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Carbonic Anhydrases II, IX and XII in Astrocytic Gliomas

Their relationship with clinicopathological features and proliferation

ACADEMIC DISSERTATION
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III Haapasalo J, Nordfors K, Järvelä S, Bragge H, Rantala I, Parkkila AK, Haapasalo H, Parkkila S (2007); Carbonic anhydrase II in the endothelium of glial tumours: a potential target for therapy. Neuro Oncol. 9:308-13.

IV Haapasalo J, Hilvo M, Nordfors K, Haapasalo H, Parkkila S, Hyrskyluoto A, Rantala I, Waheed A, Sly WS, Pastoreková S, Pastorek J, Parkkila AK (2008); Identification of an alternatively spliced isoform of carbonic anhydrase XII in diffusely infiltrating astrocytic gliomas. Neuro Oncol. 10:131-8.

These articles are referred to in the text by studies I – IV. The original publications have been reproduced with the permission of the copyright holders.

Abbreviations

ACTH	adrenocorticotropic hormone
AE	anion exchanger
ALL	acute lymphocytic leukaemia
CA	carbonic anhydrase
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
CNS	central nervous system
CT	computer tomography
DAB	diaminobenzidine
EGFR	endothelial growth factor reseptor
FasL	Fas-Fas ligand
FDA	Food and Drug Administration
GABA	gamma-aminobutyric acid
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GLUT	glucose transporter
GTR	gross total resection
H&E	hematoxylin-eosin
HIF	hypoxia-inducible factor
HNPCC	hereditary non-polyposis colorectal cancer
HRE	hypoxia response element
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
IL	interleukin
LOH	loss of heterozygosity
MCT	H+/monocarboxylate transporter
MIB-1	an antibody against Ki-67
MN	carbonic anhydrase IX
MRI	magnetic resonance imaging
NHE	Na+/H+ exchanger
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PG	proteoglycan
PHD	prolyl-4-hydroxylases
PIP3	phosphatidylinositol 3-trisphosphate
PTEN	phosphatase and tensin homolog
pVHL	von Hippel-Lindau tumour suppressor protein
RB1	retinoblastoma tumour suppressor protein
SD	standard deviation
RCC	renal cell cancer
ROC	receiver operating characteristic
RT-PCR	reverse transcription-polymerase chain reaction
TEO	National Authority for Medicolegal Affairs
TGF	transforming growth factor
TNF	tumour necrosis factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TP53	tumour protein p53
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau tumour suppressor
WHO	World Health Organization

Abstract

Background: Diffusely infiltrating astrocytomas are the most common primary brain tumours and most malignant of all brain tumours. These malignant gliomas are classified to WHO grades II to IV. Grade IV glioblastoma represents a devastating tumour type with a 5-year survival rate below 10%. The best treatment option available today, that is surgery possibly combined with radiation- and/or chemotherapy, is insufficient. Therefore, brain tumour research has focused on understanding of the pathogenesis, and accordingly, has aimed to develop better methods for cancer diagnostics and treatment. Carbonic anhydrases (CAs), investigated in this study, have previously been linked to carcinogenic processes of several malignancies, and are proposed to represent an attaractive target for cancer therapy.

Aims: The purpose of this study is to describe the expression of CAs in astrocytic gliomas and study their relationship to clinicopathological features, especially the proliferation of cancer cells measured by Ki-67 / MIB-1. Another aim is to develop methods for improved diagnostics and to predict more accurately survival of patients with astrocytic gliomas. The additional objective of this study is to characterize possible targets for future therapeutic interventions of gliomas.

Results: In the first study, a new method is introduced for intraoperative immunohistochemical staining of a proliferation marker, Ki-67 (Ultrarapid-Ki67®). This method was practical to perform and morphologically and quantitatively indistinguishable from the conventional Ki-67 / MIB-1 staining. In the following three studies, the expression of CAs II, IX, and XII is described in a large series of diffusely infiltrating astrocytomas for the first time. By immunohistochemistry, CA II was detected in the neovessels of high-grade gliomas, whereas CA IX and CA XII were mainly located in cancer cells. A short isoform of CA XII by alternative splicing was also introduced into gliomas. None of the CAs correlated to tumour cell proliferation by Ki-67 / MIB-1, whereas CA IX was associated with necrotic tumour regions. The expression of all CAs was significantly associated with higher WHO grade. Most importantly, the expression of all CAs predicted poor survival of patients in univariate analysis, and CA IX and CA XII were significant predictors of survival even in multivariate analysis.

Conclusions: CA II, IX, and XII are expressed in astrocytic gliomas and the expression increases within increasing WHO grade. Being involved in carcinogenesis, CAs could be used in predicting

poor survival of patients. CAs seem to be potential target molecules for therapy in gliomas, which should be evaluated in clinical trials. CAs were not associated with tumour cell proliferation as evaluated by Ki-67 / MIB-1 staining. In addition, Ultrarapid-Ki67® immunostaining, as a fast and reliable method for proliferation estimation, could be used in routine intraoperative diagnosis of gliomas.

Keywords: astrocytic glioma, carbonic anhydrase, diffusely infiltrating astrocytoma, Ki-67, oligodendroglioma, prognosis

Tiivistelmä

Tausta: Diffuusisti infiltroivat astrosytoomat ovat astrosyyttisiä glioomia ja primaareista aivokasvaimista yleisimpiä ja pahanlaatuisimpia. Nykyisillä hoitomuodoilla - kasvaimen kirurgisella poistolla yhdistettynä mahdollisesti sädehoitoon ja kemoterapiaan - ei pystytä sairautta parantavaan hoitotulokseen varsinkaan korkeimman pahanlaatuisuusasteen (gradus IV) glioblastoomissa, joissa potilaan ennuste on erittäin huono (viisivuotisennuste on alle 10 %). Aivokasvaintutkimuksen tavoitteena on ollut vuosien ajan ymmärtää syövän patogeneesiä ja kehittää tämän perusteella uusia menetelmiä diagnostiikkaan ja hoitoon. Tutkimuksen kohteena olevat hiilihappoanhydraasit (carbonic anhydrase, CA) ovat keskeisiä syövän patogeneesissä monissa kasvaimissa; niiden ilmentyminen kasvaimessa liittyy usein potilaiden huonoon ennusteeseen, ja niitä on pidetty mahdollisina kohdemolekyyleinä syöpähoidoille.

Tavoitteet: Tutkimuksen tavoitteena on kuvata hiilihappoanhydraasien esiintymistä glioomissa ja tutkia näiden yhteyttä kliinispatologisiin muuttujiin sekä erityisesti syöpäsolujen proliferaatioon, jota mitataan Ki-67 / MIB-1:n avulla. Tutkimuksessa on tarkoitus kehittää menetelmiä, joilla voidaan tarkentaa kasvaindiagnostiikkaa ja kuvata potilaiden eloonjäämisennustetta. Tavoitteena on myös kartoittaa tutkittavien molekyylien käytön mahdollisuutta syöpähoitojen kohdemolekyyleinä.

Tulokset: Ensimmäisessä osajulkaisussa kuvattiin proliferaatiovärjäysmenetelmä, jonka avulla voidaan neurokirurgisen leikkauksen aikana tehdä nopeasti diagnostiikkaa helpottava Ki-67 –värjäys jääleikenäytteestä (Ultrarapid-Ki67®). Värjäysmenetelmä oli helppo toteuttaa, ja sen tulokset olivat morfologisesti ja kvantitatiivisesti identtiset verrattuna tavalliseen Ki-67 / MIB-1 -värjäykseen. Muissa osajulkaisuissa kuvasimme ensimmäistä kertaa hiilihappoanhydraasien II, IX ja XII ilmentymisen laajassa astrosytoomamateriaalissa. Immunohistokemia osoitti CA II:n ilmentyvän glioblastoomien uudisverisuonien endoteelissä. CA IX sekä CA XII esiintyivät pääasiassa syöpäsoluissa. RT-PCR (käänteiskopioijapolymeraasiketjureaktio) -menetelmän avulla selvisi, että astrosytoomissa havaittu CA XII on pääasiallisesti vaihtoehtoisen silmukoinnin avulla tuotettu lyhyempi variantti. Kaikkien hiilihappoanhydraasien ilmentyminen lisääntyi tilastollisesti merkittävästi suhteessa kasvavaan WHO -gradukseen. CA -entsyymit eivät assosioituneet syöpäsolujen proliferaatioon, mutta CA IX ilmeni enemmän nekroottisilla alueilla. Endoteeliin paikantuvan CA II:n sekä syöpäsolujensisäisten CA IX:n ja CA XII:n esiintyminen ennusti

tilastollisesti merkittävästi potilaiden lyhyempää eloonjäämisaikaa. Lisäksi CA IX ja CA XII toimivat itsenäisinä ennustetekijöinä monimuuttuja-analyyseissä.

Johtopäätökset: Hiilihappoanhydraasit II, IX ja XII ilmentyvät astrosyyttisissä glioomissa, joissa niillä vaikuttaisi olevan tärkeä biologinen merkitys. Hiilihappoanhydraasien lisääntynyt ilmentyminen ennusti tilastollisesti merkittävästi potilaiden lyhyempää eloonjäämisaikaa. Hiilihappoanhydraasit eivät olleet yhteydessä syöpäsolujen proliferaatioon, jota mitattiin Ki-67 / MIB-1-värjäyksen avulla. Lisäksi kuvattu Ki-67 –pikavärjäysmenetelmä (Ultrarapid-Ki67®) on nopeutensa ja luotettavuutensa perusteella käyttökelpoinen kasvaimien leikkauksenaikaisessa jääleikediagnostiikassa.

Avainsanat: ennuste, gliooma, diffuusisti infiltroiva astrosytooma, hiilihappoanhydraasi, Ki-67, oligodendroglioma

1. Introduction

Astrocytic gliomas are a heterogeneous group of tumours which have been known to represent an important and mostly malignant entity for over a hundred years (Virchow 1863, Bailey and Cushing 1926). Traditionally, gliomas, including astrocytomas, oligodendrogliomas and ependymomas, were thought to originate from supporting cells of the central nervous system (CNS), the glia cells. Advanced genetic methods have revealed that the origin is complex; the development of these tumours includes not only the astrocytes, but also their precursor cells or even stem cells (Ohgaki et al. 2004). The current World Health Organizaion (WHO) classification of astrocytic tumours defined astrocytomas as grade I-IV tumours, of which grades II-IV are considered malignant and termed diffusely infiltrating astrocytomas (Louis et al. 2007). Indeed, they represent an important tumour entity, being the most common primary intracranial neoplasms, accounting for 60 % of all primary brain tumours. The incidence of diffusely infiltrating astrocytomas is approximately 5-7 new cases per 100 000 population, and the most common type of astrocytoma is grade IV glioblastoma multiforme (GBM) with the incidence of approximately three new cases per 100 000 population (Louis et al. 2007).

Although diffusely infiltrating astrocytomas have been intensively studied, survival of the patients still remains poor. A meta-analysis by Stewart et al. (2002) combining 12 randomized clinical trials showed that the overall survival rate was 40% at one year and only slightly higher (46%) after the addition of adjuvant therapies. This is the case especially with glioblastomas, in which the 5-year survival has been reported to be 9.8% with the latest therapeutical methods (Stupp et al. 2009).

Surgical resection, to its feasible extent, has been the gold standard for treatment of astrocytomas for decades. The present scheme also includes radiation therapy and chemotherapy in the treatment of high grade gliomas. The devastating feature of malignant astrocytomas is the infiltration of cancer cells to the adjacent normal brain tissue, thus making the full removal of the tumour impossible. Even the lower grade astrocytomas (grades II and III) tend to appear in a more malignant fashion and develop into secondary glioblastomas. In previous years, a vast number of promising molecules have been proposed to improve the diagnosis and therapeutics of astrocytic gliomas, e.g. a humanized monoclonal antibody against vascular endothelial growth factor (VEGF) has been used in recurrent gliomas (Norden et al. 2008). Unfortunately, the major breakthrough still remains to be achieved.

This study was designed to improve understanding of the pathogenesis of astrocytic gliomas. The investigations focused on cell proliferation and carbonic anhydrases, which are often overexpressed in cancer and may contribute to carcinogenic processes. New methods for diagnostics and prognosis are evaluated, and strategies for future research on target molecules proposed.

2. Review of the literature

2.1. Astrocytic gliomas

Astrocytic gliomas belong to a heterogeneous group of tumours that include both low-grade and high-grade brain tumours. Traditionally, gliomas were thought to originate from glia (*glia* gk. glue) cells, which are definied as supporting cells of the CNS. During the last two decades, it has been understood that the development of these tumours includes not only the glial cells, but also their precursor cells or even stem cells (Ohgaki et al. 2004). Based on recent genetic analyses, another major theory postulates that neural stem cells or neural progenitors can undergo transformation events, which can ultimately lead to a malignancy (Furnari et al. 2007, The Cancer Genome Atlas (TCGA) Research Network 2008).

There are four main groups of glial cells in the CNS: astrocytes, oligodendrocytes, ependymal cells, and choroid plexus cells. Astrocytes (*astron* gk. star) are process-bearing and thus star-shaped cells which are poorly visualized in light microscopy without special staining techniques. Their functions involve, for example, structural support to other CNS cells, provision of nutrients and oxygen, guidance in the developmental process, regulation of the blood-brain barrier, influence on local neurotransmitters and electrolyte concentrations, and waste disposal. Therefore, the glial cells have multiple roles and their functional mechanisms are complex.

The term astrocytoma was first used by a famous German pathologist, Rudolf Virchow (1863), and was introduced to histopathological classification of brain tumours in 1926 (Bailey and Cushing). Astrocytomas are divided into four grades according to WHO (Louis et al. 2007). The grade I pilocytic astrocytoma is a benign tumour predominately affecting children or young adults. It is more circumscribed than the other astrocytomas, is often located in the cerebellum, and has a more favourable clinical behaviour, although it may have a lethal consequence depending on location. Other low-grade astrocytic tumours include subependymal giant cell astrocytoma (WHO grade I) and pleomorphic xanthoastrocytoma (WHO grade II). Diffusely infiltrating astrocytomas are divided into WHO grades II to IV (Louis et al. 2007). They include grade II diffuse astrocytomas, grade III anaplastic astrocytomas, and grade IV glioblastomas.

Diffusely infiltrating astrocytomas are the most common primary brain neoplasms and account for approximately 60 % of all primary brain tumours (Louis et al. 2007). Incidence of diffusely

infiltrating astrocytomas varies between different regions, with approximately 5-7 new cases per 100 000 population. In addition, the most common type of astrocytoma is grade IV glioblastoma (multiforme) with an incidence of approximately three new cases per 100 000 population.

When the etiology of diffusely infiltrating astrocytomas has been studied, iatrogenic x-ray irritation has been associated with an increased risk of tumours, especially in the case of prophylactic radiotherapy of the brain for acute lymphocytic leukaemia (ALL) (Chung et al. 1981, Edwards et al. 1986). When this was examined in a cohort study containing 9720 children treated for ALL, there was a 22-fold excess of neoplasms of the CNS and the estimated cumulative proportion of children in whom a second neoplasm developed was 2.53 % (Neglia et al. 1991). The irradiation of other brain tumours, such as pituitary adenomas, craniopharyngioma, pineal parencymal tumour and germinoma, has also been associated with a higher incidence of gliomas (Kitanaka et al. 1989).

2.1.1. Diffuse astrocytoma

Diffuse astrocytoma is a WHO grade II astrocytoma and has three different histological variants: fibrillary astrocytoma, gemistocytic astrocytoma, and protoplasmic astrocytoma (Louis et al. 2007). The incidence rate is approximately 1.4 new cases per 1 million population a year and incidence in children has increased during past decades in Scandinavia (Hemminki et al. 1999, Louis et al. 2007). Diffuse astrocytoma represents 10 – 15% of all astrocytic tumours. The peak incidence is between ages 30 and 40, and the mean age is 34 years. Localisation of diffuse astrocytoma varies. Supratentorial localisation in frontal and temporal lobes is the most common, and tumours in the brain stem or spinal cord are also typical. The first symptom is usually epileptic seizure, but abnormalities in sensation, vision or motoric activity can occur depending on the tumour localisation. Macroscopically, diffuse astrocytoma infiltrates to neighbouring anatomical structures and causes enlargement. In addition, cysts are usually present and calcification can be observed. Microscopically, fibrillary or gemistocytic neoplastic astrocytes are seen, cellularity is increased and nuclear atypia may occur. In fibrillary and gemistocytic astrocytomas, proliferation labeling index by Ki-67 / MIB-1 is usually less than 4% (Watanabe et al. 1996, Kros et al. 1996) and in protoplasmic astrocytomas less than 1% (Prayson et al. 1996). *Tumour protein p53 (TP53)* mutation is one of the important genetic alterations in diffuse astrocytomas. More than 60% of these tumours contain the mutation, and the frequency of the mutation does not increase during the malignant progression, suggesting that the *TP53* mutation is an early event (Okamoto et al. 2004). Other genetic changes include increased expression of platelet-derived growth factor (PDGF), receptor

alpha and $p14^{ARF}$ and MGMT promoter methylation (Louis et al. 2007). Mean survival time varies significantly, and after neurosurgical intervention it can be between six and eight years. Diffuse astrocytoma often progresses to glioblastoma and the mean time interval for this transition is approximately five years (Ohgaki et al. 2005a). Favourable clinical prognostic factors include young age at diagnosis and neurosurgical gross total resection (GTR). In contrast, a large tumour size and high Ki-67 / MIB-1 index (>5%) predict worse prognosis (Louis et al. 2007).

2.1.2. Anaplastic astrocytoma

WHO grade III astrocytoma is defined as anaplastic astrocytoma, and represents approximately 10% of all astrocytic tumours (Louis et al. 2007). It may arise from grade II diffuse astrocytoma or occur *de novo,* and has a strong tendency to progress to glioblastoma (Ohgaki et al. 2004). It is slightly more common among males (male/female ratio 1.16:1) and the mean age of patients at biopsy is 46 years according to a population-based European study (Ohgaki et al. 2005b). The typical localisation of anaplastic astrocytoma is in the cerebral hemisphere, but it also occurs in any other region of the CNS. The clinical symptoms, e.g. headache and nausea, could be due to increased intracranial pressure. Weakness, motor dysfunction and changes in behavior are other common symptoms. Macroscopically, anaplastic astrocytoma is often difficult to differentiate from grade II diffuse astrocytoma. The tumour infiltrates to surrounding normal brain tissue. Microscopic features of grade III astrocytoma include increased cellularity, nuclear atypia and elevated mitotic activity, especially when compared with grade II astrocytomas. Microvascular proliferation and necrosis are absent. When proliferation is assessed by Ki-67 / MIB-1, the range is approximately 5-10%, but overlapping results may be found with grade II and IV astrocytomas (Raghavan et al. 1990). Nevertheless, Ki-67 / MIB-1 is widely used and helpful in differential diagnostics. From a genetic point of view, anaplastic astrocytoma can be described as an intermediate stage on the malignant progression towards grade IV glioblastoma. It has been estimated to take approximately two years to develop a grade IV astrocytoma (Ohkagi et al. 2004). The genetic alterations include a high number of *TP53* mutations (>70% of all anaplastic astrocytomas) and loss of heterozygosity (LOH) 17p (50-60%), LOH 10q (35-60%), LOH 22q (20-30%), LOH 19q (46%), LOH 6q (approximately 30%), whereas *epidermal growth factor receptor* (*EGFR*) gene amplification is uncommon (<10%) (Louis et al. 2007). In patients with anaplastic astrocytoma, older age predicts poor prognosis.

2.1.3. Glioblastoma

WHO grade IV diffusely infiltrating astrocytoma is called glioblastoma. Previously, it was commonly called "glioblastoma multiforme", describing the variable histopathology (Louis et al. 2007). It presents two main histological variants: giant cell glioblastoma and gliosarcoma. Glioblastoma is the most common primary brain tumour and comprises 12-15% of all intracranial neoplasms and 60-75% of all astrocytic tumours (Ohgaki et al. 2005b). The incidence of glioblastoma varies between three and four cases per 100 000 population per year, being similar in North America and Europe (Louis et al. 2007). The tumour is slightly more common in males (male:female ratio 1.28:1) (Ohgaki et al. 2004).

The majority of glioblastomas (approximately 95%) develop *de novo*, with a short clinical history and no known pre-lesion, and thus are considered primary glioblastomas (Ohkagi et al. 2004). Another type, secondary glioblastoma, progresses to more malignant phenotype from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). These tumours are typically diagnosed in younger patients (mean age 45 years). According to genetic analyses, primary and secondary glioblastomas are considered to be relatively distinct entities and their development involves different genetic pathways. The typical example of genetic difference is *IDH1* mutation frequently occurring in secondary glioblastomas (Ohgaki and Kleihues 2009).

Glioblastoma preferentially occurs in adults, with a peak incidence age between 45 and 75 years, and a mean age of 61.3, according to a population-based study in Switzerland (Ohgaki et al. 2004). Nevertheless, it may manifest at any age, although it is very rare in patients younger than 20 years. The most common localisation of glioblastoma is in the white matter of cerebral hemispheres, and according to the WHO classification (Louis et al. 2007), the localisation varies in different lobes as follows: temporal (31%), parietal (24%), frontal (23%), and occipital (16%). Glioblastoma is uncommon in the cerebellum and spinal cord. Tumour cells often infiltrate to adjacent lobes and a combined fronto-temporal location is typical, as well as spreading through corpus callosum into the contralateral hemisphere ("butterfly glioma"). However, glioblastoma rarely invades to subarachnoidal space or metastazises via cerebrospinal fluid, and hematogeneous spread is also uncommon (Pasquier et al. 1980).

Common symptoms of glioblastoma include nausea, vomiting, and headache due to increased intracranial pressure (Louis et al. 2007). Epileptic seizure is common and could be the first symptom prompting the patient to seek medical treatment. Sometimes large haemorrhages may occur, causing stroke-like symptoms. Personality changes may occur, especially when the tumour is located in frontal lobes.

Macroscopically, the tumour is usually large at the time of computer tomography (CT) -scan or magnetic resonance imaging (MRI), even though the first symptoms may only have occurred a few months earlier. The lesion is usually unilateral and contains grey areas of tumour tissue, necrotic tissue up to 80% of the total tumour mass, and haemorrhages. Macroscopic cysts may also occur, containing liquid necrotic tissue.

Histopathology of glioblastoma typically shows poorly differentiated and anaplastic astrocytic tumour cells, with high mitotic activity and marked nuclear atypia. Microvascular proliferation and necrotic areas of variable size are essential characteristics in differential diagnosis (**Figure 1**). The tumour is highly heterogenic, which should be considered when small biopsies are examined. (Louis et al. 2007). The Ki-67 / MIB-1 values vary between different regions of glioblastoma, and mean values of 15-20 % are typical (Burger et al. 1986, Giangaspero et al. 1987, Deckert et al. 1989).

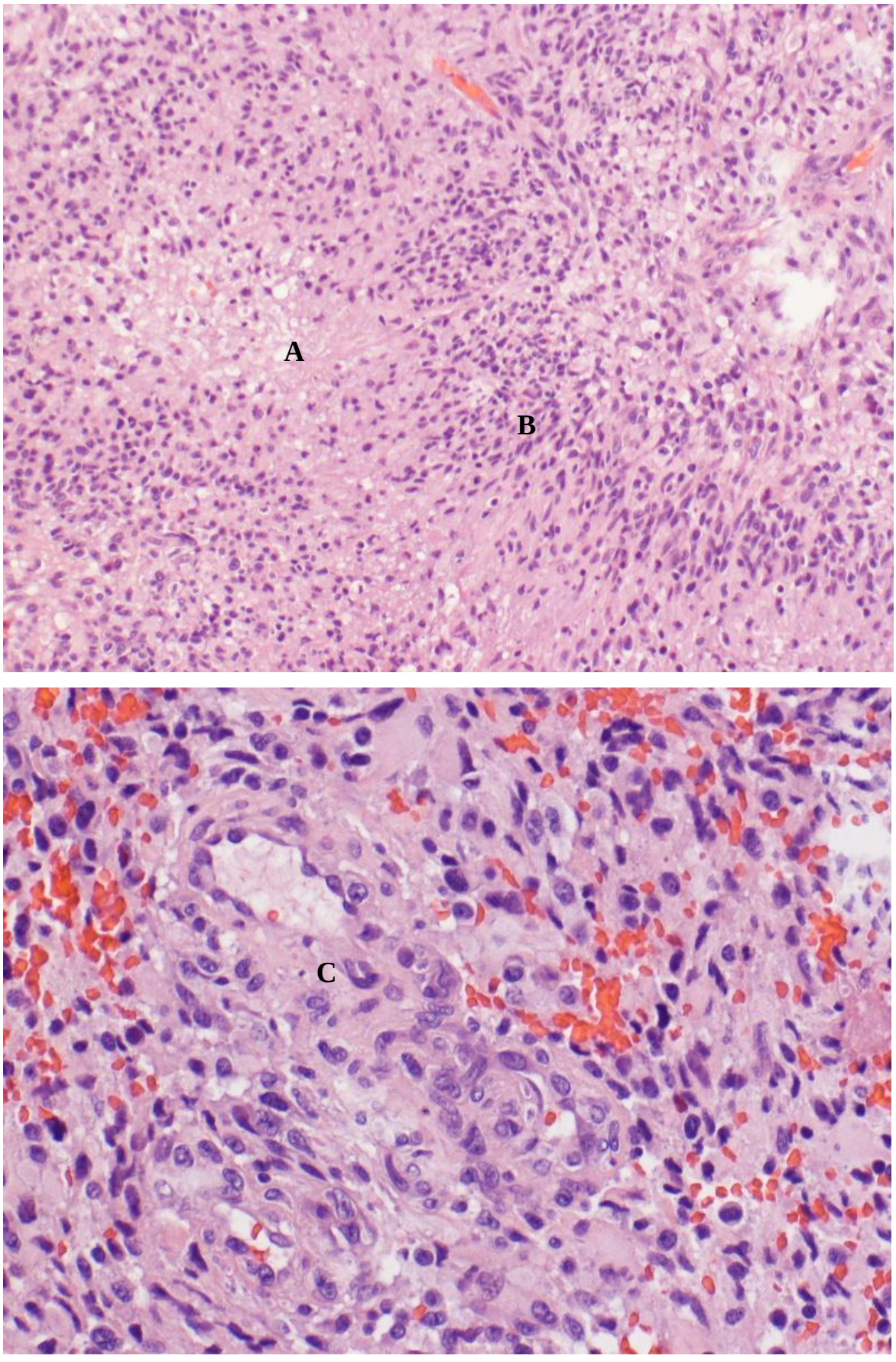

Figure 1. Essential histological features of glioblastoma. **A** Geographical necrosis surrounded by (**B)** pseudopalisading tumor cells. **C** Microvascular proliferation.

2.1.3.1. Giant cell glioblastoma

Giant cell glioblastoma, previously known as monstrocellular sarcoma (Zulch et al. 1979), is a rare histological variant of glioblastoma comprising 5% of all glioblastomas (Homma et al. 2006). They develop *de novo*, and the male:female ratio is 1:1. The histopathological features include multinucleated giant cells, small fusiform cells, and reticulin network (Margetts et al. 1989). The genetic profile includes frequent *TP53* mutations (75-90%) and *phosphatase and tensin homolog (PTEN)* mutations (33%), whereas *EGFR* amplifications (5%) and *p16* deletions (0%) are rare (Peraud et al. 1997, 1999). Giant cell glioblastoma may be predictive of a slightly better survival rate when compared to other glioblastomas (Shinojima et al. 2004)

2.1.3.2. Gliosarcoma

This glioblastoma variant constitutes approximately 1.8 – 2.8 % of all glioblastomas (Lutterbach et al. 2001). It is usually located in the cerebral hemispheres (Louis et al. 2007). Histopathologically, the tumour consists of gliomatous and sarcomatous tissue, where the glial component fulfills the cytologic criteria of glioblastoma and the mesenchymal component shows a wide variety of morphologies. Gliosarcoma affects adults in the sixth to seventh decades of life, with a male:female ratio of 1.4–1.8:1 (Lutterbach et al. 2001). Reis et al. (2000) showed that they contain *TP53* mutations (23%), *PTEN* mutations (38%), p16 deletions (38%), but low number of *MDM2* amplifications (5%) or infrequent *EGFR* amplifications (0%). When prognosis of gliosarcoma has been studied, no significant differences between gliosarcomas and ordinary glioblastomas have been reported (Meis et al. 1991).

2.1.3.3. Angiogenesis

Glioblastomas are highly vascular tumours and represent the most angiogenetic entity of all solid tumours. From a diagnostic point of view, microvascular proliferation is the essential feature of glioblastomas (Louis et al. 2007). The glioma angiogenesis is driven by a number of molecular pathways, the angiogenic process is complex and dynamic, and different mechanisms can act simultaneously. In addition to the classical concept of neoangiogenesis, involving sprouting and growth of new capillary vessels from pre-existing vessels stimulated by hypoxia-induced growth factors such as VEGF (Damert et al. 1997), glioblastoma cells adopt pre-existing vessels and migrate to them. Then, the reduced perfusion and increased metabolic activity of tumour cells causes hypoxia, and even necrosis. This in turn triggers angiogenesis by secretion and activation of various cytokines. Hypoxia is considered to be a major factor in the development of angiogenesis in

glioblastoma. Furthermore, the hypoxia inducible factor 1-alpha (HIF-1α) plays an important role, being stabilized in hypoxic conditions, and furthermore its accumulation orchestrates over 100 hypoxia regulated genes, including carbonic anhydrases (CAs) (Harris 2002). Due to this cascade, vascular permeability is increased and this, in turn, causes cerebral oedema because of an abnormal blood-brain barrier function. Another mechanism for new blood vessel growth is vasculogenesis, in which bone marrow–derived endothelial precursor cells are recruited to brain tumours in response to tumour-derived cytokines, and are incorporated into the tumour vasculature. However, the findings are still controversial and the subject of scientific debate (Machein et al. 2003, Folkins et al. 2009).

2.1.3.4. Necrosis

Another essential feature of glioblastoma is the presence of necrosis, which is commonly used as a differential diagnostic criterion when tumours are evaluated either by neuroimagining or microscopically (Burger et al. 1983). The necrotic areas can comprise up to 80% of the total tumour mass and can be seen as a non-enchancing core in MRI. Microscopically, large necrotic areas are detected. Although the conventional theory of ischaemic necrosis due to insufficient blood supply is still valid, mechanisms for the development of necrosis are still controversial and under scientific debate. The typical histological feature of glioblastoma is a pseudopalisading pattern, a configuration that surrounds the necrotic foci. (Louis et al. 2007). Brat et al. (2004) proposed that pseudopalisades represent a wave of tumour cells actively migrating away from the central hypoxia, and vaso-occlusive and prothrombotic mechanisms could explain the phenomenon.

2.1.3.5. Apoptosis

Apoptosis is defined as the process of programmed cell death which occurs in both normal physiology as well as abnormal processes, such as tumourigenesis. Tumour necrosis factor (TNF)-induced and the Fas-Fas ligand (FasL)-mediated models are theories which consider the direct initiation of apoptotic mechanisms in mammals. Tachibana et al. (1996) studied the expression of Fas by reverse transcription-polymerase chain reaction (RT-PCR) and polyclonal anti-Fas antibody: they showed that Fas is frequently expressed in malignant gliomas. 87 % of the tumours showed immunoreactivity, whereas the percentage was significantly lower in WHO grade II and III astocytomas. Furthermore, Fas expression was almost exclusively observed in glioma cells surrounding the necrotic areas and there was also an accumulation of glioma cells undergoing apoptosis, as detected by *in situ* nick-end labeling. It is also notable that high expression of Fas and

FasL correlates to WHO grade, the expression levels are higher in tumour tissue than in the normal brain, and Fas expression is more frequent in primary glioblastomas than in secondary glioblastomas (Tohma et al. 1998).

2.1.3.6. Genetics

Figure 2 shows the genetic alterations suggested to be involved in the formation of diffusely infiltrating astrocytomas. For decades, glioblastoma cells were thought to originate from de-differentiated astrocytes, which were supported by staining by different astrocyte-specific markers. The heterogeneity of glioblastoma cells prompted researchers to revisit the concept, and the recent findings, indeed, have supported the theory that the tumours originate from bipotential precursor cells or even neural stem cells (Mayer-Proschel et al. 1997). Furthermore, glioblastoma cells containing stem cell properties have been isolated and cultured. However, both histogenesis and genetic changes in tumourigenesis are complex, and further studies are warranted for a profound understanding of the detailed mechanisms. Genetic expression varies even among glioblastoma cases, and is most frequent in glioblastoma of all gliomas (Liang et al. 2005). One of these changes is *EGFR* amplification. In fact, *EGFR* is the most amplified gene in glioblastoma shown to date (Biernat et al. 2004). The encoded transmembrane protein senses extracellular ligands, such as EGF and transforming growth factor alpha (TGF-alpha), and transmits a singal for the cell to proliferate. The findings that approximately 40% of all primary glioblastomas have *EGFR* amplifications compared to 8% percent in secondary ones underlines the different genetic pathways of these tumours (Ohkagi et al. 2004). There is a clear causality between *EGFR* gene amplification and EGFR protein overexpression (Biernat et al. 2004). A similar phenomenon has been described for the *PTEN* mutation. The normal function of PTEN is to inhibit the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) signal, a signal activated by growth factors such as EGF, thus leading to inhibition of proliferation. One quarter of primary glioblastomas contain *PTEN* mutations, whereas they are nearly absent in secondary ones (Ohkagi et al. 2004). *TP53* represents another well known difference between the two glioblastoma types. *TP53* mutation is frequent in secondary glioblastomas, in approximately 65% of cases, and is present even in their precursor lower grade tumours (Louis et al. 2007). On the contrary, this event is rarer in primary glioblastomas (28%) (Ohkagi et al. 2004). The essential normal role of p53 can also be disturbed by altered expression of controlling *MDM2* or $p14^{ARF}$ genes (Kamijo et al. 1998).

Another important genetic alteration in glioblastomas is *p16*^{INK4a} mutation, which is more frequent in primary (31%) than in secondary tumours (Ohkagi et al. 2004). It participates in the signaling pathway in which retinoblastoma tumour suppressor protein (RB1) is involved. Normally, RB1 prevents the cell from replicating damaged deoxyribonucleic acid (DNA) by preventing its progression through G1 into the S phase. CDK4/cyclin D1 phosphorylates retinoblastoma proteins and inhibits their activity. Amplifications of *CDK4* genes are present in about 15% of glioblastomas (Nishikawa et al. 1995). In addition, during tumour progression, loss of p16^{INK4A} protein may be necessary for cells with wild-type RB to bypass this G1 arrest checkpoint, because it normally inhibits the CDK4/cyclin D1 complex. Finally, loss of chromosome 10 (LOH 10) is one of the typical alterations in gliomas. Loss of either the complete chromosome or some parts of it can occur. LOH 10q is common in both primary and secondary glioblastomas (70% and 63%, respectively), whereas LOH 10p is most often detected in primary glioblastoma (Ohkagi et al. 2004).

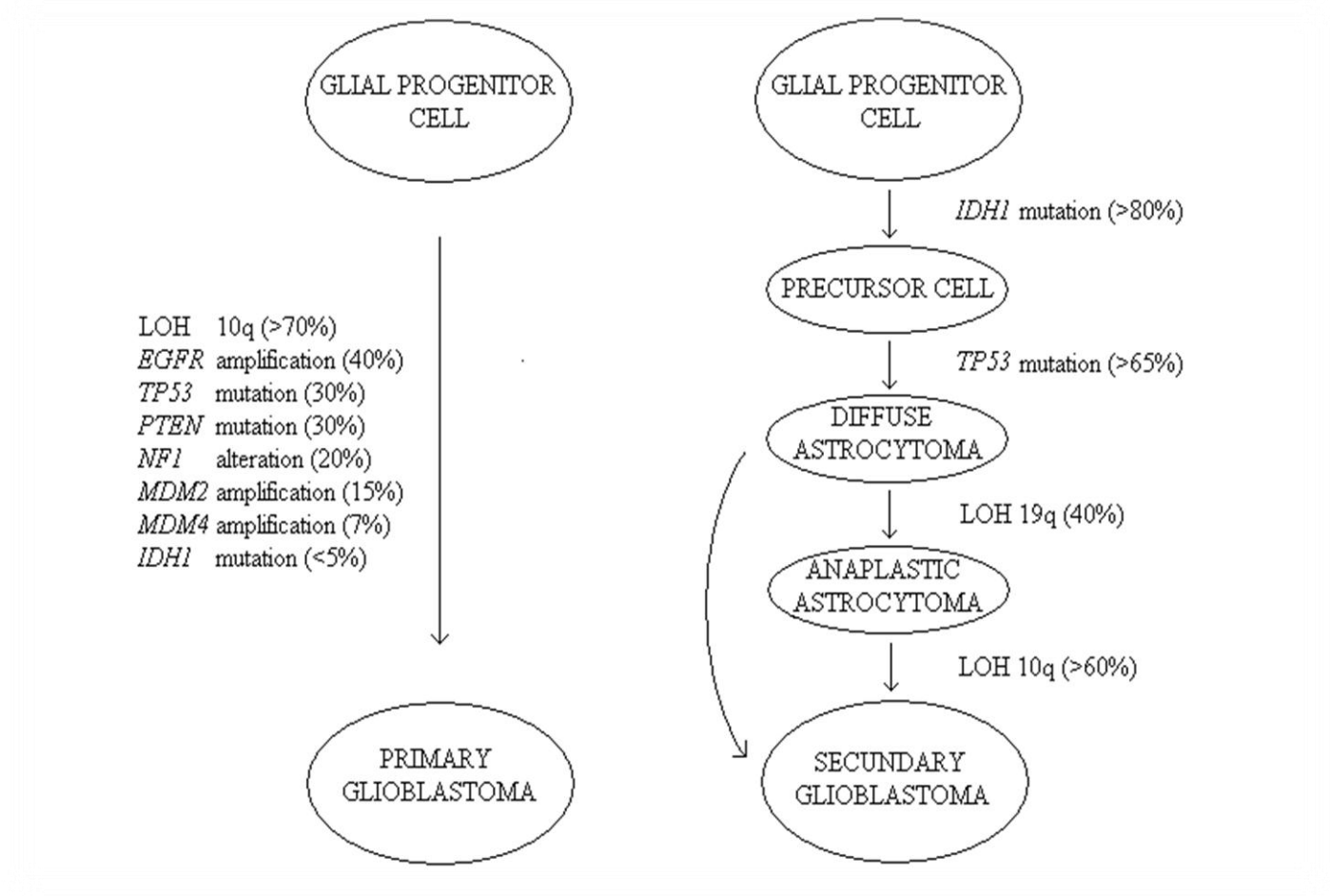

Figure 2. The genetic alterations suggested to be involved in formation of diffusely infiltrating astrocytomas. Adapted from Ohgaki and Kleihues (2009).

2.1.4. Gliomatosis cerebri

Gliomatosis cerebri is a multilobar and extensive neoplasm that corresponds typically to WHO grade III and in some cases to WHO grade IV astrocytoma (Louis et al. 2007). The term was introduced by Nevin (1938) and used today to describe this rare, diffusely infiltrating glial neoplasm with uncertain histogenesis. Two different hypotheses have been proposed. According to the first, it is a subtype of typical diffusely infiltrating glioma with the exception of vast infiltrative capacity, and according to the second, it develops by the simultaneous neoplastic transformation of the entire tissue field. Kros et al. (2002) studied a unique autopsy case and took random samples from 24 locations in the brain. They showed a wide distribution of a particular set of genetic aberrations, supporting the concept of monoclonal tumour proliferation, and highlighted the genetic aberration of exon 7 of *TP53*. This tumour features a highly variable presentation, poorly defined clinical course, and typically fatal outcome (Louis et al. 2007).

2.1.5. Treatment of astrocytic gliomas

The survival of glioblastoma patients has improved slowly but prognosis remains poor. Until recently, surgical resection followed by radiotherapy was the most optimal treatment for newly diagnosed glioblastoma. Significant survival advantage was reported when six randomised trials of radiotherapy versus no radiotherapy were pooled (Walker et al. 1980, Laperriere et al. 2002). Furthermore, an improvement of 6% in one year survival rate was reached when nitrosourea-based chemotherapy was added (a meta-analysis of 12 randomised trials of adjuvant chemotherapy) (Stewart et al. 2002). The final one year survival rate became 35%.

Today, standard therapy for newly diagnosed glioblastoma includes three different methods: surgery, radiation therapy, and chemotherapy. In a clinical setting, the most important factor to direct the treatment strategy is the correct neuropathological diagnosis. The tumour tissue needed for this evaluation is obtained either by stereotactic biopsy or open resection, but in an ideal situation, a neurosurgeon attemps to perform a gross total resection of the tumour. GTR (with resection of 98% or more of tumour volume) has been associated with prolonged survival of patients with grade IV astrocytomas (approximately 4 month survival difference) (Lacroix et al. 2001). The obstacles to gross total resection are, especially when higher grade astrocytomas are considered, the diffuse infiltration of cancer cells to adjacent tissue and the localisation of tumour in deep or eloquent brain regions, where the resection would cause severe iatrogenic neurological damage. In addition to providing tissue for neuropathological diagnosis, the goal for newly

diagnosed glioblastomas is tumour cytoreduction. This aims to reduce the symptoms and neurological deficits. For recurrent disease, the goal is usually tumour debulking alone.

Postoperative radiation therapy is normally focused on the actual tumour region plus small margins (2-3 cm). During the following six weeks, radiotherapy is typically given in fractions of 2 Gy (Stupp et al. 2005). The goal of postoperative radiotherapy and chemotherapy is to treat residual disease that is present following surgery. Stupp et al. (2005) showed that the addition of temozolomide to radiotherapy for newly diagnosed glioblastoma resulted in a clinically meaningful and statistically significant survival benefit, while only minimal additional toxicity was observed. The addition of temozolomide prolonged median overall survival by 2.5 months, and the two year survival rate for patients receiving temozolomide and radiation therapy was 26% compared with 10% for those receiving only radiotherapy. Furthermore, a recent randomized phase III trial showed a clear survival benefit for patients treated with temozolomide and radiotherapy when compared to patients with radiation therapy only (Stupp et al. 2009). Thus, concurrent temozolomide with radiotherapy followed by adjuvant temozolomide is used as a standard treatment for newly diagnosed glioblastomas.

For WHO grade II astrocytomas, the gold standard treatment is attemped GTR, and in some cases, radiation therapy is considered. The treatment strategies for patients with newly diagnosed anaplastic astocytoma (WHO grade III) are similar to glioblastoma, including both surgery and radiation therapy. A neurosurgeon performs maximal feasible resection, which is followed by radiotherapy (fractions of 2 Gy each, 30 times) (Stupp et al. 2005). The use of chemotherapy varies and many neuro-oncologists use temozolomide for newly diagnosed anaplastic astrocytomas, though its use is not comprehensively approved and further investigations are warranted.

Glioblastoma recurrence is a common phenomenon. For the treatment of recurrence it is possible to perform a second craniotomy, but the clinical state of the patient has to be reasonably well and the operation has to be justified, e.g. aiming to lowering the intracranial pressure or otherwise improving the neurological state. Often neuro-oncologists use some of the second-line chemotherapeutics, e.g. nitrosurea, etoposide or platinum compounds (Franceschi et al. 2004, Rao et al. 2005, Chamberlain et al. 2006), and radiation therapy is seldom used because of the side-effects, such as necrosis and leukoencephalopathy (Bauman et al. 1996). The use of temozolomide in the management of glioblastoma at the time of recurrence is indicated in the European Union and Canada, although clinical trials failed to show significant improvement in survival outcome (Yung

et al. 1999, Wong et al. 1999, Brada et al. 2001). When the use of temozolomide was assessed in recurrent anaplastic astrocytomas, a phase II trial showed a response rate of 35% and a six month progression-free survival of 46% (Wong et al. 1999), and thus its use was approved by the FDA and the EU.

2.2. Carbonic anhydrases

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes present in prokaryotes and eukaryotes (Sly and Hu 1995). The main function of CAs is acting as a catalyst in the reaction:

$$CO_2 + H_2O <=> HCO_3^- + H^+$$

Their fuction is, in other words, to catalyze the conversion of CO_2 to bicarbonate ion and proton. There are several unrelated CA gene families which contain a number of different isozymes. First, there are the α-CAs, which are present in vertebrates, bacteria, algae and the cytoplasm of green plants. Second, the β-CAs, which are present in bacteria, algae, and the chloroplasts of monocotyledons and dicotyledons, as well as in some invertebrate animal species (Syrjänen et al. 2010). Third, the γ-CAs, which can be found predominantly in archaea and bacteria. Fourth, the δ-CAs, which have been reported in marine diatoms. The family of mammalian α-CAs consists of thirteen active isozymes. These include five cytoplasmic (CA I, CA II, CA III, CA VII, and CA XIII), five membrane-associated (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial (CA VA and CA VB) and one secreted (CA VI) form (Sly and Hu 1995, Parkkila and Parkkila 1996, Lehtonen et al. 2004, Hilvo et al. 2005).

The role of CAs in the regulation of pH homeostasis has been known for decades (Henriques 1928, Meldrum and Roughton 1932, 1933). The functions of CAs are essential in many physiological processes, e.g., in gluconeogenesis, lipogenesis, ureagenesis, bone resorption, and formation of gastric juice and cerebrospinal fluid (Sly and Hu 1995, Pastoreková et al. 2004). Their presence and role in cancer cells has become an important research topic over the last 15 years. **Figure 3** shows the pH regulation in a cancer cell under hypoxia. The next chapters describe in more detail the isozymes which have been demonstrated in tumours and are currently considered potential diagnostic biomarkers and therapeutic targets of cancer.

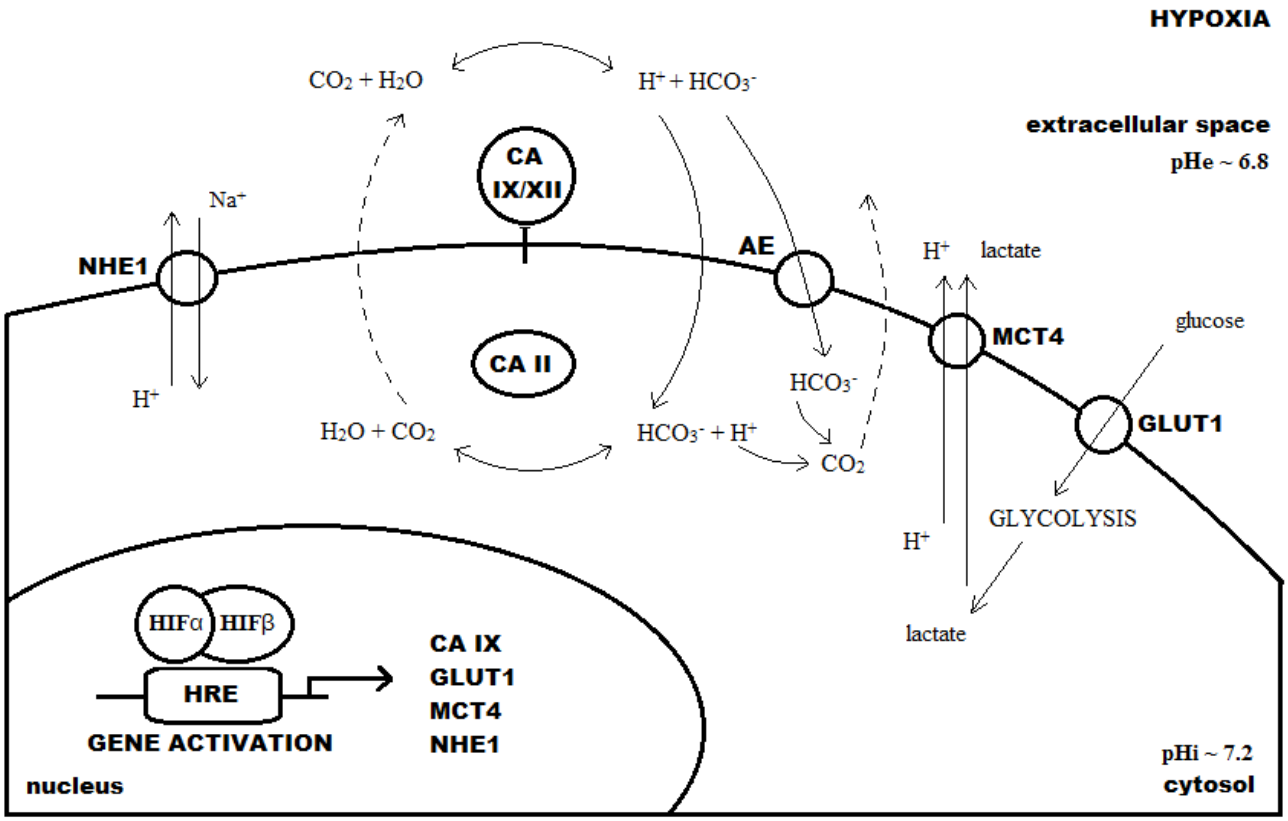

Figure 3. pH regulation in a cancer cell under hypoxia, controlled by HIF-1 mediated gene activation. The rapid metabolic rate requires glucose which is transported to the cell by the glucose transporter (GLUT1). Glycolysis produces lactate and protons, which are transported to extracellular space by the H^+/monocarboxylate transporter 4 (MCT4). The transmebrane CA IX and XII, and cytosolic CA II prevent intracellular acidification and are essential for pH maintenance. Anion exchangers (AE) transport bicarbonate to cytosol, which then buffers the protons produced by the active metabolism. Resulting CO_2 is secreted from the cell by diffusion. The Na^+/H^+ exchanger 1 (NHE1) participates in the secretion of proton. The HIF-mediated machinery and oncogenic pathways result in secretion of protons and CO_2 to extracellular space, thus promoting the breakdown of the extracellular matrix and invasion of tumour cells. Adapted from Pastoreková et al. (2008).

2.2.1. Carbonic anhydrase II

CA II is one of the most efficient enzymes known and and its enzymatic activity is comparable to CA IX. Being the most widely distributed member of the CA gene family, this cytosolic enzyme is expressed in almost every human tissue and organ (Kivelä et al. 2005). Historically, the carbonic anhydrase was found in erythrocytes where it is involved in hydration of CO_2 (Meldrum and Roughton 1932, 1933). Its functioning in gastrointestinal mucosa was first studied in the 1930´s (Davenport and Fisher 1938).

2.2.1.1. Carbonic anhydrase II in normal tissue

In humans, CA II is widely expressed in the alimentary tract, mainly in the epithelia of various organs, such as the oesophagus, small intestine, colon, and stomach (Lönnerholm et al. 1985, Parkkila et al. 1994, Parkkila and Parkkila 1996). Its expression has been studied, e.g. in the ephitelial cells of salivary glands, where CA II participates in the production of bicarbonate in saliva and thus regulates the salivary buffer capacity. CA II is highly expressed in the bile ducts and gallbladder where it conceivably participates in formation and concentration of bile (Parkkila et al. 1994). In the human pancreas, CA II has been detected in the ductal cells where it facilitates bicarbonate secretion to pancreatic juice (Kumpulainen et al. 1981, Parkkila et al. 1994). In the stomach, CA II is located in the surface epithelial cells, where it is involved in production of bicarbonate, and in the parietal cells, where it participates in production of gastric acid (Parkkila et al. 1994). Carbonic anhydrase II is present in the zona glomerulosa cells of the human adrenal gland (Parkkila et al. 1993). In the human male reproductive tract, expression of CA II has been located in the epithelia of the seminal vesicle, ampulla of the ductus deferens and distal ductus deferens (Kaunisto et al. 1990). Some epithelial cells of the corpus and cauda epididymidis were also stained for CA II.

2.2.1.2. Carbonic anhydrase II in chronic disease

CA II plays a pivotal role in several physiological processes. Its primary deficiency causes a rare autosomal recessive disorder, resulting in renal tubular acidosis, osteopetrosis, and cerebral calcification in humans (Sly et al. 1983, 1985) and growth retardation and renal tubular acidosis in mice (Lewis et al. 1988). The kidney represents one of the major locations for CA II expression. The enzyme is present in the renal tubular cells and collecting ducts (Wistrand 1980, Wåhlstrand and Wistrand 1980), and its function is essential to urinary acidification. The important role of CA

II has also been documented by correction of renal tubular acidosis with gene therapy in CA II-deficient mice (Lai et al. 1998).

CA II plays a role in different pathological processes in humans. Patients with autoimmune disorders, such as idiopathic chronic pancreatitis and Sjögren´s syndrome, have autoantibodies against carbonic anhydrase II (Kino-Ohsaki et al. 1996, Ono et al. 1999). Palminiello et al. (2008) reported increased levels of CA II in the developing brain of Ts65Dn mice, a mouse model for Down syndrome. They proposed that the increased CA II activity might be a compensatory mechanism mobilized in response to structural/functional abnormalities. On the other hand, the authors speculated that up-regulation of CA II may also increase susceptibility to seizures in Down syndrome. Elevated plasma CA II protein levels have been reported in patients with Alzheimer's disease, suggesting that CA II level may play a role in the pathogenesis (Jang et al. 2010). CA II is expressed and induced in the epidermis with various forms of eczema, including atopic dermatitis, allergic contact dermatitis and irritant contact dermatitis (Kamsteeg et al. 2009). It was proposed that CA II upregulation could be a response to restore fluid balance following impaired barrier function. These observations could be used as molecular diagnostic criteria for inflammatory skin conditions.

2.2.1.3. Carbonic anhydrase II in neoplastic tissue

The expression of CA II has been reported in several human brain tumours (Parkkila AK et al. 1995). Frazier et al. (1990) reported that CA II mRNA increases after the treatment of retinoic acid (differentiating agent) in the pancreatic adenocarcinoma cell lines. Later, the transcriptional regulation of CA II by retinoic acid was further assessed in the human pancreatic tumour cell line (Rosewicz et al. 1995). Furthermore, Parkkila S et al. (1995) detected CA II in neoplastic ductal epithelium of pancreatic tumours. CA expression has been shown to be aberrantly expressed in human erythroleukemia cells (Frankel et al. 1985) The finding of CA expression in human erythroleukemia cells was later verified by Leppilampi et al. (2002), who showed that CA II expression in hematological malignancies may result from a genetic aberration that occurs in both myeloid and lymphatic lineages or in their progenitor cell. Yoshiura et al. (2005) conducted dendritic cell therapy on malignant melanoma patients and reported shrinkage or disappearance of metastatic tumours in some patients. Importantly, they identified the CA II as a potential target antigen; half of the patients exhibited anti-CA II autoantibodies either before or after therapy and 30% of the patients had posttherapy antibody levels that were higher than pretherapy leves. By

immunohistochemistry, they showed that CA II was expressed in the endothelium of neovessels in melanoma and oesophageal, renal, and lung cancer, whereas the normal renal vessels did not express CA II. By cell culture conditions reminiscent of a cancer cell microenvironment, CA II expression was induced in the endothelial cells *in vitro*. In conclusion, the authors suggested that CA II is a tumour vessel endothelium–associated antigen, a target antigen to autoantibody response by dendritic cell therapy, and could be associated with a more favourable clinical outcome.

2.2.2. Carbonic anydrase IX

CA IX, formally known as MN, was found by Pastoreková et al. (1992) in a human carcinoma cell line. The importance of MN was discovered when it was associated to oncogenic processes, being found in human carcinomas of ovary, endometrium and uterine cervix, but not in normal tissues of corresponding organs (Závada et al. 1993). Previously, Oosterwijk et al. (1986) had described a monoclonal antibody, G250, which located G250 protein expression to the cell membranes of renal cell carcinoma cells, but not to the normal tubular epithelium. G250 was studied intensively as a tool for cancer diagnosis and treatment, and Grabmaier et al. (2000) found this antigen to be CA IX, the same protein that was detected by Pastoreková et al. (1992), and the corresponding gene was cloned by Pastorek et al. (1994). The cloning of *CA9* gene showed that it encodes a 466 amino acid-long protein composed of a proteoglycan (PG)-like domain, a central catalytic CA domain, a transmembrane anchor, and a short COOH-terminal cytoplasmic tail. Opavský et al. (1996) located the gene to chromosome 17, reported that the gene consists of eleven exons and ten introns, and also showed that the N-terminal side of CA IX contains a PG-like region, which is unique to CA IX among all other CA isoenzymes. Being the ninth mammalian CA isozyme, it was named CA IX (Hewett-Emmett et al. 1996). Furthermore, a detailed characterisation of human CA IX protein has been reported and it has been shown that the recombinant CA IX protein exhibits the highest catalytic activity ever measured for any CA isozyme (Hilvo et al. 2008). Even though Opavský et al. (1996) originally mapped *CA9* gene to 17q21.2 by fluorescence in situ hybridization, it was later shown by radiation hybrid mapping that it is localized in the chromosome 9p13-p12 (http://www.ncbi.nlm.nih.gov/gene/768).

2.2.2.1. Carbonic anhydrase IX in normal tissue

Hilvo et al. (2004) studied the CA IX expression in mouse tissue: the highest immunoreactivity was described in gastric mucosa, moderate signals were seen in the colon and brain, and low expression was detectable in the pancreas and various segments of the small intestine. CAIX -deficient mice

have been shown to have vacuolar degenerative changes and they exhibited abnormal locomotor activity and poor performance in a memory test (Pan et al. 2011). A similar expression pattern has been detected in human tissues, where high expression of CA IX has been discovered in the gastrointestinal tract, especially in the epithelia of the gastric and gallbladder mucosa (Pastorek et al. 1994; Pastoreková et al. 1997; Saarnio et al. 1998a). CA IX was detected along the cranial-caudal axis of the human gut, having the most intensive signals in the intestinal epithelium of the duodenum and jejunum and diminishing towards the large intestine (Saarnio et al. 1998b). Moreover, its expression has been shown in the male reproductive organs, whereas normal organs of the female reproductive tract contain no or low amounts of CA IX (Liao et al. 1994; Karhumaa et al. 2001). In addition, CA IX expression has been detected in the mesothelium, epithelial cells of the esophagus, and pancreatic and biliary ducts (Turner et al. 1997, Pastoreková et al. 1997, Kivelä et al. 2000, Ivanov et al. 2001).

2.2.2.2. Carbonic anhydrase IX in neoplastic tissue

Expression of CA IX has been studied in various tumour types, and it is usually highly expressed in human malignancies originating from CA IX –negative tissues (Závada et al. 1993, Liao et al. 1997, Ivanov et al. 2001). Because most of the normal tissues in the human body show only weak or no expression of CA IX, CA IX has been considered a promising target molecule in both cancer diagnostics and therapy. Liao et al. (1994) first demonstrated its potential in the diagnosis of cervical carcinomas, because normal cervical tissue did not express the antigen. Similarly, CA IX was found to be a potential biomarker for renal cell carcinoma (McKiernan et al. 1997), validating the finding of Oosterwijk E et al. (1986), even though G 250 and CA IX were not yet identified as the same molecule. Then, CA IX was found in oesophageal squamous cell carcinomas and was thought to play a role in the proliferation and regeneration of oesophageal squamous epithelium, where loss of its expression was associated with cancer progression in Barrett's-associated adenocarcinomas (Turner et al. 1997). In head and neck squamous cell carcinoma specimens, CA IX was related to the location of tumour microvessels, angiogenesis, necrosis, and tumour stage, and was considered to represent a potential target for future therapy (Beasley et al. 2001). Many colorectal tumours also overexpress CA IX (Saarnio et al. 1998a). It seems that CA IX expression is quite heterogenous across different categories of colorectal cancer. Recent results have shown that CA IX expression is most prominent in hereditary non-polyposis colorectal cancer (HNPCC) (Niemelä et al. 2007). Its high expression in premalignant lesions has further suggested that it might be a useful marker in early diagnosis of colorectal tumours (Saarnio et al. 1998a). A recent study by

Korkeila et al. (2009) showed that CA IX expression was a predictor of poor disease-free survival and disease-specific survival in rectal cancer in both univariate and multivariate analysis. On the other hand, the low expression of CA IX in gastric carcinomas is notable and strongly contrasts with the very high expression in normal gastric mucosa (Leppilampi et al. 2003, Chen et al. 2005). Interestingly, a subgroup of gastric cancers retains CA IX expression in malignant cells at the invasion front (Chen et al. 2005), suggesting that increased CA IX expression may contribute to a more advanced disease and tumour progression in a subset of gastric cancers. In general, the expression of CA IX has frequently been observed in tumours derived from different segments of the gastrointestinal tract, beginning from oral and ending with colorectal cancers (Pastoreková et al. 2006). Expression of CA IX has been reported in both biliary and pancreatic tumours. Saarnio et al. (2001) showed that immunostaining for CA IX was mainly located on the basolateral surface of the epithelial cells in biliary epithelial tumours, similar to normal biliary mucosa. CA IX could be used as a marker for biliary differentiation in hepatobiliary neoplasms: it is present in neoplastic hepatobiliary cells and absent in hepatocellular carcinomas. In pancreatic tumours, the hyperplastic ductal epithelium generally shows an increased staining for CA IX and may contribute to tumourigenesis by an unknown mechanism (Kivelä et al. 2000). The expression of CA IX in a relatively low number of malignant tumour specimens suggests that it may have a limited value in diagnostic evaluation of pancreatic carcinoma.

CA IX may also serve as a valuable marker to predict the prognosis of certain cancers. Its expression in lung tumours, for example, is a useful marker for differentiation between preneoplastic lesions and non-small cell lung cancer (Vermylen et al. 1998), and has predicted poor survival in this tumour type (Swinson et al. 2003, Kim et al. 2004). The presence of CA IX has specifically been linked to the expression of proteins that are involved in angiogenesis, inhibition of apoptosis, and disruption of cell-cell adhesion, thus explaining the strong association of this enzyme with poor clinical outcome in lung cancer (Giatromanolaki et al. 2001). In addition to the prognostic capability of CA IX in tumour tissue, a high concentration of CA IX in plasma seems to be an independent prognostic biomarker in patients with non-small cell lung cancer (Ilie et al. 2010). High CA IX expression has been associated with poor prognosis for patients with soft tissue sarcoma (Måseide et al. 2004), esophageal cancer (Birner et al. 2011), ovarian cancer (Hynninen et al. 2006, Choschzick et al. 2011), and cervical cancer. In cervical cancer, the CA IX expression also correlates to tumour hypoxia, and therefore could be used as a tool for the selection of suitable patients for hypoxia-modification therapies (Loncaster et al. 2001). In breast cancer, expression of CA IX is associated with malignant tissues and is related to overexpression of c-erbB2 (Bartosová

et al. 2002). Furthermore, it has been confirmed in several studies that CA IX correlates with poor prognosis in breast cancer (Chia et al. 2001, Brennan et al. 2006, Hussain et al. 2007), even though Span et al. (2007) recently pointed out that CA IX is more predictive than prognostic in this cancer type. CA IX has perhaps been studied most thoroughly in renal cell carcinoma (RCC), and may represent a useful marker for the most common RCC subtype, clear cell carcinoma (Liao et al. 1997, McKiernan et al. 1997, Parkkila et al. 2000a). In addition, it is considered to be a promising therapeutic target for novel oncological applications, including immunotherapy and radioisotopic methods (Pastoreková et al. 2006, Bleumer et al. 2006). Bui et al. (2003) showed that decreased CA IX levels were independently associated with poor survival in advanced RCC. Sandlund et al. (2007) found that the expression is higher in conventional (clear cell) cancer than in other renal cell cancer types, and patients with both conventional renal cell cancer and low CA IX expression had a less favourable prognosis.

The first major pathway discovered in CA IX control was the inactivating mutation of the *von Hippel–Lindau (VHL)* tumour suppressor gene (Wykoff et al. 2000). In normal tissues and normoxia, the encoded VHL protein (pVHL) binds to hydroxylated hypoxia inducible factor 1 – alpha (hydroxylation is by prolyl-4-hydroxylases (PHDs)) and causes degradation by the ubiquitin-proteasome system, thus inactivating the downstream target genes. On the contrary, under hypoxia pVHL does not recognize HIF1-alpha, which is not hydroxylated in the absence of active PHDs, and this causes the stabilization and accumulation of HIF1-alpha in cytoplasm. **Figure 4** shows the functioning of HIF in hypoxia and normoxia.

The finding that loss of functional VHL protein causes stabilization of HIF-1, leading to concomitant up-regulation of CAs with loss of regulation by hypoxia, explained the overexpression of CA IX in the majority of RCCs (Gnarra et al 1994, Wykoff et al. 2000). On the other hand, in tumours that do not contain *VHL* mutations, CA IX is expressed in focal perinecrotic areas and is induced by hypoxia. Indeed, many solid tumours contain hypoxic regions caused by rapid growth pattern and irregular and functionally defective tumour vasculature. Again, HIF plays a central role by activating genes that change the expression profile of tumour cells suffering from hypoxia; thus, either leading to adaptation to the hypoxic stress or resulting in cell death. Furthermore, the surviving tumour cell population is associated with worse prognosis and resistance to anti-cancer treatment due to increasingly aggressive behaviour involving invasion and metastases (Harris 2002). This mechanism is supported by various immunohistochemical studies in which the CA IX expression is located in in the perinecrotic regions of solid tumours (Wykoff et al. 2000).

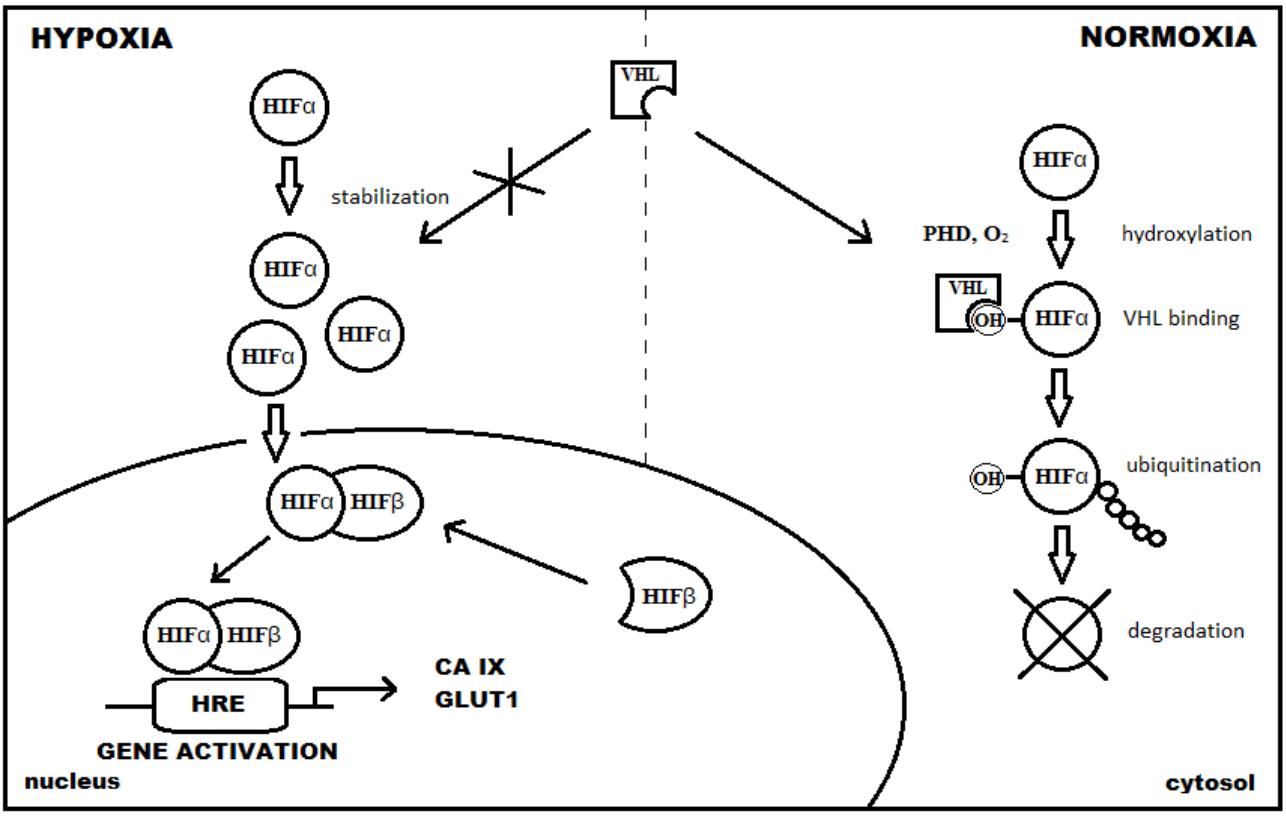

Figure 4. Activation of hypoxia-inducible genes. Under normoxia, HIFα is degraded by ubiquitin-proteasome system as follows: prolyl-4-hydroxylases (PHD) hydroxylate two conserved proline residues of HIFα, then von Hippel-Lindau protein (VHL) binds to the hydroxylated HIFα. Under hypoxia, PHDs are inactive in the absence of dioxygen, and therefore, HIFα is not recognized by VHL protein. HIFα accumulates and is translocated to the nucleus. HIFβ constitutive subunit dimerizes with HIFα, resulting in the active transcription factor, which binds to hypoxia response element (HRE). Then the transcription of target genes, such as *CA9* and *GLUT1*, is induced. Adapted from Pastoreková et al. (2008).

Many published reports have assessed the role of CA IX in neoplastic tissue. Svastová et al. (2004) showed that CA IX increases extracellular acidification by shifting the site of CO_2 hydration from intra- to extracellular, and this process can be disturbed by inhibiting CA IX with selective sulfonamide inhibitors. It was suggested that this could lead to increased capability of tumour cells to survive and invade. Furthermore, CA IX has been proposed to diminish the intracellular pH gradient in the hypoxic core of three-dimensional tumour spheroids grown from cancer-derived cell lines (Swietach et al. 2008). Interestingly, extracellular pH is substantially and consistently acidic in malignant tumours, while intracellular pH is essentially identical in the tumour compared to normal tissue (Swietach et al. 2008).

In cervical cancer, the hypoxia measured by needle electrodes correlated to high CA IX expression (Loncaster et al. 2001). It has been also shown that *CA9* gene expression is hypoxia-regulated in glioblastoma cells *in vitro*, thus promoting tumour cell survival (Said et al. 2008). Futhermore, *CA9* has been reported to be the most consistently induced gene (among the 32 identified hypoxia responsive genes, including *VEGF*) in human solid tumours adapting to the hypoxia (Lal et al. 2002). In addition, Innocenti et al. (2009) reported that the full length CA IX has an optimal catalytic activity for CO_2 hydration to bicarbonate and proton in acidic pH, having its maximal catalytic activity at pH values around 6.5. These findings suggest that CA IX is an essential factor for tumour cells in adaptation to hypoxia and survival. Even though the expression of CA IX is clearly regulated by hypoxia, Ihnatko et al. (2006) have shown that acidosis increases CA IX expression via a hypoxia-independent mechanism, suggesting regulation through modulation of the basic *CA9* transcriptional machinery. Similarly, CA IX has been proposed to be regulated by low oxygen concentrations or constitutive, oncogene-related mechanisms (Said et al. 2007a).

Svastová et al. (2003) proposed a novel potential mechanism for CA IX in tumour progression. They suggested that CA IX is involved in cancer cell migration, which was supported by the finding that CA IX modulates E-cadherin mediated cell adhesion by decreasing the binding of this cell adhesion molecule to beta-catenin. The disruption of the link between these adhesion molecules would possibly promote cell motility and invasion. Previously, when acetazolamide, a potent CA inhibitor, was studied, it was found to suppress the invasion of renal cancer cells *in vitro* (Parkkila et al. 2000b). However, the inhibition of CA IX in RNAi-treated breast cancer cells did not reduce the invasion capacity of the cells significantly, even though it appeared to be slightly reduced compared with the control (Robertson et al. 2004). The role of CA IX in resistance to chemotherapy has also been investigated in breast cancer patients: CA IX expression showed a negative predictive

role for treatment efficacy in oestrogen receptor-positive tumours which were treated with adjuvant tamoxifen after primary chemo-endocrine therapy (Generali et al. 2006). The authors suggested that effects of pH on tamoxifen uptake might explain this.

2.2.3. Carbonic anhydrase XII

Carbonic anhydrase XII (CA XII) was initially cloned and characterizated in a human RCC by Türeci et al. (1998). They showed that the *CA12* gene was located in chromozome 15q22, which was later confirmed by Ivanov et al. (1998). The corresponding CA XII protein consists of a 29-amino acid signal sequence, a 261-amino acid CA domain, an additional short extracellular segment, a 26-amino acid hydrophobic transmembrane domain, and a hydrophilic C-terminal cytoplasmic tail of 29 amino acids. Furthermore, the three-dimensional crystal structure of human CA XII has been reported (Whittington et al. 2001). Schematic structure of the domain composition of carbonic anhydrases IX and XII is shown in **Figure 5**.

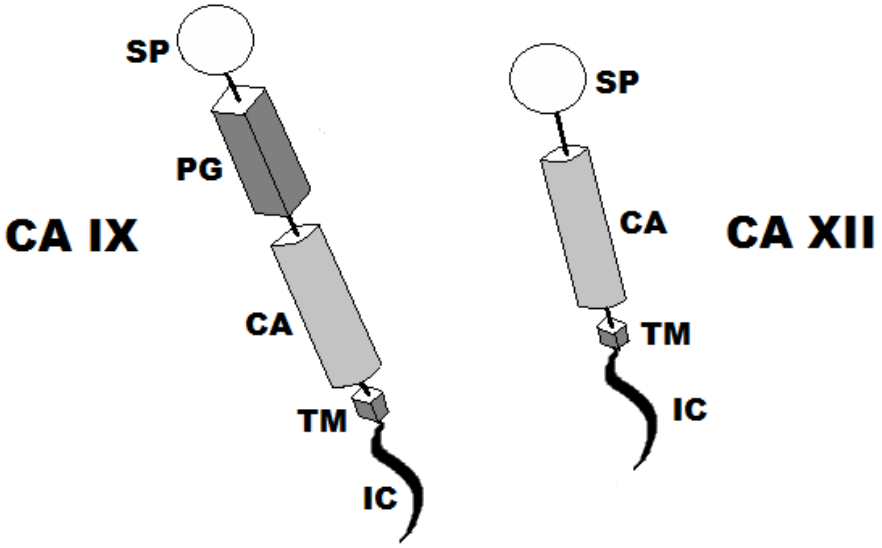

Figure 5. Schematic structure of the domain composition of carbonic anhydrases IX and XII. Proteoglycan-related region (PG) is absent in CA XII, whereas other compartments are similar (SP: signal peptides, CA: carbonic anhydrase domains, TM: transmembrane regions, IC: intracellular tails). Adapted from Ivanov et al. (1998).

2.2.3.1. Carbonic anhydrase XII in normal tissue

By the means of RT-PCR using CA XII-specific primers, CA XII mRNA was detected in many normal tissues (the lung, liver, colon, stomach, skeletal muscle, skin, kidney, spleen, tonsil, lymph node, peripheral blood lymphocytes, activated peripheral blood lymphocytes, bladder, breast, uterus, ovary, brain, prostate, and skin) (Türeci et al. 1998). In contrast, Northern blots showed a more limited distribution; the transcript was seen only in the kidney, colon and peripheral blood lymphocytes (Türeci et al. 1998). Moreover, Ivanov et al. (1998) studied the expression pattern of the *CA12* gene by Northern blot hybridization and found high expression in the colon, kidney, and prostate, moderate in the pancreas, ovary, and testis and very low expression in the lung and brain. In a more recent study, high *CA12* gene expression was also detected in the rectum, oesophagus, brain, and pancreas, whereas the mammary gland, bladder, uterus, trachea, and aorta showed low signal (Ivanov et al. 2001). By immunohistochemistry, high signals were detected in the distal convoluted tubules and the collecting duct of the kidney, the epithelium of the breast, sweat glands of the skin, seminal vesicles, and the proliferative endometrial glands, whereas low signals of CA XII expression were found focally in the salivary glands, upper respiratory system, and acinar cells of the pancreas (Ivanov et al. 2001).

When normal renal tissue was studied by immunohistochemistry, CA XII had distinct staining in the basolateral plasma membrane of the epithelial cells in the thick ascending limb of Henle and distal convoluted tubules, and in the principal cells of the collecting ducts, suggesting an important role in normal renal physiology, e.g. water homeostasis (Parkkila et al. 2000a). In addition, the expression of CA XII has been studied in the human alimentary tract, where it has been located to the epithelial cells of the salivary glands, stomach, colon, and pancreatic acini (Ivanov et al. 2001). CA XII is also expressed in the basolateral plasma membrane of the epithelial cells in the efferent ducts, as well as in the epididymal duct, suggesting a role in ion transport and the concentration processes of testicular fluid (Karhumaa et al. 2001). As for female human genitals, CA XII is expressed in the basolateral plasma membrane of the epithelial cells of the endometrium, supporting its role in pH-dependent events of fertilization (Karhumaa et al. 2000).

2.2.3.2. Carbonic anhydrase XII in neoplastic tissue

The original report of Türeci et al. (1998) showed that the CA XII transcript was expressed at higher levels in the RCC than in surrounding normal kidney tissue in approximately 10% of patients with RCC. When the location of CA XII protein in renal tumours was examined by

immunohistochemistry, moderate or strong CA XII signals were detected in plasma membranes in most oncocytomas and clear-cell carcinomas (Parkkila et al. 2000a). Studies of human tumour cell lines have shown that CA XII is strongly expressed in adenocarcinoma of the lung, non-small cell lung carcinoma, and colorectal carcinoma, whereas lymphoma cells showed low expression (Ivanov et al. 1998). Ivanov et al. (2001) further studied the expresson of CA XII by immunohistochemistry, and concluded that a wide variety of tumours showed immunoreactivity. They showed strong signals in endometrial carcinoma, breast ductal and lobular carcinoma, kidney carcinoma, colon carcinoma and adenoma, head and neck squamous cell carcinoma, and mesothelioma. Wide expression of CA XII has been reported in ovarian tumours (Hynninen et al. 2006).

Although regulation of CA XII by hypoxia has been demonstrated, the stimulation of expression is not comparable to CA IX (Ivanov et al 1998). *CA12* gene expression has been shown to be regulated by the von Hippel–Lindau (VHL) tumour suppressor protein (Ivanov et al. 1998). Consequently, CA XII is up-regulated in RCC, because many of these tumours have a defective VHL tumour suppressor function due to *VHL* mutations (Latif et al. 1993). Kinetic analysis has shown that CA XII can contribute to cellular CO_2 and HCO_3^- homeostasis, and it may be involved in the acidification of cancer cell microenvironment (Ulmasov et al. 2000). This in turn might increase the invasiveness of cancer cells and tumour growth. This theory has been supported by the finding that the effect of acetazolamide, a CA inhibitor, suppresses invasion of renal cancer cells *in vitro* particularly in cell lines expressing high levels of CA II and CA XII (Parkkila et al. 2000b). Furthermore, CA XII has been shown to promote tumour cell growth by counteracting acidosis through the regulation of the intracellular pH, and *CA9* silencing *in vivo* leads to a 40% reduction in xenograft tumour volume with up-regulation of CA12 mRNA levels, whereas invalidation of both CAIX and CAXII gave 85% reduction (Chiche et al. 2009).

Interestingly, although being most often associated with more malignant phenotypes, the expression of CA XII has been linked to lower tumour grade and good prognosis in both invasive breast carcinoma (Watson et al. 2003) and non-small cell lung carcinoma (Ilie et al. 2011). In the case of breast carcinoma, an association was found between CA XII expression and positive estrogen receptor status as well as negative EGFR status. CA XII showed high staining intensity in regions adjacent to necrosis.

2.2.4. Carbonic anhydrases in normal brain

Since the 1940s, the CAs have been investigated in the CNS of mammals. They were first demonstrated biochemically by van Goor (1940), followed by studies by Ashby (1944a, 1944b). Furthermore, localisation of CA was studied by Giacobini (1961, 1962) and the highest activity was proposed in the glial and choroid plexus cells of the rat brain by microdissection. CA activity was shown in the mouse brain in the areas containing myelinated fibres and glial cells (Korhonen et al. 1964) by histochemical means. Methodological problems in the early studies were due to insufficient purification of myelin and sorting of different cell types, and thus immunohistochemical methods later provided more accurate information about the localisation of different CAs in the brain.

The expression of CA II in the mammalian CNS has been studied in several publications (Ghandour et al. 1979, Roussel et al. 1979, Langley et al. 1980, Kumpulainen and Korhonen 1982, Kumpulainen et al. 1983, Kimelberg et al. 1982, Snyder et al. 1983, Cammer and Zhang 1992). When studied in rat tissue, Roussel et al. (1979) found CA II positivity in both oligodendrocytes and astrocytes. Indirect immunoflurescence and immunoperoxidase methods showed CA II only in the oligodendrocytes, whereas glial fibrillary acidic protein (GFAP)-positive astrocytes were CA II negative (Ghandour 1979). The oligodendroglial location of CA II was later confirmed by Langley et al. (1980). Kumpulainen et al. (1982) studied the localisation of CAs in the mouse brain and found a strong CA II-specific reactivity in the heavily myelinated nerve tracts, oligodendrocytes, myelin sheaths, and choroid plexus cells. Similarly to finding of Roussel et al. (1979), Kimelberg et al. (1982) suggested that astrocytes in primary astrocyte cultures from rat brain might contain CA II, and Snyder et al (1983) reported relatively low amounts of CA II in astrocytes isolated from the brains of developing rats. Cammer and coworkers have also demonstrated CA II-positive immunoreactivity in both mouse and rat astrocytes (Cammer and Zhang 1991, 1992) as well as in rat microglia during development (Cammer and Zhang 1996). However, *in situ* hybridization analysis has again confirmed that CA II mRNA seems to be limited to oligondendrocytes in mouse primary cultured glial cells (Ghandour and Skoff 1991).

In the human nervous system, oligodendrocytes and retinal Müller cells have shown positive immunostaining reaction for CA II, and GFAP immunofluorescence has not been found in CA II-positive cells, supporting the oligodendrocyte-specificity of CA II in the adult brain (Kumpulainen et al. 1983). Using immunoperoxidase and double immunofluorescence staining techniques, the

enzyme was located to the corticotrophs of the human pituitary gland. Its physiological role may be linked to the regulation of optimal pH in the secretory vesicles for the cleavage of ACTH from its precursor (Parkkila et al. 1996).

The study of Kida et al. (2006) provided some insights into the developmental changes of CA II expression in the human brain. By means of immunoblotting, they showed an increase in CA II levels from embryos of 17 weeks´ gestation to childhood and adolescence. By immunohistochemical methods, the CA II expression was detected in conventional sites, such as oligodendrocytes and choroid plexus epithelium, in which the expression decreased with aging in adults. Interestingly, a subset of neurons mostly with gamma-aminobutyric acidergic (GABA) phenotype as well as some microvessels was positive for CA II during brain development. A few astrocytes were also weakly stained and seen more often in brain tissues showing anoxic changes. In addition, there was decreased immunoreactivity of CA II in the choroid plexus with increasing age, and the authors speculated that this could be involved in lowered production of cerebrospinal fluid in the elderly human brain.

Expression of CA IX is absent or low in the human brain, the exception being the epithelial cells of the choroid plexus (Ivanov et al. 2001, Proescholdt et al. 2005). Accordingly, Said et al. (2007a) reported low levels of CA IX mRNA in the normal brain compared to malignant tissues. As for CA XII in the brain, Ivanov et al. (2001) showed that there were regional differences in expression; high levels of mRNA were detected in the nucleus caudatus and putamen, whereas other regions contained lower amounts. By immunohistochemistry, CA XII expression was restricted to the posterior lobe of the pituitary glands, remnants of Rathke's pouch, choroid plexus and a few neurons in the cortex (Ivanov et al. 2001). Others have shown low levels of CA XII in the normal brain by means of Western blotting and immunohistochemistry (Proescholdt et al. 2005).

2.2.5. Carbonic anhydrases in brain tumours

Parkkila AK et al. (1995) were the first to assess the expression of CA II in a series of different brain tumours. They reported CA II-positive staining in astrocytic tumours, oligodendrogliomas and medulloblastomas, and showed that the most malignant tumours exhibited the strongest staining. As for bening tumours, expression was also detected in acoustic neurinoma, plexiform neurofibroma, choroid plexus papilloma, and subependymoma. CAs are also expressed to some extent in meningiomas: 14.8% of the tumours stained positively for CA II in the endothelium, and in 11.6%

of cases cytosolic CA IX expression was detected (Korhonen et al. 2009). Endothelial CA II expression correlated with increasing histological grade, and cell proliferation rates were higher in CA II-positive than CA II-negative cases. CA IX positivity was not associated with the studied clinicopathological factors, probably indicating a smaller role for CA IX in these mostly benign tumours.

During the last ten years, it has been clearly demonstrated that CAs are highly expressed in some malignant gliomas (Ivanov et al. 2001, Proescholdt et al. 2005, Korkolopoulou et al. 2007, Flynn et al. 2008, Järvelä et al. 2008, Yoo et al. 2010). Ivanov et al. (2001) screened a large number of tumours and found CA IX expression in astrocytoma cell lines and some tumour specimens. CA IX immunoreactivity was found in gliomas, including glioblastoma multiforme. Furthermore, they also studied CA XII expression and found strong immunopositivity in low-grade gliomas. A few years later, another screening confirmed elevated levels of CA IX and XII in brain tumours, such as astrocytic gliomas, compared with the normal brain (Proescholdt et al. 2005).

As previously described in chapter 2.2.2.2, CA IX expression is strongly regulated by the hypoxia pathway, involving HIF-1 as the key transcription factor that induces the expression of CA IX in hypoxic tumours. The same phenomenon has been suggested for CA IX induction in brain tumours. When CA IX mRNA was studied in human malignant glioma cell lines, distinct patterns of hypoxic expression of CA IX were observed (Said et al. 2007b). The finding indicated that low oxygen concentration is probably the driving force for the increased CA IX expression due to the presence of a HRE in the *CA9* promoter (Wykoff et al. 2000). Recently, CA IX was studied in oligodendroglial tumours (Järvelä et al. 2008). 80% of the tumours were immunopositive for CA IX, and CA IX expression was correlated to poor patient survival in univariate and multivariate analyses. Interestingly, a correlation between CA IX positivity and decreased proliferation index by Ki-67 / MIB-1 was also reported. Furthermore, the expression of CA II, CA IX, and CA XII has been studied in 35 patients with either medulloblastomas or supratentorial primitive neuroectodermal tumours (Nordfors et al. 2010), which represent the most common highly malignant brain tumours in children. Endothelial CA II and cytoplasmic CA II were detected in 49% and 73% of the tumours, whereas 23% of the tumours were positive for CA IX, and 11% of the tumours expressed CA XII. Similar to the finding of Yoshiura et al. (2005), CA II was detected in the tumour neovessel endothelium. CA IX was mainly expressed in perinecrotic areas and remarkably, predicted poor prognosis in both univariate and multivariate analyses in the total

tumour material. When only the patients with medulloblastomas were included to the analysis, CA XII positivity predicted significantly worse prognosis.

2.3. Ki-67

Gerdes et al. (1983) were the first researchers to identify Ki-67 nuclear antigen, which is present in proliferating cells but absent in resting cells. The *Ki-67* gene is located on the long arm of human chromosome 10 (Fonatsch et al. 1991), and its complete complementary deoxyribonucleic acid (cDNA) sequence has been published (Schluter et al. 1993). The encoded protein has been characterized as a nuclear nonhistone protein (Gerdes et al. 1991). Because of the alternative splicing, there are two mRNA species which encode two isoforms of the protein, and the molecular masses are 359 kD and 320 kD (Scholzen et al. 1997). Ki-67 nuclear antigen is present in G1, S, G2, and M phases of the cell cycle, but is absent in G0 (Gerdes et al. 1984). Taken together, levels of Ki-67 are low during G1- and early S-phase and increase progressively being highest during mitosis. The cellular location of Ki-67 varies during the cell cycle (van Dierendonck et al. 1989, Kill 1996). During early G1, weak staining is found throughout the karyoplasm; during S-phase and G2 it is expressed in the nucleolus. During early mitosis, Ki-67 is intensively expressed on the surface of condensed chromosomes. Furthermore, this expression pattern rapidly disappears in anaphase-telophase. The half-life of the protein is estimated to be between 60 and 90 minutes (Bruno and Darzynkiewicz 1992, Heidebrecht et al. 1996).

Though Ki-67 has been intensively studied for decades, its function remains obscure. Little is known about the proteins it recognizes beyond the information that the protein has a critical role in cell division and is phosphorylated via serine and theronine, and that the phosphorylation of the Ki-67 proteins increases in cycling cells (Heidebrecht et al. 1996). Importantly, the discovery of the anti-Ki-67 (MIB-1) antibody (Cattoretti et al. 1992, Key et al. 1993) provided a useful method to assess not only fresh or frozen samples, but also formalin-fixed and paraffin-embedded tissues. This finding led to an extensive use of MIB-1 antibody for routine histopathological diagnostics.

The clinical value of Ki-67 / MIB-1 labeling index has been intensively studied in human cancer, including tumours derived from the CNS (Burger et al. 1986, Sallinen et al. 1994, Karamitopoulou et al. 1994, Wakimoto et al. 1996, Di et al. 1997, McKeever et al. 1997 and 1998, Hsu et al. 1997, Khalid et al. 1997, Hilton et al. 1998, Eneström et al. 1998, Ralte et al. 2001, Torp 2002, Neder et al. 2004). In astrocytomas, the Ki-67 / MIB-1 index usually increases with increasing grade of

malignancy and could be used to identify low-grade astrocytomas from higher grade ones, although indices vary for different articles. Sallinen et al. (1994) showed that MIB-1 LI differentiates WHO grade II astrocytomas from grade III astrocytomas. This finding has been confirmed by more recent studies (Ralte et al. 2001, Torp 2002), and there are also some reports showing significant differences when grade III tumours have been compared to grade IV tumours (Di et al. 1997, Enström et al. 1998). This difference is probably due to overlapping indices between different WHO grades, and Ki-67 / MIB-1 could be used as an additional tool, along with histological criteria, when defining the malignancy grade.

As a diagnostic tool, Ki-67 immunopositivity was first used in frozen sections of brain tumours (Burger et al. 1986, Shibata and Burger 1987). The use of Ki-67 immunostaining in an intraoperative setting has been suggested to be useful in astrocytomas, because the WHO diagnostic criteria, such as the presence or absence of atypia and mitoses, endothelial proliferation, and necrosis, are not always easy to interpret due to the limited size and relatively poor morphology of frozen sections. The long staining procedure has been the main problem, which has hampered the implementation of Ki-67 immunostaining for intraoperative use. Typical immunohistochemical techniques for Ki-67 staining can take several hours to perform. Therefore, there has been a demand for a useful and fast staining method (Tsutsumi et al. 1995, Kämmerer et al. 2001).

3. Aims of the study

1) **To study the distribution of CAs in gliomas**

2) **To correlate CA II, CA IX, CA XII and Ki-67 with conventional and molecular pathological features in gliomas**

3) **To study the diagnostic and clinical value of CA II, IX, XII and Ki-67 in immunostaining of gliomas**

4. Materials and methods

4.1. Patients and tumours

The tumour tissue specimens for all four articles were obtained from neurosurgical patients treated at the Tampere University Hospital, Tampere, Finland. The tumours were radically resected if possible and most patients with high grade gliomas also received radiotherapy and/or chemotherapy. The tumour tissues were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. Then the slides were stained with haematoxylin and eosin (H&E). Typing and grading of tumours were performed by a neuropathologist according to the criteria presented by the WHO 2000 guidelines (Kleihues, Cavenee, eds. 2000). These include the presence of atypia, mitotic activity, necrosis, and endothelial proliferation, and divide diffusely infiltrating astrocytomas into three grades (II-IV). The multitissue blocks were used in the second, third and fourth article. The neuropathologist pinpointed one histologically representative tumour region in each H&E -slide, of which a tissue sample was included in the multitissue blocks. The multitissue blocks were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) and the sample diameter of the tissue cores was 600 µm. The study design of the orginal articles was approved by the Ethics committee of Pirkanmaa Hospital Distrinct and by the National Authority for Medicolegal Affairs (TEO).

4.1.1. Patients and tumours in study I

Tumour material for the first article of this thesis was collected between 1996 and 2000 at Tampere University Hospital. This included specimens from 34 patients (14 males and 20 females) neurosurgically treated for astrocytoma, of which intraoperative consultation to a neuropathologist was made by a neurosurgeon. The age of the patients ranged from 2 to 81 years (median, 48 years). Follow-up data was available for at least 36 months in all cases. These tumours consisted of nine pilocytic astrocytomas (grade I) and 25 diffuse astrocytomas (nine grade II astrocytomas, four grade III anaplastic astrocytomas, and 12 grade IV glioblastomas). Intraoperately, frozen sections were cut from a sample selected by the neurosurgeon and stained with H&E. After confirming the intraoperative diagnosis, additional sections were cut, sealed airtight, and stored unfixed at -70˚C.

4.1.2. Patients and tumours in study II

Tumour material for the second article of this thesis was collected from tissue archives from 1983 to 2001 at Tampere University Hospital. The study material consisted of 362 diffusely infiltrating astrocytoma samples. The analyses were performed using multitissue blocks, which were composed of 362 astrocytic tumours and included 281 primary tumours and 81 recurrences. The grades were as follows: 52 cases of grade II, 45 cases of grade III, and 265 cases of grade IV. The age of patients with primary tumours varied from 15 to 89 years (median 64 ± SD 15 years) and recurrent tumours from 15 to 89 years (median 52 ± SD 12 years). Overall survival was known for 186 patients (26 of grade II, 21 of grade III, and 139 of grade IV) and the median follow-up time for 40 survivors was 809 days (146 patients died during follow-up).

4.1.3. Patients and tumours in study III

The material for the third article of this thesis was obtained from surgical patients treated at the Tampere University Hospital in 1983–2002. There were 255 astrocytoma samples (27 of grade II, 39 of grade III, and 189 of grade IV), of which 189 were primary tumours and 66 were recurrences. The ages of patients with primary astrocytic tumours varied from 1 to 82 years (mean 48 ± SD 20), and with recurrent tumours from 1 to 76 years (mean 40 ± SD 18). Overall survival was known for 195 patients, of whom 161 had died during the five-year follow-up period. In the case of oligodendroglial tumours, there were 71 tumour samples included in the analyses (pure oligodendrogliomas: 31 of grade II, and 12 of grade III; and oligoastrocytomas: 18 of grade II, and 9 of grade III), of which 52 were primary tumours and 19 were recurrences. The ages of patients with primary oligodentroglial tumours ranged from 8 to 76 years (mean 43 ± SD 14), and those of the patients with recurrent tumours from 12 to 69 years (mean 41 ± SD 14). Overall survival was known for 52 patients. The median follow-up time for the 26 survivors was 8 years 2 months, and 26 patients died during follow-up.

4.1.4. Patients and tumours in study IV

The study material for the fourth article of this thesis was collected between 1983 and 2001 at Tampere University Hospital. The material consisted of 370 diffusely infiltrating astrocytic gliomas. In the multitissue blocks, there were 287 primary tumours (39 grade II, 30 grade III, 218 grade IV) and 83 recurrences (15 grade II, 17 grade III, 51 grade IV). Ages of patients with primary tumours ranged from 3 to 82 years (median 55 ± SD 15 years), and ages of patients with recurrent tumours from 3 to 75 years (median 44 ± SD 12 years). Overall survival was known for 287

patients (39 grade II, 30 grade III, and 218 grade IV). During the 5-year follow-up, 237 patients died and 50 patients were still alive.

4.2. Immunohistochemistry

4.2.1. Ultrarapid Ki-67® and Ki-67 / MIB-1 immunostaining

Ultrarapid-Ki67® IHC kit (Immuno Diagnostic Inc, Hämeenlinna, Finland) was used in immunohistochemical staining of tumour slides and performed according to the manufacturer's instructions. The slides were first fixed in 100% acetone/0.03% hydrogen peroxide for one minute at room temperature and air dried. Tissue sections were encircled with a hydrophobic pen (PAP-pen) to help keep antibody droplets on the sections. The sections were first incubated with ready to use mouse Ki-67 antibody for three minutes, followed by a short rinse in phosphate buffered saline (10 seconds with continuous slide agitation). The detection antibody (antimouse–horseradish peroxidise polymer) was applied and the sections were incubated for three minutes before rinsing with phosphate buffered saline for 10 seconds. Diaminobenzidine (DAB) was used as a chromogenic substrate and was prepared by adding one drop of reagent A and one drop of reagent B to 500 ml of DAB diluent. The slides were incubated in DAB solution for one minute and washed with distilled water. Antibody and DAB incubations were carried out on a thermal plate set at 41˚C. The slides were counterstained with haematoxylin (10 seconds), dehydrated, and embedded according to routine frozen section protocols.

"Control MIB-1" staining was performed on the remaining frozen tissue block, after inraoperative consultation. The block was fixed in 10% buffered formalin overnight and embedded in paraffin wax. Then, sections were cut and immunostained with MIB-1 antibody (DakoCytomation, Glostrup, Denmark). Heat induced epitope retrieval (Tris/EDTA buffer (pH 9.0) 2 x 7 minutes in a microwave oven) and an automated immunostaining protocol (TechMate immunostainer) were used. Then, the haematoxylin counterstained slides were analysed visually. As described previously by Sallinen et al. (2000), MIB-1 staining was also carried out using a tissue block showing the highest mitotic activity and cellularity, as evaluated by neuropathologist from H&E stained slides. These tissue sections were stained with MIB-1, as described above, and counterstained with methyl green, and termed "highest-MIB-1".

Evaluation of Ultrarapid-Ki67® and control MIB-1 slides was carried out in the area of highest immunoreactivity. Visual scoring was carried out by two observers. In total, 300–500 neoplastic tumour cells were analysed and necrotic and haemorrhagic tumour areas were ignored, and only definitely brown nuclei were recorded as positive. The results were expressed as a percentage of immunoreactive tumour cell nuclei. Computer assisted image analysis of immunopositivity of the highest MIB-1 slides was carried out by observer II using a CAS-200 image analysis system (CAS-200 Software; Becton Dickinson, Mountain View, California, USA), and described as the percentage of immunopositive nuclei (proliferation index).

4.2.2. Immunohistochemistry for CA II

Automated immunostaining for CA II was performed using Power Vision Poly-HRP IHC Kit (horseradish peroxidase immunohistochemistry kit; ImmunoVision Technologies, Brisbane, CA, USA) reagents. The immunostaining method was performed as follows: (a) rinsing in wash buffer; (b) treatment in 3% hydrogen peroxide in double-distilled water (ddH_2O) for 5 minutes and rinsing in wash buffer; (c) blocking with Universal IHC Blocking/Diluent (ImmunoVision) for 30 minutes and rinsing in wash buffer; (d) incubation with rabbit anti-human CA II serum or normal rabbit serum diluted 1:2,000 in Universal IHC Blocking/Diluent for 30 minutes; (e) rinsing in wash buffer for 3 x 5 minutes; (f) incubation in poly- HRP–conjugated anti-rabbit immunoglobulin G for 30 minutes and rinsing in wash buffer for 3 x 5 minutes; (g) incubation in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (1 drop of DAB solution A and 1 drop of DAB solution B with 1 ml ddH_2O) for 6 minutes; (h) rinsing with ddH_2O; (i) copper(II) sulfate treatment for 5 minutes to enhance the signal; and (j) rinsing with ddH_2O. All procedures were carried out at room temperature. The sections were mounted in Entellan Neu (Merck, Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

4.2.3. Imunohistochemistry for CA IX

Immunohistochemistry of CA IX was performed using the monoclonal M75 anti-human CA IX antibody, originally described by Pastoreková et al. (1992). The antibody has shown no cross-reactivity with other CAs and the signal is easily detectable in routine formalin-fixed paraffin-embedded tissue sections. The CA IX enzyme was immunostained by the biotin-streptavidin complex method by following this procedure: (a) pretreatment of the sections with undiluted cow colostral whey (Biotop Oy, Oulu, Finland) for 30 minutes and washing in PBS, (b) incubation for

one hour with M75 antibody (1:10) in 1% bovine serum albumin-PBS, (c) incubation for one hour in biotinylated goat anti-mouse IgG (Zymed Laboratories, Inc., South San Francisco, California) diluted 1:300 in 1% bovine serum albumin-PBS, and then (d) incubation for 30 minutes with peroxidise-conjugated streptavidin (Zymed) diluted 1:500 in PBS; (e) thereafter, incubation was performed for two minutes in DAB solution containing 9 mg 3,3´-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 mL PBS and 5 μL 30% H_2O_2. The sections were rinsed three times for ten minutes in PBS after incubation steps (b) and (c), and four times for five minutes in PBS after step (d). The tumour sections were counterstained with hematoxylin after immunostaining.

4.2.4. Immunohistochemistry for CA XII

Rabbit anti-human CA XII serum used for both immunohistochemistry and Western blotting has been characterized by Karhumaa et al. (2000). The polyclonal antiserum was raised against the truncated form of recombinant human CA XII, and its specificity was confirmed under both denaturing and native conditions. The recombinant CA XII protein did not include the segment that is potentially deleted due to the alternative mRNA splicing process according to our present data. Therefore, the produced antibody should recognize both the intact CA XII and the spliced form. The automated staining method for CA XII was essentially the same as described for CA II. Normal rabbit serum was used for the control stainings.

4.2.5. Evaluation of CA immunohistochemistry

Immunohistochemical evaluation of CAs (II, IX, and XII) was performed under a light microscope. In terms of the staining intensity, the scores were evaluated as follows: 0, no reaction; +, weak reaction; ++, moderate reaction and +++, strong reaction. A four-step evaluation was used in the estimation of the extent of the highest staining intensity: 0, no positive cells; +, < 25 % positive cells; ++, 25 % to 50 % positive cells; and +++, > 50 % positive cells. The tumours were also divided into two groups based on the nuclear staining of the CA IX: 0 no nuclear reaction; 1, positive nuclear reaction.

In the statistical analyses of the study III, the specimens were grouped into two categories based on the staining intensity: CA II+ve tumours, with moderate or strong reaction in the endothelium; and CA II–ve tumours with weak or negative immunostaining results.

In the study IV, the simultaneous expression of CAs was evaluated in survival analysis. On each separate enzyme (CA II, CA IX, and CA XII), negative and weak stainings were considered CA-negative (CA–ve), and moderate and strong stainings were considered CA-positive (CA+ve). Analyses on simultaneous expression of transmembrane CAs (CA XII and CA IX) were categorized as follows: 0, CA XII and CA IX were both CA–ve; 1, only one enzyme was CA+ve; 2, both enzymes were CA+ve. The variable obtained was called the CA IX/XII index. Simultaneous expression of tumour-associated CAs (CA XII, CA IX, and CA II) was evaluated as follows: 0, all three enzymes were CA–ve; 1, one of the three enzymes was CA+ve; 2, two of the three enzymes were CA+ve; and 3, all three enzymes were CA+ve. This variable was termed the CA II/IX/XII index.

4.3. mRNA analysis

4.3.1. mRNA analysis for CA IX

mRNA analysis included seven brain tumours and one normal brain sample and the isolation was carried out using RNeasy Mini-Kit (Qiagen, Hilden, Germany). Reverse transcription was performed with Moloney murine leukemia virus Mo-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) and random primers (400 μg/mL) were used. The published information on CA IX mRNA in GenBank (accession no. NM_001216) was used in the design of the primers for the PCR reaction. To produce an amplification product of 457 bp, the forward primer (F1) was 5´-GTTGCTGTCTCGCTTGGAAGAAA- 3´ (nucleotides 915-937) and the reverse primer (R1) 5´-GCGGTAGCTCACACCCCCTTT-3´ (nucleotides 1392-1372). The control PCR reaction was performed with the following primers for human β2-microglobulin (accession no. NM_004048): the forward primer was 5´-TATCCAGCGTACTCCAAAGATTCA-3´ (nucleotides 120-143) and the reverse primer was 5´-GAAAGACAAGTCTGAATGCTCCAC-3´ (nucleotides 288-265). The deoxynucleotide triphosphate mix for PCR reaction was from Finnzymes and other reagents were from BD Biosciences (Palo Alto, CA). Twenty nanograms of cDNA were used as the template. The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, California). The protocol consisted of the following steps: a 94ºC denaturation step for 1 minute, then 33 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 90 seconds, and final extension at 72ºC for 3 minutes. The results of the PCR reaction were analyzed using 1.5% agarose gel containing 0.1 μg/mL ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA).

4.3.2. mRNA analysis for CA XII

RNeasy Mini-Kit (Qiagen, Hilden, Germany) was used in RNA extraction for tumour material from a total of seven tumours and one normal brain sample. Mo-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) was used in RT reaction using random primers (400 μg/ml). The primers for the PCR were designed using the published information on *CA12* mRNA in Gen-Bank (accession nos. NM_001218 and NM_206925; these represent alternatively spliced CA XII isoforms). Both spliced forms were identified as separate bands in the samples. The forward primer (F1) was 5'-CAACTTCCGGCAGGTCCAGA-3' (nucleotides 972 – 991 in NM_001218), and the reverse primer (R1) was 5'-TTGAGGTGTCGCAAGTGTCCAG-3' (nucleotides 1287 – 1308 in NM_001218 and 1254 – 1275 in NM_206925). The predicted amplification products were 336 bp for isoform 1 and 303 bp for isoform 2. The control PCR reaction was performed with the following primers for human b-actin (accession no. NM_001101): forward primer (F2) 5'-CACGGCATCGTCACCAACTG-3' (nucleotides 290 – 309) and reverse primer (R2) 5'-GCCTGGATAGCAACGTACATGGC-3 (nucleotides 464 – 486), producing an amplification product of 197 bp. The PCR was performed using 1.13 ReddyMix PCR Master Mix (Abgene, Epsom, UK), and 20 ng cDNA was used as a template in a 25-μl reaction. The PCR reaction was performed on a thermal cycler (PTC 200 Thermal Cycler, MJ Research, Inc., Waltham, MA, USA); the protocol included one minute 94°C denaturation step and was followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 90 seconds, followed by final extension at 72°C for three minutes. 1.5% agarose gel containing 0.1 μg/ml ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA, USA) was used in the analysis of the PCR reaction. In addition, RNA analysis was performed on glioblastoma cell lines (U-87 MG, U-373 MG, and CCF-STTG1 (European Collection of Cell Cultures, Salisbury, UK), two renal cancer cell lines (Caki-1 and A-498; American Type Culture Collection, Manassas, VA, USA)), and two normal tissues (colon and kidney). RNeasy Mini-Kit was used in RNA isolation, and RT-reaction was performed with the Fermentas First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The PCR reaction was performed as described for tumour samples with a few exceptions. The control PCR reaction was performed with the following primers for human β-2-microglobulin (NM_004048): forward primer (F3) 5'-TCCAGCGTACTCCAAAGATTCAGG-3' (nucleotides 122 – 145), reverse primer (R3) 5'-ATGCGGCATCTTCAAACCTCC-3' (nucleotides 431 – 451); the resulting PCR product was 330 bp. Two nanograms of cDNA were used as a template in the PCR reactions performed using the commercial cDNA panel (Human MTC panel I, BD Biosciences, Palo Alto, CA, USA). 1.5%

agarose gel was used in the analysis of the PCR. A grade IV astrocytoma sample showing a double band was used to confirm the presence of alternatively spliced mRNA. PCR products were purified from gel with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Poole, UK). Then, the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) was used in sequencing, which was performed in both directions with primers F1 and R1. Purified PCR product (5 µl) was mixed with 2 µl Big Dye mix and 2 µl sequencing buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), and 1.6 pmol of primers was added. The reactions were amplified by cycle sequencing on a PTC 200 Thermal Cycler according to the manufacturer's protocol. The products were purified by ethanol precipitation, resuspended in HiDi formamide (Applied Biosystems), and denatured according to the manufacturer's instructions. The sequencing was performed with an ABI PRISM Genetic Analyser 9100 (Applied Biosystems).

4.4. Other analysis

4.4.1. Western blotting for CA XII

Five cell lines (U-87 MG, CCF-STTG1, Caki-1, A-498, and UMRC6 (generously provided by Dr. Sergey V. Ivanov)) were used in Western blotting, which was performed to evaluate the presence of CA XII isoforms. The cells were cultured under normoxia for four days in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and isolated from cell culture plates. Total cell homogenates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described.

4.4.2. Other immunohistochemistry

Apoptosis by TUNEL, epidermal growth factor receptor (EGFR) amplification with chromogenic *in situ* hybridization and p53 immunohistochemistry was performed as described in original articles. 1p/19q analysis by fluorescence *in situ* hybridization was performed as previously described (Järvelä et al. 2006).

4.4.3. Statistical analysis

All statistical analyses were performed using SPSS for Windows (SPSS 11.0, Chicago, IL, USA). The significance of associations was defined using the chi-square test, Mann-Whitney test, and Kruskal-Wallis test. The log-rank test and Kaplan-Meier curves were used in univariate survival analyses, and Cox multivariate regression analysis was used in multivariate survival analyses. Pearson's correlation coefficient (r) was used to study concordance of the IHC tests. Optimal cutoffs for proliferation indices in survival analyses were defined using receiver operating characteristics (ROC) curves, as described previously (Sallinen et al. 1994).

5. Results

5.1. Immunohistochemistry

5.1.1. Ultrarapid Ki-67®

The positive staining for Ultrarapid Ki-67® was seen in proliferating astrocytoma nuclei, which were scarce in low grade tumours and abundant in glioblastoma (Figure 1 of study I). The staining pattern of Ultrarapid Ki-67® frozen sections of gliomas was similar to that of MIB-1 staining in paraffin wax embedded tumour sections.

The indices of Ultrarapid Ki-67® in frozen sections and corresponding MIB-1 staining in paraffin embedded sections (control MIB-1) are shown in study I, Figure 2A. When these two were compared, they were similar and correlated very significantly ($r = 0.916$, Pearson's correlation coefficient, $p < 0.001$). Furthermore, a strong correlation was found between the highest proliferation indices in paraffin wax embedded sections (highest MIB-1) and Ultrarapid Ki-67® indices ($r = 0.790$, Pearson's correlation coefficient, $p < 0.001$; study I, Figure 2B). Interobserver variation between observers I and II was minimal, as indicated by a correlation coefficient of $r = 0.946$ ($p < 0.001$, data not shown). All mean and median Ki-67 indices (Ultrarapid Ki-67®, control MIB-1, highest MIB-1) by WHO grade are shown in Table 1 (study I). The mean and median values for Ultrarapid Ki-67® and control MIB-1 were comparable, whereas the highest MIB-1 indices yielded higher values as these were assumed to concentrate in the most proliferative areas. Proliferation indices by all three methods correlated significantly with histological grade (Ultrarapid Ki-67®: $p < 0.001$; control MIB-1: $p = 0.001$; highest MIB-1: $p < 0.001$, Kruskal-Wallis test). The results of a comparison between intraoperative tumour grade, Ki-67 indices and final tumour grade are shown in study I, Table 2. The astrocytomas were divided into low grade (grades I and II) and high grade (grades III and IV) tumours according to the original intraoperative frozen section interpretation (based on H&E staining). A significant difference ($p < 0.001$, Mann-Whitney test) and only a minor overlap were found when the proliferation indices (Ultrarapid Ki-67®) were compared between these groups (Study I, Table 2).

The ROC analysis was used to study the prognostic value of Ultrarapid Ki-67® and the optimal cutoff points were determined. The prognostic sensitivity (true positive; that is, deceased patient with Ki-67 index above cutoff point) and specificity (100 false positive; that is, a living patient with

Ki-67 index above cutoff point) are shown in study I, Figure 3 during the three year follow-up period for Ultrarapid Ki-67® and the two MIB-1 assays. These ROC curves indicate that Ultrarapid Ki-67®, control MIB-1, and the highest MIB-1 were of similar prognostic value. Optimal cutoffs yielding the best sensitivity and specificity for the three tests were 7.5%, 10%, and 12.5 %, respectively (study I, Figure 3). Using these cutoffs, a highly significant distinction was found in survival analysis (Ultrarapid Ki-67®: $p < 0.0001$; control MIB-1: $p = 0.0047$; highest MIB-1: $p = 0.0001$, log rank test). A similar prognostic significance was also found when only diffusely infiltrating astrocytomas (grades II–IV) were included in the analysis (Ultrarapid Ki-67®: $p = 0.0008$; control MIB-1: $p = 0.0167$; the highest MIB-1: $p = 0.005$).

5.1.2. Immunohistochemistry for CA II in gliomas

The whole tumour material consisted of 261 diffusely infiltrating astrocytoma cases in study III. Some brain tumours clearly appeared to express CA II in the vascular endothelium (study III, Figure 1). Endothelial CA II immunopositivity was observed in 117 (45%) cases and the frequencies of endothelial CA II staining were as follows: strong in 45 cases, moderate in 36, and weak in 36, and 144 tumours (55%) were negative. Normal brain tissues studied showed no endothelial CA II expression (data not shown). When cytoplasmic expression of CA II was evaluated in tumour cells, strong staining was detected in only six cases. Moderate cytoplasmic staining was observed in 35 tumours and weak reactions in 52 cases, whereas 168 cases (64%) were negative.

Figure 1 of study III shows positive CA II immunostaining in some tumours. The grade II astrocytomas did not express CA II at all in the endothelium, whereas positive endothelial staining was most often seen in the proliferative endothelium and in small neovessels of grade IV astrocytomas. The positive cells in the proliferative endothelium were usually located near the vascular lumen. No significant differences in CA II immunostaining were detected between primary tumours and recurrences (p = n.s., chi-square test). Then, the specimens were grouped into categories according to the endothelial staining intensity; strongly and moderately stained specimens were considered CA II+ve and tumours containing weak or no staining were considered CA II-ve. Of the total 261 tumours, 81 cases (31%) were CA II+ve and 180 cases (69%) were CA II–ve. Endothelial CA II expression was not associated with the area of necrosis in the same tissue section ($n = 37$, p = n.s., Mann-Whitney test) and no correlation was found between the CA II reactivity and necrosis when the cut-off point of the latter was set to the median value (p = n.s., chi-

square test). Positive endothelial staining of CA II was significantly associated with a higher tumour grade ($p < 0.001$, chi-square test) (study III, Figure 2A). Importantly, the presence of CA II in the endothelium and CA IX in the tumour cells showed a significant correlation ($p = 0.006$, chi-square test).

In addition to astrocytomas, the materials of study III included oligodendrogliomas and mixed oligoastrocytomas. 26 (60%) cases of pure oligodendrogliomas contained positive endothelial staining for CA II and 15 (58%) of the mixed oligoastrocytomas were positive. Figure 2B of study III shows CA II immunostaining intensity in different grade categories. Generally, the oligodendroglial tumours showed weaker endothelial expression of CA II than the astrocytomas (study III, Figures 2B and 4), the staining intensities being strong in nine, moderate in one, and weak in 31 cases. Cytoplasmic tumour cell–associated CA II expression was only detected in a minority of the specimens. The signals were strong in four, moderate in one, and weak in 23 cases. A total of 43 oligodendroglial tumours (61%) showed no immunoreactions in the tumour cells. In oligodendroglial tumours, no significant differences in CA II expression were observed between primary tumours and recurrences (p = n.s., chi-square test).

Of the oligodendroglial tumours, 10 (14%) cases were CA II+ve (strong or modereate staining) in the endothelium. Furthermore, endothelial CA II was more often expressed in high-grade mixed oligoastrocytomas than low-grade tumours ($p = 0.018$, chi-square test) (study III, Figure 2B). When 1p 19q loss and CA II expression were evaluated together, no association was found (p = n.s., chi-square test). In patient survival analysis, neither endothelial nor cytoplasmic staining for CA II was a significant predictor of survival in the primary oligodendrogliomas (p = n.s., log-rank test).

5.1.3. Immunohistochemistry for CA IX in astrocytic tumours

The expression of CA IX was studied in diffusely infiltrating astrocytomas by immunohistochemistry and 284 of 362 cases (78%) were positive. Cellular CA IX immunopositivity was detected in tissue sections as follows: 57 (16%) were strongly, 84 (23%) moderately, and 143 (39%) weakly stained. 78 (22%) tumour specimens were completely negative (study II, Figure 1). When studied according to WHO grade, 65% of grade II astrocytomas were CA IX positive (15% moderately, 50% weakly), 73% of grade III astrocytomas were positive (9% strongly, 33% moderately, and 31% weakly), and 82% of grade IV astrocytomas stained positively (20% strongly, 23% moderately, 39% weakly). The signal for CA IX was usually unevenly

distributed within the tumour and was associated with nercosis. The strongly stained areas were often located close to the necrotic regions (study II, Figure 1), and CA IX intensity correlated significantly with the presence of necrosis in the same tissue section ($P < 0.001$, chi-square test).
In addition to the typical membrane-associated staining of CA IX, weak cytoplasmic staining was occasionally detected in the infiltrative zone of neoplastic cells of lower grade tumours (study II, Figure 1C). The expression of CA IX was not associated with the distribution of blood vessels or the endothelial cell proliferation of blood vessels (volume percentage of endothelial cells, p = n.s., chi-square test). The cell cytoplasm of tumours with anaplastic features was often more intensely stained (study II, Figure 1D). The comparison of cytoplasmic CA IX intensity and tumour grade revealed significantly higher CA IX signal in the tumours with higher malignancy grade ($p < 0.001$, chi-square test; study II, Table 1). When nuclear staining for CA IX was evaluated, 211 (58%) of the tumours were positive and 151 (42%) negative. The lower grade gliomas showed more frequently nuclear staining for CA IX in contrast to glioblastomas, which were often negative ($p < 0.001$, chi-square test). CA IX extent, describing the relative area of the positive staining, was found to be high in 184 (51%), moderate in 72 (20%), scant in 28 (8%), and negative in 78 (22%) cases, and increasing CA IX extent correlated significantly with increasing histological grade ($p= 0.027$, chi-square test).

When statistical comparisons with patient age were performed, the study population was divided into two subgroups according to whether the patient was over or under 50 years old (<50 years, n = 164; ≥50, n = 200). A significant association was found between increasing CA IX intensity and age ($p = 0.003$, chi-square test), as well as increasing CA IX nuclear staining and patient age ($p = 0.004$, chi-square test). When tested separately in different WHO grade tumours, similar correlation was found in grade II and III tumours ($p = 0.010$, $p = 0.013$, respectively, chi-square test) but not in grade IV tumours (p = n.s., chi-square test).

5.1.4. Immunohistochemistry for CA XII in astrocytic tumours

CA XII was another isozyme expressed in most specimens of diffusely infiltrating astrocytomas. 363 (98%) of 370 cases showed positive immunostaining and the reactions were as follows by four-category assessment: 39 (11%) were strongly stained, 169 (46%) moderately stained, 155 (42%) weakly stained, and 7 (2%) cases were negative. The extent of CA XII immunoreaction was found to be 3 (>50% positive cells) in 207 (56%) cases, 2 (25%–50% positive cells) in 47 (13%) cases, 1 (<25% positive cells) in 109 (29%) cases, and 0 (no positive cells) in 7 (2%) cases. The expression

of CA XII was homogenous and usually unevenly distributed within the tumour (study IV, Figure 3). Some immunoreactivity was also detected in the nuclei and cytoplasm of tumour cells, but the nuclear staining was considered unspecific, because similar nuclear reactions were occasionally seen in control stainings performed using normal rabbit serum. Neither endothelial proliferation nor necrosis was significantly associated with CA XII intensity (p = n.s., respectively, chi-square test).

Increasing patient age and CA XII intensity correlated significantly with each other when primary tumours and recurrences were pooled (p = 0.022, variance analysis). Similar association was found when only primary tumours were studied (p = 0.016, variance analysis). Importantly, CA XII intensity was found to be higher in tumours with higher WHO grade (p = 0.006, chi-square test; study IV, Table 1) and the analysis revealed similar significant association even when the intensities were grouped as CA-positive and CA-negative (p = 0.032, chi-square test).

5.2. mRNA analysis of CAs

5.2.1. mRNA analysis for CA IX

The presence of CA IX in diffuse astrocytomas was verified from seven tumour samples (grades II-IV, two samples of each grade and hemangioblastoma, which was a positive control) by means of RT-PCR and the results are shown in Figure 3 of study II. A strong band for CA IX mRNA was detected from one grade IV astrocytoma (glioblastoma) and from hemangioblastoma. Weak positive signals were found in two grade II, one grade III and one grade IV specimen.

5.2.2. mRNA analysis for CA XII

All six astrocytoma samples (grades II-IV, two samples of each grade) except for one grade II tumour were positive for CA XII in the RT-PCR reaction (study IV, Figure 1). It was notable that grade IV samples showed the strongest bands for *CA12* mRNA. The hemangioblastoma, known to have defects in VHL protein and up-regulation of CA XII, served as a positive control for the PCR reaction and, as expected, showed strong bands for *CA12*. Two very faint bands were also detected by RT-PCR in the normal brain sample, suggesting expression of both alternative isoforms. When these bands were reamplified with the original primers, the two bands became clearly visible.

Of the tumour samples studied, only one grade IV sample contained a double band, but in all the other positive samples the shorter form of CA XII was predominant. Confirmation by sequencing showed that these two bands represented the two variants of *CA12* mRNA whose sequences were available in GenBank.

The results from RT-PCR are shown in Figure 1B (study IV). As positive controls, the experiment was performed for the normal human kidney and colon, and both tissues produced only the longer (full-length) isoform of CA XII. When two renal carcinoma cell lines were examined, the A-498 cell line also produced the longer isoform, whereas the Caki-1 cell line showed bands representing both isoforms. We also studied three grade IV astrocytoma (glioblastoma) cell lines (U-373 MG, U-87 MG, and CCF-STTG1). The U-373 MG glioblastoma cell line was almost negative for CA XII, while the other two cell lines expressed both isoforms of CA XII. As a conclusion, the normal human brain used in our study weakly expressed both isoforms, whereas the brain tumours expressed greater quantities of mRNAs of the spliced isoform. Furthermore, Western blotting was performed to evaluate the translation of *CA12* mRNA variants into protein isoforms in two glioblastoma cell lines and a Caki-1 cell line (study IV, Figure 2) U-87 MG cell line showed two isoforms of CA XII. A VHL-defective cell line, A-498, showed an intense broad polypeptide band, which may include the shorter form in addition to the intact longer form of CA XII protein. Another cell line with VHL deletions, UMRC6, also showed an intense band, but only the longer isoform was evident.

5.3. CAs and molecular pathological features

CA II expression did not correlate to proliferation by MIB-1 / Ki-67 proliferation index (n = 234, p = n.s., Mann-Whitney test). The positive endothelial CA II staining correlated significantly with the presence of EGFR amplification (n = 172, p = 0.008, chi-square test), whereas CA II did not correleate with p53 expression (n = 103, p = n.s., chi-square test).

CA IX immunohistochemistry did not correlate with proliferation by Ki-67 / MIB-1 (n = 329, p = n.s., Kruskall-Wallis test). Similarly, when compared to apoptosis by terminal nucleotidyl transferase–mediated nick end labeling index (TUNEL), no association was seen (n = 29, p = n.s., Mann-Whitney test). Furthermore, CA IX intensity showed no significant association with p53 (n =

141, p = n.s., chi-square test) nor did it correlate with EGFR amplification (n = 183, p = n.s., chi-square test).

Immunohistochemical studies on CA XII showed no association between proliferation by Ki-67 / MIB-1 and CA XII expression (n = 359, p = n.s., Mann-Whitney test). There was a significant association between positive CA XII intensity and positive VEGF status (n = 322, p = 0.032, chi-square test). Neither EGFR amplification (n = 249) nor p53 (n = 141) correlated with CA XII intensity (p = n.s., chi-square test).

5.4. CAs and patient survival

5.4.1. CA II and patient survival

Overall survival data was known for 237 patients. Patient survival was tested by log-rank test in relation to CA II intensity divided into two categories (CA II-ve: no and weak reactions; CA II+ve moderate and strong reactions). In a five-year survival analysis of patients with primary tumours, the patients having tumours with strong or moderate endothelial CA II staining showed significantly worse survival (p = 0.032, log-rank test) (study III, Figure 3). All except one of the total 81 patients having CA II+ve endothelial staining died during the follow-up period, whereas 26 (17%) of the 156 patients with CA II–ve tumours were still alive at the end of follow-up. Endothelial CA II did not predict patient survival in univariate survival analysis when grades II, III, and IV were tested separately (p = n.s., log-rank test). When patients with grade IV astrocytomas were evaluated separately, all the patients with CA II+ve endothelial staining (n = 58) died during the five-year follow-up, whereas 11 (13%) of 87 patients with CA II-ve tumours were still alive. In Cox multivariate analysis, however, endothelial CA II staining was not an independent prognosticator of survival, when conventional prognostic markers (tumour grade, patient age, MIB-1) were also included in the analysis. Only tumour grade (p < 0.001; odds ratio, 2.268) and patient age (p < 0.001; odds ratio, 2.011) showed independent prognostic value.

5.4.2. CA IX and patient survival

Overall survival data was known for 186 patients. When tested by log-rank test, CA IX intensity divided the tumours into four significantly differing prognostic subsets (p = 0.0011; study II, Figure 2). When evaluated separately within different tumour grades, significant correlations were found in grade II (p = 0.0331, log-rank test) and grade IV (p = 0.0163 log-rank test), but not in grade III

tumours (p = n.s., log-rank test). The extent of CA IX immunostaining also showed a significant prognostic value (p = 0.030, log-rank test). Then Cox multivariate analysis was performed. Patient age (cut points: 45 and 60 years), tumour histologic grade (II-IV), CA IX intensity (0-3), CA IX extent (0-3), CA IX nuclear staining (0-1), and MIB-1 were included in the analysis. Remarkably, patient age, tumour grade, and CA IX intensity all showed independent prognostic value (study II, Table 2). However, MIB-1 index did not seem to be an independent prognosticator, although it correlated with overall survival (p = 0.0047, log-rank test) and WHO grade (p < 0.001, Kruskal-Wallis test). No correlation was found between p53 status, apoptotic rate, and survival.

5.4.3. CA XII and patient survival

Overall survival data was known for 287 patients. In primary tumours, CA XII intensity divided the samples into four significantly differing prognostic subsets (p = 0.010, log-rank test). Because the number of negatively stained tumours for CA XII was limited, the intensities 0 and 1 were pooled. When the remaining three categories were tested by log-rank test, the difference was even more significant (p = 0.004, log-rank test) (study IV, Figure 4A). Then, Cox multivariate analysis was performed to test the prognostic significance of CA XII, and important clinicopathological factors, such as patient age, WHO grade, and proliferation by MIB-1 were included in the analysis. Patient age (p < 0.001; odds ratio, 1.959;), tumour grade (p < 0.001; odds ratio, 2.202), and remarkably, CA XII intensity (p= 0.033; odds ratio, 1.250) all had independent prognostic value.

5.4.4. Simultaneous expression of CAs and patient survival

In study IV, simultaneous expression of CA II, CA IX and CA XII was compared to survival of patients with diffuse astrocytomas. Both CA XII and CA IX intensities were evaluated in parallel in 352 cases. CA XII positively correlated with CA IX intensity (p < 0.001, chi-square test). No significant association was found when the expression of CA XII and endothelial CA II were studied (n = 258, p = n.s., chi-square test), and similar results were found between CA XII expression and cytoplasmic CA II (n = 260, p = n.s., chi-square test). Then, CA IX/XII and CA II/IX/XII indices describing simultaneous expression of CA II, CA IX, and CA XII were established as described in the materials and methods. Higher CA IX/XII index significantly correlated with the higher WHO tumour grade (p = 0.002, chi-square test). In addition, CA IX/XII index divided the tumours into three significantly differing prognostic subsets (p = 0.003, log-rank test; study IV, Figure 4B). Furthermore, the simultaneous expression of transmembrane CAs (CA XII and CA IX) was significant when evaluated in Cox multivariate analysis. The analysis revealed that patient age

($p < 0.001$; odds ratio, 1.992), WHO grade ($p < 0.001$; odds ratio, 2.176), and the CA IX/XII index ($p = 0.048$; odds ratio, 1.196) had independent prognostic value. When the prognostic significance of the CA II/IX/XII index was studied in 191 cases, it classified the tumours into four prognostically differing subsets ($p = 0.002$, log-rank test; study IV, Figure 4C), although the CA II/IX/XII index was not significant in multivariate analyses when the variables mentioned above were included.

6. Discussion

6.1. Endothelial carbonic anhydrase II predicts poor prognosis

The endothelial expression of CA II has been found to be induced in the neovessels of several malignant tumours (Yoshiura et al. 2005). These interesting findings led us to investigate endothelial CA II expression in malignant astrocytomas and oligodendrogliomas (study III). Previously, the expression of CA II had been studied in brain tumours (Parkkila AK et al. 1995), but the emphasis was on the localisation of the enzyme in cancer cells instead of the endothelium. Therefore, the results demonstrated for the first time the expression of CA II in the endothelial cells of neovessels in diffusely infiltrating astrocytomas and oligodendrogliomas. As shown in the results, 31% of the malignant astrocytomas were CA II +ve, and the immunopositivity correlated significantly to increasing tumour grade. The oligodendroglial tumours studied seemed to have lower endothelial CA II expression, and the astrocytic component was positively associated with the presence of CA II, which was more often expressed in high-grade mixed oligoastrocytomas than low-grade tumours. All grade II astrocytomas were CA II-ve, while positive CA II endothelial staining was more associated with highly neovascularized grade IV tumours, the most angiogenic of all solid tumours. These findings suggested the involvement of CA II in the malignant progression of astrocytic tumours. Furthermore, the patients having moderate or strong endothelial CA II positivity had worse prognosis when evaluated in univariate analysis.

Endothelial CA II has also been associated with aggressive tumour features in other brain tumours. In meningiomas, endothelial staining of CA II positively correlated to increasing tumour grade (Korhonen et al. 2009). However, CA II reactions do not always correlate to the severity of brain tumours. There is clear evidence that neither endothelial nor cytosolic CA II expression associates with patient survival or other important clinicopathological features in medulloblastomas and primitive neuroectodermal tumours (Nordfors et al. 2010). In fact, the association seems to be most significant in the case of astrocytic gliomas, which indeed represent the most malignant and hypoxic brain tumours, and thus, involve high metabolic stress as a result e.g. from a switch from aerobic metabolism to anaerobic glycolysis (Yamagata et al. 1998). Similarly, Kida et al. (2006) showed the expression of CA II in microvessels of the immature human brain, and after birth the intensity of staining and the number of immunoreactive microvessels declined. They suggested a role for CA in the regulation of intracellular pH in proliferating and differentiating endothelial cells. Furthermore, the rapidly changing environment plays a crucial role in tumourigenesis. Thus, the CA

II enzyme in the tumour vessel endothelium might serve as an essential factor for survival and adaption of proliferating and hypoxic cancer cells.

6.2. Carbonic anhydrase IX is associated to poor prognosis

While the expression of CA IX in brain tumours had been studied earlier (Ivanov et al. 2001, Proescholdt et al. 2005), the analysis of CA IX in a large series of malignant astrocytomas and correlation to clinical features was lacking. In the present study (study II), the expression of CA IX was observed in 78% of astrocytic tumours. As with the previous reports, positive areas were often located in close proximity to necrotic regions of grade IV astrocytomas, and a significant association was found with the presence of necrosis. Also, some cytoplasmic staining was seen in the neoplastic cells of the infiltrative zone. In glioblastomas, these pseudopalisades represent a wave of tumour cells actively migrating away from the central hypoxia. Thus, it seemed possible that activity of CA IX is essential to tumour cells struggling under high metabolic stress. Importantly, the cytoplasmic expression of CA IX had a strong association with increasing tumour WHO grade; 65% of grade II astrocytomas were CA IX-positive, whereas the positivity increased along with the grade, being 73% in grade III astrocytomas and 82% in glioblastomas. The correlation of CA IX with prognosis was then studied in survival analysis, which importantly showed that increasing cytoplasmic CA IX intensity correlated significantly to poor prognosis of the patients in univariate analysis, and this difference was significant for both grade II and grade IV astrocytomas, as well. Finally, Cox multivariate survival analysis showed that CA IX intensity, along with patient age and WHO tumour grade, was an independent prognostic factor. These findings supported the essential role of CA IX in tumourigenesis and malignancy of diffusely infiltrating astrocytomas. CA IX could be clinically used to assess the prognosis of astrocytoma patients.

The correlation of CA IX with prognosis has been confirmed by others. Korkolopoulou et al. (2007) showed that increasing CA IX immunopositivity was associated with a shortened survival in univariate analysis, and when tested in multivariate analysis, they reported similar independent prognosticators; CA IX, tumour grade and patient age were the only parameters entered into the model. In accordance with the present study, perinecrotic distribution of CA IX immunostaining was detected and intensity increased in parallel with the extent of necrosis and histological grade. Furthermore, in a phase II trial, patients with recurrent malignant astrocytomas treated with bevacizumab and irinotecan (a combination of VEGF -neutralizing antibodies) were retrospectively

evaluated (Sathornsumetee et al. 2008). Again, the high expression of CA IX was associated with poor survival outcome in the patient cohort (**Figure 6**). Another hypoxia-regulated marker, VEGF, was associated with radiographic response but not with survival. Additionally, when both CA IX and HIF-2α were simultaneously included in a Cox model as separate factors, only CA IX remained as a statistically significant factor.

In addition to the studies mentioned above, Flynn et al. (2008) investigated hypoxia-regulated protein expression, including CA IX, in another cohort of patients with glioblastomas. They found no significant correlation between CA IX expression and patient survival or tumour grade when low-grade astrocytomas were included in the analysis. Though being statistically insignificant, the patients with CA IX-positive tumours seemed to have a trend towards a worse prognosis. The discrepancy between this and the three studies mentioned above could be due to different immunostaining method used. In this study and also in the study by Korkolopoulou et al. (2007) the M75 antibody is used, which represents an extensively used and reliable CA IX antibody which has no cross-reactivity with other CAs (Saarnio et al. 1998b). Another important issue is the smaller number of patients in the study of Flynn et al. (2008). The latest finding of Yoo et al. (2010) showed that CA IX expression was a predictive factor for poor survival and, again, correlated positively with increasing WHO grade. Thus, the initial finding of the study II seems to be steadily supported by others.

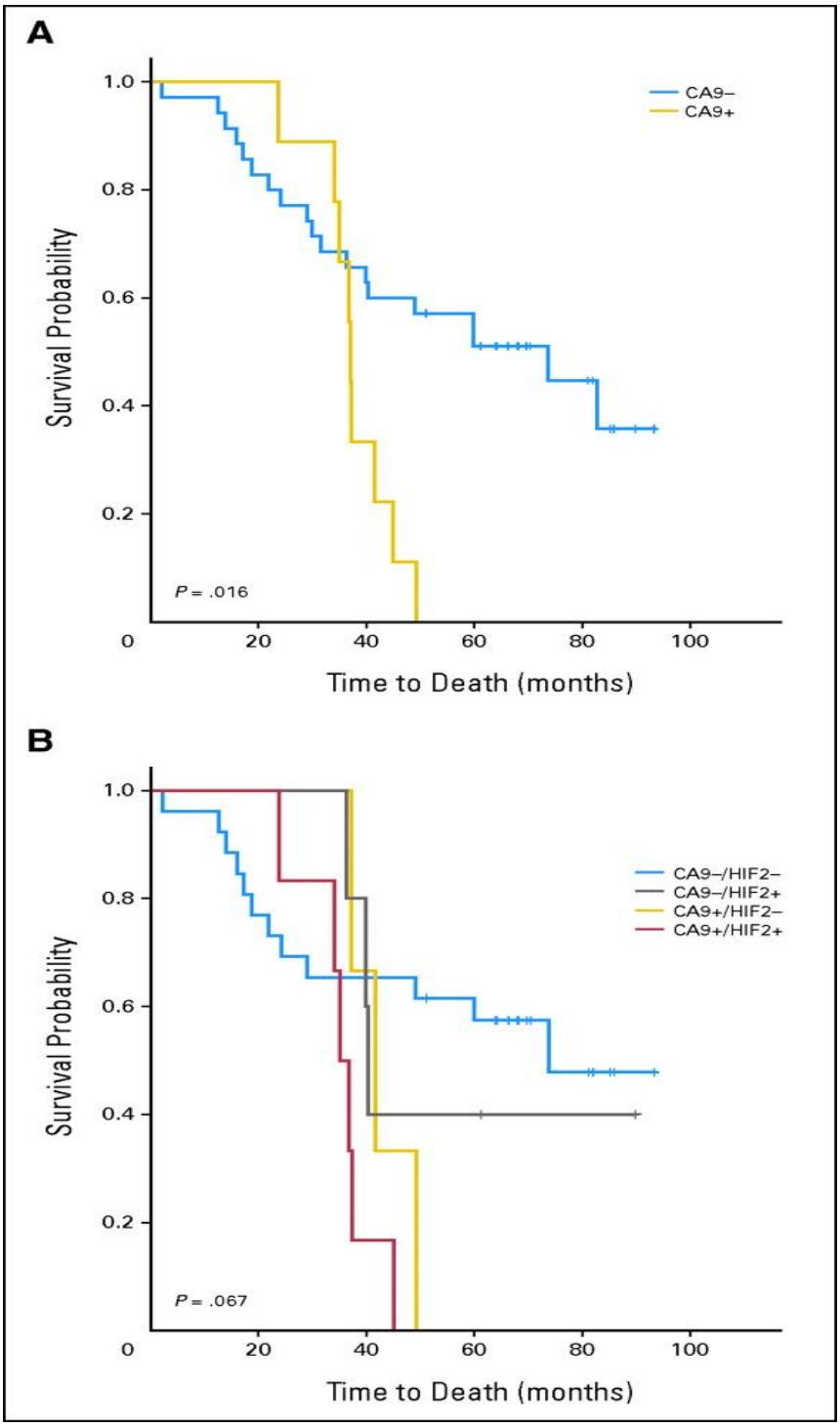

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Figure 6. Kaplan-Meier survival curves stratified by biomarker status. (A) Survival stratified by carbonic anhydrase 9 (CA9) status. (B) Survival stratified by CA9 and hypoxia-inducible factor-2α (HIF-2) status**.** Figure: Sathornsumetee et al. (2008). Reprinted with permission.

For several years, CA IX has been linked to tumour hypoxia and necrosis, and several investigations have been conducted to assess this issue in detail (Ivanov et al. 2001, Proescholdt et al. 2005). Furthermore, *CA9* has been reported to be one of the most consistently hypoxia-inducible genes *in vitro* in human glioblastoma cell lines (Said et al. 2007b). Indeed, CA IX is strongly regulated by hypoxia, and thus, could be used as an additional tool when defining the WHO grade. The small biopsies of gliomas are often a challenge to the neuropathological diagnosis of glioblastoma, especially when the necrotic areas are absent. In these cases, CA IX expression could be useful to aid clinical diagnostics, because it significantly associates with increasing WHO grade and necrosis. However, the regulation of CA IX seems to be more complex and the immunopositivity has also been reported in tumour cells located near the blood vessels (Proescholdt et al. 2005). Correspondingly, it has been suggested that CA IX induction in gliomas may involve hypoxia-independent mechanisms, and acidosis also induces CA IX independently of pericellular hypoxia in glioblastoma cell lines (Ihnatko et al. 2006). Finally, *in vitro* studies on glioblastoma cells have suggested that CA IX expression can result from low oxygen concentrations or constitutive, oncogene-related overexpression (Said et al. 2007a).

6.3. Carbonic anhydrase XII is associated to poor prognosis

Almost all of the diffusely infiltrating astrocytomas showed positive immunostaining reaction for CA XII. The distribution of staining was homogenous within the tumour tissue, and it did not correspond to the presence of endothelial proliferation or necrosis. Furthermore, high expression of CA XII correlated significantly to increasing WHO grade and patient age. Most importantly, CA XII expression divided the tumours into four significantly differing subsets, and was an independent prognostic marker of poor prognosis when evaluated in multivariate analysis. The results presented suggest an important role for CA XII in the pathogenesis of astrocytic gliomas and the enzyme could clinically be used to assess the prognosis of the patients. CA XII has been proposed to be a hypoxia-inducible enzyme, and the significant correlation between positive CA XII and VEGF status supports this. In addition, we characterized the alternatively spliced isoform of CA XII in astrocytic gliomas. Interestingly, CA XII was mainly encoded by a shorter mRNA variant in gliomas. Although the biological significance of the alternatively spliced CA XII remains to be studied in future, the alternatively spliced proteins have been linked previously to carcinogenesis and more aggressive phenotypes of tumours (Venables et al. 2004). In the present study, it was not defined which isoform was expressed in each tissue section. This kind of information would require anti-peptide or monoclonal antibodies which might recognize only one isoform of CA XII.

6.4. Ultrarapid Ki-67® predicts survival in astrocytic gliomas

The prognostic significance of Ultrarapid Ki-67® was studied by setting the optimal cutoff points for best sensitivity and specifity (ROC curve). When cutoff points 7.5%, 10%, and 12.5% were selected, significant results were found in survival analyses. The results obtained support the use of Ultrarapid Ki-67® in predicting the prognosis of patients with astrocytic gliomas. Furthermore, the different proliferation tests were comparable and thus the results can be considered reliable. According to the results of study I, Ultrarapid Ki-67® is a useful additional tool in differential diagnosis of gliomas. As a fast test, it can be routinely performed during surgical operations and the proliferation indices could be used to direct the radicality of operation as well. The results were encouraging and the usefulness of Ultrarapid Ki-67® should be tested also for histopathological diagnostics of other neoplasms.

6.5. Carbonic anhydrases II, IX, and XII are not associated to proliferation measured by Ki-67 / MIB-1 staining

When the endothelial and cytosolic expression of CA II was evaluated in astrocytic gliomas, CA II immunoreactivity did not correlate significantly with cell proliferation determined by Ki-67 / MIB-1. This is in line with the majority of previous studies on CA II, in which CA II has not been associated with proliferartion with the exception of meningiomas (Korhonen et al. 2009). In contrast, CA IX has occasionally been associated with proliferation by Ki-67 / MIB-1, e.g. in colorectal (Saarnio et al. 1998) and lung cancers (Kim et al. 2004, 2005), and even in brain tumours (Proescholdt et al. 2005, Korkolopoulou et al., 2007). In the study II, the proliferation assessed by Ki-67 / MIB-1 was not significantly associated with CA IX expression. In the study of Korkolopoulou et al. (2007), the association was significant even though the correlation was not strong, and their analysis included only limited number of cases. They speculated that the tumour cell population would benefit from the activity of CA IX; the hypoxia-triggered CA IX would assist in the maintenance of intracellular pH and thus permit continued proliferation. Even though this speculation is reasonable, the results of the present study can be considered more reliable, because it included a study group that was seven times larger and the investigation was performed using a computer-assisted proliferation analysis from the most histologically representative areas. In addition, when Proescholdt et al. (2005) found the association between the tumour grade and proliferation by Ki-67, all histologies were pooled into one group in the analysis, including pilocytic astrocytomas, grade I menigiomas, metastases, primitive neuroectodermal tumours, and hemangioblastomas. The fact that they had a more limited number of specimens and a heterogenous

group of tumours with totally different biology suggests that results of the present study are, indeed, more accurate. The addition of meningiomas to the analysis has probably had a significant effect on the results (Korhonen et al. 2009), and the inclusion of low grade tumours, in general, might have led to a misinterpretation of the results. One can also assume that the highly hypoxic and necrotic cell population of diffusely infiltrating astrocytoma would proliferate more slowly if capable of living in the oxidative stress and nutrient-poor state. The association of CA IX with cell proliferation remains complex and was further highlighted in oligodendroglial tumours where CA IX positivity inversely correlated to Ki-67 / MIB-1 staining (Järvelä et al. 2008). Therefore, the CA IX expression seems to be regulated by multiple mechanisms and the expression probably contributes to different biological pathways depending on the tumour type.

The expression of CA XII has also been linked to proliferation of the brain tumours (Proescholdt et al. 2005). The same limitations mentioned above, regarding the tumour materials, were present and the correlation coefficient was again rather low, although statistically significant. The results of the present study suggested that CA XII expression did not correlate to proliferation by Ki-67 / MIB-1. The association of CA XII with proliferation has not been assessed in many articles, and although being mostly referred to as a hypoxia inducible protein, the hypoxia response element has not been characterized for *CA12* gene. Thus, strong causalities between the hypoxia, CA XII expression and proliferation are still questionable.

In the present study, the building of multitissue blocks used in the assessment of CAs was done by a neuropathologist, after careful selection of the most representative tumour area based on H&E staining. Other strategies for the evaluation of immunohistochemistry are widely used, as well. Histological evaluation using larger tissue sections is the conventional method, and can also be preferred as the most reliable method, especially for Ki-67 / MIB-1 staining (Persson and Englund 2008). However, being rather gruelling and time-consuming, it is mainly used for routine diagnostics as well as for experimental studies with a smaller number of cases. In a previous study on Ki-67, a statistically significant survival difference was found in glioblastomas when large tissue sections were evaluated. However, the investigators reported no correlation to survival when they evaluated the areas showing the highest Ki-67 index or analyzed only the histologically most representative areas (Persson and Englund 2008). The methods used in that study were comparable to the methods of our first study, where whole section slides (“control MIB-1”) and areas showing the highest mitotic activity and cellularity (highest MIB-1) were used. We found a strong association with survival when grade II-IV astrocytomas were included in the analysis, suggesting

the usefulness of all our methods in the prediction of survival. As expected, the "highest MIB-1" values were higher than those determined for whole sections. When Ki-67 index was evaluated in relation to the expression of different CAs, the selected tumour tissue was from the most representative area. Another factor contributing to the possible association of CAs with the proliferation index is the intratumoural heterogeneity of diffusely infiltrating astrocytomas. Iakovlev et al. (2007) assessed the possible sampling error due to distributional heterogeneity of CA IX in invasive cervical carcinoma. Indeed, the sampling error of heterogeneous tumours might also represent a challenge in our study. In our opinion, careful selection of the histologically most representative area and the high number of cases studied should correct the error. In future studies, it would be of interest to evaluate the expression of CA IX from additional multiple cores from the same tumours, as suggested by Iakovlev et al. (2007).

6.6. Future prospects

After the publication of studies I and II, both Ultrarapid Ki-67® and CA IX immunohistochemistry have been used in clinical histopathological diagnostics of brain tumours. Intraoperative evaluation of proliferation has been recognised as an important additional tool in the diagnostics of astrocytomas. Furthermore, the expression of hypoxia-inducible and necrosis-associated CA IX helps in decision making when evaluating the aggressivity of the tumour. This has been true especially when it has been difficult to decide the best treatment strategies concerning whether the tumour is operable or not. CA IX has also been found to represent a reliable marker of hypoxia even with small biopsies where necrosis is often absent.

Primary (*de novo*) and secondary glioblastomas differ in their genetic background as well as in clinical behaviour (Ohkagi et al. 2004, Louis et al. 2007). For example, the activation of HIF-1 by *IDH1* mutations in secondary glioblastomas has been recently studied and proposed to provide new insights into the metabolism of cancer cells (Reitman et al. 2010). One interesting subject to study further would be a comparison of the expression of different CAs and HIF in primary and secondary glioblastomas, and furthermore between primary and secondary lower grade tumours. This would increase knowledge of the different regulation pathways in gliomas as well as possible differeces in CA regulation. In our material, there was no correlation between CA II, CA IX, and CA XII expression levels and the subtype of glioblastoma (total number of glioblastomas was 401 of which only 15 were classified as secondary) (p = n.s., chi-square test, data not shown). Because there was such a limited number of secondary glioblastomas, it is difficult to make conclusions from the

present data. There were only four patients in whom we studied the expression of all CAs in both the pre-existing, less malignant precursor lesion and the subsequent higher grade astrocytoma. Due to the small number of specimens, statistical comparison between these tumours and the CA expression could not be made. A larger series of newly conducted studies is required to evaluate this issue in detail.

Based on new theories of cancer progression, cancer stem cell populations have the potential to play an important role in tumour progression due to their self-renewal capacity and strong proliferative manner. Malignant gliomas have tumour subpopulations which share characteristics with neural stem cells. Bao et al. (2006) studied the potential of stem cell–like glioma cells to support tumour angiogenesis. The stem cell-like glioma cells isolated from human glioblastoma and xenografts, generated tumours more aggressively into the brains of immunocompromised mice. These tumours were characterised by widespread angiogenesis, necrosis, and haemorrhage, in strong contrast to the brains implanted with non-stem-cell populations. They found out that stem cell-like glioma cells also secreted markedly elevated levels of VEGF and this was strongly induced by hypoxic conditions. Interestingly, the study of Li et al. (2009) showed that glioma stem cells differentially respond to hypoxia with distinct HIF induction patterns in comparison to normal neural progenitors. Furthermore, Folkins et al. (2009) studied tumour vascular development, by comparing tumour xenografts of the C6 glioma cell line containing either a low or high fraction of cancer stem cells. High fraction xenografts exhibited increased microvessel density and blood perfusion and induced increased mobilization and tumour recruitment of bone marrow–derived endothelial progenitor cells. Higher levels of endothelial cell proliferation and tubule organization *in vitro* were also reported, and cancer stem cell-high cultures and tumours expressed increased levels of the proangiogenic factors including VEGF. Malignant gliomas are highly aggressive cancers and tumour cell populations suffer from great hypoxia, thus being dependent on angiogenesis. According to this study, CA II is expressed in tumour vessel endothelium and might be involved in the development of tumour microvessels. Added to this, the findings of CA IX and CA XII expression, induced by hypoxia, support the idea that the association between cancer stem cells and CAs might also be of importance. It would be interesting to evaluate the expression and regulation of CAs in cancer stem cells isolated from gliomas.

Brat et al. (2004) proposed that pseudopalisades represent differing stages and histologic samplings of astrocytoma cells migrating away from a hypoxic/anoxic focus. They concluded that pseudopalisading cells are 5-50% less proliferative and 6-20 times more apoptotic than the cells in

adjacent astrocytoma, indicating that cell accumulation does not result from increased proliferation or resistance to apoptosis. Furthermore, pseudopalisading cells showed nuclear expression of HIF-1α, consistent with their hypoxic nature. Singh et al. (2003) also showed that CD133-positive glioblastoma stem cells formed pseudopalisading pattenrs *in vivo*. Study II has also shown that CA IX is expressed intensively in the border of the necrotic area and could also be of importance in the complex biological process, possibly including glioblastoma stem cells. Indeed, hypoxic upregulation has been proposed to be an essential factor in tumour angiogenesis and microvascular proliferation.

CA IX has been linked to several cancer tissues, whereas the normal tissue is mostly negative. This also seems to be the case for astrocytic gliomas. Thus, several treatment strategies have been suggested based on CA expression. One possible option is to use cancer-specific antibodies. CA IX is an attractive target protein because it has a unique extracellular structure composed of the two different domains: the CA and proteoglycan domains. In this regard, renal cell carcinoma has been the most extensively studied neoplasia, because CA IX is highly overexpressed in these tumours (Liao et al. 1997). Although therapeutic methods for renal cell cancer have been under discussion for several years, therapeutic trials with high-dose radiolabeled CA IX antibody (cG250) and CA IX-loaded dendritic cells are still in phase 1 or 2 (Stillebroer et al. 2010). A significant breakthrough in clinical trials remains to be achieved. Another strategy which could be beneficial is to use CA-selective inhibitors (Pastoreková et al. 2004). Glioma cells probably use CAs as key enzymes to adapt to the hostile enviroment caused by metabolic stress of cancer cells. The enhanced secretion of protons to the extracellular space causes the extracellular acidification, which can further facilitate the spread and invasion of cancer cells (Svastová et al. 2004). As in brain tumours, the presence of highly active CA in cancer cells could also increase the capability of glioma cells to infiltrate the neighboring tissue. The inhibition of this process would potentially disturb the invasion processes of cancer cells.

Vaccination has been proposed as a possible therapeutic intervention strategy in cancer therapy. Greiner et al. (2006) found a significant correlation between high mRNA levels of CA IX and longer overall survival in acute myeloid leukemia. The authors state that CA IX could induce a strong antileukemic immune response. This finding supports the use of CA IX as a potential target for immune therapy. Additionally, Uemura et al. (2006) performed a phase I peptide vaccination procedure in patients with progressive cytokine-refractory metastatic RCC. Vaccination with tumour-RNA pulsed dendritic cells led to increased numbers of CA IX peptide-specific cytotoxic T-

lymphocytes and IgG levels without any major adverse event. Three patients with multiple lung metastases showed partial responses with disappearance and shrinking of metastatic lesions, and a stable disease for over six months was observed in six patients. However, further studies are required to confirm these findings in larger study cohorts. Autoimmunity against tumour antigens is one strategy to fight against malignant diseases. The presence of serum CA autoantibodies has been studied in patients with Hodgkin's disease and multiple myeloma, who relapsed/progressed after high dose therapy and autologous stem cell transplantation (Lakota et al. 2008). Immunoreactions against CA enzymes were different in Western blots using sera obtained from patients with various types of diseases. Sera of patients with Hodgkin's disease reacted with CA I, II and XII; sera of multiple myeloma patients reacted with CA I, II, XII and IX. In addition, patients with CA autoantibodies had a significant survival benefit over those who did not develop CA autoantibodies. The authors suggested that the high CA autoantibody levels may keep tumour cells in a dormant state. To our knowledge, levels of CA autoantibodies have not been assessed in patients with brain tumours.

In renal cancer, overexpression of CA IX is associated with loss of functional pVHL and the expression levels usually decrease with advancing stage (Bui et al. 2003). It has been shown that patients with increased CA IX expression may be more likely to respond to IL-2 based therapy in renal cancer (Atkins et al. 2005). The biological mechanism behind these studies has been proposed by Wang et al. (2008), suggesting that CA IX has chaperone-like functions and CA IX shed from tumours may play a direct role in stimulating an adaptive immune response in clear-cell renal tumours. They also showed that a soluble form of CA IX, found to be shed from the surface of tumour cells (Závada et al. 2003) and reported to be regulated by a metalloprotease dependent process (Zatovicova et al. 2005), had the same chaperone-like functions. Moreover, under hypoxic conditions, soluble CA IX may alter the microenvironment and facilitate an immune response against extracellular antigens. Although the expression of CA IX is regulated by a slightly different mechanism in gliomas (increased HIF-1α due to hypoxia) than in RCC (mutation in *VHL* gene), the immunomodulation strategy seems interesting and should be studied further. A recent study by Pedretti et al. (2010) assessed the therapeutic properties of temozolomide in combination with F16-IL2, a clinical-stage immunocytokine consisting of human interleukin (IL)-2 fused to the human antibody F16. Their results showed that the drug induced complete remission in animals, led to a consistent size reduction of intracranial xenografts and resulted in a longer survival of animals. They concluded that the combined use of temozolomide with F16-IL2 should be clinically investigated.

The CAs II, IX, and XII have previously been linked to many malignancies and thus referred to as "tumour-associated" enzymes. It seems that they are all important in the tumourigenesis of astrocytic gliomas according to the individual analyses. When the simultaneous expression of CAs was evaluated in this study, the presence of transmembrane CAs (CA XII and CA IX) appeared as a significant predictor of survival in both univariate and multivariate analyses. Furthermore, the model including all CAs was prognostically significant in univariate analysis. These results suggest that all tumour-associated CAs are valuable markers in the prediction of survival in astrocytomas.

Astrocytic gliomas remain devastating tumours despite being studied intensively for decades. The key factor for achieving a breakthrough in the treatment of gliomas is to increase knowledge of the biology of these heterogenous and complex tumours. Indeed, glioblastomas are characterized by strong regional phenotypic heterogeneity, and have been proposed to represent the multicellular hierarchical unit comprising not only the astrocytic cells and cancer stem cells, but also different communicating cell populations. CAs have been shown here to be associated with hypoxia, necrosis, and angiogenesis – features traditionally linked to the tumourigenesis of brain tumours. According to this study, CAs seem to be potential target molecules in gliomas, which should be evaluated in clinical trials aiming to either eradicate the tumour cell population or turn the astrocytoma into a more chronic and stable disease.

7. Summary and conclusions

1) The expression of CAs was a common phenomenon in astrocytic gliomas. CA II was expressed in cancer cell cytoplasm in 36% of cases, whereas endothelial expression was detected in 45% of cases. Interestingly, endothelial CA II positivity was located on the proliferative neovessels of glioblastomas. Positive staining for CA IX was detected in 78% of tumours. In glioblastomas, the highest signals for CA IX were usually found in regions near the necrosis. Almost all tumors showed positive immunostaining for CA XII and the distribution of immunostaining was usually homogeneous.

2) Endothelial CA II expression was neither associated with p53 expression nor the presence of necrosis in the same tissue section. CA IX intensity correlated significantly with the presence of necrosis, but it was neither associated with apoptosis nor with endothelial cell proliferation. Neither endothelial proliferation nor necrosis correlated with CA XII intensity. None of the CAs studied (endothelial CA II, CA IX and CA XII) were associated with proliferation by MIB-1 / Ki-67. Positive endothelial CA II staining significantly correlated with the presence of EGFR amplification. CA IX intensity showed no significant association with p53 nor did it correlate with EGFR amplification. CA XII intensity correlated significantly with positive VEGF status, whereas it showed no association to EGFR amplification or p53 expression.

3) All CAs showed clinical value in astrocytic gliomas. Increasing expression of endothelial CA II was significantly associated with a higher tumour grade, and the patients whose tumours had strong or moderate endothelial CA II staining showed significantly worse survival in univariate analysis. Similarly, both CA IX and CA XII were associated with increasing WHO grade. Their presence in tumour cells predicted poor survival in univariate and multivariate survival analyses. CAs seem to be potential target molecules for therapy in gliomas, which should be evaluated in clinical trials. When Ultrarapid Ki-67® staining in frozen sections and MIB-1 immunostaining in paraffin embedded sections were compared, very high quantitative correlation was found in astrocytic gliomas. The Ultrarapid Ki-67® indices were significantly higher in high grade tumours (grades III and IV) than in low-grade astrocytomas (grades I and II). As a fast and reliable test, intraoperative Ultrarapid Ki-67® immunostaining was found to be a useful adjunct to morphological diagnosis and grading of astrocytic tumours.

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Joonas Haapasalo

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10. Original communications I - IV

ORIGINAL ARTICLE

Ultrarapid Ki-67 immunostaining in frozen section interpretation of gliomas

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Background: Astrocytic tumours, the most common gliomas, are often classified intraoperatively using standard morphological staining. The final diagnosis and grading of gliomas on paraffin wax sections is often assisted by Ki-67 immunohistochemistry, but standard immunostaining protocols take too long to be used intraoperatively.
Aims: To investigate a new rapid Ki-67 immunohistochemical test for its use in an intraoperative setting.
Methods: The new Ki-67 immunostaining (Ultrarapid-Ki67®) method on frozen sections can be carried out in 10 minutes. Thirty four pilocytic and diffuse astrocytomas were immunostained by rapid Ki-67 and results were compared with corresponding MIB-1 staining, histological grading, and prognosis.
Results: The staining protocol was practical to perform and the results were morphologically and quantitatively indistinguishable from those after immunostaining with MIB-1, an antibody recognising Ki-67 in paraffin wax embedded tissue. A comparison of Ultrarapid-Ki67 and MIB-1 immunostaining of paraffin wax sections showed almost identical quantitative correlation in astrocytic gliomas ($r=0.916$; $p<0.001$). The Ultrarapid-Ki67 indices (percentage of positive cells) of low grade (I/II) astrocytomas ranged from 0% to 6.1%, whereas those of representative high grade (III/IV) tumours were significantly higher (range, 5.6–45%; $p<0.001$). The best prognostic cutoff point for Ultrarapid-Ki67 was 7.5%, which divided diffuse grade II–IV astrocytomas into significantly differing subsets ($p=0.0008$).
Conclusion: Ultrarapid-Ki67 immunostaining is a useful adjunct to morphological diagnosis and grading of astrocytic tumours, and as a fast test (~10 minutes for staining plus three to four minutes for scoring), it could be used in routine intraoperative diagnosis of gliomas and other neoplastic diseases.

Intraoperative frozen section diagnosis of astrocytic tumours is valuable to neurosurgeons for several reasons. First, the neoplastic nature of the removed tissue needs to be confirmed, because surgery is adjusted according to the histopathological nature of the tissue. For example, glioblastomas, metastases, and granulomas may resemble each other macroscopically, but their surgical treatments differ from each other. Second, it is necessary to distinguish benign neoplasms and gliotic tissue from anaplastic and grade IV gliomas. Neurosurgeons may adjust the extent of the resection, and in cases of malignant tumours, apply BCNU (carmustine) wafers in the resected tumour bed or use gene therapeutic approaches in clinical trials.

The interpretation of intraoperative frozen sections of gliomas is demanding and requires the evaluation of subtle histological features not always familiar to the general pathologist, whose daily diagnostic routine rarely includes central nervous system tumours. In some cases, the nature of an astrocytic tumour may remain uncertain even when investigated by an experienced neuropathologist. It has been estimated that intraoperative diagnostic accuracy with regard to central nervous system tumours is approximately 90%.[1] The World Health Organisation (WHO) 2000[2] grading criteria of the most common gliomas—diffuse astrocytomas—include the presence or absence of atypia, mitoses, endothelial proliferation, and necrosis. However, strict interpretation of these criteria may not always be straightforward in frozen sections. For example, diffuse astrocytomas without necrosis or endothelial proliferation are divided into grade II and III tumours according to the absence or presence of mitotic figures, respectively. However, solitary mitotic figures may be seen in grade III astrocytomas prognostically resembling grade II astrocytomas.[3] Furthermore, as a result of the relatively poor morphology of frozen sections, mitotic figures are often difficult to identify with certainty.

> "Unfortunately, standard immunohistochemical procedures typically take two to four hours to perform, precluding their use during intraoperative frozen section diagnosis"

The proliferation activity of tumours can also be assessed by means of Ki-67 immunohistochemistry, which is now widely used in clinical pathology and neuropathology diagnostics. Burger and co-workers were the first to show the tight association between Ki-67 immunopositivity and glioma grade in freshly frozen samples of astrocytomas.[4] We and others have described the prognostic value of immunostaining with MIB-1,[5–7] an antibody recognising the Ki-67 antigen in paraffin wax embedded tissue.[8] Based on a large number of studies, the added prognostic information provided by Ki-67 immunostaining is now well established with regard to astrocytic tumours. The latest WHO classification of central nervous system tumours[2] includes Ki-67 as an additional tool in histological typing and grading, although it cannot be regarded as being entirely prognostic in individual cases.

On the basis of abundant data and the widespread clinical use of paraffin wax embedded material, Ki-67 immunostaining could also be applied to the intraoperative diagnosis of astrocytic tumours. Unfortunately, standard immunohistochemical (IHC) procedures typically take two to four hours to

Abbreviations: DAB, diaminobenzidine; HE, haematoxylin and eosin; IHC, immunohistochemistry; ROC, receiver operating characteristics; WHO, World Health Organisation

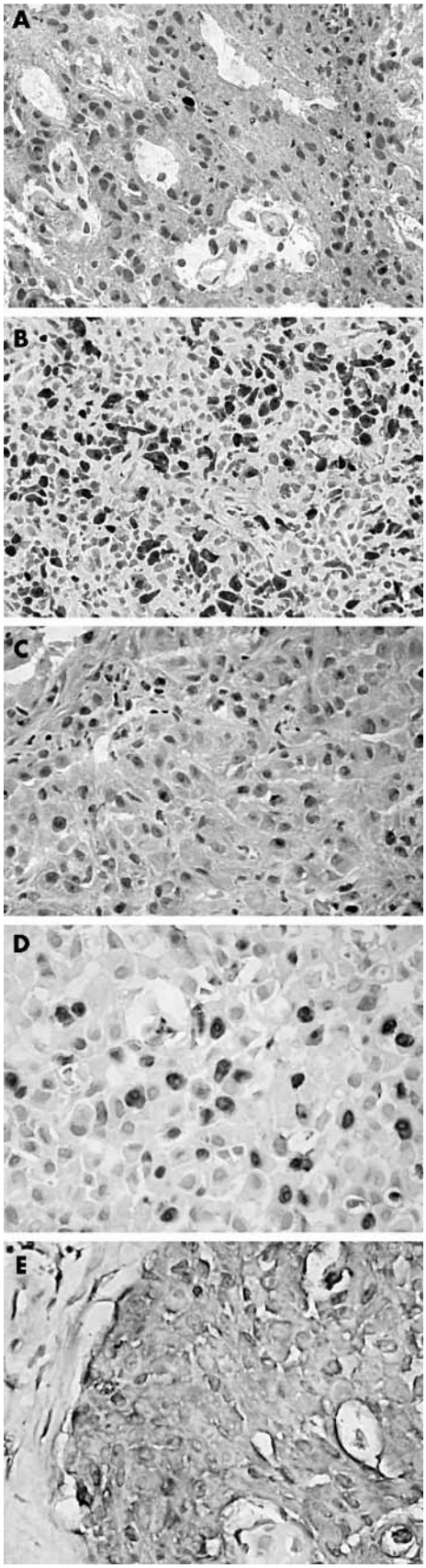

Figure 1 Immunostaining in a frozen section of (A) low grade and (B) high grade glioma using the Ultrarapid-Ki67 kit. An additional clinical case demonstrating the usefulness of intraoperative Ki-67 is shown in (C–E). (C) Haematoxylin and eosin staining of a cortical tumour was inconclusive, leaving the differential diagnosis of primary brain tumour (preferentially meningioma, one mitosis/10 high power fields) and metastatic carcinoma. (D) A high Ki-67 labelling index ruled out low grade and benign tumours. (E) Additional rapid intraoperative pan-cytokeratin staining defined the tumour finally as a metastatic carcinoma.

perform, precluding their use during intraoperative frozen section diagnosis. Rapid or semirapid immunostaining protocols have been described,[9 10] but so far they have not gained wide acceptance in routine practice. In our study, we introduce an ultrarapid immunostaining protocol, which makes it possible to perform Ki-67 immunostaining intraoperatively. We compared rapid frozen section Ki-67 immunostaining with the corresponding MIB-1 staining of formalin fixed, paraffin wax embedded samples. Proliferation indices were also compared with regard to their ability to predict the final histological diagnosis and prognosis of patients with grade I–IV astrocytomas.

METHODS

Patients and tumours

Tumour material for our study was collected from 34 patients surgically treated for astrocytoma at Tampere University Hospital, Finland from 1996 to 2000. At the time of intraoperative consultation, frozen sections (6–8 μm thick) were cut from a sample selected by the neurosurgeon and stained with haematoxylin and eosin (HE). After confirming the intraoperative diagnosis, additional sections were cut, sealed airtight, and stored unfixed at −70°C. Histopathological typing and grading were performed by an expert neuropathologist according to the criteria presented by the WHO.[2] The material from the 34 patients (14 male and 20 female) consisted of nine pilocytic astrocytomas (grade I) and 25 diffuse astrocytomas (nine grade II astrocytomas, four grade III anaplastic astrocytomas, and 12 grade IV glioblastomas). The age of the patients ranged from 2 to 81 years (median, 48). The tumours were radically resected and most patients with high grade gliomas also received radiotherapy. Follow up data were available for at least 36 months in all cases.

Ultrarapid Ki-67 immunostaining

Slides were immunostained using the Ultrarapid-Ki67® IHC kit (Immuno Diagnostic Inc, Hämeenlinna, Finland), according to the manufacturer's instructions. Briefly, the slides were first fixed in 100% acetone/0.03% hydrogen peroxide for one minute at room temperature and air dried. Tissue sections were encircled with a hydrophobic pen (PAP-pen) to help keep antibody droplets on the sections. The sections were first incubated with ready to use mouse Ki-67 antibody for three minutes, followed by a short rinse in phosphate buffered saline (10 seconds with continuous slide agitation). The detection antibody (antimouse–horseradish peroxidase polymer) was applied and the sections were incubated for three minutes before rinsing with phosphate buffered saline for 10 seconds. Diaminobenzidine (DAB) was used as chromogenic substrate and was prepared by adding one drop of reagent A and one drop of reagent B to 500 μl of DAB diluent. The slides were incubated in DAB solution for one minute and washed with distilled water. Antibody and DAB incubations were carried out on a thermal plate set at 41°C. The slides were counterstained with haematoxylin (10 seconds), dehydrated, and embedded according to routine frozen section protocols.

Control Ki-67 (MIB-1) immunostaining

After intraoperative consultation, the remaining frozen tissue block was fixed in 10% buffered formalin overnight and embedded in paraffin wax. Sections cut from this block were immunostained with MIB-1 antibody (DakoCytomation,

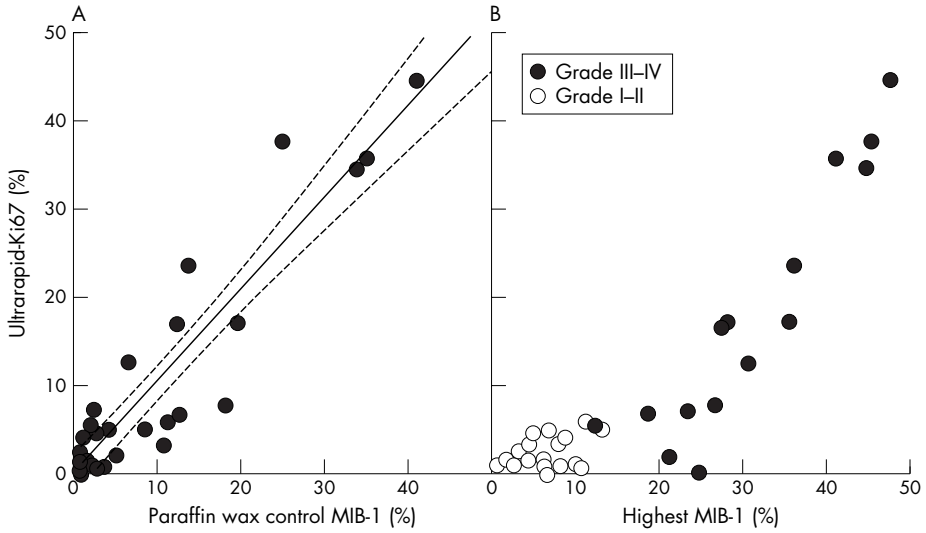

Figure 2 Growth fractions (percentage of Ki-67 positive cells) by rapid frozen section and standard paraffin wax embedded section Ki-67 immunostaining in matched pairs of human astrocytoma samples. (A) Comparison between Ultrarapid-Ki67 values of frozen sections and corresponding MIB-1 values of paraffin wax embedded control sections analysed by the same observer ($r = 0.916$; $p < 0.001$). The regression line and its 95% confidence intervals are plotted in solid and dashed lines, respectively. (B) Comparison between Ultrarapid-Ki67 index of frozen sections and selected tumour areas showing highest MIB-1 index of paraffin wax embedded tumour material analysed by computer assisted image analysis ($r = 0.790$ $p < 0.001$). Open symbols, low grade tumour in final diagnosis; filled symbols, high grade tumour in final diagnosis.

Glostrup, Denmark). Heat induced epitope retrieval (Tris/EDTA buffer (pH 9.0) 2 × 7 minutes in an microwave oven) and an automated immunostaining protocol (TechMate immunostainer) were used. These slides were counterstained with haematoxylin and analysed visually. This staining was termed ''control MIB-1''. MIB-1 staining was also carried out using a tissue block showing the highest mitotic activity and cellularity, as described previously.[11] These sections were identified from all the HE stained sections by a neuropathologist. These tissue sections were stained with MIB-1, as described above, and counterstained with methyl green. In the comparisons this staining was termed ''highest MIB-1''.

Scoring of immunopositivity

Scoring of Ultrarapid-Ki67 and control MIB-1 slides was carried out in the area of highest immunopositivity by two observers (I and II). In total, 300–500 neoplastic tumour cells were analysed. Necrotic and haemorrhagic tumour areas were ignored. In the visual estimation only definitely brown nuclei were recorded as positive. The results were expressed as percentage of immunoreactive tumour cell nuclei. Analysis of immunopositivity of the highest MIB-1 slides was carried out by observer II using a CAS-200 image analysis system (CAS-200 Software; Becton Dickinson, Mountain View, California, USA), as described previously.[5] The proliferation index obtained by computer assisted image analysis was also reported as the percentage of immunopositive nuclei.

Statistical methods

Pearson's correlation coefficient (r), and Mann-Whitney and Kruskal-Wallis tests were used to study concordance of the IHC tests. Optimal cutoffs for proliferation indices in survival

Table 1 Ki-67 indices by histological grade

			Ultrarapid-Ki67		Control MIB-1	Highest MIB-1
Grade	N		Observer 1	Observer 2	Observer 1	Observer 2
I	9	Mean	2.69	2.59	3.1	6.92
		SD	2.20	3.00	3.92	3.87
		Median	1.50	1.00	1.05	6.20
		Range	0–6.10	0–9.40	0.30–10.50	0.40–13.00
II	9	Mean	2.41	2.68	1.74	5.47
		SD	1.52	1.55	1.24	3.07
		Median	1.70	2.30	1.35	5.80
		Range	0.90–5.10	1.20–5.3	0.30–3.80	1.30–10.30
III	4	Mean	3.73	5.05	4.78	19.08
		SD	3.05	3.62	5.37	5.22
		Median	3.85	5.40	3.3	19.75
		Range	0.30–6.90	0.30–9.10	0.20–12.30	12.20–24.60
IV	12	Mean	21.92	19.33	18.29	36.71
		SD	13.43	10.89	12.64	10.10
		Median	17.25	17.55	15.65	35.65
		Range	5.90–45.00	7.20–41.20	2.10–40.60	23.20–55.50
Total material	34	Mean	9.52	8.81	8.67	18.48
		SD	12.21	10.31	11.02	15.68
		Median	4.90	5.10	3.55	11.60
		Range	0–45.00	0–41.20	0.20–40.60	0.40–55.50

analyses were defined using receiver operating characteristics (ROC) curves, as described previously.[5] The significance of survival differences was determined by the log rank test. Statistical analyses were carried out using SPSS for Windows software (SPSS Inc, Chicago, Illinois, USA).

RESULTS

The staining pattern of Ultrarapid-Ki67 frozen sections of gliomas was similar to that of MIB-1 staining in paraffin wax embedded tumour sections. Ultrarapid-Ki67 reagents revealed proliferating astrocytoma nuclei (fig 1), which were scarce in low grade tumours and abundant in glioblastoma (fig 1A, B). During the preparation of our article intraoperative IHC was used in the clinical setting. A clinical case demonstrating the usefulness of intraoperative Ki-67 is shown in fig 1C–E. HE staining of a cortical tumour was inconclusive, leaving the differential diagnosis of primary brain tumour (preferentially meningioma) and metastatic carcinoma. A high Ki-67 labelling index (30–40% Ki-67 positive tumour cells) ruled out low grade and benign tumours. Additional rapid pan-cytokeratin staining (1E) defined the tumour finally as a metastatic carcinoma.

Figure 2A shows the proliferation indices of Ultrarapid-Ki67 frozen sections and corresponding MIB-1 staining in paraffin wax embedded sections (control MIB-1). The correlation between the two methods was nearly perfect ($r = 0.916$; $p < 0.001$). When the highest proliferation indices in paraffin wax embedded sections (highest MIB-1) were used for comparison, the correlation remained substantial ($r = 0.790$; $p < 0.001$; fig 2B). Interobserver variation between observers I and II was minimal, as indicated by a correlation coefficient of $r = 0.946$ ($p < 0.001$, data not shown).

Table 1 shows the mean and median Ultrarapid-Ki67 and MIB-1 proliferation indices by final histological grade. Proliferation indices by all three methods differed significantly with histological grade (Ultrarapid-Ki67: $p < 0.001$; control MIB-1: $p = 0.001$; highest MIB-1: $p < 0.001$, Kruskal-Wallis test). The mean and median values for Ultrarapid-Ki67 and control MIB-1 were comparable. The analysis concentrating in the most proliferative areas (highest MIB-1) yielded higher proliferation indices.

Table 2 shows a comparison of intraoperative tumour grade, Ki-67 indices, and final tumour grade. The tumours were divided into low grade (grades I and II) and high grade (grades III and IV) astrocytomas according to the original intraoperative frozen section interpretation based on HE staining. A significant difference ($p < 0.001$, Mann-Whitney test) and only a minor overlap were found when the proliferation indices (Ultrarapid-Ki67) were compared between these groups (table 2).

Histopathological differential diagnosis of grading (low grade versus high grade) could not be performed in one case intraoperatively (uncertain frozen section grading, mostly necrotic sample) (table 2). The final diagnosis in this patient, after thorough investigation of all paraffin wax embedded tissue, was glioblastoma (grade IV). Interestingly, the Ultrarapid-Ki67 index of 300–400 non-necrotic cells present in this frozen section was 16.9%, which would have indicated a high grade tumour. There were two cases misinterpreted as low grade tumour intraoperatively, although the final diagnosis was anaplastic astrocytoma (table 2). The original intraoperative diagnoses were based on the lack of mitoses. In concordance with mitotic counting, the Ultrarapid-Ki67 and control MIB-1 data showed low proliferation indices in both cases (0.3% and 2.1%, 0.2% and 4.8%, respectively; table 2). More representative and extensive postoperative analysis using all the resected material revealed tissue areas with mitotic figures and higher MIB-1 indices (highest MIB-1: 24.6% and 21.0%, respectively), resulting in the diagnosis of grade III anaplastic astrocytoma in both cases.

The prognostic value of Ultrarapid-Ki67 and the optimal cutoff points were studied by means of ROC analysis. Figure 3 shows the prognostic sensitivity (true positive; that is, deceased patient with Ki-67 index above cutoff point) and specificity (100 false positive; that is, a living patient with Ki-67 index above cutoff point) during a three year follow up period for Ultrarapid-Ki67 and the two MIB-1 assays. The almost superimposed ROC curves indicate that Ultrarapid-Ki67, control MIB-1, and highest MIB-1 were of similar prognostic value. Optimal cutoffs yielding the best sensitivity and specificity for the three tests were 7.5%, 10%, and 12.5 %, respectively (fig 3). Using these cutoffs, a highly significant distinction was found in survival analysis (Ultrarapid-Ki67: $p < 0.0001$; control MIB-1: $p = 0.0047$; highest MIB-1:

Table 2 Comparison of intraoperative frozen section grading, Ki-67 indices, and final grading using paraffin wax embedded sections

	Intraoperative frozen section grading			
	Low grade (N = 20)	High grade (N = 13)	Uncertain (N = 1)	p Value*
Ultrarapid-Ki67				
Mean	2.4	19.9	16.9	
Median	1.7	17.2	16.9	<0.001
Range	0–6.1	5.6–45.0		
Control MIB-1				
Mean	2.5	17.6	†	
Median	1.1	13.4		<0.001
Range	0.2–10.5	1.8–40.6		
Highest MIB-1				
Mean	7.9	34.1	27.3	
Median	6.3	35.3	27.3	<0.001
Range	0.4–24.6	12.2–55.5		
Final grade				
I	9	0		
II	9	0		0.004
III	2‡	2		
IV	0	11	1	

*Mann-Whitney test for proliferation indices and χ^2 test for grades; †insufficient tumour tissue for control MIB-1 staining; ‡two tumours with Ultrarapid-Ki67 indices of 0.3 and 2.1, control MIB-1 of 0.2 and 4.8, and highest MIB-1 of 24.6 and 21.0, respectively.

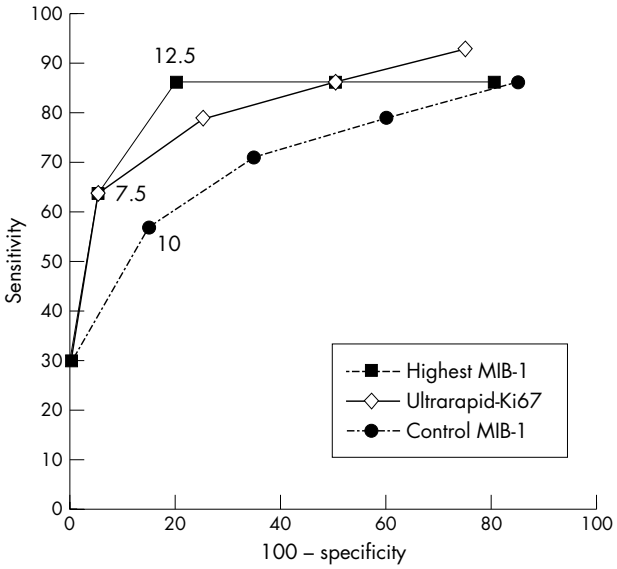

Figure 3 Receiver operating characteristics curve showing prognostic true and false positivity rates of the Ki-67 indices. The predictive properties (%) are calculated on the basis of three year survival of the 34 patients divided into six subgroups of approximately equal size. The patient groups are placed in the curve according to decreasing proliferation: patients with the highest tumour proliferation are at the bottom left corner of the curve, and patients with the lowest tumour proliferation are at the top right corner of the curve. The most efficient cutoff point of each Ki-67 index is shown.

$p = 0.0001$; log rank test). Prognostic significance was also seen when only diffuse astrocytomas (grades II–IV) were included in the analysis (Ultrarapid-Ki67: $p = 0.0008$; control MIB-1: $p = 0.0167$; highest MIB-1: $p = 0.005$).

DISCUSSION

It is well known that intraoperative grading of astrocytomas is a demanding task when based on frozen section HE staining alone. In our study, we evaluated an ultrarapid immunostaining protocol for Ki-67, which can be used intraoperatively to aid frozen section interpretation—typing and grading of gliomas. Using material from 34 patients, we found an almost perfect correlation between Ultrarapid-Ki67 and MIB-1 staining, with MIB-1 staining being carried out in matched paraffin wax embedded samples. The prognostic value of Ultrarapid-Ki67 was as good as that of the paraffin wax embedded section MIB-1 procedure. The mean and median values of the Ultrarapid-Ki67 data in each glioma grade matched well with the cutoff points suggested in the WHO grading scheme,[2] further indicating the validity of the method. Thus, the results indicate that estimation of the tumour proliferation rate could aid pathologists not only postoperatively, but also in the intraoperative differential diagnosis of borderline cases.

> ''Using material from 34 patients, we found an almost perfect correlation between Ultrarapid-Ki67 and MIB-1 staining, with MIB-1 staining being carried out in matched paraffin wax embedded samples''

When considering the usefulness of immunohistochemical tumour proliferation assessment, overlapping Ki-67 indices in different histopathological categories and considerable intratumorous regional variation of proliferation are well known problems. The advantage of Ki-67 comes from its wider coverage of the cell cycle, resulting in higher percentages of positively labelled proliferating cells when compared with proliferating cell nuclear antigen or mitotic indices. This provides a better statistical distinction between different histological grades.[5 11 12] At the practical level, another important issue to consider is sampling. Variation in tumour proliferation indices is much smaller when evaluation is systematically performed in the area of highest proliferation.[11] Unfortunately, samples from these tumour areas are not always sent for intraoperative diagnostic assessment. This fact was illustrated by the two discordant cases showing very low indices in the Ultrarapid-Ki67 and control MIB-1 tests, and absence of mitotic figures in histology. However, postoperative analysis using all the formalin fixed and paraffin wax embedded material revealed mitoses and high MIB-1 indices, resulting in a final diagnosis of grade III anaplastic astrocytoma. These false negative cases show that frozen section based grading is always preliminary only. Regional intratumorous heterogeneity may result in underestimation of malignancy intraoperatively, irrespective of which method of tumour proliferation assessment is used.

The usefulness of Ultrarapid-Ki67 immunostaining was highlighted in one case in which intraoperative diagnosis remained uncertain as a result of extensive tissue necrosis. If available at the time of intraoperative consultation, a high Ultrarapid-Ki67® index (16.9%) would have suggested a high grade astrocytoma. The final diagnosis in this case was glioblastoma with the highest MIB-1 index of 27.3%. As presented with the clinical case example, we believe that intraoperative Ki-67 assay could also be of great help in cases where the distinction between low grade glioma and non-neoplastic tissue processes (gliosis) or primary and metastatic malignancy remains uncertain.

The present ultrarapid immunostaining method for Ki-67 follows the same methodology as the one in which a pan-cytokeratin antibody is used in the intraoperative evaluation of sentinel lymph nodes removed in connection with breast cancer (CytoNel; Immunodiagnostic Inc). Other techniques for rapid Ki-67 and other rapid methods in IHC have been presented previously,[9 10 13–15] but further reports on their clinical usefulness are lacking. In our study, we showed that the novel Ultrarapid-Ki67 immunostaining method can be carried out in 10 minutes and visual scoring of one slide takes an additional three to four minutes. In our series, Ultrarapid-Ki67 IHC was found to be highly concordant, both quantitatively and qualitatively, with conventional MIB-1 immunostaining of paraffin wax embedded sections of the same tumours.

Technical precautions in Ultrarapid-Ki67 immunostaining are similar to those used in any technique involving frozen sections. Tissue material is often fragmented and can be

Take home messages

- We describe a rapid method for the immunohistochemical staining of Ki-67 in frozen glioma sections intraoperatively
- This method was found to be practical to perform and the results were morphologically and quantitatively indistinguishable from those after immunostaining with MIB-1, an antibody recognising Ki-67 in paraffin wax embedded tissue
- Thus, Ultrarapid-Ki67 immunostaining is a useful adjunct to the morphological diagnosis and grading of astrocytic tumours, and because of its speed could be used in the routine intraoperative diagnosis of gliomas and other neoplastic diseases

difficult to cut with a cryomicrotome. Therefore, an experienced histotechnologist is needed to prepare and cut high quality frozen sections. The most important technical detail in the staining protocol is the immediate acetone fixation of the slide. In other respects, intraoperative Ki-67 staining is relatively simple to perform, and does not require special equipment other than a thermal plate.

The results of the histopathological examination of tumours must be reported in a timely fashion to be clinically useful at the time of surgery. Immunohistochemical methods that can be performed in 10 minutes or less clearly fulfil this criterion, and will probably be used in other areas of tumour pathology as soon as immunostaining protocols for other diagnostically useful antibodies are developed and optimised. In our study, we describe a method for Ki-67 immunostaining that is likely to become a useful adjunct to the intraoperative frozen section evaluation of astrocytic tumours.

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Expression of Carbonic Anhydrase IX in Astrocytic Tumors Predicts Poor Prognosis

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Abstract

Purpose: Carbonic anhydrase IX (CA IX) is a hypoxia-inducible enzyme, which is associated with neoplastic growth. Ectopic CA IX expression has been observed in several tumors, whose normal counterparts do not express this enzyme. Normal human brain tissue shows only slight or no expression of CA IX.

Experimental Design: We describe CA IX expression in human diffusely infiltrating astrocytomas. The association of CA IX is evaluated with clinicopathologic and molecular factors including cell proliferation and apoptosis as well as the expression of p53 and epidermal growth factor receptor.

Results: CA IX immunopositivity was observed in 284 cases of 362 (78%) tumors. The positive areas were often located in close proximity to necrotic regions ($P < 0.001$). The CA IX immunoreactivity showed strong association with tumor malignancy grades ($P < 0.0001$). CA IX showed no association with p53 expression nor did it correlate with epidermal growth factor receptor–amplification, apoptosis, or cell proliferation. CA IX intensity had significant prognostic value in univariate ($P = 0.0011$, log-rank test) and multivariate survival analysis ($P = 0.038$, Cox analysis).

Conclusions: CA IX expression is common in diffusely infiltrating high-grade astrocytomas. Our results suggest that CA IX is a useful biomarker for predicting poor prognosis of astrocytic tumors. It may also be a promising target molecule for the improvement of therapeutic interventions in astrocytomas.

Carbonic anhydrases (CA) are zinc-containing metalloenzymes that catalyze reversible hydration of carbon dioxide in a reaction $CO_2 + H_2O \Longleftrightarrow HCO_3^- + H^+$. CAs are produced in a variety of tissues where they participate in several biological processes such as acid-base balance, carbon dioxide, and ion transport, respiration, bone resorption, ureagenesis, gluconeogenesis, and formation of cerebrospinal fluid (1–3). The mammalian α-CA family consists of at least 12 active isoenzymes with different structural and catalytic properties (4). In addition to their essential biochemical functions in pH regulation, the expression of some CA family members is also associated with neoplastic process (2, 3, 5–7).

CA IX is a catalytically active plasma membrane isoform containing a central catalytic CA domain, a transmembrane part, and a short COOH-terminal cytoplasmic tail. The structure that makes CA IX different from other isozymes is a proteoglycan-like region in the NH_2-terminal side suggested to be capable of acting in cell adhesion (8, 9). CA IX plays a dual role in normal tissues: in the stomach, it supports differentiation, and in the intestine, it is connected to the proliferation of epithelial cells (5). In a mouse model, CA IX seems to be involved in the control of differentiation and proliferation of epithelial cell lineages of the gastric mucosa during morphogenesis of the stomach (10). CA IX is present in few normal tissues but is ectopically expressed in a wide spectrum of human tumors. Expression is observed, e.g., in tumors of kidney, breast, head and neck, lung, bladder, and cervix uteri, i.e., in tissues where CA IX expression is normally absent (5). In a recent study, expression of CA IX was reported in the mouse brain (11). The normal human brain tissue shows only a slight or no expression of CA IX except the epithelial cells of the choroid plexus (6).

As to brain tumors, there is no large study to describe the expression of CA IX in human diffusely infiltrating astrocytomas, which are highly malignant tumors derived from glial cells. In these tumors, the surgical treatment is often impossible or inadequate, and thus, the prognosis of patients is poor. Here, we evaluated the association between CA IX expression

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and several clinicopathologic and molecular factors including cell proliferation, apoptosis, p53, and epidermal growth factor receptor (EGFR).

Materials and Methods

Study material. The study material consisted of 362 diffusely infiltrating astrocytoma samples, which were obtained from surgically operated patients in Tampere University Hospital, Tampere, Finland, during 1983 to 2001. First, the brain tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. On the basis of H&E-stained slides, a neuropathologist (H. Haapasalo) did an evaluation of the tumors according to the WHO 2000 criteria (12). These criteria divide diffusely infiltrating astrocytomas into three grades (2-4) according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The neuropathologist pinpointed one histologically representative tumor region in each sample specimen. From this region, a sample was included to multitissue blocks. The multitissue blocks were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD) and the sample diameter of the tissue cores was 600 μmol/L. The multitissue blocks were composed of 362 astrocytic tumors [grade 2 (52), grade 3 (45), and grade 4 (265)] and consisted of 281 primary tumors and 81 recurrences. The age of patients with primary tumors varied from 15 to 89 (median ± SD, 64 ± 15) and recurrent tumors from 15 to 89 (median ± SD, 52 ± 12). Overall survival was known for 186 patients [grade 2 (26), grade 3 (21), and grade 4 (139)]. The median follow-up time for 40 survivors was 809 days, and 146 patients died during the follow-up. The tumors were radically resected if possible and most patients with high-grade gliomas also received radiotherapy.

Immunohistochemistry. The monoclonal M75 antibody against human CA IX has been described previously (13). The antibody has been characterized for specificity and it has shown no cross-reactivity with other CAs (14). The CA IX enzyme was immunostained by the biotin-streptavidin complex method from the multitissue blocks by following this procedure: (*a*) pretreatment of the sections with undiluted cow colostral whey (Biotop Oy, Oulu, Finland) for 30 minutes and washed in PBS, (*b*) incubation for 1 hour with M75 antibody (1:10) in 1% bovine serum albumin-PBS, (*c*) incubation for 1 hour in biotinylated goat anti-mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) diluted 1:300 in 1% bovine serum albumin-PBS, and then (*d*) incubation for 30 minutes with peroxidase-conjugated streptavidin (Zymed) diluted 1:500 in PBS; (*e*) thereafter, incubation was done for 2 minutes in DAB solution containing 9 mg 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 mL PBS and 5 μL 30% H_2O_2. The sections were rinsed thrice for 10 minutes in PBS after incubation steps (*b*) and (*c*), and four times for 5 minutes in PBS after step (*d*). The tumor sections were counterstained with hematoxylin after the immunostaining.

The extent and the intensity of the staining reaction for CA IX were scored from the multitissue blocks on a scale from 0 to 3. In terms of the staining intensity, the scores were evaluated as follows: 0, no reaction; +, weak reaction; ++, moderate reaction; +++, strong reaction. The extent of the signal was also scored by a four-step assessment: 0, no positive cells; +, <25% positive cells; ++, 25% to 50% positive cells; +++, >50% positive cells. The tumors were also divided into two groups based on the nuclear staining of the CA IX as follows: −, no nuclear reaction; +, positive nuclear reaction. Gastric mucosa served as a positive control (15).

Proliferation by Ki-67 (MIB-1), apoptosis by terminal nucleotidyl transferase–mediated nick end labeling and p53 immunohistochemistry were done as previously described (16). EGFR amplification was detected with chromogenic *in situ* hybridization (17).

mRNA analysis. mRNA was isolated from seven brain tumors and one normal brain sample with RNeasy Mini-Kit (Qiagen, Hilden, Germany). Reverse transcription was done with Mo-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random primers (400 μg/mL). The primers for the PCR reaction were designed by using the published information on CA IX mRNA in GenBank (accession no. NM_001216). To produce an amplification product of 457 bp, the forward primer (F1) was 5′-GTTGCTGTCTCGCTTGGAAGAAA-3′ (nucleotides 915-937) and the reverse primer (R1) 5′-GCGGTAGCTCACACCCCCTTT-3′ (nucleotides 1392-1372). The control PCR reaction was done with the following primers for human β2-microglobulin (accession no. NM_004048): the forward primer was 5′-TATCCAGCGTACTCCAAAGATTCA-3′ (nucleotides 120-143) and the reverse primer was 5′-GAAAGACAAGTCTGAATGCTCCAC-3′ (nucleotides 288-265). The reagents for the PCR reaction were from BD Biosciences (Palo Alto, CA), except for the deoxynucleotide triphosphate mix which was from Finnzymes. Twenty nanograms of cDNA were used as the template. The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Foster City, CA), and the protocol consisted of a 94°C denaturation step for 1 minute followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 90 seconds, followed by final extension at 72°C for 3 minutes. The results of the PCR reaction were analyzed using 1.5% agarose gel containing 0.1 μg/mL ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA).

Statistical analysis. All statistical analyses were done using SPSS for Windows (Chicago, IL). The significance of associations was defined using χ^2 test, Mann-Whitney test, and Kruskal-Wallis test. The log rank test, Kaplan-Meier curves, and Cox multivariate regression analysis were used in the survival analysis.

Results

CA IX and clinicopathologic features. Cellular CA IX immunopositivity was observed in 284 of 362 cases (78%) of diffusely infiltrating astrocytomas. CA IX expression was detected in tissue sections as follows: 57 (16%) were strongly, 84 (23%) moderately, 143 (39%) weakly stained, and 78 (22%) were negative (Fig. 1). Within WHO grade 2 astrocytomas, there were 65% CA IX-positive cases (15% moderately, 50% weakly). Similarly, 73% of grade 3 astrocytomas (9% strongly, 33% moderately, and 31% weakly) and 82% of grade 4 astrocytomas (20% strongly, 23% moderately, 39% weakly) were CA IX-positive. The strongly positive areas were often located close to the necrotic regions (Fig. 1E) and CA IX intensity correlated significantly with the presence of necrosis in the same tissue section ($P < 0.001$, χ^2 test). Weak, mainly cytoplasmic, staining was occasionally seen in the neoplastic cells located in the infiltrative zone of lower grade tumors (Fig. 1C). The positive staining was usually unevenly distributed within the tumor. The positive immunostaining did not correlate clearly with the distribution of blood vessels, nor did it associate with endothelial cell proliferation in vessels (volume percentage of endothelial cells, P = n.s., χ^2 test). It also seemed that the cell cytoplasm was more intensively stained in the tumors with anaplastic features (Fig. 1D). The statistical comparison of cytoplasmic CA IX intensity and tumor grade also revealed significantly higher CA IX intensity in tumors of higher malignancy grade ($P = 0.000$, χ^2 test; Table 1).

Nuclear staining for CA IX was observed in 211 (58%) tumors, whereas 151 (42%) cases remained negative. There was more frequent nuclear staining in gliomas of better differentiation in contrast with the grade 4 glioblastomas, in which the nuclear staining was rarely discovered ($P < 0.001$, χ^2 test). CA IX extent, which describes the relative area of the highest intensity, was found to be high in 184 (51%), moderate in

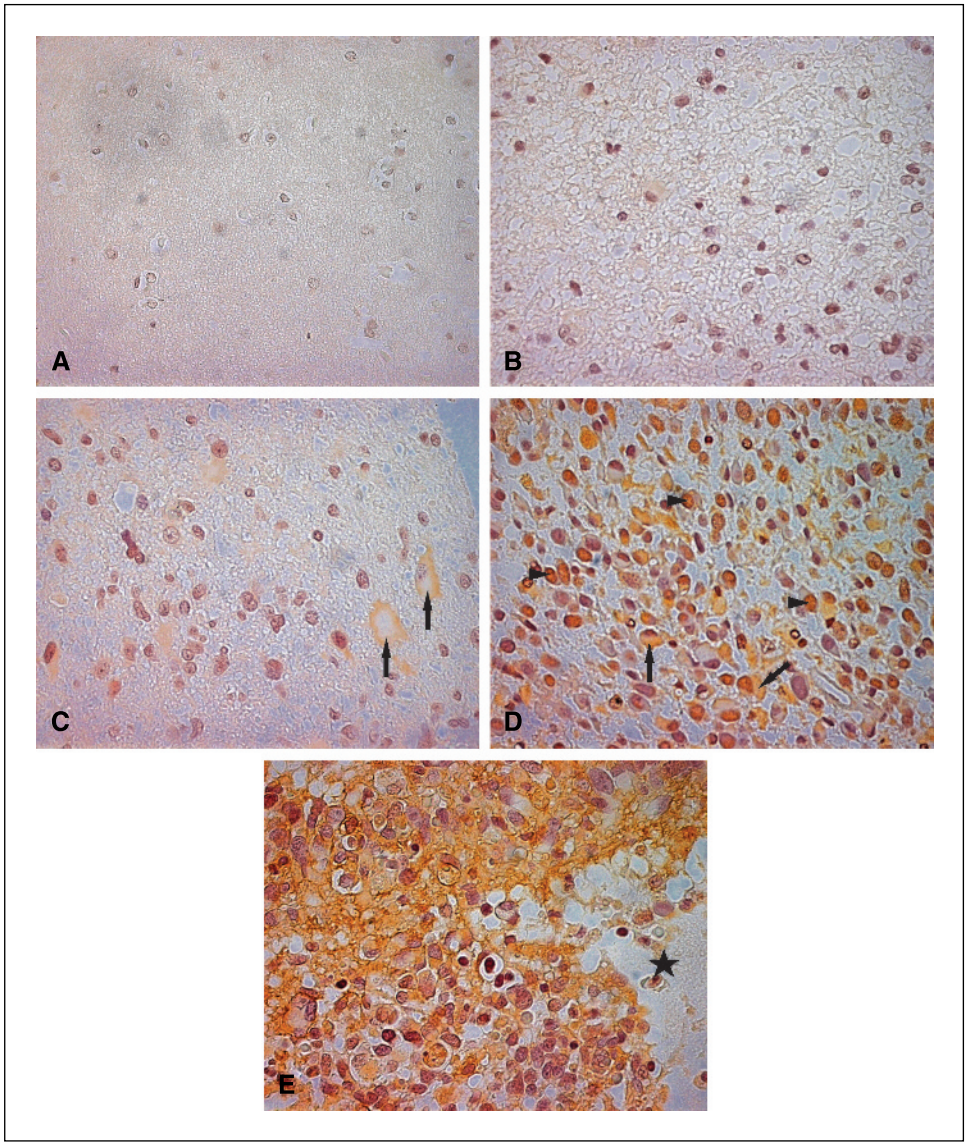

Fig. 1. Negative CA IX immunostaining of white matter in normal human brain (*A*) and grade 2 astrocytoma (*B*; magnification, ×630). *C,* weakly positive cytoplasm (*arrow*) in grade 2 astrocytoma (magnification, ×630). *D,* moderate cytoplasmic (*arrow*) and nuclear (*arrowhead*) CA IX immunostaining in an anaplastic astrocytoma (grade 3; magnification, ×630). *E,* strongly CA IX – immunopositive border of necrotic area (★) of glioblastoma (grade 4). Tumor cells and especially their plasma membrane stain positively, whereas most nuclei are negative (magnification, ×630).

72 (20%), scant in 28 (8%), and negative in 78 (22%) cases. A significant association was found between the increasing CA IX extent and increasing histological grade ($P = 0.027$, χ^2).

The associations of the patient age and CA IX intensity were studied by dividing the group of patients by the age of 50 into two subgroups (<50 years, $n = 164$; ≥50, $n = 200$). The χ^2 test showed significant correlations between increasing CA IX intensity and patient age ($P = 0.003$) as well as increasing CA IX nuclear staining and patient age ($P = 0.004$). Similarly, CA IX intensity correlated with patient age within grade 2 ($P = 0.010$) and grade 3 ($P = 0.013$) but not in grade 4 astrocytomas.

CA IX and molecular pathologic features. When molecular pathologic features typical of astrocytomas were compared with CA IX immunohistochemistry, no association was seen between proliferation by Ki-67/MIB-1 (329 cases studied: grade 2, median 3.1, range 0-35.8; grade 3, median 5.0, range 0-50.9; grade 4, median 13.6, range 0-55.6) or apoptosis by terminal nucleotidyl transferase–mediated nick end labeling index (29 cases studied: grade 2, median 0.35, range 0-0.80; grade 3, median 0.15, range 0-0.3; grade 4, median 0.3, range 0-2.5; Mann-Whitney test). CA IX intensity showed no significant association with p53 (141 cases studied, 69 positive and 72 negative) nor did it correlate with EGFR amplification (183 cases studied, 83 positive and 100 negative; χ^2 test).

CA IX and survival. Overall survival data was known for 186 patients. Patient survival was tested by log-rank test in relation to CA IX intensity. Interestingly, CA IX intensity divided the tumors into four significantly differing prognostic subsets ($P = 0.0011$; Fig. 2). The survival difference was even significant within grade 2 ($P = 0.0331$) and grade 4 ($P = 0.0163$), but not in grade 3 tumors. The extent of CA IX immunostaining also had a significant prognostic value ($P = 0.030$, log-rank test).

Most importantly, statistical analysis of the data revealed that the patient age, tumor grade, and CA IX intensity all had independent prognostic value (Cox multivariate analysis; Table 2). However, MIB-1 index did not seem to be an independent prognosticator although it correlated with overall survival ($P = 0.0047$, log-rank test; see cut points; Table 2) and WHO grade ($P < 0.001$, Kruskal-Wallis test). There was no correlation between p53 status, apoptotic rate, and survival.

Table 1. Association between CA IX immunointensity with WHO grade ($P < 0.001$, χ^2 test)

	WHO grade			Total
	2	3	4	
CA IX intensity				
0	18	12	48	78
1	26	14	103	143
2	8	15	61	84
3	0	4	53	57
Total	52	45	265	362

mRNA analysis. Immunohistochemical results of seven tumor samples (grades 2-4, two samples of each grade and hemangioblastoma) were verified by means of RT-PCR. A strong band for CA IX mRNA was found from one sample of grade 4 astrocytoma. Very weak positive signals were detected in two grade 2 specimens as well as in one grade 3 sample (Fig. 3).

Discussion

According to our study, CA IX expression is common in diffusely infiltrating astrocytomas, especially in high-grade tumors. The CA IX expression was associated with older patient age and poor prognosis. Importantly, CA IX expression was found to be an independent prognostic factor in addition to the patient age and histologic grade of astrocytomas. Even though the highest expression was observed in perinecrotic areas, it was not significantly associated with cell proliferation or apoptotic activity.

CA IX is ectopically expressed at relatively high levels and with high prevalence in tumors whose normal counterparts do not contain this protein (5). In addition to carcinomas of the cervix uteri, esophagus, kidney, lung and breast (18–23), this also seems to be true in human astrocytomas. Similar to carcinomas of the breast, cervix uteri, and lung, CA IX expression is localized to the perinecrotic regions of astrocytic tumors, suggesting that CA IX expression in brain tumors is induced by hypoxic conditions. Hypoxia triggers architectural and phenotypic rearrangements of tumor tissue that results in the development of necrotic areas surrounded by the zones of surviving hypoxic cells. It is these cells which could particularly acquire the most aggressive behavior. When a cell is exposed to hypoxic or anoxic conditions, transcriptionally active HIF-1 is bound to the hypoxia response element in the promoter area of the *CA9* gene, thus, inducing CA IX expression (24). HIF-1-regulated pathway is modulated by VHL protein (pVHL), which, under normoxic conditions, targets the α subunits of HIF for proteosomal destruction (25, 26). Because *CA9* is a target gene of HIF-1, its expression is increased in tumors associated with von Hippel-Lindau disease in which pVHL is defective due to mutations or deletions in *VHL* gene. von Hippel-Lindau disease is characterized clinically by vascular tumors, including central nervous system hemangioblastomas located in the cerebellum, spinal cord, or brain stem (27). Interestingly, somatic mutations in the *VHL* gene have also been reported in other central nervous system tumors including gliomas (28).

Table 2. The independent prognostic indicators of astrocytomas as evaluated by Cox's stepwise regression model

	Significance	Hazard Ratio	95.0% CI for Hazard Ratio	
			Lower	Upper
Age	0.000	1,854	1,486	2,315
Grade	0.000	1,980	1,498	2,616
CA IX intensity	0.038	1,188	1,010	1,397

NOTE: Patient age (cut points, 45 and 60 years), histologic grade (2-4), CA IX intensity (0-3), CA IX extension (0-3), CA IX nuclear staining (0.1), and MIB-1 (cut points 3%, 10%, and 20%) were included into the analysis (enter limit $P < 0.05$).

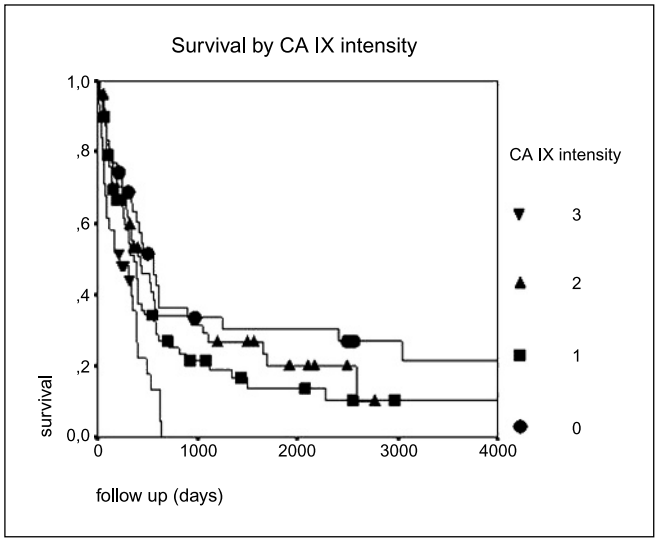

Fig. 2. Overall patient survival by CA IX intensity. Kaplan-Meier curves are shown ($P = 0.0011$, log-rank test).

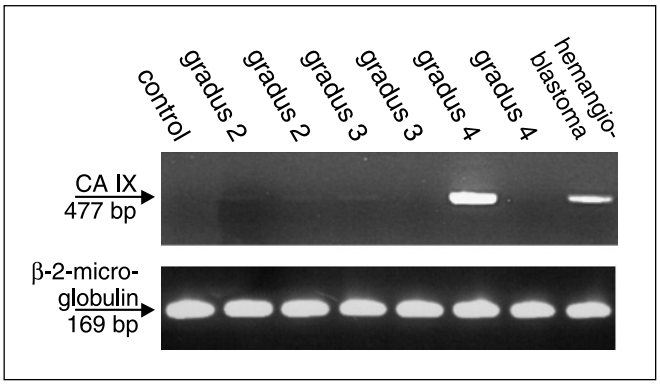

Fig. 3. mRNA analysis. Immunohistochemical results of six tumor samples (grades 2-4, two samples of each grade) were verified by means of RT-PCR. A strong band for CA IX mRNA was found from one sample of astrocytoma grade 4. Only weak bands were observed in two grade 2, one grade 3, and another grade 4 astrocytoma samples. A hemangioblastoma was included in the analysis as a positive control.

A recent study has indicated that hypoxia has a dual role in the regulation of CA IX (29). In addition to transcriptional induction, hypoxia also activates the kinetic properties of CA IX enzyme in hypoxic conditions. This may have important implications for tumor progression, because the enzymatic activation probably enhances extracellular acidification in tumors. In addition, experimental data suggests that increased cell density could influence CA IX expression. Both hypoxia and high cell density may contribute to increased CA IX expression in astrocytomas because the most malignant type (glioblastoma multiforme) represents a category of highly hypoxic and cellular tumors.

Coexpression of CA IX with c-ErbB2 and EGFR has been suggested to be regulated by the same oncogenic pathways, e.g., in non–small cell lung cancer (22, 30). However, we did not find an association between CA IX expression and EGFR amplifications which are commonly associated with astrocytic tumors. The differences in oncogenic pathways between different tumors may explain the observed discrepancy.

An infiltrative growth pattern is typical for astrocytic tumors. Instability and disorganization of cadherin-mediated junctions are required to promote migration and invasiveness in glioblastoma cell lines (31). CA IX has been also suggested to play a role in intercellular adhesion. In polarized epithelial Madin-Darby canine kidney cells transfected with the human *CA9* cDNA, CA IX protein colocalizes with a key adhesion molecule, E-cadherin, and destabilizes E-cadherin-mediated cell-cell contacts via a mechanism that involves competitive interaction with β-catenin (9). This capability of CA IX is reminiscent of some oncoproteins such as EGFR and c-ErbB2, and makes CA IX a candidate contributor to tumor invasion. Our results suggest that this phenomenon might also take place in diffusely infiltrating astrocytomas. For example, cytoplasmic CA IX staining was also seen in the infiltrative border of lower grade tumors. In terms of cancer therapy, the aggressive character of astrocytoma cells is probably the most problematic feature. This might also explain the strong association between increased CA IX expression and poor survival in these tumors.

In summary, CA IX expression is a common phenomenon in high-grade astrocytomas. Our results suggest that CA IX is a useful biomarker for predicting poor prognosis in these tumors. It may also be a promising target molecule for the improvement of therapeutic interventions.

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Carbonic anhydrase II in the endothelium of glial tumors: A potential target for therapy

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Carbonic anhydrase isozyme II (CA II) is a cytosolic enzyme that is highly expressed in most organs, including the brain, where it is mainly located in the oligodendrocytes. Recent studies have shown that its expression is induced in the endothelium of neovessels in melanoma and esophageal, renal, and lung cancer. Immunological studies further indicate that CA II represents a major target antigen stimulating an autoantibody response in melanoma patients. These results prompted us to investigate endothelial CA II expression in two types of brain cancer: oligodendrogliomas and astrocytomas. A series of 255 astrocytoma and 71 oligodendroglial tumor specimens was immunostained for CA II. The staining results were correlated with a number of different clinicopathological factors and survival data. CA II showed weak or no expression in low-grade tumors, while grade 3 mixed oligoastrocytoma and glioblastoma multiforme were the most positively stained tumor types. Survival analysis indicated that endothelial CA II staining is significantly associated with a poor prognosis in patients with astrocytomas. About 17% of patients with CA II–negative tumors (weak or no endothelial signal) were still alive at the end of the follow-up period of five years. The presence of CA II in the tumor endothelium suggests that it may play an important functional role in tumor metabolism. From a clinical perspective, the results also open new avenues for selecting tumor types for dendritic cell therapy trials. *Neuro-Oncology 9, 308–313, 2007 (Posted to Neuro-Oncology [serial online], Doc. D06-00091, April 13, 2007. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2007-001)*

Keywords: brain, cancer, carbonic anhydrase, endothelium, tumor

A recent study on dendritic cell therapy for melanoma patients identified carbonic anhydrase isozyme II (CA II) as a potential target antigen.[1] Half of the patients exhibited anti–CA II autoantibodies either before or after therapy, and 3 of 10 patients had posttherapy antibody levels that were higher than the pretherapy levels. Immunohistochemical analysis further revealed that CA II is expressed in the endothelium of neovessels in some cancer tissues, including melanoma and esophageal, renal, and lung cancer. It was also shown that cell culture conditions reminiscent of a cancer cell microenvironment induced CA II expression in endothelial cells in vitro. Based on these results, the authors suggested that CA II is a tumor vessel endothelium–associated antigen, and that elicitation of an anti–CA II autoantibody response by dendritic cell therapy could be associated with a more favorable clinical outcome.

As the members of the CA isozyme family contribute to a number of important physiological functions, it is not surprising that their insufficient or excessive expression has been implicated in several diseases.[2,3] The functional involvement of CAs in cancer has become

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evident after the identification of two membrane-bound isoforms, CA IX and XII, that are overexpressed in several types of cancer.[3–5] These two CAs have become attractive research topics, given the hope that an understanding of their roles in tumor physiology could lead to the development of novel strategies for cancer detection, prognosis, and treatment.[5] Only few studies have been conducted on the CA II enzyme in tumors, however, and this isoform has not been considered very attractive because most results regarding epithelial tumors have indicated that malignant cells contain no CA II or only low levels.[6,7] In contrast, it has been reported that some, but not all, malignant blast cells in leukemia patients express CA II.[8] The expression of CA II in the tumor vessel endothelium had never been investigated, however, until the recent article by Yoshiura et al.[1] The results of that study will probably attract researchers to explore CA II in more detail in connection with various cancers, and it may be that the generation of autoantibodies to it in some patients could represent an important step in the natural defense system against cancer, and, even more important, as suggested recently,[1] CA II associated with the endothelium of neovessels could serve as a potential target for cancer therapy. The present study was designed to analyze endothelial CA II expression in a large series of malignant gliomas. These brain tumors usually represent highly vascularized neoplasias with very poor clinical prognoses. Our results showed that 31% of diffuse astrocytomas and 14% of oligodendrogliomas show moderate or strong CA II expression in the tumor vessel endothelium.

Materials and Methods

Materials

The materials were obtained from surgical patients at Tampere University Hospital, Tampere, Finland, in 1983–2002. The study was approved by the research ethics committee of Tampere University Hospital. The brain tumor specimens were fixed in 4% phosphate-buffered formaldehyde and processed into paraffin blocks. On the basis of hematoxylin and eosin–stained slides, a neuropathologist (H.H.) evaluated the tumors according to the WHO 2000 criteria. One histologically representative tumor region was selected in each specimen, and a sample from this region was included in multitissue blocks constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The tissue cores were 600 μm in diameter.

Astrocytic Tumors

There were 255 astrocytoma samples included in the analyses (27 of grade 2, 39 of grade 3, and 189 of grade 4), of which 189 were primary tumors and 66 were recurrences. The WHO criteria divide diffusely infiltrating astrocytomas into three grades (2–4) according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. The ages of the patients with primary tumors varied from 1 to 82 years (mean ± SD: 48 ± 20), and those of the patients with recurrent tumors varied from 1 to 76 years (mean ± SD: 40 ± 18). Overall survival was known for 195 patients, of whom 161 had died during the five-year follow-up period. The tumors were resected if possible (95%), and most patients with high-grade gliomas also received radiotherapy (55%) and/or chemotherapy (24%).

Oligodendroglial Tumors

There were 71 oligodendroglial tumor samples included in the analyses (pure oligodendrogliomas: 31 of grade 2, and 12 of grade 3; and oligoastrocytomas: 18 of grade 2, and 9 of grade 3), of which 52 were primary tumors and 19 were recurrences. The WHO criteria group oligodendroglial tumors into two main categories: pure oligodendrogliomas and mixed oligoastrocytomas, which are further divided into two grades (2 and 3) according to the presence of increased cellularity, atypia, and mitotic activity. The ages of the patients with primary tumors varied from 8 to 76 years (mean ± SD: 43 ± 14), and those of the patients with recurrent tumors varied from 12 to 69 years (mean ± SD: 41 ± 14). Overall survival was known for 52 patients: The median follow-up time for the 26 survivors was 8 years 2 months, and 26 patients died during follow-up. Detailed information about therapies was known for 31 patients. Sixteen patients received radiotherapy, and two received chemotherapy and radiation therapy. 1p/19q analysis by fluorescence in situ hybridization was performed as previously described.[9]

Immunohistochemistry and In Situ Hybridization

Proliferation was examined by Ki-67 (MIB-1), apoptosis was assessed by TUNEL, and p53 immunohistochemistry was performed as previously described.[10] Epidermal growth factor receptor (EGFR) amplification in the astrocytic tumors was detected with chromogenic in situ hybridization.[9] The immunostaining for CA IX was performed as previously described.[11]

Automated immunostaining for CA II was performed using Power Vision+ Poly-HRP IHC Kit (polymerized horseradish peroxidase immunohistochemistry kit; ImmunoVision Technologies, Brisbane, CA, USA) reagents. The immunostaining method included the following steps: (a) rinsing in wash buffer; (b) treatment in 3% hydrogen peroxide in double-distilled water (ddH_2O) for 5 min and rinsing in wash buffer; (c) blocking with Universal IHC Blocking/Diluent (ImmunoVision) for 30 min and rinsing in wash buffer; (d) incubation with rabbit anti-human CA II serum (produced and characterized earlier[12]) or normal rabbit serum diluted 1:2,000 in Universal IHC Blocking/Diluent for 30 min; (e) rinsing in wash buffer for 3 × 5 min; (f) incubation in poly-HRP–conjugated anti-rabbit immunoglobulin G for 30 min and rinsing in wash buffer for 3 × 5 min; (g) incubation in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (1 drop of DAB solution A and 1 drop of DAB solution B with 1 ml ddH_2O) for 6 min; (h) rinsing

with ddH_2O; (i) copper(II) sulfate treatment for 5 min to enhance the signal; and (j) rinsing with ddH_2O. All procedures were carried out at room temperature. The sections were mounted in Entellan Neu (Merck, Darmstadt, Germany) and finally examined and photographed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany).

The intensity of the staining reaction for endothelial and cytoplasmic CA II was scored from the multitissue blocks on a scale from 0 to 3. The scores were evaluated in terms of staining intensity as follows: 0, no reaction; +, weak reaction; ++, moderate reaction; and +++, strong reaction. In the statistical analyses, the specimens were grouped into two categories based on the staining intensity: CA II+ve tumors, including those exhibiting moderate or strong reactions in the endothelium; and CA II–ve tumors, with weak or negative immunostaining results.

Statistical Analysis

All of the statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA). The significances of associations were defined using the chi-square test, Mann-Whitney test, and Kruskal-Wallis test. The log-rank test, Kaplan-Meier curves, and Cox multivariate regression analysis were used in the survival analysis.

Results

Astrocytic Tumors

Some brain tumors clearly appeared to express CA II in the vascular endothelium (Fig. 1), whereas the normal brain tissues showed no endothelial CA II expression (data not shown). Out of 261 diffusely infiltrating astrocytoma cases, endothelial CA II immunopositivity was observed in 117 (45%). The signal was strong in 45 cases, moderate in 36, and weak in 36. A total of 144 tumors (55%) were completely negative. High cytoplasmic CA II expression within the tumor cells appeared to be rare among these tumors, with only six specimens showing a strong signal. Moderate cytoplasmic staining was observed in 35 tumors and weak reactions in 52. A total of 168 cases (64%) showed no CA II expression within the tumor cells. No significant differences in CA II immunostaining were detected between primary tumors and recurrences (p = NS, chi-square test).

When the specimens were grouped into two categories according to the endothelial staining intensity, 81 cases (31%) of 261 diffusively infiltrating astrocytomas were CA II+ve (moderate or strong reaction in the vascular endothelium) and 180 cases (69%) were CA II–ve (weak or no endothelial staining). The endothelial staining was most often seen in the proliferative endothelium and in small neovessels of grade 4 astrocytomas. The positive cells in the proliferative endothelium were usually located near the vascular lumen. The grade 2 astrocytomas did not express CA II at all in the endothelium (Fig. 1).

When the important clinicopathological factors were also evaluated, positive endothelial CA II staining was found to correlate significantly with the presence of EGFR amplification (n = 172, p = 0.008, chi-square test), whereas it showed no association with p53 expression (n = 103, p = NS, chi-square test) or MIB-1/Ki-67 proliferation (n =234, p = NS, Mann-Whitney test). Endothelial CA II expression did not correlate with the area of necrosis in the same tissue section (n = 37, p = NS, Mann-Whitney test). Nor did we find significant association between CA II reactivity and necrosis when the cut point of the latter was set to the median value (p = NS, chi-square test). We previously analyzed the expression of CA IX in the same diffuse astrocytomas,[11] and comparison of the immunostaining results indicated

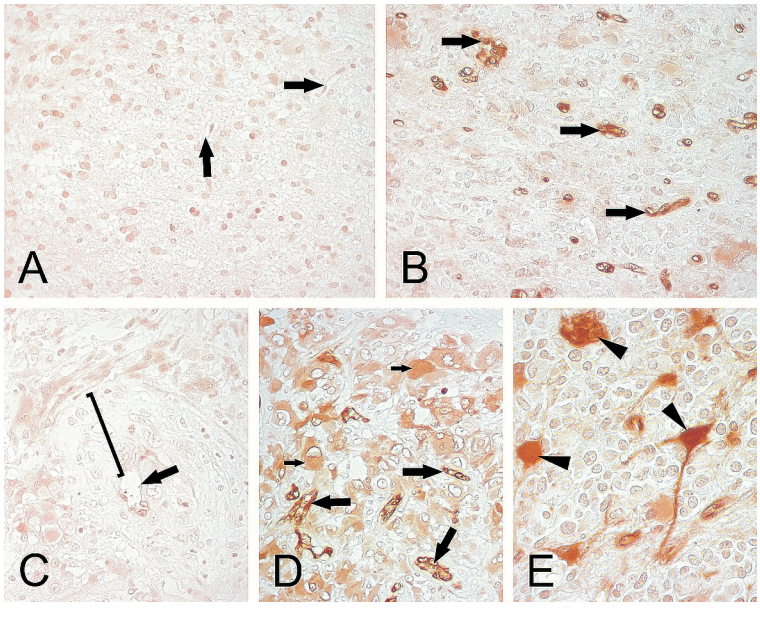

Fig. 1. Expression of carbonic anhydrase isozyme II (CA II) in astrocytomas. (A) There was no endothelial (arrows showing capillaries) or tumor cell immunopositivity in grade 2 astrocytoma. (B) In grade 3 astrocytoma, the capillary endothelium (arrows) was moderately immunopositive. (C) There was only weak immunopositivity close to the lumen (arrow) in the endothelial layers (column) of a glioblastoma (astrocytoma grade 4). (D) The capillaries (large arrows) were strongly immunopositive and the tumor cells (small arrows) moderately positive in a glioblastoma. (E) Some neurons (arrowheads) intermingled within glioblastoma cells of another tumor were also strongly immunopositive.

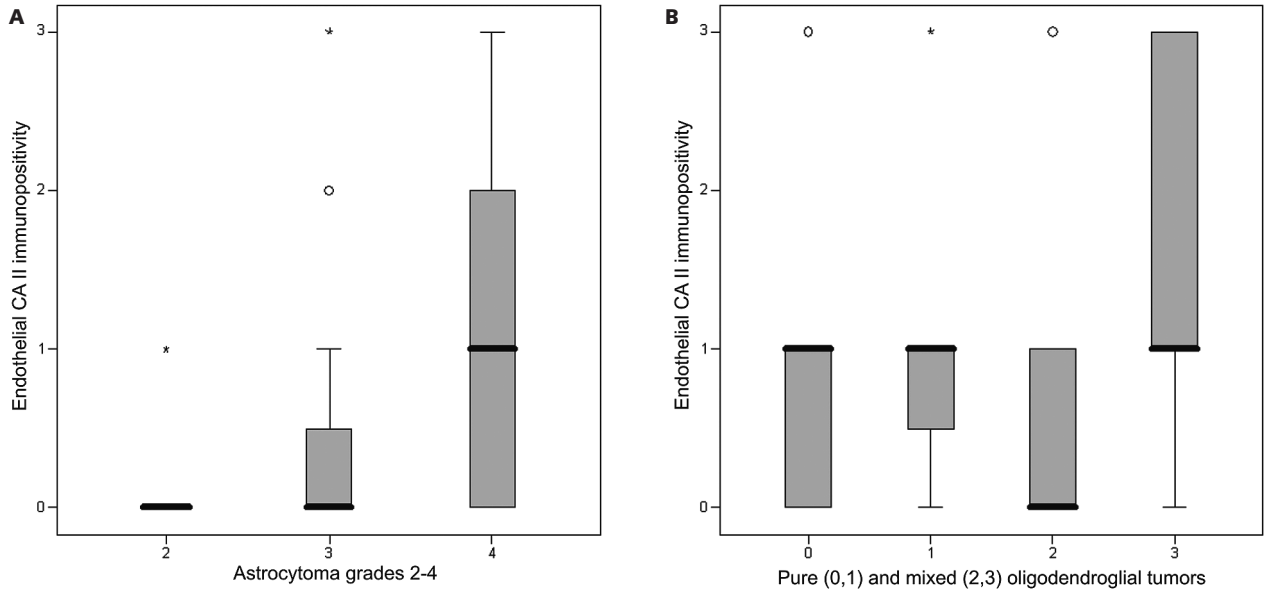

Fig. 2. Box plots describing endothelial carbonic anhydrase isozyme II (CA II) immunoreactivity in astrocytic (A) and oligodendroglial (B) tumors. Astrocytomas are divided into three WHO grades (2–4) and oligodendroglial tumors into pure oligodendroglioma grade 2 (category 0) and grade 3 (category 1) and into mixed oligoastrocytoma grade 2 (category 2) and grade 3 (category 3). There is a significant difference in the immunoreactivity between the astrocytoma grades ($p < 0.001$, chi-square test) and the mixed oligodendroglial tumor grades ($p = 0.018$, chi-square test). Endothelial CA II immunopositivity: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. Each box shows the median, quartiles, and extreme values within a category.

a significant association between the presence of CA II in the endothelium and CA IX in the tumor cells ($p = 0.006$, chi-square test).

Positive endothelial staining of CA II was significantly associated with a higher tumor grade ($p < 0.001$, chi-square test) (Fig. 2A). In a five-year survival analysis of patients with primary tumors, the patients having strong or moderate endothelial CA II staining showed significantly poorer survival ($p = 0.032$, log-rank test) (Fig. 3). In fact, 80 (99%) of the 81 patients having strong or moderate endothelial staining for CA II (CA II+ve) died during the follow-up period, whereas about 26 (17%) of the 156 patients with weakly stained or negative tumors (CA II–ve) were still alive at the end. Endothelial CA II was not of prognostic significance in univariate survival analysis when grades 2, 3, and 4 were tested separately. When only patients with grade 4 astrocytomas were examined after the five-year follow-up, all the patients having strong or moderate endothelial staining ($n = 58$) died during the five-year follow-up, whereas 11 (13%) of 87 patients with weakly stained or negative tumors were still alive. However, when endothelial CA II staining (CA–ve versus CA+ve) and conventional prognostic markers (tumor grade; patient age, cutoff points 50 and 65 years; MIB-1, cutoff points 5% and 15%) were included into the Cox multivariate analysis of primary tumors, only tumor grade ($p < 0.001$; odds ratio, 2.268) and patient age ($p < 0.001$; odds ratio, 2.011) showed independent prognostic value.

Oligodendroglial Tumors

Of the 43 pure oligodendrogliomas, 26 (60%) showed immunoreactions for CA II in the endothelium, and 15 (58%) of the 26 mixed oligoastrocytomas were positive. Figure 2B shows CA II immunostaining intensity in different grade categories. It is significant that the oligodendroglial tumors generally showed weaker endothelial expres-

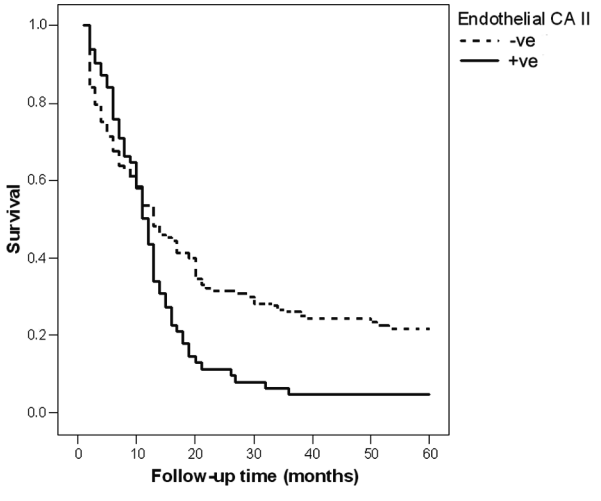

Fig. 3. Survival in astrocytomas by endothelial carbonic anhydrase isozyme II (CA II) expression. The patients with CA II–ve tumors had significantly better survival than those with CA II+ve tumors ($p = 0.032$, log-rank test).

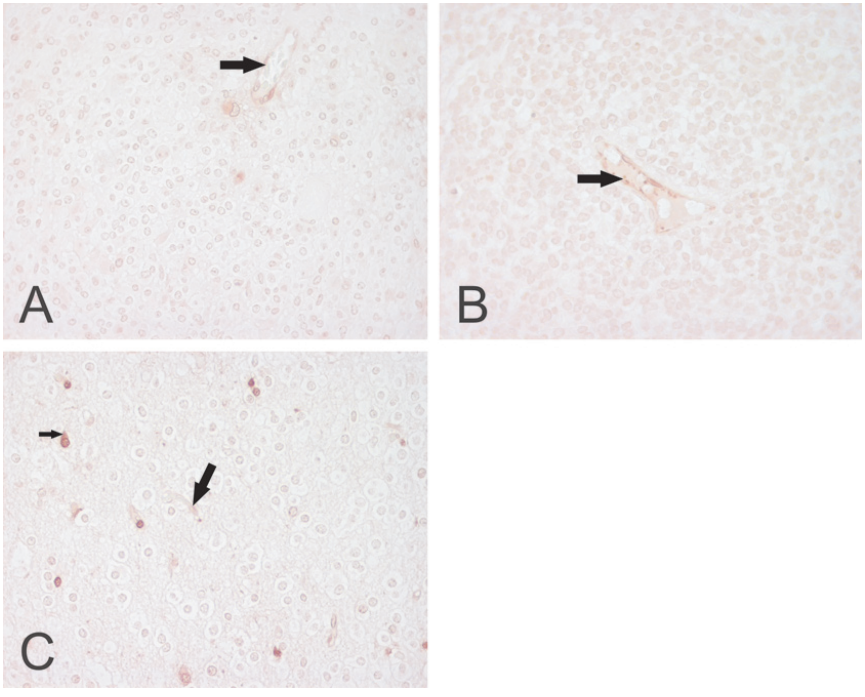

Fig. 4. Expression of carbonic anhydrase isozyme II (CA II) in oligodendroglial tumors. The endothelial expression was generally negative or weak in these cases (large arrows in an oligodendroglioma of grade 2 [A], an oligodendroglioma of grade 3 [B], and a mixed oligoastrocytoma of grade 2 [C]). The mixed oligoastrocytoma had moderate cytoplasmic immunopositivity in some tumor cells as well (small arrow in C).

sion of CA II than the astrocytomas (Figs. 2B and 4), the staining intensities being strong in 9, moderate in 1, and weak in 31 of 71 cases. Cytoplasmic tumor cell–associated CA II expression was detected in only a minority of the specimens. The signals were strong in 4, moderate in 1, and weak in 23 cases. A total of 43 oligodendroglial tumors (61%) showed no immunoreactions in the tumor cells. In oligodendroglial tumors, no significant differences in CA II expression were observed between primary tumors and recurrences (p = NS, chi-square test).

Of the 71 oligodendroglial tumors, 10 (14%) showed strong or moderate immunoreactions (CA II+ve) in the endothelium, while endothelial CA II was more often expressed in high-grade mixed oligoastrocytomas than low-grade tumors (p = 0.018, chi-square test) (Fig. 2B). There was no association between 1p or 19q loss and CA II expression (n = 17, p = NS, chi-square test), and neither endothelial nor cytoplasmic staining for CA II was a significant predictor of survival in this series of primary oligodendrogliomas (n= 52, p = NS, log-rank test).

Discussion

A recent attractive finding has indicated that CA II expression is induced in the endothelial cells of neovessels in melanoma and esophageal, renal, and lung cancers.[1] It was proposed that the presence of CA II in the endothelium could contribute to the generation of an autoantibody response that would in turn be a desired outcome of immune therapy for cancer. The original finding raised the question of whether CA II expression is induced in other cancers, as well. We investigated here the expression of CA II in two major types of brain cancer: astrocytomas and oligodendrogliomas. A high-grade astrocytoma is typically a highly neovascularized tumor that confers a dismal prognosis with a median survival of only 1–2 years, while the other common adult CNS tumors (i.e., low-grade astrocytomas and oligodendrogliomas) carry a less ominous, yet still poor, prognosis.

According to our findings, endothelial CA II expression seems to be a common phenomenon among high-grade diffusely infiltrating astrocytomas and is also present to some extent in high-grade mixed oligoastrocytomas and less so in pure oligodendrogliomas. This finding suggests that the astrocytic component within the tumor associates with higher CA II expression in the endothelium. It would be of interest to investigate possible paracrine or other factors that potentially induce CA II expression in the tumor endothelium. Increased endothelial CA II expression was associated with a higher malignancy grade in both tumor types, and, consequently, endothelial CA II expression was also found to correlate with poor prognosis in diffuse astrocytomas.

The present results indicate an association between endothelial CA II expression and EGFR amplification. There have been suggestions that some CA isozymes may be regulated by similar oncogenic pathways to EGFR.[13] Based on our finding, this could also be the case with CA II expression in malignant brain tumors. The *EGFR* gene is amplified in more than one-third of glioblastomas and in a few anaplastic astrocytomas, but not in grade 2 astrocytomas.[9] We did not find any correlation between endothelial CA II and p53 protein expression, possibly because all grades of diffuse astrocytomas contain p53 mutations, which is an early event in a major subset of diffuse astrocytomas.[14] It was of interest that

CA II immunoreactivity did not correlate significantly with cell proliferation determined by MIB-1 immunostaining although both showed the highest signals in the grade 4 astrocytomas. Even though this finding cannot be explained at the present stage of the studies, it is notable that CA II has never been reported to associate with cell proliferation.

The recent study by Yoshiura et al.[1] suggested that endothelial CA II could be a potential target antigen for dendritic cell therapy. Since all solid brain tumors show high neovascularization,[15] this kind of therapy might be beneficial in patients suffering from glial tumors. Significantly, this strategy has already been used successfully in the treatment of diffuse astrocytomas,[16] although the investigators did not report the antigen(s) responsible for dendritic cell activation. Based on the present findings, it is reasonable to hypothesize that CA II could represent a promising candidate target of dendritic cell therapy for astrocytoma, as was the case for melanoma.

Yoshiura et al.[1] also proposed that anti–CA II antibody status could be a useful marker for monitoring the clinical response to dendritic cell therapy. If CA II were to represent a target for therapy in malignant brain tumors as well, immunohistochemical analysis of the tumor specimen could be used as a guide in assessing the appropriateness of the treatment. Endothelial CA II staining could also be used to help in the evaluation of neovascularization in the histological diagnosis of astrocytic tumors.

The presence of at least two CA isozymes—CA II and CA IX—in diffuse astrocytomas adds another option for therapeutic applications; that is, via inhibition of CA enzymatic activity. Recent drug developments have produced a number of compounds with efficient CA inhibition profiles,[3] and it has been shown that some of these can markedly reduce the invasive capacity of cancer cells[3,17,18] and may also disturb neoangiogenesis, thus reducing tumor growth.[19] The present findings suggest that it could be worth testing the effect of some potent and well-tolerated CA inhibitors such as acetazolamide and topiramate on brain tumor angiogenesis. Because of the extremely high mortality rate associated with glial tumors, urgent efforts should be made to discover novel treatment strategies that might prolong the survival time of patients.

Acknowledgments

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Identification of an alternatively spliced isoform of carbonic anhydrase XII in diffusely infiltrating astrocytic gliomas

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Carbonic anhydrase XII (CA XII) is a transmembrane enzyme that is associated with neoplastic growth. CA XII has been proposed to be involved in acidification of the extracellular milieu, creating an appropriate microenvironment for rapid tumor growth. Because RNA sequence databases have indicated that two isoforms of CA XII might exist in human tissues, and because alternatively spliced protein forms have been linked to aggressive behavior of cancer cells, we designed a study to evaluate the presence of the two forms of CA XII in diffuse astrocytomas, a tumor type known for its aggressive and often noncurable behavior. Reverse transcription PCR of tumor samples surprisingly revealed that CA XII present in diffuse astrocytomas is mainly encoded by a shorter mRNA variant. We further showed by Western blotting that anti-CA XII antibody recognized both isoforms in the glioblastoma cell lines, and we then evaluated the expression of CA XII in astrocytomas using immunohistochemistry and correlated the results with various clinicopathological and molecular factors. Of 370 diffusely infiltrating astrocytomas, 363 cases (98%) showed immunoreactions for CA XII. Importantly, CA XII expression correlated with poorer patient prognosis in univariate (p = 0.010, log-rank test) and multivariate survival analyses (p = 0.039, Cox analysis). From these results, we conclude that CA XII is commonly expressed in diffuse astrocytomas and that it might be used as a biomarker of poor prognosis. The absence of 11 amino acids in the shorter isoform, which seems to be common in astrocytomas, may affect the normal quaternary structure and biological function of CA XII.
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Alternative splicing, a process in which identical pre-mRNA molecules are spliced in different ways, is a fundamental mechanism in differential gene expression. Variations in expression and/or activity of splicing factors could lead to changes in the splicing patterns of certain mRNAs whose protein products are

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involved in different stages of tumor progression. The alternative splicing may affect cell growth, adhesion, migration, invasion, and apoptosis as well as the connections between signaling pathways, all important steps in tumorigenesis. Recently, there have been several reports on alternative splicing in different cancers and the effect of splicing on patient prognosis.[1,2]

Carbonic anhydrase XII (CA XII) is a transmembrane enzyme that was originally identified by the overexpression of its mRNA in human renal cancer cells.[3,4] Since then, it has been associated with several human neoplasms, as has the other transmembrane isoenzyme, CA IX.[5–10] Both CA XII and CA IX are regulated by similar mechanisms. They are both induced at the transcriptional level via the hypoxia-inducible factor-1 (HIF-1)–mediated pathway, which is activated in tumor cells by hypoxia or by a mutation in the gene encoding the von Hippel-Lindau (VHL) tumor-suppressor protein.[4,11] High expression of CA IX and CA XII in certain tumors has supported the idea that these isozymes may functionally participate in the invasion process. Acidification of the extracellular milieu surrounding the cancer cells, a process in which CAs ultimately participate, creates a microenvironment that leads to activation of proteolytic enzymes and favors tumor growth and spread.[4,12] In favor of this hypothesis, it has been shown in vitro that CA inhibitors can reduce the invasion capacity and/or proliferation of cancer cells.[12,13]

The information obtained from GenBank suggested that CA XII may have two alternative isoforms. The spliced mRNA was predicted to lack exon 9, which has 33 nucleotides (coding for 11 amino acid residues). Because these alternative isoforms had not been studied in any of the previous publications on CA XII, we wanted to investigate whether they are detectable in diffuse astrocytoma samples, which are known to represent a highly malignant tumor type with an extremely poor prognosis. The forward and reverse primers for the PCR reaction were designed in a way that the predicted PCR amplification product covered exon 9, and therefore the alternatively spliced isoforms became identifiable by the difference in the length of the PCR product. The expression of *CA12* mRNA was analyzed from one normal brain sample, six diffuse astrocytomas (grades II–IV, two samples of each grade), and one hemangioblastoma by reverse transcription (RT)-PCR. We chose diffuse astrocytomas, because they are the most common highly malignant glial cell–derived brain tumors. They are difficult to remove surgically because of their infiltrating and diffuse growth pattern, and thus the prognosis of patients is still very poor.[14,15]

Materials and Methods

mRNA Analysis

Total RNA was extracted from seven brain tumors and one normal brain sample using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). RT was performed with Moloney murine leukemia virus reverse transcriptase (Finnzymes, Espoo, Finland) using random primers (400 μg/ml). The primers for the PCR were designed using the published information on *CA12* mRNA in GenBank (accession nos. NM_001218 and NM_206925). These two accession numbers represent alternatively spliced CA XII isoforms. The primers were designed in a way that both spliced forms could be identified from the samples as separate bands. The forward primer (F1) was 5'-CAACTTCCGGCAGGTCCAGA-3' (nucleotides 972–991 in NM_001218), and the reverse primer (R1) was 5'-TTGAGGTGTCGCAAGTGTCCAG-3' (nucleotides 1287–1308 in NM_001218 and 1254–1275 in NM_206925). The predicted amplification products were 336 bp for isoform 1 and 303 bp for isoform 2. The control PCR reaction was performed with the following primers for human β-actin (accession no. NM_001101): forward primer (F2) 5'-CACGGCATCGTCACCA-ACTG-3' (nucleotides 290–309) and reverse primer (R2) 5'-GCCTGGATAGCAACGTACATGGC-3 (nucleotides 464–486), producing an amplification product of 197 bp. The PCR was performed using 1.1× ReddyMix PCR Master Mix (Abgene, Epsom, UK), and 20 ng cDNA was used as a template in a 25-μl reaction. The PCR reaction was performed on a thermal cycler (PTC 200 Thermal Cycler, MJ Research, Inc., Waltham, MA, USA); the protocol consisted of a 94°C denaturation step for 1 min followed by 33 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 90 s, followed by final extension at 72°C for 3 min. The results of the PCR reaction were analyzed using 1.5% agarose gel containing 0.1 μg/ml ethidium bromide with DNA standard (100 bp DNA Ladder, New England Biolabs, Beverly, MA, USA).

Because the RT-PCR results showed evidence of CA XII splicing variants in human astrocytomas, we further expanded our PCR studies to include three glioblastoma cell lines, two renal cancer cell lines, and two normal tissues (colon and kidney) that are known to express CA XII. Total RNA was extracted from the human glioblastoma cell lines U-87 MG, U-373 MG, and CCF-STTG1 (European Collection of Cell Cultures, Salisbury, UK). The renal cancer cell lines used for the study were Caki-1 and A-498 (American Type Culture Collection, Manassas, VA, USA). RNA was isolated using the RNeasy Mini-Kit, and reverse transcription was performed with the Fermentas First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The PCR reaction was performed as described for tumor samples with a few exceptions, as follows. The control PCR reaction was performed with the following primers for human β-2-microglobulin (NM_004048): forward primer (F3) 5'-TCCAGCGTACTCCAAAGAT-TCAGG-3' (nucleotides 122–145), reverse primer (R3) 5'-ATGCGGCATCTTCAAACCTCC-3' (nucleotides 431–451); the resulting PCR product was 330 bp. Two nanograms of cDNA was used as a template in the PCR reactions performed from the commercial cDNA panel (Human MTC panel I, BD Biosciences, Palo Alto, CA, USA). The results of the PCR reaction were analyzed using 1.5% agarose gel.

To confirm the presence of the alternatively spliced

mRNA, PCR products from a grade IV astrocytoma sample (showing a double band) were purified from the gel with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Poole, UK). The sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reactions Kit, version 3.1 (Applied Biosystems, Foster City, CA, USA). The sequencing was performed in both directions with primers F1 and R1. Purified PCR product (5 µl) was mixed with 2 µl Big Dye mix and 2 µl sequencing buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), and 1.6 pmol primers was added. The reactions were amplified by cycle sequencing on a PTC 200 Thermal Cycler according to the manufacturer's protocol. The products were purified by ethanol precipitation, resuspended in HiDi formamide (Applied Biosystems), and denatured according to the manufacturer's instructions. The sequencing was performed with an ABI PRISM Genetic Analyser 9100 (Applied Biosystems).

Western Blotting

Western blotting was performed to evaluate the presence of CA XII isoforms in five cell lines: U-87 MG, CCF-STTG1, Caki-1, A-498, and a human renal cell carcinoma cell line (UMRC6; generously provided by Dr. Sergey V. Ivanov). The cells were cultured under normoxia for 4 days in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and isolated from the cell culture plates, and total cell homogenates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting as previously described.[16]

Study Materials for Immunohistochemistry

Because there have been no earlier reports on alternative spliced isoforms of CA XII, and because we found that malignant astrocytomas and cell lines derived from glioblastomas expressed mainly the shorter form of CA XII, we wanted to study a large series of diffuse astrocytomas and correlate the level of CA XII expression with clinicopathological features and tumor-relevant molecular factors, including CA IX and CA II, cell proliferation (Ki-67/MIB-1), p53, vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR).

The study material consisted of 370 diffusively infiltrating astrocytic gliomas (grades II, III, and IV) obtained from patients who underwent surgery in Tampere University Hospital (Tampere, Finland) between 1983 and 2001. There were 287 primary tumors (39 grade II, 30 grade III, 218 grade IV) and 83 recurrences (15 grade II, 17 grade III, 51 grade IV). The tumors were radically resected when possible, and most patients with high-grade astrocytomas were also treated with radiotherapy. Ages of patients with primary tumors ranged between 3 and 82 years (median 55 ± SD 15 years), and ages of patients with recurrent tumors, between 3 and 75 years (median 44 ± SD 12 years). Overall survival was known for 287 patients (39 grade II, 30 grade III, and 218 grade IV), of whom only two were younger than 18 years. During the 5-year follow-up, 237 patients died and 50 patients were still alive.

After fixation in 4% phosphate-buffered formaldehyde, brain tumor specimens were processed into paraffin blocks. A neuropathologist (H.H.) evaluated the hematoxylin and eosin–stained slides on the basis of the WHO criteria,[17] which divide diffusely infiltrating astrocytomas into three grades (II–IV). Evaluation was made according to the presence of atypia, mitotic activity, necrosis, and endothelial proliferation. Then samples from histologically representative tumor regions were placed into the recipient multitissue blocks, which were constructed with a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The diameter of the tissue cores was 600 µm.

Antibodies and Immunohistochemistry

Monoclonal antibody M75, which recognizes the N-terminal domain of human CA IX, has been described previously.[18] The rabbit antihuman CA XII serum used for both immunohistochemistry and Western blotting has been characterized by Karhumaa et al.[16] The polyclonal antiserum was raised against the truncated form of recombinant human CA XII, and its specificity was confirmed under both denaturing and native conditions. The recombinant CA XII protein did not include the segment that is potentially deleted due to the alternative mRNA splicing process according to our present data. Therefore, the produced antibody should recognize both the intact CA XII and the spliced form. Rabbit antiserum against human CA II had been produced and characterized previously.[19] Normal rabbit serum was used for control staining.

Immunohistochemical analysis for CA II, CA IX, and CA XII was performed as previously described.[9,20,21] The stained sections were examined and photographed using a Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany). The intensity of the staining reaction for CA XII was scored from the sections using a four-category assessment: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. The extent of the CA XII staining was also scored on a scale from 0 to 3: 0, no positive cells; 1, <25% positive cells; 2, 25–50% positive cells; 3, >50% positive cells.

When simultaneous expression of CAs was evaluated in survival analysis, negative and weak stainings were considered CA-negative (CA –ve), and moderate and strong stainings were considered CA-positive (CA +ve), on each enzyme separately. Analyses on simultaneous expression of transmembrane CAs (CA XII and CA IX) were categorized as follows: 0, CA XII and CA IX both were CA –ve; 1, only one enzyme was CA +ve; 2, both enzymes were CA +ve. The variable obtained we refer to as the CA IX/XII index. Simultaneous expression of tumor-associated CAs (CA XII, CA IX, and CA II) was evaluated as follows: 0, all three enzymes were CA –ve; 1, one of the three enzymes was CA +ve; 2, two of the three enzymes were CA +ve; and 3, all three enzymes were CA +ve. This variable is called the CA II/IX/XII index.

Proliferation by Ki-67 (MIB-1) immunostaining,[22,23] VEGF immunostaining,[24] EGFR amplification by chromogenic in situ hybridization,[25] and p53 immunostaining[26] were performed as previously described.

Statistical Analysis

All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). The significance of associations was defined using the chi-square test, Mann-Whitney test, and Kruskal-Wallis test. The log-rank test and Kaplan-Meier curves were used in univariate survival analyses, and Cox multivariate regression analysis was used in the multivariate survival analyses.

Results

CA12 *Variant mRNA Is Expressed in Astrocytic Tumors*

All astrocytoma samples except for one grade II tumor were positive for CA XII in the PCR reaction (Fig. 1). The two grade IV samples showed especially strong bands for *CA12* mRNA. The hemangioblastoma sample served as a positive control for the PCR reaction, since defects in VHL protein often cause hemangioblastomas and also up-regulate the expression of CA XII.[4] Also, the hemangioblastoma sample was positive for *CA12* mRNA, as expected. The normal brain sample showed a very faint doublet, suggesting that it expresses both alternative isoforms. When it was reamplified with the original primers, the two bands became clearly visible. The tumor samples expressed mainly the shorter isoform of CA XII. Only one grade IV sample showed both bands. The bands from this sample were isolated from the gel and sequenced in order to confirm their identity. The sequencing results showed that these two bands indeed represented the two variants of *CA12* mRNA whose sequences can be found in GenBank.

We also analyzed some additional samples to see how common the spliced form is in different tissues known to express CA XII, for example, in the normal kidney and colon.[8,10] CA XII is also overexpressed in renal carcinomas and in several other tumors.[3–5] Therefore, RT-PCR was performed for the normal human kidney and colon and two renal carcinoma cell lines (Caki-1 and A-498). We also examined three glioblastoma multiforme (grade IV astrocytoma) cell lines (U-373 MG, U-87 MG, and CCF-STTG1). The results are shown in Fig. 1B. Normal human kidney and colon produced only the longer isoform of CA XII. The A-498 renal carcinoma cell line also produced the longer isoform, whereas the Caki-1 cell line showed bands representing both isoforms. One glioblastoma cell line was almost negative for CA XII, while the other two cell lines expressed both isoforms of CA XII.

Our conclusion from the PCR results is that the normal human colon and kidney express only the longer isoform of CA XII. The longer isoform may be dominant also in renal carcinoma cell lines, although Caki-1 cells also contained a faint band representing the shorter isoform. The normal human brain used in our study weakly expressed both isoforms, whereas the brain tumors expressed greater quantities of the spliced isoform.

Western blotting of two glioblastoma and the Caki-1 cell lines suggests that both *CA12* mRNA variants are translated into protein isoforms in these cell lines (Fig. 2). A VHL-defective cell line, A-498, showed an intense broad polypeptide band, which may include the shorter form in addition to the intact longer form of CA XII protein. Another cell line with VHL deletions, UMRC6, also showed an intense band, but only the longer isoform was evident.

CA XII Protein Is Expressed in Most Astrocytic Tumors

Because we found by RT-PCR and Western blotting the spliced isoform of CA XII in malignant astrocytomas and cell lines derived from glioblastomas, we wanted to study by immunohistochemistry whether CA XII is expressed in many or most astrocytomas, and whether the levels of CA XII expression correlate with clinico-

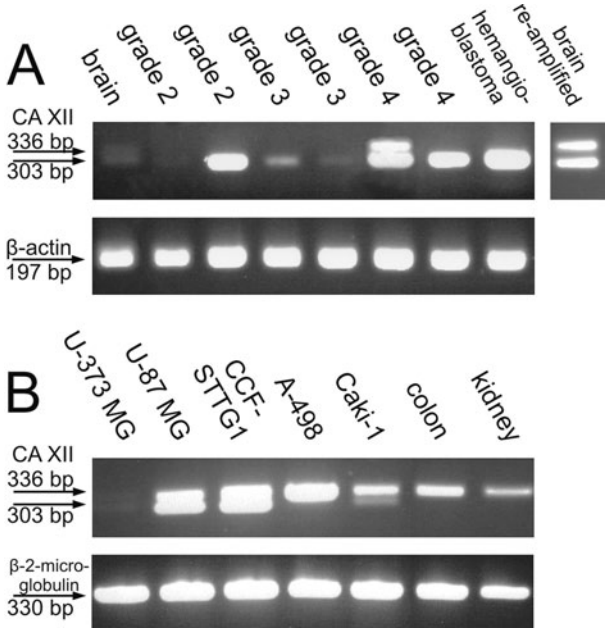

Fig. 1. Reverse transcription PCR results showing the expression of *CA12* mRNA in diffuse astrocytomas, hemangioblastoma, and normal brain (A) and in human tissues and cancer cell lines (B). The normal brain sample contains a faint double band representing alternatively spliced isoforms of carbonic anhydrase XII (CA XII; A). Hemangioblastoma, which served as a positive control, confirms that the PCR reaction has succeeded. The result of one grade II sample can be considered negative, whereas all the other astrocytoma samples contain *CA12* mRNA. One grade IV sample contained a double band, but in all the other positive samples the shorter form of CA XII was expressed. The last lane in A shows reamplified PCR products from the normal brain. Normal human tissues (colon and kidney) expressed the longer isoform of CA XII (B). This was also the dominant form in renal carcinoma cell lines (A-498 and Caki-1). However, Caki-1 also produced the shorter isoform. Two of the glioblastoma cell lines expressed both isoforms, and one was almost negative (the gel contained a very faint double band that is barely detectable in the picture taken from the gel).

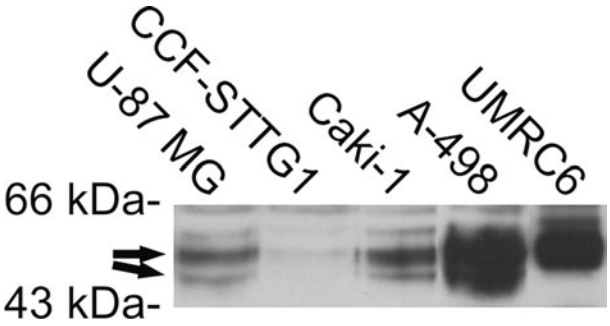

Fig. 2. Western blotting of carbonic anhydrase XII (CA XII) in glioblastoma and renal cancer cell lines. The arrows indicate the polypeptide bands representing the two isoforms of CA XII.

pathological features and with tumor-relevant molecular factors in diffuse astrocytoma specimens. In our large series of diffusely infiltrating astrocytomas, 363 (98%) of 370 cases showed positive immunostaining for CA XII. When the tissue sections were evaluated by four-category assessment, CA XII intensities were as follows: 39 (11%) were strongly stained, 169 (46%) moderately stained, 155 (42%) weakly stained, and 7 (2%) cases were negative. The score for CA XII extent describing the relative area of the positive staining was found to be 3 (>50% positive cells) in 207 (56%), 2 (25%–50% positive cells) in 47 (13%), 1 (<25% positive cells) in 109 (29%), and 0 (no positive cells) in 7 (2%) cases. The distribution of CA XII immunostaining was usually quite homogeneous in tumor tissue (Fig. 3). In some cases, immunoreactivity was also observed in the nuclei and cytoplasm of tumor cells. The nuclear staining was considered unspecific. The cytoplasmic staining most probably represents newly produced enzyme and was taken into account in the present scoring method. When morphologically evaluated, neither endothelial proliferation nor necrosis correlated statistically with CA XII intensity ($p = 0.264$ and $p = 0.619$, respectively, chi-square test).

The present results indicate that the CA XII intensity was higher in tumors with higher malignancy grade ($p = 0.006$, chi-square test; Table 1). The association was significant even when the intensities were grouped as CA-positive and CA-negative tumors ($p = 0.032$, chi-square test). Increasing patient age and CA XII intensity significantly correlated with each other in all tumors combined (primary tumors and recurrences) ($p = 0.022$, variance analysis) as well as in primary tumors ($p = 0.016$, variance analysis). This finding may be related to the higher age of patients with the most malignant gliomas.

When we compared the typical molecular pathological features of astrocytomas with CA XII immunohistochemistry, we found no association between proliferation by Ki-67/MIB-1 and CA XII expression (359 cases, Mann-Whitney test). There was a significant association between positive CA XII intensity and positive VEGF status (322 cases, $p = 0.032$, chi-square test). Neither EGFR amplification (249 cases) nor p53 (141 cases) correlated with CA XII intensity (chi-square test).

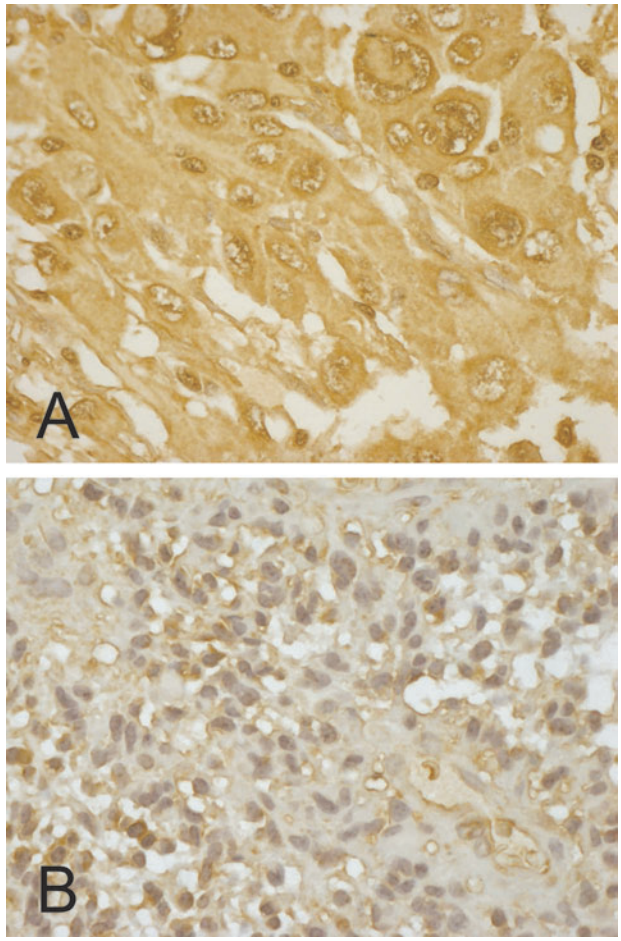

Fig. 3. Immunohistochemical staining of carbonic anhydrase XII in two astrocytomas. A and B show strong and weak immunoreactions, respectively. Original magnification, ×400.

CA XII Expression Correlates with Poor Prognosis

Overall survival data were known for 287 patients. When the patient survival was tested by the log-rank test in primary tumors, CA XII intensity divided the tumors into four significantly differing prognostic subsets ($p = 0.010$, log-rank test). The difference was even more significant when the cases with intensities 0 (negatively stained) and 1 (weakly stained) were pooled to make only three categories ($p = 0.004$, log-rank test) (Fig. 4A).

The prognostic significance of CA XII intensity was also evaluated in a multivariate analysis when important

Table 1. Comparison between carbonic anhydrase XII intensity and WHO grade ($p = 0.006$, chi-square test)

Carbonic Anhydrase XII Intensity	WHO Grade			Total
	II	III	IV	
0	4	0	3	7
1	28	22	105	155
2	19	23	127	169
3	3	2	34	39
Total	54	47	269	370

clinicopathological factors such as patient age, WHO grade, and proliferation by MIB-1 were included in the analysis. Most important, Cox multivariate analysis revealed that patient age ($p < 0.001$; odds ratio, 1.959; 95% confidence intervals [95% CIs] for odds ratio, 1.629–2.356), tumor grade ($p < 0.001$; odds ratio, 2.202; 95% CI, 1.697–2.858), and CA XII intensity ($p = 0.033$; odds ratio, 1.250; 95% CI, 1.018–1.535) all had independent prognostic value.

The expression levels of other tumor-associated CA isozymes, CA II and CA IX, were recently studied in the same patients.[20,21] In the present study, we evaluated both CA XII and CA IX intensities in parallel in 352 cases. When we compared the intensities of these enzymes, we found that CA XII positively correlated with CA IX intensity ($p < 0.001$, chi-square test). Simultaneous expression of CA XII and endothelial CA II was evaluated in 258 cases as well as in 260 cases between CA XII and cytoplasmic CA II, and no association was seen ($p = 0.872$ and $p = 0.778$, respectively, chi-square test). As described in "Materials and Methods," CA IX/XII and CA II/IX/XII indices describing simultaneous expression of CA II, CA IX, and CA XII were established. The CA IX/XII index significantly correlated with the WHO grade of the tumors ($p = 0.002$, chi-square test). When the CA IX/XII index was evaluated by the log-rank test, it classified the tumors into three prognostically differing subsets ($p = 0.003$, log-rank test; Fig. 4B). Remarkably, the simultaneous expression of transmembrane CAs (CA XII and CA IX) was significant when evaluated in Cox multivariate analysis. The analysis revealed that patient age ($p < 0.001$; odds ratio, 1.992; 95% CI for odds ratio, 1.655–2.397), WHO grade ($p < 0.001$; odds ratio, 2.176; 95% CI, 1.674–2.829), and the CA IX/XII index ($p = 0.048$; odds ratio, 1.196; 95% CI, 1.002–1.427) had independent prognostic value. The prognostic significance of the CA II/IX/XII index was studied in 191 cases. This index resulted in four significant prognostic subsets of tumors ($p = 0.002$, log-rank test; Fig. 4C). However, the CA II/IX/XII index was not significant in multivariate analyses that included the abovementioned variables.

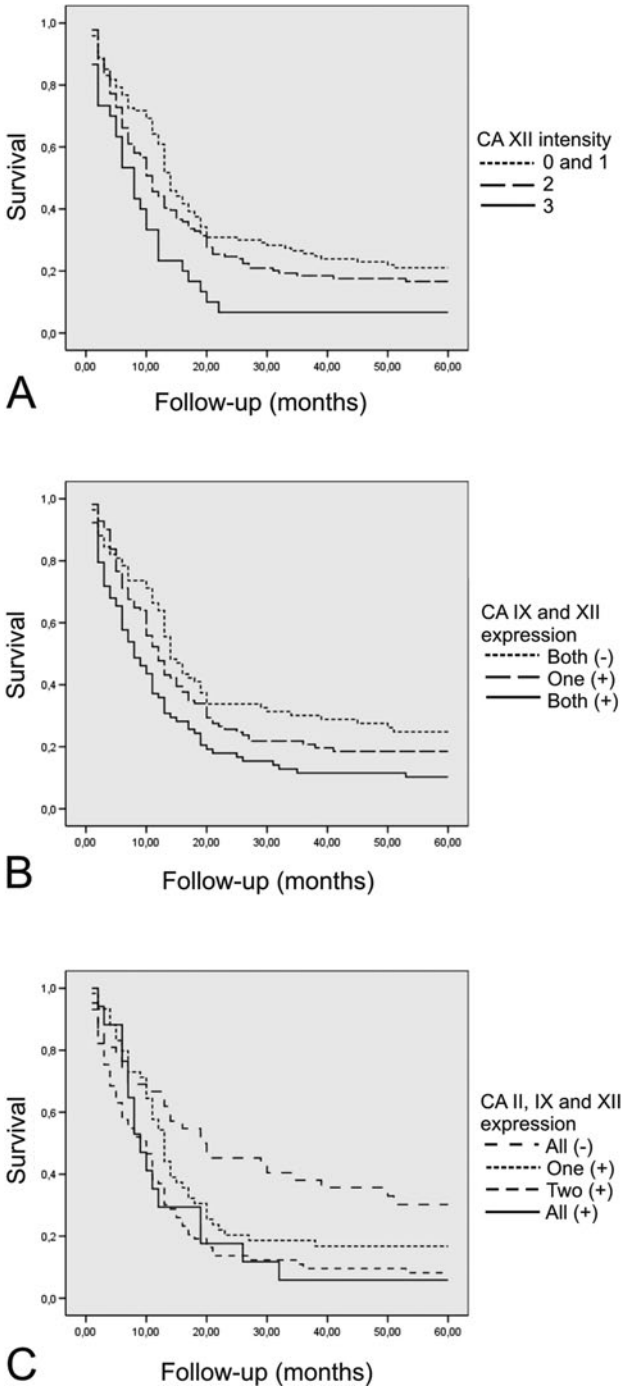

Fig. 4. Survival of patients with diffuse astrocytomas grouped according to carbonic anhydrase immunostaining intensity. (A) Carbonic anhydrase XII (CA XII) expression. Reaction strength: 0, no reaction; 1, weak; 2, moderate; 3, strong. (B) Simultaneous expression of transmembrane CA IX and CA XII. (C) Simultaneous expression of tumor-associated CA II, CA IX, and CA XII.

Discussion

The present studies were initiated when we found novel sequence data on an alternatively spliced CA XII isoform from GenBank. This information seemed important, because alternative mRNA splicing has been linked with oncogenic processes and found to correlate with patient survival.[1,2] In addition, CA XII overexpression has been associated with several types of cancer, but its prognostic significance has not been evaluated thoroughly. The only published data available are from analyses of breast cancer patients, in whom CA XII expression was found to be associated with good prognosis.[6]

The present RT-PCR analyses were designed to evaluate whether an alternatively spliced variant of *CA12* mRNA was detectable in astrocytoma samples. Normal human brain showed faint positive signals for both isoforms. All astrocytoma tumor samples except for one grade II sample contained the smaller, spliced isoform. Only one astrocytoma tumor sample and two glioblastoma cell lines contained the longer isoform in addition to the shorter one, whereas normal human kidney and colon contained only the longer isoform of CA XII. In conclusion, normal human tissues samples expressed mainly the longer isoform and little or none of the shorter isoform.

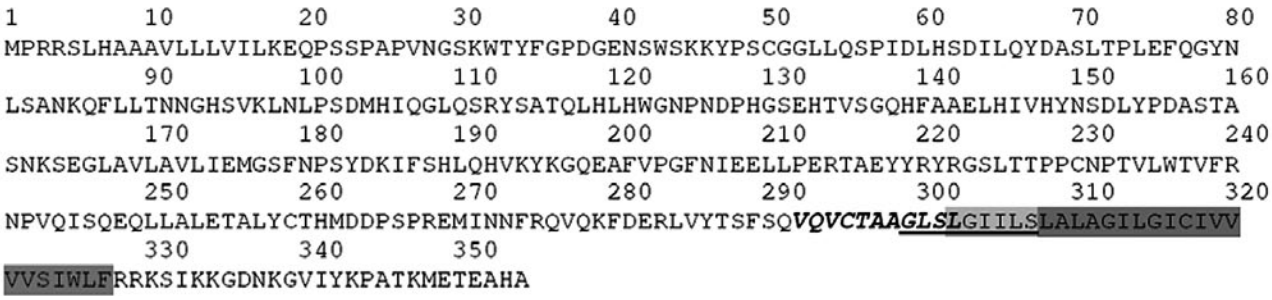

Fig. 5. The complete sequence of carbonic anhydrase XII. The 11 amino acid residues missing in the shorter isoform are shown in boldface and italics. The GXXXG and GXXXS motifs are underlined. The transmembrane region was predicted with three programs (TMHMM [http://www.cbs.dtu.dk/services/TMHMM-2.0/], TMpred [http://www.ch.embnet.org/software/TMPRED_form.html], TopPred [http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html]), and the N-terminal helix prediction of these programs differed slightly: the area that showed differences is shown in light gray, and the common transmembrane sequence prediction by these programs is shown in dark gray.

In contrast, the shorter isoform of CA XII appeared to be the predominant form in the brain tumors examined.

The results obtained in the present study raise a question about the possible functional consequences related to the alternatively spliced CA XII transcript. Exon 9, which is missing from the shorter isoform, has 33 nucleotides and encodes for 11 amino acid residues. Thus, the alternative splicing does not alter the reading frame in this splicing variant. Interestingly, the shorter isoform lacks residues that are located adjacent to the predicted membrane-spanning helix on the extracellular side of the membrane (Fig. 5). The CA XII transmembrane domain contains the GXXXG and GXXXS motifs that have been considered to be important for the dimerization of the native enzyme.[27–29] Importantly, the GXXXG motif is disrupted in the shorter isoform, and therefore these alternatively spliced protein isoforms could have different properties in the oligomerization of CA XII. The structural prediction by Motifscan software (http://scansite.mit.edu) suggests that the intracellular C-tail of CA XII contains a binding site for protein kinases C and A. Disruption of the GXXXG motif could potentially change the quaternary structure of dimeric CA XII protein and thereby affect the signaling cascades involving protein kinase–driven phosphorylation in the C-tail of CA XII molecule.

The alternative splicing event in the *CA12* gene seems to be conserved during evolution. Messenger RNAs representing these alternatively spliced isoforms can be found in the mouse databases (e.g., accession no. BC035941 codes for isoform 1 and BC031385 for isoform 2). Interestingly, the mouse and human 9th exons do not contain the same amino acids, except for the GXXXG motif (GLSLG), which is exactly the same in both species. The conservation of alternative splicing and disruption of the GXXXG motif raise the possibility that these two isoforms may have important functional roles, which can be evaluated using mammalian expression systems.

According to our immunohistochemical results, CA XII is also expressed at the protein level in most diffuse astrocytomas, and increasing cellular immunopositivity was associated with higher WHO grade and older patient age. Previous studies have established that age is an important prognostic factor in patients with primary astrocytomas and brain metastases[30–32] and is still considered in the assessment of treatment strategies.[33] Elderly patients have a worse outcome, which has been shown in numerous retrospective, prospective, and epidemiological studies.[17] Our study suggests that CA XII is another prognostic factor showing a positive correlation with patient age. Remarkably, in multivariate analyses, CA XII expression was an independent prognostic factor in addition to patient age and WHO grade.

Endothelial vascular proliferation due to angiogenic factors is one criterion that is used to distinguish grade IV astrocytomas from lower-grade tumors. We recently reported the induction of CA II expression in the tumor endothelium of diffuse astrocytomas.[20] This phenomenon was also associated with poor prognosis. Thus, we wanted to evaluate the simultaneous expression of hypoxia-inducible CA XII and CA IX as well as CA II as prognostic indicators. Simultaneous expression of CA II, CA IX, and CA XII predicted an extremely poor prognosis for the patients, and the multivariate analysis further revealed that simultaneous expression of both CA XII and CA IX was an independent prognostic factor. According to our data, parallel evaluation of these isozymes using an immunohistochemical method could be of clinical value when predicting the prognoses of astrocytomas in patients. Most notably, the survival data indicated that CA XII itself was an independent prognostic factor in astrocytomas, which seemed to express predominantly the alternatively spliced *CA12* mRNA. This finding agrees well with the previous observations that the mRNA splicing events are commonly associated with poor prognosis in cancer patients.[1,2] Further studies are warranted to evaluate whether the presence of the spliced *CA12* mRNA could be clinically useful as a prognostic marker in cancer diagnostics.

Acknowledgments

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