SATU-LEENA SALLINEN

Genetic Profiling of Astrocytic Tumors

■

ACADEMIC DISSERTATION

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To Pauli and Elias

TABLE OF CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

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 This thesis also includes unpublished data.

I Sallinen S-L, Sallinen P, Haapasalo H, Kononen J, Karhu R, Helén P, Isola J. Accumulation of genetic changes is associated with poor prognosis in Grade II astrocytomas. Am J Pathol 1997; 151:1799-1807

II Sallinen S-L, Sallinen P, Ahlstedt-Soini M, Haapasalo H, Helin H, Isola J, Karhu R. Arm-specific multi-color FISH reveals widespread chromosomal instability in glioma cell lines. Submitted for publication, 2002

III Sallinen S-L, Sallinen PK, Haapasalo HK, Helin HJ, Helén PT, Schramal P, Kallioniemi O-P, Kononen J. Identification of differentially expressed genes in human gliomas by DNA microarray and tissue chip techniques. Cancer Res 2000; 60:6617-6622

IV Sallinen S-L, Sallinen PK, Kononen JT, Syrjäkoski KM, Nupponen NN, Rantala IS, Helén PT, Helin HJ, Haapasalo HK. Cyclin D1 expression in astrocytomas is associated with cell prolifertaion activity and patient prognosis. J Pathol 1999; 188:289-293

ABBREVIATIONS

*arm*FISH	arm-specific multi-color fluorescence *in situ* hybridization
ATCC	American Type Cell Collection
BCNU	bischloroethyl-nitrosourea
CDK4	cyclin dependent kinase 4
CDKN2	cyclin dependent kinase inhibitor 2
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
CNS	central nervous system
CNTF	cilliary neurotrophic factor
Cpm	counts per minute
DAPI	4`, 6-diamino-2-phenylindole
DCC	deleted in colorectal carcinoma
dCTP	deoxycytosinetriphosphatase
DMBT1	deleted in malignant brain tumor 1
dmins	double minutes
DNA	deoxyribonucleic acid
DOP-PCR	degenerate oligonucleotide primed polymerase chain reaction
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF2	fibroblast growth factor 2
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiosyanate
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
H&E	hematoxylin and eosin
IGFBP2	insulin-like growth factor receptor binding protein 2
kDa	kilodalton
Ki-67 (MIB-1)	MIB-1 antibody directed against the Ki-67 antigen
LI	labeling index
LOH	loss of heterozygosity
Mb	megabase pairs
MDM2	murine double minute 2

mFISH	multicolor fluorescence *in situ* hybridization
mRNA	messenger ribonucleic acid
MRI	magnetic resonance imaging
NF2	neurofibromatosis 2
O2A	oligodendrocyte-Type 2 astrocyte
p-arm	short arm of the chromosome
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PTEN	phosphatase and tensin homolog
RB1	retinoblastoma type 1
RNA	ribonucleic acid
SKY	spectral karyotyping
SSC	standard saline citrate
T1A	type-1 astrocyte
T2A	type-2 astrocyte
TGF	transforming growth factor
TP53	tumor protein 53
TMA	tissue microarray
TSG	tumor suppressor gene
VEGF	vascular endothelial growth factor
WHO	the World Health Organization

INTRODUCTION

Gliomas are tumors of the neuroglia. A further subclassification of gliomas distinguishes astrocytomas, oligodendrogliomas, mixed oligo-astrocytomas, ependymomas and choroid plexus tumors on the basis of the cell origin of the tumor. Brain tumors comprise approximately 9% of all human cancers, and in 40% of cases a brain tumor is diagnosed as a glioma (Central Brain Tumor Registry of the U.S. data, Surawicz *et al.* 1999). In Finland, the age-adjusted incidence rates of gliomas are 5.0 for males and 4.1 for females per 100, 000 person years, which amounts to approximately 260 new gliomas annually (Finnish Cancer Registry, 1996). Over the past decades the incidence rates of gliomas have slightly increased, most likely due to improved diagnostic methods (computed tomography, CT, and magnetic resonance imaging, MRI), on one hand, and increased mean age of the population on the other hand. Astrocytoma is the most common type of gliomas.

The etiology of gliomas remains unclear. Different chemotoxic and nutritional agents, such as aspartame (Olney *et al.* 1996), have been suggested to account for some elevation in incidence rates, but so far only radiation has been convincingly implicated in the etiology of gliomas. Therapeutic X-radiation, e.g. prophylactic irradiation of the central nervous system (CNS) of children with acute lymphocytic leukemia (ALL) or irradiation of pituitary adenomas, has been demonstrated to increase the risk of developing gliomas (Edwards *et al.* 1986, Branda *et al.* 1992). Population-based research interest has accordingly focused on the ever increasing use of mobile phones, the effect of which on elevated brain tumor occurrences still remains to be shown. Some gliomas relate to hereditary multi-system disorders associated with specific gene defects. These *hereditary* disorders include neurofibromatosis 1 and 2, tuberous sclerosis, Li-Fraumeni syndrome and Turcot syndrome (Louis and von Diemling 1995). Occasionally, an accumulation of glioma incidences has been aggregated to families without evidence of hereditary multi-system disorders (the so-called *familial* gliomas) (Paunu *et al.* 2002b). In a recent study, a unique low-penetrance chromosome region of 15q23-q26.3 was demonstrated among Finnish glioma families by linkage and association analyses (Paunu *et al.* 2002a). The gene or genes in this chromosome region remain unknown.

The treatment of a glioma largely depends on its histopathological subtype and malignancy grade. However, gliomas may display considerable individuality in clinical behavior within the tumor entities, similar to that of patients with gliomas. New treatment strategies are constantly being developed and tested, but, in order to improve treatment, one needs to develop diagnostic techniques that better distinguish high-risk factors in individual tumors. During the past decade, the knowledge of genetic

aberrations underlying gliomas has increased enormously. Advances made in genetic research techniques together with the ongoing sequencing of the human genome (the Humane Genome Project) have provided convenient new setups for studies of cancer genetics. It has become evident that different types of gliomas ultimately develop and grow along distinct genetic pathways, characteristic of type-specific genetic alterations. Accordingly, any prognostic differences between two gliomas of similar histopathological appearance may reflect genomic variation.

This study is based on the hypothesis that the prognostication of the clinical behavior of a glioma or more specifically astrocytoma would greatly benefit from the genetic analysis of the tumor specimen. The information about genetic alterations in individual astrocytic tumors could, eventually, lead to treatment protocols targeting the cause rather than the effect of tumor growth. Here, new molecular and cytogenetic research tools have been investigated for their value and clinical suitability in the search for genetic aberrations underlying the growth of astrocytic tumors.

REVIEW OF THE LITERATURE

1. Astrocytes and Astrocytic Tumors

Neuroglial cells, i.e. the astrocytes, oligodendrocytes and ependymal cells, form the principal supporting tissue of the CNS (Burger *et al.* 1991). The neuroglia makes up about one half of the brain volume. Today, fibroblast-like type-1 (T1A) and processes bearing, neuron-like type-2 (T2A) astrocytes have been separated *in vitro.* T1As are found predominantly in the gray matter and T2As in the white matter of the brain. Both types of astrocytes express glial fibrillary acidic protein (GFAP) and S-100 markers. In addition, T2As are also positive for A2B5 antibody (reviewed by Holland 2001). In a developing brain, astrocytes migrate and continue to proliferate to form a fine branching network, characterized by numerous dendrite-like processes that connect astrocytes to neighboring neurons and blood vessels (Burger *et al.* 1991). These connections enable astrocytes to take an active part in normal brain metabolism and neuronal activity, as well as in sustaining the blood-brain barrier. The capacity of astrocytes for migration and division under stimuli persists through adult life, which reflects their pivotal role in the repair of tissue damage in the CNS.

It has been postulated that neuroepithelial stem cells are multipotential, and produce various kinds of more restricted precursors that divide a limited number of times before they terminally differentiate into either neurons or glia cells (Figure 1) (Lee *et al.* 2000, Holland 2001). Gliogenesis continues long after neurogenesis (reviewed by Goldman 1998), and astrocyte generation persists throughout life (Altman 1966, Sturrock 1982). Recently, it has been demonstrated *in vitro* that certain extracellular signals can revert oligodendrocyte precursor cells to multipotential neural stem cells which can differentiate yet again into neurons, astrocytes or oligodendrocytes (Kondo and Raff 2000).

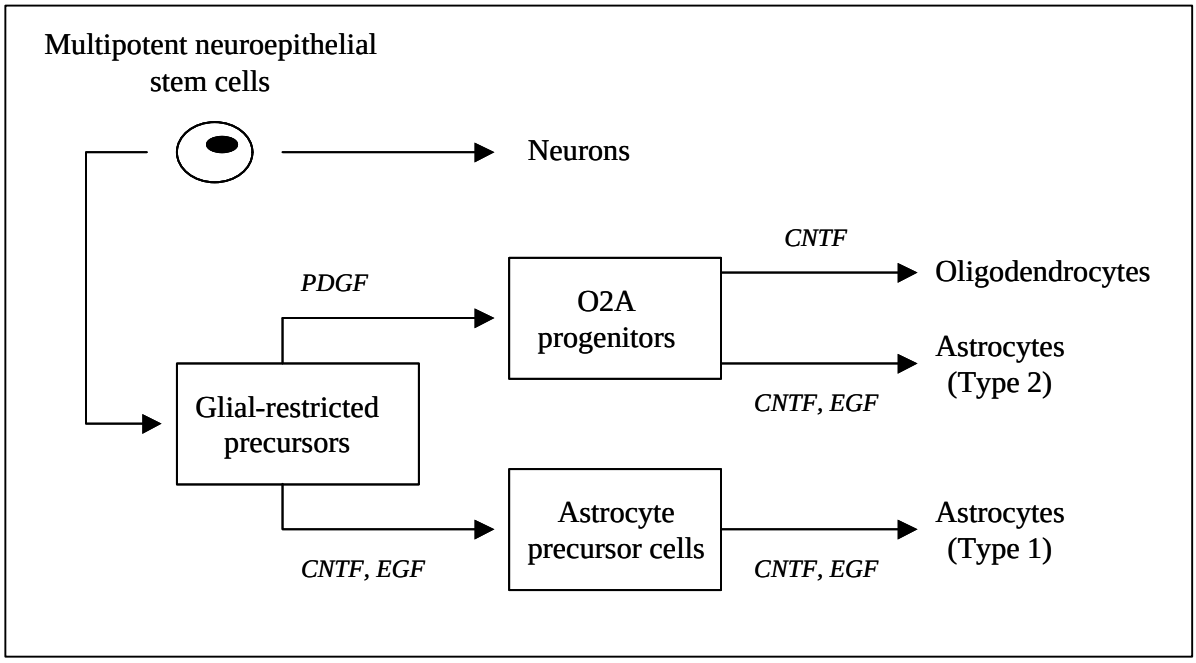

Figure 1. Multipotential neuroepithelial stem cell theory. Multipotential neuroepithelial stem cells differentiate into neurons or glia cells. Glial-restricted precursors give rise to both oligodendroglial progenitors (O2A) and astrocyte precursor cells. In the cell culture platelet-derived growth factor (PDGF) drives cells towards O2A population. Fibroblast growth factor 2 (FGF2) prevents population's further differentiation into mature oligodendrocytes. Withdrawal of PDGF and FGF2 and stimulation by cilliary neurotrophic factor (CNTF) and epidermal growth factor (EGF) in turn drives the cells towards astrocyte and oligodendrocyte differentiation (Lee *et al.* 2000, Holland 2001).

1.1 Tumors of glial origin

Tumors of the neuroglia, gliomas, are the most common type of primary neoplasms of the brain (Burger *et al.* 1991). In light-microscopy, distinct histomorphological features separate them from the other tumor entities established to occupy the brain tissue (Burger *et al.* 1991, Kleihues *et al.* 1993). In addition, various immunohistochemical stainings are in routine use to facilitate the diagnostic differentiation of the tumor type (Kleihues *et al.* 1993). According to the nomenclature presented by the World Health Organization (Kleihues *et al.* 1993, Kleihues *et al.* 2000), gliomas comprise several histological subtypes: astrocytic and oligodendroglial tumors, their mixed variants (oligo-astrocytomas), as well as ependymal and choroid plexus tumors. Considering astrocytic tumors that frequently stain positive for GFAP (Schiffer *et al.* 1986, Paetau 1989), one fundamental subdivision has been made

between diffuse astrocytomas, which grow infiltrating the surrounding brain tissue, and others (namely pilocytic astrocytomas, pleomorphic xanthoastrocytomas and subependymal giant cell astrocytomas) with generally a more circumscribed growth pattern (Table I). Not only does the infiltrative growth behavior of diffuse astrocytomas challenge the therapy of the affected patients, but it also reflects profound differences in the genetic background between diffuse and more circumscribed astrocytic lesions.

1.2 Histopathological malignancy grade of astrocytic tumors

Kernohan and Sayre (1952) proposed that the behavior of astrocytomas could better be predicted by subdividing the tumors further into four malignancy categories, Grades 1-4, on the basis of apparent anaplastic features detectable by microscopic inspection. The World Health Organization (WHO) grading scheme (Kleihues *et al.* 2000) reserves Grade I for pilocytic astrocytomas, typically tumors of the juvenile cerebellum, and subependymal giant cell astrocytomas. Instead, Grades II-IV usually refer to the diffusely infiltrating growth pattern usually found in the cerebral hemispheres of adults.

Grade II astrocytomas are homogenous or cystic tumors, indefinitely bordering on the surrounding normal brain tissue. They present nuclear atypia and pleomorphism, but mitotic figures are very rare. Patients are often under 40 years of age. **Grade III astrocytomas** are cellular tumors. Rapid growth is indicated by apparent mitotic activity that serves as the most important histopathological determinant of high-grade malignancy. Patients are usually over 40 years of age. **The Grade IV astrocytoma, i.e. the glioblastoma multiforme (GBM)**, is the most common and malignant glioma. Pronounced cytological atypia, mitotic activity and proliferating endothelial cells characterize GBMs. In addition, necrosis, densely parenthesized by (pseudopalisading) neoplastic cells, is often present. Patients are typically over 50 years of age (Burger *et al.* 1991, Kleihues *et al*. 2000). Secondary GBMs arise from a previous, less malignant glioma. The prefix "*de novo*" or primary defines a subset of GBMs in patients who do not have a previous glioma history. Clinically, patients with primary GBMs appear to be older than those with secondary Grade IV lesions (Burger and Green 1987).

Table I. Typing of gliomas by the WHO (Kleihues *et al*. 2000).

Tumor Type	**Grade**	**Variants**
1. Astrocytic tumors		
Diffuse astrocytoma	Grade II	fibrillary protoplasmic gemistocytic
Anaplastic astrocytoma	Grade III	
Glioblastoma multiforme	Grade IV	giant cell glioblastoma gliosarcoma
Pilocytic astrocytoma	Grade I	
Pleomorphic xanthoastrocytoma	Grade II	
Subependymal giant cell astrocytoma	Grade I	
2. Oligodendroglial tumors		
Oligodendroglioma	Grade II	
Anaplastic oligodendroglioma	Grade III	
3. Ependymal tumors		
Ependymoma	Grade II	cellular papillary clear cell
Anaplastic ependymoma	Grade III	
Myxopapillary ependymoma	Grade I	
Subependymoma	Grade I	
4. Mixed gliomas		
Oligo-astrocytoma	Grade II	
Anaplastic oligoastrocytoma	Grade III	
Others		
5. Choroid plexus tumors		
Choroid plexus papilloma	Grade II	
Choroid plexus carcinoma	Grade III	

1.3 Treatment and prognosis

The treatment of astrocytic tumors aims at the maximum reduction of the neoplastic tissue that 1) carries a risk of further growth and dedifferentiation and 2) originates neurological deficit. Whereas management plans may vary considerably, a standard treatment recommendation pinpoints the

histopathological verification of the diagnosis as soon as possible. Surgical resection by open craniotomy is the conventional means of obtaining tumor specimens for microscopic inspection. Another option is biopsy, the diagnostic accuracy of which has significantly improved along with the development of brain imaging by MRI, especially (Kaye and Laws Jr 1995, Rock *et al.* 1999).

Grade II astrocytomas are first treated by surgery alone, but the infiltrative growth pattern of the tumors makes surgical approaches difficult to accomplish. Grade II astrocytomas tend to recur and progress into more malignant forms, and approximately 60-80% of the patients survive the first five years after the onset of treatment (Daumas-Duport *et al.* 1988, Philippon *et al.* 1993, Kleihues *et al.* 2000). The benefits of (postoperative) radiation therapy in the treatment of low-grade astrocytomas have yet to be shown (Kaye and Laws Jr 1995). The use of radiotherapy in children has also been relatively controversial due to the maturing brain tissue, which may make the clinical role of chemotherapy significant in postponing the need for tumor irradiation (Castello *et al.* 1998). However, the blood-brain barrier challenges the systemic administering of therapeutic agents.

High-grade astrocytomas grow fast and infiltrate aggressively into the surrounding brain tissue (Burger *et al.* 1991). Therefore, postoperative radiation therapy with total tumor dose of 60 Gy is usually part of the management of Grade III astrocytomas and GBMs, and it has become current practice to restrict radiation to an image-defined area with sufficient margin in order to sustain maximum quality of survival (Kaye and Laws Jr 1995). Such image-guided (stereotactic) treatment techniques include the gamma knife (targeted external-beam radiation) and interstitially implanted radioisotopes, e.g. ^{125}Iodine and ^{192}Iridium. Postoperative adjuvant therapy also includes chemotherapy, usually with drugs that cause DNA alkylation. Intravenously administered carmustine (BCNU), bischloroethyl-nitrosurea, has been the traditional drug of choice due to its good delivery through the blood-brain barrier (Kaye and Laws Jr 1995). Approximately the same therapeutic effect could be achieved by orally administered lomustine and procarbazine, whereas some patients with Grade III astrocytomas have been shown to respond better to a combination of procarbazine, lomustine and vincristine (PCV) than carmustine treatment (Levin *et al.* 1990). Despite aggressive management, the overall prognosis has been poor. The median survival of patients with Grade III tumors has been less than two years and with GBMs one year after the onset of treatment (Daumas-Duport *et al.* 1988, Burger *et al.* 1991).

New drugs such as temozolomide (O'Reilly *et al.* 1993), BCNU-saturated biodegradable wafers in the tumor cavity (Valtonen *et al.* 1997, Subach *et al.* 1999), boron neutron capture therapy (Barth *et al.* 1999) and gene therapy (Culver and Blaese 1994, Ram *et al*. 1997, Klatzmann *et al*. 1998, Palu *et al*. 1999,

Shand *et al.* 1999, Sandmair *et al.* 2000) have been tested as promising new strategies for the local therapy of astrocytic tumors.

1.4 Prognostic factors

Emphasis has been placed on prognostic factors that could aid in communication about the treatment of astrocytoma patients. In addition to the histopathological malignancy grade, patient's age has served as a traditional clinical factor that correlates with patient outcome (Cohadon *et al.* 1985, Burger *et al.* 1991). For instance, young age has been suggested to favor the long-term survival of some GBM patients, which may reflect both good capacity to recover after aggressive treatment and good host resistance in young adults (Cohadon *et al.* 1985, Chandler *et al.* 1993). The volumetric reduction of the tumor mass and the extent of preoperative deficit, the so-called Karnofsky's performance status (Karnofsky and Burchmal 1949), have also been shown to have a significant impact on the length of the survival of astrocytoma patients (Philippon *et al.* 1993, Berger 1994). Among the quantitative histopathological parameters Ki-67 (MIB-1) labeling index, mitoses count and presence of tumor necrosis have been shown to correlate with poor clinical outcome of patients with diffusely infiltrating astrocytoma (Sallinen *et al.* 1994, Sallinen *et al.* 2000).

2. Strategies to reveal genetic alterations in human neoplasms

The formation of tumors is a complex, multi-step process resulting from an accumulation of various genetic aberrations (James *et al.* 1988, Fearon and Vogelstein 1990, Sato *et al.* 1990, Morita *et al.* 1991, reviewed by Lengauer *et al.* 1998). Despite the complexities underlining cancer formation and progression, it has been suggested that six essential acquired capabilities collectively determine malignant growth: 1) self-sufficiency in growth signals, 2) insensitivity to antigrowth signals, 3) evading of apoptosis, 4) unlimited replicative potential, 5) sustained angiogenesis and 6) tissue invasion and metastasis (reviewed by Hanahan and Weinberg 2000). In general, these changes in the genome affect three types of genes: oncogenes, tumor suppressor genes (TSGs) or `gatekeeper genes´ and DNA repair genes or `caretaker genes´ (Vogelstein and Kinzler 1998). Oncogenes are capable of inducing or maintaining neoplastic cell proliferation and tissue growth, whereas TSGs are negative regulators of growth. DNA repair genes maintain the integrity of the genome, and their inactivation increases genetic instability that promotes tumor formation and growth. Point mutations, DNA rearrangements and gene amplifications are the main mechanisms of oncogene activation, whereas inactivation of TSGs and DNA repair genes could be triggered by point mutations and DNA rearrangements as well as physical deletions in chromosomes.

Hahn *et al.* (1999) demonstrated that as few as three *specific* genetic alterations are sufficient for the malignant transformation of normal human epithelial and fibroblast cells in vitro. The observed aberrations were an ectopic expression of 1) the catalytic subunit of the telomerase enzyme (*hTERT*) in combination with 2) the simian virus 40 large-T (SV40T) oncoprotein and 3) oncogenic allele of the H-*ras* (Hahn *et al.* 1999).

It has been estimated that it usually takes decades, for cancer to develop. The role of genetic instability, e.g. an occurrence rate of mutation, in the formation and progression of cancer has been argued (reviewed by Lengauer *et al.* 1998). However, there is evidence that most solid tumors are genetically unstable, and that the instability exists at two levels. The instability in the nucleotide level (NIN) or microsatellite insatbilty (MIN) alters one or few base pairs by substitution, deletion or insertion. It is uncommon in human cancers. The second type of genetic instability, chromosomal instability (CIN), is likely to occur in most human malignancies. It results from losses and gains of whole chromosomes or large portions of them (reviewed by Lengauer *et al.* 1998).

2.1 Karyotyping analyses and comparative genomic hybridization (CGH)

With genome-wide research strategies, such as karyotyping analyses and CGH, it is possible to investigate the entire genome of one sample by a single hybridization. The karyotyping analyses provide information about both numerical and structural chromosomal aberrations. Conventional cytogenetics targets chromosomal aberrations detectable by light-microscopy in short-term cultured, metaphase arrested cells. After the first successful karyotypic analysis in the 1960`s (Steel and Breg 1966), conventional cytogenetics has been widely used for both cancer diagnostic and research purposes and approximately 27 000 cytogenetically aberrant human neoplasia samples have been collected into an accessible database (Mitelman *et al.* 1997). However, the technical difficulties in the chromosome banding analysis of solid tumors have limited their number to approximately 3200 in the database (Mertens *et al.* 1997).

Modern methods based on 24-color fluorescence *in situ* hybridization (FISH) have been developed for karyotyping analyses. Spectral karyotyping (SKY) (Schröck *et al.* 1996b) and multicolor FISH (mFISH) (Speicher *et al.* 1996) are based on the simultaneous hybridization of 24 chromosome-specific painting probes labeled with different combinations of five fluorochromes. A new technical application of mFISH, so called *arm*FISH, combines the mFISH analysis and detection of chromosome arms by the arm-specific painting probes method (Karhu *et al*. 2001).

The development of CGH in 1992 markedly enhanced the means of investigating solid tumors (Kallioniemi *et al.* 1992). The method is based on the simultaneous *in situ* hybridization of differentially labeled tumor DNA and normal reference DNA to normal lymphocyte metaphase chromosomes (Figure 2). Unlike conventional cytogenetic analyses, the CGH method requires only genomic DNA from tumors and normal tissue and can be applied to both fresh and paraffin-embedded tissue specimens (Kallioniemi *et al.* 1992, Speicher *et al*. 1993, Isola *et al*. 1994). Furthermore, even very small tumor samples with only a few hundred or thousand neoplastic cells can be studied after universal amplification of the tumor material using a degenerate oligonucleotide primed PCR (DOP-PCR) (Speicher *et al*. 1993, Speicher *et al.* 1995, Wiltshire *et al*. 1995, Kuukasjärvi *et al*. 1997b, Hirose *et al.* 2001). On the other hand, the CGH method only provides information about the chromosomal regions of gains or losses. Unlike karyotyping analyses, it cannot detect structural aberrations such as balanced chromosomal translocations, inversions or small intragenic rearrangements (reviewed by Kallioniemi *et al.* 1994b). In addition, chromosomal aberrations, which present in low frequency, amplifications smaller than 2 megabases (Mb) or deletions smaller than 5 Mb remain beyond the

resolution and detection sensitivity of the CGH method (Kallioniemi *et al.* 1992, reviewed by Kallioniemi *et al.* 1994b, reviewed by Forozan *et al.* 1997).

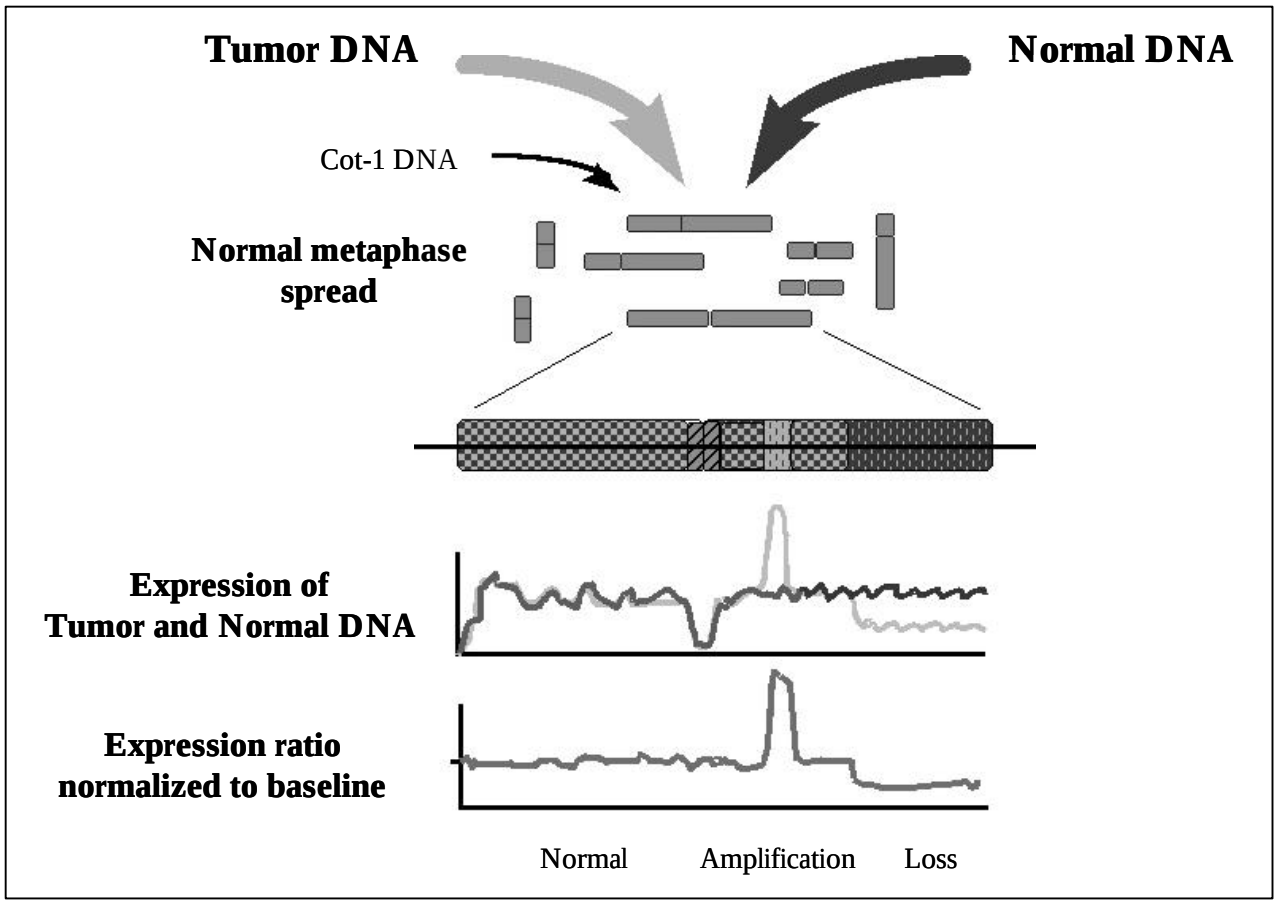

Figure 2. CGH method. Differentially labeled tumor DNA and normal reference DNA become co-hybridized with unlabeled Cot-1 DNA to normal metaphase chromosomes. Chromosomal region with a gain (or amplification) become visualized as an overexpression of labeled tumor DNA. Unbound tumor DNA, which in turn highlights the labeled reference DNA, indicates to a chromosomal loss.

CGH has been widely utilized in studies on cancer genetics. It has been widely used in the characterization of chromosomal aberrations and their progression and clonal expansion in a variety of tumors and hematological neoplasias (Kallioniemi *et al.* 1994a, Schröck *et al.* 1994, Bentz *et al.* 1995, Wiltshire *et al.* 1995, Heselmayer *et al.* 1996, Gronwald *et al.* 1997, Karhu *et al.* 1997, Kuukasjärvi *et al.* 1997a, Bigner *et al.* 1999). CGH has also been successfully used for the identification of novel genes involved in tumorigenesis (Visakorpi *et al.* 1995, Houldsworth *et al.* 1996, Anzick *et al.* 1997, Sen *et al.*

1997, Hemminki *et al.* 1998). During the past few years, a number of CGH studies have focused on identifying recurring chromosomal aberrations and their associations with clinical, pathological or prognostic factors (Isola *et al.* 1995, Tirkkonen *et al.* 1998, Hirai 1999, Larramendy *et al.* 1999, Skytting *et al.* 1999, Tarkkanen *et al.* 1999a, Tarkkanen *et al.* 1999b, Wiltshire *et al.* 2000, Kanerva 2001, Vettenranta *et al.* 2001).

A typical finding in cytogenetic studies on astrocytic tumors has been an increase in the number of chromosomal abnormalities along with increasing histopathological malignancy. Considering Grade II and III astrocytomas, losses of regions on sex chromosomes have been the most common chromosomal aberrations, whereas normal diploid stemlines have been reported in the majority of tumors (Rey *et al.* 1987a, Bigner *et al.* 1988, Jenkins *et al.* 1989, Griffin *et al.* 1992, Thiel *et al.* 1992, Magnani *et al.* 1994). In a few Grade III astrocytomas, trisomy of chromosome 7 has been detected. The majority of GBMs have been shown to harbor stemline abnormalities. The most common aberrations in GBMs have been a gain on chromosome 7 and losses on chromosomes 6, 10, 22, X and Y as well as structural abnormalities involving the short (p-) arms of chromosomes 1 and 9 (Rey *et al.* 1987b, Bigner *et al.* 1988, Jenkins *et al.* 1989, Thiel *et al.* 1992, Magnani *et al.* 1994, Mertens *et al.* 1997). Double minutes (dmins) have been demonstrated in up to 50% of the GBMs evaluated, and in most cases the dmins have contained an amplification of the *epidermal growth factor receptor* (*EGFR*) (Bigner *et al.* 1987, Bigner *et al.* 1988, Bigner *et al.* 1990, Thiel *et al.* 1992, Magnani *et al.* 1994).

CGH analysis has been widely used in the genetic characterization of astrocytomas. Figure 3 summarizes the results of the previously published CGH studies on 34 Grade II astrocytomas and 323 Grade III-IV astrocytomas (Schröck *et al.* 1994, Kim *et al.* 1995, Schlegel *et al.* 1996, Schröck *et al.* 1996a, Weber *et al.* 1996a, Weber *et al.* 1996b, Mohapatra *et al.* 1998, Nishizaki *et al.* 1998, Brunner *et al.* 1999, Maruno *et al.* 1999, Mao and Hamoudi. 2000, Wiltshire *et al.* 2000, Squire *et al.* 2001). Briefly, the total number of chromosomal aberrations has been accumulated along with increasing malignancy grade of astrocytomas. The mean number of chromosomal changes per tumor has been two in Grade II astrocytomas and five in Grade III-IV astrocytomas. In Grade II astrocytomas the chromosomal gains have outnumbered chromosomal losses (43 gains versus 33 losses). In Grade III-IV astrocytomas the majority of chromosomal alterations were losses (889 losses versus 790 gains). The most common chromosomal alterations in Grade II astrocytomas have been gains (and/or amplifications) on chromosomes 7, 8q, 12p and losses on chromosomes 19q and X. Regarding Grade III-IV astrocytomas, the most frequent alterations have been gains on chromosomes 7, 19, 20 and losses on chromosomes 9p, 10, 13, 14 and 22.

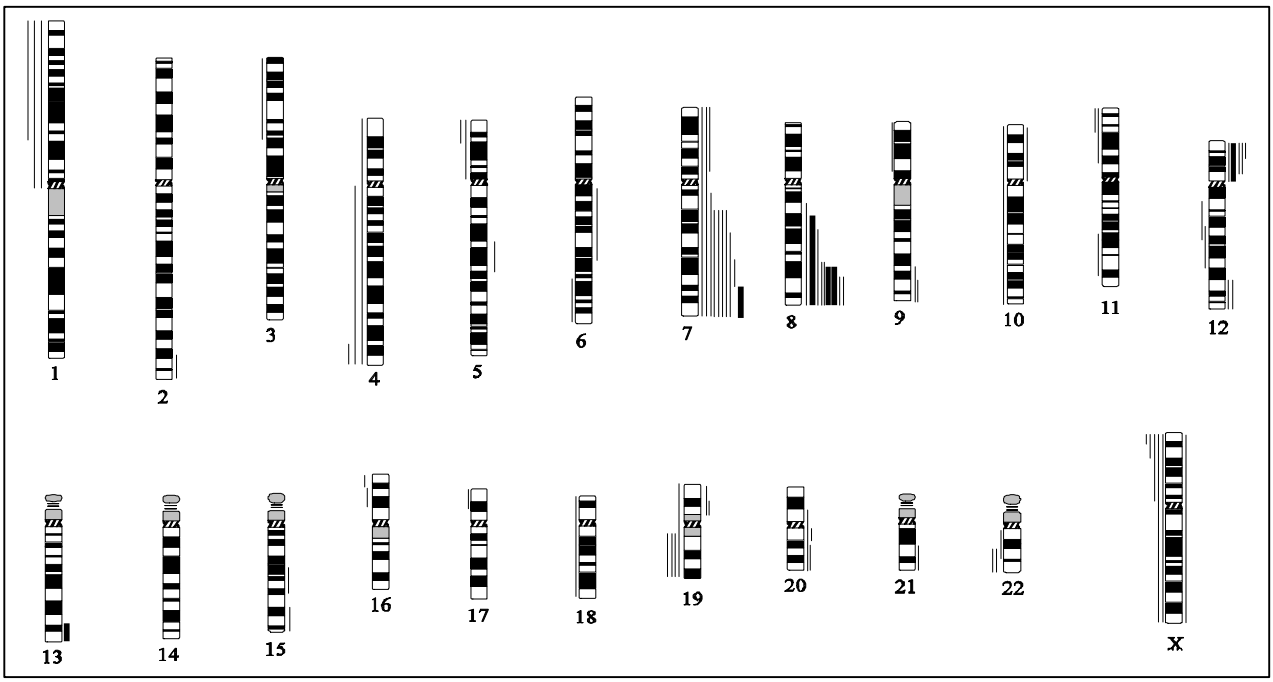

Figure 3A. Summary of literature of the chomosomal alterations in 34 sporadic Grade II astrocytomas by CGH. Lines on the left side of the idiogram represent chromosomal losses, and lines on the right side represent chromosomal gains. Chromosomal amplifications are indicated by thick lines on the right side of the chromosome idiogram.

Two recent studies have combined SKY and CGH analyses (Kubota *et al.* 2001, Squire *et al.* 2001). Kubota *et al.* (2001) studied nine GBM cell lines, and by SKY analyses demonstrated recurrent chromosomal rearrangements. In fact, three of the cell lines of different origin showed very similar karyotypes. According to CGH, the most commonly lost chromosomal regions were situated on chromosomes 4q, 10p, 13q, 14q and 18q and gains were detected most often on chromosomes 7 and X. In addition, frequent amplifications on chromosomal loci 1p13, 4q12 and 16q13 were demonstrated. Interestingly, those regions of low-level DNA amplification were found translocated and/or inserted at a very high rate in SKY analyses (Kubota *et al.* 2001). The second study used 16 cell lines, ten of which were cultured from glial tumors (Squire *et al.* 2001). The chromosomes affected most often by translocation events were chromosomes 1 and 10. In addition, translocations often also involved chromosomes 3, 5, 7 and 11. The most common alteration with CGH was gain on chromosome 7.

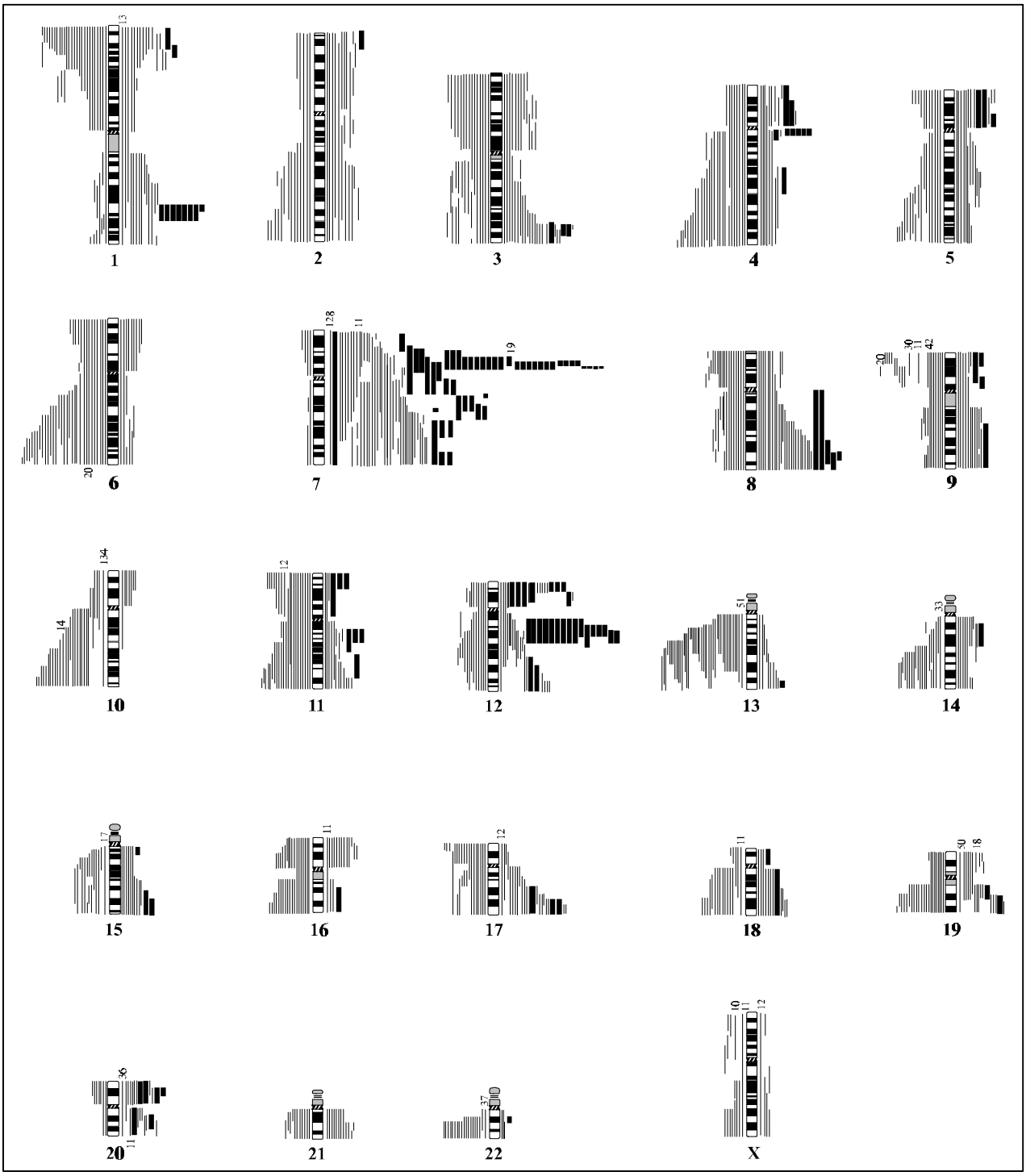

Figure 3B. Summary of chromosoml alterations in 323 Grades III-IVastrocytomas detected by CGH according to the literature. Chromosomal losses are indicated by lines on the left side of the chromosome idiogram, and lines on the right side represent gains. Chromsomal amplifications are indicated by thick lines on the right side of the idiogram.

2.2 Loss of heterozygosity (LOH) by allelotyping

It has been possible to detect distinct regions of allelic losses in tumors by allelotyping analysis. Polymorphic marker loci have been used to localize the regions of allelic losses in tumors and, subsequently, identify TSGs that are important in the pathogenesis of a variety of tumors (Cavenee *et al.* 1983, Marshall 1991).

Considering astrocytomas, LOH have now been found on all the autosomes (von Deimling *et al.* 2000). Astrocytic tumors have usually shown LOH simultaneously on multiple chromosomes, and the number of affected chromosomes has been demonstrated to correlate with the histopathological malignancy of tumors (Fults *et al.* 1990, von Deimling *et al.* 2000). The short arm (p-arm) of chromosome 17 has been one of the most commonly affected loci, found in 30-60% of Grade II-IV astrocytomas (James *et al.* 1988, el-Azouzi *et al.* 1989, Fults *et al.* 1989, Venter and Thomas 1991, Fults *et al.* 1992a, von Deimling *et al.* 1992a). LOH on chromosome 22q has been regularly detected in Grade II-IV astrocytomas (James *et al.* 1988, Rey *et al.* 1993, Hoang-Xuan *et al.* 1995, Ino *et al.* 1999, Oskam *et al.* 2000). In addition, LOH on chromosomes 1p, 13q and 19 have been demonstrated commonly in diffusely infiltrating astrocytomas of all grades (James *et al.* 1988, von Deimling *et al.* 1994a, von Deimling *et al.* 1994b, Bello *et al.* 1995, Diedrich *et al.* 1995, Ritland *et al.* 1995). LOH on chromosome 9p has been connected to Grade III-IV astrocytomas (James *et al.* 1991, Sonoda *et al.* 1995b, Maruno *et al.* 1996, von Deimling *et al.* 2000). LOH on chromosome 10 has been characterized to the GBM group, where it has been shown in up to 90% of tumors (James *et al.* 1988, Fujimoto *et al.* 1989, Venter and Thomas 1991, Fults *et al.* 1992a, Karlbom *et al.* 1993, Diedrich *et al.* 1995, von Deimling *et al.* 2000). Other less frequently observed regions of LOH in GBMs include chromosomes 6q, 11, 14q and 17q (Fults *et al.* 1992a, von Deimling *et al.* 2000).

2.3 High-throughput array strategies

DNA microarrays provide a simple and rapid vehicle for exploring the tumor genome. By complementary DNA (cDNA) microarray analysis or oligonucleotide array the expression of thousands of genes could be measured in the same tumor sample in a single hybridization (reviewed by Ramsay 1998). In a cDNA microarray, DNA probes representing cDNA clones are printed onto glass slides or nylon substrate to serve as gene-specific hybridization targets. A fluorescent or radioactive probe is prepared from total mRNA (messenger-RNA) of tumor sample and hybridized on the array. Measurement of fluorescence or radioactive intensity allows quantitation of gene expression

(Schena 1996, Schena *et al.* 1996, reviewed by Duggan *et al.* 1999). Simultaneous, two-color fluorescence detection, where a mixture of two independently labeled probes is simultaneously hybridized on the array, enables direct comparison of two independent biological samples (Schena 1996). In an oligonucleotide array, different oligonucleotides are synthesized either by *in situ* light-directed combinatorial synthesis or by conventional synthesis followed by immobilization on glass substrates. The array is exposed to labeled sample DNA, hybridized and complementary sequences are determined (reviewed by Ramsay 1998, reviewed by Lipshutz *et al.* 1999). As the cDNA microarray analysis allows gene expression studies and gene discovery, the oligonucleotide array also enables polymorphism screening and detection of mutations as well as mapping of genomic DNA clones (reviewed by Ramsay 1998, reviewed by Lipshutz *et al.* 1999).

CGH arrays have been developed to better clinical needs and to improve the resolution of conventional metaphase CGH. The main principles of CGH arrays are similar to those of conventional chromosomal CGH, but the hybridization targets vary. In matrix-based CGH, the target DNAs have been arrayed in small spots onto glass slides (Solinas-Toldo *et al.* 1997), whereas in a cDNA microarray-based CGH a single cDNA microarray serves as a hybridization target (Pollack *et al.* 1999). cDNA microarray-based CGH has been shown to have a 20-fold higher mapping resolution than conventional metaphase CGH (Pollack *et al.* 1999).

As DNA microarrays expose the expression profiles of thousands of genes in a single hybridization, tissue microarray enables the parallel *in situ* detection of DNA, RNA or protein targets in hundreds of tumors in a single hybridization (Kononen *et al.* 1998). Tissue microarrays are constructed by bringing small cylindrical tissue biopsies from different tumors into a single paraffin block. The power of the tissue array method for the rapid screening of tumor specimens is well demonstrated. As many as 1000 individual tumors can be applied on to one tissue array block within three days (Peter and Sikorski 1998). Amplification of three different oncogenes could be analyzed by FISH in almost 400 individual tumors within a week (Schraml *et al.* 1999).

So far microarray techniques, especially cDNA microarray, have been widely used in the gene expression profiling of various cancers (Khan *et al.* 1998, Anbazhagan *et al.* 1999, Sgroi *et al.* 1999, Wang *et al.* 1999, Elek 2000, Al Moustafa *et al.* 2002). In breast cancer, the clinical utility of cDNA microarray has been demonstrated, as the gene expression profiles of tumors have been associated with the clinical outcome of patients (van't Veer *et al.* 2002). However, only limited studies of array-based analyses of astrocytomas have been reported. The cDNA microarray analysis of 588 known genes revealed the overexpression of *insulin-like growth factor receptor binding protein 2* (*IGFBP2*) in GBMs,

but not in anaplastic astrocytomas (Fuller *et al*. 1999). A study of the gene expression profile of 1176 known cancer-associated genes in 11 Grade II astrocytomas demonstrated significant expression changes in 24 genes. The expressions of *TIMP3*, *c-myc*, *EGFR*, *DR-nm23*, *nm23-H4* and *GDNPF* were detected in the majority of Grade II astrocytomas, but not in nontumorous brain tissue. In addition, the *AAD14*, *SPARC*, *LRP*, *PDGFR-α (platelet derived growth factor receptor-á)*, *60S ribosomal protein L5*, *PTN*, *hBAP* were demonstrated to be up-regulated more than 2-fold in 20-60% of Grade II astrocytomas, whereas *IFI 9-27*, *protein kinase CLK*, *TDGF1*, *BIN1*, *GAB1*, *TYRO3*, *LDH-A*, *adducing 3*, *GUK1*, *CDC10* and *KRT8* were down-regulated more than 50% in the majority of the tumors (Huang *et al*. 2000). More recently, distinctive molecular profiles of low-grade and high-grade astrocytomas were demonstrated using oligonucleotide-based microarray analysis of ~6800 genes (Rickman *et al*. 2001). Of the almost 7000 genes analyzed, a total of 378 genes differed in their expression patterns between Grade II astrocytomas and normal brain tissue samples. Likewise, 1305 genes had differences in expression levels between GBMs and normal brain tissue samples. When the expression profiles of GBMs were compared with those of Grade II astrocytomas, a total of 183 genes was expressed at a higher level and 149 genes at a lower level. Many of the genes upregulated in GBMs encode proteins that are involved in cell proliferation or cell migration (Rickman *et al*. 2001). A second study of oligonucleotide-based microarray on four GBMs identified several downregulated ion and solute transport-related genes (Markert *et al*. 2001). In contrast, *aquaporin-1*, *GLUT-3*, *osteopontin*, *nicotinamide N-methyltransferase*, *MDM2 (murine double minute 2)*, *epithelin*, *cytokine* and *p53 binding protein and macrophage migration inhibitory factor (MIF)* were found to be upregulated.

Seven GBM cell lines and seven GBMs were analyzed by array CGH of 58 target oncogenes (Hui *et al*. 2001). The study revealed high-level amplifications of *cyclin dependent kinase 4* (*CDK4)*, *GLI*, *MYCN*, *MYC*, *MDM2* and *PDGFRA* and frequent gains on *PIK3CA*, *EGFR*, *CSE1L*, *NRAS*, *MYCN*, *FGR*, *ESR*, *PGY1*, suggesting their involvement in GBM tumorigenesis (Hui *et al*. 2001).

3. Tumorigenetic pathways of astrocytic neoplasms

The stepwise progression of diffuse astrocytomas from low-grade tumors to highly aggressive GBMs has been well documented (Louis 1997, Kleihues *et al.* 2000). In addition to purely astrocytic origin, the so-called secondary GBMs may develop from oligodendrocytic tumors, especially from mixed oligo-astrocytomas. The literature reports several genetic alterations that are characteristic of different astrocytoma progression pathways (Figure 4).

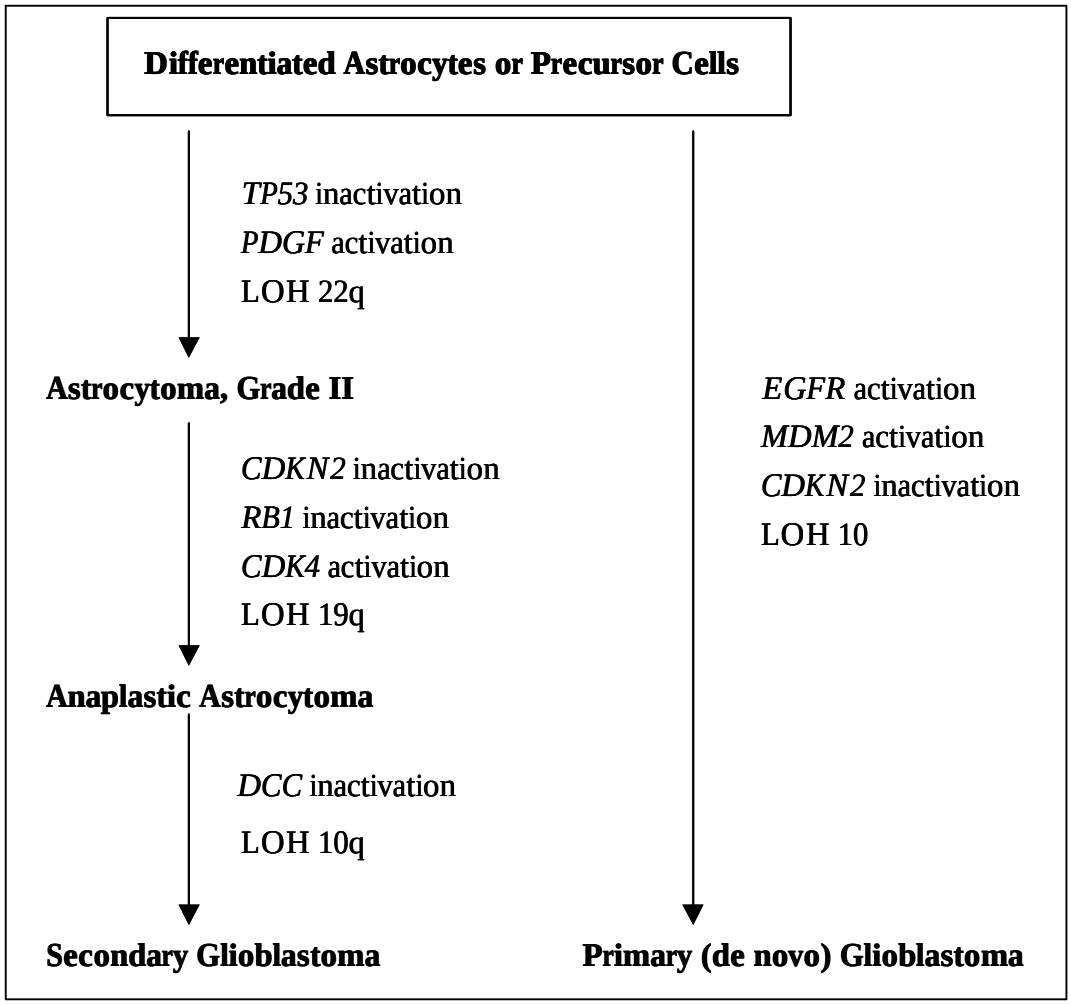

Figure 4. Molecular genetic model of tumorigenetic pathways of diffusely infiltrating gliomas.

3.1 Formation of Grade II astrocytoma

Inactivation of *tumor protein 53 (TP53)* TSG is one of the earliest genetic alterations in diffuse astrocytomas. The *TP53* gene is located on chromosome 17p13.1, and it encodes a 53 kDa (kilodalton) nuclear phosphoprotein which acts as a multi-functional transcription factor. *TP53* has been referred to as the guardian gene of the genome due to its pivotal role in the control of cell proliferation, apoptosis and neovascularization (Lane 1992, Bogler *et al.* 1995). Upregulated p53 protein in e.g. ultraviolet-irradiated skin has been suggested to demonstrate an active function of the *TP53* gene in cell response to DNA damage (Maltzman and Czyzyk. 1984). Furthermore, experiments on GBM cell lines have indicated that the wild type p53 is capable of suppressing proliferation of neoplastic cells (Mercer *et al.* 1990, Van Meir *et al.* 1995). Inactivation of *TP53* by allelic loss of chromosome 17p or mutations most commonly affecting exons 5, 7 and 8 has been found in approximately one third of Grade II astrocytomas (Fults *et al.* 1992a, Sidransky *et al.* 1992, von Deimling *et al.* 1992a, Louis *et al.* 1993, Lang *et al.* 1994b, Van Meir *et al.* 1994). It has been suggested that the inactivation of *TP53* increases genetic instability in neoplastic cells, thereby significantly increasing the likehood of further genetic aberrations occurring (Hartwell 1992, reviewed by Carson and Lois 1995).

Another early genetic aberration in astrocytomas is the activation of the platelet-derived growth factor (PDGF) system. PDGF is a powerful mitogen for glial and connective tissue cells (Richardson *et al.* 1988, Maxwell *et al.* 1990, Chaudhry *et al.* 1992). It consists of dimmers of two highly homologous peptide chains, A- and B-chains respectively. Two distinct PDGF receptors, α- and β-receptors, exist and they belong to the tyrosine kinase family and activate a cellular signaling pathway (reviewed by Heldin and Westermark 1999). PDGF and the corresponding receptors are frequently co-expressed in glioma cells, which could indicate that the system represents an autocrine stimulatory loop (Maxwell *et al.* 1990, Hermanson *et al.* 1992, Shamah *et al.* 1993, van der Valk *et al.* 1997). Overexpression of PDGF ligands and receptors, especially the A-ligand (chromosome locus 7p22) and α-receptor (chromosome locus 4q11-12), has been detected in approximately 60% of Grade II astrocytomas (Maxwell *et al.* 1990, Fleming *et al.* 1992, Hermanson *et al.* 1992, Hermanson *et al.* 1996, van der Valk *et al.* 1997).

A third, frequently detected aberration has been the LOH on chromosome 22q, found in approximately 20% of Grade II astrocytomas (James *et al.* 1988, Fults *et al.* 1990, Rey *et al.* 1993, Hoang-Xuan *et al.* 1995). The putative TSG in this chromosomal region remains to be defined. So far, researchers have eliminated the *neurofibromatosis 2* (*NF2*) gene from being the target gene, since the

affected chromosomal locus seems to be more telomeric to the *NF2* locus (Rubio *et al.* 1994, Hoang-Xuan *et al*. 1995, Ino *et al.* 1999, Oskam *et al*. 2000).

In addition, overproduction of growth factors such as FGF2 (fibroblast growth factor 2) and CNTF (cilliary neurotrophic factor) and their receptors has been shown equally in gliomas of all grades (reviewed by Holland 2001).

3.2 Transition to Grade III astrocytoma

Approximately one half of Grade III astrocytomas harbor aberrations in at least one component of the p16/CDK4/RB/E2F cell cycle regulatory system (He *et al.* 1995, Ueki *et al.* 1996). Normally, p15 and p16 proteins act as inhibitors of cyclin dependent kinases (CDKs, especially CDK4 and CDK6). The activity of CDKs is essential for the G1/S-phase transition of the cell cycle. Cyclin D1 is one of the key regulator cyclins in the G1 phase of the cell cycle (reviewed by Draetta 1994). It activates CDK 4 and 6 to phosphorylate the retinoblastoma protein (pRb), which leads to the release of E2F transcription factor and the activation of genes necessary for continued cell proliferation (reviewed by La Thangue 1994, Sherr 1994, Cordon-Cardo 1995, reviewed by Weinberg 1995). In astrocytomas, p16 is the most often affected component in this pathway. *Cyclin dependent kinase inhibitors 2A* and *2B* (*CDKN2A* and *CDKN2B*), which encode p15 and p16 proteins, have been mapped to chromosome 9p21, a region with homozygous deletions in about one third of Grade III astrocytomas (Schmidt *et al.* 1994, He *et al.* 1995, Ichimura *et al.* 1996, Ueki *et al.* 1996). In addition to deletions, the function of p15 and p16 can be inactivated by mutations and hypermethylation of the CpG island in the 5´ region of *CDKN2A* or *CDKN2B* (Merlo *et al.* 1995, Costello *et al.* 1996). Concerning astrocytomas, however, these alternative inactivation mechanisms seem to be very rare (Giani and Finocchiaro 1994, He *et al.* 1995, Li *et al.* 1995, Moulton *et al.* 1995, Sonoda *et al.* 1995b, Fueyo *et al.* 1996, Ueki *et al.* 1996, Hegi *et al.* 1997, Schmidt *et al.* 1997). *Retinoblastoma type 1 (RB1)* gene (chromosome 13q14) is altered in about 25% of Grade III astrocytomas (Henson *et al.* 1994, He *et al.* 1995, Ichimura *et al.* 1996, Ueki *et al.* 1996), and approximately 10% of Grade III astrocytomas harbor *CDK4* amplification on chromosome 12q13-14 (Reifenberger *et al.* 1994, Schmidt *et al.* 1994, He *et al.* 1994, Nishikawa *et al.* 1995, Ichimura *et al.* 1996). Cyclin D1 protein expression has been shown to increase with the histopathological malignancy grade of astrocytomas (Chakrabarty *et al.* 1996, Cavalla *et al.* 1998). However, *cyclin D1* amplification (chromosome 11q13) has been identified in only a small fraction (1.5%) of Grade III-IV astrocytomas studied (He *et al.* 1995, Büschges *et al.* 1999).

The human *CDKN2A* locus also contains an alternative reading frame that encodes $p14^{ARF}$. ARF functions independent of the RB-pathway. It modulates TP53 function distinct from those activated by DNA damage as part of a checkpoint response to oncogenic and hyperproliferative signals. In primary mouse embryo fibroblasts, overexpression of Myc, E1A or E2F-1 rapidly induces *ARF* gene expression leading to TP53-dependent apoptosis. ARF may also bind to MDM2, which blocks MDM2-mediated TP53 degradation and transactivational silencing (reviewed in more detail by Sherr 1998). Homozygous deletions of $p14^{ARF}$ have been observed in 15% of Grade III astrocytomas (Ichimura *et al*. 2000).

LOH on chromosome 19q has been shown to occur in up to 50% of Grade III astrocytomas (von Deimling *et al*. 1994a, von Deimling *et al.* 1994b, Ritland *et al.* 1995, von Deimling *et al.* 2000). The putative TSG in this region is still unknown, but the gene has been mapped to the band 19q13.3 in between the genetic markers D19S412 and STD (Smith *et al.* 2000a). Other relatively frequently detected aberrations in Grade III astrocytomas include LOH on chromosomes 1p and 11p15.5, the candidate genes have not been identified (Fults *et al*. 1992b, Sonoda *et al*. 1995a).

3.3 Transition to GBM, Grade IV

The most malignant astrocytic tumor, GBM, may develop from Grade II or III astrocytomas (secondary GBM) or without any evidence of previous less malignant astrocytoma (primary or *de novo* GBM) (von Deimling *et al*. 1993, Lang *et al*. 1994a). GBMs are characterized by microvascular proliferation. The most important regulator of the vascular function in glioma induced-angiogenesis is vascular endothelial growth factor (*VEGF*, also known as the vascular permeability factor or *VPF*). It is located on chromosome 6p21 and encodes an angiogenic mitogen, which also has the ability to induce microvascular permeability (Dvorak *et al.* 1995). VEGF is induced by hypoxia and signals through two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, which are expressed specifically on endothelial cells (de Vries *et al*. 1992, Shweiki *et al.* 1992). During glioma progression, VEGF and its receptors have been shown to increase along with the increasing histopathological malignancy grade of astrocytoma, and it is particularly highly expressed in GBMs (Pietsch *et al.* 1997, Abdulrauf *et al.* 1998, Chan *et al.* 1998, Miyagami *et al*. 1998, Takekawa and Sawada 1998, Carroll *et al*. 1999, Lafuente *et al*. 1999, Oehring *et al.* 1999). In addition, a set of other endothelial cell receptor tyrosine kinases or their ligands such as PDGFR-β, EGF (epidermal growth factor), FGF, TGF-β (transforming growth factor beta), Tie-1, Tie-2 and c-met has also been associated with angiogenesis and vascular remodeling of gliomas (Kleihues *et al*. 2000).

Secondary GBM. In secondary GBMs, the frequency of *TP53* mutations and TP53 protein accumulation is high (>65% and >90% respectively). The percentage of cells with accumulated TP53 protein have been shown to increase from the first biopsy to tumor recurrence, although over 90% of the mutations had already occurred at the time of the first surgical intervention (Reifenberger *et al.* 1996). A significant correlation between LOH 17p and high expression levels of PDGFR-α has been reported, which indicates that PDGFR-α alterations are typical on the pathway leading to secondary GBMs (Hermanson *et al.* 1996). Although the overexpression of PDGF-α has been well documented, *PDGFR-a* has been found amplified in only few GBMs (Fleming *et al.* 1992, Hermanson *et al.* 1996). In a recent study of 167 Grade III-IV astrocytomas and 70 anaplastic oligodendroglial tumors, no *PDGFR-a* amplification could be detected in any of the astrocytomas, whereas 10% of the anaplastic oligodendrogliomas and one Grade III oligo-astrocytoma had *PDGFR-a* amplification (Smith *et al.* 2000b). Finally, LOH 19q, a frequent hallmark of Grade III astrocytomas, has been rather associated with the tumorigenesis of secondary (54%) than of primary (6%) GBMs (Nakamura *et al.* 2000).

LOH on chromosome 10q is characteristic of the conversion from a Grade III astrocytoma to a GBM (Louis 1997, Fujisawa *et al.* 2000, Kleihues *et al.* 2000). A number of potential TSGs are mapped on this chromosomal locus: the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*, also called mutated in multiple advanced cancer, (*MMAC1*) located at 10q23.3 (Li *et al.* 1997, Steck *et al.* 1997), the deleted in malignant brain tumors 1 (*DMBT1*) on chromosome 10q25-26 (Mollenhauer *et al.* 1997), the *h-neu* on chromosome 10q25.1 (Nakamura *et al.* 1998) and the *MXI1* on chromosome 10q24 (Eagle *et al.* 1995).

Two research groups identified the *PTEN* gene simultaneously (Li *et al.* 1997, Steck *et al.* 1997). The phosphatase homology of *PTEN* indicates that the gene may suppress tumor cell growth by antagonizing protein tyrosine kinases. In addition, the resemblance to tensin may point to a possible role of the gene in the regulation of tumor cell invasion and metastasis, since tensin normally helps cells to stay in their physiological locations within a tissue (Li *et al.* 1997). *PTEN* has been shown to be inactivated in GBMs either via deletion combined with mutation of the remaining allele or by homozygous deletion. Heterozygous deletions of *PTEN* have been detected in the majority of all GBMs, *PTEN* mutation having ranged from 27% to 44% (Rasheed *et al.* 1997, Wang *et al.* 1997, Liu *et al.* 1997, Bostrom *et al.* 1998, Fults *et al.* 1998, Maier *et al.* 1998, Schmidt *et al.* 1999, Zhou *et al.* 1999). Regarding the subset of secondary GBMs, however, a *PTEN* mutation seems to be a rare event

(somewhat 4%) (Tohma *et al.* 1998), and no homozygous deletions have been detected in secondary GBMs (Liu *et al.* 1997, Tohma *et al.* 1998).

DMBT1 has homology to members of the scavenger receptor cystein-rich (SCRC) family. *DMBT1* encodes a protein with at least two different functions, one that is associated with the immune defense system and the other with epithelial differentiation (Mollenhauer *et al.* 2000). Homozygous deletions of *DMBT1* have been detected in 23-38% of all GBMs, whereas no *DMBT1* mutations have been reported (Mollenhauer *et al.* 1997, Somerville *et al.* 1998).

Another interesting gene on chromosome 10q is the *h-neu,* which encodes a protein with strong homology to Drosophilia neuralized (D-neu) protein. D-neu protein has a critical function in neurogenesis in Drosophilia. Studies on astrocytomas have suggested that *h-neu* may have an important role as a TSG during astrocytoma progression (Nakamura *et al.* 1998). Normal human brain tissue expresses h-neu, but the expression levels have been found to be very low in human malignant astrocytoma specimens and in the majority of glioma cell lines studied. Furthermore, *h-neu* point mutation has been confirmed in a U251MG GBM cell line (Nakamura *et al.* 1998). The TSG *MXI1* on chromosome 10q24 has been shown to carry several mutations in prostate cancer (Eagle *et al.* 1995). However, mutations on *MXI1* have not been observed in gliomas (Albarosa *et al.* 1995, Fults *et al.* 1998).

The loss of DCC (deleted in colon carcinomas) expression has been suggested to be a late event in the tumorigenesis of astrocytomas (Scheck and Coons 1993, Reyes-Mugica *et al.* 1997). *DCC* is located on chromosome 18q21.1, and the gene induces apoptosis and G2/M cell cycle arrest in tumor cells. A reduction of *DCC* expression has been demonstrated to occur during progression from low-grade (93% positive) to high-grade (47% positive) astrocytomas (Reyes-Mugica *et al.* 1997). Accordingly, secondary GBMs have been found more often to be DCC negative than primary GBMs (53% negative versus 23% negative) (Reyes-Mugica *et al.* 1997).

Primary GBM (*de novo* GBM). *EGFR* gene (c-erbB) maps to chromosome 7p11.2. EGFR is a transmembrane glycoprotein, with intrinsic tyrosine kinase activity (Ullrich *et al.* 1984). It can bind specific ligands, EGF and TGF-α, and transmit their signals to the cell (Sporn and Roberts 1985). The ligands for EGFR are expressed along with an overexpressed receptor gene, indicating an autocrine or paracrine growth-stimulatory loop involving the EGFR and its ligands (Ekstrand *et al.* 1991). In a normal cell, the expression of these growth factors and their receptors is highly regulated; inadequate

regulation allows uncontrolled cell proliferation and tumor formation. In primary GBMs, overexpression of EGFR has been detected in 60% and gene amplification in 30-40% of tumors (Libermann *et al*. 1985, Wong *et al*. 1987, Strommer *et al.* 1990, Ekstrand *et al*. 1991, Chaffanet *et al*. 1992, Fuller and Bigner 1992, Schlegel *et al*. 1994, Schwechheimer *et al*. 1995, Sauter *et al*. 1996, Waha *et al*. 1996). Half of the amplified genes are also rearranged, the most common mutant variant being deltaEGFR (also called EGFRVIII or de2-7EGFR), which lacks a portion of the extracellular ligand-binding domain due to 801 base pair deletion (Humphrey *et al*. 1990, Sugawa *et al*. 1990, Ekstrand *et al*. 1991, Ekstrand *et al*. 1992, Wong *et al*. 1992, Schwechheimer *et al*. 1995, Frederick *et al*. 2000). One third of the GBMs with *EGFR* amplification show multiple types of *EGFR* mutations (Frederick *et al*. 2000). Mutated receptors are incapable of binding their ligands and they are constitutively autophosphorylated (Ekstrand *et al*. 1991, Ekstrand *et al*. 1994, Nishikawa *et al*. 1994, Nagane *et al*. 1996, Huang *et al*. 1997).

EGFR amplification is allied with loss of chromosome 10 (von Deimling *et al*. 1992b). In primary GBMs, the whole chromosome 10 is typically lost (Fujisawa *et al*. 2000). PTEN mutations have been detected in 32% of primary GBMs (Tohma *et al*. 1998), and up to 5% of *de novo* GBMs have been shown to carry homozygous deletions of *PTEN* (Liu *et al*. 1997, Tohma *et al*. 1998). Homozygous deletions of *DMBT1* have been found in up to 38% of primary GBMs (Somerville *et al*. 1998). The target TSGs on chromosome 10p have not yet been identified, but the deletion mappings of 10p in human gliomas have been demonstrated two distinct chromosomal regions, 10p14 and 10p15, which are involved in tumorigenesis on astrocytomas (Kon *et al*. 1998). The majority of primary GBMs with EGFR amplification also show homozygous deletion of *CDKN2* (Hayashi *et al*. 1997, Hegi *et al*. 1997). This occurs in over one third (36%) of primary GBMs, but is rarely seen in secondary GBMs (4%).

Interestingly, *EGFR* amplification rarely occurs in tumors with *TP53* mutations. Only 10% of primary GBMs harbor *TP53* mutations (Watanabe *et al*. 1996). On the other hand, overexpression of MDM2 has been shown in up to 50% and amplification of *MDM2* in approximately 10% of primary GBMs without *TP53* mutations (Reifenberger *et al*. 1993, Biernat *et al*. 1997). The *MDM2* gene is located on chromosome 12q14.3-q15, and MDM2 protein forms a complex with TP53 abolishing its transcriptional activity (Momand *et al*. 1992, Oliner *et al*. 1992). It also promotes the degradation of TP53 (Haupt *et al*. 1997). On the other hand, the transcription of *MDM2* is induced by wild-type TP53 (Barak 1992, Zauberman *et al*. 1995). Thus, *MDM2* offers an alternative mechanism for escaping TP53-regulated control of cell growth.

Approximately 20% of primary GBMs harbor losses of heterozygosity on chromosomes 6q, 14q and/or 17q (von Deimling *et al*. 2000). The putative TSGs in these chromosome regions have not yet been identified.

AIMS OF THE STUDY

The aims of the study were:

1. To characterize chromosomal aberrations in astrocytomas.

2. To investigate the utility of CGH in the clinical prognostication of Grade II astrocytomas.

3. To evaluate the utility of cDNA microarray and tissue microarray for screening for genetic alterations in astrocytomas.

4. To evaluate the expression and prognostic significance of *cyclin D1* oncogene in astrocytomas.

MATERIALS AND METHODS

1. Patients, Tumor Samples and Cell Lines

This study is based on tumor material obtained from patients operated on at Tampere University Hospital during the years 1983-1998. Systematic collection of freshly frozen samples of brain tumors for storage at -70°C started in 1992. All patients received treatment that included gross total tumor removal, followed by adjuvant radiotheraphy and/or chemotherapy according to the principles described in the Treatment and Prognosis section (see page 9) and in the original publications. The gliomas had been classified and graded by a neuropathologist according to the criteria by WHO 1993 and 2000 systems (Kleihues *et al.* 1993, Kleihues *et al.* 2000). A consensus meeting with another neuropathologist was held for the interpretation of some cases. In addition, a total of eleven glioma cell lines from the American Type Culture Collection (ATCC, Rockville, MD, USA) were utilized for the study. The cells were cultured in mediums according to the instructions of the ATCC.

Table II. The glioma cell lines analysed by CGH and/or *arm*FISH.

Cell Line	Type	Initiation of the Cell Line*
A172	Glioblastoma	1973 (first reference)
CCF-STTG1	Astrocytoma	1983 (first reference)
DBTRG-05MG	Glioblastoma	1992 (first reference)
Hs 683	Glioma	1976 (first reference)
SW 1088	Astrocytoma	1975 (operation year)
SW 1783	Astrocytoma, Grade III	1977 (operation year)
T98G	Glioblastoma	1979 (first reference)
U-87 MG	Glioblastoma	1968 (first reference)
U-118 MG	Glioblastoma	1968 (first reference)
U-138 MG	Glioblastoma	1968 (first reference)
U-373 MG	Glioblastoma	1985 (first reference)

*Initiation of the cell line according to the patients' operation year or first references presented in the catalogue of ATCC.

Study I. Eleven formalin-fixed, paraffin-embedded diffusely infiltrating grade II astrocytomas were used for CGH experiments. The Grade II astrocytomas were from the years 1988-1992 in order to achieve sufficient follow-up for survival analyses. Five of the patients died of the disease during the 5-year follow-up. Eight patients were male and three were female. Patient age ranged from 3 to 56 years (median age 38 years). The patients were categorized into two different prognostic groups on the basis of survival using a cut-off value of 2.5 years. Seven of the patients were included in the group of "good" prognosis and the remaining four patients in the group of poor prognosis. In addition, five Grade III astrocytomas (one paraffin-embedded and four freshly frozen) and eight Grade IV GBMs (four paraffin-embedded and four freshly frozen) were randomly selected from the same pathology archive for the CGH analyses. Nine glioma cell lines: A172, CCF-STTG1, DBTRG-05MG, Hs683, SW 1088, SW 1783, T-98G, U-87 MG and U-138 MG (Table II) were also included.

Study II. Eleven established glioma cell lines: A172, CCF-STTG1, DBTRG-05MG, Hs683, SW 1088, SW 1783, T-98G, U-87 MG, U-118 MG, U-138 MG and U-373 MG (Table II) were used for the *arm*FISH analyses.

Study III. cDNA microarrays were done on freshly frozen samples of two Grade II astrocytomas, four Grade III astrocytomas and three GBMs randomly selected from years the 1996-1998. Of the four Grade III astrocytomas, one sample represented the primary tumor occurrence and one its reoccurrence 8 months later. Commercially available pooled total RNA from normal human brain (Clontech Laboratories Inc., Palo Alto, CA, USA) was used for comparison analyses. For the study, a high-density tissue array of 364 gliomas and 54 other types of brain tumors (mainly meningiomas and neuronal or mixed neuronal-glial tumors) was constructed from the standard formalin-fixed, paraffin-embedded tumor blocks from the years 1983-1996. The gliomas comprised 256 primary and 88 recurrent tumors. In addition, 20 tumors were arrayed twice on the tissue array block to evaluate intratumoral heterogeneity. Of the primary tumors, 192 represented astrocytic tumors. In 29 cases, patients had both their primary and one or more recurrent astrocytic tumor in tissue array. In nine cases, the histopathological malignancy grade had been upgraded at the time of recurrence. A more detailed description of the primary astrocytomas is presented in Table III.

Study IV. Paraffin-embedded, primary tumors of 46 patients (from the years 1988-1992) were collected for *cyclin D1* expression analyses. The tumor material included 21 Grade II astrocytomas (14 male and 7 female), 9 Grade III astrocytomas (7 male and 2 female) and 16 GBMs (7 male and 9

female). The median age of the patients was 35 years for Grade II astrocytomas (range 3-56 years), 50 years in Grade III astrocytomas (range 27-75 years) and 59 years for GBMs (range 29-76 years). The median survival of the patients was approximately 2 years.

Table III. Tissue array of 192 primary astrocytic tumors.

Tumor Type	**N of Tumors**	Sex M/F	Median Age* (range)	**Mean Survival*** [95% CI for MS]
Grade II astrocytoma	24	14/10	35 (3-55)	9.5 [7.4-11.8]
Grade III astrocytoma	16	9/7	41 (25-64)	3.0 [1.8-4.1]
Glioblastoma, Grade IV	129	64/65	58 (17-80)	1.6 [1.0-2.1]
Pilocytic astrocytoma, Grade I	18	10/8	7 (0-66)	12.4 [10.8-14.0]
Pleomorphic Xantho-astrocytoma, Grade II-III	3	3/0	(11-28)	All alive
Subependymal Giant Cell Astrocytoma, Grade I	2	2/0	(10-20)	All alive

* Median age and mean survival have been expressed as years

2. Study Protocols

2.1 Immunohistochemical stainings

Immunohistochemical stainings were used in Studies I, III and IV. For immunohistochemistry, five μm formalin fixed, paraffin-embedded tissue sections were cut onto poly-L-lysine (Sigma Chemical CO, St. Louis, USA) or Vectabond-treated (Vector Laboratories Inc., CA, USA) or SuperFrost+ slides. Standard indirect immunoperoxidase procedures were used. Briefly, microwave oven heating was used for antigen retrieval. The bound antibody was visualized with a streptavidin-biotin peroxidase technique (Zymed Laboratories Inc., CA, USA) using diaminobenzidine as a chromogen. The sections were counterstained with hematoxylin and eosin (H&E) or ethyl/methyl green.

Cell proliferation (Studies I, IV) was analyzed by a mouse monoclonal antibody MIB-1 (IgG, Immunotech, S.A. Marseilles, France) recognizing the Ki-67 antigen. The MIB-1 antibody was used at dilution 1:40. The tissue sections were counterstained with ethyl green. The assessment score was reported as the percentage of immunopositive nuclei in the analysis area (Ki-67 (MIB-1) labeling index).

In p53 immunostaining (Studies I, III and IV), DO-7 antibody (Novocastra Laboratories, Newcastle, United Kingdom) was used. In Studies I and IV, the antibody was used at a dilution 1:300, and sections were counterstained with hematoxylin. In Study III, the dilution for DO-7 antibody was 1:40 and methyl green was used for counterstaining. Tumor cells with unequivocal staining of neoplastic nuclei were recorded as immunopositive.

Cyclin D1 (Study IV) expression was evaluated using mouse monoclonal antibody (IgG, Novocastra Laboratories) at dilution 1:40. The slides were counterstained with ethyl green. The tumor areas analyzed for cyclin D1 mRNA expression were used for the analysis of cyclin D1 immunoreactivity. The tumors were categorized cyclin D1 immunonegative and cyclin D1 immunopositive tumors on the basis of the presence of distinct nuclear immunoreactivity.

IGFBP2 immunoreactivity (Study III) was studied with a goat polyclonal antibody C-18 (Santa Cruz Biotechnology, Inc., CA, USA) at dilution 1:1000 using a brain tumor tissue microarray. The sections were counterstained with hematoxylin. The results were evaluated semiquantitatively. Three observers

placed the tumors in the categories of negative (no staining or weakly positive tumor areas) or strong positive (intense staining covering the majority of the neoplastic cells) immunostaining.

Vimentin (Study III) expression was evaluated using monoclonal antibody (Boeheringer Mannheim, Germany) at dilution 1:160. The sections were counterstained with hematoxylin. Three observers evaluated the results semiquantitatively as described above in the chapter on IGFBP2 immunohistochemistry.

2.2 Comparative Genomic Hybridization (CGH)

CGH was used for studying genetic aberrations in Grade II astrocytomas (Study I). The histological representatives of the formalin-fixed, paraffin-embedded tumor samples were verified by 5 μm, hematoxylin and eosin-stained diagnostic sections. The genomic DNA was extracted from the paraffin-embedded tumor samples and freshly frozen tissue sections as well as cell lines according to a published standard method (Sambrook *et al.* 1989, Isola *et al.* 1994). Agarose gel electrophoresis and ethidium bromide staining were used to estimate the size, distribution and DNA concentration. If the extracted DNA concentration was not sufficient for direct labeling with nick-translation, it was amplified and labeled by DOP-PCR using UN1 primers (UN1 primer, 5´-CCG ACT CGA G*NN NNN N*AT GTG G-3´, with *N* = A, C, G or T, Telenius *et al.* 1992) as described elsewhere (Kuukasjärvi *et al.* 1997b). Briefly, approximately 5 ng of extracted tumor DNA was used for two-step DOP-PCR: four cycles of the preamplification step were carried out with unspecific conditions followed by 30 cycles of the amplification step with more stringent conditions. Finally, the amplified DNA was fluorescein isothiocyanate (FITC)-labeled (FITC-dUTP, NEN Life Science Products, Boston, MA, USA) in a set of reactions similar to the amplification step. A negative control was included in each amplification batch. Genomic DNAs from freshly frozen tumor sections and cell lines were labeled with FITC-12-dUTP and normal reference DNA with TexasRed-5-dUTP (NEN Life Science Products) by standard nick-translation protocol (Sambrook *et al.* 1989).

CGH was carried out as described elsewhere (Kallioniemi *et al.* 1992, Isola *et al.* 1994, Kallioniemi *et al.* 1994b). Briefly, FITC-labeled test DNA (5 μl of the labeled DOP-PCR product or 600 ng of nick-translated DNA) and Texas-Red labeled normal DNA (600 ng) together with 10 μg of unlabeled human Cot-1 DNA (Life Technologies, Gaithersburg, MD, USA) was denatured and applied to denatured normal lymphocyte metaphase preparations (Vysis Inc., Downers Growe, IL, USA). The

hybridization was performed in a moist chamber at 37°C for 48 hours. For each batch of hybridization, two control experiments were performed: hybridization of normal male against normal female and hybridization of DNA from previously characterized breast cancer cell line, MCF-7, against normal female DNA.

The hybridizations were analyzed using a digital image analysis system as described previously (Kallioniemi *et al.* 1994b). The hybridization results were visualized using an epifluorescence microscope (Olympus BX, Olympus Co., Tokyo, Japan) equipped with a cooled charge coupled device camera (CCD, Xillix Technologies, Vancouver, Canada) and interfaced to a Sun LX workstation (Sun Microsystems, Mountain View, CA, USA). Interpretation of the results and quality control followed previous guidelines (Kallioniemi *et al.* 1994a). Chromosomal regions for which the mean green to red ratio (minus one standard deviation of this ratio) fell below 0.85 were considered to be losses, whereas gains were defined as the mean ratio (plus one standard deviation of this ratio) was above 1.15.

2.3 Arm-specific multicolor-FISH (*arm*FISH)

*Arm*FISH, a new modification of the mFISH method, was used in Study II to evaluate chromosomal aberrations in eleven glioma cell lines. The slides with metaphase cells were prepared according to the standard protocol and stored at -20°C. Before hybridization, the slides were kept at room temperature for 1-2 days.

The *arm*FISH hybridization was done as described previously (Karhu *et al.* 2001) with minor modifications. Briefly, the *arm*FISH was analyzed in two steps. First, the conventional mFISH image analysis with commercially available mFISH-kit (24XCyte, MetaSystems GmbH, Altslussheim, Germany) was performed, followed by an analysis with a set of chromosome arm-specific painting probes (Guan *et al*. 1996). The *arm*FISH hybridization cocktail contained 5 µl of mFISH-kit probe reagent and 0.5 µl of digoxigenin-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) labeled chromosome arm-specific painting probes (hereafter called the arm-kit). The arm-kit consisted of painting probes specific for all human p- *or* q-chromosome arms (except acrocentric chromosomes and Y): 1q, 2p, 3p, 4p, 5p, 6q, 7p, 8q, 9p, 10q, 11q, 12q, 16p, 17p, 18p, 19p, 20p and Xp. Heterochromatin regions were blocked in mFISH hybridization and therefore remained unhybridized. The arm-kit hybridization was detected by horseradish peroxidase-conjugated anti-digoxigenin (Roche Molecular Biochemicals) diluted (1:300) in the blocking reagent (NEN™, Boston, MA, USA). The

procedure was followed by a signal amplification step with biotinyl tyramide (NEN™) (diluted 1:100). The hybridization results were visualized by LaserPro™ IR790 (1:300) (Molecular Probes, Inc., Eugene, OR, USA). The slides were counterstained with DAPI (4´, 6-diamino-2-phenylindone) in antifade solution (MetaSystems GmbH).

Digital images were captured by a Zeiss Axioplan II epifluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) with filters for DAPI, DEAC, FITC, Cy3, TexasRed, Cy5, and Cy7 in an 8-position motorized reflector turret (all filters were from Chroma Technology Corp, Brattleboro, VT, USA). The *arm*FISH analysis was performed stepwise by using ISIS 3.2.0 mFISH software (MetaSystems, GmbH). First, the chromosomes were classified and translocations were evaluated according to the standard mFISH analysis. Second, the chromosome arms involved in rearrangements were identified by comparing the arm-kit hybridization pattern (+ or -) present on the chromosomes with the chromosome classification. The International System for Human Cytogenetic Nomenclature (ISCN)(1991, 1995) was used for the mFISH karyotype with minor exceptions. Chromosomes or chromosome arms present in the derivative chromosome were listed from the p-arm to the q-arm (long arm of the chromosome).

2.4 C-banding

In Study II, C-banding was used for the evaluation of the number of centromeres, analysis of the marker chromosomes unhybridized in mFISH to indicate their heterochromatic origin, and for the evaluation of the morphology of interphase nuclei in eleven glioma cell lines. C-banding was done using the standard method. Briefly, three-months-old slides stored at -20°C were first treated with 0.2 N HCl for 60 minutes followed by $Ba(OH)_2$ for 2.5 minutes before the incubation in SSC (standard saline citrate) at +60°C for one hour. The chromosomes were stained with 2% Giesma solution.

2.5 cDNA microarray

In Study III, cDNA microarray (Schena *et al.* 1996) was used in order to pinpoint differentially expressed genes between normal brain and diffuse astrocytomas as well as between one patient's primary tumor (grade III) and recurrence tumor eight months later (also grade III astrocytoma).

Differentially expressed genes between normal brain and diffuse astrocytomas. Total cellular RNAs from freshly frozen tumor samples were extracted according to the manufacturer´s instructions using RNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany).

The cDNA membranes of 588 individual cDNA clones as targets (Atlas Human Cancer cDNA Expression Array 7742-1; Clontech Laboratories, Inc.) were used for the analysis of differentially expressed genes between normal brain and Grade II-IV astrocytomas. Equal amounts (2.5 μg) of total RNA from the two Grade II astrocytomas were pooled for the cDNA microarray analysis. The total RNAs from the two Grade III astrocytomas were similarly pooled together as well as total RNAs from the two GBMs. In addition, 5 μg of pooled total RNA from human brain (Clontech Laboratories, Inc.) was used for cDNA analysis. Labeled cDNA probes were prepared from the pooled sample RNAs by single-pass reverse transcription reaction with SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA) using [α-^{32}P]dCTP as a labeled nucleotide. The probes were purified by gel chromatography (BioSpin 6, Bio-Rad, Hercules, CA, USA), after which the residual RNA was degraded with alkaline hydrolysis in 1 M NaOH at 68°C for 20 minutes. The probes were neutralized with 1 M NaH_2PO_4 at 68°C for 20 minutes. The membranes were prehybridized in an Express Hybrid solution (Clontech Laboratories, Inc.) containing 100 μg/ml freshly cooked shared salmon sperm DNA at 68°C for two hours. The probes were hybridized together with Cot-1 DNA (Clontech Laboratories, Inc.) onto the membranes at 68°C overnight. After hybridization, the membranes were washed four times in low stringency wash buffer (2x SSC-1% SDS) and twice in high stringency wash buffer (0.1x SSC-0.5% SDS) at 68°C for 20 minutes each. The membranes were exposed to phosphoimager plates (Phosphoimager 2 SI; Molecular Dynamics, Sunnyvale, CA, USA) for 48 hours. The plates were scanned with a phosphoimager at a 50-μm resolution and analyzed with Image Quant software (Molecular Dynamics). A gene was regarded as overexpressed if the intensity of the subjectively visible signal in the tumor membrane was ≥ 1.8x higher than the signal of the corresponding spot in the normal brain membrane. In turn, a gene was regarded as downregulated if the intensity of the visible signal in the normal brain membrane was ≥ 1.8x higher than the signal of the corresponding spot in the tumor membrane. In addition, the hybridization results were semiquantitatively inspected to categorize signals for 1) no signal, 2) a visible signal and 3) a strong-intensity signal.

The differentially expressed genes between normal brain and GBMs were also studied using the membranes of 5760 individual cDNA clones (Human GeneFilter. Release 1, GF200; Research

Genetics, Inc., Huntsville, AL, USA) as targets for cDNA microarray hybridization. Equal amounts of total RNA from the three GBMs were pooled (52.5 μg total). A similar amount of pooled total RNA from human brain (Clontech Laboratories, Inc.) was used for cDNA microarray hybridization. The cDNA microarray hybridization was performed as described above, with some modifications. [α-^{33}P]dCTP was used as a labeled nucleotide in the preparation of cDNA probes. Hybridization and washings were done at a lower temperature of 60°C. The membranes were exposed to phosphoimager plates for 24 hours, scanned with a phosphoimager at a 50-μm resolution and analyzed using Pathways Software (Research Genetics, Inc.). Images were normalized using all spots on the membrane as reference spots. A cut-off point of 1.8 intensity ratio for up-regulated and down-regulated genes was determined from a histogram analysis of the intensity ratios of all of the spots on the membrane.

Differentially expressed genes between one patient's primary and recurrent astrocytoma. Gene expression profiles of one patient's primary astrocytoma (Grade III) and recurrence (also Grade III) were analyzed using membranes of 588 individual cDNA clones (Atlas Human cDNA expression Array 7740-1, Clontech Laboratories, Inc.) as described above.

2.6 Tissue microarray

For Study III, a high-density tissue microarray (TMA) of 418 brain tumors was constructed and used for clinical validation of gene expression changes pinpointed in the cDNA microarray analysis. The construction of the TMA was done as described elsewhere (Figure 5) (Kononen *et al.* 1998). Briefly, a neuropathologist first evaluated the tumors using H&E-stained standard slides to pinpoint the histologically most representative tumor area in each tumor. Second, the TMA block was constructed from these most representative tumor regions with a custom-built instrument (Beecher Instruments, Silver Spring, MD, USA). The tumor specimens were obtained from the diagnostic, formalin-fixed paraffin-embedded tumor blocks with a diameter of 600 μm. They were placed in the microarray block at regular intervals of 100 μm. In 20 randomly selected tumor cases multiple samples were collected from different sites of the subjectively most representative tumor region to evaluate intratumor heterogeneity within one selected tumor area.

Five-μm tissue microarray sections were cut using an adhesive-coated tape system (Instrumedics, Hackensack, NJ, USA) for IGFBP2, p53 and vimentin immunohistochemistry. To control the histology in the tissue microarray sections, H&E-stained slides were used. The immunohistochemistry

of IGFBP2, p53 and vimentin is described in more detail above in the chapter on immunohistochemical staining. The results of the p53 immunoreactivity in the tissue microarray were compared to our previous analysis of p53 immunoreactivity in deparaffinized whole tumor sections in the case of 42 tumor samples.

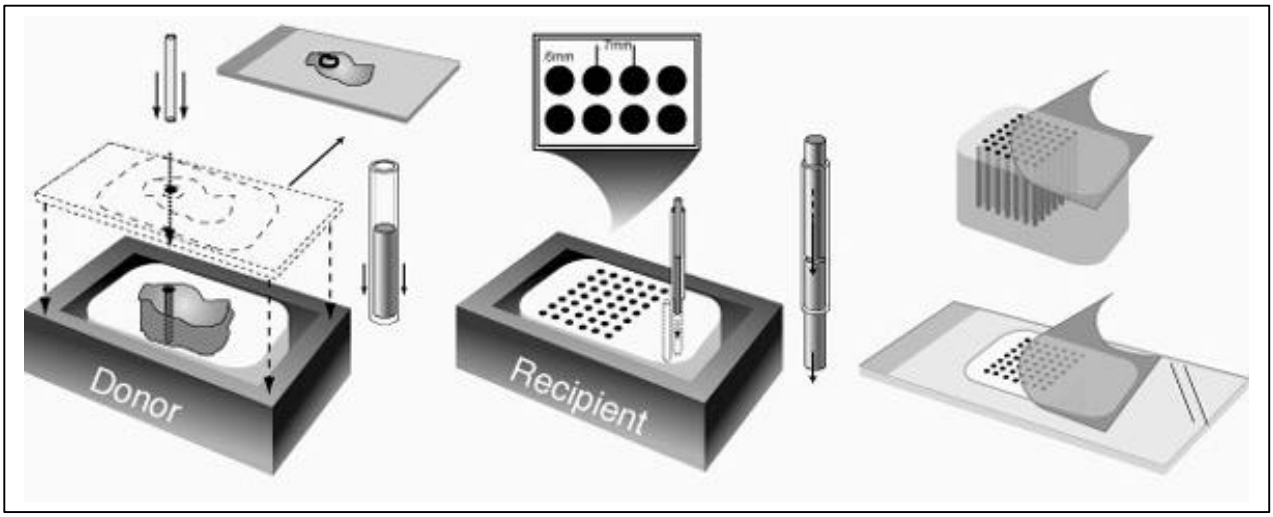

Figure 5. Construction of the tissue array. Cylindrical tissue biopsy with a diameter of 0.6 mm was obtained from the morphologically representative site of donor tissue block. A hole for a new specimen in the growing array (recipient block) was created, where the tissue core was deposited. The array block was sectioned using adhesive-coated tape sectioning technique.

2.7 Messenger-RNA *in situ* hybridization

Messenger-RNA *in situ* hybridization was used in Study IV to evaluate the *cyclin D1* expression in diffusely infiltrating astrocytomas as described previously (Dagerlind *et al.* 1992). Cyclin D1 cDNA was cloned to the *Bst*XI restriction site of the pRcD1/CMV. Riboprobe System-SP6/T7 (Promega, Madison, WI, USA) was used for *in vitro* transcription of sense and antisense cRNA probes in the presence of ^{35}S-UTP (DuPont, New England Nuclear research Products, Boston, MA, USA). The hybridization mixture included the labeled probe [10^7 counts per minute (cpm)/ml], 50% formamide, 2x SSC, 20 mM Tris (pH 8), 1 X Denhardt´s solution, 1 mM EDTA, 10% dextran sulphate and 500 µg/ml yeast RNA. Five µm tissue sections were digested with proteinase-K (Sigma) prior to hybridization at 55°C overnight. After hybridization, the slides were washed and exposed to

phosphoimager plates (Molecular Dynamics, CA, USA) for 24 hours. Using Image Quant™ software the tumor area with the most intense hybridization signal was anlyzed. The cyclin D1 mRNA expression was recorded as the total radioactivity per day of exposure in 1 mm^2 of the tumor tissue ($cpm/day/mm^2$). As negative controls, a sense cyclin D1 cRNA probe and RNase treatment were utilized.

2.8 Fluorescence in situ hybridization (FISH)

Dual-color FISH of tumor interphase nuclei was performed as described previously (Hyytinen *et al.* 1994). In Study I, CGH results were validated with dual-color FISH using a chromosome locus 11q13 specific probe D11S3935. In Study IV, a locus-specific PAC-probe for cyclin D1 was obtained by screening the PAC-library by PCR with primers specific to cyclin D1 (Ioannou *et al.* 1994). In both studies, the reference probe was targeted on pericentromeric repeat regions of chromosome 11 (pLC11A).

The probes were labeled indirectly by nick-translation using digoxigenin-11-dUTP (locus specific probe) or biotin-14-dATP (reference probe). A mixture of 30 ng digoxigenin-labeled locus specific probe, 10 ng biotin-labeled reference probe and 10 μg unlabeled human placental DNA (Sigma Chemical Co., St Louis, MO, USA) were denatured and applied to denatured tumor interphase nuclei on the slides. The hybridization was performed in a moist chamber at 37°C for 48 hours. The probes were visualized with 5 μg/ml avidin-FITC (Vector) and 2 μg/ml anti-digoxigenin-rhodamine (Boehringer Mannheim, Indianapolis, IN, USA), and the FITC signal was amplified with biotinylated anti-avidin antibody (5 μg/ml, Vector) and avidin-FITC.

In addition, the tumor array was utilized in FISH analyses using locus specific identifier DNA probe for *cyclin D1* (LSI®, Vysis Inc.). 5 μm paraffin-embedded, formalin-fixed tissue sections were used for the analyses. The hybridization was done according to the manufacturer´s instructions using a commercially available Paraffin Pretreatment Kit (Vysis Inc.).

The hybridization results were scored using either an Olympus BX (Olympus Co.) or a Zeiss Axioplan II (Carl Zeiss Jena GmbH, Jena, Germany) epifluorescence microscope equipped with a multiband pass filter system (Chroma Technology Corp., Brattleboro, VT, USA) and 60x and 100x objectives. Approximately one hundred non-overlapping nuclei (conventional interphase FISH) or cells (tumor

array) in every sample were analyzed. Amplification of the corresponding gene was recorded if the gene-specific probe signals outnumbered at least two-fold those of the reference probe. Normal lymphocytes served as a negative control.

2.9 Statistical methods

The statistical methods used in Studies I, III and IV included the chi-square test, the Mann-Whitney test and univariate survival analysis (log rank) as described in more detail in Studies I, III and IV. The best prognostic cut-off points for the survival analyses were determined by the receiver operating characteristic (ROC) curve. Pearson's correlation coefficient was used for comparison of the data between arrayed samples and standard sections. All the analyses were performed with SPSS for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Chromosomal aberrations in astrocytomas

By CGH, gains on chromosomes 1p34-pter, 11q13 and X were most frequently shown in eleven Grade II astrocytomas by CGH (Figure 6). In 13 higher malignancy grade astrocytomas, Grades III-IV, gains on chromosomes 7q, 8q, 10p13-pter and 17q and loss on chromosome 13q were the most common CGH findings (Figure 7). Losses on chromosomes 4q, 6q, 9p, 10, 13q and 14q as well as gains on chromosomes 7, 19 and 20/20q characterized chromosomal aberrations in nine glioma cell lines (Figure 8a).

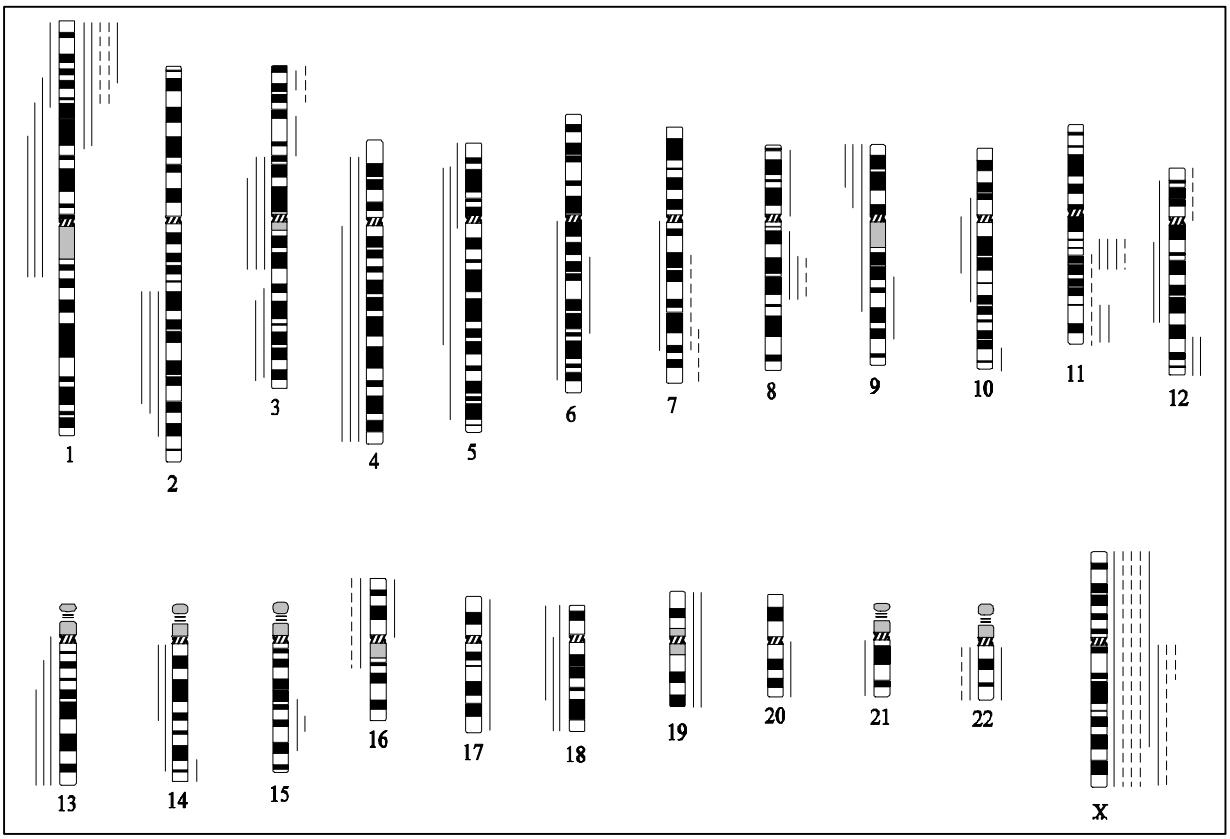

Figure 6. Summary of chromosomal aberrations in 11 Grade II astrocytoma detected by CGH. Continuous lines represent the tumors with poor prognosis (n = 4) and discontinuous lines represent the tumors with more conventional or "good" prognosis (n = 7). Losses are indicated by lines on the left side of the chromsome idiogram, and lines on the right side represent gains.

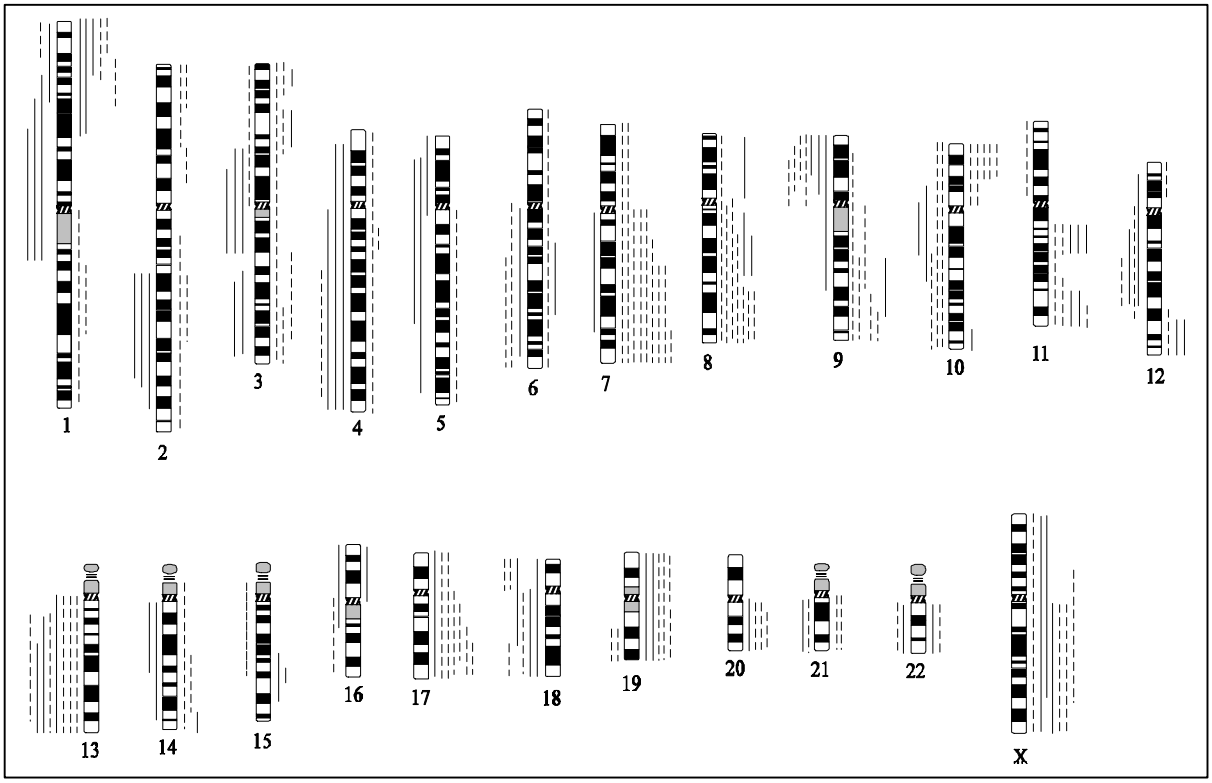

Figure 7. Comparison of the chromsomal changes detected by CGH between aggressively behaving Grade II astrocytomas (n = 4, continuous lines) and Grade III-IV astrocytomas (n = 13, discontinuous lines). Losses are indicated lines on the left side of the chromosome idiogram and lines on the right side represent gains.

All of the eleven glioma cell lines analyzed by *arm*FISH showed both numerical and structural aberrations. The total number of chromosomal changes varied between 14 and 65 per cell line. The most common numerical changes were extra copies of chromosomes 1, 7 and 20 and losses of chromosomes 10, 13 and 14 (Figure 8b). The comparison of the number of cells with extra copies of chromosomes to those with losses on corresponding chromosomes (or vice versa) pinpointed the extra copies of chromosomes 5, 7 and 20 as well as losses of chromosomes 4, 10, 14 and 22. The numerical aberrations were in good concordance with the results of the CGH analyses in nine of the glioma cell lines (Figure 8).

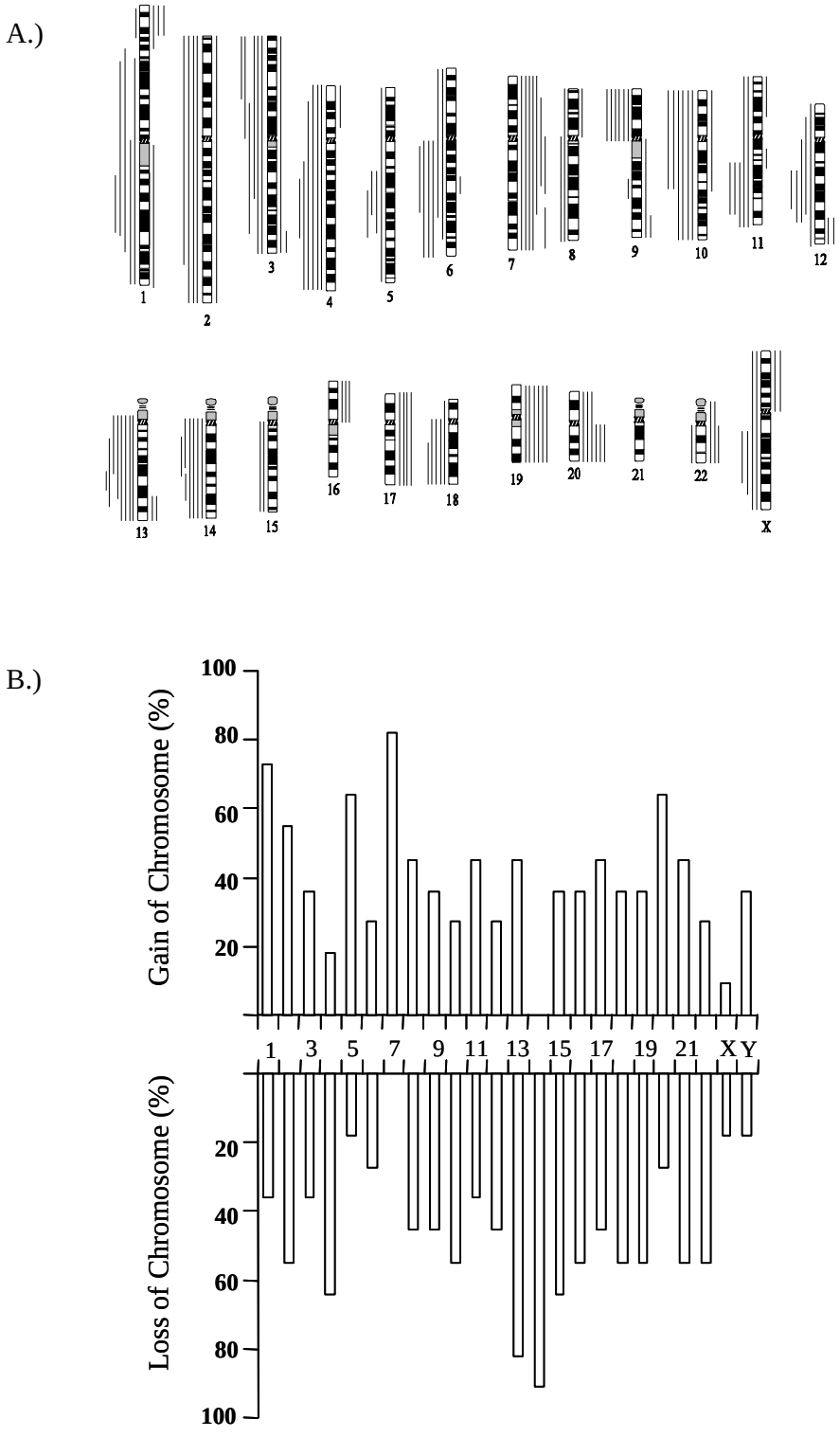

Figure 8. Numerical aberrations in glioma cell lines detected by CGH and *arm*FISH. In A) summary of chromsomal aberrations detected by CGH in 9 glioma cell lines is presented. Chromsomal losses are indicated by lines on the left side of the chromsome idiogram, and lines on the right side represent gains. In B) summary of numerical changes detected by armFISH in 11 glioma cell lines is shown.

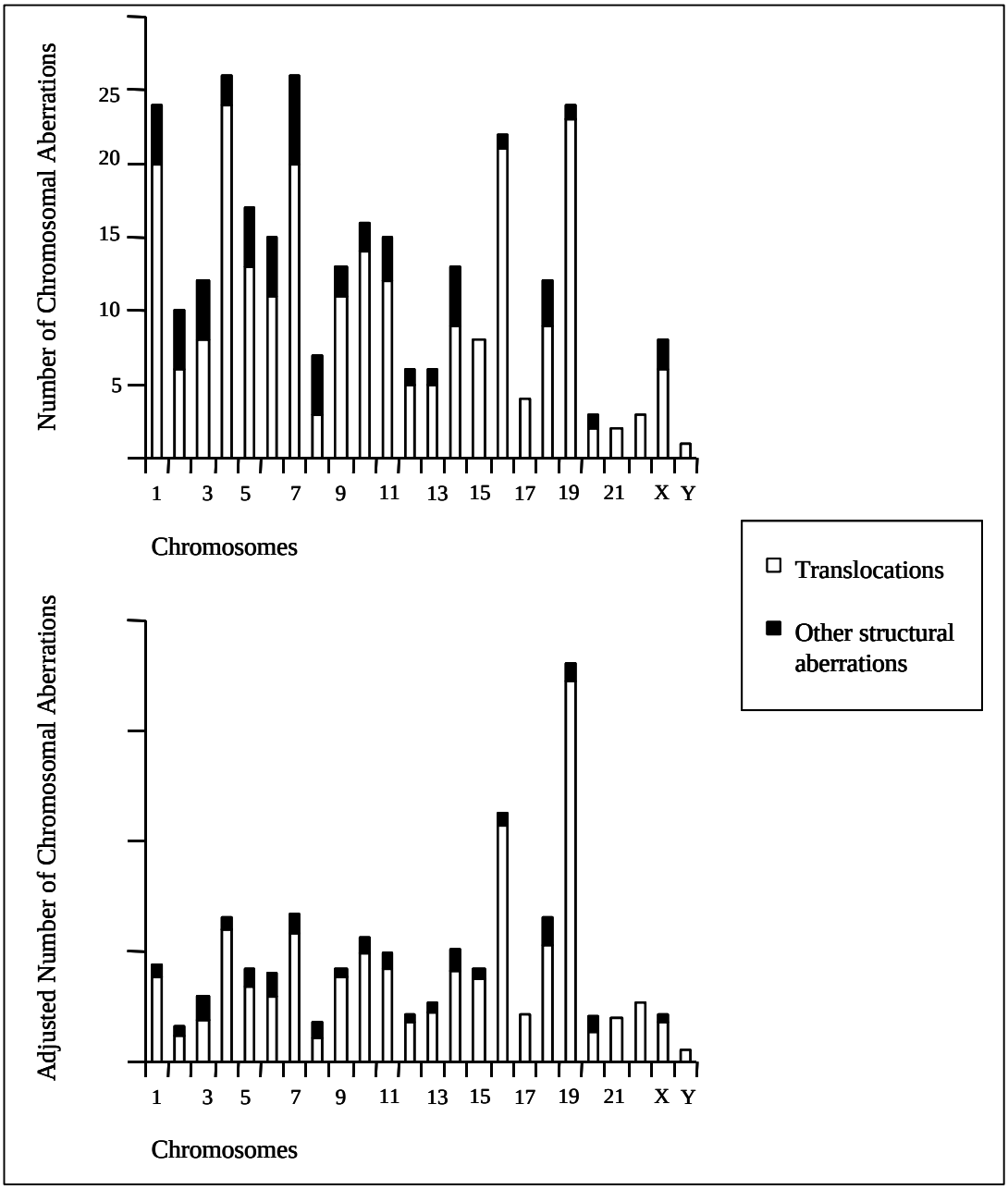

Figure 9. Structural chromsomal changes in 11 glioma cell lines by *arm*FISH. The total number of structural alterations (top) are adjusted to the lenght of correspondence chromosomes (bottom). The adjustment pinpoints involvement of chromsomes 16 and 19 in structural chromosomal aberrations in glioma cell lines

With *arm*FISH, the most common structural changes in glioma cell lines were translocation events. The majority of these were unbalanced translocations (91 unbalanced translocations versus 5 balanced translocations). Other structural aberrations obtained included chromosomal deletions, duplications, isochromosomes or isoderivative chromosomes, dicentric chromosomes and ring chromosomes. In addition, two of the cell lines harbored heterochromatin alterations. The structural changes most frequently affected chromosomes 1, 4, 7, 16 and 19 (Figure 9). The comparison of the change with the length of the corresponding chromosome pinpointed chromosomes 16 and 19 (Figure 9). Unlike the numerical changes, no structural aberration unique in most or all glioma cell lines could be found.

The eleven glioma cell lines analyzed by *arm*FISH were also evaluated by C-banding. Nuclear abnormalities (anaphase bridges, micronuclei) were found in seven cell lines, dicentric chromosomes in three cell lines and small marker chromosomes in one cell line.

2. Genetic changes in Grade II astrocytomas with typical (good) or poor prognosis

Four of the eleven Grade II astrocytomas analyzed by CGH had led to very poor patient survival (< 2.5 years). These tumors harbored significantly more chromosomal changes than those of the patients with better prognosis (median number of changes: 15.5 versus 2, range 8-28 versus 0-4, $p=0.008$, Mann-Whitney test). The prognostic cut-off point could be placed on 4 observed chromosomal abnormalities (recurrence-free survival: $p<0.001$; overall survival: $p<0.001$). The extended 10-year follow-up confirmed the prognostic significance of the original CGH results (survival time [95% CI]: 3038 days [2442-3634] versus 710 days [543-877], $p<0.001$), and only one patient died during the extended follow-up. Survival differences could not be predicted by Ki-67 cell proliferation indices.

Nearly all observed aberrations in the subgroup of Grade II astrocytomas with good patient prognosis were chromosomal gains (14/16), whereas the majority of changes in Grade II tumors with poor prognosis were chromosomal losses (38/67). Gains on chromosomes 1p, 3p, 8q, 11q and X as well as losses on chromosomes 16p and 22q were found in both prognostic tumor subgroups. Gains on chromosomes 6q, 8p, 9q, 10q, 12q, 14q, 15q, 16p, 17, 19, 20, and 22 and losses on chromosomes 1p, 2q, 3, 4q, 5, 6q, 7q, 9p, 10q, 12, 13q, 14q, 18 and 21 were only observed in Grade II astrocytomas associated with poor patient prognosis. The majority of these aberrations also characterized the high-grade astrocytic tumor group (Figure 7).

3. Genetic alterations in astrocytomas by cDNA array and TMA

3.1. cDNA microarray

cDNA microarray analyses demonstrated an accumulation of gene expression changes in GBMs, as expression alterations of over 200 individual genes were found (cDNA membrane from Clontech: 117 overexpressed and 24 downregulated genes; cDNA membrane from Research Genetics: 107 overexpressed and 111 downregulated genes). On the other hand, relatively few gene expression changes were found in Grade II and Grade III astrocytomas (Grade II: 38 overexpressed and 12 down-regulated genes; Grade III: 32 overexpressed and 10 down-regulated genes). The majority of gene expression alterations in Grade II-III astrocytomas were also detected in GBMs with a pattern of elevated expression along with increasing histopathological malignancy. In GBMs, the most intensive gene expression signals (Clontech membrane) pinpointed *IGFBP2*, *plasminogen activator inhibitor-1* (*PAI-1*), *activator 1 40 kDa subunit* (*RFC40*), *fibronectin* and *VEGF*. Downregulated genes included *p53-induced gene-10* (*PIG10*), *receptor tyrosine kinase* (*SKY*), *neuroendocrine Drosophilia discs large* (*NE-dlg*), *cyclin-dependent kinase 4 inhibitor D* (*p19INK4d*) and *T-lymphoma invasion and metastasis inducing* (*TIAM1*).

The cDNA microarray of pooled GBM samples using a membrane with 5760 individual targets (Research Genetics) revealed 107 genes that were overexpressed and 111 genes that were downregulated when compared with the analysis results of a normal brain.

A Grade III astrocytoma by phenotype and its reoccurrence of Grade III astrocytoma eight months later differed from each other according to the cDNA microarray data. Reciprocal comparison of the data revealed a number of genes that had become activated (e.g. *IGFBP2* and *VEGF*) or inactivated (e.g. *PDGFR-α* and *MacMarcks*) during tumor regrowth.

3.2. Brain Tumor TMA

The results of the IGFBP2 and vimentin immunostainings showed no variation between the 20 randomly chosen duplicative samples.

IGFBP2. Considering primary Grade II-IV astrocytomas, strong IGFBP2 immunopositivity was closely associated with the established histopathological malignancy grade ($p<0.0001$, chi-square test):

21% of Grade II astrocytomas, 53% of Grade III astrocytomas and 88% of GBMs were strongly positive for IGFBP2. IGFBP2 expression associated with poor patient survival ($p<0.0001$, log-rank test), a tendency also found within a subgroup of Grade III astrocytomas ($p=0.081$). Twenty-nine patients had samples of both their primary operation and tumor reoccurrence in the tissue array. Strong IGFBP2 immunoexpression characterized 21 of the 29 reoccurring tumors. An increase in the histopathological malignancy grade was recorded in nine tumor reoccurrences. Six of the nine reoccurring tumors showed an increase in and two equally strong IGFBP2 expressions when compared to the primary tumors.

Vimentin. Nearly all primary Grade II-IV astrocytomas (100% of Grade II-III astrocytomas and 98% of GBMs) were strongly positive for vimentin. For oligo-astrocytomas (mixed gliomas), strong vimentin immunopositivity characterized rather tumors with predominant astrocytic than oligodendroglial component.

Arrayed samples vs. corresponding standard sections. Comparison of data obtained from tissue array analysis with that of analyses on standard sections revealed similarly recorded TP53 results in 33 of the 42 tumors ($p<0.001$, chi-square test). Qualitative analyses of TP53 immunohistochemical staining (negative *versus* positive) in primary astrocytomas of tissue array showed a significant statistical correlation with tumor malignancy grade (Grades I-II versus Grades III-IV; $p<0.001$, chi-square test).

4. Expression and Prognostic Significance of *Cyclin D1* Expression

Cyclin D1 mRNA and protein expression levels varied significantly between different tumors and different regions of individual tumors. Cyclin D1 expression was clearly elevated in the GBM group when compared to Grade II-III tumors (mRNA expression: $p<0.001$, Mann-Whitney test; immunoexpression: $p=0.013$, chi-square test). Overall 74% of the astrocytic tumors with high cyclin D1 mRNA expression were also cyclin D1-immunopositive ($p=0.011$, chi-square test). Elevated cyclin D1 expression was closely associated with poor patient prognosis (mRNA expression: $p<0.001$; immunostatus: $p=0.031$, log rank). Cyclin D1 mRNA expression was also a strong prognosticator within the subgroup of Grade II and III astrocytomas ($p<0.001$, log rank).

Cell proliferation activity (by Ki-67 (MIB-1) labeling index (LI) and mitotic count) was closely correlated with both high cyclin D1 mRNA expression ($p<0.001$, Mann-Whitney test) and cyclin D1 immunopositivity (Ki-67 (MIB-1) -LI: $p=0.002$; mitotic count: $p=0.012$, Mann-Whitney test). Aberrant

p53 immunoexpression also correlated with elevated cyclin D1 expression levels (p=0.013, Mann-Whitney test). Nonetheless, *cyclin D1* gene amplification was not detected in any of the seven astrocytomas studied by FISH (standard interphase preparates). Extended FISH analyses with the *cyclin D1* specific probe on a tissue array of 259 astrocytic tumors revealed low-level (2-3 fold) *cyclin D1* gene amplification in 20 tumors (one pilocytic astrocytoma, three Grade II astrocytomas, two Grade III astrocytomas and 14 GBMs) (unpublished data).

DISCUSSION

This study was performed during an era of innovative achievements in cancer research. While general interest has been in the Human Genome Project, a major effort has also been expended on molecular and molecular cytogenetic techniques that could facilitate diagnostic and prognostic decision-making in clinical practice. The conventional techniques such as immunohistochemical antibody demonstration, mRNA and FISH methods, are still of great importance in evaluations of specific targets of interest, but the new high-throughput screening methods, such as cDNA microarrays and tissue arrays, have been developed to rapidly gather a vast amount of information through single experiments. Genome-wide strategies, such as CGH and *arm*FISH, add to these screening methods by allowing the researcher to evaluate gross total genetic changes along all the chromosomes in a single hybridization analysis.

1. Chromosomal aberrations in astrocytomas using CGH and *arm*FISH

It should be noted that both CGH and multi-color FISH have already been adopted as aids in the diagnosis of leukemia and lymphomas (reviewed by Avet-Loiseau 1999). In addition, preliminary data suggests that the resolution of CGH could suffice for distinguishing clinically relevant subtypes of cancer (Vettenranta *et al.* 2001). Considering astrocytic tumors, this clinical utility of CGH could aim at the detection of different progression pathways, as suggested by Wiltshire *et al.* (2000). The idea of being able to point to "genetic markers" associated with tumor progression drove the focus of the present CGH study onto Grade II astrocytomas that, at some point, give rise to high-grade astrocytoma growth. An interesting extension to the CGH study was provided by the *arm*FISH analyses of glioma cell lines that appeared to conceal complex structural chromosome aberrations indicative of a highrate of genomic instability. Altogether, these two studies pinpoint an enormous genetic heterogeneity of astrocytomas: it is highly unlikely that two individual tumors with identical genomes would be found.

1.1 CGH study

Two features of astrocytomas became obvious through CGH. First, the numerical chromosome changes, i.e. chromosomal gains and/or losses, accumulated along with increasing histopathological malignancy grade of astrocytomas. Second, astrocytomas of the same histopathological malignancy

grade harbored similar chromosomal aberrations. The most frequently observed changes in Grade II astrocytomas were gains on chromosomes 1pter-p35, 11q13, and X. In Grade III-IV astrocytomas, losses on chromosomes 9p21 and 13q21-q33 and gains on chromosomes 7q, 8q, 9q33-qter, 10pter-p13, 17q23-q24, and Xcen-q25 were the most common findings. Considering the high-grade astrocytoma derived cell lines, losses of different parts of chromosomes 1, 2, 3, 4, 5, 6, 9p, 10, 11q, 12q, 13q, 14q, 18q, and Xq and gains of chromosomes 1p, 7, 17, 19, 20q and 22q were the most frequent numerical chromosome aberrations. These observations are in good concordance with the published CGH literature (Figure 3).

Considering tumor histopathology or conventional prognostic indicators, the Grade II astrocytoma group seemed quite homogeneous. Nonetheless, four of the eleven tumors had followed an unexpectedly aggressive clinical course, better matching that of high-grade astrocytomas. By CGH, the four Grade II astrocytomas differed considerably from the other seven counterparts in terms of both qualitative and quantitative chromosomal changes. In addition to those few changes detected in Grade II astrocytomas and mentioned above, the aggressive Grade II tumors harbored gains on chromosomes 6q, 8p, 9q, 10q, 12q, 14q, 15q, 16p, 17, 19, 20q, and 22q and losses on chromosomes 1p, 2q, 3, 4q, 5, 6q, 7q, 9p, 10q, 12, 13q, 14q, 18 and 21. These alterations were also frequent in high-grade (Grade III-IV) astrocytomas of the present study and listed in previous studies (see Figures 3B and 7). Interestingly, chromosomal losses appeared to accumulate along with aggressive tumor behavior. In Grade III-IV astrocytomas, the majority of the chromosomal changes detected were losses (71%). For Grade II astrocytomas, losses comprised 57% of all the detected chromosomal changes in the poor prognostic subgroup but only 12.5% in the subgroup of tumors with a more conventional clinical course. These findings are in good agreement with a previous study, in which an accumulation of chromosomal losses in a breast tumor was associated with poor patient outcome (Isola *et al.* 1995).

The CGH results demonstrate quite convincingly the clinical potential of the method in distinguishing aggressive behavior in astrocytomas. In the present context and within the resolution of the method, an overall accumulation of chromosomal aberrations or an increased rate of chromosomal losses could serve as a prognostic “genetic marker” in Grade II astrocytomas. Both these observations relate to the chromosomal instability of tumor cells, which becomes more clearly demonstrated by the *arm*FISH analyses (see below). Defects in tumor-suppressors, including inactivation of gatekeeper and caretaker genes by single point mutations or losses of chromosomal material, contribute to chromosomal instability. It would also be temptating to speculate that one or several of the above listed hot spots carry significance in the progression of astrocytic tumors from Grade II to histopathologically more malignant astrocytomas at the level of single genes. Although CGH is laborious and requires special

equipment, the screening of gross total genomic changes could be cost-effective in phenotypically low-grade astrocytomas.

1.2 *Arm*FISH study

*Arm*FISH is a technique that requires cultured specimens. The eleven commercially available cell lines investigated showed widespread chromosomal instability, which was reflected in considerable genomic heterogeneity, as chromosomal aberrations varied between the cell lines and cell-by-cell within cell lines. A vast majority of the glioma cell lines was found to be polyploid. Many of the numerical chromosome changes observed through CGH also became highlighted through *arm*FISH: losses on chromosomes 10, 13, 14 and 22 and gains on chromosomes 1, 7 and 20. Kubota *et al.* (2001) and Squire *et al.* (2001) also frequently demonstrated these numerical chromosomal changes in glioma cell lines by SKY analysis. It is also important to note that many numerical chromosome aberrations detected in glioma cell lines were also frequently observed in high-grade astrocytomas of the CGH study, giving support to the use of cultured cell lines in cancer research. Unlike numerical chromosome changes, no unique structural alteration between the cell lines could be found. In good concordance with the earlier multi-color FISH studies (Kubota *et al.* 2001, Squire *et al.* 2001), the most common structural change was a translocation event, which usually affected chromosomes 1, 4, 7, 16 and 19. When the length of the corresponding chromosome was taken into account (Morton 1991), chromosomes 16 and 19 seemed the most prone to translocations. Even though most of the translocation events (~ 95%) were unbalanced, those that appeared to better survive the genetic remodeling in cells were balanced translocations; 80% of the balanced translocations could be found in the great majority (> 80%) of the cells in a cell line, whereas only 37% of the unbalanced translocations could be detected in neighboring cells. Other structural aberrations revealed by *arm*FISH included chromosomal deletions, duplications, isochromosomes or isoderivative chromosomes, dicentric chromosomes and ring chromosomes.

The multiform structural aberrations observed in cell lines reflect chromosomal instability that, as mentioned earlier, renders tumors prone to progression. Gains or losses of chromosomal material have been recognized to underlie oncogene activation or inactivation of tumor-suppressors. Translocations may also result in aberrant gene expression by gene activation or gene fusion (Sánchez-García 1997), which, according to the results presented, makes chromosomes 16 and 19 interesting targets in the search for glioma-associated critical chromosomal breakpoints. In addition, heterochromatic alterations, including small marker chromosomes (verified to be heterochromatic by

conventional C-banding) and an insertion in the heterochromatin region found in two cell lines, may imply heterochromatin-dependent gene silencing as suggested eralier by Bannisten *et al.* (2001) as well as Ringrose and Paro (2001).

1.2 Aspects regarding CGH and *arm*FISH methods

This study describes two modern strategies, CGH and *arm*FISH, for the investigation of genetic changes underlying glioma growth. In order for a new method to gain general acceptance, the findings evinced require validation. Here among other observations, losses on chromosomes 10, 13 and 22 and a gain on chromosome 7 were frequently demonstrated by both methods. With respect to the current knowledge of the tumorigenesis of GBMs, allelic loss on chromosome 22q and activation of the platelet-derived growth factor system (*PDGFR* A-ligand at Chr 7p22) associate with early tumorigenetic changes. Inactivation of *RB1* gene on chromosome 13q14 could be found in roughly one-third of high-grade (Grade III-IV) astrocytic tumors. Inactivation of the *PTEN* gene, loss/LOH of chromosome 10 and amplification of the *EGFR* gene (epidermal growth factor receptor, Chr 7p12) have been shown to relate to the highly malignant growth acquired by GBMs (Kleihues *et al.* 2000, Holland 2001). The discrepancies in some of the results regarding numerical chromosome changes between CGH and *arm*FISH could, on the one hand, be explained by the methodological difference: CGH gives an average profile of a population of tumor cells obtained from the specimen, whereas *arm*FISH provides information about individual cells. For example, *arm*FISH analyses revealed that the same chromosome region could either contain a gain or be lost in different cells within one cell line. An average of the cell population by CGH analysis could make such a chromosome appear normal. Another reason for some of the discrepancy in results between the two methods could be the cell lines themselves, since the studies were performed on cultures at different passages.

Unlike CGH, *arm*FISH is not, as such, applicable for the investigation of genetic aberrations in solid tumors. However, *arm*FISH represents a powerful tool for the identification of chromosomal aberrations and their *formation patterns* in tumors with a complex genome at the level of chromosome arms. One such example of the applicability of *arm*FISH in revealing the progress of events during tumorigenesis comes from the analysis of the DBTRG-05MG cell line. Through multi-color FISH analysis, it became apparent that one to four copies of chromosomes 1, 4, 5, 11, 16 and 19 had been rearranged by several consecutive reciprocal translocations, resulting in ten derivative chromosomes. These derivative chromosomes could be detected in the majority of the cells analyzed. Further investigation using arm-specific painting probes revealed that chromosomes 16q and 19q had been lost

during the multiform chromosomal evolution. It is justifiable to claim that *arm*FISH, when compared to CGH or mFISH, dramatically improves the accuracy of studies of genetic abnormalities, for which reason any modification of the methodology that could make *arm*FISH more suitable for studies on astrocytic neoplasms would be appreciated.

2. cDNA Microarray and Tissue Microarray in Astrocytomas

The cDNA microarray represents a new genomic high-throughput technology that makes molecular profiling of human tumors rapid by a single hybridization analysis of thousands of genes simultaneously. High-density TMA, on the other hand, enables the resercher to screen for the expression of a gene of interest through hundreds of tumor samples on practically an overnight basis. Thus, the combination of these two high-throughput strategies provided an interesting research scenario for the present study.

2.1 Screening of gene expression in astrocytomas by cDNA microarray analysis

The study utilizing the membranes of 588 individual cDNA clones as targets (Clontech membranes) demonstrated relatively few genetic differences between normal brain and Grade II or Grade III astrocytoma, whereas a manifest accumulation of gene expression alterations was observed in GBMs. A total of 24% of the genes tested were differently expressed in GBMs from that in normal brain. The majority of the gene expression changes recorded from Grade II or Grade III astrocytomas were also present in GBMs. In addition, upregulated genes often followed an increasing expression pattern toward higher histopathological malignancy. This study also demonstrated a cDNA microarray analysis with 5760 individual targets (Research Genetics membrane) performed on pooled GBMs. The investigation revealed over 200 genes with expression differing from that in normal brain. Several gene expression alterations were common to both cDNA microarray analyses and some have been well documented in astrocytomas or other human tumors, including the upregulation of *VEGF* (Pietsch *et al.* 1997, Abdulrauf *et al.* 1998, Chan *et al.* 1998, Miyagami *et al.* 1998, Takekawa and Sawada 1998, Carroll *et al.* 1999, Laffuente *et al.* 1999, Oehring *et al.* 1999) and overexpression of *cyclin D1* (Pelosio *et al.* 1996, Shinozaki *et al.* 1996, Gansauge *et al.* 1997, Takeuchi *et al.* 1997, Inomata *et al.* 1998, Ishikawa *et al.* 1998, Kornmann *et al.* 1998, Rayson *et al.* 1998, Roncalli *et al.* 1998, Itami *et al.* 1999, Sarbia *et al.* 1999, Shimada *et al.* 1999, Dunsmuir *et al.* 2000, Yatabe *et al.* 2000).

The cDNA microarray analyses filled a database of gene expression alterations that had occurred during the tumorigenesis of a few astrocytic tumors. The function or significance to tumor evolution and progression of a number of those genes is yet to be shown, which is an appealing setup for any further investigations. However, the analyses were performed on pooled samples of two to three individual tumors, for which reason one needed to be particular about pinpointing any new "genetic marker" of astrocytoma growth. One gene that raised interest was *SPARC* (secreted protein acidic and rich in cysteine, also called osteonectin), located on chromosome 5q31.3-q32 (Le Beau *et al*. 1993). SPARC is involved in cell proliferation, repair of tissue damage, and modeling of extracellular matrix (reviewed by Brekken and Sage 2000). It has been shown to change endothelium permeability in response to certain types of injury (Goldblum *et al*. 1994). *In vitro,* increased SPARC expression has been demonstrated to promote GBM tumor invasion (Golembieski *et al*. 1999). Shortly after the publication of the original communication another study by Huang *et al*. (2000) pointed to the upregulated expression profile of *SPARC* in Grade II astrocytomas. In addition, they demonstrated positive immunoreactivity for SPARC in all diffuse astrocytomas studied. Ever since, the role of SPARC in tumorigenesis has been extensively investigated (Paley *et al*. 2000, Thomas *et al*. 2000, Sakai *et al*. 2001, Yamanaka *et al*. 2001). The comparison of the gene expression profiles of a primary Grade III astrocytoma with its later reoccurrence as a Grade III tumor directed attention to the expression pattern of *IFGBP2* (see below) through the earlier cDNA microarray analyses. The comparison study of two tumors obtained from the same patient, at the level of the expression of single genes, gave further support to those findings by CGH and *arm*FISH indicating to marked genetic heterogeneity among astrocytomas of similar histopathological appearance. Where both Grade III astrocytomas harbored many gene expression changes, the comparison of the genetic profiles of these tumors revealed considerable differences between the tumors. Furthermore, many genes that had been shown to be upregulated in GBMs were found to be upregulated in the recurrent Grade III astrocytoma. Such genes included *tumor necrosis factor receptor 2*, *IGFBP2* and *VEGF*.

2.2 Screening of astrocytomas for expression of candidate genes by TMA

The construction of a brain tumor array of 418 targets followed a careful practice of identification of the best representative tumor region for histopathological malignancy from which the tissue specimen was removed and placed on the array block. Considering that an astrocytoma may show marked genetic heterogeneity within the tumor sample as well as the very small size of the inserted specimen, a genetic marker of the growth would greatly facilitate sampling of truly representative tumor specimens. Immunohistochemical demonstration of areas under active cell proliferation could serve as such a

marker, but estimated cell proliferation activity does not necessarily predict aggressive behavior of low-grade astrocytomas according to the CGH study. Although sampling is an important issue and has raised some debate about the use of tissue arrays (reviewed by Rimm *et al.* 2001), it needs to be emphasized that a tissue array is merely a tool for screening. Here, the candidate genes collected for tumor array analyses were *IGFBP2*, *vimentin* and *cyclin D1*.

2.2.1 TMA of IGFBP2 and vimentin immunohistochemistry

According to the results of cDNA microarray analyses, *IGFBP2* becomes overexpressed during astrocytoma progression. A previous study has also demonstrated overexpression of *IGFBP2* in GBMs (Fuller *et al.* 1999). Here, the observation was validated by immunohistochemical demonstration of IGFBP2 in the brain tumor array. The results of the analysis revealed that most GBMs show strong IGFBP2 immunopositivity, whereas approfimately one half of the Grade III astrocytomas on the array remained IGFBP2 immunonegative. The tendency of IGFBP2 to correlate with poor survival among Grade III astrocytomas was particularly interesting, since mitotic activity as defined by the WHO for the grading of Grade III astrocytomas leaves plenty of room for interpretation. Considering that only 20% of Grade II astrocytomas and 28% of pilocytic (Grade I) astrocytomas expressed IGFBP2, the clinical value of IGFBP2 as a marker of high-grade astrocytoma malignancy needs further investigation. The insulin-like growth factor system (IGF) is composed of a family of ligands, receptor and binding proteins. In the circulation and extracellular space IGFs form normally complexes with their binding proteins (IGFBPs), which modulate their effects (Clemmons 1991). *IGFBP2* has been mapped to chromosomal region 2q33-q34 (Agarwal *et al.* 1991). Its gene expression is localized in astroglia, and it has a role in brain development as well as in neuroprotection after the injury to CNS (Lee *et al.* 1992, Woods *et al.* 1995, Breese *et al.* 1996).

In the present context, vimentin was selected in order to validate by a widely utilized immunohistochemical marker the combined use of a cDNA microarray and a TMA. *Vimentin* in the cDNA microarray analyses appeared to be a uniform marker of astrocytoma growth. Accordingly, immunohistochemical demonstration of vimentin in the brain tumor array showed strong immunopositivity in nearly all tumors of astrocytic origin (Grades I-IV). In addition, all ependymal and choroid plexus tumors were found to be immunopositive for vimentin. Oligodendrogliomas showed relatively large variation in vimentin expression, whereas those oligo-astrocytomas with a predominant astrocytic component often were positive for vimentin. These findings are in good agreement with the knowledge of vimentin immunoreactivity in gliomas (Graham and Lantos, 1997).

2.2.2 Analysis of cyclin D1 expression and amplification

Cyclin D1 was one of the overexpressed genes in the cDNA microarray assay of nearly 6000 gene targets performed on GBMs. In addition, CGH profiles of high-malignancy Grade III-IV astrocytic tumors (Figures 3 and 7) as well as those four Grade II astrocytomas associated with poor clinical prognosis (Figure 6) pointed to a frequent gain on chromosomal loci 11q13, which has been established to harbor the *cyclin D1* gene. Cyclin D1 expression pattern in astrocytomas was investigated by mRNA *in situ* hybridization (mRNA-ISH) analysis combined with immunohistochemistry on standard whole section specimens. According to these analyses, high-level cyclin D1 expression characterized the GBM group. This is in line with earlier studies that have shown an increasing expression pattern of cyclin D1 along with increasing histopathological malignancy of astrocytomas (Chakrabarty *et al.* 1996, Cavalla *et al.* 1998). In this study, elevated expression of cyclin D1 was also detected in those Grade II-III astrocytomas associated with poor patient prognosis.

The observed parallel distribution pattern of proliferating cells (by Ki-67 (MIB-1) and mitotic figures) and cyclin D1 expressing cells in tumor samples were of importance. This finding relates to the role of cyclin D1 as one of the key regulatory cyclins during the progression of cells through the G1 phase of the cell cycle (reviewed by Draetta in 1994). It was also possible that active cell proliferation of neoplastic cells merely modulated cyclin D1 expression, and other genetic disturbances rather than those directly affecting the *cyclin D1* gene were the engine of the increased rate of cell proliferation. One such alteration could be the abrogation of the *TP53* gene, as indicated by the close correlation between an accumulation of abnormal TP53 protein and elevated expression of cyclin D1 in astrocytomas. Therefore, FISH analysis was performed in order to investigate whether astrocytomas expressed amplification of *cyclin D1*. The first FISH analysis on seven astrocytic tumors did not reveal amplified *cyclin D1*. The brain tumor array, including 259 primary astrocytic tumors, was then utilized for an extended screening of the aberration. This investigation demonstrated an infrequent, low-level *cyclin D1* amplification in only 9% of the Grade II astrocytomas (3/24) and 7% of the Grade III astrocytomas (2/16) and in 8% of the GBMs (14/129). In addition to the diffusely infiltrating astrocytomas, one pilocytic astrocytoma (3%) harbored low-level *cyclin D1* amplification. According to the literature, amplification of *cyclin D1* is a rare event in astrocytic tumors. He *et al.* (1995) demonstrated *cyclin D1* amplification in one of the 29 GBMs investigated. Büschges and co-workers (1999) could not point to amplified *cyclin D1* in any of the 102 GBMs studied. Instead, one Grade III astrocytoma appeared to harbor *cyclin D1* amplification (12.5%). These two studies argue against the somewhat higher frequency of *cyclin D1* amplifications observed in this study. Here, a more sensitive

dual-color FISH analysis was used, in contrast to the Southern blot analysis of the previous studies. Altogether, it could be suggested that mechanisms other than *cyclin D1* amplification are responsible for the increased expression cyclin D1 observed by mRNA *in situ* hybridization analyses or immunohistochemical stainings.

3. Future prospects

Molecular genetic characterization of diffusely infiltrating astrocytomas will provide insights into astrocytoma biology and diagnosis and, eventually, lead to new treatment strategies. Today, the classification of astrocytomas, giving guidelines for patient treatment, is mainly based on the histopathological evaluation of the tumors (Kleihues *et al*. 2000). However, individual astrocytomas, even with the same established histopathological malignancy grade, differ in clinical behavior. It could be suggested that within each histopathological malignancy grade of diffusely infiltrating astrocytomas there are many genetic tumor entities with different growth rates and responses to therapy. Thus a new classification scheme of diffusely infiltrating astrocytomas that combines both histopathological and genetic evaluations of the tumors is highly warranted (Louis *et al*. 2001). Genome-wide, high-throughput methods could serve as a convenient tool for the subclassification of diffusely infiltrating astrocytomas as well as other human tumors. At present, it is possible to build up a cDNA microarray according to the way the question is put, e.g. a astrocytoma specific gene array.

Knowledge of the genetic aberrations underlying the development and growth of astrocytoma is needed for the development of new treatment strategies. Where conventional treatment of neoplasms relies on the cytotoxic effects of treatment agents, the novel treatment strategies would be targeted at specific gene defects in tumors. The tyrosine kinase family has become especially interesting during the development of new therapeutic agents for different cancers. Herceptin® treatment for *ERBB2* positive breast cancer patients is today clinical practice (Ross and Fletcher 1999). A tyrosine kinase inhibitor, imantinib (Glivec® or Gleevec®), has been demonstrated to be an effective treatment agent for patients with chronic myeloid leukemia and gastrointestinal stromal tumor (Cohen *et al*. 2002, Joensuu 2002). Considering diffusely infiltrating astrocytomas, *EGFR* targeted therapy has aroused special research interest (Sampson *et al*. 2000).

SUMMARY AND CONCLUSIONS

1. This study shows that an accumulation of genomic aberrations is associated with an increase in the histopathological malignancy of astrocytic tumors. The pattern could be detected by CGH analysis of routine histological specimens. CGH analysis revealed aberrations in chromosome regions that have been demonstrated to be affected or harbor genes that become affected during the tumorigenesis of astrocytomas. In addition, a number of other chromosome regions were found to be altered in CGH. These results show that CGH is a powerful tool for screening numerical chromosome aberrations genome-wide on diagnostic samples. Unlike CGH, *arm*FISH is not suitable as such for diagnostic routine due to the requirement of cultured cells. However, this study demonstrates the advantage of *arm*FISH over CGH in the analysis of complex chromosomal aberrations, as both numerical and structural chromosome alterations can be simultaneously analyzed at the level of chromosome arms. In addition, *arm*FISH allows the researcher to outline the progress of events through which alterations take place. Where *arm*FISH and CGH quite uniformly point to several common numerical chromosome aberrations in glioma cell lines, no unique structural aberration common to glioma cell lines could be found.

2. With CGH analysis, genetic heterogeneity was observed among Grade II astrocytomas. An accumulation of chromosomal aberrations was associated with unexpectedly poor prognosis that could not be predicted by histopathological appearance nor conventional prognostic markers such as cell proliferation index. Most of those gross total chromosomal changes found in Grade II astrocytomas with poor prognosis, whereas chromosomal gains predominated the subgroup of tumors with more conventional clinical outcome. In addition, a number of genetic alterations detected in Grade II tumors with poor prognosis were also frequent occurrences among Grade III-IV astrocytomas. These results confirm the view that genetic changes precede histopathological ones. Therefore, CGH could significantly increase the accuracy of diagnostic and prognostic settlement, especially of Grade II astrocytomas.

3. A few cDNA microarray analyses provided rapid screening of thousands of differentially expressed genes in astrocytomas. One of those genes that aroused our interest due to the increasing expression pattern along with higher-grade astrocytoma was *IGFBP2*. The immunoexpression of IGFBP2 was subsequently analyzed in 192 primary astrocytic tumors on a TMA. The results of the TMA analysis verified the accumulation of IGFBP2 expression in high-malignancy astrocytomas. The strategy of combining cDNA microarray and TMA analyses in the

present context demonstrates a whole new ideology of high-throughput techniques in the fast search for momentous genetic alterations in tumors.

4. Both CGH and cDNA microarray analyses implicated c*yclin D1* gene in astrocytoma growth. Analyses of whole tissue sections of astrocytoma specimens revealed that an elevated expression level of cyclin D1 mRNA and a positive cyclin D1 immunostatus associated with high malignancy. In tissue array analysis, amplification of *cyclin D1* was an infrequent event. In addition, low-level amplifications were found in all categories of histopathological malignancy, including one pilocytic (Grade I) astrocytoma. The FISH analyses indicate that mechanisms other than gene amplification are responsible for increased cyclin D1 expression in astrocytic tumors.

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