PAULI SALLINEN

Quantitative Pathology in Astrocytic Tumors

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ACADEMIC DISSERTATION

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Quantitative Pathology in Astrocytic Tumors

Special Reference to Image Analysis and Proliferation Capacity of Cancer Cells

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi
Lenkkeilijänkatu 6, Tampere,
on December 17th, 1999 at 12 o'clock.

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LIST OF ORIGINAL COMMUNICATIONS

The study is based on the following publications, which are referred to in the text by the Roman numerals I-V. This thesis also includes unpublished data.

I Haapasalo HK, Sallinen PK, Helén PT, Rantala IS, Helin HJ, Isola JJ. Comparison of three quantitation methods for PCNA immunostaining: applicability and relation to survival in 83 astrocytic neoplasms. J Pathol 1993; 171:207-214

II Sallinen P, Haapasalo H, Kerttula T, Rantala I, Kalimo H, Collan Y, Isola J, Helin H. Sources of variation in the assessment of cell proliferation using proliferating cell nuclear antigen immunohistochemistry. Analyt Quant Cytol Histol 1994; 16:261-268

III Sallinen PK, Haapasalo HK, Visakorpi T, Helén PT, Rantala IS, Isola JJ, Helin HJ. Prognostication of astrocytoma patient survival by Ki-67 (MIB-1), PCNA, and S-phase fraction using archival paraffin-embedded samples. J Pathol 1994; 174:275-282

IV Sallinen P, Miettinen H, Sallinen S-L, Haapasalo H, Helin H, Kononen J. Increased expression of telomerase RNA component is associated with increased cell proliferation in human astrocytomas. Am J Pathol 1997; 150:1159-1164

V Sallinen PK, Sallinen S-L, Helén PT, Rantala IS, Rautiainen E, Helin HJ, Kalimo H, Haapasalo HK. Diffusely infiltrating astrocytomas by quantitative histopathology, cell proliferation and image cytometric DNA analysis: Comparison of 133 tumors in the context of WHO 1979 and WHO 1993 grading schemes. Submitted for publication, 1999.

ABBREVIATIONS

AgNOR	nuclear organizer regions detected by silver binding argyrophilic techniques
apoptosis	"genetically programmed" cell death
BrdU	bromodeoxyuridine
BCNU	bischloroethyl-nitrosurea
CDK	cyclin-dependent kinase
CI	confidence interval
CNS	central nervous system
cpm	counts per minute
CT	computed tomography
CV	coefficient of variation
DAB	diaminobenzidine
dATP	deoxyadenosinetriphosphate
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FCM-DNA	flow cytometric DNA analysis
GFAP	glial fibrillary acidic protein
hEST2	human ever shorter telomeres protein 2
HPF	high-power microscopic field
hTRT	human telomerase reverse transcriptase
ICM-DNA	image cytometric DNA analysis
IOD	integrated optical density
KI-67(MIB-1)	MIB-1 antibody directed against the Ki-67 antigen
LI	labeling index
LOH	loss of heterozygosity
M/V-index	volume corrected mitotic index
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NOR	nuclear organizer region
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PTEN	phosphatase and tensin homolog
RNA	ribonucleic acid
ROC	receiver operating characteristics
TGF-α	transforming growth factor alpha
TmR	messenger ribonucleic acid of the telomerase RNA component
TRAP	telomeric repeats amplification protocol
TRF1	telomeric-repeat binding factor 1
VPN	area fraction covered by neoplastic nuclei
WHO	the World Health Organization

INTRODUCTION

Astrocytes form the astroglia that constitutes the principal supporting tissue of the central nervous system (CNS). The fine branching network, created by numerous dendrite-like processes of astrocytes, ramifies among nerve fibers and connects them to blood vessels and neighboring neurons. Astrocytes have an important role in normal brain metabolism and neuronal activity, as well as in sustaining the blood-brain barrier (Burger *et al.* 1991). Tumors of the glial tissue, gliomas, are the most common type of primary neoplasms of the brain (Burger *et al.* 1991). In most instances, gliomas are of astrocytic origin.

The etiology of brain tumors has remained unknown. Only some of the increase in the incidence rates can be explained by improved diagnostic techniques. The magnitude of the risk of developing a brain tumor, as a result of a long-term exposure to electromagnetic fields, has been an issue of controversy, but various studies have observed relatively consistently that the risk could be present (Brem *et al.* 1995). The causality of environmental and nutritional hazards for the occurrence of a brain tumor remains to be shown. Approximately 5 % of brain tumors, including astrocytic tumors, have been related to a hereditary gene defect (Louis and von Deimling 1995).

In the United States during the years 1990-1994, gliomas accounted for approximately 40% of all primary brain tumors defined by histology, and the second largest group, meningiomas, for 24% (Central Brain Tumor Registry of the U.S. data, Surawicz *et al.* 1999). In Finland, over 1260 new gliomas were diagnosed between 1988 and 1992. The age-adjusted incidence rates (5.0 for males and 4.1 for females per 100 000 person-years during 1988-1992), as in the US, have shown a gradual increase over the past decades (Finnish Cancer Registry 1996). At that time, slightly over 150 primary astrocytic tumors were operated on at the Tampere University Hospital (Figure 1). The number of the patients totalled over 170 during the years 1993-1997. Despite substantial advances in brain imaging and surgical technology, the five-year cumulative survival rates of the patients have not essentially changed (Figure 2).

The advent of computed tomography (CT) and magnetic resonance imaging (MRI) has essentially improved the diagnosis of brain tumors, characterized clinically by neurological symptoms from mild indisposition or headaches to severe impairment. The diagnosis is done by microscopic evaluation of the tumor histology, on the basis of prevailing classification and grading systems and for decisions on treatment. Astrocytic tumors are primarily treated by surgery. The initial histological diagnosis is usually done from the available tumor material during surgery, after which the resected tumor is processed for more thorough inspection. Radiotherapy is combined post-operatively in the treatment of histologically highly malignant lesions. In cases of less aggressive histopathological appearance, decisions on tumor radiation vary, balancing the benefits against the risk of secondary radionecrosis (Pollack *et al.* 1995). The use of systemic chemotherapy or radiosensitisers is challenged by the blood-brain barrier, and the more targeted approaches are still to date very experimental. Most astrocytic tumors possess a strong capacity to progress, and, if left untreated, the tumors behave in a malignant manner. An exception is made by some pilocytic astrocytomas that typically occupy the cerebellum of children (Burger *et al.* 1991).

The vast biological variation between individual astrocytic tumors, even of the same histopathological malignancy grade, challenges therapy. The conventional histopathological inspection of the tumor tissue has provided only crude distinctions between typical tumors of the most extreme prognostic categories (Vandenberg 1992). In addition, the subjectivity in recognition and appreciation of the established grading criteria has greatly affected the reproducibility of

different observers (Coons *et al.* 1997). This has stressed the need for approaches that could improve the accuracy in prognostic evaluation of astrocytic tumors. The current knowledge of the phenomena leading to and involved in the genesis and progression of human cancer has resulted in an increasing collection of commercially available analysis procedures. Subsequently, new aspects have been pinpointed for future diagnostic and prognostic determinations in neuropathology.

This retrospective study was carried out in search of new prognostic factors which, alone or in combination with conventional methods, could aid in communication about the treatment of astrocytoma patients. Factors closely related to the established histopathological grading criteria have been investigated in a quantitative perspective. Immunohistochemically determined cell proliferation activity and cytometric DNA measurements have been studied in the present series of astrocytic tumors for their applicability in routine clinical neuropathology. In addition, the potential role of telomerase enzyme activity in sustaining cell proliferation and continued growth of astrocytic tumors has been evaluated. Emphasis has been put on the measurement techniques in order to improve the objectivity and reproducibility of the results analyzed. For this purpose, computer-assisted image analysis has been utilized in parallel with the more conventional visual approaches.

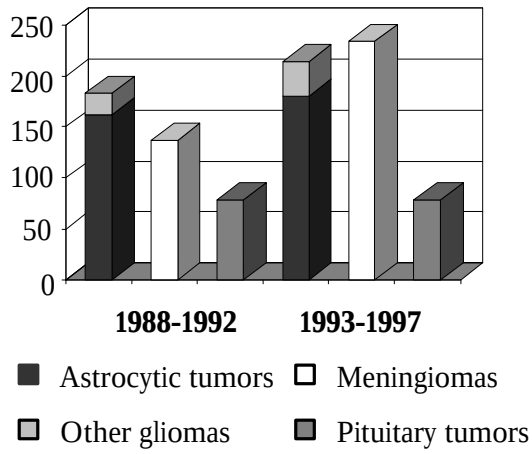

Figure 1. The most common primary neoplasms of the brain operated on at Tampere University Hospital 1988-1997.

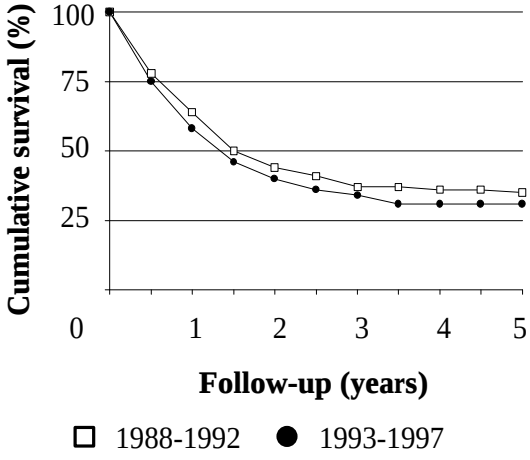

Figure 2. Cumulative survival rates of astrocytic tumor patients operated on at Tampere University Hospital 1988-1992 and 1993-1997.

1. Histology

Bailey and Cushing (1926) were pioneers in the histological typing of glial tumors (gliomas). The evaluation of the tumor tissue focused on the resemblance of adult glioma cells with cells at various stages of embryogenetic differentiation. Kernohan and Sayre (1952) favored a more simplified classification system which subdivided gliomas into astrocytomas, oligodendrogliomas and ependymomas on the basis of cell differentiation.

Astrocytic tumors (Table I) comprise a number of cytological and histological varieties (Vandenberg 1992, Kleihues *et al.* 1993a). One fundamental subdivision has been made between diffuse astrocytomas, pilocytic astrocytomas, pleomorphic xanthoastrocytomas and subependymal giant cell astrocytomas (Kleihues *et al.* 1993a, Kleihues *et al.* 1993b). The latter three tumors generally represent a more circumscribed growth and favorable prognosis. Diffuse astrocytomas show a growth pattern that infiltrates the surrounding tissue. The tumor entity has been termed as the diffusely infiltrating astrocytomas. They are the most common of the astrocytic lesions and encompass three histological variants: the fibrillary type, the protoplasmic type and the gemistocytic type. The fibrillary type is the most typical variant. Gemistocytic differentiation, also as a composition, has been associated with a relatively rapid tumor progression, whereas protoplasmic astrocytomas have been shown to represent a more benign tumor behavior (Watanabe *et al.* 1997, Prayson and Estes 1996).

Table I. The histological typing of gliomas by the WHO (Kleihues *et al.* 1993a).

1. Astrocytic tumors
- Astrocytoma, Grade II
  - *variants:* *fibrillary*, *protoplasmic*, *gemistocytic*
- Grade III (anaplastic) astrocytoma
- Glioblastoma, Grade IV
  - *variants:* *giant cell glioblastoma*, *gliosarcoma*
- Pilocytic astrocytoma, Grade I
- Pleomorphic xanthoastrocytoma, Grade II-III
- Subependymal giant cell astrocytoma, Grade I

2. Oligodendroglial tumors
- Oligodendroglioma, Grade II
- Anaplastic oligodendroglioma, Grade III

3. Ependymal tumors
- Ependymoma, Grade II
  - *variants:* *cellular*, *papillary*, *clear cell*
- Anaplastic ependymoma, Grade III
- Myxopapillary ependymoma, Grade I
- Subependymoma, Grade I

4. Mixed gliomas
- Oligo-astrocytoma, Grade II
- Anaplastic oligo-astrocytoma, Grade III
- Others

5. Choroid plexus tumors
- Choroid plexus papilloma, Grade II
- Choroid plexus carcinoma, Grade III

The vast number of tumor entities established to occupy the central nervous system (Kleihues *et al.* 1993a) has challenged the diagnostic determination of an astrocytoma. In addition to the recognition of distinct histomorphological features, neuropathologists have become to rely on immunohistochemical methods for the differential diagnosis. The staining for the glial fibrillary acidic protein (GFAP) has been of the most significant value. The GFAP is a type of cytoplasmic intermediate filaments and is present in normal, reactive and neoplastic astrocytes (Paetau 1989, Burger *et al.* 1991). GFAP expression in astrocytomas has been shown to decrease along with the dedifferentiation process (Schiffer *et al.* 1986). Other routinely used immunohistochemical stainings in brain tumor diagnosis include in alphabetical order: carcino-embryonic antigen (CEA, positive e.g. in metastatic carcinomas), chromogranin (neuroendocrine tumors), cytokeratin (CK, metastatic carcinomas), epithelial membrane antigen (EMA, meningiomas), HMB-45 (melanomas), leukocyte common antigen (LCA, lymphomas), placental alkaline phosphatase (PLAP, germ cell tumors), S-100 (schwannomas), and synaptophysin (primitive neuroectodermal tumors) (Ackerman 1996).

1.1 Histopathological malignancy grade

The malignancy grading of astrocytomas has been based on the recognition of anaplastic histopathological features during the microscopic inspection of the tumor tissue, and various grading schemes have served as prognostic tools for therapeutic purposes (Vandenberg 1992). Kernohan and Sayre (1952) proposed a glioma classification system in which the astrocytoma group was further divided into four grades. The significant features included cellular anaplasia, cellularity, presence of mitotic figures, endothelial proliferation, presence of necrosis and the extent of tumor transition zone to normal brain. Since Kernohan's four-tiered grading scheme, several other grading systems have modified the diagnostic evaluation of astrocytomas in search of improved prognostic accuracy, as well as reproducibility of the evaluation (Daumas-Duport *et al.* 1988). The diagnostic histopathological determinants have remained basically the same. However, the simultaneous use of various grading schemes, different from each other in nomenclature and the way the significance of the determinants have been considered, has confused the comparisons of different studies. The consensus meeting, held in 1988 (Kleihues *et al.* 1993a), took into consideration the tumorigenetic and prognostic concept of astrocytomas. It was proposed that diffusely infiltrating astrocytomas, characterized by their invasive growth behavior and a typical stepwise anaplastic progression (Figure 4), should be separated from the more circumscribed pilocytic and subependymal giant cell astrocytomas regarded as Grade I. The separation seemed warranted not only for the relatively good overall prognosis of patients with circumscribed tumors, but also for the distinct differences in the genetic background of the tumor entities. Furthermore, new emphasis was placed on the immunohistochemical differential diagnosis of brain tumors. In the new three-tiered scheme (Grades II - IV), adapted by the World Health Organization (WHO), tissue cellularity was removed from the list of the pivotal grading determinants, and mitotic activity was considered to characterize anaplastic astrocytoma growth (Grade III), as well as glioblastomas (Grade IV). The presence of tumor necrosis, accompanied with endothelial cell proliferation, had earlier upgraded tumors into the Grade IV category. In the revised grading scheme, the glioblastoma diagnosis was proposed also to be possible on the basis of the detection of endothelial proliferation. This has also been suggested by Daumas-Duport *et al.* (1988) in their grading scheme, denoted as the St. Anne-Mayo System for diffusely infiltrating astrocytomas. The revision was supported by Barker II *et al.* (1996), who found necrosis present in 88% of the glioblastomas studied. Survival of patients with tumors containing endothelial proliferation, but no necrosis, was considered to resemble rather that of patients with glioblastomas than of those with anaplastic astrocytomas. However, the detection of tumor necrosis, unlike endothelial proliferation, has been shown to be of independent prognostic value in diffuse astrocytomas, and remained a pivotal grading criterion in some institutes (Cruz-

Sánches *et al.* 1997, Newcomb *et al.* 1998, Giannini *et al.* 1999). Table II summarizes the significant grading criteria of the WHO 1993 grading system.

Table II. The histopathological grading of diffuse astrocytomas according to the WHO 1993 classification system (Kleihues *et al.* 1993a).

ASTROCYTOMA	**Grade II**	**Grade III** (anaplastic)	**Grade IV** (glioblastoma)
Features of anaplasia			
cytological atypia	+	+	+
mitoses		+	+
endothelial proliferation			+
tumor necrosis			+

The following characterization of the fibrillary-type astrocytoma malignancy grades is presented with respect to Burger *et al.* (1991) and the WHO 1993 grading scheme (Kleihues *et al.* 1993a).

Grade II astrocytomas are most prevalent in patients under 40 years of age. Macroscopically, the homogenous or cystic tumor is indefinitely boundered with the surrounding tissue. Microscopically, cellular density is increased, and cells are irregularly distributed. In more compact areas, cytoplasmic processes form fibrillary and often microcystic networks. Neoplastic astrocytes may be seen to be clustered around neurons, the phenomenon also known as "satellitosis". The tumors are marked by nuclear atypia and pleomorphism. Mitotic activity is very rare or non-existent. The extent of surgical removal is an important prognostic factor, providing a 5-year survival rate of 60-80% (Daumas-Duport *et al.* 1988, Philippon *et al.* 1993). Most patients die within ten years after the onset of treatment. During this time, tumors tend to re-occur and progress into more malignant forms.

Anaplastic (Grade III) astrocytoma. Mitoses serve as the most important histopathological determinant of high-malignancy astrocytomas. In addition, cells are more densely packed, when compared with Grade II tumors. Anaplastic astrocytomas give rise to glioblastomas and are likewise treated aggressively. Therefore, the tumor category has been considered more of prognostic than therapeutic significance. Tumors usually appear some ten years later than Grade II astrocytomas and ten years earlier than glioblastomas. The median patient survival is less than two years (Daumas-Duport *et al.* 1988).

Glioblastoma (Grade IV) is the most common glioma and represents the culmination of the malignant transformation of astrocytomas. Patients are typically over 50 years of age. Necrosis, often present and characterized by densely parenthesizing neoplastic cells (pseudopalisading), has been an important diagnostic criterion, but recently the detection of endothelial proliferation together with mitotic activity have been proposed to be sufficient for the diagnosis (Kleihues *et al.* 1993a). Glioblastomas show a strong tendency to re-occur, usually as highly undifferentiated small-

cell neoplasms with poor prognosis. Despite aggressive tumor therapy, median survival is often less than one year. However, a small number of patients have been reported to survive in the long-term (Vertosick and Selker 1992, Chandler *et al.* 1993). Such a positive relationship with survival has been described in the presence of unusually large and multinucleated cells, defining a rare variant of glioblastomas, the giant cell glioblastoma (Burger and Vollmer 1980).

2. Clinical Factors

2.1 Location and size

Astrocytomas usually involve the cerebral hemispheres, and the topographic incidence is in relation to the amount of white matter that contains the highest number of fibrillary astrocytes (Burger *et al.* 1991). Brain stem lesions are relatively rare and occur most commonly in children (Packer *et al.* 1992). Hemispheric location has been shown to provide a survival advantage over deep-seated astrocytomas, e.g. in the thalamic region, and those of the brain stem (McCormack *et al.* 1992, Packer *et al.* 1992).

Although some controversy exists about the prognostic value of the preoperative size of an astrocytoma, the extent of its volumetric reduction has been considered to play an important role in therapy (Laws *et al.* 1984, Cohadon *et al.* 1985, Wood *et al.* 1988, Soffietti *et al.* 1989, Philippon *et al.* 1993, Berger 1994, Laws 1995). The contrast enhancing property of highly malignant astrocytomas at computed tomography (CT) has been used as an aid in determination of the tumor volume and the extent of its resection (Berger 1994). Berger (1994) proposed that an attempt at radical tumor resection should be made. This was not only because of the improvement achieved in functional outcome of the patients, as a result of the relief from the mass effect. Berger also pinpointed the significant risk of the residual tumor, left within the brain after surgery, of tumor transition to high-grade malignancy. The volume of the residual tumor was demonstrated to be inversely correlated with the length of the survival. Similarly, McCormack *et al.* (1992) suggested that the residual tumor cells of low-grade astrocytomas could have the potential for dedifferentiation and progression of the original tumor. The authors reported enhancement with administered contrast in 30% of low-grade astrocytomas. They suggested that contrast enhancement may reflect aggressive components within the tumor tissue, since a significant prognostic difference was found between contrast enhancing and non-enhancing astrocytomas. Philippon *et al.* (1993) reported an 80% 5-year cumulative survival of patients with completely removed low-grade astrocytomas. The survival rates for incomplete resection and biopsy were 50% and 45%. The authors found contrast enhancement in 40% of low-grade astrocytomas. However, no survival difference between the patient groups could be demonstrated.

2.2 Patient age

The strong influence of patient age on survival has been explained, to some extent, by the typical occurrence of aggressively behaving high-grade astrocytomas at a somewhat later age, and by decreased host resistance with age (Cohadon *et al.* 1985, Burger *et al.* 1991). However, patient age has been shown also to have a role within the tumor malignancy categories (Burger *et al.* 1985, Burger and Green 1987). McCormack *et al.* (1992) reported a distinct survival difference in adults with low-grade astrocytomas between patient groups of under and over 40 years of age. Philippon *et al.* (1993) extended the influence of age on therapeutic results in a series of adult low-grade

astrocytomas. They showed that patients older than 40 years gained significant benefit from radiotherapy after incomplete tumor resection. Vertosick and Selker (1992) evaluated a series of 21 high-grade astrocytomas in patients surviving for more than four years after the diagnosis (survival ranged from 4.2 to 15.8 years). They concluded that young age was a key factor favoring the good prognosis. A confirming conclusion was drawn by Chandler *et al.* (1993), who reported a subgroup of glioblastoma (Grade IV) patients with a long-term survival of at least five years. In the case of children, young age limits the use of adjuvant radiotherapy due to a possible penalty in long-term cognitive function (Pollack *et al.* 1995). It has been suggested that the potentially deleterious effect of radiotherapy on children could be postponed, or even avoided, by means of chemotherapy (Castello *et al.* 1998).

2.3 Karnofsky's performance status

The patient's activity or ability to work can be evaluated by Karnofsky's ten-tiered scale (Karnofsky and Burchmal 1949). Cohadon *et al.* (1985) suggested that the very poor survival of patients with high-grade astrocytomas could be explained by the interactions between aggressive tumor behavior, old age and poor functional status at the time of the diagnosis. Philippon *et al.* (1993) reported that 37 % of the adult low-grade astrocytoma patients studied had a normal life (score > 70) and 48 % only moderate neurological deficit (score 60 - 70) before the operation. Some 15% of the patients needed assistance in daily activities (score < 60). They found that Karnofsky's score had a significant impact on the length of survival, but it was also stated that surgery did not essentially alter the prognostic subgroups. A high baseline Karnofsky score could offer a good response to the tumor therapy (Chandler *et al.* 1993). However, Sachsenheimer *et al.* 1992 suggested that, especially in the final stages of the disease, a generally short-lived post-operative improvement in neurological function also needs to be considered with regard to the patient's well-being when planning the onset of aggressive adjuvant therapy.

3. Treatment of Astrocytomas

As a general oncologic rule, maximum reduction of tumor tissue should be carried out by surgery as early as possible (Kaye and Laws Jr 1995). First, surgery serves to make precise pathological diagnosis. Second, tumor cells left within the brain possess a risk of tumor growth and dedifferentiation. Third, the elimination of the mass effect could reverse neurological deficit. However, the infiltrative growth of most astrocytomas and the capacity of individual tumor cells to migrate distances make radical surgical approaches difficult to accomplish. In addition, surgical intervention increases the potential risk of a new or aggravated neurological deficit as well as postoperative morbidity. Therefore, management plans for patients with astrocytic tumors have varied (Kaye and Laws Jr 1995).

A *standard* treatment recommendation pinpoints the histopathological verification of the diagnosis (Kaye and Laws Jr 1995, Rock *et al.* 1999). Radical resection of the tumor tissue is the traditional means of obtaining tumor specimens for the diagnostic inspection. Considering non-life-threatening mass lesions, such as low-grade astrocytomas in neurologically intact patients, one option is biopsy, the diagnostic accuracy of which has significantly improved with the development of brain imaging by MRI especially. Surgical management could alternatively be delayed if frequent radiological check-ups do not indicate any change in the appearance of the lesion (Rock *et al.* 1999). In this case the diagnosis remains inconclusive and the decision usually involves patients with deep-seated astrocytomas and/or with an increased risk of poor postoperative recovery.

Radiation therapy is usually administered in the management of high-malignancy (Grade III-IV) astrocytomas, whereas the benefits of its use in the treatment of low-grade tumors remain to be shown (Kaye and Laws Jr 1995). Considering the deleterious effects of radiation on normal brain tissue, it has become the current practice to restrict radiation therapy to an image-defined tumor mass with sufficient margin in order to sustain maximum quality of survival (Kaye and Laws Jr 1995). Such stereotactic (image-guided) treatment includes targeted external-beam radiation with e.g. the gamma knife and the application of interstitially implanted radioisotopes (^{125}Iodine and ^{192}Iridium) in the tumor cavity.

Chemotherapy may have a significant clinical role in postponing the need for tumor irradiation in the treatment of low-malignancy astrocytomas of children (Castello *et al.* 1998). In general, however, chemotherapy in combination with radiation therapy has not been shown to essentially improve the survival of astrocytoma patients. Carmustine (BCNU, bischloroethyl-nitrosourea) is often the first drug of choice in planning chemotherapy, because of its good delivery through the blood-brain barrier (Kaye and Laws Jr 1995). As carmustine has been traditionally administered intravenously, carmustine saturated biodegradable polymers have been applied in the treatment of high-grade astrocytomas, as locally delivered into the tumor cavity, providing high local concentration of the drug with minimal systemic toxicity (Brem 1990, Valtonen *et al.* 1997). The clinical effectiveness of carmustine has been suggested to be limited by inherent drug resistance and the sensitivity of the surrounding brain (Kaye and Laws Jr 1995). Considering the latter, Subach *et al.* (1999) reported recently that patients did not benefit from locally implanted BCNU-wafers, rather the treatment increased the risk of postoperative complications, especially those related to infection and wound healing. Orally administered lomustine and procarbazine have approximately the same effect as intravenous carmustine therapy (Kaye and Laws Jr 1995). In addition, some grade III astrocytoma patients may better respond to a combination of procarbazine, lomustine and vincristine (PCV) than carmustine treatment (Levin *et al.* 1990).

Some astrocytic tumors respond to the radiation therapy or chemotherapy better than others, which has been suggested to result from the genotypic variation between individual tumors (Shapiro *et al.* 1993, Scheck *et al.* 1993, Belanich *et al.* 1996, Haas-Kogan *et al.* 1999, Huhn *et al.* 1999). New drugs, such as the temozolomide (O'Reilly *et al.* 1993), and technical achievements, such as the boron neutron capture therapy (Barth *et al.* 1999), have been introduced and tested for enhanced local therapy. In addition, the genotype of cancer has initiated clinical trials for the development of new therapeutic strategies (Culver and Blaese 1994). In a rat model, the results of gene therapy (induced apoptosis in a malignant glioma) have been promising (Hakumäki *et al.* 1998). However, a Finnish multicenter study on human gliomas shows that the strategy needs further development (unpublished data).

4. Quantitative Pathology

The histopathological malignancy grade of an astrocytoma is established on the basis of subjective recognition and appreciation of the grading criteria during microscopic inspection. However, subjectivity in the evaluation of tumor samples has been shown to affect the value of the morphology-based grading as an interlaboratory reference standard (Coons *et al.* 1997). Considering the intratumor heterogeneity in histopathological malignancy, especially of high-grade astrocytomas, a correctly assigned tumor grade is heavily dependent on the experience of the inspector and the size of the tumor sample (Burger and Kleihues 1989). Furthermore, the prognostic value of histopathological grading has been essentially derived from the gross survival differences between typical astrocytomas of the extreme malignancy groups (Vandenberg 1992). Regarding both the

revised WHO 1993 and the St. Anne-Mayo astrocytoma grading schemes, Coons *et al.* (1997) reported an initial grading concordance of 54 % (59 % for the St. Anne-Mayo system) between four neuropathologists having independently reviewed a set of 66 gliomas. With adjustments regarding the interpretation of individual grading criteria, the concordance rates were shown to improve to 86% and 89% respectively. Coons suggested that the presence of a histological criterion was easier to evaluate than its degree. On the other hand, quantitative assessment of histomorphological features has been proposed to increase both the objectivity and reproducibility of tumor evaluation, as well as the sensitivity in identifying minimal changes of biological relevance (True 1996).

Quantitative cytopathology and histopathology have offered a numerical scale for a range of features obtained from tumor tissues, including number or area fraction of analysis objects, linear distance between objects as well as various form factors (e.g. size and shape) characterizing the objects (True 1996). Whereas the conventional assessments in tumor pathology have focused on the readily visible features (such as the neoplastic nuclei and the chromatin texture within the nuclei, or cell density and mitotic figures), a variety of techniques have been introduced as tools for improved diagnostic accuracy and prognostic settlement. True (1996) reviewed the potential sources of variation in morphometric analyses, and attention was devoted to sample acquisition and fixation that may cause substantial changes in tissue architecture and cell integrity. In cytometric analyses of the nuclear DNA content, for example, autolysis has been demonstrated to increase DNA staining, which resulted in apparent but false DNA aneuploidy (Alanen *et al.* 1989). As for immunohistochemical analyses, the cross-linking of proteins by formalin fixation may significantly alter the immunodetectability of the molecules, which has been approached by various modifications in the use of antigen retrieval techniques, antibody concentrations and intensification of the bound antibodies (Munakata and Hendricks 1993, McCormick *et al.* 1993, Pileri *et al.* 1997). Whereas changes related to the conditions of the material analyzed may have a significant effect on the reliability and reproducibility of different results, the extensive use of various analysis techniques has caused concern regarding the lack of carefully defined and universally followed standards (Wied *et al.* 1989). This also accounts for sampling in measurements of either traditional diagnostic or investigative nature, or biologically heterogeneous tissues (Collan *et al.* 1987).

The efficiency, accuracy and objectivity of various quantitative analyses depend largely on the choice of the measurement technique (True 1996). Manual assessment in quantitative pathology is done from the microscopic image by counting the expression of a variable either directly or with tools, such as overlaid grids. The method provides targeted object analysis involving relatively hard work, and it requires well-prepared schemes for sampling and data collection in order to sustain sufficient accuracy and reproducibility. Fully automated analysis, on the other hand, offers large and objective data collection. Without intervention by the inspector, however, fully automatic technique is only rarely appropriate for the diagnostic routine in pathology. The best diagnostic measurement technique of quantitative pathology has been suggested to take advantage of the control in manual analysis and the potential provided by computer-assisted image analysis (Wied *et al.* 1989). The computerized image analysis workstation in pathology has basically four distinct elements: the microscope for the identification of the object, the video or digital camera for scanning of the microscopic image, the lookup table ("frame grabber") for the separation of the object from the background data, and the computer for storage and analysis of the digitized data (Bacus and Grace 1987, Erler *et al.* 1993).

4.1 DNA cytometry

In cytophotometry, or cytometry, the relative concentration of substances in cells can be evaluated by light absorbance (DNA image cytometry, ICM-DNA) or specific fluorescence (DNA flow

cytometry, FCM-DNA)(Atkin 1970, Auer *et al.* 1989). Within the limits of resolution, the technique has been widely used for the measurement of nuclear DNA content (Caspersson 1987, Wied *et al.* 1989). Detectable alterations in DNA content may result from changes in number or size of chromatids during replication, chromosomal gain or deletion, gene amplification, viral infection or polyploidization (e.g. endomitosis). The results of DNA cytometric analyses are usually displayed in frequency histograms, in which the DNA distribution is evaluated for tumor DNA ploidy (a type of the distribution with reference to known standard, e.g. 2C = two sets of 23 chromosomes) or DNA index (a quantitative estimate of the relative DNA content with reference to the DNA diploid, i.e. euploid, cells). The coefficient of variation (CV) has been used for determinations of the quality of the analyses (Wied *et al.* 1989). Cytometry has provided means for the detection of gross total genetic aberrations in tissues, but the method has also been utilized for cell proliferation estimates and concentration analyses of labeled intracellular (e.g. cell proliferation associated) markers (Koss *et al.* 1989, Cohen 1996). Image cytometers have also been used for simultaneous analysis of various cytomorphometric and histomorphometric parameters.

4.1.1 Image cytometric DNA analysis

In 1935, Caspersson described a cytophotometric experiment that measured nuclear DNA content of unstained cells (Caspersson 1987). Along with development in instrumentation, the Feulgen reaction, introduced by Feulgen and Rossenbech (1924) for staining of DNA, has become the basis for modern absorbance cytometry. The technique measures the difference in intensity between the light transmitted through Feulgen stained DNA and uninterrupted light passing through the surrounding medium. Basically, a conventional microscopic image is captured by a digital camera, converted into a series of light points or pixels and transmitted to a computer for presentation and analysis. The gray level, numerical value for the light intensity, in all the pixels within the target object (e.g. a nucleus) is quantified as an integrated optical density (IOD) corresponding to the amount of DNA in the nucleus. The measurement is relative, and the system needs to be calibrated for the linearity of the IOD values (DNA scaling for arbitrary unit) with external reference cells. In addition, internal control, such as lymphocytes intermingled in the tissue, is utilized for the establishment of the DNA diploid (euploid) cell population. The ICM-DNA analysis is interactive, enabling the observer to select the nuclei to be measured. However, the control over the analysis material is compromised by the time consumed during measurements, which is reflected in results based on a relatively small number of cells.

The results of Salmon *et al.* (1992) showed DNA aneuploidy, i.e. the ICM-DNA index equal or greater than 1.30 IOD, in 47% of Grade II astrocytomas, 61% of anaplastic astrocytomas and 50% of glioblastomas. In a group of patients with anaplastic astrocytomas, DNA aneuploidy was found to be positively correlated with survival, as opposed to DNA diploid tumors. The observation was further supported by the characterization of the tumors by DNA histogram type, which showed that DNA hypertriploid astrocytomas, regardless of the established histopathological malignancy grade, had a significant survival advantage. This was suggested to result, at least partly, from the detected decrease in cell proliferation activity in these tumors (Salmon and Kiss 1993). Yoshii *et al.* (1995) demonstrated a positive correlation between the mean DNA indices and the established proliferation fraction of gliomas. DNA hypotetraploid tumors were found to have a trend toward longer tumor progression than those associated with DNA tetraploidy or hypertetraploidy. Large and deformed nuclei were more often observed in the DNA hypertetraploid tumor group that also showed high cell proliferative activity. Decaestecker *et al.* (1997) evaluated nuclear chromatin pattern and DNA ploidy in a series of astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas. They concluded that the image cytometric parameters could be used for the differentiation of the

glial tumor types. In addition, they suggested that the parameters could provide an aid in identifying the two glial phenotypes in mixed oligo-astroglial tumors.

4.1.2 Flow cytometric DNA analysis

Flow cytometric DNA analysis (FCM-DNA) represents a fully automated measurement technique of quantitative pathology. The method provides a DNA content analysis from approximately 10 000 cells per second (Koss *et al.* 1989, Wersto *et al.* 1991). Single fluorochrome-stained cells in a liquid suspension are directed through a laser beam, an excitation source, and the emitted fluorescence level is captured, digitally converted and stored in a computer unit for measurement. Because of the quantitative binding of the fluorochrome to DNA, the registered fluorescence levels are directly proportional to the amount of DNA in cells. An external control is used for the determination of tumor DNA ploidy, whereas cells with a constant DNA content can be added to the cell suspension as an internal control for the measurement of the DNA index. In addition to the measurement of DNA content in neoplastic cells, the FCM-DNA computer software can be used for dividing the cells into three basic cell cycle compartments (S, G2 and M phases described in greater detail below), offering a convenient technique for the analysis of cell proliferation activity in tumors.

A number of studies have shown a significant correlation between FCM-DNA aneuploidy and an elevated histopathological malignancy grade of astrocytomas or shortened survival rates of patients (Zapryanov and Christov 1988, Nishizaki *et al.* 1989, Danova *et al.* 1991, Vavruch *et al.* 1996). Similar results were initially also presented by Coons *et al.* (1988). Some years later in a series of 230 astrocytoma cases, however, Coons and co-workers (1994) were unable to demonstrate a statistical association of tumor ploidy with the established malignancy grade. Furthermore, survival advantage was shown to have shifted toward DNA aneuploid astrocytomas. Ganju *et al.* (1994) suggested that DNA aneuploidy in glioblastomas of patients under the age of 66 years may yield an improved prognosis.

FCM-DNA is an objective and fast technique for the quantitative measurement of large cell populations in tumors. The lack of morphological control over the analysis material and subjective interpretation of the results have been major pitfalls in the method and are assumed to account for some of the discrepancies in the literature (Koss *et al.* 1989, Wersto *et al.* 1991, Coons *et al.* 1994). As measurements have been shown to vary significantly between different regions of an astrocytoma sample, and local clonal cell populations have been demonstrated both in low-grade and high-grade lesions, it has been suggested that such regional heterogeneity has a negative effect on the usefulness of the method in astrocytomas (Coons and Johnson 1993a, Coons *et al.* 1994). In a comparison study utilizing the FCM-DNA and ICM-DNA techniques, in a series of astroglial and oligodendroglial tumors, Ullén *et al.* (1991) showed that investigations could be done equally with both methods. However, the advantages of the ICM-DNA were emphasized when the choice was to be made between the techniques.

4.2 Measurement of cell proliferation activity

Malignant tumors arise as a consequence of the production of daughter cells that have escaped the quality control limiting the growth of differentiated cells (Dirks and Rutka 1997). Glial cells have been thought to be relatively quiescent, but their proliferative capacity has been shown to be retained as in the processes of reactive and reparative gliosis (Miyake *et al.* 1992). Under appropriate stimuli, and/or lack of regulation, a proliferating cell enters and passes through a sequence of

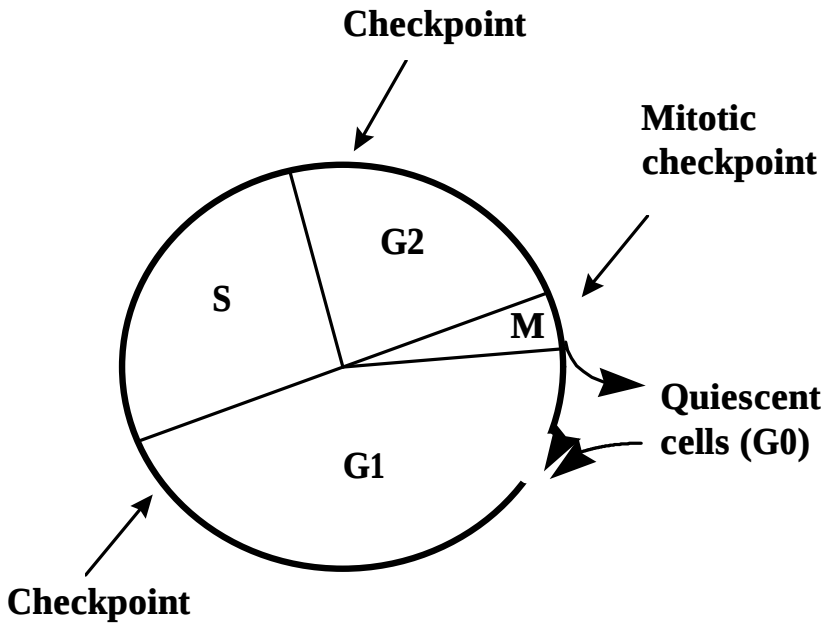

Figure 3. The cell cycle. The size of the sectors correspond to the relative duration of each phase of the cell cycle.

consecutive steps, or phases, that comprise the cell cycle (Baserga 1981) (Figure 3). The progress in the cell cycle is controlled at various checkpoints that ensure the completion of the previous phase before the onset of the next. The first phase is the Gap 1 (G1) phase, during which the cell grows and is prepared for DNA synthesis. There is a checkpoint at late G1, called the restriction point, for the decisions of the regulatory cell cycle machinery to progress. The replication of the chromosomal material takes place in the DNA synthetic phase (S), which is followed by the second Gap (G2) phase. During the G2 phase, DNA replication is checked for errors before the onset of mitosis (M), in which the duplicated DNA material is equally separated between the two daughter cells. The mitotic checkpoint ensures the proper separation and migration of the sister chromatids. After mitosis, the daughter cells exit the cell cycle to enter the state of quiescence (G0 phase) or re-enter the cell cycle for further division. In neoplasia, e.g. in the process of glioma growth, abnormal expression of positive regulators (activation of oncogenes) or loss of negative regulators (inactivation of tumor suppressor genes) have abrogated stringent cell cycle checkpoints (Louis 1997). As a result, glioma cells have acquired a growth advantage over normal cells. Furthermore, the likelihood for subsequent errors in the genome has increased, which eventually leads to the accumulation of genetic changes in daughter cells and further dedifferentiation and malignant behavior of the tumor tissue. Schröder *et al.* (1991) estimated the median duration of the cell cycle to be 544 hours (range 287 - 1338) for Grade II and 180 hours (range 108 - 354) for Grade III astrocytomas, and 88 hours (range 19 - 352) for glioblastomas. The duration of mitosis seemed to remain constant and independent of tumor type (approximately 1 - 2 hours).

4.2.1 Mitotic activity

In the histopathological grading of astrocytomas, an increased number of mitoses, widespread or focally detected, is the most important diagnostic determinant of progressive tumor growth and serves, therefore, as an indication for aggressive tumor therapy. For grading purposes, mitoses have been evaluated only for their presence (Kernohan and Sayre 1952, Kleihues *et al.* 1993a). The interpretation has been supported by the rarity of detectable mitotic figures due to the relatively short duration of mitosis in the cell cycle (Schröder *et al.* 1991, Dirks and Rutka 1997). In addition, the number of mitoses has been shown to decrease with delay in tissue processing, e.g. into the fixative (Graem and Helweg-Larsen 1979, Donhuijsen *et al.* 1990). On the other hand, a solitary observed mitosis, strictly interpreted as "mitotic activity", has been suggested to lead to overgrading of astrocytoma malignancy and tumor growth (Giannini *et al.* 1999).

Instead of semi-quantitatively recording mitotic figures as existing or non-existing, the number of mitotic figures has been a traditional and widely used diagnostic method to express mitotic activity in human tumors (Hilsenbeck and Allred 1992). Fulling and Garcia (1985) reported that anaplastic astrocytomas with mitotic activity of more than one mitosis per 10 microscopic high-power fields (HPF) carry a significantly weaker prognosis than corresponding tumors containing equal or less than one detectable mitosis per 10 HPF. Consistent with the result, Giannini *et al.* (1999) suggested that the distinction between Grade II and III astrocytomas by a "solitary mitosis" may not be optimal for malignancy grading. Schiffer *et al.* (1988) showed that one or more mitoses per 10 HPF was significantly associated with a shortened survival of patients with well-differentiated or anaplastic astrocytomas. A more dramatic decrease in survival was found, as the number exceeded four mitoses per 10 HPF. The quantitative assessment of mitoses is susceptible to variation in sampling (Donhuijsen 1986, Montironi *et al.* 1988), and it has been suggested that the estimate of mitotic index, based on a limited number of microscopic fields, is an adequate tool only for distinguishing large differences in mitotic activity between tumors (O'Leary and Steffes 1996). In addition to sampling, variation in size of microscopic fields may cause a significant difference in established mitotic indices (Haapasalo *et al.* 1989). A correction could be achieved by a field size specific coefficient and by relating the mitosis counts to the volume of the underlying neoplastic tissue (volume corrected mitotic index, M/V index). A volume corrected mitotic index could also reduce the effect of the variation in cell density, e.g. between different tumor areas, on the measurements. Simpson *et al.* (1992) suggested that, for improved reproducibility, the mitotic index needs to be established on the basis of a given number of tumor cells, excluding section areas of the non-neoplastic tissue compartment. Sørensen *et al.* (1991) reported a significant increase in mitotic figures per 1000 neoplastic cells in glioblastomas when compared with anaplastic astrocytomas. They also showed that the established mitotic index had prognostic value in high-grade astrocytomas. The results of Cruz-Sánchez *et al.* (1997) showed that the volume corrected mitotic index could be used for prognostic evaluation of a range of astrocytoma malignancies. They suggested, however, that other techniques of assessing cell proliferation, such as immunohistochemical stainings with antibodies against proliferation-associated nuclear proteins, could provide a better prognostic discrimination of astrocytomas.

4.2.2 Flow cytometric proliferation analysis

The nuclear DNA content of proliferating cells increases along the phases of the cell cycle, having doubled at the G2/mitosis. These changes can be detected by flow cytometry. With appropriate software, it is possible to estimate the percentage of cells at each phase of the cell cycle (Koss *et al.* 1989). Danova *et al.* (1987) showed that malignant brain tumors expressed a higher percentage of cells in the S-phase than benign tumors, but the finding did not correlate with the histological

tumor subtype. Zaprianov and Christov (1988) reported that patients with astrocytomas of low S-phase cell fraction survived longer than those with increased S-phase fractions. Coons *et al.* (1988) demonstrated a strong association of high-level G/M fraction with short-term survival, which was shown to parallel the distribution of malignancy of the astrocytoma cases. In a number of instances, flow cytometric analysis has been shown to provide valuable diagnostic and prognostic information about the proliferative potential of astrocytomas (Coons *et al.* 1994, Ganju *et al.* 1994, Vavruch *et al.* 1996, Struikmans *et al.* 1998), but the lack of histological control over the analyzed material has been regarded as a major pitfall and for the benefit of other assessment techniques.

4.2.3 Bromodeoxyuridine labeling and nuclear organizer regions

Exogenously incorporated bromodeoxyuridine (BrdU), a thymidine analogue, is taken up specifically by cells at the S-phase of the cell cycle, and the method has been used for radiosensitization in tumor therapy as well as for cell proliferation analyses by immunohistochemistry (Gratzner 1982, Morstyn *et al.* 1983, Weingart and Brem 1992). Hoshino *et al.* (1988) suggested that aggressive adjuvant therapy, postoperative radiation or chemotherapy, may be beneficial for patients with low-grade astrocytomas of high cell proliferation activity as determined by the BrdU labeling index (BrdU-LI). Fujimaki *et al.* (1991) reported a significant correlation between the BrdU-LI and the histopathological malignancy grade of astrocytomas, and residive-free survival. The prediction power of the BrdU-LI in astrocytomas together with its applicability in cell kinetic studies on tumor growth has also been found significant by others (Nishizaki *et al.* 1989, Labrousse *et al.* 1991, Hoshino *et al.* 1993, Ito *et al.* 1994). The BrdU-LI has been shown to correlate with a variety of other cell proliferation estimates, but the technical demands, including "*in vivo*" administration or "*in vitro*" incubation of fresh tissue, have favored the use of other markers than BrdU in cell proliferation analyses of astrocytomas (Morimura *et al.* 1989, Allegranza *et al.* 1991, Sasaki *et al.* 1992, Shibuya *et al.* 1993, Onda *et al.* 1994).

Nuclear organizer regions (NORs) represent loops of DNA which transcribe to ribosomal RNA and associated non-histone, partly argyrophilic proteins with an affinity for silver (Crocker *et al.* 1988). Although not a pure cell proliferation marker, counting of silver stained NORs (AgNORs) has been suggested to assist the evaluation of astrocytoma malignancy, and AgNOR counts have been shown to parallel the cell proliferation activity, determined by Ki-67 immunolabeling, in these tumors (Hara *et al.* 1990, Hara *et al.* 1991). Maier *et al.* (1990) found counting of AgNORs to be of only limited value in astrocytomas. They concluded that the shapes and patterns of intranucleolar and extranucleolar staining made it difficult to correlate the counting results with the established histopathological malignancy status. Shibuya *et al.* (1993) suggested that the number and configuration of AgNORs may not measure cellular proliferation directly but may reflect other features of biological malignancy in tumors.

4.2.4 Ki-67 antigen

Gerdes *et al.* (1983) isolated a mouse monoclonal antibody that detected an epitope on a nuclear antigen, termed Ki-67 and present only in proliferating cells. The Ki-67 antigen expression was demonstrated, by immunohistochemical labeling, throughout the cell cycle from late G1 to mitosis (M), but not in quiescent (G0) cells (Gerdes *et al.* 1984). In constantly proliferating cells the Ki-67 was also detectable in early G1 after mitosis, in contrast to those cells having entered the cell cycle as a result of mitogen triggered transition from G0. After several years of extensive and well-documented use of the monoclonal antibody, the Ki-67 antigen was shown to be a non-histone protein assembled by polypeptide chains with apparent molecular weights of 345 and 395

kilodaltons (Gerdes *et al.* 1991). The gene encoding for the Ki-67 was assigned to chromosome 10q25ter (Fonatsch *et al.* 1991). The cloning of the cDNA was completed in 1993 (Schlüter *et al.* 1993). The function of the Ki-67 protein is still unclear. The highly conserved repeat structure throughout mammals and the suggested DNA binding properties of the protein indicate a very important role during cell proliferation (Falini *et al.* 1989, Lopez *et al.* 1994). The half-life of the detectable antigen is short, which reduces the possibility of erroneous immunostaining in cells having re-entered G0, but it may also lead to underestimates of cycling cells in late G1 (Bruno and Darzynkiewicz 1992). The expression of the Ki-67 antigen has also been shown to be lost in nutritionally deprived, cycling cells (Verheijen *et al.* 1989).

Of the endogenous molecules, the Ki-67 antigen has been the best recognized operational marker for cell proliferation. However, the epitope against which the monoclonal antibody was first generated by Gerdes *et al.* (1983) is destroyed by fixation (Hall and Woods 1990). This permitted the use of only fresh or quick frozen tissue. Cattoretti *et al.* (1992) applied the assessment of Ki-67 expression successfully to formalin-fixed, paraffin-embedded tissues by immunostaining with a mouse monoclonal antibody, designated MIB-1 and raised against the recombinant parts of the antigen (Key *et al.* 1993a). Shortly after, a rabbit polyclonal antiserum equivalent to the MIB-1 was introduced, which enabled double immunostaining in combination with other monoclonal antibodies (Key *et al.* 1993b). The antigen retrieval was based on microwave oven heating, which had earlier been shown to increase immunostaining with a number of both monoclonal and polyclonal antibodies in routinely fixed tissues (Shi *et al.* 1991).

4.2.5 Proliferating cell nuclear antigen

The proliferating cell nuclear antigen (PCNA) gained considerable attention during the "first chapter" (fresh tissue analyses) of the Ki-67 immunohistochemistry. Miyachi *et al.* (1978) described in patients with systemic lupus erythematosus an auto-antibody that recognized the PCNA. The antigen was found to be a highly conserved, 36 kD acidic non-histone protein functioning as a cofactor to DNA polymerase δ and an absolute requirement for DNA synthesis (Bravo *et al.* 1987, Prelich *et al.* 1987, Jaskulski *et al.* 1988a). The PCNA gene has been located on chromosome 20 (Ku *et al.* 1989). Two populations of the PCNA exist during the S-phase of the cell cycle: one that is nucleoplasmic as in quiescent cells of proliferative capacity and easily extracted by detergent, and another that is detergent resistant and associated with specific nuclear structures (Bravo and Macdonald-Bravo 1987). The DNA binding of the latter before the initiation of DNA replication (Takahashi and Caviness 1993), i.e. the late G1 phase, and a marked increase in expression in proliferating cells (Morris and Mathews 1989) have indicated a fundamental relationship of the PCNA with cell cycle progression and cell proliferation. However, the PCNA has also been demonstrated to be involved in DNA nucleotide excision-repair unrelated to cell proliferation (Celis and Madsen 1986, Shivji *et al.* 1992). Growth factors, such as the platelet derived growth factor (PDGF), epidermal growth factor (EGF) or transforming growth factor (TGF-α), and oncogene activation have also been suggested to upregulate PCNA expression in non-cycling cells (Jaskulski *et al.* 1988b, Hall *et al.* 1990, Baserga 1991, Hall *et al.* 1994). Furthermore, elevated expression of the PCNA has been detected in non-proliferating cells adjacent to tumor growth and inflammation (Harrison *et al.* 1993).

The advantage of the PCNA immunohistochemistry over the Ki-67 immunolabeling was initially based on the resistance of some of the epitopes of the PCNA to conventional fixation and paraffin-embedding. This also extended immunohistochemically determined cell proliferation estimates on archived material. Several commercially available monoclonal antibodies against the PCNA have been introduced, including 19A2, 19F4 and PC10 (Ogata *et al.* 1987, Waseem and Lane

1990). However, the fixative and the duration of fixation have been observed to affect the immunodetection of the different epitopes recognized by the antibodies (Gelb *et al.* 1992, Yu and Filipe 1993). The long half-life of the antigen has been suggested to result in a gross overestimation of the growth fraction (Scott *et al.* 1991). In addition, the complex regulation of the PCNA has cast doubt on the reliability of PCNA immunohistochemistry in assessments of cell proliferation.

4.2.6 Ki-67 and PCNA labeling indices in astrocytomas

Burger *et al.* (1986) were the first to show an increasing Ki-67 labeling index (Ki-67-LI) along with increasing histopathological grade in astrocytomas. They found that Ki-67 staining was particularly useful for distinguishing anaplastic admixture of tumor cells, especially in better differentiated tumor regions. Subsequently, a number of studies have demonstrated a close correlation of the Ki-67-LI with astrocytoma grade, and suggested that the method has potential clinical value in assisting the diagnostic evaluation of the tumors (Shibata *et al.* 1988, Zuber *et al.* 1988, Patsouris *et al.* 1988, Deckert *et al.* 1989, Morimura *et al.* 1989, Raghavan *et al.* 1990, Louis *et al.* 1991, Schröder *et al.* 1991, Shibuya *et al.* 1993). Ki-67 immunohistochemistry has also been proposed to add objective *prognostic* information about astrocytomas of different grades (Jaros *et al.* 1992, Montine *et al.* 1994), or tumors within one established malignancy category but different tumorigenetic background (Torp *et al.* 1992).

Increased Ki-67 immunoexpression has been found to be associated with dedifferentiation of the astrocytic lesion (Table III). However, a marked overlapping in assessment scores, particularly between the high-grade categories, has cast doubt on whether the Ki-67-LI could be useful for the evaluation of high-grade astrocytomas (Pigott *et al.* 1991, Montine *et al.* 1994). Coons and Johnson (1993b) suggested that the regional heterogeneity in Ki-67 immunolabeling could be one of the main reasons affecting interobserver reproducibility. They recorded three patterns of Ki-67 expression in astrocytomas: (1) diffuse labeling, (2) a single area with distinctly increased labeling, and (3) scattered, multifocal distribution of a number of proliferative areas. The low-grade tumors exhibited only little intratumor heterogeneity in Ki-67 expression, but this increased consistently along with tumor grade. In addition, regions with the highest LIs were found to cluster with decreasing gradient toward surrounding tissue areas. Alterations in Ki-67-LIs were observed to parallel the variation in histopathological malignancy in different regions of the tumor tissue.

The "second chapter" of Ki-67 immunohistochemistry, defining the period of the new polyclonal Ki-67 and monoclonal Ki-67(MIB-1) antibodies, meant not only access to routinely fixed and paraffin-embedded astrocytoma specimens. It became possible to utilize large tumor archives for studies on the prognostic utility of the Ki-67 labeling index in heterogeneously behaving astrocytomas. In a series of 90 cerebral gliomas, Onda *et al.* (1994) showed that the new method with the Ki-67(MIB-1) antibody seemed to be advantageous over the previously used Ki-67 immunohistochemistry. The stained nuclei were more clearly visualized. In addition, the Ki-67(MIB-1) method was found to detect a larger number of cells than the previous technique, which was suggested to result from better preservation of the epitope. Torp *et al.* (1995) reported that the novel polyclonal Ki-67 antibody provided an excellent immunostaining on both frozen and paraffin-embedded glioblastoma samples, whereas no attempts to utilize the first monoclonal Ki-67 antibody in microwave-oven pretreated tumor specimens could be recommended.

Table III. Ki-67 immunohistochemistry in astrocytic tumors (from freshly frozen tissue samples, SE = standard error, med = median value).

ASTROCYTOMA		**Grade I** (pilocytic)		**Grade II**		**Grade III** (anaplastic)		**Grade IV** (glioblastoma)	
Ki-67 Labeling Index (%)	Dilution	n	Mean range	n	Mean range	n	Mean range	n	Mean range
Burger *et al.* 1986	undiluted	3	1.1 0.6-1.4	4	2.2 0.8-3.2	1	1.6	9	6.2 1.3-12.4
Shibata *et al.* 1988	undiluted	13	0.9 0.3-1.6	12	1.8 0.6-3.2	6	4.3 1.6-9.0	53	9.8 1.3-27.6
Zuber *et al.* 1988*	1:10	in II		16	1.0 0.1-4.5	8	3.5 0.7-7.4	27	11.1 1.7-32.2
Raghavan *et al.* 1990*	1:15			26	0.5 0-1.9	26	4.1 0.6-10.9	38	6.4 0.9-16.2
Louis *et al.* 1991	1:10			1	< 1.0	7	3.9 2-10	7	21.4 17-26
Schröder *et al.* 1991*	1:10			16	med 4.8 2.4-9.0	20	med 9.7 4.0-15.5	32	med 13.9 5.8-39.5
Jaros *et al.* 1992*†	1:25	3	0.9 0.5-1.2	7	1.2 0-3.1	13	8.3 0.1-36.7	20	13.4 0.6-30.9
Torp *et al.* 1992†	1:50							20	5.2 0.2-24.6
Shibuya *et al.* 1993*	1:25			12	1.9 ±0.5 SE	11	6.0 ±1.4 SE	34	15.0 ±1.8 SE
Montine *et al.* 1994*†	1:100			11	3.1 0.5-7.5	12	14.3 3.9-30.6	13	18.9 5.7-42.7
Onda *et al.* 1994	1:25			3	0.7 ±0.8 SE	1	7.0	17	13.8 ±9.9 SE

* statistical association with histological grade; † statistical association with survival

In general, numerous studies have been consistent in conclusions about the positive impact of the new Ki-67 method in astrocytoma diagnosis (Table IV), especially in distinguishing low-grade and high-grade malignancy (Onda *et al.* 1994, Ellison *et al.* 1995, Wakimoto *et al.* 1996, Kordek *et al.* 1996, Hsu *et al.* 1997, Cunningham *et al.* 1997, Giannini *et al.* 1999). Both Ellison *et al.* (1995) and Cunningham *et al.* (1997) demonstrated the significant prognostic value of the Ki-67(MIB-1)-LI in astrocytomas, which was not, however, equally clear after adjustments for tumor grade and patient age. Hsu *et al.* (1997) showed that the Ki-67(MIB-1)-LIs in Grade II astrocytomas differed significantly from those in either of the high-grade categories, whereas the indices between the Grade III and IV tumors were essentially alike. Furthermore, they found that the Ki-67(MIB-1)-LI was a significant *independent* predictor of patient outcome. Wakimoto *et al.* (1996) showed that the three histopathological malignancy groups of astrocytomas differed significantly in terms of the Ki-67(MIB-1) labeling index. In addition, the prognostic value of the Ki-67(MIB-1)-LI was found to be superior to the histopathological grade in highly malignant tumor cases (astrocytoma grades III-IV).

The results were supported by Pollack *et al.* (1997), who suggested that the Ki-67(MIB-1)-LI was a better predictor of both overall and progression-free survival than histology in high-grade astrocytomas in children. Furthermore, Nakamura *et al.* (1997a) showed that the Ki-67 expression could be used for the prognostic distinction of anaplastic astrocytomas (Grade III). Another finding of clinical importance was achieved in a series of fifty Grade II astrocytomas, in which a significant survival difference was reported between tumors of low and high Ki-67(MIB-1)-LI (Schiffer *et al.* 1997). Such a survival difference by cell proliferation was not observed by Giannini *et al.* (1999).

Table IV. Ki-67 immunohistochemistry in astrocytic tumors (from the era of paraffin-embedded tissues, SD = standard deviation, med = median value).

ASTROCYTOMA		**Grade I** (pilocytic)		**Grade II**		**Grade III** (anaplastic)		**Grade IV** (glioblastoma)	
Ki-67 Labeling Index (%)	Anti-body	n	Mean range	n	Mean range	n	Mean range	n	Mean range
Onda *et al.* 1994	1:50 MIB-1			23	4.2 ±4.1 SD	22	7.3 ±7.2 SD	30	23.9 ±13.5 SD
Ellison *et al.* 1995*†	1:50 MIB-1			24	med 0.4	31	med 2.5	68	med 5.7
Wakimoto *et al.* 1995*†	1:50 MIB-1			19	3.8 ±2.7 SD	25	18.4 ±9.7 SD	28	31.6 ±12.9 SD
Kordek *et al.* 1996*	1:50 poAb	8	0.8 0.2-2.3	9	1.9 0.3-3.2	9	6.3 0.6-13.2	30	9.8 2.1-36.8
Ono *et al.* 1996	1:50 MM1	2	1.1-3.7	15	2.4 0.5-7.5	20	11.7 3.5-25.2	13	15.1 5.1-27.3
Cunningham *et al.* 1997*†	1:60 MIB-1			15	9.0 ±10.9 SD	19	17.9 ±13.9 SD	86	23.3 ±16.5 SD
Hsu *et al.* 1997*†	1:75 MIB-1			16	0.9 0-3.5	31	8.8 0-32.4	33	9.1 0-29.8
Khalid *et al.* 1997*	1:100 MIB-1	9	1.2 ±1.6 SD	24	1.8 ±3.2 SD	20	13.5 ±11.2 SD	33	15.7 ±15.4 SD
Nakamura *et al.* 1997†	1:100 MIB-1					50	19.7 ±11.4 SD		
Pollack *et al.* 1997*†	1:100 MIB-1					10	11.9 1.8-31.5	19	27.3 3.8-62.0
Schiffer *et al.* 1997†	1:100 MIB-1			50	cut-off at 8.0 %				
McKeever *et al.* 1998†	1:200 MIB-1			50	cut-off at 2.0 %				
Giannini *et al.* 1999*†	1:100 MIB-1			45	2.3 0-7.6	50	6.0 0.1-25.7	45	9.1 0.3-36

* statistical association with histological grade; † statistical association with survival

A number of studies have reported of a positive correlation of the PCNA-LI with both the BrdU and Ki-67 labeling indices in astrocytomas as well as with tumor grade and patient prognosis (Allegranza *et al.* 1991, Schiffer *et al.* 1993, Kim *et al.* 1993, Revesz *et al.* 1993, Kordek *et al.* 1996, Cruz-Sánchez *et al.* 1997, Cunningham *et al.* 1997) (Table V). However, analysis results and conclusions on the clinical applicability of the PCNA method have varied considerably. This has been considered to result, to some extent, from the use of a range of different antibodies that detect different epitopes of the antigen (Cunningham *et al.* 1997). Louis *et al.* (1991) suggested that, when compared with Ki-67 immunodetection, factors such as good specificity for S-phase cells but less sensitivity for proliferating cells, variation in staining pattern from intense to light as well as somewhat inferior correlation with the established histopathological malignancy grade may not favor the use of the PCNA immunohistochemistry in astrocytomas.

Table V. PCNA immunohistochemistry in astrocytic tumors (SD = standard deviation, SE = standard error).

ASTROCYTOMA		**Grade I** (pilocytic)		**Grade II**		**Grade III** (anaplastic)		**Grade IV** (glioblastoma)	
PCNA Labeling Index (%)	Anti-body	n	Mean range	n	Mean range	n	Mean range	n	Mean range
Louis *et al.* 1991	1:40 19F4			1	$\leq$ 1.0	7	$\leq$ 1.0	7	10.0 6.0-15.0
Figge *et al.* 1992	1.5µl PC10							40	24.7 0.5-75
Kim *et al.* 1993*	1:4000 19A2			7	0.6 $\pm$0.2 SE	6	1.2 $\pm$0.1 SE	18	1.8 $\pm$0.2 SE
Revesz *et al.* 1993*	1:10 PC10	6	5.6 2.9-7.8	8	4.1 0.2-10.5	16	8.1 1.0-26.8	25	26.8 3.3-72.7
Schiffer *et al.* 1993	1:300 PC10			10	4.8 3.4-6.4	10	33.7 13.2-52.9	10	65.1 58.0-74.1
Kordek *et al.* 1996*	1:50 PC10	8	3.7 0.8-8.3	9	6.3 0.4-32.7	9	33.2 2.7-86.3	30	33.4 2.7-86.3
Cruz-Sánchez *et al.* 1997*†	1:200 PC10	7	8.7 $\pm$9.7 SD	12	4.6 $\pm$3.0 SD	23	19.5 $\pm$12.7 SD	38	21.4 $\pm$10.0 SD
Cunningham *et al.* 1997*†	1:500 PC10			15	10.9 $\pm$10.5 SD	19	14.7 $\pm$12.5 SD	86	26.0 $\pm$20.8 SD

* statistical association with histological grade; † statistical association with survival

4.3 Prognostic scoring of astrocytomas

Martin and Voss (1982) proposed that a computer-assisted microscopic image analysis of a number of quantitative features pertaining to the number, size, shape or chromatin texture of the tumor cell nuclei enhances the reliability of the histological diagnosis of individual astrocytomas. This was

supported by Scarpelli *et al.* (1994), who were able to distinguish low- and high-grade astrocytoma malignancy by a continuous grading model that combined the image analysis data of both nuclear and nucleolar features. Similarly, Kiss *et al.* (1997) showed that a combined analysis of tissue cellularity and cell proliferation activity (Ki-67[MIB-1]) could add significant prognostic information about the growth pattern of high-grade astrocytomas. Such multiple analyses of various prognostic factors have been integrated into artificial neural networks, i.e. computerized decision-tree models, in search for improved accuracy in the prediction of cancer behavior (Cross *et al.* 1995, Baxt 1995, Burke *et al.* 1997). A decision-tree model is composed of a set of variables that are interconnected. Thus, the evaluation of the analysis data proceeds in a stepwise manner, as a decision regarding one variable leads to the decision on another. In artificial neural networks, a large amount of statistically optimized background data (training with true occurrences, such as correct classification or true patient outcome) has been stored in a computer. The analysis data (input variables) are then fed into the model, which compares the interactions of the variables on basis of the preset networks and provides a probability score (output) of an occurrence.

With biopsies of 86 astrocytic tumors, Kolles *et al.* (1995) demonstrated a grading system with neural networks that utilized morphometric analysis in combination with cell proliferation estimates (Ki-67[MIB-1]). The computer was provided with a three-tiered grading scheme which included statistically tested variables of growth pattern, cellularity, cell proliferation tendency and nuclear pleomorphism. An overall concordance of 42% was reported between the WHO 1993 and computer-assisted grading systems. The poor correlation of the systems was considered for the benefit of the neural networks, as the new model revealed subsets of structurally similar astrocytomas which differed from the subdivision by conventional malignancy grading. McKeown and Ramsay (1996) recorded 14 histological features of Grade II-IV astrocytomas as several sets of principal components. The authors suggested that the flexibility of artificial neural networks could provide a significant aid for the standardization process of astrocytoma diagnosis, as well as for decisions on the management of the tumors. Decaestecker *et al.* (1998) combined morphonuclear and chromatin texture-related features with image cytometric DNA analysis, in order to detect atypical prognosis of patients within the established WHO Grade II and III malignancy categories. They concluded that a systematic determination of markers related to molecular biology will significantly influence the art of neural networks in the prediction of astrocytoma behavior.

5. Astrocytoma, a Genetic Disease

Astrocytoma malignancy develops along a biological continuum (Figure 4) that is driven by an accumulation of genetic changes during the stepwise progression from low-grade to high-grade lesions (Bigner and Vogelstein 1990, von Deimling *et al.* 1993, Kleihues *et al.* 1993b, Louis 1997). Abrogation of the p53 tumor suppressor gene on chromosome 17p, activation of the platelet-derived growth factor (PDGF) system and allelic loss of chromosome 22q have been suggested to account for the earliest changes, possibly representing an initial disturbance in the ratio between cell gain (cell proliferation activity) and cell loss (cell death or apoptosis) (Louis 1997). The transformation from a Grade II to anaplastic (Grade III) astrocytoma has been associated with an increasing capacity of tumor cells to proliferate, as a result of the loss of critical regulation in the cell cycle. The related genetic changes have included inactivation of tumor suppressor genes on chromosomes 13q (the retinoblastoma gene), 9p (the cyclin dependent kinase inhibitor p16 encoding CDKN2 gene), 19q (a possible glioma tumor suppressor gene), and 11p (a possible glioma tumor suppressor gene), as well as the activation of cyclin dependent kinase 4 (CDK4) oncogene on chromosome 12q. Approximately 60-85% of the glioblastomas arise from a previous astrocytoma (secondary glioblastomas) via a loss on chromosome 10. Recently, the PTEN gene (called for phosphatase and tensin homolog deleted on chromosome 10) has been located at 10q23 (Li *et al.*

1997). There is also molecular genetic evidence of primary glioblastomas that develop without a prior less malignant lesion. These tumors appear to be characterized by a loss on chromosome 10, particularly the short arm (p) of the chromosome, and a subsequent amplification of the epidermal growth factor receptor (EGFR) gene (von Deimling *et al.* 1993, Watanabe *et al.* 1996, Louis 1997).

Rapid advances have been made in techniques of molecular biology and cancer cytogenetics, such as the possibility to detect gene-specific abnormalities by in situ hybridization (Gray *et al.* 1991),

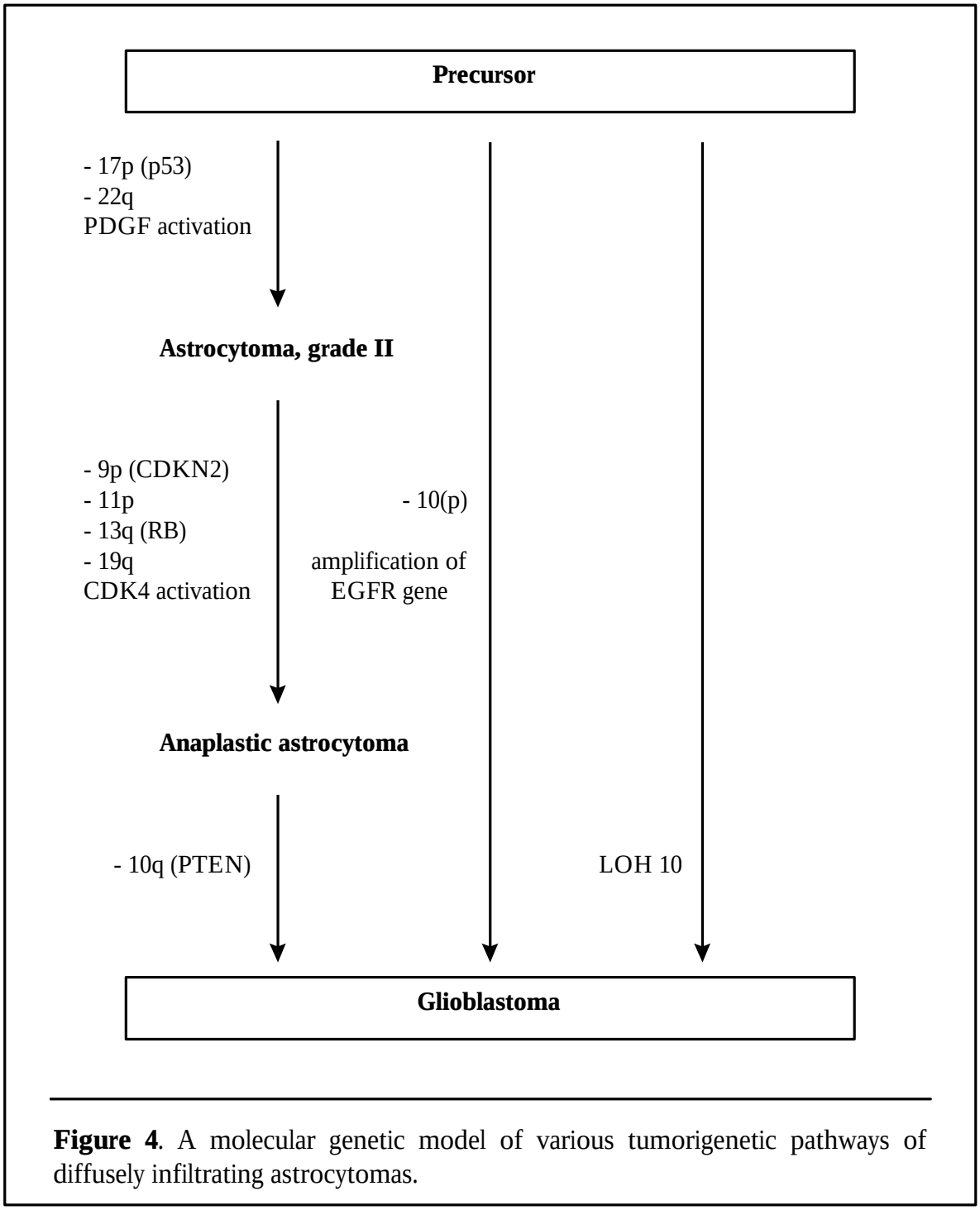

Figure 4. A molecular genetic model of various tumorigenetic pathways of diffusely infiltrating astrocytomas.

gross-total genome alterations by comparative genomic hybridization (Kallioniemi *et al.* 1992) and genetic alterations comprehensively by high-throughput array techniques (Schena *et al.* 1995, Kononen *et al.* 1998). Subsequently, a vast (commercial) interest has arisen in developing new therapy strategies that would target genetic alterations underlying the tumorigenesis of astrocytomas (Whittle 1995, Karpati *et al.* 1996).

5.1 Telomerase and progressive tumor growth

Blackburn and Gall (1978) showed that the chromosome ends, or telomeres, are composed of sequences of six nucleotides, TTAGGG (thymidine, adenosine and guanine), repeated in tandem arrays with associated proteins. In humans, the size of the telomeric ends has been shown to vary between cells, approximations ranging up to 15 kilobases (kb) per telomere (Moyzis *et al.* 1988, Morin 1989). Telomeres have been suggested to carry a stabilizing function that protects the chromosome ends from degradation, recombination and end-to-end fusion during DNA replication, as well as ensuring the completion of the replication of the DNA sequences (Müller 1938, McClintock 1941, Blackburn 1991, Zakian 1995). The chromosome ends become incompletely replicated after each cell cycle, which results in shortened telomeres in daughter cells (Olovnikov 1973, Harley *et al.* 1990). The shortening of telomeres has been suggested to represent a pathway of replicative aging (Harley 1991), and explained by the inability of conventional DNA polymerases to start the replication without an existing stretch of the complementary polynucleotide chain (Zakian 1997) (Figure 5). Furthermore, it has been proposed that alterations in the telomeric region may be a uniform feature underlying the genomic instability of neuroepithelial tumors (Manuelidis 1994).

The telomere hypothesis (Harley *et al.* 1992) states two critical checkpoints, mortality stages 1 (M1) and 2 (M2), in which the telomere length plays an important role (Figure 6). The M1 represents the endpoint of the replicative life of cells. However, disturbed control of the cell cycle, e.g. inactivation of critical tumor suppressor genes or active viral oncogenes (Shay *et al.* 1991, Counter *et al.* 1992), may sustain cell proliferation activity beyond the protective function of the telomeres, and cells die (M2). The discovery of maintained telomeric DNA (Shampay *et al.* 1984) and the identification of the telomerase enzyme, a terminal transferase capable of adding telomeric sequences to chromosome ends (Greider and Blackburn 1985), provided the first biological evidence of active upregulation of telomere length. Telomerase is a ribonucleoprotein DNA polymerase that utilizes its internal RNA component as a template for the synthesis of TTAGGG repeats (Greider and Blackburn 1989) (Figure 5). Thereby the activation of telomerase has been suggested to ensure infinitive cell proliferation capacity and life (Morin 1989, Harley *et al.* 1990). Counter *et al.* 1992 observed that in some immortal cells, having escaped the checkpoints M1 and M2, the telomere length appeared stable and the immortalization coincided with the onset of telomerase activity.

Feng *et al.* (1995) cloned the human telomerase RNA component (hTR) and mapped the single-copy gene to the distal quarter of the long arm (q) of chromosome 3. They showed that cells with expected telomerase activity expressed more hTR than observed in normal somatic tissues. Upregulated telomerase RNA expression has also been detected in the absence of telomerase, which has led to a suggestion that telomerase activity may also be regulated by other mechanisms (Avilion *et al.* 1996, Blasco *et al.* 1996). One regulatory pathway has been proposed to involve the telomeric-repeat binding factor TRF1, which may function as a negative feedback to telomerase activity (van Steensel and de Lange 1997). The identification and cloning of the human telomerase catalytic subunit gene simultaneously by two separate study groups (Meyerson *et al.* 1997, Nakamura *et al.* 1997b) demonstrated another regulatory pathway. The gene encoding the human ever shorter telomeres protein 2 (hEST2, Meyerson *et al.* 1997) or the human telomerase reverse transcriptase

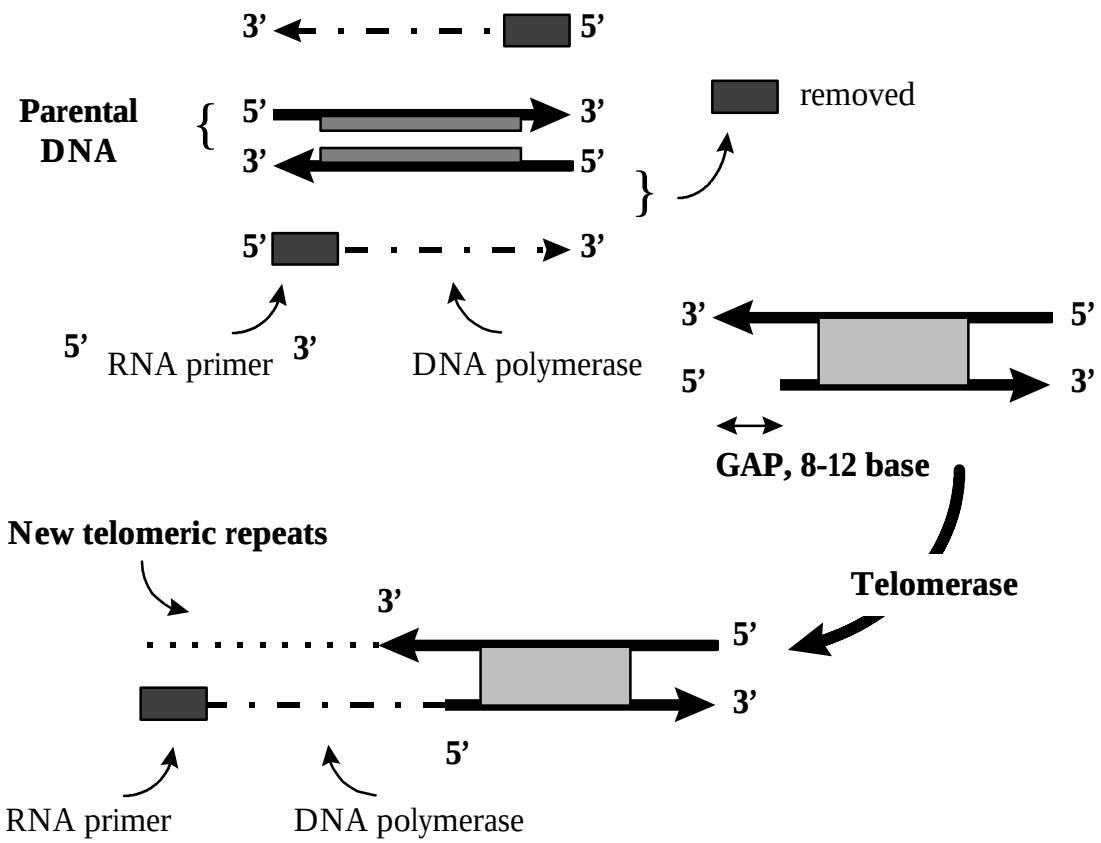

Figure 5. Model of replicative senescence. The transcription of a terminal RNA primer (8-12 nucleotides) is needed to provide a stretch of the complementary polynucleotide chain for conventional DNA polymerases to start the replication. Since DNA polymerases synthesize DNA strictly in the 5'-to-3' direction, the removal of the RNA primer leaves a 5'-terminal gap and a 3'-overhang in daughter molecules that is lost after every other S-phase of the cell cycle. The length of the telomeres corresponds to the number of cell divisions possible. The amount of telomeric DNA lost in the replication process may increase significantly, if the transcription of the RNA primer does not take place at the very end of the chromosome. By contrast, telomerase activity may significantly increase the length of the replicative life of cells.

(hTRT), as termed by Nakamura *et al.* (1997b), was located to the short arm (p) of chromosome 5. The hTRT messenger-RNA (mRNA) levels, characterizing the expression of the gene, correlated better with human telomerase activity than the hTR mRNA expression levels.

Telomerase is absent in the majority of normal human non-reproductive tissues, whereas high activity detected in germ-cells has been explained by the extensive proliferation requirement of the cells during differentiation (Harley 1991). In addition, a number of human tumors have been shown to attain telomerase activity (Kim *et al*. 1994), which has raised speculations of its possible role in targeted cancer therapy (Morin 1995). On the other hand, it has been proposed that tumors could achieve over a million-fold increase in cell number before telomerase is required, which would make

the enzyme synthesis only a late stage event in tumorigenesis (Kipling 1995). Langford *et al.* (1995) demonstrated telomerase activity in 75% of glioblastomas and in a few anaplastic astrocytomas studied. The lack of telomerase activity, especially in the anaplastic astrocytoma group, was suggested to result from alternative mechanisms that could sustain telomere length. Nakatani *et al.* (1997) and DeMasters *et al.* (1997) reported that 53-60% of the glioblastomas studied showed enzyme activity. Both authors found Grade II and anaplastic astrocytomas to be telomerase negative. However, Le *et al.* (1998) demonstrated telomerase activity in 33% of Grade II and 45% of anaplastic astrocytomas, as well as in 89% of the glioblastoma samples studied. They suggested that the expression of telomerase could demonstrate a shortening of the telomeres during malignant progression of astrocytomas. Kleinschmidt-DeMasters *et al.* (1998) showed telomerase activity in nearly all high-grade astrocytomas (Grades III-IV). The expression of telomerase associated closely with Ki-67(MIB-1) cell proliferation activity, tumor grade, patient age and endothelial proliferation. Furthermore, the enzyme activity was observed to vary between different tumor regions. They suggested that the lack of detectable telomerase in highly malignant astrocytomas could result from the poor sensitivity of the applied detection technique in tumor tissues that show marked intratumor heterogeneity in malignancy.

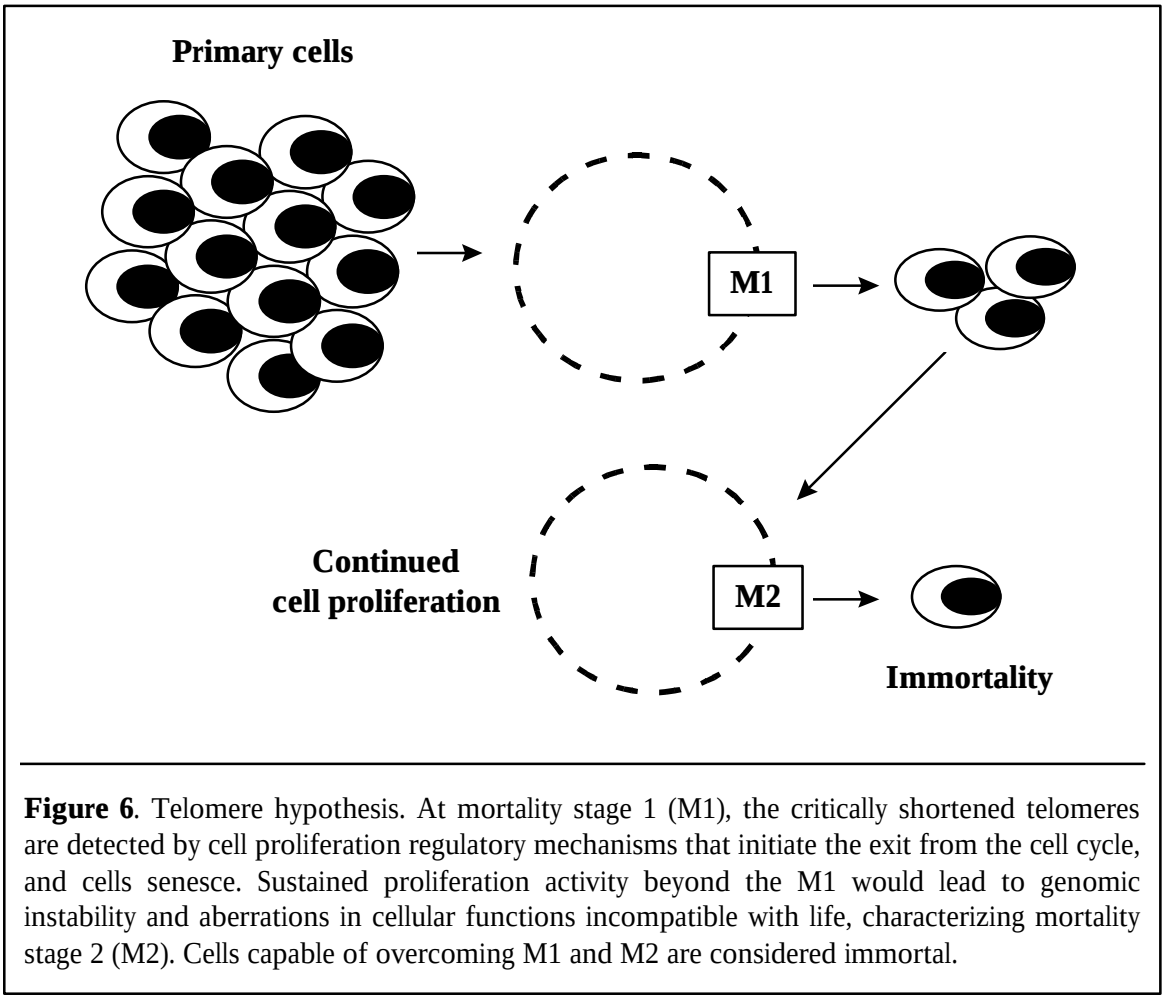

Figure 6. Telomere hypothesis. At mortality stage 1 (M1), the critically shortened telomeres are detected by cell proliferation regulatory mechanisms that initiate the exit from the cell cycle, and cells senesce. Sustained proliferation activity beyond the M1 would lead to genomic instability and aberrations in cellular functions incompatible with life, characterizing mortality stage 2 (M2). Cells capable of overcoming M1 and M2 are considered immortal.

AIMS OF THE STUDY

The aims of the study were:

1. To improve the prognostic evaluation of astrocytomas by the assessment of cell proliferation activity in tumors (Studies I and III).

2. To improve the accuracy, objectivity and reproducibility of cell proliferation estimates by computer-assisted image analysis, for their potential applicability in routine brain pathology (Studies I and II).

3. To investigate the potential role of telomerase enzyme activation in upregulated and continued cell proliferation activity in astrocytomas (Study IV).

4. To evaluate whether image analysis based quantitation of cell proliferation and histopathological factors closely related with the established WHO grading criteria, as well as nuclear DNA content could assist the conventional malignancy grading and prognostication of astrocytomas (Study V).

MATERIALS AND METHODS

1. Patients and Tumors

A total of 115 astrocytic tumors were originally collected for this study. The patients had been operated consecutively at the Tampere University Hospital, Finland, between December 1987 and March 1992. Open craniotomy was used for gross radical tumor resection. The patients were monitored by computed tomography (CT) or magnetic resonance (MRI) prior to surgery and during the clinical check-ups. One neuropathologist, and in most instances two neuropathologists (Studies I, III and V), classified and graded the primary tumors according to the nomenclature and criteria presented by the WHO 1979 and WHO 1993 classification systems (Zülch 1979, Kleihues *et al.* 1993a). In the present context, the subdivision of the tumor material is based on the diagnostic recognition of the WHO 1993 criteria, unless indicated otherwise, and presented in Table VI. Very small and/or necrotic tumor specimens, as well as those tumors that did not represent the primary occurrence, were excluded from this study. This accounted for 13 astrocytic tumors. Most of the patients with highly malignant astrocytomas (Grades III-IV) received post-operative radiation and/or chemotherapy. All the patients were followed-up until death, or for at least five years.

Pilocytic astrocytomas (Grade I). Pilocytic astrocytomas have been proposed to comprise a tumor entity of astrocytic neoplasms, and thus are presented here only as the most benign astrocytoma type for comparison with the diffusely infiltrating type. Eleven cerebellar pilocytic astrocytomas were obtained from seven male and four female patients. The median age was 11 years (range 1 - 54 years). One patient died of the tumor and one tumor reoccurred within five years after the primary surgery.

Diffusely infiltrating Grade II astrocytomas. Fifteen male and eight female patients had Grade II astrocytomas. The median age was 32 years (range 3 - 56 years). Two tumors expressed major gemistocytic and three tumors additional oligodendrocytic (mixed gliomas) differentiation. During the follow-up, twelve patients were re-operated on due to tumor reoccurrence which was accompanied with a malignant transformation of the tumor, i.e. an increase in tumor malignancy grade, in ten cases.

Anaplastic (Grade III) astrocytomas. Fourteen male and four female patients had anaplastic astrocytomas. The median age 42 years (range 8 - 75 years). Five tumors expressed an additional oligodendrocytic differentiation. Six patients were re-operated on for the tumor reoccurrence, accompanied with a malignant dedifferentiation in two cases.

Glioblastomas (Grade IV). A total of 50 glioblastomas were obtained from 29 male and 21 female patients. The median age was 58 years (range 27 - 77 years). Five glioblastomas had an additional sarcomatous component (gliosarcomas).

Study II also included cryostat sections (n = 28) of various other tumors of the nervous system (mainly meningiomas, neuroblastomas and brain metastases), obtained from patients operated on at the Tampere University Hospital or Turku University Hospital, Finland. For Study IV, freshly frozen samples of six additional glioblastomas and one Grade II astrocytoma from patients more recently operated on (Tampere University Hospital) were collected. Study V included an additional series of 42 consecutive diffusely infiltrating astrocytomas operated on at the Tampere University Hospital during May 1992 - March 1994 (included in the "testing set").

Table VI. The astrocytic tumor material (1987-1992) presented in the original publications (Studies I-V) and collected for this study (dissertation). The revised nomenclature in the WHO 1993 scheme designates diffuse Grade I astrocytomas (the Studies I-II) into the Grade II astrocytomas category.

Study, n	Grading scheme	Grade I	Grade II	Grade III	Grade IV	Protocols
Study I, 83	WHO 1979	11	14	19	39	PCNA (visual)
Study II, 104*[1]	WHO 1979	13	23	23	45	PCNA (visual vs. image analysis)
Study III, 50	WHO 1993	5	8	13	24	PCNA, Ki-67, Flow cytometry
Study IV, 46*[2]	WHO 1993	2	16	4	24	Telomerase, Ki-67
Study V, 67 (+66)*[3]	WHO 1979	4	14 (12)	14 (15)	35 (39)	Morphometry, Ki-67, ICM-DNA
	WHO 1993*[4]		14 (7)	11 (16)	42 (43)	
Dissertation, 102	WHO 1993	11	23	18	50	

*[1] The original publication includes an additional series of 28 tumors of the nervous system.
*[2] The original publication includes an additional series of 7 diffuse astrocytomas.
*[3] The latter 66 astrocytomas include 42 cases operated on during 1992-1994 (in parenthesis).
*[4] "Strict" interpretation of the WHO 1993 criteria (e.g. one mitosis sufficient for grade III).

2. Study Protocols

In addition to that in the original publications (Studies I-V, Table VI), some study protocols have been described here in more detail. In the case of paraffin-embedded tumor material, only one tissue block per tumor sample was used for the analyses. The block was chosen by a neuropathologist for the diagnostic determination of the tumor case. The specimens were fixed in 4% phosphate-buffered formaldehyde (median fixation time less than 24 hours) before processing into paraffin.

2.1 Cell proliferation activity

2.1.1 Immunohistochemical stainings

For immunohistochemistry, five-µm tissue sections were collected onto poly-L-lysine or Vectabond-treated (Vector Laboratories Inc., CA, USA), or SuperFrost+ slides. Deparaffinization was done with xylene, after which the sections were rehydrated through a descending series of ethanol into water. The antigenicity was retrieved by incubation in a 1:3 diluted target unmasking

fluid (TUF, Sanbio, Uden, The Netherlands) or by microwave oven heating. Endogenous peroxidase activity was inhibited by incubation in 0.3% hydrogen peroxide (H_2O_2) in methanol for fifteen minutes.

A streptavidin-biotin-enzyme complex (SABC) method was used for the immunohistochemical stainings (Zymed Laboratories Inc., CA, USA). After the antigen retrieval and washings in 0.1M phosphate-buffered saline (PBS, pH 7.4), the tissue sections were incubated in normal goat serum (Zymed) in a humidified chamber for fifteen minutes. The incubation in the primary serum (primary antibodies) took place in a humidified chamber at +4°C overnight. Washings in PBS were followed by the incubation in the biotinylated secondary serum (goat anti-primary antibodies, Zymed) for 30 minutes. After washing in PBS, the sections were incubated in a humidified chamber with the streptavidin-biotin-peroxidase complex (Zymed) for 30 minutes. Peroxidase was then visualized with H_2O_2 and 3'-diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml, the chromogen) to obtain a brown end-product by a five-minute incubation. Positive controls included samples from the skin or gut.

Proliferating Cell Nuclear Antigen (PCNA, Studies I-III). Three different methods were used for the evaluation of PCNA labeling index in sections. The first immunostaining was performed in a separate university laboratory (Tampere, Finland) using the mouse monoclonal antibody 19A2 (IgM subclass, Coulter Immunology, FL, USA) at a 1:1000 dilution (Studies I and II). Visualization of the PCNA-immunoreactive nuclei was enhanced by silver intensification of the peroxidase reaction product. Counterstaining was done with Harris's hematoxylin. The second staining utilized the same primary serum at a dilution of 1:200, without enhancement (Studies II and III). The third PCNA immunochemical staining (randomly selected cases, Study II) was done with the anti-$PCNA_{19A2}$ primary serum, obtained from BioGenex (IgM subclass, BioGenex, CA, USA) and diluted 1:70. Because of the computer-assisted image analysis, the counterstaining in the latter two stainings was done with 0.2-0.4% ethyl green in 0.1M sodium acetate buffer for 15-30 minutes.

Ki-67 antigen (Studies III-V). A mouse monoclonal antibody MIB-1 (IgG_1, Immunotech, S.A. Marseille, France) recognizing the Ki-67 antigen, was used at a dilution of 1:40. The tissue sections were counterstained with ethyl green.

2.1.2 Assessment of immunohistochemical staining results

The cell proliferation analyses were targeted on those tumor areas expressing the highest number of immunoreactive neoplastic nuclei. Section borders, endothelial cells and necrotic tumor areas were omitted. A total of 20 adjacent high-power microscopic fields (HPFs) were analyzed at 400x magnification. In the case of large tumor areas, the assessment proceeded first along the longest axis of the tumor sample, and then perpendicularly through the midpoint of the analyzed axis along another axis.

Visual scoring of the PCNA immunopositivity was performed with a 25-point square lattice eyepiece (Studies I-II). The visual analysis resulted in four types of quantitative estimates:

Volume percentage of nuclei (VPN, %) = area fraction covered by neoplastic nuclei
PCNA labeling index (LI, %) = percentage of PCNA positive nuclei
Uncorrected PCNA-LI = number of PCNA positive nuclei per 1 mm^2 of neoplastic tissue
Corrected PCNA-LI = number of PCNA positive nuclei per 1 mm^2 of neoplastic nuclei

A computer-assisted image analysis system with associated software (CAS-200™, Becton Dickinson, CA, USA) was used for the evaluation of cell proliferation activity in tissue sections counterstained with ethyl green (Studies II-V). The image analysis system was an integrated unit of a standard microscope and two video cameras connected to a computer. Of the microscopic image, the cameras registered two different wavelengths of the emitted light: brown color (620 nm, diaminobenzidine) of the immunopositive nuclear areas and green color (500 nm, ethyl green) of the immunonegative nuclear areas. The registered microscopic image (0.012 mm^2 of neoplastic tissue at 400x magnification) was transmitted to a frame grabber, which digitized the picture for computer processing and storage. Each image was displayed in brown and green colors by a computer monitor for the observer. The threshold for the edge detection of the colored nuclear areas was kept constant during the analyses. The cell proliferation analyses utilizing the image analysis procedure (PCNA and Ki-67) provided four parameters characterizing the immunostained tissues:

- percentage of immunopositive nuclei (count index, LIc, %)
- percentage of immunopositive nuclear area (area index, LIa, %)
- total number of neoplastic nuclei
- total nuclear area (mm^2)

For the evaluation of PCNA immunopositivity, the computer-assisted image analysis and the visual assessment with a square lattice were performed simultaneously from 20 microscopic HPFs as described above (Study II). This combined evaluation method was used for the analysis of the second PCNA staining (senior observer), and by two observers (senior versus junior) regarding the third PCNA staining.

2.1.3 Flow cytometric DNA analysis

The FCM-DNA (Study III) was performed on nuclear suspensions obtained from 50 µm deparaffinized tissue sections. Only data regarding the cell cycle distribution of the nuclei were utilized.

2.1.4 Mitotic activity

Mitotic figures (Study V) were counted from *light* Harris's hematoxylin stained sections. The counting was performed from an area of highest mitotic activity. Parallel Ki-67(MIB-1) immunostained tissue sections were used as an aid in identification of most proliferative tumor areas. Counting of mitotic figures was done with the microscope attached to the image analysis system (40 HPFs, 400x magnification). This provided a simultaneous recording of the neoplastic nuclei in the analysis area (see below). Mitoses were identified on the basis of the criteria presented by Baak and Oort (1983). The following parameters were recorded:

- mitosis count recorded as mitoses per 1 mm^2 of neoplastic tissue
- volume corrected mitotic index (M/V-index, mitoses per 1 mm^2 of nuclear area)

2.2 Neoplastic nuclei by image analysis

During mitosis counting (Study V), the image analysis system was used for recording the total area (mm^2) and number of the underlying neoplastic nuclei. This provided quantitative estimates of the area fraction of neoplastic nuclei (%) as well as the mean nuclear size (μm^2) in the tissue area analyzed.

2.2.1 Image cytometric DNA analysis

The ICM-DNA (Study V) was done with the computer-assisted image analysis system and associated software (CAS-200™). Deparaffinized 50-100 µm tissue sections were incubated in a preheated solution of Carlsberg (0.1M TRIS/0.07M NaCl, pH 7.2) with admixture of protease XXVII (Sigma Chemical Company, MO, USA) at 37°C for one hour (digestion). Clear liquid containing the nuclei, was removed and centrifuged at 1200 rounds per minute for 10 minutes. The supernatant was removed and nuclear suspensions were prepared from the remaining pellets (one million nuclei/ 1 ml of 1% bovine serum albumin in PBS). Nuclear counting was done in a Bürker's chamber in suspension containing crystal purple. The nuclear suspensions (an amount corresponding to approximately 150 000 nuclei per sample) were centrifuged (500 rounds per minute for 10 minutes) onto poly-L-lysine treated slides, air-dried, fixed in 10% phosphate-buffered formaldehyde for 30 minutes and rinsed in running deionized water. The Feulgen staining was performed with the CAS™ DNA Staining Kit (Becton Dickinson) optimized for the image analysis system. The kit was provided with calibration slides that contained cells (rat hepatocytes) with a standard DNA content. One of the video cameras in the image analysis system was used for registering the blue color of the Feulgen stained (wavelength of 630 nm) nuclei. The analysis of the tumors was performed through adjacent microscopic HPFs (400x magnification) across the sample area. All the nuclei analyzed (200 per tumor case) were visualized on a computer monitor and stored on an optical disk. The neoplastic cells and the tumors were classified into three categories as described in Study V: DNA diploid, non-diploid and aneuploid.

2.3 Endothelial cells

The area fraction of endothelial cells (endothelial cellularity, %) was estimated in a tissue area of the most pronounced vascularity/ endothelial cell proliferation. Twenty adjacent HPFs were counted using a 100-point square lattice attached to the microscope (Study V).

2.4 Tumor necrosis

The area fraction of obvious tumor necrosis in samples (%, Study V), large foci or small often multiple regions (detectable by the eye at 40x magnification) but not individual cell necrosis, was estimated interactively by image analysis (Scion Image for Windows, Scion Corp., Maryland, USA). Based on the results of the Study V, the tumors in this study have been evaluated for the presence or absence of detectable tumor necrosis.

2.5 Telomerase enzyme

The mRNA in situ hybridization. The mRNA in situ hybridization analysis (Study IV) was applied for studying telomerase RNA gene expression in tumors. Five μm tissue sections were hybridized with a synthesized oligonucleotide probe (nucleotides 29-76, GenBank accession number S79400), labeled at the 3'-end with [^{33}P]dATP (DuPont New England Nuclear Research Products, MA, USA) using terminal deoxyribonucleotidyltransferase (Amersham, UK). The hybridization solution included 50 ml of deionized formamide, 20 ml of 20x standard saline citrate (SSC), 1 ml of 100x Denhardt's solution, 10 ml of 0.2M $NaPO_4$, 10 g of dextran sulphate, 5 ml of 20% sarcosyl (Sigma L5125), denatured salmon DNA, and the probe (10^7 counts per minute [cpm]/ml) in a humidified chamber at 42°C for 18 hours. After removal of the unbound probe, and dehydration through an ascending series of ethanol (up to 96%), the slides were exposed to phosphorimager plates (Molecular Dynamics, CA, USA) for nine days. The tumor area with the most intense hybridization signal, and representative for the histopathological diagnosis, was analyzed. The telomerase mRNA (TmR) expression was recorded as the total radioactivity per day of exposure in 1 mm^2 of the tumor tissue (cpm/d/mm^2). The image analysis system performed the slide background correction automatically. A part of the slides were covered with autoradiographic emulsion (Kodak NTB2). After nine days' exposure, the emulsion was developed and counterstained with hematoxylin to visualize the TmR expression at the cellular level.

The telomeric repeats amplification protocol (TRAP). The TRAP assay was used for the detection of telomerase enzyme activity in the seven supplemented astrocytic tumors. Freshly frozen tissue material (100 μm cryostat sections) and a commercially available telomerase detection kit were utilized for the experiment (TRAP-eze Telomerase Detection Kit, Oncor, MD, USA) as described in Study IV.

2.6 Statistical methods

The Pearson correlation coefficient (r), one-way analysis of variance (ANOVA), Mann-Whitney test, chi-square test, the univariate survival analysis (Mantel-Cox) and the Cox proportional multivariate analysis were used for the statistical evaluations as described in Studies I-V. The best prognostic cut-off points for the survival analyses were determined by the receiver operating characteristics (ROC) curve as described in Study III.

RESULTS

The results in this study are presented with respect to the prevailing practice in histopathological malignancy grading of astrocytic tumors (WHO 1993) unless otherwise indicated. The distribution of the tumor material in histopathological malignancy categories is presented in Table VI. This study contains previously unpublished data. Therefore, some results may differ slightly, but not significantly, from those in the original publications. It is also important to mention that the histopathological malignancy grading of diffuse astrocytomas in Study V followed a "strict" interpretation of the WHO 1993 grading criteria. This practice altered the grading of some tumor cases presented in this study.

1. Histopathological Malignancy Grade and Patient Age

The 91 diffusely infiltrating astrocytomas comprised 23 Grade II and 18 anaplastic (Grade III) astrocytomas, and 50 glioblastomas (Grade IV) (Table VI). Patient age differed significantly between the histopathological malignancy groups ($F = 25.2$, $p < 0.001$, ANOVA).

2. Quantitative Pathology

2.1 Cell proliferation activity

Visual assessment of PCNA immunoreactivity (Studies I-II). The assessment scores of the first and the second PCNA immunostaining by visual inspection (25-point eye-piece lattice) are presented in Table VII.

PCNA scores by image analysis (Studies II-III). The computer-assisted PCNA cell proliferation estimates (staining 2) in the three histopathological malignancy categories are presented in Table VIII.

Reproducibility of PCNA estimates (Study II). One observer (senior) evaluated all three PCNA immunostainings. The application of either the uncorrected or corrected PCNA-LI improved the correlation between the visual estimates of staining batches 1 and 2 (PCNA-LI: $r = 0.678$; uncorrected PCNA-LI: $r = 0.776$; corrected PCNA-LI: $r = 0.702$, $n = 82$). The comparison of visual and computer-assisted assessments of PCNA-LI and VPN from staining batches 2 and 3 is presented in Table IX, as in the original publication. A total of 38 cases were randomly selected from staining batch 2 for a junior observer for the PCNA cell proliferation analysis. The comparison of the results by the two observers is presented in Table IX, as in the original publication. The application of the uncorrected or corrected PCNA-LI slightly improved the reproducibility of visual assessment scores between the two observers (uncorrected PCNA-LI: $r = 0.634$; corrected PCNA-LI: $r = 0.630$). When the assessment scores of the senior observer were compared between visual and computer-assisted techniques, the correlation was substantial (PCNA-LI: $r = 0.887$; VPN: $r = 0.823$, staining 2, $n = 88$). For the junior observer, the correlation coefficients were 0.800 for the PCNA-LIs and 0.940 for the VPN scores ($n = 38$). When utilizing the prognostic cut-off point of 2% (see below) for the computer-assisted PCNA-LIc, the two

observers concurred in placing the tumors into categories of low and high PCNA-LI in 29 of the 38 cases ($p < 0.001$, chi-square test).

Table VII. PCNA assessment scores by visual inspection in the three histopathological malignancy groups (WHO 1993) of diffuse astrocytomas (SD = standard deviation).

	Median	**Mean ± SD**	**Range**	**p***
Staining 1 (visual inspection)				
PCNA-LI (%)	**2.4**	**3.7 ± 3.8**	**<0.5 - 21.1**	**< 0.001** (23.7)
Grade II (n=21)	*0.8*	*0.9 ± 0.7*	*<0.5 - 2.8*	
Grade III (n=17)	*2.3*	*2.7 ± 2.3*	*<0.5 - 8.6*	*0.006*
Grade IV (n=44)	*4.8*	*5.5 ± 4.1*	*<0.5 - 21.1*	*0.007*
Uncorrected PCNA-LI (/1mm^2 tissue)	**104**	**165 ± 200**	**<1 - 1166**	**< 0.001** (28.4)
Grade II (n=21)	*25*	*29 ± 25*	*<1 - 73*	
Grade III (n=17)	*61*	*115 ± 153*	*4 - 661*	*0.004*
Grade IV (n=44)	*195*	*248 ± 221*	*9 - 1166*	*0.002*
Corrected PCNA-LI (/1mm^2 nuclei)	**379**	**532 ± 480**	**<1 - 2096**	**< 0.001** (17.6)
Grade II (n=21)	*173*	*167 ± 130*	*<1 - 375*	
Grade III (n=17)	*387*	*425 ± 389*	*13 - 1599*	*0.008*
Grade IV (n=44)	*752*	*747 ± 503*	*38 - 2096*	*0.014*
Staining 2 (visual inspection)				
PCNA-LI (%)	**1.8**	**3.9 ± 4.9**	**<0.5 - 23.8**	**< 0.001** (36.6)
Grade II (n = 23)	*0.3*	*0.4 ± 0.4*	*<0.5 - 1.6*	
Grade III (n = 18)	*1.1*	*1.9 ± 1.8*	*<0.5 - 6.3*	*< 0.001*
Grade IV (n = 47)	*4.4*	*6.3 ± 5.5*	*<0.5 - 23.8*	*< 0.001*
Uncorrected PCNA-LI (/1mm^2 tissue)	**93**	**208 ± 272**	**<1 - 1093**	**< 0.001** (60.1)
Grade II (n = 23)	*9*	*14 ± 13*	*<1 - 55*	
Grade III (n = 18)	*56*	*97 ± 118*	*7 - 462*	*< 0.001*
Grade IV (n = 47)	*238*	*345 ± 303*	*<1 - 1093*	*< 0.001*
Corrected PCNA-LI (/1mm^2 nuclei)	**324**	**597 ± 684**	**<1 - 2657**	**<0.001** (34.3)
Grade II (n = 23)	*66*	*78 ± 76*	*<1 - 237*	
Grade III (n = 18)	*224*	*341 ± 300*	*23 - 981*	*< 0.001*
Grade IV (n = 47)	*793*	*949 ± 750*	*<1 - 2657*	*< 0.001*

* Analysis of variance (F-value), the statistical significance of the difference between consecutive malignancy categories has also been presented (Mann-Whitney test)

Ki-67(MIB-1) labeling index (Studies III-V). The Ki-67(MIB-1) cell proliferation estimates in the three histopathological malignancy categories are presented in Table VIII. The reciprocal correlation between the PCNA-LIc and Ki-67(MIB-1)-LIc was poor ($r = 0.484$, $n = 88$, Pearson).

FCM-DNA (Study III). The estimates of the S-phase fraction and combined fractions of S- and G2/M- phase cells by flow cytometric DNA analysis are presented in Table VIII. The correlation

between the PCNA-LIc and S-phase fraction (FCM-DNA) was poor (r = 0.398, n = 62), and slight between the KI-67(MIB-1)-LIc and S+G2/M fraction (r = 0.564, n = 62).

Table VIII. Cell proliferation scores by image analysis in the three histopathological malignancy groups (WHO 1993) of diffusely infiltrating astrocytomas (SD = standard deviation).

	Median	**Mean ± SD**	**Range**	**p***
PCNA-LIc (count-%)	**2.6**	**4.6 ± 5.9**	**<0.5 - 30.2**	**< 0.001** (33.1)
Grade II (n = 23)	*0.2*	*0.7 ± 1.2*	*<0.5 - 5.6*	
Grade III (n = 18)	*1.2*	*2.0 ± 1.6*	*<0.5 - 5.2*	*0.002*
Grade IV (n = 47)	*5.5*	*7.5 ± 6.8*	*<0.5 - 30.2*	*< 0.001*
PCNA-LIa (area-%)	**1.8**	**3.1 ± 3.9**	**<0.5 - 21.9**	**< 0.001** (29.3)
Grade II (n = 23)	*0.3*	*0.5 ± 0.7*	*<0.5 - 3.4*	
Grade III (n = 18)	*1.1*	*1.5 ± 1.0*	*<0.5 - 3.3*	*< 0.001*
Grade IV (n = 47)	*3.5*	*4.9 ± 4.6*	*<0.5 - 21.9*	*< 0.001*
Ki-67(MIB-1)-LIc (count-%)	**22.3**	**23.1 ± 13.7**	**< 0.5 - 53.8**	**< 0.001** (54.5)
Grade II (n = 23)	*8.0*	*8.0 ± 3.9*	*< 0.5 - 16.7*	
Grade III (n = 18)	*15.5*	*18.3 ± 10.9*	*0.6 - 50.0*	*< 0.001*
Grade IV (n = 50)	*31.4*	*31.7 ± 10.3*	*10.4 - 53.8*	*< 0.001*
Ki-67(MIB-1)-LIa (area-%)	**15.1**	**17.8 ± 13.3**	**<0.5 - 58.4**	**< 0.001** (59.6)
Grade II (n = 23)	*4.7*	*5.1 ± 2.7*	*<0.5 - 10.8*	
Grade III (n = 18)	*9.5*	*12.9 ± 10.8*	*<0.5 - 49.8*	*< 0.001*
Grade IV (n = 50)	*23.1*	*25.3 ± 11.9*	*6.4 - 58.4*	*< 0.001*
S-phase fraction (%, FCM-DNA)	**7.6**	**8.8 ± 5.1**	**2.0 - 21.7**	**< 0.001** (23.7)
Grade II (n = 17)	*4.3*	*4.9 ± 2.6*	*2.0 - 11.9*	
Grade III (n = 12)	*5.0*	*7.0 ± 4.8*	*3.6 - 21.1*	*0.057*
Grade IV (n = 33)	*10.0*	*11.4 ± 4.8*	*4.6 - 21.7*	*< 0.001*
S+G2/M fraction (%, FCM-DNA)	**11.8**	**13.6 ± 7.5**	**2.2 - 31.0**	**< 0.001** (17.3)
Grade II (n = 17)	*7.6*	*8.1 ± 4.3*	*2.2 - 17.0*	
Grade III (n = 12)	*7.6*	*11.0 ± 7.8*	*4.1 - 31.0*	*0.352*
Grade IV (n = 33)	*16.1*	*17.4 ± 6.6*	*6.7 - 28.7*	*0.003*

* Analysis of variance (F-value), the statistical significance of the difference between consecutive malignancy categories has also been presented (Mann-Whitney test)

Table IX. Comparison of PCNA labeling indices (LIs) and VPN (area fraction of nuclei) scores in astrocytic tumors assessed by two observers (n = 38) and one (senior) observer from two different staining batches (n = 44) using visual inspection and computer-assisted image analysis (Pearson's correlation coefficient, LIc = count-%, LIa = area-%).

	Senior observer (staining 2)				
	Visual assessment		**Computer-assisted analysis**		
	PCNA-LI	VPN	PCNA-LIc	PCNA-LIa	VPN
Senior observer (staining 3)					
Visual assessment	0.879	0.831			
Computer-assisted analysis			0.857	0.874	0.693
Junior observer (staining 2)					
Visual assessment	0.624	0.810			
Computer-assisted analysis			0.904	0.927	0.934

Mitotic activity (Study V). The mitosis count and the M/V-index in the three histopathological malignancy categories are presented in Table X.

2.2 Quantitative histopathology

Study V. The mean nuclear size, area fraction of endothelial cells, and area fraction of neoplastic nuclei in the three histopathological malignancy categories are presented in Table X, together with the data of tumor necrosis.

The subdivision of tumors (diploid, non-diploid, aneuploid) by image cytometric DNA analysis (ICM-DNA, n = 72) was not associated with the established histopathological tumor malignancy (p = 0.241, chi-square test). The estimates of the quantitative histological variables did not differ between the established DNA ploidy categories (Mann-Whitney tests). This was also the case when DNA aneuploid astrocytomas were compared with the other tumors.

Table X. Assessment scores of histological features in the three histopathological malignancy groups (WHO 1993) of diffusely infiltrating astrocytomas (SD = standard deviation).

	Median	**Mean ± SD**	**Range**	**p***
Mean nuclear size (μm^2)	**36.9**	**39.6 ± 7.6**	**30.8 - 57.7**	**< 0.001** (8.4)
Grade II (n = 23)	*34.4*	*35.3 ± 5.5*	*30.9 - 55.9*	
Grade III (n = 18)	*36.3*	*37.8 ± 7.3*	*30.8 - 55.6*	*0.134*
Grade IV (n = 50)	*40.8*	*42.3 ± 7.6*	*32.2 - 57.7*	*0.009*
Mitosis count (per mm^2 of tissue)	**5.8**	**8.5 ± 8.2**	**0 - 32.5**	**< 0.001** (138.2)
Grade II (n = 23)	*0*	*0.6 ± 0.8*	*0 - 2.5*	
Grade III (n = 18)	*3.3*	*4.0 ± 3.4*	*0.8 - 15.8*	*< 0.001*
Grade IV (n = 50)	*13.3*	*13.8 ± 7.4*	*3.3 - 32.5*	*< 0.001*
Area fraction of endothelial cells (%)	**2.6**	**2.9 ± 1.4**	**0.1 - 7.4**	**0.001** (8.4)
Grade II (n = 18)	*1.8*	*2.0 ± 0.6*	*1.1 - 3.2*	
Grade III (n = 13)	*2.4*	*2.5 ± 1.2*	*0.1 - 4.9*	*0.138*
Grade IV (n = 38)	*3.3*	*3.4 ± 1.6*	*0.7 - 7.4*	*0.056*
Tumor necrosis (number of cases)				
Grade II (n = 23)			*0*	
Grade III (n = 18)			*4 #*	
Grade IV (n = 50)			*49*	
Area fraction of nuclei (%)	**15.6**	**18.2 ± 10.1**	**4.8 - 52.7**	**< 0.001** (12.2)
Grade II (n = 23)	*11.1*	*11.7 ± 6.2*	*5.2 - 34.8*	
Grade III (n = 18)	*15.3*	*15.2 ± 7.0*	*5.1 - 33.6*	*0.055*
Grade IV (n = 50)	*19.2*	*22.3 ± 10.5*	*4.8 - 52.7*	*0.010*
M/V-index (mitoses/ mm^2 of nuclei)	**82**	**115 ± 109**	**0 - 485**	**< 0.001** (99.1)
Grade II (n = 23)	*0*	*11 ± 15*	*0 - 47*	
Grade III (n = 18)	*66*	*76 ± 64*	*8 - 239*	*< 0.001*
Grade IV (n = 50)	*170*	*177 ± 103*	*20 - 485*	*< 0.001*

* Analysis of variance (F-value), the statistical significance of the difference between consecutive malignancy categories has also been presented (Mann-Whitney test)

\# Small groups of necrotic cells

3. Pilocytic Astrocytomas

Mean nuclear size in the 11 pilocytic astrocytomas (Grade I) ranged between 30.7 and 38.2 μm^2 (mean = 33.8, median = 32.8). Mitotic figures were not observed. The mean value for the area fraction of endothelial cells was 3.3% (median = 2.8, range: 2.0 - 6.0). VPN scores ranged between 4.1 and 20.0 % (mean = 11.3, median = 9.6). Tumor necrosis was not observed. Mean PCNA-LI was 1.4% (median = 0.6, range: <0.5 - 5.5), and mean Ki-67(MIB-1)-LI was 5.6% (median = 3.0, range: <0.5 - 16.7). One tumor was recorded as DNA aneuploid. This tumor also showed the highest cell proliferation activity among pilocytic tumors, and it recurred. At least one parameter

score was relatively high (over the prognostic cut-offs in diffuse astrocytomas, see below) in eight tumor cases.

4. Telomerase Enzyme

Study IV. The results of the telomerase mRNA (TmR) in situ hybridization analysis (n = 46, includes 2 pilocytic astrocytomas) in astrocytic tumors correlated closely with the established histopathological malignancy grade of the tumors (WHO 1993 Grade I-II versus Grade III-IV: p = 0.024, Mann-Whitney test). TRAP assays (seven supplemented freshly frozen tumors) revealed telomerase enzyme activity in four of the six glioblastomas and in the Grade II astrocytoma studied. Increased production of TmR was detected in six of the seven cases. Mitotic activity and cell proliferation scores by the Ki-67(MIB-1) immunohistochemistry were significantly increased in the subgroup of tumors with increased production of TmR (mitoses: p = 0.017; Ki-67: p = 0.004, Mann-Whitney test). This was also the case among Grade II astrocytomas with increased production of TmR (Ki-67(MIB-1)-LI: p = 0.047, Mann-Whitney test). TmR expression did not differ between DNA diploid/non-diploid and DNA aneuploid tumors (p = 0.95, n = 37, Mann-Whitney test), and relatively high levels of TmR expression (> 173 cpm/mm^2/day of exposure) were observed in both of the categories (p = 0.89, chi-square test).

5. Survival Analyses

The best prognostic cut-off point values (the receiver operating characteristics curve, 2-year follow-up) were used for dividing the tumors into two subgroups of low versus high parameter status (Table XI). For visual analyses of staining 2, the corrected PCNA-LI (sensitivity 81%, specificity 80%) provided a better distinction between poor and relatively good prognosis when compared with the uncorrected PCNA-LI (sensitivity 76%, specificity 80%) or the PCNA-LI (sensitivity 74%, specificity 77%). The nuclear area-related cell proliferation index (LIa), provided by the image analysis system, did not improve the prognostic significance of the PCNA nor the Ki-67(MIB-1) estimates. This was also the case with the M/V-index, when compared with the mitosis count. Of the quantitative variables (mean nuclear size, mitosis count, tumor necrosis, VPN, PCNA-LIc and Ki-67(MIB-1)-LIc included), tumor necrosis was the single independent prognosticator in the Cox proportional multivariate analysis (exp(B) = 16.38, 95% confidence interval for exp(B): 7.81 - 34.38, p < 0.001). The result was not changed when the histopathological malignancy grade (Grade II versus Grades III-IV) and patient age (cut-off = 50 years) were included. When only the cell proliferation estimates were included in the Cox model, mitosis count (exp(B) = 3.03, 95% confidence interval for exp(B): 1.22 - 7.51, p = 0.017) and Ki-67(MIB-1)-LI (exp(B) = 2.62, 95% confidence interval for exp(B): 1.15 - 5.98, p = 0.023) were independently associated with survival.

Among Grade II astrocytomas, tumor DNA ploidy (ICM-DNA) was the only variable strongly associated with prognosis. The mean survival for the patients with DNA aneuploid tumors was 35 months (95% confidence interval for mean survival: 20 - 51, n = 8), compared with 94 months for those with DNA non-aneuploid tumors (95% confidence interval for mean survival: 84 - 103, n = 15, p < 0.001). Tumor DNA ploidy also remained significantly associated with survival in the Grade II and anaplastic (Grade III) astrocytoma group (p = 0.002, n = 38). In addition, mitotic activity gained statistical significance among Grade II and anaplastic astrocytomas. The mean survival of the patients with tumors of mitotic activity less than 3 mitoses in one mm^2 of neoplastic tissue was 70 months (95% confidence interval for mean survival: 57 - 83, n = 30), and 39 months for those with

mitotic activity equal or more than 3 mitoses (95% confidence interval for mean survival: 17 - 60, n = 11, p = 0.043).

Table XI. Survival of patients with diffusely infiltrating astrocytomas divided into subclasses on the basis of morphometric and cell proliferation scores, and patient age (CI = confidence interval).

		Survival (months)				
		Mean	95 % CI for Mean	Sensitivity (%)	Specificity (%)	p*
Mean nuclear size (μm^2)						**< 0.001**
< 36	(n = 37)	49	36 - 62			
≥ 36	(n = 54)	21	13 - 29	75	69	
Mitosis count (1 mm^2 of tissue)						**< 0.001**
< 3	(n = 30)	70	57 - 83			
≥ 3	(n = 61)	14	9 - 19	92	78	
Area fraction of endothelia (%)						**< 0.001**
< 2.5	(n = 32)	49	35 - 63			
≥ 2.5	(n = 37)	15	9 - 22	68	72	
Tumor necrosis						**< 0.001**
absent	(n = 38)	67	55 - 78			
present	(n = 53)	8	7 - 10	86	94	
Area fraction of nuclei (%)						**< 0.001**
< 16	(n = 47)	49	37 - 60			
≥ 16	(n = 44)	15	8 - 21	66	84	
PCNA-LIc (%)						**< 0.001**
< 2	(n = 41)	55	42 - 67			
≥ 2	(n = 47)	15	9 - 21	73	81	
Ki-67(MIB-1)-LIc (%)						**< 0.001**
< 20	(n = 39)	61	48 - 73			
≥ 20	(n = 52)	12	8 - 15	80	84	
S-phase fraction (%, FCM-DNA)						**< 0.001**
< 6	(n = 23)	70	54 - 86			
≥ 6	(n = 39)	19	10 - 27	84	68	
S+G2/M fraction (%, FCM-DNA)						**< 0.001**
< 11	(n = 28)	58	42 - 74			
≥ 11	(n = 34)	21	11 - 31	73	72	
Patient age						**< 0.001**
< 50	(n = 44)	55	43 - 67			
≥ 50	(n = 47)	12	7 - 16	71	84	

* univariate survival analysis

According to the receiver operating characteristics curve, the sensitivity of the established cut-off point for the TmR expression (173 cpm/mm^2/day of exposure) was 58% and the specificity 60%. The subdivision of the tumors by TmR expression was not associated with survival (p = 0.316).

6. Decision-Tree Model

Study V. The best prognostic factors were collected in a decision-tree model (Figure 7). Twig I included diffusely infiltrating astrocytomas with low (less than 3 mitoses in one mm^2 of neoplastic tissue) mitotic activity. Twig II was composed of tumors with elevated mitotic activity but no detectable tumor necrosis, and Twig III included tumors with even a small focus of incipient necrosis. The three established tumor categories were closely associated with the survival of the patients (p < 0.001), and the model was superior to the WHO 1979 and WHO 1993 grading schemes in the multivariate survival analysis (exp(B) = 4.488, 95% confidence interval for exp(B): 2.989 - 6.738, p < 0.001, n = 91). The subdivision of Twig I into DNA diploid or non-diploid tumors (Twig IA) and DNA aneuploid tumors (Twig IB) improved the identification of aggressively behaving tumors (p < 0.001, Table XII, Figure 7).

Table XII. Comparison of age and survival of patients with astrocytomas divided into three malignancy categories on basis of the former WHO 1979 and revised WHO 1993 grading schemes, and into four subgroups by a decision-tree model (SD = standard deviation, CI = confidence interval).

	AGE (years)	***SURVIVAL (months)***		
	Mean ± SD	**Mean**	**95% CI for Mean**	**p***
WHO 1979				**< 0.001**
I-II (n = 23)	36 ± 16	67	52 - 82	
III (n = 25)	44 ± 16	41	26 - 56	(0.046)
IV (n = 43)	56 ± 10	9	7 - 11	(< 0.001)
WHO 1993				**< 0.001**
II (n = 23)	34 ± 14	74	60 - 89	
III (n = 18)	44 ± 17	45	28 - 62	(0.023)
IV (n = 50)	56 ± 11	9	7 - 11	(< 0.001)
Decision-tree model				**< 0.001**
Twig IA (n = 16)	29 ± 13	93	83 - 102	
Twig IB (n = 13)	40 ± 10	42	28 - 56	(< 0.001)[#]
Twig II (n = 9)	41 ± 18	48	25 - 71	(0.900)
Twig III (n = 53)	57 ± 10	8	7 - 10	(< 0.001)

* Mantel-Cox (also between consecutive categories)

[#] The age difference between patients in Twigs IA and IB was statistically significant (p = 0.012, Mann-Whitney test), but patient age (cut-off at 35 years) was not significantly associated with survival within Twig I (p = 0.219).

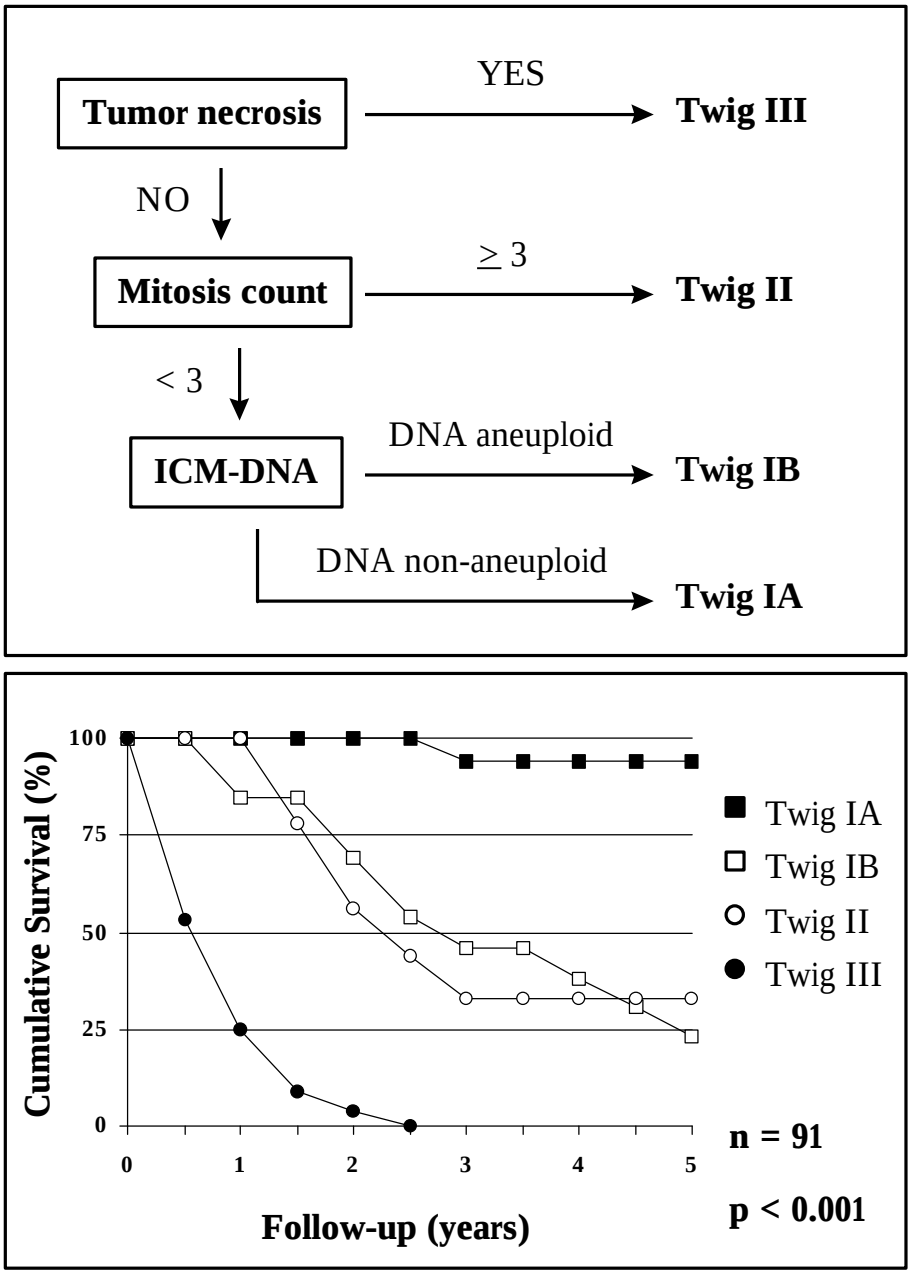

Figure 7. The subdivision of astrocytomas illustrated by a decision-tree model. The detection of even a small focus of (incipient) tumor necrosis, but not individual cell necrosis, provides sufficient grounds for assigning the tumor to Twig III. If absent, mitotic figures are counted from the most proliferative tumor area, determined by Ki-67(MIB-1) immunolabeling. If the mitosis count does not exceed 2 mitoses per one mm^2 of neoplastic tissue, a further prognostic characterization of the tumor could be made by image cytometric DNA analysis (ICM-DNA). The survival of the patients in the corresponding categories is presented on the bottom..

DISCUSSION

The treatment of patients with astrocytic tumors has largely been based on the histopathological malignancy grade established by the neuropathologist. Over the years, the grading criteria have remained essentially the same, and various astrocytoma classification systems have made only slight modifications for the appreciation of the criteria (Vandenberg 1992, Kleihues *et al.* 1993a). Nonetheless, each grading criterion has been evaluated ultimately for its presence, which has, on the one hand, simplified the grading models for universal use. On the other hand, the dichotomous practice has concealed potential information about the behavior of individual tumors. Upregulated cell proliferation activity is characteristic of human malignancies (Dirks and Rutka 1997). Thus, a strong hypothesis of the present study was that the assessment of cell proliferation could explain some of the heterogeneity in prognosis of astrocytic tumors, especially the diffusely infiltrating type.

1. Cell Proliferation Analysis by Immunohistochemistry

1.1 Sources of variation

Unstandardized use of various measurement techniques characterizes the considerable variation in immunohistochemical cell proliferation estimates between studies presented in Tables III-V. The subjectivity of the evaluation is evidently one factor, influencing the reproducibility of the estimates. Astrocytomas may show significant variation in cell proliferation activity between regions of the tumor sample (Coons and Johnson 1993b, Figure 8). Thus, the determination of the representative tumor area to be analyzed depends on the experience of the observer and the quality of immunostaining. The latter, influencing the recognition of immunoreactive cells, pinpoints the laboratory technique. Routine tissue processing by fixation affects the immunodetection of cell proliferation associated antigens, which has led to the development of various alternatives for an improved antigen demonstration (McCormick *et al.* 1993, Munakata and Hendricks 1993, Pileri *et al.* 1997).

The application of different antibodies (even on a mutual target) makes direct comparisons of various studies difficult, or inadequate. Of the three PCNA stainings, the detectability of immunopositive cells varied between the staining batches (data shown in Study II). The best visualization of immunoreactive cells, as suggested earlier by Louis *et al.* (1991) and Rose *et al.* (1994), was achieved by the Ki-67(MIB-1) method, since the Ki-67 staining was more intense and restricted to cell nuclei with less background in surrounding areas when compared with any of the PCNA protocols. In addition, Ki-67(MIB-1) immunoreactive nuclei outnumbered those detected by the PCNA methods (Table VII), which made the selection of analysis areas less laborious. This observation is in line with Louis *et al.* (1991), but it contradicts the findings presented by Kordek *et al.* (1996). These two study groups utilized antibodies (Tables III-V) different from those described in this study.

In this study, the cell proliferation analyses were performed in tumor regions with subjectively the highest number of immunoreactive cells (sampling). The effect of sampling on the reproducibility of cell proliferation estimates could be demonstrated in the present context by the discrepancy in visually assessed PCNA-LI and VPN (tissue cellularity) scores between two observers (Table IX). It seems, however, that experience in distinguishing immunoreactive cells from the immunonegative ones had more impact on reproducibility than the selection of representative tumor areas, since the

correlation coefficient of the VPN scores was significantly higher than that of the PCNA-LIs. Furthermore, the correlation of both the PCNA and VPN estimates between the two observers improved significantly when the counting was done objectively from the same microscopic high-power fields with computer assistance. The lack of condense proliferation foci in tumors with relatively low cell proliferation activity (e.g. pilocytic or Grade II astrocytomas) remain problematic for sampling.

Considering immunohistochemical cell proliferation estimates, it is important to define possible sources of error in order to develop the methodology for improved applicability, reliability and reproducibility. In this study, simple adjustments to the quantitation technique improved both the prognostic value and reproducibility of visually determined cell proliferation estimates in astrocytomas (uncorrected and corrected PCNA labeling indices). Such standardization of visual assessment techniques could have importance for the applicability of cell proliferation measurements if computer-assisted analysis were not possible. However, the potential of computer-assisted quantitation has to be emphasized, since the objectivity in the recognition and assessment of immunoreactive nuclei could be increased by an image analysis technique. The time consumed for measurements could be reduced greatly by computer guidance, and the highly optimized staining conditions required for image analysis could be considered beneficial for the standardization process of immunohistochemical proliferation analyses. Of the studies presented in Tables III-V, computer-assisted analysis technique was employed by Montine *et al.* (1994), Cunningham *et al.* (1997) and Giannini *et al.* (1999).

1.2 Cell proliferation activity in astrocytomas

The present study shows that immunohistochemically determined cell proliferation activity in astrocytomas increases significantly along with the histopathological malignancy. Furthermore, the cell proliferation estimates provide statistically significant prognostic information about the tumors. These findings are in general concordance with the majority of the studies collected into Tables III-V, although the estimates, both those presented here (Tables VII-VIII) and those of other studies, show considerable fluctuation within established histopathological malignancy groups. Considering the selection of the antibody for cell proliferation estimates, the wider coverage of proliferating cells by the Ki-67(MIB-1) method, when compared with the PCNA method, provided a better statistical distinction between the cell proliferation activity of typical Grade II and anaplastic astrocytomas (image analysis, Table VIII). Furthermore, the application of the Ki-67(MIB-1) method improved the prognostic sensitivity and specificity of cell proliferation analysis in astrocytomas from that achieved by the PCNA method (Table XI). The significant differences in cell proliferation activity (PCNA and Ki-67) between different astrocytoma malignancy categories indicate that cell proliferation estimates could aid pathologists in the diagnostic determination of astrocytic tumors, especially in cases of borderline grade. The results presented by Giannini *et al.* (1999) support this point of view, but the authors seriously questioned the predictive value of low Ki-67(MIB-1)-LIs in Grade II astrocytomas. The cell proliferation estimates by Giannini *et al.* (1999) in diffuse astrocytomas were generally at a lower level than those presented in this study. Nonetheless, the present results show that the prognostic significance of the Ki-67(MIB-1) method is largely derived from the very high labeling indices in glioblastomas and aggressively behaving anaplastic astrocytomas (prognostic cut-off at 20%, Tables VIII and XI), while the mean score of the Grade II astrocytoma group remained at 8%. The potentially valuable diagnostic aid, provided by cell proliferation estimates, in borderline astrocytic tumors needs prospective prognostic inspection, since neither the present study nor Giannini *et al.* (1999) evaluated the effect of the possible change in therapeutic strategies on patient survival.

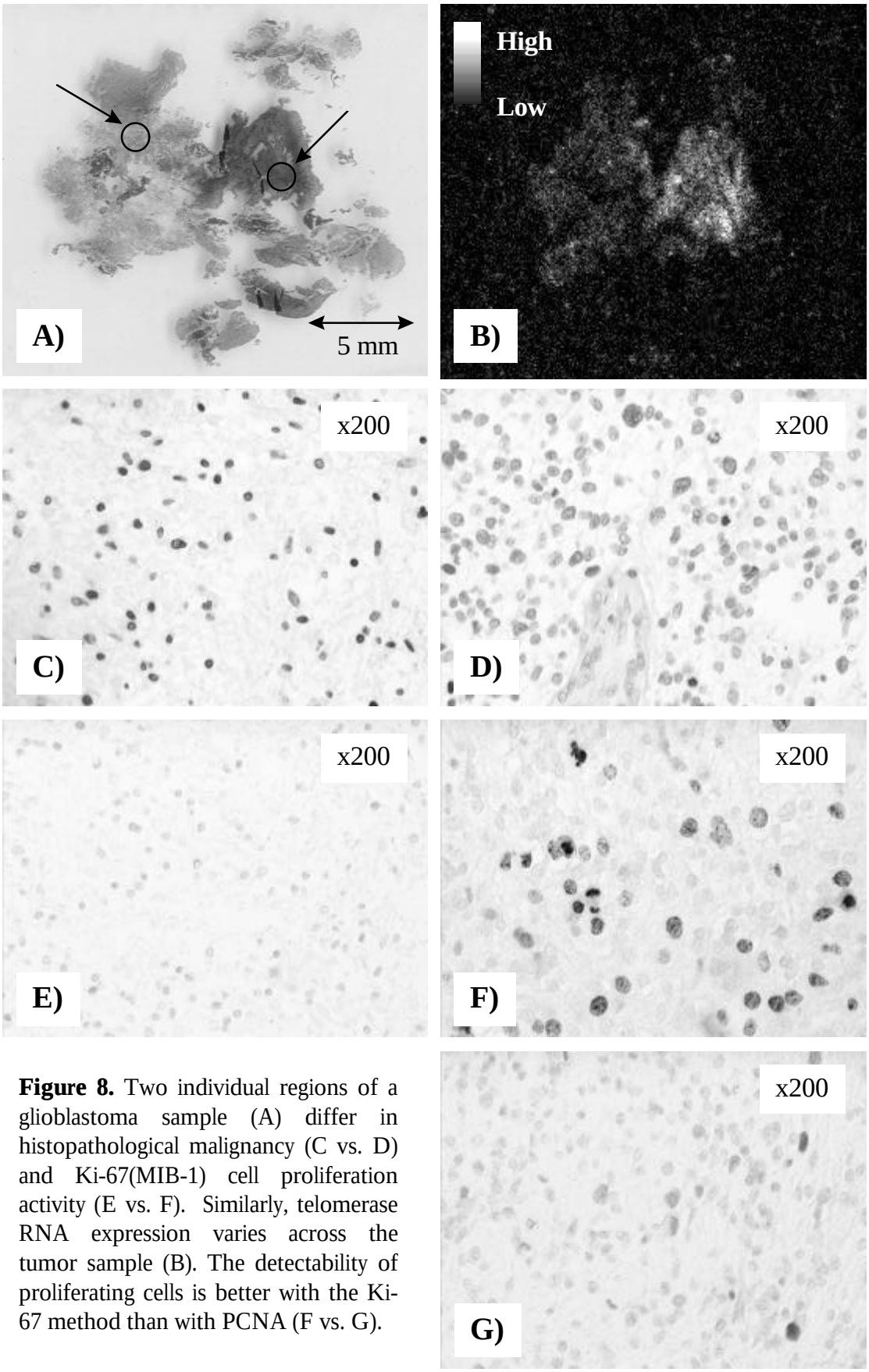

Figure 8. Two individual regions of a glioblastoma sample (A) differ in histopathological malignancy (C vs. D) and Ki-67(MIB-1) cell proliferation activity (E vs. F). Similarly, telomerase RNA expression varies across the tumor sample (B). The detectability of proliferating cells is better with the Ki-67 method than with PCNA (F vs. G).

2. Cytometric DNA Analysis

Cell proliferation estimates by flow cytometric DNA analysis supported the present results of the immunohistochemical cell proliferation analyses. However, the FCM-DNA was not successful in the majority of tumor cases, mainly due to reasons related to small/necrotic sample size and the intratumor heterogeneity in astrocytoma samples (large coefficients of variation, CV values). The latter most probably also had a negative effect on the FCM-DNA results when the comparison was made between different cell proliferation assessment techniques (Table VIII). Coons and Johnson (1993) demonstrated by regional FCM-DNA that most gliomas show pronounced variation in the S-phase fraction and tumor DNA ploidy among tumor areas. Since the conventional FCM-DNA lacks morphological control over the tumor tissue, other means for assessing astrocytomas seem more adequate.

In this study, the image cytometric DNA analysis (ICM-DNA) was used for a targeted analysis of visually detected, highly abnormal-like neoplastic nuclei (nuclear DNA content exceeding four sets of 23 chromosomes). Due to technical limitations in resolution, no more than three clearly separate tumor DNA ploidy categories were established. Tumor DNA ploidy did not correlate with the established histopathological malignancy grade of astrocytomas. Nor did the cell proliferation activity differ between the established DNA ploidy categories. These findings support the results present by Salmon *et al.* (1992), but they contradict those by Yoshii *et al.* (1995). The ICM-DNA, however, was the only method that provided significant prognostic information about Grade II astrocytomas in this study. An elevated number of DNA aneuploid cells in Grade II tumors was closely associated with an unusually aggressive clinical outcome, which indicated that Grade II astrocytomas present genotypic variation of potential prognostic significance. Salmon *et al.* (1992) and later in Decaestecker *et al.* (1998), utilizing quite a different approach from that presented in this study, demonstrated that such gross total genotypic variation in astrocytic tumors could be of assistance in the identification of aggressively behaving tumors, regardless of the established histopathological malignancy grade. Thus, it seems that the prognostic evaluation of low-malignancy astrocytomas especially will need to be focused on the evolved aberrations of the genome. It is evident that new techniques of molecular biology and cytogenetics will provide more accurate information about these genetic changes than the ICM-DNA.

3. Tumor Growth by Telomerase Activity

Normal astrocytes only rarely proliferate. Thus, upregulated proliferation, according to the telomeric hypothesis (Harley *et al.* 1992), could result in early replicative senescence of these cells. In this study, glioblastomas especially showed very intense cell proliferation activity, which indicates active mechanisms in sustaining the proliferation capacity. Telomerase enzyme activity was detected in the majority of the analyzed glioblastomas, which is in line with the 53-100% estimates presented in the literature (Langford *et al.* 1995, Nakatani *et al.* 1997, DeMasters *et al.* 1997, Le *et al.* 1998, Kleinschmidt-DeMasters *et al.* 1998). In addition, the expression of telomerase RNA (TmR) was significantly increased in anaplastic astrocytomas and glioblastomas. The TmR expression was observed to follow the distribution pattern of cell proliferation activity in these high-malignancy astrocytomas, and it correlated closely with the Ki-67(MIB-1)-LIs. These findings lend support to the speculation that telomerase enzyme activation may have an important role in continued cell proliferation of neoplastic cells (Kim *et al.* 1994, Kipling 1995). The upregulated TmR expression could signal an ongoing production of telomerase. Such a capacity for telomerase activation was also observed in this study in some low-malignancy astrocytomas, characterized by either relatively

high TmR expression or telomerase activity. Le *et al.* (1998) reported that some Grade II astrocytomas show telomerase activity which becomes more frequent in high-malignancy lesions. It is possible that the active telomerase could represent one progression pathway of astrocytomas, which does not necessarily require major changes in the genome as indicated by the present results of the similar TmR expression patterns in different tumor DNA ploidy categories (ICM-DNA). The lack of detectable telomerase activity in some glioblastomas could be explained by the lack of sufficient sensitivity of the analysis protocol in heterogeneous tumor tissue, as suggested by Le *et al.* 1998, Kleinschmidt-DeMasters *et al.* 1998.

4. Malignancy Grade of Astrocytomas

A new diagnostic or prognostic factor is usually compared with the conventional prognosticators to determine whether it has independent prognostic value or if it is to be used along with the conventional factors. This may result in less attention being paid to the traditional prognostic evaluation of tumors. In this study, histopathological features, closely related to the established grading criteria of astrocytomas, were inspected in a quantitative perspective. Each histological estimate (mean nuclear size, mitosis count, area fraction of endothelial cells and tumor necrosis) provided statistically significant prognostic information about astrocytomas (Table XI). The association with survival was, however, often derived from the pronounced difference in estimates between Grade II astrocytomas, characterized in general by relatively good survival, and glioblastomas characterized by poor survival. Interestingly, both the mitotic activity, the traditional cell proliferation estimate and a feature of high-grade astrocytoma malignancy, and tumor necrosis, formerly the pivotal criterion of glioblastomas, were superior to the Ki-67(MIB-1)-LI according to the ROC curve (Table XI).

Giannini *et al.* (1999) questioned the assignment of anaplastic astrocytoma diagnosis on the basis of a "solitary detected mitosis", since they found a significant survival difference between anaplastic astrocytoma patients with one observed mitotic figure, as opposed to those patients with Grade III tumors containing many mitoses. Furthermore, the Ki-67(MIB-1)-LIs were found to differ between the tumor groups. According to the results of the present study, the detection of one or two mitotic figures may not, indeed, provide sufficient grounds for a Grade III diagnosis. It is noteworthy, however, that the present results do not take the effect of tumor therapy (on patient survival) into account. In this study, the mitosis counting was done from the most proliferative tumor areas selected under the guidance of Ki-67(MIB-1) immunolabeling. This may have increased the prognostic threshold of mitotic activity by a degree, when compared with the proposed cut-off points in the literature (Fulling and Garcia 1985, Schiffer *et al.* 1988, Cruz-Sánchez *et al.* 1997, Giannini *et al.* 1999). Nevertheless, the Ki-67(MIB-1) method facilitates the search for mitotic figures, and, thus, offers good means for standardizing mitosis counting in astrocytic tumors.

Tumor necrosis was the strongest prognosticator of survival among all the variables tested in this study (Table XI). In addition, tumor necrosis, whether incipient or widely spread, was the only feature that did not eventually require quantitative description. As a typical histological parameter of the high-malignancy astrocytoma group and from the perspective of other study results (Cruz-Sánchez *et al.* 1997, Giannini *et al.* 1999), the role of tumor necrosis as a pivotal histological criterion of glioblastomas should be re-considered.

As both the histological features had independent prognostic value in the multivariate survival analysis of the present study, mitosis counts and tumor necrosis were used in assigning the tumors into three subgroups of a decision-tree model, demonstrated in Figure 7. Twig I of the model included astrocytomas with mitosis counts of less than 3 per 1 mm^2 of analyzed tissue. Twig II was

established on the basis of the detection of mitotic activity equal to or exceeding 3 mitoses per 1 mm^2 of tissue, and Twig III on the basis of the detection of tumor necrosis. The established decision-tree model was superior to the WHO 1979 and prevailing WHO 1993 grading schemes. It is important to mention that both the WHO classification systems have served as universally accepted reference standards (Zülch 1979, Kleihues *et al.* 1993a), with considerable weakness in their capacity to distinguish aggressive behavior in Grade II and anaplastic astrocytomas (Table XII). The tumor subgroups newly established by the decision-tree model did not provide solid improvement in accessing the diagnostic dilemma, since the low-malignancy category (Twig I) still included very heterogeneously behaving astrocytomas. Therefore, a further subdivision of the low-malignancy Twig I was made on the basis of the ICM-DNA, which represents an unspecific means for the identification of genomic variation in tumors. Twigs IA and IB significantly improved the prognostic value of the model (Figure 7).

The patient with an astrocytic tumor comprises one biological entity. The better we could characterize this individuality, the more accurately could we predict the clinical course of the patient and improve therapy. New strategies lead the process toward the truth in diagnosis. For the evaluation of the diagnostic or investigative utility of the new potential prognosticators, it is important to constantly develop the reference standard, here cell proliferation estimation for further characterization of astrocytic tumors as well as the histopathological malignancy grading.

SUMMARY AND CONCLUSIONS

1. Elevated cell proliferation activity, measured either by immunodetection of proliferation related antigens or flow cytometric DNA analysis, correlated significantly with the aggressive behavior of astrocytomas. Of the cell proliferation markers, the Ki-67(MIB-1) method showed the best prognostic sensitivity and specificity in placing the patients into subgroups of relatively good and poor clinical outcome (2-year follow-up).

2. Objectivity and reproducibility of immunohistochemical cell proliferation estimates improved significantly through computer-aided image analysis. Such standardization of the analysis technique provides a basis for an accurate and reliable use of quantitative cell proliferation measurements in brain tumor pathology.

3. The telomeric hypothesis proposes that normal cells have a varying but limited proliferation capacity. Glioblastomas especially grow rapidly and very high cell proliferation estimates were observed in the majority of the tumors. This could indicate an active role of the telomerase enzyme in sustaining the cell proliferation capacity of glioblastomas. The close association of cell proliferation activity with TmR expression could also indicate that an increased cell proliferation activity triggers telomerase activation by mechanisms involving an upregulated telomerase RNA component, even at early stages of the progression pathway.

4. The subdivision of astrocytomas by quantitatively assessed mitosis counts and dichotomously by tumor necrosis provided a better identification of aggressive tumor behavior than achieved by either the WHO 1979 or WHO 1993 grading schemes. For standardizing the mitosis counting in grading of the tumors, the Ki-67(MIB-1) immunohistochemistry was used as a guide to the most intensively proliferating tumor regions. Some low-malignancy astrocytomas followed an aggressive clinical course that was difficult to predict by aberrations in the phenotype. Rather, it seems that these tumors present genotypic variation, the identification of which may play an important role in the prognostic characterization of these tumors in the future.

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REFERENCES

Ackerman (1996): Surgical Pathology. 8th edn. St. Louis, Missouri: Mosby - Year Book.

Alanen KA, Joensuu H, Klemi PJ (1989): Autolysis is a potential source of false aneuploid peaks in flow cytometric DNA histograms. Cytometry 10:417-425.

Allegranza A, Girlando S, Arrigoni GL, Veronese S, Mauri FA, Gambacorta M, Pollo B, Dalla Palma P, Barbareschi M (1991): Proliferating cell nuclear antigen expression in central nervous system neoplasms. Virchows Arch A Pathol Anat Histopathol 419:417-423.

Atkin N (1970): Principles and application of the Deeley integrating microdensitometer. In: Introduction to quantitative cytochemistry. Vol 2, New York and Sonder Academic Press pp. 1-26.

Auer G, Askensten U, Ahrens O (1989): Cytophotometry. Hum Pathol 20:518-527.

Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S, Greider CW (1996): Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res 56:645-650.

Baak JPA and Oort J (1983): Obtaining quantitative data. In: Baak JPA and Oort J (eds.). A manual of morphometry in diagnostic pathology. Berlin: Springer-Verlag.

Bacus JW and Grace LJ (1987): Optical microscope system for standardized cell measurements and analyses. Appl Optics 26:3280-3293.

Bailey P and Cushing H (1926): A classification of the tumors of the glioma group on a histogenetic basis with a correlated study of prognosis. Philadelphia: Lippincott.

Barker II FG, Davis RL, Chang SM, Prados MD (1996): Necrosis as a prognostic factor in glioblastoma multiforme. Cancer 77:1161-1166.

Barth RF, Soloway AH, Goodman JH, Gahbauer RA, Gupta N, Blue TE, Yang W, Tjarks W (1999): Boron neutron capture therapy of brain tumors: an emerging therapeutic modality. Neurosurgery 44:433-451.

Baserga R (1981): The cell cycle. New Eng J Med 304:453-459.

Baserga R (1991): Growth regulation of the PCNA gene. J Cell Sci 98:433-436.

Baxt WG (1995): Application of artificial neural networks to clinical medicine. Lancet 346:1135-1138.

Belanich M, Pastor M, Randall T, Guerra D, Kibitel J, Alas L, Li B, Citron M, Wasserman P, White A, Eyre H, Jaeckle K, Schulman S, Rector D, Prados M, Coons S, Shapiro W, Yarosh D (1996): Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. Cancer Res 56:783-788.

Berger MS (1994): Malignant astrocytomas: Surgical aspects. Seminars in Oncology 21:172-185.

Bigner SH and Vogelstein B (1990): Cytogenetics and molecular genetics of malignant gliomas and medulloblastoma. Brain Pathol 1:12-18.

Blackburn EH and Gall JG (1978): A tandemly repeated sequence at the termini of extrachromosomal RNA genes in *Tetrahymena*. J Mol Biol 120:33-53.

Blackburn EH (1991): Structure and function of telomeres. Nature 350:569-573.

Blasco MA, Rizen M, Greider CW, Hanahan D (1996): Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nat Genet 12:200-204.

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987): Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature 326:515-517.

Bravo R and Macdonald-Bravo H (1987): Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle. Association with DNA replication sites. J Cell Biol 105:1549-1554.

Brem H (1990): Polymers to treat brain tumours. Biomaterials 11:699-701.

Brem S, Rozental JM, Moskal JR (1995): What is the etiology of human brain tumors? A report on the First Lebow Conference. Cancer 76:709-713.

Bruno S and Darzynkiewicz Z (1992): Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif 25:31-40.

Burger PC and Vollmer RT (1980): Histologic factors of prognostic significance in the glioblastoma multiforme. Cancer 46:1179-1186.

Burger PC, Vogel FS, Green SB, Strike TA (1985): Glioblastoma multiforme and anaplastic astrocytoma. Pathologic criteria and prognostic implications. Cancer 56:1106-1111.

Burger PC, Shibata T, Kleihues P (1986): The use of the monoclonal antibody Ki-67 in the identification of proliferating cells: application to surgical neuropathology. Am J Surg Pathol 10:611-617.

Burger PC and Green SB (1987): Patient age, histological features, and length of survival in patients with glioblastoma multiforme. Cancer 59:1617-1625.

Burger PC and Kleihues P (1989): Cytologic composition of the untreated glioblastoma with implications for evaluation of needle biopsies. Cancer 63:2014-2023.

Burger PC, Scheitauer BW, Vogel FS (1991): Surgical pathology of the nervous system and its coverings. 3rd edn. New York: Churchill Livingstone.

Burke HB, Goodman PH, Rosen DB, Henson DE, Weinstein JN, Harrell FE, Marks JR, Winchester DP, Bostwick DG (1997): Artificial neural networks improve the accuracy of cancer survival prediction. Cancer 79:857-862.

Caspersson T (1987): History of the development of cytophotometry from 1935 to the present. Analyt Quant Cytol Histol 9:2-6.

Castello MA, Schiavetti A, Varrasso G, Clerico A, Cappelli C (1998): Chemotherapy in low-grade astrocytoma management. Childs Nerv Syst 14:6-9.

Cattoretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J (1992): Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB-1 and MIB-3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168:357-363.

Celis JE and Madsen P (1986): Increased nuclear cyclin/PCNA antigen staining of non S-phase transformed human amnion cells engaged in nucleotide excision DNA repair. FEBS Lett 209:277-283.

Chandler KL, Prados MD, Malec M, Wilson CB (1993): Long-term survival in patients with glioblastoma multiforme. Neurosurgery 32:716-720.

Cohadon F, Aouad N, Rougier A, Vital C, Rivel J, Dartigues JF (1985): Histologic and non-histologic factors correlated with survival time in supratentorial astrocytic tumors. J Neurooncol 3:105-111.

Cohen C (1996): Image cytometric analysis in pathology. Hum Pathol 27:482-493.

Collan Y, Torkkeli T, Kosma V-M, Pesonen E, Kosunen O, Jantunen E (1987): Sampling in diagnostic morphometry. The influence of variation sources. Path Res Pract 182:401-406.

Coons S, Davis J, Way D (1988): Correlation of DNA content and histology in prognosis of astrocytomas. Am J Clin Pathol 90:289-293.

Coons S and Johnson P (1993a): Regional heterogeneity in the DNA content of human gliomas. Cancer 72:3052-3060.

Coons SW and Johnson PC (1993b): Regional heterogeneity in the proliferative activity of human gliomas as measured by the Ki-67 labeling index. J Neuropathol Exp Neurol 52:609-618.

Coons S, Johnson P, Pearl D (1994): Prognostic significance of flow cytometry deoxyribonucleic acid analysis of human astrocytomas. Neurosurgery 35:119-126.

Coons SW, Johnson PC, Scheithauer BW, Yates, AJ, Pearl DK (1997): Improving diagnostic accuracy and interobserver concordance in the classification and grading of primary gliomas. Cancer 79:1381-1393.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S (1992): Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 11:1921-1929.

Crocker J, McCartney JC, Smith PJ (1988): Correlation between DNA flow cytometric and nucleolar region data in non-Hodgkin's lymphoma. J Pathol 154:151-160.

Cross SS, Harrison RF, Kennedy RL (1995): Introduction to neural networks. Lancet 346:1075-1079.

Cruz-Sánchez FF, Ferreres JC, Figols J, Palacin A, Cardesa A, Rossi ML, Val-Bernal JF (1997): Prognostic analysis of astrocytic gliomas correlating histological parameters with the proliferating cell nuclear antigen labelling index (PCNA-LI). Histol Histopathol 12:43-49.

Culver KW and Blaese RM (1994): Gene therapy for cancer. TIG 10:174-178.

Cunningham JM, Kimmel DW, Scheithauer BW, O'Fallon JR, Novotny PJ, Jenkins RB (1997): Analysis of proliferation markers and p53 expression in gliomas of astrocytic origin: relationships and prognostic value. J Neurosurg 86:121-130.

Danova M, Riccardi A, Mazzini G, Ucci G, Gaetani P, Silvani V, Knerich R, Butti G, Ascari E (1987): Ploidy and proliferative activity of human brain tumors. A flow cytofluorometric study. Oncology 44:102-107.

Danova M, Giaretti W, Merlo F, Mazzini G, Gaetani P, Geido E, Gentile S, Butti G, Di Vinci A, Riccardi A (1991): Prognostic significance of nuclear DNA content in human neuroepithelial tumors. Int J Cancer 48:663-667.

Daumas-Duport C, Scheithauer B, O'Fallon J, Kelly P (1988): Grading of astrocytomas. A simple and reproducible method. Cancer 62:2152-2165.

Decaestecker C, Lopes BS, Gordower L, Camby I, Cras P, Martin JJ, Kiss R, VandenBerg SR, Salmon I (1997): Quantitative chromatin pattern description in Feulgen-stained nuclei as a diagnostic tool to characterize the oligodendroglial and astroglial components in mixed oligo-astrocytomas. J Neuropathol Exp Neurol 56:391-402.

Decaestecker C, Camby I, Nagy N, Brotchi J, Kiss R, Salmon I (1998): Improving morphology-based malignancy grading schemes in astrocytic tumors by means of computer-assisted techniques. Brain Pathol 8:29-38.

Deckert M, Reifenberger G, Wechsler W (1989): Determination of the proliferative potential of human brain tumors using the monoclonal antibody Ki-67. J Cancer Res Clin Oncol 115:179-188.

DeMasters BK, Markham N, Lillehei KO, Shroyer KR (1997): Differential telomerase expression in human primary intracranial tumors. Am J Clin Pathol 107:548-554.

Dirks PB and Rutka JT (1997): Current concepts in neuro-oncology. The cell cycle - a review. Neurosurgery 40:1000-1015.

Donhuijsen K (1986): Mitosis counts: Reproducibility and significance in grading of malignancy. Hum Pathol 17:1122-1125.

Donhuijsen K, Schmidt U, Hirche H, van Beuningen D, Budach V (1990): Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol 21:709-714.

Ellison DW, Steart PV, Bateman AC, Pickering RM, Palmer JD, Weller O (1995): Prognostic indicators in a spectrum of astrocytic tumours: an immunohistochemical study with Ki-67 and p53 antibodies. J Neurol Neurosurg Psych 59:413-419.

Erler BS, Chein K, Marchevsky AM (1993): An image analysis workstation for the pathology laboratory. Mod Pathol 6:612-618.

Falini B, Flenghi L, Fagioli M, Stein H, Schwarting R, Riccardi C, Manocchio I, Pileri S, Pelicci PG, Lanfrancombe L (1989): Evolutionary conservation in various mammalian species of the proliferation associated epitope recognized by the Ki-67 monoclonal antibody. J Histochem Cytochem 37:1471-1478.

Feng J, Funk WD, Wang S-S, Weinrich SL, Avilion AA, Chiu C-P, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW, Villeponteau B (1995): The RNA component of human telomerase. Science 269:1236-1241.

Feulgen R and Rossenbeck H (1924): Mikroskopisch-chemischer Nachweis einer Nukleinsäure vom Typus der Thymonukleinsäure und die darauf beruhende elektive Färbung von Zellkernen in mikroskopischen Präparaten. Physiol Chem 135:203.

Figge C, Reifenberger G, Vogeley KT, Messing M, Roosen N, Wechsler W (1992): Immunohistochemical demonstration of proliferating cell nuclear antigen in glioblastomas: pronounced heterogeneity and lack of prognostic significance. J Cancer Res Clin Oncol 118:289-295.

Finnish Cancer Registry (1996): Cancer incidence in Finland 1994. Helsinki.

Fonatsch C, Duchrow M, Rieder H, Schlüter C, Gerdes J (1991): Assignment of the human Ki-67 gene (MKI67) to 10q25ter. Genomics 11:476-477.

Fujimaki T, Matsutani M, Nakamura O, Asai A, Funada N, Koike M, Segawa H, Aritake K, Fukushima T, Houjo S, Tamura A, Sano K (1991): Correlation between bromodeoxyuridine-labeling and patient prognosis in cerebral astrocytic tumors of adults. Cancer 67:1629-1634.

Fulling KH and Garcia DM (1985): Anaplastic astrocytoma of the adult cerebrum: Prognostic value of histologic features. Cancer 55:928-931.

Ganju V, Jenkins R, O'Fallon J, Scheithauer B, Ransom D, Katzmann J, Kimmel D (1994): Prognostic factors in gliomas. A multivariate analysis of clinical, pathologic, flow cytometric, cytogenetic, and molecular markers. Cancer 74:920-927.

Gelb AB, Kamel OW, LeBrun DP, Warnke RA (1992): Estimation of tumor growth fractions in archival formalin-fixed, paraffin-embedded tissues using two anti-PCNA/cyclin monoclonal antibodies. Am J Pathol 141:1453-1458.

Gerdes J, Schwab U, Lemke H, Stein H (1983): Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13-20.

Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H (1984): Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133:1710-1715.

Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad H-D (1991): Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 138:867-873.

Giannini C, Scheithauer BW, Burger PC, Christensen MR, Wollan PC, Sebo TJ, Forsyth PA, Hayostek CJ (1999): Cellular proliferation in pilocytic and diffuse astrocytomas. J Neuropathol Exp Neurol 58:46-53.

Graem N and Helweg-Larsen K (1979): Mitotic activity and delay in fixation of tumor tissue. Acta Pathol Microbiol Scand [A] 87:375-378.

Gratzner HG (1982): Monoclonal antibody to 5-bromo and 5-iodo-deoxyuridine; a new reagent for detection of DNA replication. Science 218:474-475.

Gray JW, Lucas J, Kallioniemi O, Kallioniemi A, Kuo WL, Straume T, Tkachuk D, Tenjin T, Weier HU, Pinkel D (1991): Applications of fluorescence in situ hybridization in biological dosimetry and detection of disease-specific chromosome aberrations. Prog Clin Biol Res 372:399-411.

Greider CW and Blackburn EH (1985): Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. Cell 43:405-413.

Greider CW and Blackburn EH (1989): A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 337:331-337.

Haapasalo H, Collan Y, Pesonen E (1989): Volume corrected mitotic index (M/V-index) - the standard of mitotic activity in neoplasms. Path Res Pract 185:551-554.

Haas-Kogan DA, Kogan SS, Yount G, Hsu J, Haas M, Deen DF, Israel MA (1999): p53 function influences the effect of fractionated radiotherapy on glioblastoma tumors. In J Radiat Oncol Biol Phys 43:399-403.

Hakumäki JM, Poptani H, Puumalainen AM, Loimas S, Paljärvi LA, Ylä-Herttuala S, Kauppinen RA (1998): Quantitative 1H nuclear magnetic resonance diffusion spectroscopy of BT4C rat

glioma during thymidine kinase-mediated gene therapy in vivo: identification of apoptotic response. Cancer Res 58:3791-3799.

Hall PA, Levison DA, Woods AL Yu CC-W, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP (1990): Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162:285-294.

Hall PA and Woods AL (1990): Immunohistochemical markers of cellular proliferation: achievements, problems and prospects. Cell Tissue Kinet 23:505-522.

Hall PA, Coates PJ, Goodlad RA, Hart IR, Lane DP (1994): Proliferating cell nuclear antigen expression in non-cycling cells may be induced by growth factors *in vivo*. Br J Cancer 70:244-247.

Hara A, Hirayama H, Sakai N, Yamada H, Tanaka T, Mori H (1990): Correlation between nucleolar organizer region staining and Ki-67 immunostaining in human gliomas. Surg Neurol 33:320-324.

Hara A, Sakai N, Yamada H, Hirayama H, Tanaka T, Mori H (1991): Nucleolar organizer regions in vascular and neoplastic cells of human gliomas. Neurosurgery 29:211-215.

Harley CB, Futcher AB, Greider CW (1990): Telomeres shorten during aging of human fibroblasts. Nature 345:458-460.

Harley CB (1991): Telomere loss: mitotic clock or genetic time bomb? Mutat Res 256:271-282.

Harley CB, Vaziri H, Counter CM, Allsopp RC (1992): The telomere hypothesis of cellular aging. Exp Gerontol 27:375-382.

Harrison RF, Reynolds GM, Rowlands DC (1993): Immunohistochemical evidence for the expression of proliferating cell nuclear antigen (PCNA) by non-proliferating hepatocytes adjacent to metastatic tumours and in inflammatory conditions. J Pathol 171:115-122.

Hilsenbeck SG and Allred DC (1992): Improved methods of estimating mitotic activity in solid tumors. Hum Pathol 23:601-602.

Hoshino T, Rodriguez LA, Cho KG, Lee KS, Wilson CB, Edwards MSB, Levin VA, Davis RL (1988): Prognostic implications of the proliferative potential of low-grade astrocytomas. J Neurosurg 69:839-842.

Hoshino T, Ahn D, Prados MD, Lamborn K, Wilson CB (1993): Prognostic significance of the proliferative potential of intracranial gliomas measured by bromodeoxyuridine labeling. Int J Cancer 53:550-555.

Hsu DW, Louis DN, Efird JT, Hedley-White ET (1997): Use of MIB-1 (Ki-67) immunoreactivity in differentiating grade II and grade III gliomas. J Neuropathol Exp Neurol 56:857-865.

Huhn SL, Mohapatra G, Bollen A, Lamborn K, Prados MD, Feuerstein BG (1999): Chromosomal abnormalities in glioblastoma multiforme by comparative genomic hybridization: correlation with radiation treatment outcome. Clin Cancer Res 5:1435-1443.

Ito S, Chandler KL, Prados MD, Lamborn K, Wynne J, Malec MK, Wilson CB, Davis RL, Hoshino T (1994): Proliferative potential and prognostic evaluation of low-grade astrocytomas. J Neurooncol 19:1-9.

Jaros E, Perry RH, Adam L, Kelly PJ, Crawford PJ, Kalbag RM, Mendelow AD, Sengupta RP, Pearson ADJ (1992): Prognostic implications of p53 protein, epidermal growth factor receptor, and Ki-67 labelling in brain tumours. Br J Cancer 66:373-385.

Jaskulski D, deRiel JK, Mercer WE, Calabretta B, Baserga R (1988a): Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. Science 240:1544-1546.

Jaskulski D, Gatti C, Travali S, Calabretta B, Baserga R (1988b): Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. J Biol Chem 263:10175-10179.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldeman F, Pinkel D (1992): Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818-821.

Karnofsky DA and Burchmal JH (1949): The clinical evaluation of chemotherapeutic agents in cancer. In: MacLeod CM (ed) Evaluation of chemotherapeutic agents, Columbia Univ Press 191-205.

Karpati G, Lochmüller H, Nalbantoglu J, Durham H (1996): The principles of gene therapy for the nervous system. Trends Neurosci 19:49-54.

Kaye AH and Laws Jr ER (1995): Brain tumors. An encyclopedic approach. Edinburgh, Hong Kong, London, Madrid, Melbourne, New York and Tokyo: Churchill Livingstone.

Kernohan JW and Sayre GP (1952): Atlas of tumor pathology: Tumors of the central nervous system. Washington: Armed Forces Institute of Pathology.

Key G, Becker MHG, Baron B, Duchrow M, Schlüter C, Flad H-D, Gerdes J (1993a): New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bacterially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. Lab Invest 68:629-636.

Key G, Petersen L, Becker MHG, Duchrow M, Schlüter C, Askaa J, Gerdes J (1993b): New antiserum against Ki-67 antigen suitable for double immunostaining of paraffin wax sections. J Clin Pathol 46:1080-1084.

Khalid H, Shibata S, Kishikawa M, Yasunaga A, Iseki M, Hiura T (1997): Immunohistochemical analysis of progesterone receptor and Ki-67 labeling index in astrocytic tumors. Cancer 80:2133-2140.

Kim DK, Hoyt J, Bacchi C, Keles GE, Mass M, Mayberg MR, Berger MS (1993): Detection of proliferating cell nuclear antigen in gliomas and adjacent resection margins. Neurosurgery 33:619-626.

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

Kipling D (1995): Telomerase: immortality enzyme or oncogene? Nat Genet 9:104-106.

Kiss R, Dewitte O, Decaestecker C, Camby I, Gordower L, Delbeque K, Pasteels J-L, Brotchi J, Salmon I (1997): The combined determination of proliferative activity and cell density in the prognosis of adult patients with supratentorial high-grade astrocytic tumors. Am J Clin Pathol 107:321-331.

Kleihues P, Burger PC, Scheithauer BW (1993a): Histopathological typing of tumours of the central nervous system. Geneva, World Health Organization.

Kleihues P, Burger PC, Scheithauer BW (1993b): The new WHO classification of brain tumors. Brain Pathol 3:255-268.

Kleinschmidt-DeMasters BK, Hashizumi TL, Sze CI, Lillehei KO, Shroyer AL, Shroyer KR (1998): Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling. J Clin Pathol 51:284-293.

Kolles H, von Wangenheim A, Vince GH, Niedermayer I, Feiden W (1995): Automated grading of astrocytomas based on histomorphometric analysis of Ki-67 and Feulgen stained paraffin sections. Classification results of neuronal networks and discriminant analysis. Anal Cell Pathol 8:101-116.

Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP (1998): Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 7:844-847.

Kordek R, Biernat W, Alwasiak J, Liberski PP (1996): Proliferating cell nuclear antigen (PCNA) and Ki-67 immunopositivity in human astrocytic tumors. Acta Neurochir (Wien) 138:509-513.

Koss L, Czerniak B, Herz F, Wersto R (1989): Flow cytometric measurements of DNA and other cell components in human tumors: A critical appraisal. Hum Pathol 20:528-548.

Ku DH, Travali S, Calabretta B, Huebner K, Baserga R (1989): Human gene for proliferating cell nuclear antigen has pseudogenes and localizes to chromosome 20. Somat Cell Mol Genet 15:297-307.

Labrousse F, Daumas-Duport C, Batroski L, Hoshino T (1991): Histological grading and bromodeoxyuridine labeling index of astrocytomas. Comparative study in a series of 60 cases. J Neurosurg 75:202-205.

Langford LA, Piatyszek MA, Xu R, Schold SC Jr, Shay JW (1995): Telomerase activity in human brain tumors. Lancet 346:1267-1268.

Laws ER Jr, Taylor WF, Clifton MB, Okazaki H (1984): Neurosurgical management of low-grade astrocytoma of the cerebral hemispheres. J Neurosurg 61:665-673.

Le S, Zhu JJ, Anthony DC, Greider CW, Black PM (1998): Telomerase activity in human gliomas. Neurosurgery 42:1120-1125.

Levin VA, Silver P, Hannigan J, Wara WM, Gutin PH, Davis RL, Wilson CB (1990): Superiority of post-radiotherapy adjuvant chemotherapy with CCNU, procarbazine, and vincristine (PCV) over BCNU for anaplastic gliomas: NCOG 6G61 final report. Int J Rad Oncol Biol Physics 18:321-324.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R (1997): PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943-1947.

Lopez F, Belloc F, Lacombe F, Dumain P, Reiffers J, Bernard P, Boisseau MR (1994): The labeling of proliferating cells by Ki67 and MIB-1 antibodies depends on the binding of a nuclear protein to the DNA. Exp Cell Res 210:145-153.

Louis DN, Edgerton S, Thor AD, Hedley-White ET (1991): Proliferating cell nuclear antigen and Ki-67 immunohistochemistry in brain tumors: a comparative study. Acta Neuropathol 81:675-679.

Louis DN and von Deimling A (1995): Hereditary tumor syndromes of the nervous system: Overview and rare syndromes. Brain Pathol 5:145-151.

Louis DN (1997): A molecular genetic model of astrocytoma histopathology. Brain Pathol 7:755-764.

Maier H, Morimura T, Öfner D, Hallbrucker C, Kitz K, Budka H (1990): Argyrophilic nucleolar organizer region proteins (AgNORs) in human brain tumors: relations with grade of malignancy and proliferation indices. Acta Neuropathol 80:156-162.

Manuelidis L (1994): Genomic stability and instability in different neuroepithelial tumors. A role for chromosome structure? J Neurooncol 18:225-239.

Martin H and Voss K (1982): Automated image analysis of glioblastomas and other gliomas. Acta Neuropathol (Berl) 58:9-16.

McClintock B (1941): The stability of broken ends of chromosomes in *Zea mays*. Genetics 41:234-282.

McCormack BM, Miller DC, Budzilovich, Voorhees GJ, Ransohoff J (1992): Treatment and survival of low-grade astrocytoma in adults - 1977-1988. Neurosurgery 31:636-642.

McCormick D, Yu C, Hobbs C, Hall PA (1993): The relevance of antibody concentration on the immunohistological quantification of cell proliferation-associated antigens. Histopathology 22:543-547.

McKeever PE, Strawderman MS, Yamini B, Mikhail AA, Blaivas M (1998): MIB-1 proliferation index predicts survival among patients with grade II astrocytoma. J Neuropathol Exp Neurol 57:931-936.

McKeown MJ and Ramsay DA (1996): Classification of astrocytomas and malignant astrocytomas by principal components analysis and a neural net. J Neuropathol Exp Neurol 55:1238-1245.

Meyerson M, Counter CM, Ng Eaton E, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, Weinberg RA (1997): hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 90:785-795.

Miyachi K, Fritzler MJ, Tan EM (1978): Autoantibody to a nuclear antigen in proliferating cells. J Immunol 121:2228-2234.

Miyake T, Okada M, Kitamura T (1992): Reactive proliferation of astrocytes studied by immunohistochemistry for proliferating cell nuclear antigen. Brain Research 590:300-302.
Montine TJ, Vandersteenhoven JJ, Aguzzi A, Boyko OB, Dodge RK, Kerns B-J, Burger PC (1994): Prognostic significance of Ki-67 proliferation index in supratentorial fibrillary astrocytic neoplasms. Neurosurgery 34:674-679.
Montironi R, Collan Y, Scarpelli M, Sisti S, Barbatelli G, Carnevali A, Pisani E, Mariuzzi GM (1988): Reproducibility of mitotic counts and identification of mitotic figures in malignant glial tumors. Appl Pathol 6:258-265.
Morimura T, Kitz K, Budka H (1989): In situ analysis of cell kinetics in human brain tumors. A comparative immunocytochemical study of S phase cells by a new in vitro bromodeoxyuridine-labeling technique, and of proliferating pool cells by monoclonal antibody Ki-67. Acta Neuropathol (Berl) 77:276-282.
Morin GB (1989): The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59:521-529.
Morin GB (1995): Is telomerase a universal cancer target. J Natl Cancer Inst 87:859-861.
Morris GF and Mathews MB (1989): Regulation of proliferating cell nuclear antigen during the cell cycle. J Cell Biol 264:13856-13864.
Morstyn G, Hsu SM, Kinsella T, Gratzner A, Russo A, Mitchell JB (1983): Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. J Clin Invest 72:1844-1850.
Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu J-R (1988): A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci USA 85:6622-6626.
Munakata S and Hendricks JB (1993): Effect of fixation time and microwave oven heating time on retrieval of the Ki-67 antigen from paraffin-embedded tissue. J Histochem Cytochem 41:1241-1246.
Müller HJ (1938): The remaking of chromosomes. The collecting net. Woods Hole 13:181-198.
Nakamura M, Konishi N, Tsunoda S, Hiasa Y, Tsuzuki T, Inui T, Sakaki T (1997a): Retinoblastoma protein expression and MIB-1 correlate with survival of patients with malignant astrocytoma. Cancer 80:242-249.
Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB, Cech TR (1997b): Telomerase catalytic subunit homologs from fission yeast and human. Science 277:955-959.
Nakatani K, Yoshimi N, Mori H, Yoshimura S, Sakai H, Shinoda J, Sakai N (1997): The significant role of telomerase activity in human brain tumors. Cancer 80:471-476.
Newcomb EW, Cohen H, Lee SR, Bhalla SK, Bloom J, Hayes RL, Miller DC (1998): Survival of patients with glioblastoma multiforme is not influenced by altered expression of p16, p53, EGFR, MDM2 or Bcl-2 genes. Brain Pathol 8:655-667.
Nishizaki T, Orita T, Furutani Y, Ikeyama Y, Aoki H, Sasaki K (1989): Flow-cytometric DNA analysis and immunohistochemical measurement of Ki-67 and BUdR labeling indices in human brain tumors. J Neurosurg 70:379-384.
Ogata K, Kurki P, Celis JE, Nakamura RM, Tan EM (1987): Monoclonal antibodies to a nuclear protein (PCNA/Cyclin) associated with DNA replication. Exp Cell Res 168:475-486.
O'Leary TJ and Steffes MW (1996): Can you count on the mitotic index? Hum Pathol 27:147-151.
Olovnikov AM (1973): A theory of marginotomy. The complete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol 41:181-190.
Onda K, Davis RL, Shibuya M, Wilson CB, Hoshino T (1994): Correlation between the bromodeoxyuridine labeling index and the MIB-1 and Ki-67 proliferating cell indices in cerebral gliomas. Cancer 74:1921-1926.
Ono Y, Tamiya T, Ichikawa T, Kunishio K, Matsumoto K, Furuta T, Ohmoto T, Ueki K, Louis DN (1996): Malignant astrocytomas with homozygous CDKN2/p16 gene deletions have higher Ki-67 proliferation indices. J Neuropathol Exp Neurol 55:1026-1031.

O'Reilly SM, Newlands ES, Glaser MG, Brampton M, Rice-Edwards JM, Illingworth RD, Richards PG, Kennard C, Colquhoun IR, Lewis P, Stevens MFG (1993): Temozolomide: A new oral cytotoxic chemotherapeutic agent with promising activity against primary brain tumours. Eur J Cancer 29A:940-942.

Packer RJ, Nicholson S, Vezina LG, Johnson DL (1992): Brain stem gliomas. Pediatr Neurooncol 3:863-879.

Paetau A (1989): Intermediate filaments and fibronectin in cells of glial origin. Academic dissertation. Helsinki.

Patsouris E, Stocker U, Kallmeyer V, Keiditsch E, Mehraein P, Stavrou D (1988): Relationship between Ki-67 positive cells, growth rate and histological type of human intracranial tumors. Anticancer Res 8:537-544.

Philippon JH, Clemenceau SH, Fauchon FH, Foncin JF (1993): Supratentorial low-grade astrocytomas in adults. Neurosurgery 32:554-559.

Pigott TJD, Lowe JS, Palmer J (1991): Statistical modeling in analysis of prognosis in glioblastoma multiforme: A study of clinical variables and Ki-67 index. Br J Neurosurg 5:61-66.

Pileri SA, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, Ascani S, Santini D, Piccaluga PP, Leone O, Damiani S, Ercolessi C, Sandri F, Pieri F, Leoncini L, Falini B (1997): Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol 183:116-123.

Pollack IF, Claassen D, al-Shboul Q, Janosky JE, Deutsch M (1995): Low-grade gliomas of the cerebral hemispheres in children: an analysis of 71 cases. J Neurosurg 82:536-547.

Pollack IF, Cambell JW, Hamilton RL, Martinez AJ, Bozik ME (1997): Proliferation index as a predictor of prognosis in malignant gliomas of childhood. Cancer 79:849-856.

Prayson RA and Estes ML (1996): MIB1 and p53 immunoreactivity in protoplasmic astrocytomas. Pathol Int 46:862-866.

Prelich G, Tan C-K, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987): Functional identity of proliferating cell nuclear antigen and DNA polymerase delta auxiliary protein. Nature 326:517-520.

Raghavan R, Steart PV, Weller RO (1990): Cell proliferation patterns in the diagnosis of astrocytomas, anaplastic astrocytomas and glioblastoma multiforme: a Ki-67 study. Neuropathol Appl Neurobiol 16:123-133.

Revesz T, Alsanjari N, Darling JL, Scaravilli F, Lane DP, Thomas DGT (1993): Proliferating cell nuclear antigen (PCNA): expression in samples of human astrocytic gliomas. Neuropathol Appl Neurobiol 19:152-158.

Rock JP, Rosenblum ML, Shaw EG, Cairncross JG (1999): The practical management of low-grade primary brain tumors. Philadelphia: Lippincott Williams & Wilkins.

Rose DSC, Maddox PH, Brown DC (1994): Which proliferation markers for routine immunohistochemistry? A comparison of five antibodies. J Clin Pathol 47:1010-1014.

Sachsenheimer W, Piotrowsky W, Bimmler T (1992): Quality of life in patients with intracranial tumors on the basis of Karnofsky's performance status. J Neuro-Oncol 13:177-181.

Salmon I, Kiss R, Dewitte O, Gras T, Pasteels J-L, Brotchi J, Flament-Durand J (1992): Histopathologic grading and DNA ploidy in relation to survival among 206 adult astrocytic tumor patients. Cancer 70:538-546.

Salmon I and Kiss R (1993): Relationship between proliferative activity and ploidy level in a series of 530 human brain tumors, including astrocytomas, meningiomas, schwannomas, and metastases. Hum Pathol 24:329-335.

Sasaki A, Naganuma H, Kimura R, Isoe S, Nakano S, Nukui H, Suzuki K, Kawaoi A (1992): Proliferating cell nuclear antigen (PCNA) immunostaining as an alternative to bromodeoxyuridine (BrdU) immunostaining for brain tumors in paraffin embedded sections. Acta Neurochir (Wien) 117:178-181.

Scarpelli M, Bartels PH, Montironi R, Galluzzi CM, Thompson D (1994): Morphometrically assisted grading of astrocytomas. Analyt Quant Cytol Histol 16:351-356.

Scheck AC, Mehta BM, Beikman MK, Shapiro JR (1993): BCNU-resistant human glioma cells with over-representation of chromosomes 7 and 22 demonstrate increased copy number and expression of platelet-derived growth factor genes. Genes Chromosomes Cancer 8:137-148.

Schena M, Shalon D, Davis RW, Brown PO (1995): Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467-470.

Schiffer D, Giordana MT, Germano I, Mauro A (1986): Anaplasia and heterogeneity of GFAP expression in gliomas. Tumori 72:163-170.

Schiffer D, Chiò A, Giordana MT, Leone M, Soffietti R (1988): Prognostic value of histological factors in adult cerebral astrocytoma. Cancer 61:1386-1393.

Schiffer D, Chiò A, Giordana MT, Pezzulo T, Vigliani MC (1993): Proliferating cell nuclear antigen expression in brain tumors, and its prognostic role in ependymomas: an immunohistochemical study. Acta Neuropathol 85:495-502.

Schiffer D, Cavalla P, Chio A, Richiardi P, Giordana MT (1997): Proliferative activity and prognosis of low-grade astrocytomas. J Neurooncol 34:31-35.

Schlüter C, Duchrow M, Wohlenberg C, Becker MHG, Key G, Flad H-D, Gerdes J (1993): The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle maintaining proteins. J Cell Biol 123:513-522.

Schröder R, Bien K, Kott R, Meyers I, Vössing R (1991): The relationship between Ki-67 labeling and mitotic index in gliomas and meningiomas: demonstration of the variability of the intermitotic cycle time. Acta Neuropathol 82:389-394.

Scott RJ, Hall PA, Haldane JS, van Noorden S, Price Y, Lane DP, Wright NA (1991): A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. J Pathol 165: 173-178.

Shampay J, Szostak JW, Blackburn EH (1984): DNA sequences of telomeres maintained in yeast. Nature 310:154-157.

Shapiro JR, Pu PY, Mohamed AN, Galicich JH, Ebrahim SA, Shapiro WR (1993): Chromosome number and carmustine sensitivity in human gliomas. Cancer 71:4007-4021.

Shay JW, Pereira-Smith OM, Wright WE (1991): A role of both RB and p53 in the regulation of human cellular senescence. Exp Cell Res 196:33-39.

Shi S-R, Key ME, Kalra KL (1991): Antigen retrieval in formalin-fixed, paraffin-embedded tissue: an enhancing method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741-748.

Shibata T, Burger PC, Kleihues P (1988): Ki-67 immunoperoxidase stain as marker for the histological grading of nervous system tumours. Acta Neurochir Supll (Wien) 43:103-106.

Shibuya M, Ito S, Miwa T, Davis RL, Wilson CB, Hoshino T (1993): Proliferative potential of brain tumors. Analyses with Ki-67 and anti-DNA polymerase alpha monoclonal antibodies, bromodeoxyuridine labeling, and nucleolar organizer region counts. Cancer 71:199-206.

Shivji KK, Kenny MK, Wood RD (1992): Proliferating cell nuclear antigen is required for DNA excision repair. Cell 69:367-374.

Simpson JF, Dutt PL, Page DL (1992): Expression of mitoses per thousand cells and cell density in breast carcinomas: A proposal. Hum Pathol 23:608-611.

Soffietti R, Chio A, Giordana MT, Vasario E, Schiffer D (1989): Prognostic factors in well-differentiated cerebral astrocytomas in the adult. Neurosurgery 24:686-692.

Struikmans H, Rutgers DH, Jansen GH, Tulleken CAF, van der Tweel I, Battermann JJ (1998): Prognostic relevance of cell proliferation markers and DNA-ploidy in gliomas. Acta Neurochir (Wien) 140:140-147.

Subach BR, Witham TF, Kondziolka D, Lunsford LD, Bozik M, Schiff D (1999): Morbidity and survival after 1,3-*bis*(2-chloroethyl)-1-nitrosurea wafer implantation for recurrent glioblastoma: a retrospective case-matched cohort series. Neurosurgery 45:17-23.

Surawicz TS, McCarthy BJ, Kupelian V, Jukich PJ, Bruner JM, Davis FG (1999): Descriptive epidemiology of primary brain and CNS tumors: Results from the Central Brain Tumor Registry of the United States, 1990-1994. Neuro-Oncol [serial online], Doc. 98-13, January 19.

Sørensen FB, Brændgaard H, Chistiansen AO, Bojsen-Møller M, Reske-Nielsen E (1991): Stereological estimates of nuclear volume and other quantitative variables in supratentorial brain tumors. J Neuro-Oncol 10:253-262.

Takahashi T and Caviness VS (1993): PCNA-binding to DNA at the G1/S transition in proliferating cells of the developing cerebral wall. J Neurocytol 22:1096-1102.

Torp SH, Helseth E, Dalen A, Unsgaard G (1992): Relationships between Ki-67 labelling index, amplification of the epidermal growth factor receptor gene, and prognosis in human glioblastomas. Acta Neurochir (Wien) 117:182-186.

Torp SH, Johannesen E, Lindboe CF (1995): Comparison of different Ki-67 antibodies in human glioblastomas. J Clin Pathol 48:M191-M193.

True LD (1996): Morphometric applications in anatomic pathology. Hum Pathol 27:450-467.

Ullén H, Falkmer UG, Collins VP, Auer GU (1991): Methodologic aspects of nuclear DNA assessment of gliomas with astrocytic and/or oligodendrocytic differentiation. Correlation of image and flow cytometric studies on paraffin-embedded specimens. Analyt Quant Cytol Histol 13:168-176.

Wakimoto H, Aoyagi M, Nakayama T, Nagashima G, Yamamoto S, Tamaki M, Hirakawa K (1996): Prognostic significance of Ki-67 labeling indices obtained using MIB-1 monoclonal antibody in patients with supratentorial astrocytomas. Cancer 77:373-380.

Valtonen S, Timonen U, Toivanen P, Kalimo H, Kivipelto L, Heiskanen O, Unsgaard G, Kuurne T (1997): Interstitial chemotherapy with carmustine-loaded polymers for high grade gliomas - a randomized, double-blind study. Neurosurgery 41:44-49.

van Steensel B and de Lange T (1997): Control of telomere length by the human telomeric protein TRF1. Nature 385:740-753.

Vandenberg SR (1992): Current diagnostic concepts of astrocytic tumors. J Neuropathol Exp Neurol 51:644-657.

Waseem NH and Lane DP (1990): Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of the nucleolar form. J Cell Sci 96:121-129.

Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H (1996): Overexpression of the EFG receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol 6:217-224.

Watanabe K, Tachibana O, Yonekawa Y, Kleihues P, Ohgaki H (1997): Role of gemistocytes in astrocytoma progression. Lab Invest 76:277-284.

Vavruch L, Nordenskjöld B, Cartensen J, Eneström S (1996): Prognostic value of flow cytometry and correlation to some conventional prognostic factors: a retrospective study of archival specimens of 134 astrocytomas. J Neurosurg 85:146-151.

Weingart J and Brem H (1992). Biology and therapy of glial tumors. Curr Opin Neurol Neurosurg 5:808-812.

Verheijen R, Kuijpers HJ, van Driel R, Beck JL, van Dierendonck JH, Brakenhoff GJ, Ramaekers FC (1989): Ki-67 detects a nuclear matrix-associated proliferation-related antigen. Part II. Localization in mitotic cells and association with chromosomes. J Cell Sci 92:531-540.

Wersto R, Liblit R, Koss L (1991): Flow cytometric DNA analysis of human solid tumor: A review of the interpretation of DNA histograms. Hum Pathol 22:1085-1098.

Vertosick FT and Selker RG (1992): Long-term survival after the diagnosis of malignant glioma: a series of 22 patients surviving more than 4 years after diagnosis. Surg Neurol 38:359-363.

Whittle IR (1995): Gene therapy for brain tumours. Br J Neurosurg 9:717-720.

Wied GL, Bartels PH, Bibbo M, Dytch HE (1989): Image analysis in quantitative cytopathology and histopathology. Hum Pathol 20:549-573.

von Deimling A, von Ammon K, Schoenfeld D, Wiestler OD, Seizinger BR, Louis DN (1993): Subsets of glioblastoma multiforme defined by molecular genetic analysis. Brain Pathol 3:19-26.

Wood JR, Green SB, Shapiro WR (1988): The prognostic importance of tumor size in malignant gliomas: A computed tomographic scan study by the Brain Tumor Cooperative Group. J Clin Oncol 6:338-343.

Yoshii Y, Saito A, Nose T (1995): Nuclear morphometry and DNA densitometry of human gliomas by image analysis. J Neuro-Oncol 26:1-9.

Yu CC-W and Filipe MI (1993): Update on proliferation-associated antibodies applicable to formalin-fixed paraffin-embedded tissue and their applications. Histochem J 25:843-853.

Zakian VA (1995): Telomeres: beginning to understand the end. Science 270:1601-1607.

Zakian VA (1997): Life and cancer without telomerase. Cell 91:1-3.

Zapryanov Z and Christov K (1988): Histological grading, DNA content, cell proliferation and survival of patients with astroglial tumors. Cytometry 9:380-386.

Zuber P, Hamou MF, de Tribolet N (1988): Identification of proliferating cells in human gliomas using the monoclonal antibody Ki-67. Neurosurgery 22:364-368.

Zülch KJ (1979): Histological typing of tumors of the central nervous system. Geneva, World Health Organization.

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