmic HuR immunoreactivity was present in 44 gliomas (20%, Table 6). We analysed cytoplasmic HuR expression because the shuttling of HuR between nucleus and cytoplasm is considered the main mechanism of its mRNA stabilisation activity (Doller et al., 2008). Furthermore, elevated cytoplasmic HuR content is linked to patient outcome in breast carcinoma and ovarian carcinoma (Erkinheimo et al., 2003; Heinonen et al., 2005). The immunohistochemical method is suitable for analysing HuR expression in brain tumours because it en-

ables the study of subcellular localization of the protein.

Few reports have been published on the role of HuR in brain tumours. Nabors et al. observed HuR expression at the mRNA level in glioblastomas, pilocytic astrocytomas, ependymomas, medulloblastomas and meningiomas (Nabors et al., 2001). In their material, immunohistochemistry revealed a predominantly nuclear staining pattern with weak cytoplasmic staining in glioblastomas (Nabors et al., 2001). In another a series of 27 astrocytomas, HuR mRNA expression and nuclear immunoreactivity were found in all tumours (Ido et al., 2008). Cytoplasmic immunoreactivity was associated with higher tumour grade (Ido et al., 2008).

In the present study, cytoplasmic HuR expression correlated with higher tumour grade in the whole material ($p<0.001$) and in astrocytomas ($p<0.001$) but not in oligodendrogliomas or oligoastrocytomas. Thus, our results suggest that cytoplasmic localization of HuR could be involved in glioma progression. However, no significant association was observed between histological glioma type and cytoplasmic HuR staining indicating that upregulation of HuR may play a role in the pathogenesis of both astrocytomas and oligodendrogliomas. A significant difference was seen in cytoplasmic HuR expression between primary and secondary glioblastomas in the recurrent tumour group ($p=0.01$, Table 6), which reflects divergent pathogenesis of these tumours.

Table 6   Tumour histology, WHO grade and immunoreactivity of HuR and COX-2 (study IV).

| | Cytoplasmic HuR | | | | | | COX-2 | | | | | |
| | First diagnosis | | | Recurrence | | | First diagnosis | | | Recurrence | | |
Tumour histology and grade	N	– (%)	+ (%)	N	– (%)	+ (%)	N	Low (%)	High (%)	N	Low (%)	High (%)
Astrocytoma												
Grade II	40	38 (95)	2 (5)	14	14 (100)	0 (0)	42	38 (90)	4 (10)	14	11 (79)	3 (21)
Grade III	19	17 (89)	2 (11)	20	19 (95)	1 (5)	20	16 (80)	4 (20)	20	13 (65)	7 (35)
Glioblastoma, primary	15	8 (53)	7 (47)	13	4 (31)	9 (69)	15	8 (53)	7 (47)	13	3 (23)	10 (77)
Glioblastoma, secondary		n.a.		21	16 (76)	5 (24)		n.a.		22	13 (59)	9 (41)
Oligodendroglioma												
Grade II	13	12 (92)	1 (8)	5	5 (100)	0 (0)	15	15 (100)	0 (0)	6	6 (100)	0 (0)
Grade III	9	7 (78)	2 (22)	17	12 (71)	5 (29)	9	7 (78)	2 (22)	17	11 (65)	6 (35)
Oligoastrocytoma												
Grade II	8	7 (88)	1 (13)	5	5 (100)	0 (0)	8	7 (88)	1 (13)	5	4 (80)	1 (20)
Grade III	4	2 (50)	2 (50)	19	12 (63)	7 (37)	4	4 (100)	0 (0)	19	8 (42)	11 (58)
Total	108	91	17	114	87	27	113	95	18	116	69	47

n.a. not applicable

58

Association of COX-2 and HuR expression in gliomas (study IV)

We found a significant correlation between cytoplasmic HuR staining and a high level of COX-2 expression (p<0.001). A similar association between HuR and COX-2 has been observed in carcinomas of the breast, ovary, colon and stomach (Erkinheimo et al., 2003; Denkert et al., 2004b; Mrena et al., 2005; Denkert et al., 2006). Experimental studies have shown that HuR regulates COX-2 mRNA and protein expression in cell lines (Dixon et al., 2001; Sengupta et al., 2003; Mrena et al., 2005; Young et al., 2009). Moreover, HuR is involved in the stabilisation of mRNAs of many other genes related to cancer, such as cyclins, HIF-1α and VEGF (Lopez de Silanes et al., 2005). There is evidence that HuR regulates the expression of VEGF in gliomas as well. Ido and co-workers have shown that the inhibition of cytoplasmic translocation of HuR reduces VEGF expression astrocytoma cell lines (Ido et al., 2008). Despite the substantial evidence that links HuR and cancer, it is still not known if HuR has a causal role in tumourigenesis.

Prognostic value of COX-2 and HuR in gliomas (study IV)

In the present study, univariate survival analysis showed a significant correlation between overall survival and histological tumour type (p<0.001), WHO grade (p<0.001), age at diagnosis (p=0.02), cytoplasmic HuR expression (p<0.001), high COX-2 expression (p=0.001) and combination of HuR/COX-2 expression (p<0.001). Next, we performed multivariate survival analysis to evaluate the independence of HuR and COX-2 as prognostic factors. In Cox multivariate analysis, histological tumour type (p<0.001), WHO grade (p<0.001) and patient age (p=0.03) were independent prognostic markers for overall survival. Expression of HuR and COX-2 were not independently associated with patient outcome probably due to their strong association with WHO grade. In a previous study, COX-2 was associated with poor patient outcome independent of tumour grade in astrocytomas (Shono et al., 2001). However, in the material by Shono and co-workers, WHO grade was not associated with prognosis, which is contrary to current knowledge (Louis et al., 2007). The correlation between COX-2 expression and prognosis has been observed in astrocytomas (Hara and Okayasu, 2004) and in oligodendrogliomas (Castilla et al., 2003) in univariate survival analysis. Cytoplasmic HuR expression is a prognostic marker in carcinomas of the breast and ovary (Erkinheimo et al., 2003; Denkert et al., 2004a; Heinonen et al., 2005). The role of HuR as a prognostic factor in gliomas has not been reported previously.

Molecular changes during glioma progression (studies II-IV)

Results of the current study show that the immunohistochemically detected expression of ezrin ($p<0.05$) and COX-2 ($p<0.001$) increased significantly between the first and second glioma operation. However, no significant difference was seen in the cytoplasmic expression of HuR between gliomas at first diagnosis and recurrence. At DNA level, amplification of the KIT gene was present more frequently in recurrent gliomas compared to their primary tumours ($p<0.05$). Amplification frequencies of PDGFRA, VEGFR2 and EGFR were not significantly different between primary and recurrent gliomas, but the number of tumours with these alterations was small.

Progression of gliomas from low-grade to high-grade tumours is characterised by the sequential accumulation of genetic alterations, epigenetic changes and dysregulation of proteins related to cell proliferation. These changes may be directly involved in tumourigenesis or they may reflect secondary alterations due to dysfunction of common pathways. Previous studies have identified several genetic alterations that are present in recurrent gliomas but not in the corresponding primary tumours. Deletion of the tumour suppressor gene CDKN2A is involved in the development of tumour recurrences in both astrocytomas and oligodendrogliomas (Hulsebos et al., 1998; Jeuken et al., 2002; Idbaih et al., 2008; Jeuken et al., 2010). Other tumour suppressor genes that are implicated in glioma progression include Rb1 (Hulsebos et al., 1998) and PTEN (Jeuken et al., 2010). According to recent literature, MGMT promoter methylation status may change from unmethylated to methylated during progression of oligodendrogliomas (Lavon et al., 2007) and in glioblastomas after the initial diagnosis (Brandes et al., 2010; Jung et al., 2010).

At present, it is not known if the alterations we detected in the expression of ezrin and COX-2 and if the amplification of the KIT gene are directly linked to the tumourigenesis of gliomas. It is possible that they merely represent dysregulation of the glioma cell proteome and genomic instability. However, these alterations could be used as surrogate markers of glioma progression together with other genetic and epigenetic aberrations of gliomas. Currently, the state-of-the-art molecular markers that are used in diagnostic and prognostic assessment of gliomas include 1p/19q deletions, MGMT promoter methylation, IDH1 mutation and EGFR amplification as reviewed above.

CONCLUDING REMARKS

In the first part of this thesis, we focused on the correlation of contrast enhancement in MRI and immunohistochemical analysis of gliomas. Based on our results, preoperative MRI may be used to estimate tumour cell proliferation activity and vascular density in grade II-IV diffuse gliomas. This information is of clinical value because the tissue sample submitted for histopathological analysis may not represent the most proliferative part of the tumour, whereas by MRI the tumour can be evaluated as a whole. However, contrast enhancement should be evaluated cautiously together with clinical information and other MRI features to exclude pilocytic astrocytomas and other enhancing brain lesions.

Secondly, we studied gene amplifications and proteins related to the recurrence and progression of gliomas. We confirmed that ezrin is expressed at protein level in both astrocytomas and oligodendrogliomas, and ezrin may play a role in the progression of these tumours. High ezrin expression correlates with poor patient outcome although it is not an independent prognostic factor.

Amplifications of PDGFRA, KIT and VEGFR2 are present in both low-grade and malignant gliomas. PDGFRA and KIT amplifications occur most frequently in high-grade astrocytomas and in secondary glioblastomas. Since the frequency of KIT amplification increases in recurrent gliomas, KIT appears to be linked to tumour progression.

We found that COX-2 expression is upregulated in gliomas in a grade dependent manner. Cytoplasmic HuR expression correlates with COX-2 expression in gliomas. Therefore, HuR is a potential regulator of COX-2 in gliomas, as in many epithelial cancers. Expression of COX-2 and HuR are prognostic factors for glioma patient survival in univariate analysis but not in multivariate analysis.

The strength of this study is the long follow-up time of the patients (median of 10 years for low-grade gliomas). A follow-up of several years is essential, because the median survival time of patients with low-grade gliomas is over 7 years (Okamoto et al., 2004). There are two limitations in this study. First, the tumour material was collected retrospectively. Second, we included only tumour pairs from primary and recurrent glioma operations. This approach may cause selection bias to the results. We used multivariate analysis to control the effects of patient age, histological type and grade that are known to have a strong association with the recurrence and malignancy of gliomas. Despite these limitations, our results give some new insights into the progression of gliomas.

The main future targets in the treatment of gliomas are the elimination of glioma stem cells and migrating tumour cells, which are

eventually fatal to the patient. Glioma stem cells maintain the tumour cell proliferation and growth but they are resistant to current therapies. Development of new treatments also requires novel imaging tools capable of monitoring glioma stem cells and dissemination of tumour cells.

Management of patients with low-grade diffuse gliomas is a challenge to a neuro-oncological team. The patients undergo several recurrences and treatment modalities during the course of the disease that may span over 10 years. Continuing research of the molecular biology of gliomas is needed to find markers that would identify patients at risk for early malignant transformation and to reveal possible targets for therapeutic interventions. These markers and new treatment strategies should be validated in prospective trials to determine optimal management strategies for glioma patients.

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