Genetic Alterations Associated with Development of Human Diffusely Infiltrating Astrocytomas

Alterações Genéticas Associadas com o Desenvolvimento dos Astrocitomas Infiltrativos Difusos Humanos

Rui Manuel Vieira Reis

Porto 2001

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Aos meus pais, a quem devo o que sou. A eles, à Paula, Gaspar, Cláudia e Sara, obrigado pela indespensável presença.

Ao abrigo do Art. 8º do Decreto-Lei nº 388/70 fazem parte integrante desta dissertação os seguintes trabalhos:

I. Rui M. Reis, Akira Hara, Paul Kleihues, Hiroko Ohgaki. Genetic evidence of neoplastic nature of gemistocytes in astrocytomas. *Acta Neuropathologica*, 2001, 102:422-425.

II. Hironori Fujisawa, Rui M. Reis, Mitsutoshi Nakamura, Stefano Colella, Yasuhiro Yonekawa, Paul Kleihues, Hiroko Ohgaki. Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. *Laboratory Investigation,* 2000, 80: 65-72.

III. Rui M. Reis, Mitsutoshi Nakamura, Jun Masuoka, Takao Watanabe, Stefano Colella, Yasuhiro Yonekawa, Paul Kleihues, Hiroko Ohgaki. Mutation analysis of *hBUB1, hBUBR1* and *hBUB3* genes in glioblastomas. *Acta Neuropathologica*, 2001, 101:297-304.

IV. Rui M. Reis, Riita Herva, Sebastian Brandner, John Koivukangas, Nikolai Mironov, Walter Bär, Paul Kleihues, Hiroko Ohgaki. Second Primary Glioblastoma. *Journal of Neuropathology and Experimental Neurology,* 2001, 60:208-215.

V. Rui M. Reis, Dilek Könü-Leblebicioglu, José M. Lopes, Paul Kleihues, Hiroko Ohgaki. Genetic profile of the gliosarcoma. *American Journal of Pathology,* 2000, 156:425-432.

Em cumprimento do disposto no referido Decreto-Lei declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a colaboração dos outros autores. Inclui também resultados, não publicados, relativos a mutações do gene *p53* em astrocitomas difusos.

NOTA EXPLICATIVA

A presente dissertação está escrita em inglês na sua quase totalidade, exceptuando o Sumário e Conclusões, pelo facto de a Doutora Hiroko Oghaki e o Professor Doutor Paul Kleihues terem sido seus co-orientadores.

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ARF – Alternative reading frame

ATM – Ataxia-telangiectasia mutated

ATR - Ataxia-telangiectasia and Rad3-related

BCNU – 1,3-bis(2-chloroethyl)-1-nitrosourea

bp – Base pair

BUB – Budding-uninhibited-by-benzimidazole

CDK - Cyclin dependent kinase

CDKN2A - Cyclin dependent kinase inhibitor 2A

CIP – CDK-interacting proteins

CNS - Central nervous system

CNTF – Cilliary neutrophic growth factor

CpG – Cytosine phospho guanine

CT – Computed tomography

DMBT1 – Deleted in malignant brain tumours 1

DNA – Deoxyribonucleic acid

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

FGF2 – Fibroblast growth factor 2

GAB1 – GRB2-associated binder-1

GADD45 – Growth arrested-and DNA damage-inducible gene

GDNPF – Glial derived neurite promoting factor

GFAP – Glial fibrillar acidic protein

GRB2 – Growth factor receptor-bound protein 2

H&E –Haematoxylin and eosin

HDM2 – Human double minute 2

HGF- Hepatocyte growth factor

HIF – Hypoxia-inducible factor

INK4 – Inhibitors of CDK4

LOH – Loss of heterozygosity

MAD – Mitotic-arrest-deficient

MAX – Myc-associated factor X

MBD4 – Methyl-CpG binding domain 4

MDM2 – Mousse double minute 2

MGMT – O^6-Methylguanine-DNA methyltransferase

MMAC1 – Mutated in multiple advanced cancers-1

MPS1 – Monopolar spindle-like 1

MRI – Magnetic resonance imaging

mRNA – Messenger RNA

MTS1 – Multiple tumour suppressor 1

MXI1 – Max interacting protein 1

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PIP-3 - Phosphatidylinositol (3,4,5)-triphosphatase

PKB – Protein kinase B

PTEN – Phosphatase and tensin homologue deleted on chromosome ten

Rb – Retinoblastoma

RNA – Ribonucleic acid

RTK - Receptor tyrosine kinase

RT-PCR – Reverse transcription-polymerase chain reaction

SPARC – Secreted protein acidic and rich in cysteine precursor

SSCP – Single strand conformation polymorphism

SV40 – Simian virus 40

TDG – Thymine-DNA glycosylase

TEP1 – TGF-regulated and epithelial cell-enriched phosphatase

TGF – Transforming growth factor

TIMP-3 – Tissue inhibitor of metalloproteinase 3

TSP-1- Trombospondin type 1

TYRO3 – Tyrosine protein kinase SKY

UV – Ultraviolet

WAF1 – Wild-type p53-activated fragment 1

INTRODUCTION

1-Epidemiological, clinical and histological features of diffusely infiltrating astrocytomas

1.1-Epidemiological features

Diffusely infiltrating astrocytomas constitute a distinct group within the astrocytic tumour types, including the following tumours subtypes: diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiforme [1,2].

Diffusely infiltrating astrocytomas are far the most frequent intracranial tumours and account for more than 60% of all primary brain tumours [2]. These tumours can develop at any age but with a peak of frequency in the fourth and fifth decades, and affect men more frequently than women, with overall male/female ratios ranging from 1.1 to 1.6 [1,2].

The incidence of tumours of the central nervous system (CNS) ranges between 7 to 9 new cases annually per 100,000 population, corresponding to less than 2% of all new cancers [1,3]. The highest rates are observed in developed areas (North America, Europe, and Oceania) and lowest in Africa and Pacific Islands (Fig. 1) [4,5]. The incidence of CNS tumours in Portugal is among the highest in the world, with 4.7 cases per 100,000 for women and 7 cases per 100,000 for men (Fig. 1) [5].
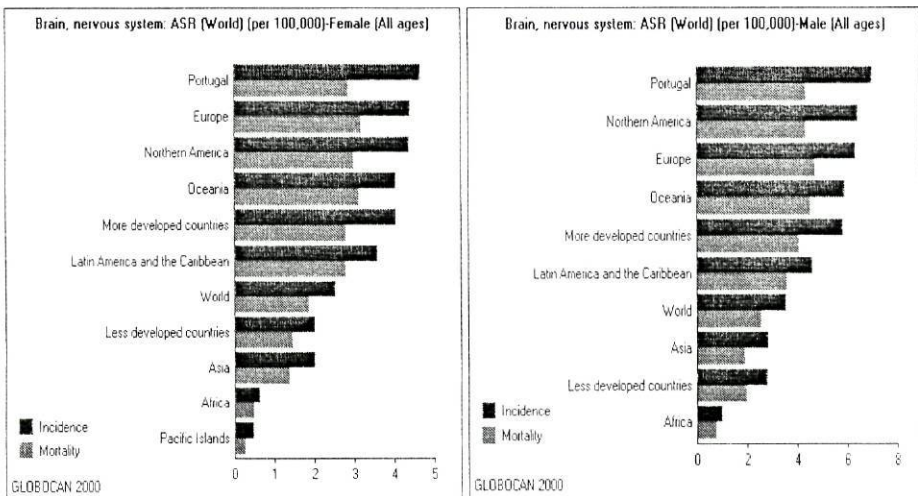
Fig. 1 - Incidence and mortality data of central nervous system tumours in the world (reproduced from Globocan, 2000 [5]).

Numerous studies reported an increase in brain tumour rates over the past several decades, particularly among the elderly [6-9]. However, several authors argued that this increase is largely artifactual, mainly reflecting improvements on the diagnosis and reporting of brain tumours [3,10,11]. For the same reason, brain tumour incidence may be

underestimated in many less developed countries and this may well explain, at least in part, the observed geographic differences [4].

1.2-Clinical and histological features

Diffusely infiltrating astrocytomas have the following features: they occur anywhere along the neuro-axis, but they are most frequent in the white matter of the cerebral hemisphere in adults and may extend to the cortex or basal ganglia; they have a wide range of histological features and biological behaviour; they tend to infiltrate the surrounding brain, and consequently, their complete removal is very difficult and they tend to recur. Frequently, the recurrence is associated with progression to more malignant lesions [1,2]. The factors associated with a more favourable clinical course in infiltrative diffuse astrocytomas are; young age of patient; high preoperative Karnofsky performance score; gross total tumour resection; and adjuvant radiotherapy and chemotherapy [1]. Unlike other solid tumours, these neoplasms very rarely metastasise outside the CNS. Diffusely infiltrating astrocytomas should be clearly differentiated from the other astrocytic tumours, namely, pilocytic astrocytoma, pleomorphic xanthoastrocytoma and subependymal giant cell astrocytoma that are typically encountered in children and young adults. These tumours lack the diffusely infiltrating growth pattern, have different biological ground and a more favourable prognosis [1,2].

Around 1950, Kernohan *et al*, [12] and Ringertz et al [13] introduced a grading system for astrocytic tumours to indicate prognosis, with four grades of increasing malignancy (grade I to IV). Currently, the most widely used is the World Health Organisation (WHO) classification of astrocytic tumours, which also includes the malignant grade and is organised in a malignancy scale (Table 1) [2].

Table 1 - Histological Classification of Diffusely Infiltrating Astrocytomas

WHO Designation	WHO Grade
Diffuse astrocytomas	II
Fibrillary astrocytoma	II
Gemistocytic astrocytoma	II
Protoplasmic astrocytoma	II
Anaplastic astrocytoma	III
Glioblastoma	IV
Giant cell glioblastoma	IV
Gliosarcoma	IV

1.2.1-Diffuse astrocytoma

Diffuse astrocytomas (WHO grade II) are also termed low-grade diffuse astrocytoma or "well-differentiated" astrocytoma. They are slow growing tumours, with a high degree of cellular differentiation, that diffusely infiltrate the normal brain structures and have a tendency for recurrence and progression to anaplastic astrocytoma (WHO grade III) and ultimately glioblastoma multiforme (WHO grade IV) [14-16]. Diffuse astrocytoma represents 10-15% of all astrocytic brain tumours and typically manifest in young adults with a peak of incidence between 30 and 40 years of age [14,15]. The meantime interval to progression to glioblastoma is 4-5 years with a considerable individual variation, with some cases remaining relatively latent for several years and others progressing rapidly [14].

Histologically three different variants can be distinguished: *fibrillary*, *gemistocytic* and *protoplasmic astrocytomas*.

1.2.1.1-Fibrillary astrocytoma

Fibrillary astrocytoma (Fig. 2A) is the most common variant and is composed of fibrillary neoplastic astrocytes, with elongated glial process expressing glial fibrillary acidic protein (GFAP). Nuclear atypia, including enlarged, elongated or irregular hyperchromatic nuclei, is a histological criterion differentiating neoplastic cells from normal and reactive astrocytes. Cell density is low to moderate [14-16].

1.21.2-Gemistocytic astrocytoma

Gemistocytic astrocytoma (Fig. 2B) is a rare variant with predominant presence of gemistocytes. Gemistocytes are characterised by voluminous, eosinophilic cytoplasm, eccentric nuclei, plump cell processes and marked expression of GFAP [14,16,17]. These tumours are particularly prone to progress to anaplastic astrocytoma and glioblastoma multiforme [14-18]. However, according to new WHO classification of brain tumours [14], this does not justify a general classification of the gemistocytic astrocytoma as anaplastic astrocytoma [14,16].
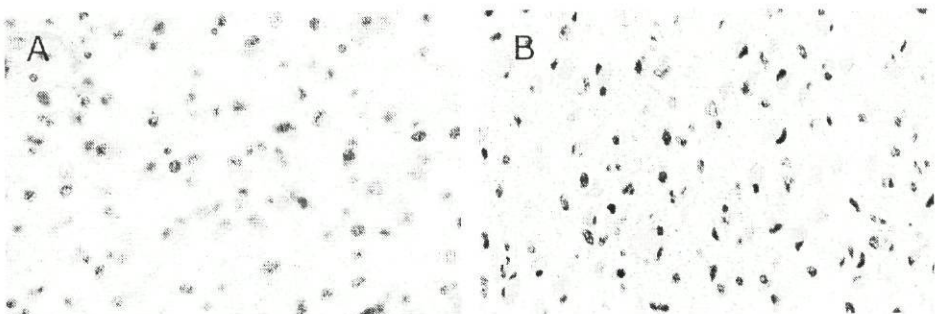
Fig. 2 - Histological appearance of fibrillary astrocytoma (A) and gemistocytic astrocytoma (B) (H&E staining).

It remains to clarify the cut off value of gemistocyte fraction associated with a worse prognosis. Krouwer et al. [18] in a series of 28 gemistocytic astrocytomas, reported that patients containing more than 20% gemistocytes showed a considerably lower 5-year survival rate when compared to those astrocytomas containing less gemistocytes. In the new WHO classification of brain tumours [14], a cut off value of 20% of gemistocytes fraction has been proposed for the diagnosis of gemistocytic astrocytoma. However, Watanabe et al [17] have reported that diffuse astrocytoma patients containing more than 5% gemistocytes had a significantly shorter time till progression to more malignant lesions when compared to those containing less than 5% gemistocytes. In addition, Mahler-Araujo et al (unpublished results) also observed that diffuse astrocytomas containing more than 5% gemistocytes had a significant tendency to progress to higher malignancies, than those with less than 5% gemistocytes. The biological basis underlying the poor outcome of gemistocytic astrocytomas remains unclear, since gemistocytes themselves have a low proliferative activity [17,19,20], and resemble to some extent reactive astrocytes encountered in a variety of other non-neoplastic CNS diseases [19,22,23].

1.2.1.3-Protoplasmic astrocytoma

Protoplasmic astrocytoma, a rare variant, is characterised by neoplastic astrocytes with small cell bodies with few, flaccid processes, with a low content of glial filaments and scant GFAP expression [14,15].

1.2.2-Anaplastic astrocytoma

Anaplastic astrocytoma (WHO grade III) may arise from progression of diffuse astrocytoma or occur de novo without evidence of a less malignant precursor lesion. It is characterised by a high cellularity, presence of mitotic figures and nuclear atypia. As does diffuse astrocytoma, anaplastic astrocytoma exhibits considerable variation in cellularity as well as in morphologic heterogeneity. They have a tendency for progression to glioblastoma multiforme with a mean time till progression of 2 years, however, with a considerable variability [24]. The incidence is highest in patients between 40 and 50 years old [24,25].

1.2.3-Glioblastoma

Glioblastoma (WHO grade IV), previously known as glioblastoma multiforme, is the most malignant and most common brain tumour, accounting for approximately 12-15% of all intracranial neoplasms and 50 to 60% of all astrocytic tumours [26,27]. In 1940, Scherer [28] used the terms primary and secondary glioblastoma to subdivide glioblastoma based on their clinical and biological features. Primary (de novo) glioblastoma constitutes the vast majority of glioblastomas (approximately 80%). They develop rapidly, with a short clinical history (usually less than 3 months) and without clinical, histologic or radiologic evidence of

a less malignant precursor lesion. Secondary glioblastomas are less frequent, develop more slowly, through progression from diffuse astrocytoma or anaplastic astrocytoma [27,28]. Primary glioblastoma occur in older patients (mean, 55 years), whereas secondary glioblastoma arises in younger patients (mean, 40 years) [27,29,30]. It is still a matter of discussion whether patients with secondary glioblastoma harbour a better prognosis than primary glioblastoma. This classification of primary and secondary glioblastoma has been merely conceptual, since both subtypes are similar histologically.

Despite multimodal treatment, including surgery, radiotherapy, and chemotherapy, the prognosis of glioblastoma patients remains dismal. The mean survival time is less than 1 year and has changed little over the past two decades [26,27,31]. Less than 2% of patients survive more than 3 years [32], and a recent review reported only 30 cases of patients that had survived up to 10 years [33].

Histologically, glioblastoma is characterised by poorly differentiated neoplastic astrocytes, nuclear atypia and presence of high mitotic activity. The presence of necrosis, which can appear with a pseudopalisading pattern or in massive areas, and exuberant microvascular proliferation, are histological hallmarks of glioblastomas (Fig. 3). The histology reveals an architectural pleomorphism, not only between patients but also within the same tumour. There are two histological glioblastoma variants, *giant-cell glioblastoma* and *gliosarcoma* [15,16].

Fig. 3 - Histological appearance of glioblastoma (H&E staining).

1.2.3.1-Giant-cell glioblastoma

Giant-cell glioblastoma (WHO grade IV) represents less than 1% of all brain tumours and up to 5% of glioblastomas [34]. Histologically it is characterised by the predominance of bizarre and multinucleated giant cells [34,35] (Fig. 4). The giant cell glioblastomas typically arise *de novo*, i.e., without the presence of a less malignant precursor lesion. They occur in patients with a mean age of 42 years and have a male/female ratio of 1.6 [34]. Therefore, this variant shares some features with both primary and secondary glioblastoma.
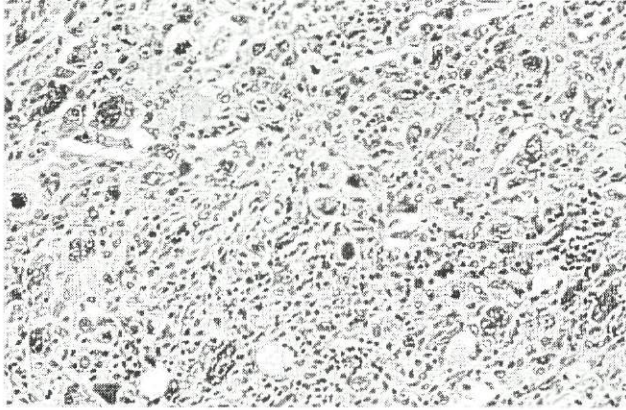
Fig. 4 - Histological appearance of giant cell glioblastoma (H&E staining).

1.2.3.2-Gliosarcoma

Gliosarcoma is a rare variant and accounts for approximately 2% of all glioblastomas. Gliosarcoma presents a clinical profile characteristic of primary glioblastoma [36]. The majority of cases develop *de novo*, occur in old patients (mean of 53 years), and have a sex ratio of 1.4. Phenotypically it is characterised by a biphasic tissue pattern with alternating areas of sarcomatous and gliomatous differentiation (Fig. 5) [36].

The histogenesis of the sarcomatous component of gliosarcomas has been a controversial issue. Earlier morphological and immunohistochemistry studies suggested an evolution of the sarcomatous component from microvascular proliferation within a highly malignant glioblastoma [37-39]. However, more recent molecular studies suggested a monoclonal origin of both gliomatous and sarcomatous components [40,41].
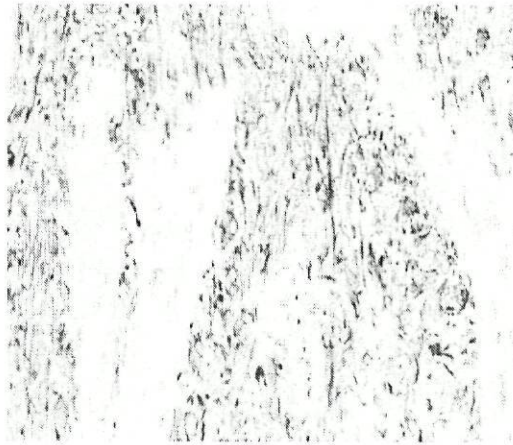
Fig. 5 – Immunohistochemical appearance of gliosarcoma (GFAP staining).

2-Genetic alterations of diffusely infiltrating astrocytomas

Carcinogenesis is a multistep process characterised by deregulation of growth, loss of differentiation, sustained angiogenesis, absence of apoptosis, limitless replicative potential and tissue invasion and metastisation. These steps reflect acquisition of genetic alterations in oncogenes (promoters of cancer initiation and progression) and tumour suppressor genes (inhibitors of cancer initiation and progression) that drive the progressive transformation of normal human cells into highly malignant cells [42,43]. Diffusely infiltrating astrocytomas biology is complex, and a full description of genetic alterations involved in all tumourigenic processes is far from complete and beyond the scope of this section. We here will briefly focus on several genetic abnormalities involved in cell-cycle control, growth factor signalling, and genetic instability of diffusely infiltrating astrocytomas.

2.1-Alterations of genes involved in cell-cycle control

2.1.1-Rb pathway (p15^{INK4b}/p16^{INK4a}/CDK4/6/Rb)

The mammalian cell-cycle is divided into four distinct phases: G_1, S, G_2 and M. G_1 phase is the interval in which cells respond to extracellular signals that ultimately determine whether cells will replicate DNA and divide or, alternatively, to exit the cell-cycle into a quiescent state (G_0). Once cells make the decision to begin DNA replication, they are

irreversibly committed to complete the cycle, and the time late in G_1 phase at which this decision is made was designated the "restriction checkpoint" [44,45]. When cells are stimulated by mitogen factors to enter the cycle from G_0, they generally require continuous mitogenic stimulation to be driven to the restriction checkpoint, after which mitogens can be withdrawn and the cells will enter S phase and complete the cycle in their absence. Conversely, antiproliferative compounds, such as transforming growth factor-β or drugs such as rapamycin, can arrest the proliferation of cells that are progressing through G_1 phase but have not yet reached the restriction checkpoint.

A complex network co-ordinates the cell cycle. In a simplified manner, progression of cells from G_1 to S phase is positively regulated by proteins such as cyclins (cyclin D and E) and cyclin-dependent kinases (CDKs) (CDK4, CDK6 and CDK2), and negatively by their inhibitors that can be of two major families; the CIP/Kip family, that includes the p21$^{Waf1/Cip1}$, p27^{kip1}, and p57^{KIP2} proteins; and the INK4 family, including the founding member p16^{INK4a} and the other closely related proteins p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. These positive and negative factors regulate ultimately the phosphorylation status of the retinoblastoma protein (Rb). The phosphorylation of Rb protein induces the release of the E2F transcription factor family that in turn activates genes involved in the late G_1 and S phase leading to cell-cycle progression (Fig. 6) (For review see [45-47]).
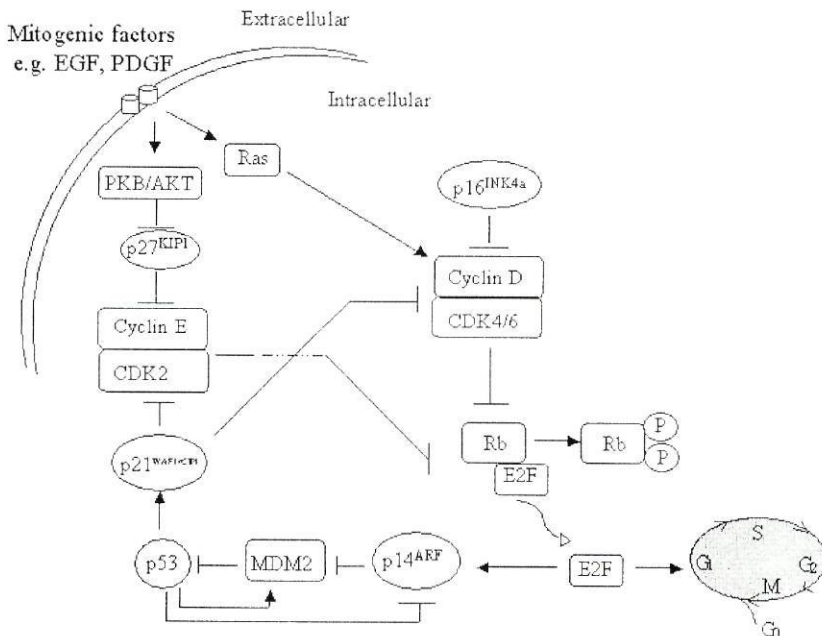
Fig. 6 - Simplified model of mammalian G_1/S phase regulation of cell cycle.

The $p16^{INK4a}$ (p16, CDKN2A, MTS1) and $p15^{INK4b}$ genes map to chromosome 9p21, a locus commonly deleted in diffusely infiltrating astrocytomas. This locus also encodes another distinct protein through alternative reading frame, the p14[ARF] protein (Fig. 7) [48]. $p16^{INK4A}$ is encoded by the unique exon 1α and common exon 2 and 3 (Fig. 7). The p16[INK4a] and p15[INK4b] proteins bind to CDK4/6 and inhibit the formation of CDK4/6-cyclin D complex. When activated, this complex phosphorylates the Rb protein, causing it to dissociate from E2F and freeing E2F to induce cell-cycle progression (Fig. 6).

In diffusely infiltrating astrocytomas, $p16^{INK4a}$ mutations are rare and the loss of $p16^{INK4a}$ expression is mainly due to homozygous deletion [49-51] or promoter methylation [52,53]. $p16^{INK4a}$ homozygous deletion has been frequently detected in glioblastomas, principally primary glioblastomas (30-40%) [27,50,54,55]. In anaplastic astrocytomas the frequency is lower (13-25%) [2,50], and absent in diffuse astrocytomas [2,50,51]. Recently, studies have reported that loss of expression of $p16^{INK4a}$ can be due to methylation of the CpG island in the promoter or first exon region. This mechanism is associated with delayed replication, condensed chromatin and inhibition of transcription initiation, resulting ultimately in gene silencing [52,56]. Promoter methylation is responsible for $p16^{INK4a}$ loss of expression in 7-20% of diffuse astrocytomas [51,53], in 17% of anaplastic astrocytomas [53] and in 25% of non-specified glioblastomas [53]. Recently, Nakamura et al [51], found that promoter methylation is preferentially associated with the secondary glioblastoma variant. These results suggest that $p16^{INK4a}$ deletion is a late event, while $p16^{INK4a}$ promoter methylation constitutes an early event in diffusely infiltrating astrocytoma tumourigenesis.
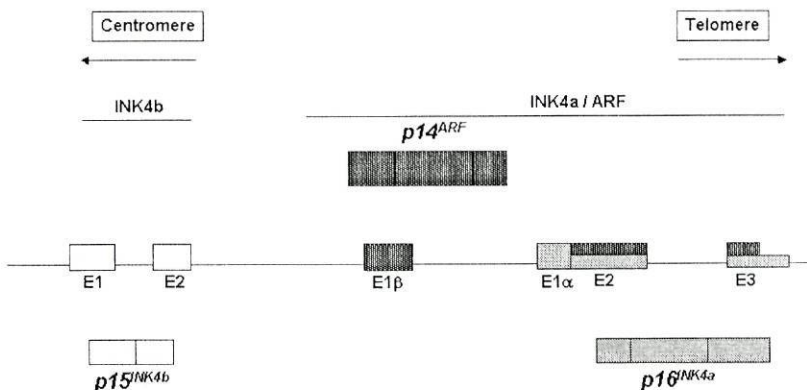
Fig. 7 - Scheme of human genomic structure of INK4a/ARF and INK4b loci on 9p21 region.

The $p15^{INK4b}$ ($p15$, $CDKN2b$, $MTS2$) gene is structurally and functionally homologous to the $p16^{INK4}$ gene (Fig. 7) [57]. Concomitant deletion of $p16^{INK4}$ and $p15^{INK4b}$ occurs in approximately 30% of astrocytic tumours [50,58,59], suggesting that both genes may be targets of 9p21 deletions [60]. Like for $p16^{INK4a}$, loss of $p15^{INK4b}$ expression can be due to methylation of the CpG island in the promoter region [61].

The retinoblastoma (Rb) protein is a central element in the control of G_1 checkpoint and inactivation of Rb would lead to unrestrained cell cycle [45,46]. Rb tumour suppressor gene is located on chromosome 13q14, a locus that is altered in about one-third of high-grade astrocytic tumours [2]. Rb inactivation can be due to a variety of mechanisms, such as mutations, homozygous deletion and promoter methylation [50,51,54,62].

Genetic alteration of Rb has been found to occur in 14-43% of glioblastomas [50,51,54,62]. A clear correlation has been found between loss of expression of Rb by immunohistochemistry, and promoter methylation [63], which was found to occur more frequently in secondary than primary glioblastomas (43% and 14%, respectively) [63]. Approximately 20% of anaplastic astrocytomas have Rb alterations, whereas in diffuse astrocytomas no alterations were found [24,50,54,62,63].

$CDK4$ and $CDK6$ genes map to chromosome 12q13-14 and 7q21-22, respectively. Both proteins can form a complex with the cyclin D family, and phosphorylate the Rb protein, leading to release of the E2F transcription factor and cell cycle progression (Fig. 6). Therefore, overexpression of $CDK4/6$ would lead to abnormal cell progression [46]. Amplification of $CDK4$ has been detected in approximately 8-15% of glioblastomas [50,55,64] and in around 10% in anaplastic astrocytomas [50]. No amplification of $CDK4$ has been found in diffuse astrocytomas [50]. Recently, $CDK6$ amplification has been detected in 6% of high-grade astrocytic tumours (anaplastic astrocytomas and/or glioblastomas) [65], and overexpression of CDK6 protein has been found in 44% of glioblastomas [66]. No amplification or overexpression of $CDK6$ has been detected in diffuse astrocytomas, suggesting that both $CDK4$ and $CDK6$ amplification/overexpression are late events in astrocytic tumourigenesis [65,66].

Amplification and overexpression of cyclin D family is rarely (approximately 3%) observed in diffusely infiltrating astrocytomas [67].

Notably, alterations of $p16^{INK4}$, Rb and $CDK4$ genes are almost mutually exclusive, suggesting that alteration of any one of these components is adequate to sufficiently abrogate the G_1/S checkpoint [50,54,55,62]. Altogether, deregulation of the Rb pathway occurs in 7-20% of diffuse astrocytomas [51,53], a significant proportion of anaplastic astrocytomas (20 to 40%) [50,55,62], and in the majority of glioblastomas (50% to 70%) [50,55,62].

2.1.2-p53 pathway (p53/MDM2/p14^{ARF})

The *p53* (*TP53*) tumour suppressor gene is located on chromosome 17p13.1. The human p53 protein has been divided structurally and functionally into four domains; N-terminus that constitutes a transcriptional activation domain; a central sequence-specific DNA binding domain; a nuclear localisation domain; and a C-terminal oligomerization domain. p53 protein forms a tetramer and acts as transcriptional activator through binding to specific sequences on target genes and thereby activates or represses transcription from a large, and ever increasing number of genes [68-70]. p53 can also exert its effects through transcription-independent mechanisms, such as direct binding of p53 protein to several cellular proteins [70,71].

p53 is involved in a diversity of cellular processes in mammalian cells. It plays an important role in the maintenance of genomic stability, hence its denomination as "genome guardian" [72]. In the cell, p53 exists at low concentration in a latent form that becomes stable (through several mechanisms, such as phosphorylation and acetylation) and is activated in response to various signals, including carcinogen-induced DNA damage, telomere erosion, changes in redox potential, aberrant proliferative signals, hypoxia, radiation, and loss of adhesion or survival signals. The best understood function of p53 is the capacity to inhibit growth of potential tumour or stressed cells. This ability is associated with cell-cycle arrest (at G_1 and/or G_2/M phases), differentiation, senescence, or apoptosis. Among the genes transcriptionally controlled by p53, *p21$^{Waf1/Cip1}$* constitutes a central effector in G_1 arrest, through the binding and inhibition of cyclinD/CDK4/6 and most important to cyclin E/CDK2 complex (Fig. 6) [69,70,73]. p53 can also contribute directly to DNA repair, and to inhibition of angiogenesis [69,70,74] (Fig. 8). The large degree of overlap between these cellular processes, as well as the fact that none of them is exclusively p53-dependent, suggests that the function of p53 is to control, co-ordinate and integrate these various pathways [69-71].

Inactivation of *p53* by mutation is a key molecular event, detected in approximately 50% of all neoplasms, including brain tumours [75]. In most of the cases, mutations in one allele are associated with loss of the other allele. These mutations impair the ability of mutant protein to carry out its activities and in some cases bestow a new dominant negative or gain of function properties [76]. The great majority of these mutations (approximately 95%) are localised to the DNA-binding domain (exon 4 to exon 8) of the protein [77].
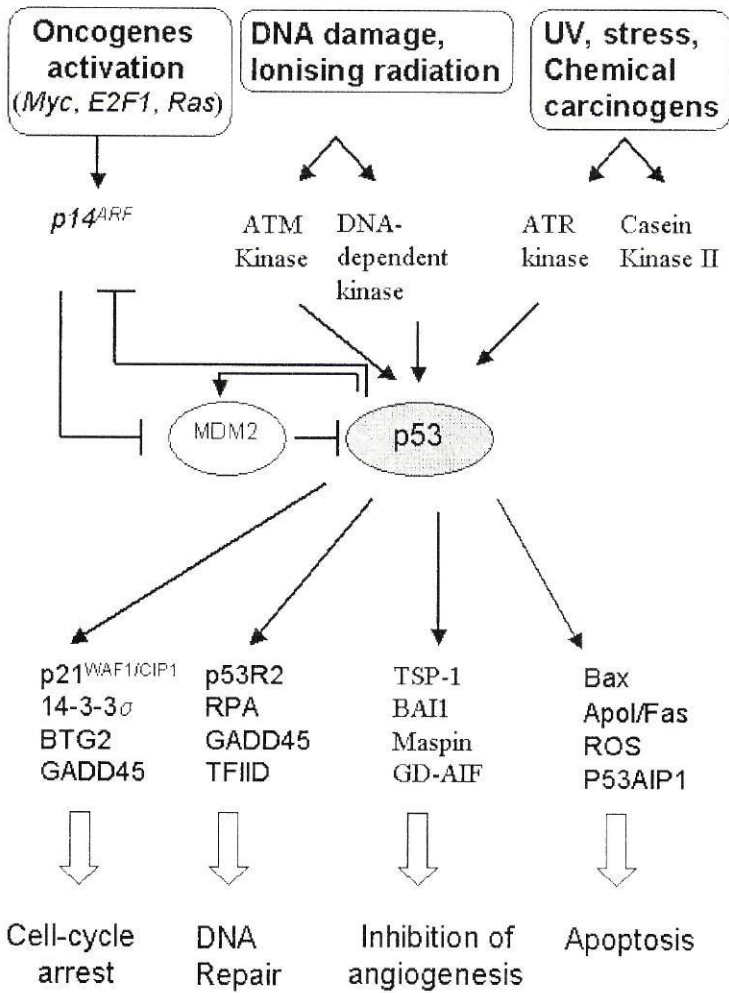
Fig. 8 - Simplified representation of *p53* signalling networks.

In diffusely infiltrating astrocytomas approximately 45% of mutations are localised in several codon "hotspots", such as codon 158, 175, 179, 245, 248, 273, and 282. All classes of mutations (deletions, insertions, transitions and transversions) can occur in the *p53* gene, but the most common are (G:C→A:T transitions at CpG sites) (data from IARC *p53* database, R5 version [77])(Fig. 9).

p53 mutations have been reported to occur in approximately 40% of diffusely infiltrating astrocytomas of all grades. An extremely high frequency of *p53* mutations, (60 - 82%) and

85%, has been observed in gemistocytic astrocytomas and giant cell glioblastomas, respectively [34,78].

There is compelling evidence that *p53* mutations constitute an early event in astrocytic tumourigenesis. Although *p53* mutations are rare in primary glioblastomas (<10%), they are frequent in secondary glioblastomas (>65%) and in 90% of cases the mutation was already present in the first biopsy [25,30,68]. Patients with Li-Fraumeni syndrome, which is caused by a germline *p53* mutation, are characterised by the frequent occurrence of brain tumours in early life, including astrocytomas [79]. This suggests that in astrocytic tumours *p53* can act as a *gatekeeper* gene, directly preventing tumour initiation [80]. However, Sidranski *et al* [81] and more recently Ishii *et al* [82] demonstrated that the fraction of *p53* mutated cells increases in the same patient during the progression of diffuse astrocytoma to higher malignancies. These facts suggest that *p53* is an early progression event rather than an initiation event.
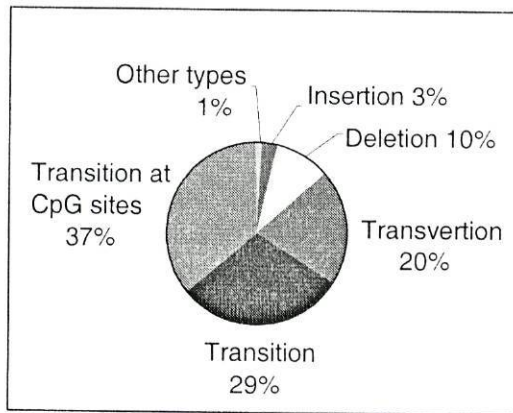
Fig. 9 - Spectrum of *p53* somatic mutations in diffusely infiltrating astrocytomas (data from IARC *p53* database, R5 version [77]).

In addition, experimental animal models, as well as in human and animal cell lines studies also support the important role of *p53* in astrocytic tumourigenesis. Astrocytes derived from *p53* -/- mice show an increased growth rate and become spontaneously immortalised in successive cultures [83,84]. In human glioblastoma cell lines lacking p53 function, the exogenous expression of wild-type *p53* results in growth arrest and/or apoptosis [85,86].

The *MDM2* gene (mouse double minute 2) also called *HDM2*, contains a *p53* DNA binding site and is localised on chromosome 12q14.3-q14. MDM2 is a multifunctional protein that binds to and abrogates the *p53* function by either targeting p53 for degradation

in the cytoplasm by the proteasome, or by repressing p53-mediated transcriptional activity in the nucleus [87]. It has been demonstrated that, under a variety of conditions, MDM2 transcription is induced by wild-type *p53* [88,89] (Fig. 6 and 8). This autoregulatory feedback loop regulates the activity of the p53 protein as well as the expression of MDM2 [90]. Amplification of *MDM2* is absent in diffuse astrocytomas and anaplastic astrocytomas [91], but present in up to 10% of primary glioblastomas that lack a *p53* mutation [91]. However, overexpression of MDM2 has been observed in more than 50% of primary glioblastomas and less frequently in secondary glioblastomas (<10%) [92,93]. Thus, alteration of *MDM2* by amplification/overexpression constitutes an alternative mechanism for escaping the *p53*-regulated cell-cycle control.

The $p14^{ARF}$ (human homologue of the mouse $p19^{ARF}$) has been recently identified as another important regulator in this pathway. The $p14^{ARF}$ protein is encoded by the *CDKN2A/INK4a* locus that maps on chromosome 9p21 (Fig. 7). It has been shown that $p14^{ARF}$ stabilises p53 by antagonising the p53-negative regulator MDM2 [94,95]. The expression of $p14^{ARF}$ is down-regulated by p53, which would establish an autoregulatory feedback loop between p53, MDM2 and $p14^{ARF}$ [96] (Fig. 6 and 8). $p14^{ARF}$ expression is activated by abnormal mitogenic signals induced by overexpression of oncoproteins such as Myc [97], Ras [98] and E2F1 [96] (Fig. 8). In this manner $p14^{ARF}$ induces p53 activity under abnormal mitogenic signals and serves to connect the *Rb* pathway with the *p53* pathway.

Homozygous deletion and promoter methylation of $p14^{ARF}$ have been reported in astrocytic brain tumours. Homozygous deletions were reported in 40% to 58% of glioblastomas [50,51], in 13% of anaplastic astrocytomas [50] but in none of diffuse astrocytomas [50,51]. In contrast, promoter methylation has been found at high frequency in diffuse astrocytomas (33%) and secondary glioblastomas (31%) [51].

Overall, impairment of *p53* pathway occurs in approximately 74% of diffuse astrocytomas that progressed to higher malignancies, and this frequency increases to approximately 88% in secondary glioblastomas (Nakamura *et al*, unpublished results). In primary glioblastomas the frequency was 70% (Nakamura *et al*, unpublished results). About 75% of non-specified glioblastomas were reported to exhibit abnormalities of *p53* pathway [50]. Unlike the Rb pathway, several findings suggest that alterations of the $p14^{ARF}$, *MDM2* and *p53* are not always mutually exclusive. Some studies showed that $p14^{ARF}$ loss of function is not restricted to tumours with wild-type *p53* [99,100]. In addition, each protein may interfere with other cellular pathways, such has been reported for the regulation of E2F1 by MDM2 [101], and the above mentioned regulation of $p14^{ARF}$ by *c-myc*, *ras*, and *E2F1* oncogenes [96-98].

2.2-Alterations of Growth Factors and Growth Factor Receptors

Normal cells require mitogenic growth signals to proliferate. These growth factors bind to their respective receptors and their signal is transmitted into the cells. One of the most important classes of receptors belongs to the family of receptor tyrosine kinase (RTK) that have an intracellular tyrosine kinase domain. Alteration of this homeostatic mechanism is frequently observed in diffusely infiltrating astrocytomas, leading to self-sufficiency in growth signals.

2.2.1-Epidermal growth factor (EGF) / Epidermal growth factor receptor (EGFR)

EGFR is a transmembrane glycoprotein composed of an extracellular ligand-binding domain, a single hydrophobic membrane-spanning domain, and a cytoplasmic tyrosine kinase domain [102]. The major ligands are the epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) and their binding to the EGFR results in receptor dimerization, autophosphorylation of the receptor itself and phosphorylation of the downstream cellular substrates leading ultimately to cell division and proliferation [102].

EGFR gene is located on chromosome 7p12.1-p12.3, and it was the first gene to be found amplified in astrocytic tumours. Approximately 40% of primary glioblastomas show EGFR amplification, which results in overexpression of the EGFR transcript [103-105]. EGFR amplification has rarely been detected in diffuse astrocytoma, anaplastic astrocytoma and secondary glioblastoma [2,106,107]. The observation of both ligands and receptors expression in gliomas suggest an autocrine stimulation loop. Approximately half of glioblastomas with gene EGFR amplification also contain gene rearrangements [2]. The most common rearrangements, called EGFRvIII or Δ EGFR, result in the elimination of exon 2-7 from the extracellular domain, which leads to a truncated receptor [108,109]. This mutant variant displays constitutive ligand-independent kinase activity [110,111], and overexpression of this mutant variant in human glioblastoma cell lines leads to enhanced tumourigenicity in vivo by stimulating proliferation and inhibiting apoptosis [112].

2.2.2-Platelet-derived growth factor (PDGF) / Platelet-derived growth factor receptor (PDGFR)

The platelet-derived growth factor (PDGF) is a major mitogen for connective tissue cells and glia, involved in normal development, especially of the CNS [113,114]. For almost two decades, PDGF homodimers (PDGF-AA and PDGF-BB) and the heterodimer (PDGF-AB) were thought to be the only ligands for the PDGF α-receptor (PDGFR-α) and PDGF β

receptor (PDGFR-β) [115]. Recently, two additional ligands were identified, PDGF-C and PDGF-D [116-118]. Like EGFR, PDGFRs belong to the receptor tyrosine kinase family. Despite the low frequency of *PDGFR-α* amplification [119,120], overexpression is frequently observed in all grades, suggesting that this alteration is important for the development of diffusely infiltrating astrocytomas [120]. The mechanism that leads to such overexpression remains unclear. Tumours frequently express both ligands and receptors, suggesting autocrine growth stimulation [2,120]. The newly identified PDGF-C and PDGF-D seem to have some functional analogy to the classic PDGF-A and –B, but their precise role is unknown [116-118].

2.3-Chromosomal instability

Genetic instability is a critical phenomenon in the development of malignant human neoplasms and occurs at least in two different forms, microsatellite instability and chromosomal instability [121-123]. The genes involved in DNA repair and maintenance of genomic stability, are classified as *caretakers* [80]. Inactivation of caretaker genes indirectly promotes cancer development, by favouring mutations in gatekeepers and other cancer-related genes [80]. Microsatellite instability is characterised by widespread insertions and deletions in microsatellite repeats [121,123]. In adult diffusely infiltrating astrocytomas the frequency of microsatellite instability is low, approximately 3% [124,125]. Tumours with microsatellite instability usually have a normal complement of chromosomes, i.e. a diploid or near-diploid karyotype [123].

In diffusely infiltrating astrocytomas, as well as in the majority of other neoplasms, the most common form is chromosomal instability, that is characterised by gains and/or losses of whole chromosomes, referred as aneuploidy [123,126-130]. At molecular level, aneuploidy is reflected in allelic imbalance and is often associated with loss of heterozygosity (LOH) [123].

One of the possible mechanisms leading to aneuploidy is the inactivation of the mitotic spindle checkpoint [123,126,130]. This is a highly conserved mechanism, which monitors the proper assembly of mitotic spindles, which consist of microtubules and ensures stable attachment of chromosomes (at the kinetochore), to microtubules. In the presence of spindle problems or unattached chromosomes the mitotic checkpoint delays the onset of anaphase and prevents improper segregation of sister chromatids to the two daughter cells, avoiding in this way abnormal chromosomal segregation and chromosomal instability [131-133].

Seven gene products, essential for mitotic checkpoint, were initially identified in budding yeast, including BUB (budding uninhibited by benomyl)-family genes (*Bub1*, *Bub2* and *Bub3*) and MAD (mitotic arrest defective)-family genes (*Mad1*, *Mad2* and *Mad3*) and *Mps1* [134-136]. Several human homologues have been identified, including *hBUB1* at chromosome

2q14 [137], *hBUBR1 (hBUB1B/MAD3-L)* at 15q14 [137], and *hBUB3* at chromosome 10q26 [138,139], *hMAD1L1* at chromosome 7pter-p15 [139,140], *hMAD2 (hMAD2L1)* at chromosome 4q27 [139], *hMAD2B (hMAD2L2)* at chromosome 1p36 [139] and *hMPS1 (TTK/MPS1L1)* at chromosome 6q13-21 [139].

Although still limited, there is some evidence suggesting that mutational inactivation of mitotic spindle checkpoint genes is involved in the evolution of a fraction of human neoplasms, in particular, those with aneuploidy. Cahill *et al.* [137] has detected both *hBUB1* and *hBUBR1* mutations in 2 out of 19 colon cancer cell lines with chromosomal instability. Moreover, ectopic expression of *hBUB1* mutant genes in euploid cells resulted in disruption of the mitotic checkpoint control, in a dominant negative fashion, supporting the notion that aneuploidy may be the result of defects in the mitotic checkpoint [137]. In addition to the human mutational data, animal models support the importance of such genes in chromosomal stability. In Drosophila and mice, *BUB1* inactivation induces chromosome missegregation and fail to block apoptosis [141,142]. *BUB3* [−/−] deficient mice showed several mitotic errors, including lagging chromosomes and irregular nuclear morphology leading to embryonic death [143].

2.3.1-LOH and inactivation of tumour suppressor genes

2.3.1.1-Chromosome 10, PTEN and other tumour suppressor genes

LOH of chromosome 10 (LOH#10) is the most frequent alteration in glioblastomas, occurring in approximately 80% of this neoplasm [2,144-146]. In anaplastic astrocytomas the frequency of LOH is lower (about 30%) [24,145-148], while in diffuse astrocytomas it is rarely observed [145,146,149]. Most glioblastomas exhibit loss of an entire copy of chromosome 10, and in cases with partial deletion, a complex pattern of LOH occurs on both arms of the chromosome [144,145,147,150]. A frequent deleted locus is the 10q25-qter [144,145,147,150]. Fujisawa *et al* [151] have found an association between LOH in this locus and neoplasms with an abrupt histological transition of diffuse astrocytoma or anaplastic astrocytoma to glioblastoma. Other frequently deleted loci include the 10p14-pter and the 10q23-24 [144,145,147,150]. These frequent and complex deleted regions indicate the presence of several tumour suppressor genes on chromosome 10.

The tumour suppressor gene *PTEN (MMAC1* or *TEP1)* was recently identified and it maps to the common deleted region, 10q23.3 [152-154]. The *PTEN* gene has homology with dual-specificity phosphatases, which are capable of dephosphorylating both tyrosine phosphate and serine/threonine phosphate residues on proteins [152-154]. The N-terminal domain of the protein also has extensive similarity to two cytoskeletal proteins tensin and

auxilin [152-154]. Besides the protein phosphatase activity [155-157], PTEN has an important lipid phosphatase activity. In particular, PTEN is a phosphatidylinositol-3 phosphatase and dephosphorylates phosphatidylinositol (3,4,5)-triphosphatase (PIP-3), a product of phosphoinositol-3-kinase (PI3K), which is required for the phosphorylation and activation of PKB/Akt [158-160] (Fig. 10). PKB/Akt is a important survival factor that protects various cell types against apoptosis and is involved in cell proliferation by mediating cell-cycle progression [161,162]. Consistent with the role of PTEN in the PI3K/Akt pathway, multiple studies have shown that PTEN negatively regulates cell survival [159,162,163]. In addition, PTEN plays a role in other cellular processes. It is considered to be involved in G_1 cell-cycle arrest, down-regulation of focal adhesion kinase (FAK) and inhibition of angiogenesis [156,157,164-168] (Fig. 10).
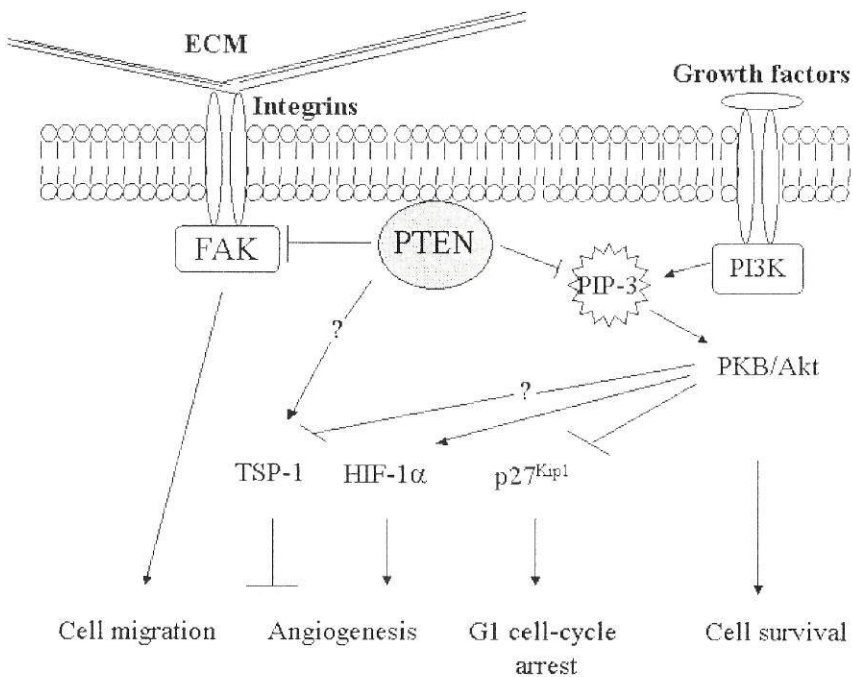
Fig. 10 - Simplified scheme of *PTEN* signalling pathways.

Germline mutations of *PTEN* are found in several rare inherited benign tumour (hamartoma) syndromes, including Cowden syndrome, which is associated with a high risk of breast, thyroid and others cancers [169,170], Bannayan-Zonana syndrome [171,172], Lhermitte-Duclos disease [170,173] and Proteus-like syndrome [174]. In addition, sporadic *PTEN* mutations are frequent in several types of tumours, including primary glioblastoma (24-44%) and

anaplastic astrocytomas (approximately 23%), while in diffuse astrocytomas they are generally absent [107,144,175,176]. Germline and somatic mutations occur mostly in the phosphatase domain [175], supporting the importance of this domain for normal development and tumour suppressor activity of *PTEN*. In addition to deletion/mutations, recent reports suggest that *PTEN* inactivation can also be due to *PTEN* promoter methylation [177] (and Baeza *et al*, in preparation).

A potential tumour suppressor gene, *DMBT1*, has been identified on the region 10q25.3-26.1 [178] and encodes a member of the scavenger receptor cystein-rich family. Homozygous deletions in the gene have been reported in both glioblastoma and medulloblastomas [178,179]. In another study, the gene was homozygously deleted in a small number of recurrent astrocytic tumours [180]. However, because no mutations were detected and its loss does not correlate with changes in tumour cell growth *in vitro* or with increased aggressiveness of tumours *in vivo* [181,182], the target of deletions may be another closely located gene other than *DMBT1*.

MXI1 gene, located on 10q25.1, is another putative glioblastoma suppressor gene [183]. MXI1 protein is thought to function as a negative regulator of the *myc* oncogene [184]. High frequency of *MXI1* LOH (approximately 65%) has been found in glioblastomas, however, sequence analysis revealed no somatic mutations [144,183]. Re-introduction of *MXI1* into human glioblastoma cell lines lacking endogenous *MXI1* expression resulted in cell growth inhibition, supporting *MXI1* as a candidate tumour suppressor gene in glioblastomas [183].

Several other putative tumour suppressor genes have been identified on 10q, including *LGI1* at 10q24 [185], *hBUB3* at 10q26 [138], *FGFR2* at 10q26 [186] and *hours-neu* at 10q25.1 [187], however, their actual role in astrocytic tumourigenesis is still unclear.

2.3.1.2-Other common deleted chromosomal regions

LOH#1p – Allelic loss of short arm of chromosome 1 occurs in less than 10% of diffuse astrocytomas, approximately in 20% of anaplastic astrocytomas and in around 10% of glioblastomas [125,188-191]. The most common deleted region was localised on 1p36 [189,192], where several potential tumour suppressor genes are mapped, including the *p73* [193], *p18^INK4C* [194] and *hMAD2B* [139]. However, there is no evidence for their role in development and progression of diffusely infiltrating astrocytomas.

LOH#11p – Allelic deletion of 11p occurs in less than 13% of diffuse astrocytomas and in 15-28% of high grade astrocytomas [125,195,196]. Based on a limited number of mapping studies, the common deleted region has been localised on 11p15. The *c-H-ras* gene that maps on this locus has been excluded as a candidate based on the absence of mutations [197].

LOH#19q - Although the presence of a glioma 19q tumour suppressor gene was first suggested a decade ago [198,199], this putative gene remains unidentified. Deletions of 19q occur in 11-25% of diffuse astrocytomas, in 32-50% of anaplastic astrocytomas and in 13-32% of non-specified glioblastomas [125,189,199]. Recently, Nakamura *et al* [188] showed that LOH19q occurs frequently in secondary glioblastomas (54%) and is rarely observed in primary glioblastomas (6%).

LOH#22q - LOH on chromosome arm 22q occurs in 20-30% of diffuse infiltrating astrocytomas, regardless of malignancy grade, suggesting the presence of a tumour suppressor gene involved in early diffuse astrocytic development [200-202]. Recent reports have defined a minimal region to 22q13 [203,204], however, no clear candidate gene has been identified hitherto.

2.4-Diffuse astrocytoma genetic profile

Karyotyping analysis of diffuse astrocytomas showed that diffuse astrocytomas are diploid and the majority of them have a normal karyoptype [205]. Comparative genomic hybridisation (CGH) and LOH studies have shown some non-random alterations, with some regions known to be associated with specific gene targets, such as LOH#17p (*p53* gene) (Table 2). Recently, using cDNA arrays several genes were found abnormally expressed in diffuse astrocytomas [206,207] (Table 2).

Recently, Costello *et al* [208] assessed the methylation status of thousands of genes in diffuse astrocytomas and found non-random aberrant methylation in a significant proportion of genes. Indeed, overexpression of DNA-methyltransferase, enzyme responsible for methylation, is already present in diffuse astrocytomas [209]. Nakamura *et al* found frequent aberrant promoter methylation of *p14^ARF* [51] and *p16^INK4a* [51] and *MGMT* [277] genes in diffuse astrocytomas (Table 2).

Table 2 - Summary of genetic abnormalities in diffuse astrocytomas

---

Chromosomal alterations

+ 7q, +8q, -9p (*p16*[INK4a]), -10p, -11p, -13q, **-17p (p53)**, -19q, **- 22q**, - X

Gene alterations

Overexpression of **PDGFR-**α, **PDGF-A**, *FGF2*, *CNTF*, *SPARC*, *GDNPF*, *TIMP-3*, DNA-Mtase

Downregulation of IFI 9-27, GAB1, TYRO3

Mutations of ***p53***

Epigenetic alterations

Methylation of *p16*[INK4a], **p14**[ARF], **MGMT**

+, chromosomic gains; -, chromosomic losses; data described in bold indicates the most frequent alterations; data from [14,21,51,145,205-207,209-213,277].

The understanding of the molecular factors that are involved in diffuse astrocytoma recurrence and malignant progression have been elusive [1,14], and few studies have focused on this issue. Although not consensual, the presence of *p53* mutations has been associated with recurrence and progression of diffuse astrocytomas [14,25,82,210,214]. Despite the low frequency of 9p losses reported in unspecified diffuse astrocytomas [215], 8 out of 14 (57%) diffuse astrocytoma cases that progressed to a higher grade of malignancy disclosed LOH#9q21 (INK4A/ARF locus) [210,216,217]. It remains to be determined whether this and others genetic alterations mentioned in Table 2 have a prognostic role in diffuse astrocytoma outcome.

2.5-Primary and secondary glioblastoma genetic profile

Recent studies showed that primary and secondary glioblastomas have not only distinct clinical features [28], but also evolve through distinct genetic pathways that lead ultimately to the same phenotype [27,29,218]. During the last decade these molecular pathways started to be unveiled, although the whole picture of the molecular events is far from complete (Fig. 11).

Primary glioblastoma is characterised by high frequency of *EGFR* amplification, *p16*[INK4a] deletion, and *PTEN* mutations, while secondary glioblastoma is characterised by high frequency of *p53* mutations, LOH#19q, and *Rb* and *MGMT* methylation. However, some genetic alterations can occur at similar extension in both subtypes, such as homozygous deletion of *p14*[ARF]. Another observation that can be drawn from this picture is the frequent

association between abnormal promoter methylation mechanisms with secondary glioblastomas (Fig. 11).
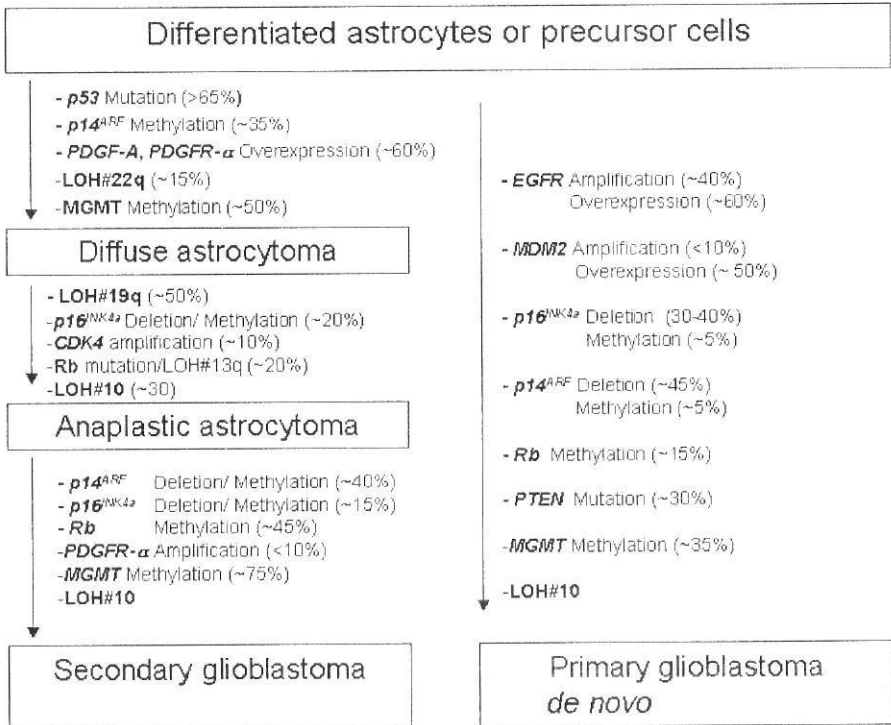
Fig. 11 - Summary of some major genetic alterations in primary and secondary glioblastomas. Adapted from Kleihues et al[27].

2.6-Giant cell glioblastoma genetic profile

Recent studies showed that the genetic profile of giant cell glioblastoma, as well as their clinical features, occupies a hybrid position between primary and secondary glioblastoma [34,35,219]. Giant cell glioblastomas are characterised by extremely high frequency of p53 (85%) and high frequency of PTEN (27%) mutations, and low or absence of EGFR amplification (5%) and $p16^{INK4a}$ deletion (<5%) [34].

2.7-Gliosarcoma genetic profile

The known genetic alterations associated with gliosarcomas are limited. Cytogenetic studies showed that the most frequent abnormalities included gains of chromosome 7, losses of chromosome 10 and deletion of short arm of chromosome 9 [41,220,221]. One study has reported *p53* mutations in 1 out of 12 gliosarcomas [40].

AIMS

Diffusely infiltrating astrocytomas are the most common primary tumours of the CNS. Regardless of the significant advances in surgical techniques, radiation and drug therapies, the prognosis of affected patients remain dismal.

There is increasing evidence that the phenotypic features of tumours are a reflection of genetic events underlying them. Therefore, the combination of histological features with the identification and characterisation of relevant genetic alterations, would lead to a more objective and reliable classification of diffusely infiltrating astrocytomas. This may also contribute to the development of new treatment strategies through the identification of novel therapeutic targets and to the improvement of the current used chemotherapeutic and radiotherapeutic agents.

In the last decade the genetic alterations responsible for the initiation and progression of these tumours have been extensively studied, but remain far from being clarified.

For practical reasons – pre-eminence of translational research based upon clinical cases and leaving apart experimental models – we decided to address the aforementioned problems using human specimens as the unique source of material of the present thesis.

The study of human pathology curbs the possibility of flowing smoothly from a question to another. Since it depends upon the availability of appropriate material ("individual cases") it is prone to create fragmented pieces of evidence. The challenge in this setting is to be able to integrate into a coherent frame the data obtained in such diverse studies.

General aim

The general aim of the study was to progress in the understanding of the genetic alterations underlying the development of diffusely infiltrative astrocytoma subtypes.

Specific aims

To achieve our purpose the following specific topics were addressed:

1- *p53* mutations and gemistocytes in diffuse astrocytomas

Diffuse astrocytomas have an inherent tendency for recurrence and progression to anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (WHO grade IV). However, time till progression varies considerably among individuals, and some cases remain dormant for several years, while others progress rapidly.

Mutations of *p53* gene are considered a genetic hallmark of diffuse astrocytomas. In this study we aimed to determine the degree of *p53* mutated tumour cells expansion in an attempt to clarify the role and timing of *p53* mutations appearance in common diffuse astrocytomas. For that purpose we screened all tumour areas of a fibrillary astrocytoma, as

well as adjacent non-tumour tissue of a whole brain section for the presence of *p53* mutations.

Gemistocytic astrocytoma (WHO grade II) is particularly prone to undergo malignant progression. The role of gemistocytes in tumour evolution is enigmatic, since their presence is associated with poor prognosis. However, gemistocytes themselves do not proliferate and may also occur in a variety of other non-neoplastic CNS diseases. These findings raise the question whether gemistocytes are true neoplastic cells or represent reactive glial cells. To elucidate this issue, we performed *p53* mutation analysis of pure populations of gemistocytes and non-gemistocytic neoplastic cells in a series of gemistocytic astrocytomas.

2- Loss of heterozygosity (LOH) on chromosome 10 and mutations in human *BUB* gene family in glioblastomas

Genetic instability, particularly chromosomal instability, is a common feature in glioblastomas and is characterised by an alteration in chromosome number, aneuploidy. At the molecular level, chromosomal instability can be identified by LOH of defined chromosomal regions. LOH on chromosome 10 (LOH#10) is the most common genetic event in glioblastoma.

It has been established that primary and secondary glioblastomas evolve through distinct genetic pathways. The first aim of this topic was to assess LOH#10 in both primary and secondary glioblastomas and elucidate whether the frequency and extension of LOH differ between both subtypes.

Mutations in the human *BUB* mitotic checkpoint genes have been associated with aneuploidy in a subset of colorectal cancer. Thus, we attempted to examine the mutation status of *hBUB1*, *hBUBR1* and *hBUB3* genes in glioblastomas and integrate it with the LOH data.

3- Second primary glioblastoma

The prognosis of patients with glioblastomas is extremely poor. Less than 2% of patients survive more than 3 years irrespectively of aggressive radio or chemotherapy. We studied a case of a patient who was diagnosed with a glioblastoma and had survived 10 years before developing a second glioblastoma in the opposite frontal lobe site. Given the unusual prolonged survival, we performed a genetic characterisation of both glioblastomas aiming to shed light on the molecular events involved in such peculiar behaviour.

4- Genetic characterisation of gliosarcomas

The knowledge of molecular events underlying the development of gliosarcomas is limited. We aimed to analyse the genetic lesions that are frequently involved in glioblastomas using immunohistochemistry and molecular biology methods. We also performed a comparative genetic analysis of both gliomatous and sarcomatous tumour components, to further elucidate the histogenesis of the sarcomatous component.

MATERIAL AND METHODS

Materials

The clinical and histological features of diffusely infiltrating astrocytomas used in the different studies of this thesis are detailed in papers I to V.

Summary of the clinical-pathological data concerning the unpublished study of the role of *p53* in diffuse astrocytomas:

A 37-year-old male patient was diagnosed with a fibrillary astrocytoma on the right frontal lobe, following a stereotactic biopsy. The patient did not receive any further treatment for the brain neoplasm. Six weeks before death, the patient had a septic shock after a necrotizing pancreatitis, resulting in apallic syndrome. A posterior post-mortem whole brain paraffin section was performed.

The tumour mass was relatively well demarcated and mostly localized in the deep temporal lobe. Histologically, it was a homogenous and fairly cellular tumour, composed of well-differentiated fibrillary neoplastic astrocytes with small areas showing a microcystic matrix. Most tumour cells have scant and slightly eosinophilic cytoplasm. No mitoses were observed. Scattered atypical cells were depicted. Based on these features a diagnose of diffuse fibrillary astrocytoma (WHO grade II) was made.

Methods

The techniques performed in papers I to V will be briefly mentioned:

Laser assisted microdissection – Used for single cell microdissection of gemistocytes and non-gemistocytic neoplastic cells (Paper I).

Histochemistry – Used for the study of reticulin expression (Paper V).

Immunohistochemistry – Avidin-biotin-peroxidase method used for expression study of glial fibrillar acidic protein (GFAP) (Papers I, II, III, IV, V), epidermal growth factor receptor (EGFR) (Paper V), p53 (Paper V), retinoblastoma (RB) (Papers IV and V), and MDM2 (Paper V) proteins.

Polymerase chain reaction (PCR) – Used for microsatellite markers analysis (Papers II and IV), amplification of *p53* exon 5 to 8 (unpublished study and papers I, II, IV and V), *PTEN* exon 1 to 9 (Papers II, IV, and V), and amplification of *hBUB1* exon 1 to 25, *hBUBR1* (entire coding sequence) and *hBUB3* (entire coding sequence) (Paper III).

Reverse transcription-polymerase chain reaction (RT-PCR) – Used to obtain cDNA from glioblastomas for LOH analysis and to perform analysis of *hBUB1*, *hBUBR1* and *hBUB3* genes (Papers II and III).

Differential PCR – Used for analysis of *p16^INK4a* homozygous deletion (Papers IV and V), and amplification of *EGFR* (Papers II, IV and V), *CDK4* (Paper V) and *MDM2* (Paper V) genes.

Single strand conformation polymorphism (SSCP) – Used for mutation screening of *p53* (unpublished study and Papers I, II, IV, and V), *PTEN* (Papers II, IV, and V) and *hBUB1*, *hBUBR1* and *hBUB3* genes (Paper III).

Direct DNA sequencing – Manual sequencing for *p53* (unpublished study and Papers II, IV and V), *PTEN* (Papers II, IV and V), *hBUB1*, *hBUBR1* and *hBUB3* genes (Paper III), and automated sequencing for *p53* gene (Paper I).

Statistical analysis – Unpaired Student's *t* test (Papers II, III and V).

Other methods used additionally (unpublished study):

Whole brain section division and DNA extraction and amplification – A grid divided in squares of 4mm^2 area each was placed under and attached to the whole brain section. Under a stereomicroscope, each of the 4mm^2 square areas were scraped off, using a 23G needle, to a 0.5ml-eppendorf tube containing 20 μl lysis buffer (100 mmol./L Tris-HCl pH 7.5 and 500 μg/ml proteinase K) and briefly vortexed and spun down by centrifugation. After incubation at 55°C overnight, proteinase K was inactivated at 99°C for 10 minutes.

Mutation Detection by Restriction Endonuclease Analysis – Fifteen microliters of *p53* exon 8 PCR products were digested with restriction endonuclease NlaIII (New England Biolabs, Hertfordshire, UK), according to the manufacturer's instructions. The mutation in codon 273, CGT→CAT, Arg→His (Arg273→His) induce a CATG sequence that is recognized by the NlaIII enzyme that cleaves the sequence generating two fragments of 74 bp and 136 bp from the 210-bp product of the exon 8 PCR. The absence of a mutation in codon 273 results in an uncleaved 210-bp fragment (full-length PCR product). These fragments were separated and identified on 2% agarose gel stained with ethidium bromide.

RESULTS

In this chapter a summary of the most relevant results is presented. A detailed description of results are documented in papers I to V.

1-*p53* mutations and gemistocytes in diffuse astrocytomas (unpublished results and Paper I)

1.1-Distribution of *p53* mutated cells in a diffuse astrocytoma (unpublished results)

A representative tumour area of a whole brain section from a diffuse astrocytoma was first pre-screened for mutations of the *p53* tumour suppressor gene. A missense mutation in codon 273, CGT→CAT, Arg→His (Arg273→His) at exon 8 was identified. This mutation creates a restriction site recognised by the endonuclease *NlaIII*, that cleaves the sequence, generating two smaller fragments. Therefore, we used a PCR, followed by restriction endonuclease digestion to detect the presence of *p53* Arg273→His mutation. Control experiments (using DNA from a homozygous *p53* Arg273→His mutated glioblastoma cell line, U251, and wild-type *p53* from blood DNA) showed a sensitivity of mutated versus wild-type DNA sequence of ≥ 0.25.

A total of 475 areas of $4mm^2$ each, were individually analysed for the presence of *p53* Arg273→His mutation. The method did not allow to precisely quantify the proportion of the mutated versus wild-type sequences. Thus, the results were described as positive, (red colour) when the mutated sequences were observed, and negative, (blue colour) when only the wild-type sequence was detected. When both mutated and wild-type sequences were observed, the result was also considered as positive (red colour). The results showed the presence of the mutant sequence in all tumour regions and absence of mutation in normal surrounding tissue (Fig. 12).

Twenty samples were submitted to a second and third independent PCR-restriction endonuclease analysis to confirm the reproducibility of the method. Similar results were found in almost all samples, except in few samples localised in the infiltrating areas.

Wild-type *p53*

Mutated *p53*

1 cm
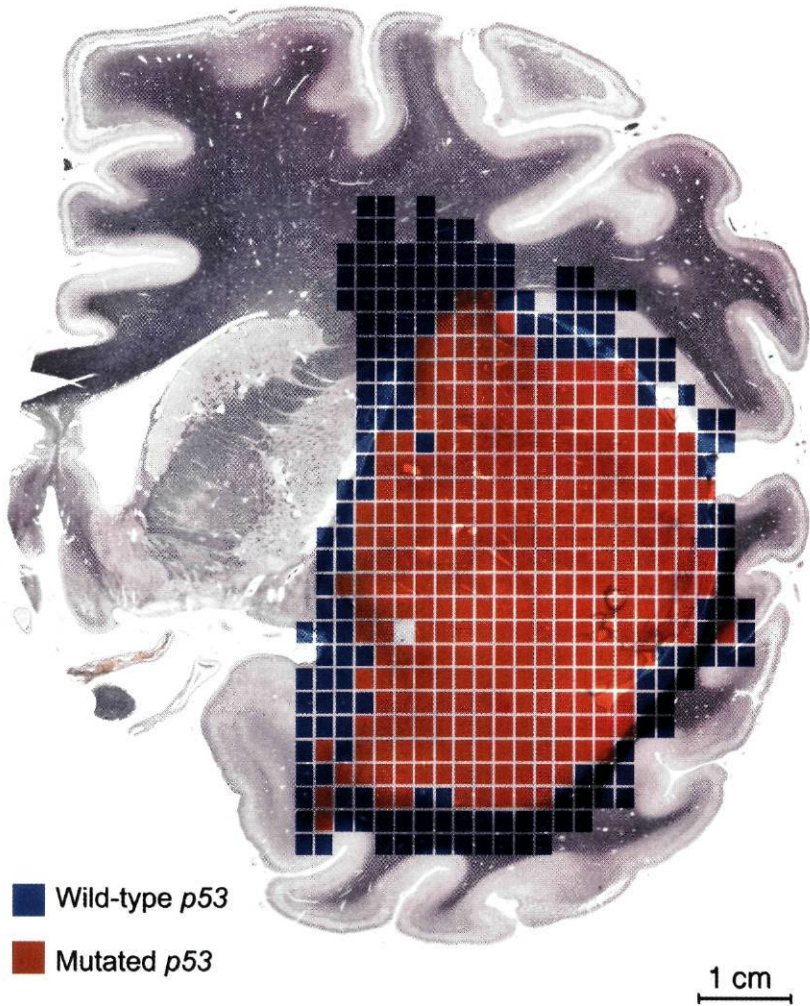
Fig. 12 - Distribution of *p53* mutations in a diffuse astrocytoma (WHO grade II).

1.2-Analysis of *p53* mutations in gemistocytes of gemistocytic astrocytomas (Paper I).

With the assistance of a laser microdissection system, individual gemistocytes and non-gemistocytic neoplastic cells were separately isolated and DNA was extracted for comparative analysis of *p53* mutations in 6 gemistocytic astrocytomas. The results demonstrated that in all 6 cases, both cell types harboured identical *p53* mutations (Table 3). These mutations were somatic and in all cases the wild-type sequence was absent.

Table 3 - *p53* mutations in gemistocytic astrocytomas.

Patient ID	Age/ Sex	WHO grade	*p53* mutation	
			Gemistocytes	Non-gemistocytic neoplastic cells
142	54/M	II	codon 242, TGC→GGC	codon 242, TGC→GGC
145	57/M	II	codon 248, CGG→CAG	codon 248, CGG→CAG
147	20/F	II	codon 175, CGC→CAC	codon 175, CGC→CAC
148	38/M	II	codon 248, CGG→CAG	codon 248, CGG→CAG
150	32/M	II	intron 7, G→C	intron 7, G→C
151	33/F	III	codon 273, CGT→TGT	codon 273, CGT→TGT

2-LOH on chromosome 10 and human *BUB* gene family mutations in glioblastomas (Papers II and III)

2.1-LOH on chromosome 10 in glioblastomas (Paper II)

Using a panel of 28 microsatellite markers spanning the entire chromosome 10, 17 primary glioblastomas and 13 secondary glioblastomas were investigated for LOH status.

The frequency of LOH#10 was similar in both primary and secondary glioblastomas, 47% and 54%, respectively. However, the pattern of deletion was different. Seven primary glioblastoma cases demonstrated LOH at all informative loci, indicating loss of whole copy of chromosome 10, and in one case (Patient ID: 294) the deletion region was restricted to the 10p arm (Table 4).

In secondary glioblastomas, the deleted loci were almost exclusively restricted to the 10q arm. With one case (Patient ID: 25) showing complete loss of 10q arm, and the other 6 cases showing partial deletions of 10q arm. Only one case (Patient ID: 70), showed LOH at 10p arm (Table 4). The common deleted region identified was located in the region 10q25-qter. In 4 cases in which normal tissue was used as reference, three of them showed

identical allelic pattern between diffuse astrocytoma and normal tissue, and one showed that a partial deletion at 10qter had already occurred in the diffuse astrocytoma.

The analysis of *p53* and *PTEN* mutations, as well as amplification of *EGFR* in both primary and secondary glioblastoma was also performed. No significant associations have been found between LOH#10 and the other genetic abnormalities, although, there was a tendency for correlation between complete LOH#10 and *EGFR* amplification (P=0.10).

Table 4 - LOH on chromosome 10 and other genetic alterations in primary and secondary glioblastomas.

Patient ID	LOH		Other genetic alterations		
	10p	10q	*p53* mutations	*PTEN* mutations	*EGFR* amplification
Primary glioblastomas					
233	-	-	-	-	-
234	+	+	-	+	-
256	-	-	+	-	-
257	-	-	-	-	-
258	-	-	-	-	-
287	+	+	-	-	-
288	+	+	-	-	-
289	+	+	-	-	+
292	-	-	-	-	-
294	+	-	-	-	-
296	-	-	-	-	-
300	+	+	-	-	+
301	+	+	-	-	+
302	+	+	-	+	+
303	-	-	-	-	-
314	-	-	+	-	+
344	-	-	-	-	-
Secondary glioblastomas					
11	-	-	+[*]	-[*]	-
25	-	+	+[*]	-[*]	-
26	-	+	+[*]	-[*]	-
35	-	-	+[*]	-[*]	-
57	-	-	+[*]	-[*]	-
58	-	-	+[*]	-[*]	-
59	-	-	+[*]	-[*]	-
60	-	+	-[*]	-[*]	-
64	-	+	+[*]	-[*]	-
68	-	+	+[*]	-[*]	-
70	+	+	-[*]	-[*]	-
72	-	-	+	-	-
295	-	+	+	-	-

[*], previously reported [30,107].

2.2-Mutation analysis of *hBUB1*, *hBUBR1* and *hBUB3* mitotic checkpoint genes in glioblastomas (Paper III)

PCR-SSCP followed by direct DNA sequencing of all 25 exons of the *hBUB1* gene showed a silent mutation at codon 144, CAG→CAA, Gln→Gln (Gln144→Gln) in one out of 22 glioblastomas (Table 5). We further screened 18 giant cell glioblastomas, and found that 2 of them also disclosed the same silent mutation at codon 144.

Table 5 - Genetic alterations of mitotic checkpoint genes in glioblastomas.

Patient ID	Age/ Sex	*hBUB1* alterations	*hBUBR1* alterations		*hBUB3* alterations
			Codon 349	Other codons	
72˙	28/F	-	Gln/Arg	-	Position -6, C→T
201	63/M	-	Gln/Arg	Ala388→Ala	-
233	59/M	-	Gln/Gln	Ala388→Ala	-
234	43/M	-	Gln/Arg	-	-
256	56/F	-	Gln/Gln	-	-
257	50/M	-	Gln/Arg	-	Position -6, C→T
258	65/F	-	Gln/Gln	Asp952→Asp	Position -6, C→T
287	47/F	-	Gln/Gln	Ala388→Ala	-
288	69/M	-	Gln/Gln	Ala388→Ala	-
289	58/F	-	Gln/Gln	Ala388→Ala	-
290	63/F	-	Gln/Gln	-	-
292	62/M	-	Gln/Arg	Ala388→Ala	-
295˙	47/M	-	Gln/Gln	-	-
296	63/F	-	Gln/Gln	Ala388→Ala	-
298	30/M	-	Gln/Gln	-	-
299	50/F	-	Gln/Gln	-	-
300	71/F	Gln144→Gln	Arg/Arg	-	-
301	68/F	-	Gln/Arg	-	-
302	64/M	-	Gln/Gln	Ala388→Ala	-
303	67/M	-	Arg/Arg	-	-
314	40/M	-	Gln/Arg	-	-
344	47/M	-	Gln/Gln	-	Position -6, C→T

˙, secondary glioblastomas.

The screening of the entire coding sequence of the *hBUBR1* gene revealed a silent mutation at codon 388, GCG→GCA, Ala→Ala (Ala388→Ala) in 8 glioblastomas and another silent mutation at codon 952 GAC→GAT, Asp→Asp (Asp952→Asp) in one glioblastoma (Table 5). A known polymorphism at codon 349 CAA→CGA, Gln→Arg (Gln349→Arg) was also detected with an allele frequency of 0.75 for Gln and 0.25 for Arg.

The analysis of peripheral blood leukocytes DNA from Caucasian individuals showed similar allele frequencies (0.73 for Gln and 0.27 for Arg) (Table 6).

Table 6 - Frequency of *hBUBR1* and *hBUB3* polymorphisms.

	hBUBR1 codon 349 Gln / Arg	*hBUB3* position (-6) C→T
Glioblastomas (n=22)	0.75 / 0.25	0.18
Random population (n=60)	0.73 / 0.27	0.15

The analysis of the entire coding sequence of *hBUB3* gene did not show any mutation in the coding region, but a base substitution was found at 6 nucleotides upstream the ATG initiator codon (C→T) in four glioblastomas (Table 5). These mutations were also found in peripheral blood leukocytes DNA from these patients, suggesting that they are polymorphism. The further analysis of Caucasian individuals for this polymorphism, showed the presence of the mutation at similar frequency (Table 6).

The *hBUB3* gene is localised on a common deleted region on chromosome 10q26. Of the 18 glioblastomas previously analysed for LOH#10, 12 were informative, and four of them were LOH positive for the 10q26 locus. In addition to the results described in Paper III, the expression of *hBUB3* mRNA by differential RT-PCR analysis was further studied (unpublished results). All samples expressed *hBUB3*, at similar levels to those obtained from non-tumour brain tissue (cortex and medulla), and no differences were observed between samples with or without LOH#10q26.

3-Second primary glioblastoma (Paper IV)

In an attempt to elucidate the ground genetic events of a patient that developed a second glioblastoma 10 years after the surgical excision of a first glioblastoma, both glioblastomas were screened for some genetic alterations usually found in glioblastomas, including *p53* and *PTEN* mutations, $p16^{INK4a}$ homozygous deletion, *EGFR* amplification and Rb expression. Further analysis of LOH on chromosomes 10, 19q and additional markers to determine microsatellite instability status was performed.

The results showed the presence of multiple *p53* and *PTEN* gene mutations, both in the first and second tumour biopsy. None of the mutations was common to both tumours. Homozygous deletion of $p16^{INK4a}$ was detected in both tumours (Table 7).

The microsatellite analysis of chromosome 10 and 19q did not show LOH in both tumours. In addition to the results described in Paper IV, LOH status was also assessed on 1p35-36 region and on 3q21 (*MBD4* locus) with 4 microsatellite markers each (unpublished results). All 8 markers were informative but no LOH was found in any of them. Except for one marker on chromosome 10, no allelic shift was detected in both tumours. A forensic DNA test with polymorphic DNA markers confirmed that both tumours were indeed from the same patient.

Table 7 - Multiple genetic alterations in first and second tumour biopsies.

Biopsy	First	Second
Age	54 years	64 years
Diagnosis	glioblastoma	glioblastoma
p53 mutations	codon 213, CGA→TGA	codon 158,CGC→CAC
	codon 306,CGA→TGA	codon 273, CGT→TGT
PTEN mutations	codon 154, TTC→TTT	codon 233, CGA→TGA
	codon 257, TTC→TTA	
p16^{INK4a} deletion	Yes	Yes
EGFR amplification	No	No
RB expression	Yes	Yes
LOH 10 and 19q	No	No
Microsatellite instability	No	No

4-Genetic characterisation of gliosarcomas (Paper V)

To define the genetic profile of gliosarcomas, an extensive molecular analysis in 19 *bona fide* cases was carried out. All tumours showed a typical biphasic pattern with alternating areas of gliomatous and sarcomatous neoplastic tissue. The gliomatous component showed GFAP positive cells without reticulin stroma, while the sarcomatous component was rich in reticulin stroma and the tumour cells lacked GFAP (see Fig. 5).

By PCR-SSCP followed by direct DNA sequencing, *PTEN* and *p53* mutations were found with a frequency of 37% and 26%, respectively. Differential PCR showed a high frequency of *p16*^{INK4a} homozygous deletion (37%). Amplification of *CDK4* and *MDM2* occurred with a frequency of 10% and 5%, respectively. None of the cases showed *EGFR* amplification (Table 8).

Immunohistochemistry analysis of Rb, p53, MDM2 and EGFR proteins was also carried out. Rb immunohistochemistry showed lack of expression in only one case (5%) (Patient ID: 210). Four cases with *p53* miscoding mutations showed p53 protein accumulation and

one case with a stop codon induced mutation did not show immunoreactivity. MDM2 overexpression (>50% of positive cells) was detected in both glial and mesenchymal components in one case that also showed amplification of *MDM2*. No overexpression of EGFR was found.

In seven gliosarcoma cases, it was possible to dissect separately the gliomatous and the sarcomatous component. In five cases, identical genetic alterations in both tumour components were found, such as *p53* mutations (Patient ID: 210), *PTEN* mutations (Patient ID: 215 and 218), *p16^{INK4a}* homozygous deletion (Patient ID: 214) and co-amplification of *CDK4* and *MDM2* (Patient ID: 220). One case (Patient ID: 213) showed *p16^{INK4a}* homozygous deletion only in the sarcomatous component, whereas the gliomatous component showed no *p16^{INK4a}* deletion (Table 8).

Table 8 - Genetic alterations of gliosarcomas.

Patient ID	Biopsy/ diagnosis	Area	p53 mutation	PTEN mutation	$p16^{INK4a}$ /sts ratio	CDK4 /IFGN ratio	MDM2 /DR ratio	EGFR /CF ratio
210	1st/GS	S	Pro190→Ser	-	1.10	0.92	1.40	0.96
210[#]	2nd/GS	S	Pro151→Ser	-	0.32	0.83	1.22	1.08
		G	Pro151→Ser	-	0.50	0.44	0.78	0.85
213[#]	1st/GS	S	-	-	**0.08**	0.61	0.79	0.75
		G	-	-	0.31	0.56	1.06	0.81
214[#]	1st/GS	S	-	-	**0.04**	0.74	0.59	1.23
		G	-	-	**0.01**	0.79	0.60	1.18
215[#]	1st/GS	S	-	Asp107→Ala	0.75	1.19	1.09	1.35
		G	-	Asp107→Ala	0.65	1.08	1.02	0.83
218[#]	1st/GS	S	-	Trp274→stop	0.60	1.23	1.29	0.88
		G	-	Trp274→stop	0.78	1.24	1.47	1.28
220[#]	1st/GS	S	-	-	0.62	**3.08**	**5.78**	1.15
		G	-	-	0.45	**3.44**	**9.24**	1.18
223[#]	1st/GS	S	-	-	0.48	0.71	0.74	1.13
		G	-	-	0.58	0.80	0.68	1.06
200	1st/GS	S	-	-	0.38	0.95	1.17	1.25
206	1st/GS	S	Gly245→Asp	-	**0.04**	0.63	0.79	0.8
207	1st/GS	S	Val197insGT	Trp111→stop	0.44	1.06	1.02	0.99
208	1st/GS	S	- *	-	**0.16**	2.33	1.04	1.25
209	1st/GS	S	- *	Tyr88→Ser	0.51	1.30	1.03	0.94
211	1st/GS	S	- *	-	**0.17**	1.34	1.49	1.67
212	1st/GS	S	nd	Gln17→Pro	nd	nd	nd	nd
	2nd/GS	S	- *	Gln17→Pro	0.31	0.95	0.88	0.76
216	1st/AA	G	-	nd	nd	nd	nd	nd
	2nd/GBM	G	-	nd	nd	nd	nd	nd
	3rd/GS	S	Val274→Ala	-	0.59	0.96	1.90	0.73
217	1st/GS	S	-	-	0.33	2.3	1.43	0.83
219	1st/GS	S	-	Trp274→stop	**0.17**	0.34	0.49	0.58
221	1st/GBM	G	nd	nd	**0.14**	nd	nd	nd
	2nd/GS	S	-	-	**0.02**	0.98	0.96	0.83
222	1st/GS	S	Met237→Ile	Asn48→Asp	0.8	**3.10**	0.97	1.08

[#], Samples suitable for comparative analysis of both tumour components; S, sarcomatous component; G, gliomatous component; *, previously reported by Biernat et al [40]; - negative; differential PCR data described in bold indicates amplification or deletion; nd, not determined; AA, anaplastic astrocytoma; GBM, glioblastoma; GS, gliosarcoma.

DISCUSSION

In this part the results described in the previous section will be discussed and analysed according to the proposed aims.

1-*p53* mutations and gemistocytes in diffuse astrocytomas

1.1-Distribution of *p53* mutated cells in a diffuse astrocytoma

Although *p53* mutations are considered a genetic hallmark of diffuse astrocytomas, their precise role and timing of appearance remains unclear. *p53* mutations in diffuse astrocytomas (WHO grade II) might be associated with a poor prognosis but the results of several studies are inconsistent [14,222]. In non-specified diffuse astrocytomas, the reported frequencies of *p53* mutation varies among studies (25 to 40%) [25,68,223], and in cases that progress to higher-grade malignancies, more than 65% harbour *p53* mutations [25,210,216,224]. Ishii *et al* [82] found that diffuse astrocytoma patients with *p53* mutations had higher risk of malignant progression and shorter time till progression. Watanabe *et al* [25] also observed a trend toward a shorter time till progression in patients with diffuse astrocytomas carrying *p53* mutations. In contrast, other studies found no association between *p53* mutations and clinical course of diffuse astrocytoma patients [225,226]. Moreover, one study showed that diffuse astrocytoma patients containing *p53* mutations had a survival time nearly twice as long as those without *p53* mutations [214]. Thus, *p53* mutation is not the only progression associated event in diffuse astrocytomas. Other genetic alterations must be involved in progression of diffuse astrocytomas.

In contrast to other neoplasms, such as colorectal carcinomas where *p53* mutations occur in the late stage of tumour progression, *p53* mutations occur at similar frequencies in diffuse astrocytomas, anaplastic astrocytomas and secondary glioblastomas [2,68]. In *p53* mutated positive anaplastic astrocytomas or glioblastomas that had developed from diffuse astrocytomas, more than 90% (22/24) of cases already disclosed the mutation in the first biopsy [25]. These evidences suggest that when *p53* mutations occur they are an early event in astrocytomas. *In vitro* experiments have suggested the clonal expansion of *p53* mutated cells during diffuse astrocytoma progression to anaplastic astrocytoma and glioblastoma [81,82]. These results support the possibility that *p53* mutation is an early progression event, rather than "the" initiation event, since only a fraction of cells in the primary diffuse astrocytoma harboured *p53* mutations. However, the high risk of astrocytoma development early in life observed in patients with Li-Fraumeni syndrome, points toward an initiation role of *p53* in the tumours of these patients [79].

In our study we conducted an extensive *p53* mutation analysis of a diffuse astrocytoma (fibrillary variant) whole brain section. The results showed the presence of a *p53* missense mutation (Arg273→His) in all tumour regions. Due to the low sensitivity of the method used

in this study, it was not possible to precisely determine the proportion of mutated versus wild-type *p53* allele in the different tumour regions. The codon 273 is the most frequently mutated in diffusely infiltrating astrocytomas [68], and this amino acid change (arginine to histidine) is believed to inactivate the transcriptional activity of p53 [227]. In some adjacent normal tissue, it was also found the presence of mutated *p53*. This observation is likely due to the infiltrating neoplastic cells into surrounding normal brain tissue, as it was confirmed by the histological examination of the respective regions. The absence of mutated sequence in non-tumour tissue excludes the possibility of a germline mutation.

In our study the only molecular alteration analysed was the presence of *p53* mutation. It remains to be clarified whether *p53* mutation is associated with other genetic abnormalities. It is interesting that LOH of *p53* locus was significantly associated with PDGFR-α overexpression in astrocytomas [228]. Moreover, primary cortical astrocytes without functional *p53* become transformed only in the presence of basic fibroblast growth factor [83], suggesting that concomitant loss of *p53* and growth factor(s) overexpression are involved in tumour development of diffuse astrocytoma.

To the best of our knowledge, these results document for the first time that a *p53* mutation is present in all tumour regions of low-grade astrocytoma (WHO grade II). These findings suggest that, at least a subset of sporadic diffuse astrocytomas evolve from clonal expansion of *p53* mutated cells, supporting the role of *p53* as a gatekeeper in diffuse astrocytoma.

1.2-Analysis of *p53* mutations in gemistocytes of gemistocytic astrocytomas

The role of gemistocytes in diffusely infiltrating astrocytomas has been a disputable issue. Gemistocytic astrocytomas have a poorer prognosis than fibrillary astrocytomas [2,15,17,18,229], leading Krouwer *et al* [18] to suggest the classification of gemistocytic astrocytomas as anaplastic astrocytomas. Indeed, according to the WHO classification [24], although rarely, some tumours are diagnosed as anaplastic gemistocytic astrocytoma. Gemistocytic astrocytomas, WHO grade IV, (probably, glioblastomas with predominant gemistocytic component) have also been described [230].

Several factors have been proposed to explain the poor prognosis associated with gemistocytic astrocytomas. This diffuse astrocytoma variant has a high frequency of *p53* mutations (60-82%) [78,230]. Watanabe *et al* [17] reported that diffuse astrocytomas containing more than 5% gemistocytes have significant shorter time till progression, and that all of these cases have *p53* mutations, whereas only 61% of cases containing less than 5% gemistocytes were *p53* mutated positive and had a significant longer time till progression [17]. However, the prognostic value of *p53* mutations in gemistocytic astrocytomas remains controversial, as previously mentioned for diffuse astrocytomas.

The mechanism responsible for gemistocyte differentiation remains obscure. Some reports suggested that this type of differentiation is caused by deprivation of nutrients and oxygen, due to competition with surrounding neoplastic cells [19]. In fact, gemistocytes are also found in a variety of central nervous system disorders associated with ischemic or hypoxic conditions [19,22,23]. Thus, the hypoxic/anoxic tumour environment may be responsible not only for the phenotype of gemistocytes, but also for a worse prognosis associated with their presence in tumours. Hypoxia has been shown to increase genomic instability, and up-regulation of proteins involved in tumour invasiveness, as well as of growth factors (e.g. fibroblast growth factor, vascular endothelial growth factor) [231,232]. Interestingly, some studies have reported the expression of basic and acid fibroblast growth factor by gemistocytes in astrocytic tumours [233,234]. Thus, the anoxic conditions could act as a selective force to induce the development of more resistant and malignant cells, leading to a more aggressive behaviour of the tumour [235-237]. Graeber et al [235] have reported the association of anoxic conditions with the selection and expansion of apoptotic resistant p53 mutated cells in solid tumours. Furthermore, Watanabe et al [17] also reported a marked bcl-2 expression in gemistocytes, suggesting that the gemistocyte accumulation within diffuse astrocytomas may be due to bcl-2-mediated apoptosis escape. Increased bcl-2 expression under p53 inactivation was suggested to provide cells with a selective survival advantage in cervical carcinoma cell lines [238].

It is interesting to stress that gemistocytes do not seem to proliferate, even if present in anaplastic astrocytomas or glioblastomas, which lead to speculate their possible terminal differentiation status [17,19,20]. The aforementioned evidence raises the question of whether gemistocytes are neoplastic cells or reactive astrocytic cells. This question was the main objective in our study of gemistocytic astrocytomas.

Kros et al [239] analysed astrocytomas, oligodendrogliomas and mixed oligoastrocytomas with gemistocytes for abnormalities of chromosomes 1, 7, 10 and 17 by in situ hybridisation. There was no difference in spot distribution between gemistocytes and non-gemistocytic neoplastic cells in six out of nine cases with abnormalities. In the remaining cases, the concordance was incomplete [239]. In our study we observed identical p53 mutations in both gemistocytes and non-gemistocytic neoplastic cells in all six gemistocytic astrocytomas analysed. The use of a laser-assisted microdissection in our study, guaranteed a pure histologic population for DNA analysis. Notably, in all 6 gemistocytic astrocytomas analysed only the mutated allele was detected, indicating the loss of the wild-type allele. In a previous study of the same tumours, in which tumour areas were dissected manually, the wild-type p53 allele was detected in four of the samples [78], probably due to contamination with entrapped non-neoplastic cells. These data stress the importance of use of laser microdissection system to more precisely determine the loss of wild-type allele in primary tumour samples, namely, p53.

In summary, our findings strongly support the monoclonal origin of both gemistocytes and non-gemistocytic neoplastic cells and that gemistocytes are indeed neoplastic cells in gemistocytic astrocytomas.

2-LOH on chromosome 10 and human *BUB* gene family mutations in glioblastomas

2.1-LOH on chromosome 10 in glioblastomas

Deletions of chromosome 10 are the most frequent genetic abnormality in glioblastomas [27,145,147]. Although the majority of glioblastomas have deletion of the entire chromosome 10 (monosomy 10), a number of attempts have been made to identify the smallest commonly deleted region, which would harbour tumour suppressor genes. The pattern of deletions observed suggests at least three putative tumour suppressor loci, i.e., 10p14-ter, 10q23-24, and 10q25-qter [144-147,240].

The results of our study showed that LOH#10 occurs at similar frequency in both primary (47%) and secondary glioblastomas (54%), but with different patterns of deletion. Primary glioblastomas are characterised by loss of an entire copy of the chromosome 10, while secondary glioblastomas typically display partial loss on 10q.

Ichimura *et al* [145] studied 198 astrocytic tumours for LOH#10 and showed that most glioblastomas lost the entire copy of the chromosome 10, while diffuse astrocytomas preferentially lost the 10p. However, the astrocytomas and glioblastomas analysed derived from distinct patients [145]. Contrasting with this study, we and others authors, observed the absent of LOH#10p on secondary glioblastomas; Weber *et al* [224] reported that 4 of 10 anaplastic astrocytomas and glioblastomas that had progressed from diffuse astrocytomas showed 10q deletions using comparative genomic hybridisation (CGH) analysis of [224]. Further evidence is provided by Fujisawa *et al* [151] that observed deletion of 10q in 4 of 5 secondary glioblastomas.

Like in previous reports [241-243], we found that *EGFR* amplification is associated with chromosome 10 monosomy. Of the several putative tumour suppressor genes that have been identified on chromosome 10, including *DMBT1* [178], *MXI1* [183], *hBUB3* [138], *LGI1* [185], only *PTEN* (10q23) was established as a tumour suppressor gene involved in glioblastoma tumourigenesis. Tohma *et al* [107] reported that *PTEN* mutations are frequent in primary glioblastomas (32%) and rarely observed in secondary glioblastomas (4%). These findings, together with the observed LOH#10q frequencies in both primary and secondary glioblastomas, indicate that inactivation of other gene(s) on chromosome 10q may be involved in the development of secondary glioblastomas. Indeed, in our study, as well as in

a previous study [151], the commonly deleted locus detected in secondary glioblastomas lies between the 10q25-qter region, distal to the *PTEN* region.

Overall, this study shows that LOH#10 is more extensive in primary than in secondary glioblastomas, suggesting that while putative tumour suppressor gene(s) on 10q may be involved in both subtypes, the gene(s) located on 10p may be preferentially associated with the primary glioblastomas.

2.2-Mutation analysis of *hBUB1*, *hBUBR1* and *hBUB3* mitotic checkpoint genes in glioblastomas

A mutational analysis of the *hBUB1*, *hBUBR1* and *hBUB3* genes was performed in order to determine whether inactivation of mitotic checkpoint genes is involved in the evolution of glioblastomas. The analysis of *hBUB1* showed only a silent mutation at codon 144 in one glioblastoma and two giant cell glioblastomas. This silent mutation has been previously detected in colon and bladder tumours, as well as from blood of the corresponding patients [137,244], indicating to be a polymorphism.

The screening of the entire coding sequence of *hBUBR1* gene revealed two silent mutations at codons 388 and 952, both of which have previously been detected in colon, breast and lung tumours [139,245,246]. It was also identified a known polymorphism on codon 349 (CAA→CGA, Gln→Arg). The allelic frequency was 0.75 for the amino acid glutamine (Gln) and 0.25 for the amino acid arginine (Arg), similar to the frequency detected in Caucasian individuals. However, these values were significantly different from the ones obtained for Japanese individuals (Table 9), suggesting differences in ethnical allelic frequencies. Nevertheless, from the molecular epidemiological point of view, this polymorphism may be of interest because of its possible association with different genetic susceptibility in different populations to the occurrence of aneuploidy. Interestingly, the incidence of CNS tumours in Switzerland is 6.1 cases per 100,000 for men, whereas in Japan is 3.0 cases per 100,000 for men [4]. Further studies should be developed to address the biological effects of this polymorphism.

The analysis of the entire coding *hBUB3* sequence revealed no mutations in any of the cases. However, a substitution mutation, C→T 6 bp upstream from the initiation codon was found in four glioblastomas, as well as in blood DNA from the corresponding patients, suggesting a polymorphism. The screening of Caucasian individuals was conducted to determine whether this polymorphism was over-represented in patients with glioblastomas. The frequency of T base in patients with glioblastoma was not statistically different from the frequency observed in the control individuals (Table 9). Moreover, Haruki *et al*, [247] have found no significant differences of allelic frequencies in patients with lung cancer (Table 9). Nevertheless, this polymorphism is localised near the Kozak's consensus sequence [249],

and its functional significance remains to be clarified in terms of translation efficiency. Together with lack of *hBUB3* mutations, the absence of *hBUB3* transcript downregulation in all of our glioblastoma cases, even the ones with LOH#10q, showed that *hBUB3* gene is not genetically deregulated, suggesting the exclusion of *hBUB3* as a candidate tumour suppressor gene in glioblastoma development.

Table 9 - Frequency of *hBUBR1* and *hBUB3* polymorphisms.

	hBUBR1 codon 349 Gln / Arg	*hBUB3* position (-6) C→T
Caucasian		
Glioblastoma patients (n=22)	0.75 / 0.25	0.18
Random population (n=60)	0.73 / 0.27	0.15
Japanese		
Lung cancer patients (n=44)*	0.39 / 0.61	0.27
Random population (n=224)**	0.31 / 0.69	nd

*,Haruki *et al* [247]; **, Katagiri *et al* [248]; nd, not determined.

Considering the absence of mutational inactivation of the *hBUB1*, *hBUBR1* and *hBUB3* genes in glioblastomas together with other mutational analysis (Table 10), it is unlikely that mutations in these mitotic checkpoint genes are associated with the genetic instability in most human tumours. Additional studies are also necessary to clarify whether mutations of other mitotic checkpoint genes, such as *hMAD1L1*, *hMAD2*, and *hMPS1* play any role in glioblastoma tumourigenesis. However, previous studies have also reported rare or absent mutations of these genes in a variety of other tumour types [139,244,250-253,253]. Indeed, a recent work reported absence of inactivation mutations of *hMAD1L1* in 5 glioblastoma cell lines [254].

Further studies will be necessary to determine whether these genes are inactivated by other mechanisms, such as promoter methylation, defects at post-transcriptional regulation, or microsatellite instability. Indeed, the later mechanism was recently detected within the *hBUB1* gene in a subset of colorectal cancers [259].

Table 10 - Summary of miscoding mutations in *hBUB*-family gene in human tumours

Tumours	N° Cases	hBUB1	hBUBR1	hBUB3	Reference
Brain	22	0	0	0	Present study
Digestive tract	32	1	nd	nd	Imai [250]
	19	2	2	0	Cahill [137,139]
	5	0	nd	nd	Shigeishi [255]
Lung	102	1	0	nd	Sato [246]
	44	0	0	0	Haruki [247]
	60	1	nd	nd	Gema [256]
	25	0	nd	nd	Yamaguchi [257]
Breast	19	0	0	nd	Myrie [245]
Bladder	21	0	0	0	Olesen [244]
	52	0	nd	0*	Hernando [251]
Leukaemia	10	1	3	nd	Oshima [258]
Head and Neck	6	0	nd	nd	Yamaguchi [257]

nd, not determined; *, analysed in only 9 out of the 52 samples.

3-Second primary glioblastoma

Glioblastomas remain one of the deadliest human neoplasms, despite advances in neurosurgical techniques, radiation and drug therapies [1,260]. Long-term (more than 3 years) survival patients with glioblastomas are uncommon, and clinical-pathological parameters associated with long-term survivors are: young age (approximately 40 years old), high Karnofsky performance score, low proliferative index, presence of an oligodendroglial component, and multimodal treatments [32,33,261-264].

In our study we reported a peculiar case of a 54 years old male patient that survived 10 years after the diagnosis of a glioblastoma. After this period the patient developed a second glioblastoma. Both tumours depicted a high proliferative activity. Clinically, both glioblastomas of our patient developed *de novo*, after a short clinical history and without evidence of a less malignant precursor lesion. Surprisingly, both biopsies presented multiple genetic alterations in the *p53* and *PTEN* tumour suppressor genes, and most strikingly, none of *p53* and *PTEN* mutations occurred in both tumours. These findings indicate that the second tumour is not a recurrence of the first neoplasm. To rule out the hypothesis of sample identity error a forensic DNA analysis was carried out and confirmed the same identity of both tumour samples.

An important observation emerged from our results; the second tumour represents a second primary glioblastoma.

Except for organs with significant exposure to environmental carcinogens, such as oesophagus and oropharynx following many years of excessive tobacco and alcohol consumption [265], and hereditary syndromes, second primary tumours are considered to be rare. There is currently no evidence for significant exogenous risk factor for human brain tumours, with the exception of ionising radiation [2]. Our patient was not submitted to radiotherapy before the development of the first tumour. Since the patient was submitted to radiotherapy after surgical resection of the first tumour, one may argue that the second glioblastoma could have been caused by the radiotherapy treatment. However, it is more likely that the development of the second tumour is associated with the genetic background of the patient since the first tumour contained already multiple mutations and deletion of tumour suppressor genes.

A recent review of the literature found only 30 cases of patients that survived 10 or more years [33]. Of the 30 cases, 2 had an oligodendroglial component, one was a Turcot syndrome case, and a fourth was possibly a pleomorphic xanthoastrocytoma [33]. The mean age of these 30 cases was 32 years [33]. At present it is not known whether long-term survival patients with glioblastomas have a distinct pattern of genetic alterations. The few reported molecular studies available are summarised in Table 11. No specific genetic alterations can be drawn from these studies. In contrast with Ino et al [261], none of the tumours of our patient disclosed LOH of 1p35-36 region, which has been associated with long-term survival patients with glioblastomas [261] (Table 11).

Table 11 – Summary of clinical, genetic and immunohistochemistry features of glioblastoma from long-term survivors.

Age/ Sex	Survival (years)	Genetics							Immunohistochemistry			Reference
		p53	PTEN	p16^{INK4a}	EGFR	1p	10	19q	p53	EGFR	Rb	
54/M	10	Mut	Mut	Del	-	-	-	-	nd	nd	+	Present study
41/M	11	-	nd	nd	nd	nd	nd	nd	-	+	nd	Morita [262]
28/F	8	-	nd	nd	nd	nd	nd	nd	+	-	nd	Morita [262]
62/M	7	-	nd	nd	nd	nd	nd	nd	+	+	nd	Morita [262]
24/M	15	-	nd	nd	nd	nd	nd	nd	-	-	nd	Morita [262]
25/F	7	-	nd	nd	nd	nd	nd	nd	+	-	nd	Morita [262]
51/M	7	-	nd	nd	nd	nd	nd	nd	-	+	nd	Morita [262]
30/M	7	-	nd	nd	nd	nd	nd	nd	+	+	nd	Morita [262]
51/M	7	-	nd	nd	nd	nd	nd	nd	-	-	nd	Morita [262]
40/M	9	-	nd	nd	nd	nd	nd	nd	-	-	nd	Morita [262]
40/F	7	-	nd	nd	nd	nd	nd	nd	-	-	nd	Morita [262]
69/M	18	Mut	nd	nd	-	-	LOH10p	-	nd	nd	nd	Sabel [33]
44/M	6	-	nd	nd	-	LOH	-	-	nd	nd	nd	Ino [261]
61/F*	6	Mut	nd	nd	nd	nd	nd	nd	nd	nd	nd	Kosel [230]
45/F	15	-	nd	Del	-	LOH	LOH10q	-	nd	nd	nd	Ino [261]

nd, not determined, Mut, mutation, Del, homozygous deletion, -, no alteration, *, gemistocytic astrocytoma (WHO grade IV).

Considering the unremarkable familiar history of the patient, it is unlikely that this case represents some of the known cancer familiar syndromes; the absence of p53 germline

mutations (Reis *et al*, unpublished results) excluded the possibility of Li-Fraumeni syndrome; absence of microsatellite instability phenotype in both tumour biopsies also excluded the possibility of Turcot syndrome. This syndrome is characterised by colorectal carcinomas and glioblastomas caused by microsatellite instability phenotype due to germline mutations of the mismatch repair enzymes and is associated with a very good prognosis [266].

A remarkable finding of our study was the high frequency of transition mutations, G:C→A:T (6 out of 7), and 5 of them were located at CpG sites. This type of mutation is frequent in astrocytic tumours [68] and is thought to be due to deamination of 5-methylcytosine, which occurs spontaneously or induced through the action of oxygen radicals or nitric oxide in all cell types and is normally corrected by DNA repair mechanisms [267]. Thymine-DNA glycosylase (TDG) and MDB4 are enzymes involved in the initiation repair of this type of errors [268,269]. Mutations in the *TDG* gene have not been identified. However, decreased levels of mRNA were reported in pancreatic tumour cell lines [270] and a high frequency of LOH (42%) was observed in the *TDG* locus in gastric carcinomas [271]. Mutations of the *MBD4* gene have only been detected in colorectal and endometrial tumours with microsatellite instability [272,273]. The LOH analysis of *MBD4* locus, did not disclose any allelic deletion in both glioblastomas of the present case (Reis *et al*, unpublished results). O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that specifically removes promutagenic alkyl groups from the O^6 position of guanine in DNA [274]. Several lines of evidence have showed that inactivation of MGMT is also associated with the generation of G:C→A:T transition mutations [275,276]. In addition, Nakamura *et al* [277] found a significant correlation between *p53* transition mutations (G:C→A:T) and promoter methylation of *MGMT* gene in diffuse astrocytomas.

Although both tumours showed clinical features of primary glioblastomas, they disclosed genetic features common to both primary (*de novo*) glioblastomas, (e.g. *PTEN* mutations and *p16^INK4a* deletion) and secondary glioblastomas, (e.g. *p53* mutations and absence of *EGFR* amplification), which raises the possibility of a distinct genetic pathway for long-term survival patients with glioblastomas.

In summary, these results provide for the first time a genetic evidence of a second primary glioblastoma, and suggest the involvement of a novel unknown disease mechanism, characterised by multiple alterations in transformation-associated genes, and a favourable prognosis.

4-Genetic characterisation of gliosarcomas

The genetic alterations associated with gliosarcomas were largely unknown. In this study we showed that gliosarcomas contain several genetic alterations similar to those encountered in primary glioblastomas, including high frequency of $p16^{INK4a}$ homozygous deletion (37%) and *PTEN* mutations (37%). Twenty-six percent of gliosarcomas had *p53* mutations. This *p53* mutation frequency is higher than the usually observed in primary glioblastomas (11%), but is significant lower than the one found in secondary glioblastomas (67%) (Table 12) [27].

Table 12 - Clinical and genetic data of glioblastoma subtypes

	Primary Glioblastoma	Gliosarcoma	Giant cell glioblastoma	Secondary glioblastoma
Clinical onset	*de novo*	*de novo*	*de novo*	secondary
Preoperative clinical history	1.7 [30]	3 [a]	1.6 [35]	53 m. [30'] 25 m. [30'']
Sex ratio M/F	1.4[30]	1.4[a] 1.8[36]	1.2[35, 219]	0.8[30]
Age of diagnosis	56 [30, 218]	56[a] 53[36]	44 [35,219]	40 [30, 218]
p53 mutation	2/19 (11%) [30]	8/35 (23%) [a, 40, 290]	31/37 (84%)[35, 219]	20/30 (67%) [30]
PTEN mutation	9/28 (32%)[107]	8/21 (38%) [a, 291]	6/22(27%) [35, 291]	1/25 (4%) [107]
$p16^{INK4a}$ deletion	10/28 (36%)[55]	7/19 (37%) [a]	1/37(3%) [35, 219]	1/23 (4%) [55]
MDM2 amplification	2/29 (7%)[92]	1/19 (5%) [a]	0/18 (0%) [35]	0/27(0%) [92]
EGFR amplification	11/28 (39%)[107]	1/22 (4%) [a, 292]	2/37(5%) [35, 219]	0/22(0%) [107]
CDK4 amplification	1/28 (4%) [55]	2/19 (10%) [a]	1/19 (5%)[219]	3/23 (13%) [55]
Rb loss expression	2/28 (14%) [55]	1/19 (5%) [a]	nd	5/22 (23%) [55]

[a], present study; superscripts are numbers from the the list of references; *, from diffuse astrocytomas; **, from anaplastic astrocytoma; nd, not determined

The only significant difference between gliosarcomas and primary glioblastomas is the absence of *EGFR* amplification or overexpression in the former. This finding is particularly interesting, since the most frequent genetic alterations in gliosarcomas detected by cytogenetic studies are gains of chromosome 7 (*EGFR* gene location) and loss of chromosome 10 [220,221,278]. Early studies raised the possibility that amplification of *EGFR* was one of the targets of chromosomal 7 alterations in gliosarcomas [220,221,278]. The absence of *EGFR* amplification reported in our study, suggests the existence of other oncogenes on chromosome 7 involved in the development of this tumour type. *CDK6*, *PDGF-A*, *c-MET* and his respective ligand HGF/scatter factor are genes reported to be amplified or overexpressed in glioblastomas [65,120,279,280]. The immunohistochemistry analysis of HGF/c-MET in 10 gliosarcomas showed expression of both proteins only in the gliomatous component, suggesting an autocrine stimulation loop not involved in the acquisition of the sarcomatous phenotype (Reis *et al*, unpublished results). The role of *CDK6* and *PDGF-A* genes in development of gliosarcomas remains to be clarified.

Deregulation of the Rb pathway ($p16^{INK4a}$ deletion, CDK4 amplification and Rb loss of expression) was found in more than a half of gliosarcomas (10/19, 53%). The alterations identified were mutually exclusive as previously described in glioblastomas [50,54,55,62], indicating that deregulation of one of these genes may lead to uncontrolled cell cycle in gliosarcomas.

The clinical onset of gliosarcomas is mainly in *de novo* fashion. Perry *et al* [282] reported that 25 of 32 gliosarcoma cases (78%) were diagnosed as *de novo*, whereas 7 (22%) were diagnosed after irradiation for glioblastomas. In this study a gliosarcoma appeared as a recurrence of a glioblastoma (patient ID 221). Rarely, gliosarcomas develop through progression from diffuse [281] or anaplastic astrocytoma [283] and a patient (ID 216) of this study.

Gliosarcoma is defined as a glioblastoma variant composed of two distinct, gliomatous and sarcomatous components [36,37]. It was initially assumed that the sarcomatous component developed secondary from hyperplasia of endothelial or non-endothelial constituents of the vessel wall within glioblastoma [37,39,284-286]. Recently, molecular studies have suggested a common origin of both tumour components. Boerman *et al* [41] found identical genetic changes in both tumour components using comparative genomic hybridisation, cytogenetics and microsatellite instability. Similar results have been found by Biernat *et al*, [40], who described identical *p53* mutations in both gliomatous and sarcomatous tumour components in 2 cases.

In our study of both sarcomatous and gliomatous components only one case showed discordant results; the sarcomatous component showed a value clearly below the 0.2-ratio threshold of homozygous deletion, while the gliomatous component had a ratio of 0.3, not reaching the threshold limit. Since the manual dissection method used does not avoid all normal cells, as previously mentioned, their presence in the gliomatous component may explain this discrepancy. In all the other 5 gliosarcomas we observed in both tumour components identical genetic alterations, such as mutations of PTEN (two cases), mutation of *p53* (one case), homozygous deletion of $p16^{INK4a}$ (one case) and co-amplification of MDM2 and CDK4 (one case).

The present study gives further support to the concept of monoclonal origin of both components of gliosarcomas. This observation supports the hypothesis that the sarcomatous portion of a gliosarcoma arise by a process of divergent differentiation either from the same common precursor cell as does the gliomatous component, or by metaplasia from the gliomatous component itself. The observation that rodent multipotent stem cells, which normally gave rise to neurons, astrocytes and oligodendrocytes, can differentiated under certain culture conditions into a smooth muscle cells [287,288], supports that sarcomatous cells evolved from a multipotent neural stem cell or an early glial progenitor that retains the capacity to differentiate into a mesenchymal phenotype. On the

other hand, numerous studies documented the frequent loss of glial and acquisition of mesenchymal features in human glioma cell cultures and xenografts [289]. In addition, a subset of gliosarcomas evolves from a primary astrocytic tumour. Interestingly, one of our gliosarcomas that evolved from a primary glioblastoma (Patient ID 221) harboured a $p16^{iNK4a}$ deletion in both primary and recurrent tumours. Therefore, it is likely that the sarcomatous portion evolves from a malignant astrocytic neoplasm, with acquisition of a mesenchymal phenotype.

Although gliosarcomas are genetically and clinical similar to primary glioblastomas, the observation of a striking differences, absence of *EGFR* amplification, raises the possibility that gliosarcomas arise through an independent genetic pathway distinct from the ones responsible for the other glioblastoma subtypes, namely, primary, secondary and giant cell glioblastoma.

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

The main conclusions of the present study are the following:

A subset of diffuse astrocytomas evolves from clonal expansion of *p53* mutated cells, supporting the role of *p53* as a gatekeeper in diffuse astrocytoma.

In gemistocytic astrocytomas, gemistocytes are not reactive astrocytes, but true neoplastic cells.

LOH#10 is frequent in both primary and secondary glioblastomas. However, the pattern of deletion is different. Primary glioblastomas are characterised by loss of the entire chromosome 10, whereas secondary glioblastomas disclose partial or complete loss of chromosome 10q but no loss of 10p.

The *hBUB1*, *hBUBR1* and *hBUB3* mitotic checkpoint genes are not mutated in glioblastomas. Several polymorphisms were detected in these genes. A Gln349→Arg polymorphism of *hBUBR1* showed different frequencies in Caucasian and Japanese populations.

Two glioblastomas of a long-term survivor were characterised by multiple and distinct mutations in *p53* and *PTEN* genes, as well as homozygous deletion of $p16^{INK4a}$ gene. This case constitutes the first report of a second primary glioblastoma confirmed by genetic analysis.

Gliosarcomas disclose not only clinical features, but also a genetic profile similar to those of primary or *de novo* glioblastomas, with frequent $p16^{INK4a}$ homozygous deletion and *PTEN* mutations. However, unlike primary glioblastomas, gliosarcomas do not present amplification/overexpression of *EGFR*. The identification of identical genetic alterations in both gliomatous and sarcomatous components strongly supports the concept of monoclonal origin of both components of gliosarcomas.

The aforementioned conclusions derive directly from the different studies that were included in the thesis. As we had foreseen (Aim section) they represent fragmented pieces of evidence that are difficult to put together, especially if one attempts to create a comprehensive model of tumour development. Nevertheless, the data we obtained provide interesting insights in several windows of the astrocytic tumourigenesis. The challenge is, as we previously mentioned, to be able to build, with those pieces-of-evidence, coherent pathways.

Based on the literature review and our own results, we feel tempted to provide the oversimplified view that is represented on Figure 13. In this scheme the most important genetic pathways associated with the histogenesis of diffuse infiltrating astrocytomas subtypes have been highlighted. We also included in Figure 13 the relative frequencies, mean age at diagnosis, WHO grade and mean survival time that reflect the behaviour of each diffusely infiltrating astrocytoma subtype.
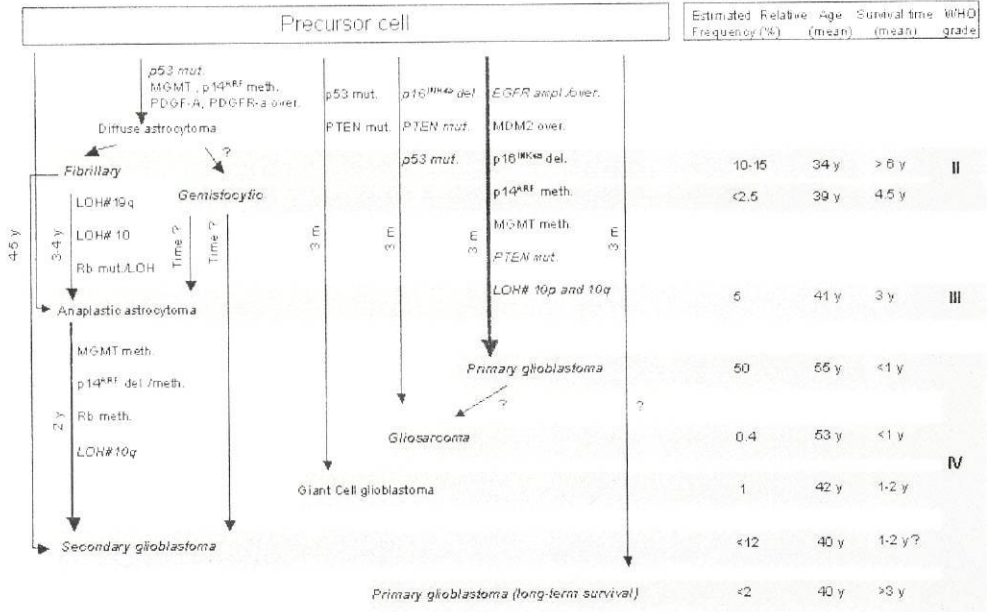
Fig. 13 – Schematic overview of diffusely infiltrating astrocytoma subtypes development. The most relevant events observed in the different studies included in this thesis are indicated in italic, and the clinico-prognostic features in dashed rows of each histologic subtype; The relative frequencies were estimated considering major reported series; y, years; m, months; mut., mutation; meth., methylation; ampl., amplification; over., overexpression; del; homozygous deletion.

Future perspectives

Several questions concerning the understanding of development of diffusely infiltrating astrocytomas are still unsolved. The present thesis also raises many additional questions, some of which are delineated:

Our results indicate that *p53* mutation represents an initiation event in diffuse astrocytomas. It will be warrant to extend this study to a higher number of diffuse astrocytomas. In fact, it is still controversial whether *p53* mutation occurs alone or in combination with other genetic alterations. Transgenic mouse models would help to clarify this issue, by the possibility of manipulation of several genes simultaneously.

It would also be interesting to study the role of gene inactivation by abnormal promoter methylation, as a possible early event in astrocytic tumour development.

Our results showed that in gemistocytic astrocytomas, gemistocytes are indeed neoplastic cells. It remains to be determined the factors involved in the gemistocyte phenotype, and why the presence of gemistocytes is associated with a poor prognosis.

Our results support the existence of several tumour suppressor genes on chromosome 10. We found a common deleted region on the 10q25-qter in secondary glioblastomas. Further studies would be necessary to refine the smallest deleted regions, and in combination with techniques for gene identification, isolate the candidate genes.

Given the importance mitotic checkpoint genes, including *BUB* family, in regulating the mitotic mechanisms and avoidance of chromosomal instability, it would be interesting to develop classic and conditional knockouts (gene deletion limited to astrocytic cells) mice to clarify the role of mitotic checkpoint genes in astrocytic tumour development.

The detection of a possible new mechanism of genetic instability in a long-term survival patient with glioblastoma, may be related with specific types of mutations / repair enzymes, namely *TDG* and *MBD4*. It would be of interest to determine their precise role in the development of our case, and in other long-term survival patients with glioblastomas.

The complete sequence of human genome will provide new tools and insights that will enhance our understanding of the genetic mechanisms underlying cancer. In recent years a variety of techniques have been developed that will allow a complete and the high-throughput analysis of genetic profile and alterations in neoplasms.

With techniques such as cDNA microarray, which allow the rapid survey of thousands of genes, it will be possible to determine the molecular fingerprints of each tumour type.

The use of such methodologies to the analysis of the diffusely infiltrating astrocytomas subtypes would help to clarify many of the aforementioned questions.

Noteworthy, the knowledge of the genetic alterations present in each tumour type will be essential to define gene therapy strategies, that may help to overcome the ominous prognosis of patients with diffuse infiltrating astrocytomas in the future.

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SUMMARY AND CONCLUSIONS

Diffusely infiltrating astrocytomas constitute the most frequent malignant primary brain tumours. These tumours exhibit an inherent tendency to progress to more malignant phenotypes and a broad range of clinical-pathological features with distinct behaviour and therapeutic implications. The understanding of the genetic alterations associated with astrocytic tumourigenesis is increasing but remains limited. The present work aimed to analyse the involvement of some genetic alterations in diffusely infiltrating astrocytomas subtypes development. This study was divided in 4 parts: **1,** *p53* mutations and gemistocytes in diffuse astrocytomas; **2,** Loss of heterozygosity (LOH) on chromosome 10 and mutations of human *BUB* genes family in glioblastomas; **3,** Second primary glioblastoma; and **4,** Genetic characterisation of gliosarcomas.

1–*p53* mutations and gemistocytes in diffuse astrocytomas

Diffuse astrocytomas (WHO grade II) are well differentiated and slow growing tumours that have a tendency for diffuse infiltration of neighbouring brain tissue, leading almost invariably to recurrence and progression to anaplastic astrocytoma, and ultimately, glioblastomas. Mutations of the *p53* gene are considered a genetic hallmark in diffuse astrocytomas. However, their degree of expansion, role and timing of appearance remain unclear. Aiming to elucidate this issue we screened all tumour areas of a fibrillary astrocytoma, as well as adjacent non-tumour tissue of a whole brain section for the presence of *p53* mutations Our observation of a *p53* mutation in all tumour areas of a diffuse astrocytoma, suggest that at least a subset of diffuse astrocytomas evolve through clonal expansion of *p53* mutated cells, supporting the role of *p53* as a gatekeeper.

Gemistocytic astrocytoma (WHO grade II), a histological variant of diffuse astrocytoma, is particularly prone to rapid malignant progression. However, gemistocytes do not proliferate and are suggested to represent a terminal differentiation status. Moreover, they resemble to some extent reactive astrocytes that are encountered under a variety of non-neoplastic CNS diseases. These facts have raised the question of whether gemistocytes are neoplastic cells or reactive astrocytes. We conducted a laser microdissection analysis of both tumour gemistocytes and non-gemistocytic neoplastic cells, and consistently observed the presence of identical *p53* mutations in both cell types. These findings strongly support their monoclonality and that gemistocytes are indeed neoplastic cells in gemistocytic astrocytomas.

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2–Loss of heterozygosity (LOH) on chromosome 10 and human *BUB* gene family mutations in glioblastomas

Glioblastomas can develop through progression of lower grade astrocytic tumours and are termed secondary glioblastomas. More frequently, they developed rapidly, with a short clinical history, without evidence of a precursor lesion, and are termed *de novo* or primary glioblastomas. These glioblastomas subtypes arise through distinct known genetic pathways. Since loss of chromosome 10 material is the most frequent genetic lesion reported in glioblastomas, we aimed to determine whether the frequency and pattern of LOH on chromosome 10 (LOH#10) differ between primary and secondary glioblastomas. The results showed that LOH#10 is frequent and it occurs at similar frequencies (approximately 50%) in both subtypes. However, the pattern of deletion was different, with primary glioblastomas exhibiting loss of the entire chromosome 10, whereas in secondary glioblastomas the deletions occur only in the long arm of chromosome 10. These findings suggest that LOH#10q is a major event in the evolution of glioblastoma as the common phenotypic end point of both genetic pathways, whereas LOH#10q is largely restricted to primary glioblastoma.

Glioblastomas are characterised by marked aneuploidy. This type of genetic instability has been associated with defects in mitotic spindle checkpoint genes in a subset of other neoplasms. We performed a mutational analysis of a family of mitotic checkpoint genes, *hBUB1*, *hBUBR1* and *hBUB3*, in a series of glioblastomas. None of the cases showed inactivating mutations of any of the genes, suggesting that mutations of these genes are not involved in the causation of chromosomal instability in glioblastomas. However, we detected several polymorphisms. The allelic frequency of the Gln349→Arg *hBUBR1* polymorphism was significantly different in Caucasian and Japanese population. Given the role of *hBUBR1* in mitotic checkpoint, this polymorphism may be of interest because of its possible association with different susceptibility in distinct populations to the occurrence of aneuploidy.

3–Second primary glioblastoma

Glioblastomas are the most malignant brain tumours and carry a dismal prognosis, with a mean survival time of less than one year. Long-term survivors with glioblastomas are rare, and their genetic background is unknown. We reported a patient who was diagnosed with a glioblastoma and had survived 10 years before developing a second glioblastoma. The molecular analysis of both glioblastomas detected the presence of multiple mutations in *p53* and *PTEN* tumour suppressor genes, as well as homozygous deletion of $p16^{INK4a}$ in both tumours. Strikingly, none of the mutations occurred in both tumours, suggesting that

the second tumour was not a recurrence of the first glioblastoma, but it was a second *de novo* glioblastoma.

Although their *de novo* fashion, genetically, both tumours share some features of primary glioblastomas (e.g. *PTEN* mutations and $p16^{INK4a}$ homozygous deletion) and with secondary glioblastomas (e.g. *p53* mutations and absence of *EGFR* amplification). There was no evidence of an inherited tumour syndrome. To our knowledge, this is the first report of a secondary primary glioblastoma confirmed by genetic analyses. These findings also suggest the possibility of a novel unknown disease mechanism, characterised by multiple alterations in transformation-associated genes and with long-term survival patient with glioblastoma.

4–Genetic characterisation of gliosarcomas

Gliosarcoma is a rare variant of glioblastomas characterised by a biphasic tissue pattern with alternating areas of sarcomatous and gliomatous differentiation. Aiming to depict the genetic alterations underlying this neoplasm, we carried out an extensive molecular analysis. We showed that this variant disclosed a genetic profile similar to primary glioblastomas, with a high frequency of *PTEN* mutations (37%) and $p16^{INK4a}$ homozygous deletion (37%); a significant fraction of gliosarcomas (26%) harboured *p53* mutations; the only but striking difference is the absence of *EGFR* amplification or overexpression that is frequently observed in primary glioblastomas.

In addition, we conducted a comparative genetic analysis of both sarcomatous and gliomatous tumour components to elucidate the histogenesis of the sarcomatous component. We identified identical genetic alterations in both tumour components. These findings strongly suggest their monoclonal origin, and that the sarcomatous component evolved due to aberrant mesenchymal differentiation in a highly malignant astrocytic neoplasm.

SUMÁRIO E CONCLUSÕES

Os astrocitomas infiltrativos difusos, são as neoplasias mais frequentes do sistema nervoso central. Caracterizam-se pela tendência para progredir para formas malignas e incluem entidades clínico-patológicas que exibem diferenças biológicas com implicações terapêuticas. Embora o conhecimento das alterações genéticas associadas ao processo de tumorigénese nos tumores astrocíticos tenha evoluído consideravelmente continua ainda longe de estar completamente elucidado. O presente trabalho tem como objectivo geral estudar o envolvimento de algumas anomalias genéticas no desenvolvimento de vários subtipos de astrocitomas infiltrativos difusos. Para a prossecução deste objectivo o trabalho foi estruturado em quatro tópicos: **1**, Mutações do gene *p53* e gemistócitos em astrocitomas difusos; **2**, Perda de heterozigotia (LOH) no cromossoma 10 e mutações da família de genes *BUB* em glioblastomas; **3**, Segundo glioblastoma primário; e **4**, Caracterização genética de gliossarcomas.

1–Mutações do gene *p53* e gemistócitos em astrocitomas difusos

Os astrocitomas difusos (grau II, OMS), são tumores bem diferenciados de baixo índice proliferativo, que infiltram de modo difuso o tecido cerebral adjacente e tendem a recidivar e progredir frequentemente para tumores de alto grau (astrocitoma anaplásico e glioblastoma). As mutações do gene *p53* são frequentes em astrocitomas difusos. No entanto, não há consenso quanto à extensão, "timing" e papel das mutações do *p53* neste tumores. Na tentativa de elucidar este problema procedemos ao mapeamento de uma mutação do gene *p53* numa secção completa de cérebro com um astrocitoma difuso. A mutação estava presente em todas as áreas tumorais. Estes resultados sugerem que pelo menos alguns astrocitomas difusos derivam da expansão clonal de células com mutação do gene *p53*, que funcionaria como gene "gatekeeper".

Os astrocitomas gemistocíticos (grau II, OMS), são uma variante histológica de astrocitomas difusos com abundantes gemistócitos, que têm particular propensão para progredir para neoplasias mais agressivas. No entanto, não há evidências de que as células gemistocíticas proliferem pelo que são consideradas células em estado de diferenciação terminal. As células gemistocíticas também ocorrem em várias patologias cerebrais não neoplásicas. Estas observações levantam dúvidas sobre a natureza neoplásica dos gemistócitos que ocorrem em tumores astrocíticos. Na tentativa de clarificar este assunto, utilizamos um microscópio com microdisseção a laser para isolar células gemistocíticas e células neoplásicas não gemistocíticas e analisamos mutações do gene *p53*. Os resultados mostraram que quer as células gemistocíticas quer as células neoplásicas não gemistocíticas dos astrocitomas gemistocíticos apresentam mutações idênticas do gene *p53*. Estes resultados dão suporte ao conceito de monoclonalidade de

ambas as células e sugerem que as células gemistocíticas dos astrocitomas gemistócitos são realmente células neoplásicas.

2-Perda de heterozigotia (LOH) no cromossoma 10 e mutações da família de genes *BUB* em glioblastomas

Os glioblastomas (grau IV, OMS) podem ser classificados em primários e secundários. Os glioblastomas secundários resultam da progressão de tumores de menor grau de malignidade. Os glioblastomas primários são mais frequentes, evoluem rapidamente, com uma curta história pré-clinica e sem evidência de neoplasia precursora. Estes 2 subtipos de glioblastomas possuem mecanismos genéticos distintos. A perda de partes ou de todo o cromossoma 10 é a alteração genética mais frequentemente observada em glioblastomas. Neste estudo pretendemos analisar a frequência e o padrão de perda de heterozigotia do cromossoma 10 (LOH#10) em glioblastomas primários e secundários. Verificámos que a frequência de LOH#10 é similar em ambos os subtipos de glioblastomas (aproximadamente 50%). No entanto o padrão de deleção observado é distinto; os glioblastomas primários perdem uma cópia inteira do cromossoma 10, enquanto que os glioblastomas secundários apenas exibem perda do braço longo (10q). Estes resultados sugerem que: a)- LOH#10q é um evento importante na evolução de ambos os subtipos de glioblastomas; b)- há gene(s) localizado(s) no 10p preferencialmente associado(s) com a evolução dos glioblastomas primários.

A grande maioria dos glioblastomas são aneuplóides. Alguns estudos mostraram uma associação entre este tipo de instabilidade genética e anomalias em genes de regulação do "checkpoint" mitótico. Neste estudo investigamos em glioblastomas a presença de mutações em genes do "checkpoint" mitótico: *hBUB1*, *hBUBR1* e *hBUB3*. Não identificamos mutações inactivantes em qualquer dos genes estudados. No entanto, detectámos vários polimorfismos; um dos polimorfismos (Gln349→Arg do gene *hBUBR1*) tinha uma frequência alélica significativamente diferente em populações Caucasiana e Japonesa utilizadas como referência. Dada a importante função do gene *hBUBR1* na regulação do "checkpoint" mitótico, é possivel que o polimorfismo observado possa estar associado a diferenças na susceptibilidade das populações para a ocorrência de aneuploidia.

3–Segundo glioblastoma primário

Os glioblastomas (grau IV, OMS), são as neoplasias cerebrais primárias mais frequentes e malignas estando associados a um prognóstico sombrio, inferior a um ano de sobrevida. São raros os casos descritos de doentes com glioblastoma com sobrevida

longa, pelo que é escassa a informação sobre as suas características biológicas. Descrevemos o caso de um doente com um glioblastoma 10 anos após tratamento por outro glioblastoma no hemisfério cerebral contralateral. Identificamos múltiplas mutações dos genes supressores tumorais *p53* e *PTEN*, assim como deleção homozigótica do gene *p16^{INK4a}* em ambos os glioblastomas. Surpreendentemente, nenhuma das mutações observadas era comum aos dois tumores, indicando que o segundo tumor não representa uma recidiva do primeiro glioblastoma, mas sim um novo tumor independente (segundo glioblastoma primário).

Embora ambos os glioblastomas descritos sejam clinicamente *de novo* (sem evidência de lesão precursora prévia), possuem alterações comuns a glioblastomas primários (ex: mutações do gene *PTEN* e deleção homozigótica do gene *p16^{INK4a}*) e a glioblastomas secundários (ex: mutações do gene *p53* e ausência de amplificação do *EGFR*). A história clínica do doente, assim como os estudo moleculares, permitem afastar a possibilidade do caso descrito representar um síndrome hereditário conhecido. Os resultados deste estudo documentam evidência genética original em glioblastomas primários metácronos e independentes num doente com sobrevida longa. Acresce que as alterações genéticas observadas sugerem a possibilidade de um novo mecanismo genético caracterizado por múltiplas alterações em genes supressores tumorais e associado a sobrevida longa.

4–Caracterização genética de gliossarcomas

Os gliossarcomas são uma variante rara de glioblastoma, caracterizados por um padrão morfológico bifásico, com um componente sarcomatoso alternando com um componente glial. Com o intuito de descrever as alterações genéticas subjacentes aos gliossarcomas, realizamos uma análise molecular extensa em 19 gliossarcomas. Verificamos que os gliossarcomas têm um perfil genético semelhante ao descrito em glioblastomas primários: elevada frequência de mutações do gene *PTEN* (37%) e deleções homozigóticas do gene *p16^{INK4a}* (37%). Um número apreciável de gliossarcomas apresentam mutações do gene *p53* (26%). A ausência de amplificação ou sobre-expressão do gene *EGFR*, que constitui uma característica genética dos glioblastomas primários, foi a diferença mais importante sistematicamente observada em todos os gliossarcomas.

Com o objectivo de clarificar a histogénese do componente sarcomatoso, realizamos uma análise genética comparativa de ambos os componentes sarcomatoso e glial dos gliossarcomas. Os resultados obtidos mostraram a presença de alterações genéticas idênticas em ambos os componentes. Estes factos suportam a hipótese de uma origem monoclonal dos gliossarcomas e sugerem que o componente sarcomatoso resulta de uma transformação mesenquimatosa de células do componente glial.

EXPRESS COMMUNICATION

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Genetic evidence of the neoplastic nature of gemistocytes in astrocytomas

Abstract Gemistocytic astrocytoma is characterized by a predominance of large astrocytes with plump processes and massive accumulation of glial fibrillary acidic protein (gemistocytes). This histological variant of low-grade diffuse astrocytoma (WHO grade II) is prone to more rapid progression to anaplastic astrocytoma and glioblastoma than the ordinary fibrillary astrocytoma. The biological basis of this unfavorable prognosis is unclear, since gemistocytes themselves have low proliferative activity, even if present in anaplastic astrocytomas or glioblastomas. This has raised the question of whether gemistocytes are neoplastic cells or dysplastic reactive astrocytes. In this study, gemistocytes and non-gemistocytic neoplastic cells were separated by laser-assisted microdissection from six gemistocytic astrocytomas carrying *TP53* mutations. In all cases, identical *TP53* mutations were identified in both cell types, indicating that gemistocytes are indeed neoplastic cells. Their lack of proliferative activity may indicate terminal differentiation.

Keywords Gemistocytic astrocytoma · Low-grade diffuse astrocytoma · *TP53* mutation · Laser microdissection

Introduction

Low-grade diffuse astrocytomas (WHO grade II) are well-differentiated, slowly growing tumors but have an inherent tendency to invade neighboring brain tissue and to progress to more malignant phenotypes, i.e., anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV) [3, 9, 17]. However, some low-grade astrocytomas progress very rapidly, while others remain as low-grade diffuse astrocytoma for long periods [3, 9, 17]. A tendency towards rapid recurrence and malignant progression is particularly observed in gemistocytic astrocytoma [9, 16, 19, 26, 29], a histological variant of diffuse astrocytoma characterized by a predominance of gemistocytes with large, eosinophilic cytoplasm, plump cell processes and marked cytoplasmic accumulation of glial fibrillary acidic protein (GFAP) [3, 9, 16, 17, 29]. Patients with gemistocytic astrocytoma show a considerably lower 5-year survival rate than patients with astrocytomas containing no or few gemistocytes [16].

Although histologically predominant, gemistocytes rarely account for more than 50% of tumor cells. In the new WHO classification of brain tumors [9], a minimum cut-off value of 20% gemistocytes has been proposed as a diagnostic criterion. We have previously shown that patients with low-grade diffuse astrocytoma containing more than 5% gemistocytes have a significantly shorter time before progression to more malignant lesions [29]. Occasionally, the fraction of gemistocytes increases during astrocytoma progression [2, 21], and this may precede an increase in proliferative activity [21].

TP53 mutations are the most frequent genetic alteration in low-grade diffuse astrocytomas [1, 9]. In gemistocytic astrocytomas, the frequency of *TP53* mutations appears to be particularly high (60–82%), irrespective of whether or not progression to a more malignant phenotype has occurred [10, 29, 30]. Despite the unfavorable outcome associated with gemistocytic astrocytoma, gemistocytes themselves show low proliferative activity [6, 14, 29], and resemble to some extent reactive astrocytes that are encountered under a variety of pathological conditions [4, 6, 12, 13, 20]. This raises the question of whether gemistocytes are truly neoplastic cells. To elucidate this issue, gemistocytes and non-gemistocytic neoplastic cells were isolated by laser microdissection and analyzed for the presence of *TP53* mutations.

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Table 1 Single-cell laser microdissection analysis of gemistocytic astrocytomas

Patient	Age/sex	WHO grade	Gemisto-cytes (%)	TP53 mutation	
				Gemistocytic cells	Non-gemistocytic neoplastic cells
142	54/M	II	15.2	Codon 242, TGC->GGC	Codon 242, TGC->GGC
145	57/M	II	30.4	Codon 248, CGG->CAG	Codon 248, CGG->CAG
147	20/F	II	43.3	Codon 175, CGC->CAC	Codon 175, CGC->CAC
148	38/M	II	25.6	Codon 248, CGG->CAG	Codon 248, CGG->CAG
150	32/M	II	40.1	Intron 7, G->C	Intron 7, G->C
151	33/F	III	32.8	Codon 273, CGT->TGT	Codon 273, CGT->TGT

Material and methods

Laser microdissection and DNA extraction

The specimens of gemistocytic astrocytomas were obtained from six patients diagnosed in the University Hospital of Zurich, Switzerland. These tumors were previously reported to carry TP53 mutations [30]. The age and sex of patients are shown in Table 1.

Gemistocytes and non-gemistocytic neoplastic cells were microdissected using the PALM Laser Microbeam System (Bernied, Germany). Gemistocytes were recognized by their large GFAP-positive cytoplasm and eccentric nuclei. Glial cells with mild atypical dense nuclei and small cytoplasm were selected as non-gemistocytic neoplastic cells. Microdissection was carried out as follows: 5-µm tissue sections of formalin-fixed, paraffin-embedded tumor samples were mounted on a 1- to 3-µm-thin, stretched polyethylene membrane, which was attached to a non-coated slide. The membrane serves as a support to facilitate laser pressure catapulting (LPC) and avoid fragmentation of tissue. After deparaffinization and staining with H&E or GFAP stain, the slides were placed on a robot-stage microscope equipped with a UV laser (wavelength 337 nm and 30 pulses/s). Individual cells were dissected with the UV laser beam and then, with a single laser shot, catapulted into a cap of an Eppendorf tube. In each specimen, 50–120 cells of each cell type were collected, then recovered in 20 µl lysis buffer (100 mmol/l TRIS-HCl pH 7.5 and 500 µg/ml proteinase K), and were spun down by centrifugation. After incubation at 55°C overnight, proteinase K was inactivated at 99°C for 10 min.

Direct DNA sequencing

Direct sequencing analysis of the TP53 gene was performed as follows: PCR was carried out with 2 µl of DNA solution, 0.2 µM of each primer, 50 µM of dNTPs, 10 mM TRIS pH 8.8, 50 µM KCl, 1.5 mM $MgCl_2$ and 1 U Platinum Taq polymerase (GIBCO-BRL,

Cergy Pontoise, France) in a final volume of 20 µl. Forty to 45 cycles of denaturation (95°C) for 50 s, annealing (60°C for exon 7, 58°C for exon 8 and 55°C for exon 5) for 60 s and extension (72°C) for 70 s, were performed in a robocycler 96 gradient temperature cycler (Stratagene GmbH, Heidelberg, Germany). Primer sequences for PCR have been previously described [25]. Care was taken to avoid contamination. In each PCR reaction, two negative controls (water and lysis buffer) were used to ensure the absence of nonspecific PCR amplification. PCR products were purified on MicroSpinTM S-300 HR columns (Pharmacia Biotech, Uppsala) and submitted to a cycle sequencing reaction using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer-Cetus) and were analyzed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer-Cetus) according to the manufacturer's instructions. Each sequencing reaction was carried out at least twice.

Results

Using a laser-assisted microdissection system, we separately isolated DNA from gemistocytes and from non-gemistocytic neoplastic cells in six cases of gemistocytic astrocytomas (Fig. 1 and Table 1). Direct DNA sequencing revealed that in each case, both gemistocytes and non-gemistocytic neoplastic cells contained an identical TP53 mutation (Fig. 2 and Table 1). In all tumors, the wild-type allele was lost. The absence of mutated TP53 in non-tu-
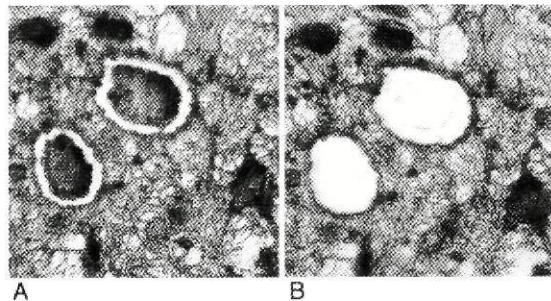
Fig. 1A, B Laser microdissection of gemistocytes (case 142). First, a laser cuts around individual gemistocytes (**A**). Then, the gemistocytes are catapulted from the slide to an Eppendorf cap (**B**)

* TP53, codon 242, TGC->GGC, Cys->Gly
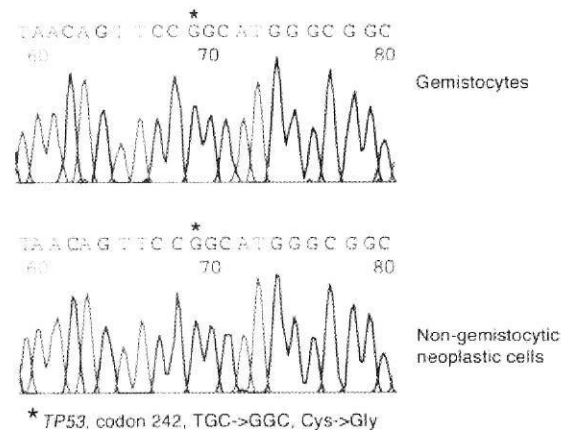
Fig. 2 Mutation analysis of codon 242 of TP53 gene after laser microdissection of gemistocytes and non-gemistocytic neoplastic cells (case 142). Identical mutations were detected in both cell types. Note the loss of wild-type base

moral tissue from the same patients excludes the possibility of a germ-line mutation.

Discussion

Gemistocytic astrocytoma, a variant of diffuse astrocytoma, is characterized by the presence of gemistocytic cells. The term gemistocytes is derived from the Greek "gemistos", which means "filled up" and describes cells characterized by voluminous, slightly eosinophilic cytoplasm, eccentric nuclei, plump cell processes and marked expression of GFAP [9, 17, 19, 31].

Kros et al. [15] screened astrocytomas, oligodendrogliomas and mixed oligoastrocytomas with gemistocytes for abnormality of chromosomes 1, 7, 10 and 17 by in situ hybridization. There was no difference in spot distribution between gemistocytes and non-gemistocytic tumor cells in six out of nine cases with abnormality; in the remaining cases, the concordance was incomplete [15]. In the present study, TP53 mutation analysis was performed in pure populations of gemistocytes and non-gemistocytic neoplastic cells from gemistocytic astrocytomas. Using laser-assisted microdissection, we isolated single cells, and demonstrated that both gemistocytes and non-gemistocytic neoplastic cells contained an identical TP53 mutation in each of six tumors analyzed, indicating that both cell types have a common monoclonal origin, and that gemistocytic cells are indeed neoplastic.

It is notable that in all samples, only the mutated allele was detectable, indicating loss of the wild-type allele, consistent with the model of two-hit inactivation of tumor suppressor genes. This observation also confirms the absence of significant DNA contamination from non-neoplastic cells. In a previous study, in which tumor areas were dissected manually, the wild-type allele was detectable in four out of six cases, probably due to contamination with entrapped non-neoplastic cells [30]. This underlines the importance of single cell microdissection in unambiguous determination of loss of wild-type allele in primary tumor samples.

The poor prognosis associated with gemistocytic astrocytomas may be due to the high frequency of TP53 mutations (60–82%) [10, 30]. Ishii et al. [7] reported that the presence of TP53 mutations in low-grade astrocytomas is associated with malignant progression and shorter progression-free survival. However, other studies failed to find any such association [10, 11, 28]. We have previously observed marked bcl-2 expression in gemistocytes [29]. In cervical carcinoma cell lines and non-Hodgkin lymphomas, increased bcl-2 expression under TP53 inactivation provides cells with a selective survival advantage [18, 23]. Other studies have shown increased expression of acid and basic fibroblastic growth factor (FGF) by gemistocytes [22, 27]. These growth factors are mitogenic for several cell types, including astrocytes, and induce angiogenesis leading to tumor growth [22, 27]. The possibility exists that gemistocytes promote tumor growth by paracrine release of FGF and related growth factors.

The biological mechanisms that lead to the gemistocyte phenotype are still unknown. Some authors have suggested that gemistocytes represent a non-proliferative pool of cells that have undergone a phenotypic change due to deprivation of nutrients and oxygen caused by competition with surrounding tumor cells [6]. Indeed, cells with a gemistocyte phenotype are encountered in a variety of central nervous system diseases associated with hypoxic or ischemic brain tissue [4, 6, 12, 13, 20]. The anoxic tumor environment could be responsible not only for the morphological changes present in gemistocytes but also for their more aggressive behavior. It has been suggested that hypoxia selects for apoptosis-resistant tumor cells with inactivated TP53. [5]. The hypoxic environment would thus favor the development of more resistant neoplastic cells and, ultimately, lead to more aggressive behavior of the tumor [5, 8, 24].

In summary, the presence of identical TP53 mutations in gemistocytes and small, non-gemistocytic astrocytoma cells provides evidence of their monoclonal origin and neoplastic nature.

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Loss of Heterozygosity on Chromosome 10 Is More Extensive in Primary (De Novo) Than in Secondary Glioblastomas

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SUMMARY: Glioblastomas develop de novo (primary glioblastomas) or through progression from low-grade or anaplastic astrocytoma (secondary glioblastomas). There is increasing evidence that these glioblastoma subtypes develop through different genetic pathways. Primary glioblastomas are characterized by *EGFR* and *MDM2* amplification/overexpression, *PTEN* mutations, and *p16* deletions, whereas secondary glioblastomas frequently contain *p53* mutations. Loss of heterozygosity (LOH) on chromosome 10 (LOH#10) is the most frequent genetic alteration in glioblastomas; the involvement of tumor suppressor genes, other than *PTEN*, has been suggested. We carried out deletion mappings on chromosome 10, using PCR-based microsatellite analysis. LOH#10 was detected at similar frequencies in primary (8/17; 47%) and secondary glioblastomas (7/13; 54%). The majority (88%) of primary glioblastomas with LOH#10 showed LOH at all informative markers, suggesting loss of the entire chromosome 10. In contrast, secondary glioblastomas with LOH#10 showed partial or complete loss of chromosome 10q but no loss of 10p. These results are in accordance with the view that LOH on 10q is a major factor in the evolution of glioblastoma multiform as the common phenotypic end point of both genetic pathways, whereas LOH on 10p is largely restricted to the primary (de novo) glioblastoma. (*Lab Invest 2000, 80:65–72*).

Glioblastoma (World Health Organization [WHO] Grade IV) is the most frequent and malignant human brain tumor, occurring at a frequency of two to three new cases per 100,000 population and per year for most European and North American countries (Lantos et al, 1996). Despite progress in surgery and adjuvant therapy, patients with glioblastoma still have a dismal prognosis and they usually succumb to the disease within 1 year after diagnosis (Galanis et al, 1998; Leenstra et al, 1998). Glioblastomas may develop rapidly, with a short clinical history (primary or de novo glioblastoma), or more slowly through progression from low-grade (WHO Grade II) or anaplastic (WHO Grade III) astrocytoma (secondary glioblastoma) (Kleihues and Ohgaki, 1999). These glioblastoma subtypes, although largely indistinguishable histologically, constitute distinct disease entities that manifest in different age groups and develop through different genetic pathways. Primary glioblastomas occur in older patients and are characterized by *EGFR* amplification/overexpression and, less frequently, *MDM2* amplification, *PTEN* (*MMAC1*) mutations, and *p16* homozygous deletion, while secondary glioblastomas occur in younger patients and contain *p53* mutations as a genetic hallmark (Kleihues and Ohgaki, 1999).

Loss of heterozygosity (LOH) on chromosome 10 (LOH#10) is the most frequent genetic alteration in glioblastomas, reportedly occurring in up to 80% of cases (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). LOH#10 is less frequent (~40%) in anaplastic astrocytomas (Albarosa et al, 1996; Bijleveld et al, 1997; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten, 1997), and rarely occurs in low-grade astrocytomas (Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). The majority of glioblastomas appear to have lost an entire copy of chromosome 10 (Albarosa, 1996; Fults et al, 1998; Ichimura et al, 1998; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Voesten et al, 1997). In glioblastomas with partial LOH#10, at least three common deletions have been identified: (a) 10p14-pter (Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Sonoda et al, 1996; Voesten et al, 1997); (b) 10q23–24 (Albarosa et al, 1996; Fults, et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996); and (c) 10q25-qter (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996), suggesting the presence of multiple tumor suppressor

genes. LOH#10 has been detected in 60% to 100% of glioblastomas with *EGFR* amplification (Lang et al, 1994; Leenstra et al, 1998; von Deimling et al, 1992) and in 40% to 80% of glioblastomas with a *p53* mutation (Lang et al, 1994; Leenstra et al, 1998), suggesting that LOH#10 is involved in the development of both primary and secondary glioblastomas.

The objective of the present study was to clarify whether the frequency and allelic patterns of LOH#10 differ between primary and secondary glioblastomas. We carried out deletion mapping on chromosome 10, using PCR-based microsatellite analysis in 17 primary glioblastomas (using normal DNA as a reference) and 13 secondary glioblastomas which progressed from low-grade astrocytomas (using normal or low-grade astrocytoma DNA as a reference). The presence and pattern of LOH#10 were correlated with other genetic alterations, including *EGFR* amplification and *p53* and *PTEN* mutations.

Results

LOH on Chromosome 10

Using 28 microsatellite markers, we examined a total of 840 polymorphic loci on chromosome 10 and obtained 621 (74%) informative results. Eight (47%) of 17 primary glioblastomas showed LOH#10. Of these, seven (88%) showed deletions at all informative loci;

this was interpreted as the loss of an entire copy of chromosome 10. Case 294 was exceptional, with deletions at all informative loci on 10p but with none on 10q (Figs. 1 and 2).

In secondary glioblastomas, LOH#10 was demonstrated in 7 of 13 (54%) cases, ie, at a frequency similar to that in primary glioblastomas ($p = 1.0$). However, in all cases, deletions were partial and typically located on 10q. One glioblastoma (case 25) showed LOH at all informative loci on 10q. In the remaining six cases, chromosomal deletions on 10q were partial. One tumor (case 70) showed an additional small deletion on 10p at D10S199. The most common deletion in all seven cases was on 10q25-qter distal to D10S1683, covering the *DMBT1* (Mollenhauer et al, 1997) and *FGFR2* (Moschonas et al, 1996) loci (Fig. 1).

For three secondary glioblastomas in which low-grade astrocytoma DNA was used as a reference, DNA from adjacent normal brain tissue (Cases 57 and 68) and peripheral blood leukocytes (Case 72) was also subjected to analysis. Allelic patterns of normal tissues and low-grade astrocytomas were concordant at all informative loci. In another case (Case 26), one of the two alleles showed significant decrease (>50%) in signal intensity when compared with the remaining allele at markers D10S587, 1723, and 1700 (Fig. 1, marked as asterisk), which may suggest that LOH had already occurred in low-grade astrocytoma.
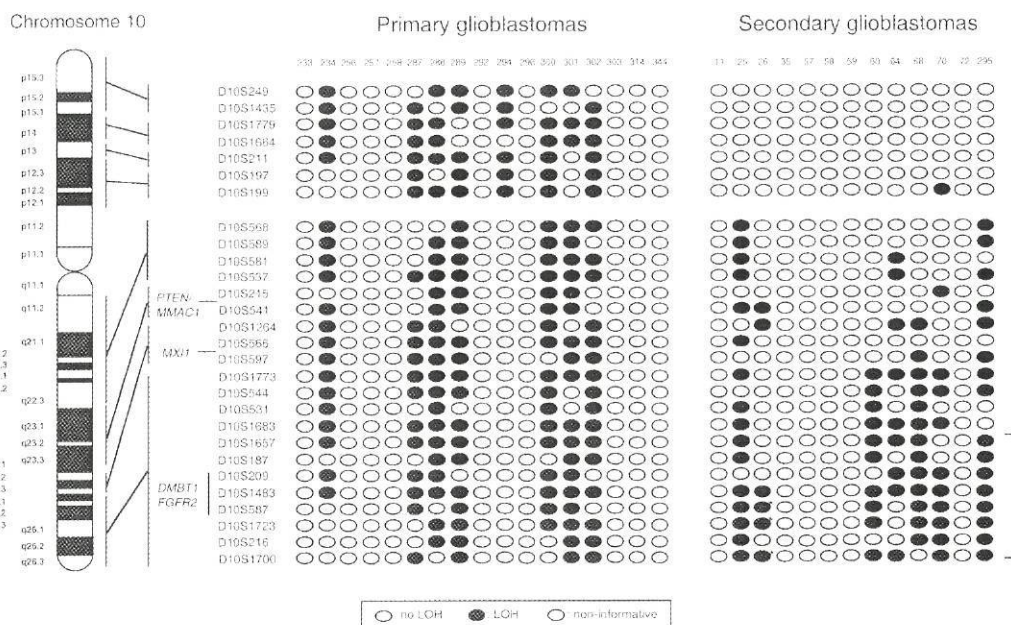
Figure 1.

Allelic patterns of chromosome 10 in 17 primary and 13 secondary glioblastomas. Case numbers are indicated at the top of each column. The overall frequency of loss of heterozygosity (LOH) on chromosome 10 is similar in both glioblastoma subtypes but the extent of chromosomal loss differs. Primary glioblastomas often show complete loss (10p, q) while in secondary glioblastomas, LOH is typically restricted to the long arm (10q).

Table 1. Loss of Heterozygosity on Chromosome 10 and Other Genetic Alterations in Primary and Secondary Glioblastomas

Patient No.	Age (year)/gender	Tumor location	Clinical history	LOH #10 p	q	p53 miscoding mutation	PTEN missense mutation	EGFR/CF ratio
Primary Glioblastomas								
233	59/M	T, BG	2 months	–	–	–	–	0.90
234	43/M	F	1 month	+	+	–	61, CAT–>CCT, His–>Pro	0.72
256	56/F	T	10 days	–	–	253, ACC–>GCC, Thr–>Ala	–	0.93
257	50/M	F, BG	2 months	–	–	–	–	1.47
258	65/F	F	1 week	+	+	–	–	0.69
287	47/F	P	1 month	+	–	–	–	0.87
288	69/M	P	2 months	+	+	–	–	1.18
289	58/F	T	3 months	+	+	–	–	**8.42**
292	62/M	P	1 month	+	–	–	–	1.50
294	36/F	P	5 weeks	+	–	–	–	1.19
296	63/F	VE	4 months	–	–	–	–	0.98
300	71/F	TO	1 month	+	+	–	–	**6.08**
301	68/F	TP	1 week	+	+	–	–	**4.54**
302	64/M	TP	3 months	+	+	–	122, ATT–>AGT, Ile–>Ser	**5.04**
303	67/M	PO	2 weeks	–	–	–	–	2.07
314	40/M	F	3 weeks	–	–	126, TAC–>TAA, Tyr–>stop	–	**14.1**
344	47/M	P	2 weeks	–	–	–	–	0.92
Secondary Glioblastomas								
11	33/F	VE	Grade II	–	–	163, TAC–>TGC, Tyr–>Cys	–	1.96
25	44/F	T	Grade II	–	+	275, TGT–>TTT, Cys–>Phe	–	1.62
26	32/F	F	Grade II	–	+	163, TAC–>TGC, Tyr–>Cys	–	1.08
35	53/M	T	Grade II	–	–	273, CGT–>TGT, Arg–>Cys	–	1.17
57	26/M	F	Grade II	–	–	175, CGC–>CAC, Arg–>His	–	1.11
58	33/M	FT	Grade II	–	–	141, TGC–>TAC, Cys–>Tyr	–	1.05
59	40/M	FT	Grade II	–	–	175, CGC–>CAC, Arg–>His	–	1.36
60	31/F	T	Grade II	–	–	–	–	1.20
64	34/M	F	Grade II	–	+	300–>89 bp del	–	1.93
68	52/F	TPO	Grade II	+	+	278, CCT–>ACT, Pro–>Thr	–	1.11
70	52/M	PO	Grade II	+	+	–	–	1.22
72	28/F	F	Grade II	–	–	301–>2 bp del	–	2.02
295	47/M	FT	Grade II	–	+	245, GGC–>AGC, Gly–>Ser	–	0.96

F, frontal; T, temporal; P, parietal; O, occipital; VE, paraventricle; BG, basal ganglia.
Results of p53 mutations and PTEN mutations in secondary glioblastomas, except for cases 72 and 295, have been previously reported (Watanabe et al, 1996; Tohma et al, 1996). An EGFR/CF ratio of >2.6 was regarded as evidence of EGFR amplification (see "Materials and Methods").
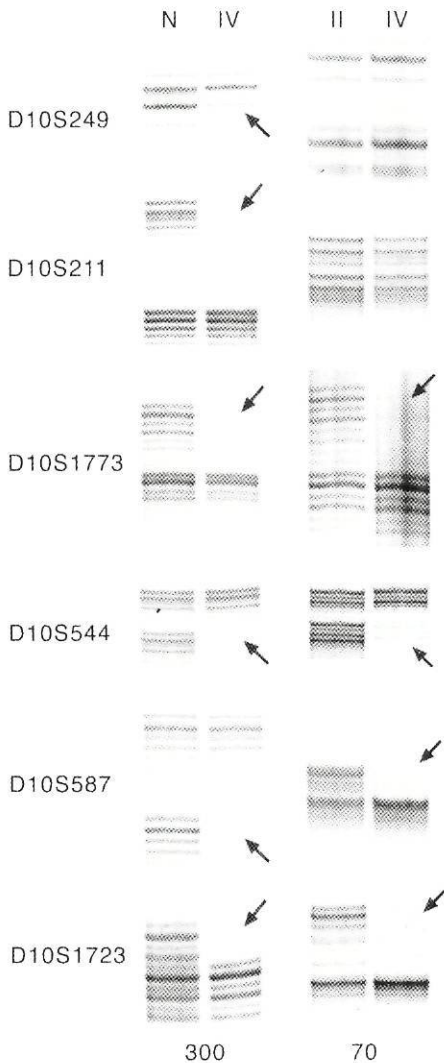
Figure 2.

Representative results of loss of heterozygosity (LOH) on chromosome 10 in a primary (case 300) and a secondary (case 70) glioblastoma. Microsatellite markers are indicated on the left side of each panel. LOH is indicated by *arrows. N*, normal (blood); *II*, low-grade astrocytoma (World Health Organization Grade II); *IV*, glioblastoma (Grade IV).

Correlation of LOH#10 with Other Genetic Alterations

PCR-SSCP, followed by DNA sequencing, demonstrated *p53* missense mutations in 2 (12%) of 17 primary glioblastomas and in 11 (85%) of 13 secondary glioblastomas ($p = 0.0001$; Table 1). *PTEN* mutations were found in 2 (12%) of 17 primary glioblastomas but none in secondary glioblastomas (Table 1). *EGFR* amplification was detected by differential PCR in 5 (29%) of 17 primary glioblastomas but in none of the secondary glioblastomas (Table 1). *EGFR* amplification tended to be associated with complete LOH#10: 4 (80%) of 5 primary glioblastomas with

EGFR amplification showed an entire loss of chromosome 10, whereas only 3 (25%) of 12 glioblastomas without *EGFR* amplification showed complete LOH#10. However, this difference was not statistically significant ($p = 0.10$). There was no significant correlation between the presence of LOH#10 and other genetic alterations.

Discussion

Entire or partial loss of chromosome 10 is a common genetic alteration in a variety of human cancers, including glioblastomas (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997), malignant meningiomas (Rempel et al, 1993; Simon et al, 1995), endometrial carcinomas (Peiffer et al, 1995), prostate carcinomas (Gray et al, 1995), renal carcinomas (Morita et al, 1991), small cell lung carcinomas (Ried et al, 1994), non-Hodgkin's lymphomas (Speaks et al, 1992), and melanomas (Herbst et al, 1994; Isshiki et al, 1993). In glioblastomas, there appear to be at least three putative tumor suppressor loci, ie, 10p14-pter, 10q23–24, and 10q25-qter (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996; Voesten et al, 1997). However, little is known whether the frequency and extent of LOH on chromosome 10 are different between primary and secondary glioblastomas. Ichimura et al (1998) carried out extensive LOH study in 198 astrocytic gliomas and showed that most glioblastomas lost one entire chromosome, while astrocytomas preferentially lost only 10p. However, the astrocytomas and glioblastomas that were analyzed were derived from different patients. Using comparative genome hybridization (CGH) analysis, Weber et al (1996) reported that 4 of 10 anaplastic astrocytomas and glioblastomas that had progressed from low-grade astrocytoma showed a reduced number of chromosome segments on 10q. Recently, we reported that LOH on 10q25-qter is often associated with abrupt morphologic transition from low-grade or anaplastic astrocytoma to a highly malignant, undifferentiated glioblastoma phenotype lacking glial fibrillary acidic protein (GFAP) expression (Fujisawa et al, 1999).

The present study is the first to demonstrate unequivocally that LOH#10 occurs at a similar frequency in primary and secondary glioblastomas but that the patterns of allelic loss are different. Entire loss of chromosome 10 was typical for primary glioblastomas, whereas partial LOH on 10q was characteristic for secondary glioblastomas. This pattern is consistent with the observation in this and previous studies (Lang et al, 1994; Leenstra et al, 1998; von Deimling et al, 1992) that glioblastomas with *EGFR* amplification, a genetic hallmark of primary glioblastomas, typically show complete loss of chromosome 10.

Several transformation-associated genes have been identified on 10q, including *PTEN* at 10q23.3 (Li et al,

1997; Steck et al, 1997), *LGI1* at 10q24 (Chernova et al, 1998), *BUB3* at 10q24-q26 (Cahill et al, 1999), *MXI1* at 10q25.1 (Eagle et al, 1995), *hours-neu at 10q25.1* (Nakamura et al, 1998), *abLIM* or *LIMAB1* at 10q25.1 (Kim et al, 1997; Roof et al, 1997) and *DMBT1* at 10q26.1 (Mollenhauer et al, 1997). The *PTEN* gene, which encodes a protein with homology to the catalytic domain of tyrosine phosphatase and to the cytoskeletal proteins tensin and auxilin (Li et al, 1997; Steck et al, 1997), is mutated frequently in glioblastomas (Boström et al, 1998; Chiariello et al, 1998; Duerr et al, 1998; Fults et al, 1998; Maier et al, 1998; Tohma et al, 1998; Wang et al, 1997), prostate carcinomas (Cairns et al, 1997; Suzuki et al, 1998), and endometrial carcinomas (Risinger et al, 1997; Tashiro et al, 1997). We previously reported that mutations in the *PTEN* gene, which is located on 10q23, are common (32%) in primary but rare (4%) in secondary glioblastomas (Tohma et al, 1998). *DMBT1* is deleted frequently in glioblastomas (Mollenhauer et al, 1997; Somerville et al, 1998) but *DMBT1* mutations have not yet been identified. Although growth suppression has been observed after microcell-mediated transfer of chromosome fragments from 10p14–15 into T98G glioblastoma cells (Kon et al, 1998), the putative tumor suppressor gene at this locus has not yet been identified.

The present study suggests that the involvement of different tumor suppressor gene(s) on chromosome 10 is between primary and secondary glioblastoma. Tumor suppressor gene(s) on chromosome 10p may play an important role in the evolution of primary, but not secondary, glioblastomas. Alternatively, the more extensive deletion on chromosome 10 in primary, rather than in secondary glioblastomas may result from greater chromosomal instability in primary glioblastomas. However, little is known about the mechanisms of chromosomal deletion, except for the general assumption that it is probably attributed to chromosomal instability caused by the disruption of mitotic checkpoints (Lengauer et al, 1998) or premature mitosis involving damaged DNA (Paulovich et al, 1997).

In conclusion, this study shows that LOH on chromosome 10 is more extensive in primary glioblastomas, rather than in secondary gliobastomas. Primary glioblastomas are characterized by an entire loss of chromosome 10, whereas in secondary glioblastomas, LOH is restricted to chromosome 10q, suggesting that different tumor suppressor genes on this chromosome are involved in the development of these glioblastoma subtypes.

Materials and Methods

Tumor and Blood Samples

Seventeen primary and 13 secondary glioblastomas were obtained from the patients operated on in the Department of Neurosurgery, University Hospital, Zürich, Switzerland. All patients with primary glioblastoma had a clinical history of <3 months and did not show any histologic or radiologic evidence of a pre-cursor lesion, whereas all patients with secondary glioblastoma had surgeries for low-grade astrocytoma >6 months before the second operation for glioblastoma. Seventeen patients were men and 13 were women (Table 1). Tumors were fixed in formalin, embedded in paraffin for routine histopathologic analysis, and were classified according to the WHO grading system (Kleihues et al, 1993).

For all primary glioblastomas and two secondary glioblastomas (cases 72 and 295), aliquots of tumors were frozen immediately in liquid nitrogen and stored at −80° C until DNA extraction. Genomic DNA was extracted using TRIZOL Reagent (GIBCO BRL, Cergy Parroise, France) according to the manufacturer's instructions. Matched peripheral blood samples of these cases were obtained and DNA was extracted using QIAamp DNA Blood Kit (QIAGEN, Courtaboeuf, France).

For the cases in which frozen tissues were not available (low-grade astrocytoma and secondary glioblastomas, except for cases 72 and 295), DNA was extracted from paraffin sections (Brüstle et al, 1992; Fujisawa et al, 1999). In two secondary glioblastomas (cases 57 and 68), DNA was also extracted from the peritumoral brain tissue in paraffin sections. In case 72, DNA was extracted from paraffin-embedded, low-grade astrocytoma, frozen glioblastoma, and blood samples.

Analysis of LOH on Chromosome 10

LOH on chromosome 10 was studied by PCR-based microsatellite analysis (Fujisawa et al, 1999). Twenty-eight microsatellite loci were selected to cover chromosome 10, including reported common deletions on 10p14-pter (Ichimura et al, 1998; Karlbom et al, 1993; Kimmelman et al, 1996; Kon et al, 1998; Sonoda et al, 1996; Voesten et al, 1997), 10q23–24 (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996), and 10q25-qter (Albarosa et al, 1996; Fults et al, 1998; Ichimura et al, 1998; Karlbom et al, 1993; Maier et al, 1997; Rasheed et al, 1995; Sonoda et al, 1996). All microsatellite markers were purchased from Research Genetics (Huntsville, AL); all were dinucleotide repeats, except for D10S1435 (tetranucleotide repeats). The size range and heterozygosity of each marker were obtained from the Genome Database (http://gdbwww.gdb.org/). The genetic map and distances of chromosome 10 were obtained from the enhanced location database at ftp://cedar.genetics.soton.ac.uk/pub/chrom 10/gmap (Collins et al, 1996).

For all primary glioblastomas and two secondary glioblastomas, DNA from peripheral blood samples from the same patient was used as a reference, whereas for secondary glioblastomas except for two cases, DNA samples from low-grade astrocytoma from the respective patients were used as a reference. After PCR amplification, the allelic pattern for each marker was determined by comparing the electrophoretic pattern of DNA from glioblastoma with that of

reference DNA, ie, blood DNA or DNA samples extracted from low-grade astrocytoma. PCR was performed according to the instructions of Research Genetics with minor modifications. Briefly, 10 ng of DNA (1 μl) samples of fresh-frozen tumors and blood samples or 1 μl of DNA solution from paraffin sections were subjected to PCR with 2 μl of 5$\times$ PCR buffer, 200 μM of each dNTP, 6 pmol of each sense and antisense primer, 1 μCi of [α-^{33}P]-dCTP (ICN Biomedicals, specific activity 3000 Ci/mmol), 0.225 units of *Taq* polymerase (Sigma Chemical, St. Louis, Missouri), and 1.5 mM of MgCl$_2$ in a final volume of 10 μl, with an initial denaturation of 95° C for 2 minutes, followed by 30 cycles (DNA from frozen tumors and blood sample) or 35 cycles (DNA from paraffin sections) of denaturation at 94° C for 45 seconds, annealing at 57° C for 45 seconds and polymerization at 72° C for 1 minute, and a final extension at 72° C for 7 minutes, using a Genius DNA Thermal Cycler (Techne, Cambridge, United Kingdom). PCR products were mixed with an equivalent volume of the denaturing solution containing 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. Immediately after heating at 95° C for 5 minutes, 4 μl of the mixture was loaded onto a 7% polyacrylamide/7 M of urea sequencing gel. Gels were run at 70 watts (W) for 3 to 6 hours, dried at 80° C, and autoradiographed for 24 to 96 hours. Gels were also exposed to Storage Phosphor screens (Molecular Dynamics, Sunnyvale, California). LOH was scored when signal intensity was reduced in glioblastoma by >50% of reference DNA, which was measured by densitometry (Bio-Rad model GS-670) or by a Phosphorimager (Molecular Dynamics).

Other Genetic Alterations

The genetic alterations in secondary glioblastomas, except for two cases, were reported previously (Tohma et al, 1998; Watanabe et al, 1996) (Table 1). For all primary glioblastomas and two cases of secondary glioblastomas (cases 72 and 295), the following genetic analyses were carried out.

PCR-SSCP Analysis and Direct DNA Sequencing for p53 and PTEN Mutations. Prescreening for mutations by PCR-SSCP analysis was carried out, as previously described in exons 5–8 of the *p53* gene (Watanabe et al, 1996) and in exons 1–9 of the *PTEN* gene (Reis et al, 1999; Tohma et al, 1998; Watanabe et al, 1998). Samples which showed mobility shifts in the SSCP gels were further analyzed by direct DNA sequencing (Reis et al, 1999; Tohma et al, 1998; Watanabe et al, 1996).

Differential PCR for EGFR Amplification. To detect EGFR amplification, differential PCR was carried out, using the cystic fibrosis *(CF)* gene as a reference (Hunter et al, 1995), with some modifications (Tohma et al, 1998). The mean *EGFR/CF* ratio, using normal blood DNA sampling, was 1.11 with a standard variation of 0.11. An *EGFR/CF* ratio >2.6 was regarded as evidence of *EGFR* amplification (Rollbrocker et al, 1996). One primary glioblastoma, which showed

EGFR amplification in our previous study (Tohma et al, 1998), was used as a positive control.

Statistical Analyses

Fisher's exact test was carried out to analyze the contingency table for frequency of *EGFR* amplification, *p53* and *PTEN* mutations, and LOH#10 between primary and secondary glioblastomas.

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lutation analysis of *hBUB1*, *hBUBR1* and *hBUB3* genes
glioblastomas

bstract Glioblastomas, the most malignant human brain
mors, are characterized by marked aneuploidy, suggest-
g chromosomal instability which may be caused by a
fective mitotic spindle checkpoint. We screened 22
ioblastomas for mutations in the mitotic spindle check-
int genes *hBUB1*, *hBUBR1* and *hBUB3*. DNA sequenc-
g revealed a silent mutation at codon 144 of *hBUB1*
AG→CAA, Gln→Gln) in one glioblastoma, a silent
utation at codon 952 of *hBUBR1* (GAC→GAT,
sp→Asp) in another glioblastoma, and a silent mutation at
don 388 of the *hBUBR1* gene (GCG→GCA, Ala→Ala)
8 glioblastomas. We also observed a known polymor-
ism at *hBUBR1* codon 349 (CAA/CGA, Gln/Arg), with
allelic frequency of 0.75 for Gln and 0.25 for Arg,
hich is similar to that among healthy Caucasian individ-
ls (0.73 vs 0.27). The coding sequence of the *hBUB3*
ne did not contain any mutation, but in 4 glioblastomas
8%), a C→T point mutation was detected at position –6
nucleotides upstream of the ATG initiator codon).
nalysis of blood DNA of these patients showed identical
quence alterations, indicating that this is a polymor-
ism. Again, the frequency in glioblastomas was similar
that in healthy Caucasians (15%). We further screened
BUB1 in 18 cases of giant cell glioblastoma, a variant
aracterized by a predominance of bizarre, multinucle-
ed giant cells. There were no changes, except for a silent
utation at codon 144 in two cases. These results suggest
at mutations in these mitotic spindle checkpoint genes
not play a significant role in the causation of chromo-
mal instability in glioblastomas.

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Introduction

Genetic instability is a critical phenomenon in the develop-
ment of malignant human neoplasms and occurs in two dif-
ferent forms, microsatellite instability and chromosomal
instability [22, 24, 27, 37]. In subsets of colorectal, gastric
and endometrial cancers, defective mismatch repair results
in increased mutation rates at the nucleotide level and, con-
sequently, widespread microsatellite instability [22, 24,
37]. Tumors with microsatellite instability usually have a
normal complement of chromosomes, i.e., a diploid or
near-diploid karyotype [27]. In most other neoplasms, in-
cluding brain tumors, genetic instability is typically ob-
served at the chromosomal level and may involve gain and
loss of whole chromosomes, leading to aneuploidy [27,
35]. The molecular basis of chromosomal instability is not
yet fully understood, but one possible mechanism is dis-
ruption of the mitotic checkpoint [27, 35], i.e., a conserved
function that delays anaphase in the presence of spindle
damage [40], so as to increase the probability of successful
delivery of an euploid genome to each daughter cell.

Three mitotic-arrest-deficient *(MAD)* genes, *Mad1*,
Mad2, and *Mad3* [14, 15, 28], three budding-uninhibited-
by-benzimidazole *(BUB)* genes, *Bub1*, *Bub2*, and *Bub3*,
[15, 39] and *MPS1* [15, 28, 48] have been identified as be-
ing required for the execution of the mitotic checkpoint in
the yeast *Saccharomyces cerevisiae*. Several human ho-
mologues have been identified, including *hMPS1* (TTK/
MPS1L1 located on chromosome 6q13–21) [6], *hMAD1L1*
(on chromosome 7pter-7p15) [6, 19], *hMAD2* (*hMAD2L1*,
on chromosome 4q27) [29], *hMAD2B* (*hMAD2L2*, on
chromosome 1p36) [6], *hBUB1* (on chromosome 2q14)
[5, 6], *hBUBR1* (*hBUB1B/MAD3-L* on chromosome 15q14)
[5, 8] and *hBUB3* (on chromosome 10q24–26) [6, 25].

Mutational inactivation of mitotic spindle checkpoint
genes appears to be involved in the evolution of some hu-
man neoplasms, in particular those with aneuploidy.

Cahill et al. [5] detected *hBUB1* and *hBUBR1* mutations in 2 each of 19 colon cancer cell lines with chromosomal instability. Mutations in the *hBUB1* gene were shown to act in a dominant-negative manner, if the exogenous expression of the mutant allele disrupts the mitotic checkpoint in euploid cell lines [5]. A somatic mutation in the *hBUB1* gene was identified in 1 of 32 sporadic digestive tract cancers [16], in 2/148 lung cancers [12, 41], and in 1/10 cases of 10 adult T cell leukemia/lymphomas [34] that, in addition, contained *hBUBR1* missense mutations and deletion in 3 cases. In contrast, no *hBUB1* mutation was found in head and neck squamous cell carcinomas and lung cancer cell lines [49]. *hBUB1* and *hBUBR1* mutations were also reported absent in 19 aneuploid breast cancer cell lines [31]. *hBUBR1* mutations were not found in 47 lung cancers [41]. In addition, no *hBUB3* mutation was detected in 19 colon cancer cell lines [5], suggesting that mutational inactivation of these checkpoint genes is a rare event in carcinomas and malignant lymphomas.

Glioblastomas are highly aneuploid tumors and frequently show loss of heterozygosity (LOH) on several chromosomes [3, 7, 11, 30, 38, 46, 47] in up to 90% of cases. LOH has frequently been observed on chromosome 10q, where *hBUB3* is located. The objective of the present study was to assess whether mutational inactivation of the *hBUB1*, *hBUBR1* and *hBUB3* genes is involved in the evolution of the glioblastoma phenotype.

Material and methods

Tumor samples

Twenty-two glioblastomas (WHO grade IV) were obtained from patients at the Department of Neurosurgery, University Hospital Zurich, Switzerland. Two glioblastomas (cases 72 and 295) were secondary glioblastomas that had progressed from low-grade astrocytoma. Other glioblastomas were primary (*de novo*) glioblastomas with a clinical history of less than 3 months. The age and sex of the patients are shown in Table 4. Twelve patients were males and ten were females. The mean age of patients at diagnosis was 55±12 years (range 28–71 years).

Tumor samples were immediately frozen in liquid nitrogen and stored at –80°C. RNA and DNA were simultaneously extracted from the same portions (about 200 mg) of tumors using the TRIZOL (Gibco BRL, Cergy Pontoise, France) according to the manufacturer's instructions.

The sources of 18 formalin-fixed, paraffin-embedded specimens of giant cell glioblastomas were reported previously [36]. Tumor samples included only typical cases showing a predominance of multinucleated monstrous, glial fibrillary acidic protein (GFAP)-positive tumor cells in at least one large area of the biopsy specimens. The occurrence of occasional giant cells in a glioblastoma was not considered diagnostic for this variant. Eleven patients were males and 7 were females. The mean age of patients was 42±20 years (range 9–78 years).

hBUB1 mutations

Screening for *hBUB1* mutations was performed by polymerase chain reaction-single-strand confirmational polymorphism (PCR-SSCP) analysis using genomic DNA. PCR amplification of the 25 exons was performed using the primers described in Table 1. Briefly, PCR was performed in a total volume of 10 μl, containing 1 μl DNA solution, 0.75 U Taq DNA polymerase (Sigma, St.

Louis, Mo.), 0.5 μCi [α-³³P]dCTP (ICN Biomedicals, N.V.S.A Belgium; specific activity, 3,000 Ci/mmol), 1.5–2.5 mM MgC 0.2 mM of each dNTP, 0.2 μM of both sense and antisen primers, 10 mM TRIS-HCl, pH 8.3, and 50 mM KCl. PCR amp fication was performed in a Genius DNA Thermal Cycler (Tech Cambridge, UK) as follows: initial denaturation for 2 min at 95° 35–40 cycles with denaturation at 95°C for 1 min, annealing 50–58°C for 1 min and extension at 72°C for 1 min, followed by final extension step for 5 min at 72°C. After PCR, 5 μl of the pro ucts was mixed with 12.5 μl of loading buffer (95% formamic 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue), d natured at 95°C for 10 min, and quenched on ice. Of the abo mixture 4 μl was run on a 6% polyacrylamide non-denaturing g containing 6% glycerol at 6 W for 14 h. Gels were dried at 80 and autoradiographed for 12–48 h.

Samples which showed mobility shifts in the SSCP analys were further analyzed by direct DNA sequencing. PCR produc were purified on MicroSpinTM S-300 HR Columns (Pharmac Biotech, Uppsala) and sequenced using the Thermo Sequenase R diolabeled Terminator Cycle Sequencing Kit (USB, Clevelan USA) according to the manufacturer's instructions.

hBUBR1 mutations

First-strand cDNA of the *hBUBR1* gene was synthesized as fo lows: 5 μg of total RNA in a total volume of 10 μl was heated 70°C for 10 min and then incubated at 42°C for 50 min followi addition of 10 μl of a mixture containing 1x 1st strand cDN buffer (Gibco BRL), 10 mM DTT, 1 mM dNTPs, 25 μg oligo-primers and 200 U moloney murine leukemia virus reverse tra scriptase (M-MuLV RT, Gibco BRL). cDNA samples were the made up to 40 μl with distilled water and kept at –20°C.

Pre-screening for *hBUBR1* mutations was carried out by PC SSCP analysis. The entire coding region of the 3,583-bp *hBUB* cDNA was amplified as 14 overlapping fragments. PCR was pe formed in a total volume of 10 μl, containing 0.5 μl cDNA sol tion, 0.75 U Taq DNA polymerase (Sigma), 0.5 μCi [α-³³P] dCT (ICN Biomedicals, specific activity, 3,000 Ci/mmol), 1.5 m MgCl₂, 0.2 mM of each dNTP, 0.2 μM of sense and antisen primers, 10 mM TRIS-HCl, pH 8.3, and 50 mM KCl, in a Therm Cycler (Stratagene, La Jolla, Calif.) as follows: initial denaturati for 2 min at 95°C, 35–40 cycles of denaturation at 95°C for 1 mi annealing at 50–58°C for 1 min followed by extension at 72°C f 1 min, and a final extension step for 5 min at 72°C. Primer s quences are shown in Table 2. After PCR, 5 μl of products we mixed with 12.5 μl loading buffer (95% formamide, 20 m EDTA, 0.05% xylene cyanol and bromophenol blue), denatured 95°C for 10 min, and quenched on ice. Samples of the above mi ture (4 μl) were run on a 6% polyacrylamide non-denaturing g containing 6% glycerol at 6 W for 14 h. Gels were dried at 80 and autoradiographed for 12–48 h. Samples that showed mobili shifts in the SSCP analysis were further analyzed by direct DN sequencing as described above.

A polymorphism at codon 349 (CAA/CGA, Gln/Arg) was d tected in this study. To assess whether particular alleles are ove represented in glioblastomas, we also analyzed blood DNA obtaine from 67 healthy caucasians individuals for this polymorphism.

hBUB3 mutations

First-strand cDNA of the *hBUB3* gene was synthesized as d scribed above.

Pre-screening for *hBUB3* mutations was carried out by PC SSCP analysis. The entire coding region of the 1,078-bp *hBUB* cDNA was amplified as 5 overlapping fragments. Primer s quences are shown in Table 3. Briefly, PCR was performed in a t tal volume of 10 μl, containing 0.5 μl cDNA solution, 0.75 U Ta DNA polymerase (Sigma), 0.5 μCi [α-³³P] dCTP (ICN Biomed icals, specific activity, 3,000 Ci/mmol), 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 μM of sense and antisense primers, 10 mM TRIS HCl, pH 8.3, and 50 mM KCl, in a Thermo Cycler (Stratagene, L

Table 1 Primers used for SSCP analyses of the *hBUB1* gene

Exon		Primer sequence	Annealing temperature (°C)	Product (bp)
1	Sense	TTCTAGTTTGCGGTT	48	75
	Antisense	TGAAGGACATTTTCC		
2	Sense	GAAGTATATCTTTTGCTTGTAGAA	52	175
	Antisense	GGGGCAGTGTATAGTTTGTT		
3	Sense	TAAACAAACTATACACTGCC	48	248
	Antisense	AAAGCAAAAGTACAACATGA		
4	Sense	TTTTTGTTTTGTTCGTGT	56	247
	Antisense	ATCACAGAGAGTTTGACTTTGTAAC		
5	Sense	ATTGATGCCTTTTCTGCTGT	52	94
	Antisense	GCATTTTAGAAAGCCTGATT		
6	Sense	ATTATGTGATAATTTTACTTAC	53	250
	Antisense	AAAGTGGATGTAGAAGGCAG		
7	Sense	TGTTTTGTGAAGTAATTTGG	54	179
	Antisense	TAAGAGCACTTAAAGGAAAC		
8	Sense	TGCTCTTAGGTACATTGTTG	48	264
	Antisense	AAGAATAACTAAAAATACAAGATT		
9	Sense	ATGCAAACCATTTCTATCTTTC	60	199
	Antisense	CATGAGGAGCACAACATACC		
10	Sense	GATGTAATGCCTGATTAGTAG	54	370
	Antisense	CACATCACTGTGATCTCTAG		
11	Sense	AGATCAAGCATTTATTCTCTAG	54	177
	Antisense	AAAATACACCTCTGAGTGATAC		
12	Sense	TTCTGATTTCCCTACTGATACCAC	62	223
	Antisense	CATTAACTTGTCATCAGTGCTAC		
13	Sense	TGATGACAAGTTAATGAAAGCTCT	56	231
	Antisense	TTTCTGTGATAACCACCTATAATG		
14	Sense	TGATATTAGTTCTTTTCTGTCAGCT	58	254
	Antisense	CGGCAGCATCCCATTAAC		
15	Sense	CAAGTGATAGCATTTATTTCCT	58	183
	Antisense	GTAGGACCCATTTCATAGATAA		
16	Sense	CTTCTTTAATCCTCATTTCTTTGG	61	331
	Antisense	GAATCAAAGTTGGCAGAAGAC		
17	Sense	ATCTTGTCTCTAATTTTTGAATCT	52	139
	Antisense	ACCAAATAAACCCTCACAAT		
18	Sense	GGTTTCTTTATTCCTTTCACAC	62	297
	Antisense	AAGGGCATAACAAAGAGTGAG		
19	Sense	TTTTTTTTTTCTCATTCCTTT	48	232
	Antisense	CGTTACCATCAACTTCTCATAG		
20	Sense	TAAAATAAAAGGCGTACATAAA	48	192
	Antisense	TCATACAAAATACAACTCAGGT		
21	Sense	ATCATTTTTCAGAATACGAG	55	248
	Antisense	ATTCATGTGCTCATCATAA		
22	Sense	AACTCAGGATTCCACTTT	56	215
	Antisense	AACCCTGTTAAAAGCATA		
23	Sense	ATAGCAGCCTTTCATCATTTGT	60	261
	Antisense	TATTTCCAAGTCCCTCACTTTA		
24	Sense	CATCTTGATATAGTCATGTGTA	44	226
	Antisense	TTAGTTATTCTCTTTTCACTAC		
25	Sense	ATCCTGCATATTGAAGGCTAC	56	289
	Antisense	CAGTGTGATTTTTAAGGACTG		

Jolla, Calif.) as follows: initial denaturation for 2 min at 95°C, 35–40 cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by a final extension for 5 min at 72°C. Samples which showed mobility shifts in the SSCP analysis were further analyzed by direct DNA sequencing as described above.

A polymorphism at position –6 was detected in glioblastomas in this study. To assess the allelic frequency, we checked for the

Table 2 Primers used for SSCP of the *hBUBR1* gene

Fragment		Primer sequence	Annealing temperature (°C)	Product (bp)
1	Sense	CGGAAGAAAGCCCAGGCGGT	58	298
	Antisense	AGGGTCATTTCCAGTGTAA		
2	Sense	CAAGAATCTGCCTGTAACA	54	269
	Antisense	AAAGGCTCATTGCATAAAC		
3	Sense	AGAAAAACGATATTATAGTGA	50	312
	Antisense	TCCTCCTCTTCTTCTTTCTC		
4	Sense	CAGCACCGACAATTCCAAGC	56	277
	Antisense	TAGAAGCCTCATCAGCATTT		
5	Sense	CCTCAACAGATGCAAAATAA	58	279
	Antisense	TGGTGTCATAACTGGCTGT		
6	Sense	CTCGTGGCAATACAGCTTC	50	316
	Antisense	CTTTGCTCTTTTAATTTCT		
7	Sense	TTTATGCAGGAGTAGGGGA	50	272
	Antisense	AGTCATTCCTGGTATTTTC		
8	Sense	TACAAAGGAGACAACTAAA	53	323
	Antisense	ATTCATCACAAACATCTGG		
9	Sense	TCAAAACCTCAGAAAGCAT	53	338
	Antisense	AATAATTGGGCTCAGCTTC		
10	Sense	CAGCTTGTGGCACTATCTA	52	313
	Antisense	TCCTTCTCAATTTCCAACT		
11	Sense	TATAGAAGACAGACCAATG	51	311
	Antisense	GAAGATCCTGAAGGGTGAA		
12	Sense	TGGCTGTATTGTTTGGCAC	58	296
	Antisense	CGCTGAGGGTAAAAACATC		
13	Sense	TTCCTACAGTGTTGACCTT	56	309
	Antisense	AGCTCCCCAAGAACAGACA		
14	Sense	CTGAATGCCAATGATGAGG	54	236
	Antisense	TAAATTACAGCACATACAG		

Table 3 Primers used for SSCP analyses of the *hBUB3* gene

Fragment		Primer sequence	Annealing temperature (°C)	Product (bp)
A[a]	Sense	GCGAGTGGCGAGTAGTGGAA	58	78
	Antisense	CCCGTCCCGCACACC		
1	Sense	CGTTGCTTCTGAGGGGAG	58	252
	Antisense	ATCTAGTCCTCCACTCCAGGC		
2	Sense	GCCTTCTACGATCCAACGC	58	259
	Antisense	GGTCTCCAGACACTGAGAGGG		
3	Sense	TCCTTGTAATGCTGGGACCT	58	267
	Antisense	TTCTGTACCTCAGGGCTTGG		
4	Sense	CCGAGTGGCAGTTGAGTATTT	58	268
	Antisense	CGTAGTCCCATCATTACTGAAGG		
5	Sense	GGTACCCCACGAGCATCG	58	234
	Antisense	ATCCACCATTGGGGAGTACG		

[a] A denotes pair of primers for part of genomic 5′ region

presence of this variant at genomic level in blood DNA obtained from 60 healthy caucasians individuals.

Results

hBUB1 mutations

The PCR-SSCP analysis of all 25 exons of the *hBUB1* gene revealed a silent mutation at codon 144 (CAG→CAA, Gln→Gln) in one of 22 glioblastomas (case 300, Table 4 and Fig. 1) and in 2 out of 18 giant cell glioblastomas. In all cases, the wild-type base G was also present.

hBUBR1 mutations

SSCP followed by DNA sequencing of the entire coding sequence of *hBUBR1* revealed a silent mutation (GAC→GAT, Asp→Asp) at codon 952 in one glioblas-

Table 4 Genetic alterations in mitotic checkpoint genes in glioblastomas

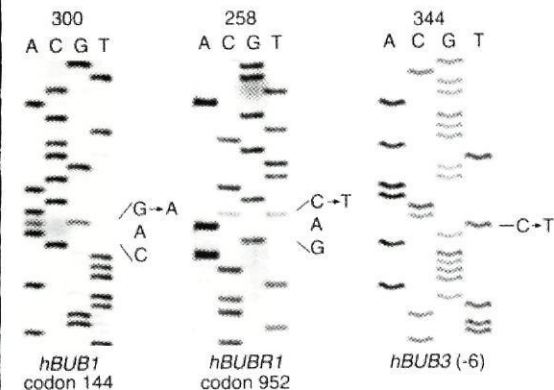| Patient ID	Age/sex	hBUB1 alteration	hBUBR1 alteration	hBUB3 alteration	
			Codon 349	Other codon	
72	28/F	–	Gln/Arg	–	Position –6, C→T[a]
201	63/M	–	Gln/Arg	GCG→GCA, Ala→Ala 388	–
233	59/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
234	43/M	–	Gln/Arg	–	–
256	56/F	–	Gln/Gln	–	–
257	50/M	–	Gln/Arg	–	Position –6, C→T[a]
258	65/F	–	Gln/Gln	GAC→GAT, Asp→Asp 952	Position –6, C→T[b]
287	47/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
288	69/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
289	58/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
290	63/F	–	Gln/Gln	–	–
292	62/M	–	Gln/Arg	GCG→GCA, Ala→Ala 388	–
295	47/M	–	Gln/Gln	–	–
296	63/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
298	30/M	–	Gln/Gln	–	–
299	50/F	–	Gln/Gln	–	–
300	71/F	CAG→CAA, Gln→Gln 144	Arg/Arg	–	–
301	68/F	–	Gln/Arg	–	–
302	64/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
303	67/M	–	Arg/Arg	–	–
314	40/M	–	Gln/Arg	–	–
344	47/M	–	Gln/Gln	–	Position –6, C→T[b]

[a] Homozygous mutation
[b] Heterozygous mutation

Fig. 1 Representative DNA sequencing autoradiographs of silent mutations in the *hBUB1* gene (codon 144, CAG→CAA, Gln→ Gln) and the *hBUBR1* gene (codon 952, GAC→GAT, Asp→Asp), and C→T change at –6 upstream of the initiator codon of the *hBUB3* gene. In cases 258 and 300, wild-type alleles are present, while in case 344, the wild-type allele is absent

toma and another silent mutation (GCG→GCA, Ala→Ala) at codon 388 in 8 glioblastomas (Fig. 1, Table 4).

We also identified a common polymorphism at codon 349 (CAA/CGA, Gln/Arg), with allelic frequencies of 0.75 for Gln and 0.25 for Arg in glioblastomas (Table 4).

These frequencies in glioblastomas were similar to those in blood DNA samples from healthy Caucasian individuals (0.73 for Gln and 0.27 for Arg).

hBUB3 mutations

A point mutation (C→T) was detected at 6 nucleotides upstream of the ATG initiator codon (position –6) in 4 of 22 (18%) glioblastomas (Table 4, Fig. 1). Of these, the wild-type base was also present together with the mutated sequence in 2 cases, whereas the other 2 showed only mutated sequences. The analysis of blood DNA from these 4 patients showed identical alterations to those detected in tumor tissues, indicating that this alteration is a polymorphism. Screening for this polymorphism in blood DNA from healthy Caucasian individuals revealed the presence of this mutation in 9/60 (15%) individuals, a similar frequency to that in the glioblastoma patients.

Discussion

Mitotic checkpoint genes play a significant role in chromosome segregation by preventing aneuploidy [1, 5, 13, 35, 40]. During mitosis, *hBUB1*, *hBUBR1* and *hBUB3* proteins localize in kinetochores before chromosome alignment and form a checkpoint kinase complex with

other proteins. The *hBuB1* and *hBUBR1* genes encode protein kinases, while the hBUB3 protein is required for kinetochore localization of hBUB1 and hBUBR1 proteins [1, 17, 42, 45]. In *Drosophila, BUB1* mutations cause chromosome missegregation and fail to block apoptosis [2]. In mice, *BUB1* is required not only for checkpoint response to spindle damage but also for normal mitotic timing, and for initiation of apoptosis when an apoptotic signal is present [44]. A recent study showed that the *BRCA*2 gene, that is associated with inherited susceptibility to breast cancer and plays a role in replication and repair of DNA during S phase, is phosphorylated by *hBUBR1* and may be involved in a mitotic checkpoint [10]. *Bub1* mutations were found in three out of four thymic lymphomas that developed in *Brca2*-deficient mice, suggesting that inactivation of these genes works synergistically in development of these tumors [26]. *Bub3*-null mouse embryos do not survive [20]; the embryos showed accumulation of several mitotic errors in the form of micronuclei, chromatin bridging, lagging chromosomes and irregular nuclear morphology, confirming that *Bub3* is essential for normal mitosis and for early embryonic development in mice [20].

In the present study, we investigated the status of the mitotic spindle checkpoint genes *hBUB1, hBUBR1* and *hBUB3* in glioblastomas. Screening of the entire coding sequence and intron-exon boundaries of the *hBUB1* gene revealed a silent mutation at codon 144 (CAG→CAA, Gln→Gln) in one glioblastoma and two giant cell glioblastomas. Although we were unable to analyze normal tissue samples from these patients, this alteration is likely to be a polymorphism, since it has been previously reported in several colon tumors [6]. To date, seven different *hBUB1* polymorphisms have been reported in human tumor cell lines [6, 12, 41, 49]. It remains to be shown whether any particular polymorphism is over-represented in cancer patients.

The analysis of the *hBUBR1* gene in this study showed two silent mutations at codons 952 and at 388, both of which have previously been detected in colon and lung tumors [6, 41], also suggesting a polymorphism. We found another polymorphism at codon 349 (CAA/CGA, Gln/Arg), which has previously been reported in colon and breast carcinomas [6, 21]. The allelic frequencies of this variant in healthy individuals in Japan are 0.31 for Gln and 0.69 for Arg [21]. We found an opposite allelic pattern in glioblastomas (0.75 for Gln and 0.25 for Arg). However, our analysis of DNA from healthy Caucasian individuals showed a frequency similar to that observed in glioblastomas. This indicates that the frequency of these alleles varies significantly between ethnically different populations, but they are not over-represented in glioblastoma patients.

In the present study, C→T mutations were detected at 6 nucleotides upstream of the ATG initiator codon of the *hBUB3* gene in 4 glioblastomas. The same mutation was found in blood DNA of these patients, suggesting that this is a new polymorphism. We also carried out screening for this allelic variant in 60 healthy Caucasian individuals, and

found its frequency to be similar to that in glioblastomas, suggesting that the presence of this polymorphism does not influence susceptibility to development of glioblastomas.

LOH on chromosome 10, in particular, loss of the 10q25-qter region, is the most frequent genetic alteration in glioblastomas, suggesting the presence of putative tumor suppressor gene(s) [38]. The *hBUB3* gene is mapped on 10q24-q26 [6, 25, 45]. However, the absence of miscoding mutations in *hBUB3* in this study excludes *hBUB3* as a candidate tumor suppressor gene involved in the pathogenesis of glioblastomas. We correlated the data of base change at 6 bp upstream of initiating codon in *hBUB3* and LOH on chromosome 10 published previously [9], but did not find any correlation (data not shown).

Occasional multinucleated neoplastic giant cells are a common histological feature in glioblastomas [4, 23]. Giant cell glioblastomas are defined by the predominance of large bizarre, multinucleated giant cells measuring up to 500 µm [33], with near-haploid or polyploid clones [4]. Our present results show that mutational inactivation of the *hBUB1* gene is not associated with giant cell phenotype. Due to unavailability of frozen samples for the generation of cDNA, we could not perform a mutational analysis of *hBUBR1* and *hBUB3* genes.

In conclusion, this study shows absence of miscoding mutations in *hBUB1, hBUBR1* and *hBUB3* genes in human glioblastomas, suggesting that mutational inactivation of these genes is not significantly involved in the aneuploidy of glioblastomas. Additional studies may be necessary to clarify whether these genes are inactivated through other mechanisms. Jaffrey et al. [18] recently detected microsatellite instability within the *hBUB1* gene in approximately 15% of colorectal cancers, raising the possibility that down-regulation of *hBUB1* may occur through instability. It also remains to be elucidated whether alterations of other mitotic spindle checkpoint genes, including *hMAD1, hMAD2, and MPS1*, are present in glioblastomas. However, lung, colon and digestive tract human neoplasms have a very low frequency or absence of inactivating mutations of these genes [6, 16, 32, 43].

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ERRATUM

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Mutation analysis of *hBUB1*, *hBUBR1* and *hBUB3* genes in glioblastomas

There were some mistakes in Table 4, which is therefore reprinted below.

Table 4 Genetic alterations in mitotic checkpoint genes in glioblastomas

Patient ID	Age/sex	*hBUB1* alteration	*hBUBR1* alteration		*hBUB3* alteration
			Codon 349	Other codon	
72	28/F	–	Gln/Arg	–	Position –6, C→T[a]
201	63/M	–	Gln/Arg	GCG→GCA, Ala→Ala 388	–
233	59/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
234	43/M	–	Gln/Arg	–	–
256	56/F	–	Gln/Gln	–	–
257	50/M	–	Gln/Arg	–	Position –6, C→T[a]
258	65/F	–	Gln/Gln	GAC→GAT, Asp→Asp 952	Position –6, C→T[b]
287	47/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
288	69/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
289	58/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
290	63/F	–	Gln/Gln	–	–
292	62/M	–	Gln/Arg	GCG→GCA, Ala→Ala 388	–
295	47/M	–	Gln/Gln	–	–
296	63/F	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
298	30/M	–	Gln/Gln	–	–
299	50/F	–	Gln/Gln	–	–
300	71/F	CAG→CAA, Gln→Gln 144	Arg/Arg	–	–
301	68/F	–	Gln/Arg	–	–
302	64/M	–	Gln/Gln	GCG→GCA, Ala→Ala 388	–
303	67/M	–	Arg/Arg	–	–
314	40/M	–	Gln/Arg	–	–
344	47/M	–	Gln/Gln	–	Position –6, C→T[b]

[a]Homozygous mutation
[b]Heterozygous mutation

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Second Primary Glioblastoma

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Abstract. Although characterized by a highly variable phenotype and multiple genetic alterations, glioblastomas are considered monoclonal in origin. We here report on a 64-yr-old patient who developed a second glioblastoma in the left frontal lobe 10 yr after surgical resection of a glioblastoma of right frontal lobe. The first tumor contained 2 *p53* mutations, in codon 213 (CGA→TGA, Arg→stop) and codon 306 (CGA→TGA, Arg→stop), further, 1 missense *PTEN* mutation (codon 257, TTC→TTA, Phe→Leu) and a silent *PTEN* mutation (codon 154, TTC→TTT, Phe→Phe). The second glioblastoma also contained multiple, but different mutations: *p53* mutations in codons 158 (CGC→CAC, Arg→His) and 273 (CGT→TGT, Arg→Cys), and a *PTEN* mutation in codon 233 (CGA→TGA, Arg→Stop). Both neoplasms had a homozygous *p16* deletion. The discordant pattern of mutations indicates that the second glioblastoma was not a recurrence but an independent second glioblastoma. The presence in these neoplasms of multiple mutations in tumor suppressor genes suggests the involvement of a novel disease mechanism but there was no indication of a DNA mismatch repair deficiency or of an inherited tumor syndrome.

Key Words: *p16* deletion; *p53* mutation; *PTEN* mutation; Second primary glioblastoma.

INTRODUCTION

Glioblastomas are among the most malignant brain tumors and carry a poor prognosis. Despite considerable efforts aiming at a more efficient adjuvant radio- and chemotherapy, less than 2% of patients survive more than 3 yr (1). Following surgical resection, recurrent growth occurs typically within a few months. Although no systematic studies have been published on the genetics of such recurrences, it is generally assumed that they share with the first glioblastoma a common monoclonal origin. Changes in the pattern of histopathological features are sometimes observed in recurrent malignant gliomas but this may be due to the acquisition of additional genetic alterations resulting from genetic instability.

There appears to be no published evidence of the independent development of a second primary glioblastoma. We here report the case history of a patient who developed a second glioblastoma after an interval of 10 yr. Both neoplasms contained multiple, but different mutations in the tumor suppressor genes *p53* and *PTEN*, excluding the possibility of a common monoclonal origin.

Case Report

The 54-yr-old male patient presented for 3–4 months with headache and temporal loss of memory. He had developed left-sided facial paresis 3 wk before the first visit to the hospital. His left eye did not close and the left

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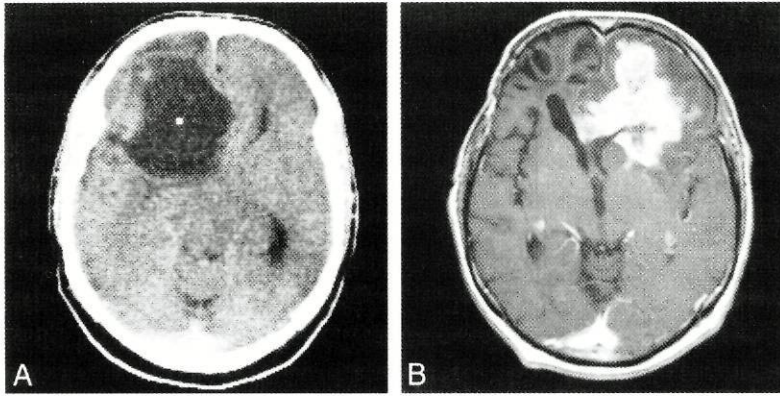
corner of the mouth was dropping. He was brought for the first time to the hospital after getting confused and being lost the whole day in his hometown. The cranial computed tomography (CT) revealed a large glioma in the right frontal lobe, which was only slightly enhanced by contrast medium and without evidence of extensive central necrosis (Fig. 1A). At the operation, a tumor with a large cyst was partially (approximately 75%) resected. Postoperative radiotherapy was first applied to the whole cranium from 2 opposite fields (2.0 Gy every weekday) for 3 wk (15 times). Three weeks later, the tumor area was treated locally with fractions of 2.0 Gy during 13 weekdays. The total dose to the tumor area was 56 Gy (5,600 cGy). No chemotherapy was given and anti-epileptic treatment was suspended several years after the operation.

Histologically, the tumor was of glial origin with medium to high cellularity, marked nuclear atypia, and numerous hyperchromatic and multinucleated tumor cells (Fig. 2A). Abundant typical and atypical mitoses were observed. Marked focal microvascular proliferation with occasional formation of fentons was also observed. There were numerous small foci of pseudopalisading necrosis (Fig. 2B). In addition, there were foci of ischemic tumor necrosis with necrotic vessels and inflammatory response. The tumor infiltrated the adjacent brain tissue, without evidence of a less malignant lesion, e.g. low-grade diffuse astrocytoma. The MIB-1 labeling index was unusually high (40%). Histologically, there was no significant oligodendroglial component. A minor fraction of tumor cells showed a clearly recognizable cytoplasmic expression of GFAP (Fig. 2C). In some areas, there was an admixture of reactive astrocytes. Based on the presence of marked nuclear atypia, brisk mitotic activity, microvascular proliferation and necrosis, the tumor was diagnosed as glioblastoma (WHO grade IV).

Fig. 1. The CT revealed a large glioma in the right frontal lobe, which was only slightly enhanced by contrast medium but no evidence of extensive central necrosis in a 54-yr-old male patient (A). Ten years after the first operation, MRI showed a large glioblastoma predominantly in the left frontal lobe with contrast enhancement, central necrosis and perifocal edema (B).

Over a period of 9 yr after the first operation, CT or magnetic resonance imaging (MRI) which were carried out every 1–2 yr, showed no significant change. Ten years after the first operation, the patient presented with memory loss, psychic changes, and aphasia. The MRI showed a large tumor predominantly in the left frontal lobe with extension into the corpus callosum, contrast enhancement, central necrosis and perifocal edema (Fig. 1B). A second surgery was performed and BCNU cytostat wafers were implanted.

Histologically, this tumor was a highly anaplastic astrocytic glioma with medium to high cellularity and marked nuclear atypia (Fig. 2D). A significant fraction of tumor cells showed a well-delineated cytoplasm with marked immunoreactivity to GFAP and peripherally located nuclei (gemistocytes, Fig. 2F). Mitoses were not very frequent, but the MIB-1 index varied focally from 5% to 40%. There were focal microvascular proliferations and large areas of ischemic necrosis (Fig. 2E). Some tumor areas lacked GFAP expression and stained positive for reticulin, but this was not extensive enough to justify the diagnosis of gliosarcoma. On the basis of its histopathological features, the tumor was diagnosed as glioblastoma (WHO grade IV) with a significant fraction of neoplastic gemistocytes. Again, there was no evidence of the presence of a less malignant precursor lesion. The patient died 3 days after the operation due to intracranial hemorrhage. The autopsy confirmed the presence of a glioblastoma with infiltration of the corpus callosum and extension into the right frontal lobe.

The family history was not remarkable. The patient's mother died at the age of 48 yr of tuberculosis and his father died at the age of 79 yr of prostate cancer. The patient had 9 sisters and brothers, none of whom developed cancer. His 3 children are alive and well.

MATERIAL AND METHODS

Screening for *p53* Mutations

DNA was extracted from formalin-fixed, paraffin-embedded histologic sections as previously described (2). Prescreening for mutations in exons 5–8 of the *p53* gene was carried out by PCR-SSCP followed by direct sequencing. Briefly, PCR was carried out in final volume of 10 μl, with 2 μl DNA solution, 2.5 pmol of each primer, 0.24 mM of dNTP, 0.5 μCi of $[\alpha\text{-}^{33}P]$-dCTP (ICN Biomedicals N.V. S.A, Belgium, specific activity, 3,000 Ci mmol), 10 mM Tris (pH 8.8), 50 mM KCl, MgCl$_2$ (1.5–2.5 mM) and 0.25 U of *Taq* polymerase (Sigma, St. Louis, MO). Forty cycles of denaturation (94°C) for 50 s, annealing (60°C for exons 6, 7 and 8, and 55°C for exon 5) for 60 s, and extension (72°C) for 70 s were carried out in a Genius DNA Thermal Cycler (Techne, Cambridge, UK). After PCR, 4 μl of PCR product were mixed with 10 μl loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue), denatured at 95°C for 10 min and immediately loaded onto a 6% polyacrylamide nondenaturing gel containing 6% glycerol. Gels were run at 40 W for 3–5 h with cooling by fan at room temperature, dried at 80°C, and autoradiographed for 24–68 h. Samples that showed a mobility shift in the SSCP analyses were further analyzed by direct DNA sequencing. Primer sequences used for SSCP and DNA sequencing were described previously (3).

Screening for *PTEN* Mutations

PCR-SSCP followed by direct sequencing was carried out in exons 1–9 of the *PTEN* gene. Briefly, PCR was carried out with 1 μl of DNA solution, 1.5–2.5 pmol of each primer, 0.2 mM of dNTPs, 0.5 μCi of $[\alpha\text{-}^{33}P]$-dCTP (ICN Biomedicals, specific activity, 3,000 Ci mmol), 10 mM Tris (pH 8.8), 50 mM KCl, MgCl$_2$ (1.5–2.5 mM) and 0.75–1.0 U of *Taq* polymerase (Sigma) in a final volume of 10 μl. PCR was performed in a robocycler 96 gradient temperature cycler (Stratagene GmbH, Heidelberg, Germany) with an initial denaturation step for 2 min at 95°C followed by 35 to 40 cycles of denaturation 95°C
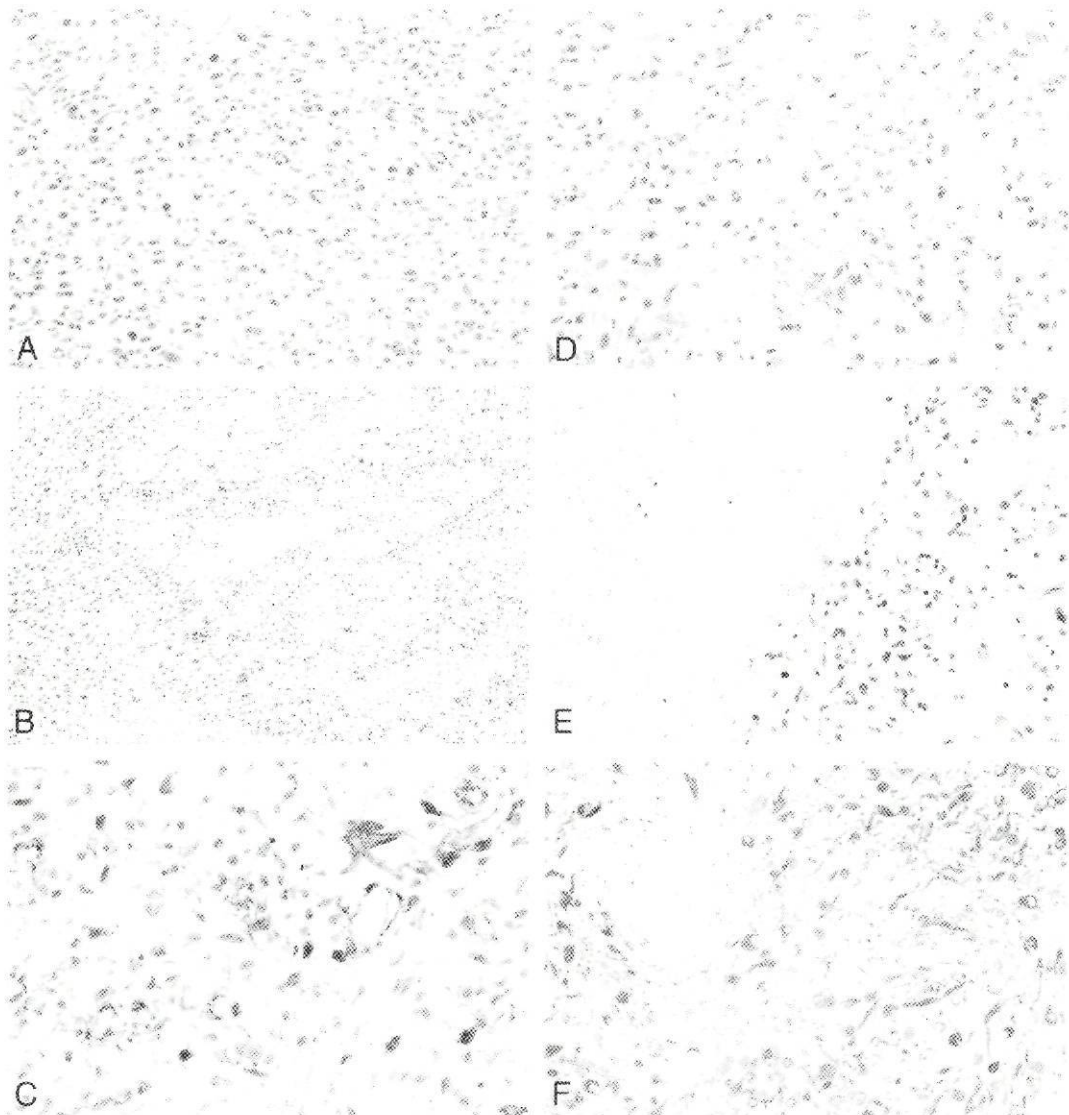
Fig. 2. Histologic appearances of the first (A–C) and second (D–F) glioblastomas. The first glioblastoma with marked nuclear atypia and numerous hyperchromatic and multinucleated tumor cells (A), and with small foci of pseudopalisading necrosis (B). Immunohistochemistry shows significant fraction of tumor cells expressing GFAP (C). The second glioblastoma with marked nuclear atypia (D), and with ischemic necrosis (E). Note marked immunoreactivity to GFAP in a significant fraction of tumor cells (F).

for 60 s, annealing at 52–58°C for 50 s, extension at 72°C for 50 s. Primer sequences for PCR amplification were previously reported (4). After PCR, 4 µl of PCR products were mixed with 10 µl loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue), denatured at 95°C for 10 min and immediately loaded onto a 6% polyacrylamide non-denaturing gel containing 6% glycerol. Gels were run at 40 W

for 3 to 5 h with cooling by fan at room temperature, dried at 80°C, and autoradiographed for 24–68 h.

Samples that showed a mobility shift in the SSCP analysis were further analyzed by direct DNA sequencing as previously described (5). Sequencing primers used were as follows: 5′-TAA AGC TGG AAA GGG ACG AA-3′ (sense) and 5′-TAT CAT TAC ACC AGT TCG TC-3′ (antisense) for

exon 5; 5′-TGA CAG TTT GAC AGT TAA AG-3′ (sense) and 5′-TGA AAT GAA AGT AAC CCT C-3′ (antisense) for exon 7.

p16 Homozygous Deletion and EGFR Amplification

To detect *p16* homozygous deletions, differential PCR was carried out using *STS* sequence as a reference, as described by Ueki et al (6), with some modifications (7). The primer sequences for differential PCR were as follows: 5′-ACAAGCTTC CTTTCCGTCAT (sense) and 5′-GCGCACGTCCAGCCG-CGCCCCGG (antisense) that amplify a 235 bp fragment of the 5′ end of *p16* exon 2, and 5′-ATTTCTGCCTGGAGA CAG-TGG (sense) and 5′-AGGCCTTGGGAGCCTCAGG (antisense) that amplify a 180 bp *STS* reference gene on the long arm of chromosome 9. After PCR, 15 μl of reaction product were electrophoresed on an 8% polyacrylamide gel and stained with ethidium bromide. Gels were photographed using Polaroid film and the intensity of the fragments was measured by means of a GS-670 densitometer (Bio-Rad, Ivry Sur Seine, France). The average *p16/STS* ratio using normal blood DNA was 1.04 with a standard variation of 0.15. Values of less than 0.2 for the *p16/STS* ratio indicated deletions of the *p16* gene (7). Two primary glioblastomas, which showed a ratio of less than 0.2 in the previous study (7), served as positive controls for *p16* homozygous deletion.

EGFR amplification was detected by differential PCR as previously described (8) using cystic fibrosis (CF) sequence as a reference. The primer sequences were as follows: 5′-AGCCAT-GCCCGCATTAGCTC (sense) and 5′-AAAGGAATGCAA-CTTCCCAA (antisense) for *EGFR* and 5′-GGCACCATTAAA-GAAAATATCATCTT (sense) and 5′-GTTGGCATGCTTTGA-TGACGCTTC (antisense) for *CF* reference gene. The size of the PCR fragments were 110 bp for *EGFR* and 79 bp for *CF* gene. The mean *EGFR/CF* ratio using DNA from peripheral blood of healthy adults was 1.2 with a standard variation of 0.20. The threshold value 2.94 was regarded as evidence of *EGFR* amplification, according to Rollbrocker et al (9). One primary glioblastoma, which showed *EGFR* amplification in a previous study (8), was used as a positive control.

LOH of Chromosome 10 and 19

We analyzed the following microsatellite markers: D10S1779, D10S211, D10S587, D10S541, D10S1731, D10S212, D10S1700, D10S209, D10S216, D10S187, D10S1657 on chromosome 10, and D19S902, D19S246, D19S596, D19S178, D19S219, D19S412 on chromosome 19. PCR was performed as previously described (10, 11). These markers cover common deletion on chromosomes 10 and 19q (10–13). Briefly, PCR was carried out with 1 μl DNA solution, 5× PCR buffer, 6 pmol of each primer, 200 mM of each dNTP, 0.5 mCi of [α-^{33}P]-dCTP (ICN Biomedicals, specific activity, 3,000 Ci mmol), 0.225 U of *Taq* polymerase (Sigma) in a final volume of 10 μl. PCR was carried out in a Genius DNA Thermal Cycler (Techne) with an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. After PCR, 10 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue) were mixed with PCR products,

denatured at 95°C for 5 min, and immediately loaded onto a 6% polyacrylamide/7 M urea sequencing gel. Gels were run at 70 W for 3–5 h, dried at 80°C, and autoradiographed for 48–62 h.

Screening for Microsatellite Instability

Microsatellite instability was analyzed in the first and second glioblastomas. The markers used were as follows: Mfd1, Mfd84 located on chromosome 12, Mfd72 (chromosome X), MSH6 (2p21), BAX (19q13.3), MSH3 (5q11-q12), BAT25 (4q11-q12) and BAT26 (2p21), TGFβRII. The PCR was performed as previously described (14). Briefly, PCR was carried out in a total volume of 25 μl containing 20 ng of DNA, 100 ng of each primer, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 0.125 μM dATP, 1mCi of [^{33}P]dATP, 0.75 U of Taq polymerase, and standard buffer. PCR was carried out on 6% denaturing polyacrylamide, 2 min at 58°C and 2 min at 72°C and additional 25 cycles with annealing temperature at 55°C. Aliquots of the PCR product were loaded onto a 8% polyacrylamide DNA sequencing gels. Gels were dried at 80°C and autoradiographed for 48–72 h.

Genetic Testing of Biopsies for Patient Identity

The following polymorphic markers were analyzed: SE33 (ACTBP2), TH01, D21S11, VWA, HumFES, D3S1358, FGA, TPOX, CSF1PO, D5S818, D13S317 and D7S820 (15–18). The PCR reactions were performed using 10 ng of template DNA. Electrophoresis was carried out on 6% denaturing polyacrylamide gels at 1,600 V and 24 mA for 8 h. Analysis and fluorescent detection of the PCR products were carried out on an ABI 372A or an ABI 377 DNA Sequencer (Perkin Elmer).

RB Immunohistochemistry

The sections were deparaffinized in xylene and rehydrated in graded ethanol. The endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 solution in methanol for 30 min. After the sections were boiled 3 times for 5 min in 10 mM sodium citrate buffer (pH 6.0) in a microwave oven, the sections were allowed to cool at room temperature. The sections were then incubated overnight at 4°C with the RB monoclonal antibody (clone G3-245, PharMingen, San Diego, CA; diluted 1:100), which recognizes an epitope between amino acids 300–380 and both phosphorylated and underphosphorylated RB protein. The reaction was visualized using the Vectastain ABC Kit and diaminobenzidine (Vector Laboratories, Burlingame, CA).

RESULTS

p53 Mutations

PCR-SSCP followed by direct sequencing revealed that first and second biopsies contain different *p53* mutations (Table; Fig. 3). In the first biopsy, double *p53* mutations were observed in codon 213 (CGA→TGA, Arg→stop) and in codon 306 (CGA→TGA, Arg→stop). On the second biopsy, double missense mutations were found in different codons, i.e. codon 158 (CGC→CGA, Arg→His) and codon 273 (CGT→TGT, Arg→Cys, Table; Fig. 3).

TABLE
Multiple Genetic Alterations in the First and Second Primary Glioblastomas

Biopsy Age Diagnosis	First 54 years Glioblastoma	Second 64 years Glioblastoma
p53 mutation	Exon 8, codon 306, CGA→TGA, Arg→Stop Exon 6, codon 213, CGA→TGA, Arg→Stop	Exon 8, codon 273, CGT→TGT, Arg→C Exon 5, codon 158, CGC→CAC, Arg→
PTEN mutation	Exon 7, codon 257, TTC→TTA, Phe→Leu Exon 5, codon 154, TTC→TTT, Phe→Phe	Exon 7, codon 233, CGA→TGA, Arg→
p16/STS ratio*	0.03 (deletion)	0.02 (deletion)
EGFR/CF ratio*	1.2 (no amplification)	0.8 (no amplification)
RB expression	No loss	No loss

* Values of less than 0.2 for the *p16/STS* ratio is considered to be homozygous deletion of the *p16* gene, and the thres
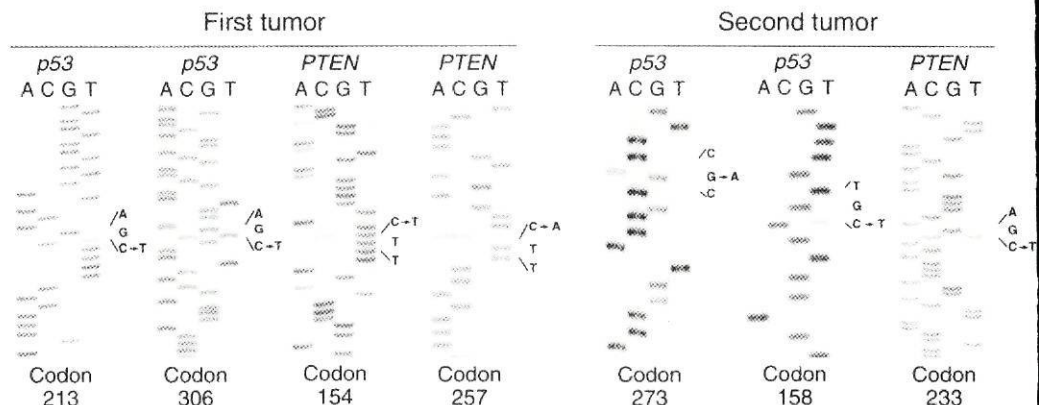value 2.94 is regarded as evidence of *EGFR* amplification (see Materials and Methods).

Fig. 3. DNA sequencing autoradiographs for *p53* and *PTEN* mutations in first and second glioblastomas in the patient.
that 2 tumors contain multiple and different mutations.

PTEN Mutations

The first and second biopsies contained different *PTEN* mutations (Table; Fig. 3). In the first biopsy, a missense mutation was detected in codon 257 (TTC→TTA, Phe→Leu) and a silence mutation (TTC→TTT, Phe→Phe) in codon 154. In the second biopsy, a nonsense mutation was found in codon 233 (CGA→TGA, Arg→stop, Table; Fig. 3).

p16 Deletion, EGFR Amplification, and RB Expression

p16 deletion was detected in both first and second tumors. *EGFR* amplification was not detected in either biopsy. Neoplastic cells in both first and second biopsies showed immunoreactivity to RB (Table).

LOH on Chromosomes 10 and 19q and Microsatellite Analysis

Of the 17 polymorphic markers analyzed, 12 (70%) were informative. There was no LOH on chromosome 10

or on 19q. There was no evidence of allelic shift i the markers studied in both first and second globla mas, except for an allelic shift found in the first g blastoma, using marker D10S211.

Patient Identity

In order to exclude the remote possibility of a mista sample identity, we carried out a forensic DNA test v polymorphic DNA markers. Identical patterns were served in the first and second tumors for 11 of the markers analyzed except for a marker SE33. For marker SE33, 1 of the alleles displayed a length di ence of 1 repeat, i.e. 29.2 instead of 30.2, suggesti mutational event in the second tumor. The mutation in SE33 for nontumorous tissue is 0.7% (19). The quency of the DNA profile is extremely rare: 1 in (× 10^{19}, applying Caucasian allelic frequencies. The l lihood of the hypothesis assuming that the 2 tumors o inate from the same patient is therefore 6.46 × 10^{19} gr er than the hypothesis, assuming that the first and

second tumor biopsy came from different individuals. Hence, it was confirmed that both biopsies were indeed from the same patient.

DISCUSSION

Several aspects of the case history of this patient are unusual. First, he survived a glioblastoma for 10 yr. This is exceptional, since the majority of patients dies within the first year and a recent population-based epidemiological study showed that less than 2% of glioblastoma patients survive more than 3 yr, irrespective of aggressive radio- and chemotherapy (1). Second, the patient developed a second glioblastoma in the opposite frontal lobe 10 yr later. Third, both glioblastomas contained multiple *p53* and *PTEN* mutations. Most significantly, none of the mutations was present in both tumors. The occurrence of 2 or more *p53* mutations has occasionally been observed (20–22) but 2 or more *PTEN* mutations are rare (5, 23). We know of no case with multiple *p53* and *PTEN* mutations.

There is convincing evidence that *p53* mutations are not lost during glioma progression; they typically constitute early genetic events and have been shown to persist during malignant progression (3, 20, 24, 25), metastatic spread (26), and long-term culture in vitro (27). Thus, the presence of multiple different mutations eliminates the possibility that the second glioblastoma constitutes a recurrence of the first tumor. In order to exclude the remote possibility of a mistaken sample identity, we carried out a forensic DNA test with polymorphic DNA markers and confirmed that both biopsies were indeed from the same patient.

Both glioblastomas developed clinically *de novo* after a short history and there was no evidence of a less malignant precursor lesion. Genetically, they displayed some features typical of primary (*de novo*) glioblastoma (*PTEN* mutations, *p16* deletion) and others characteristic of secondary glioblastoma (*p53* mutations, absence of *EGFR* amplification). This suggests that the underlying disease mechanism characterized by multiple mutations in transformation-associated genes puts these neoplasms into a separate category.

Since the patient received radiotherapy after the first surgery resection of the first glioblastoma, one may argue that the second glioblastoma was caused by ionizing radiation treatment (28). However, it is more likely that the development of the second tumor is associated with the genetic background of the patient since the first glioblastoma contained already multiple mutations and deletion of tumor suppressor genes.

To our knowledge, this is the first report of a second primary glioblastoma confirmed by genetic analyses. The development of 2 independent glioblastomas and the presence of multiple mutations in the *p53* and *PTEN*

genes in both lesions suggest genetic instability as possible underlying cause. Genetic instability in human cancers exists at 2 different levels: nucleotide (microsatellite) and chromosomal instability (29). Microsatellite instability is characterized by the presence of random contraction or expansion in the length of simple sequence repeats, and is a key feature of the hereditary non-polyposis colorectal cancer (HNPCC) syndrome (30). In HNPCC kindreds, the defect is associated with germline mutations in 1 of the mismatch repair genes *hMSH2*, *hMLH1*, *hPMS1*, or *hPMS2* (31). Some sporadic tumors, including colorectal, gastric, and endometrial carcinomas also exhibit microsatellite instability in a significant fraction of cases. Genes commonly developing microsatellite instability through this mechanism include *TGFRβII* (32, 33), *IG-FIIR* (34), *BAX* (35), *MSH3*, and *MSH6* (36). We carried out microsatellite analyses in the first and second glioblastoma of this patient, but failed to detect microsatellite instability, suggesting that the unusual pattern of multiple mutations in tumor suppressor genes are unrelated to known defects in DNA mismatch repair.

Another possible cause of instability at the nucleotide level is deficient nucleotide-excision repair that is mainly responsible for repairing damage caused by exogenous mutagens (29). Skin tumors are among the most frequent neoplasms in patients with nucleotide-excision repair defects (29), but the patient reported in this study did not show any sign of skin neoplasms.

It is notable that 6 of 7 point mutations detected in *p53* or *PTEN* genes in glioblastomas from the present patient were G:C→A:T transitions, and of these, 5 were located at CpG sites (Table). This type of mutation is frequent in brain tumors (37) and is considered to be caused endogenously as a result of deamination of 5-methylcytosine, which occurs spontaneously in all cell types but is usually corrected by DNA repair mechanisms (38). Deamination of 5-methylcytosine generates a T:G mispair, and a thymine-DNA glycosylase (TDG) has been described that initiates T:G mismatch repair by specifically excising the mismatch T (39). Although mutations in the *TDG* gene have not yet been identified in human neoplasms (40, 41), decreased levels of mRNA expression were detected in pancreatic tumor cell lines (41) and a high frequency of LOH (42%) was observed in the TDG locus in gastric carcinomas (40). Recently, another thymine-DNA glycosylase, MBD4, was found to bind to the product of deamination at methylated CpG sites (39), suggesting that it acts as a new DNA repair gene. Riccio et al (42) detected somatic *MBD4* mutations in colon (26%) and endometrial (22%) carcinomas with microsatellite instability. Similarly, Bader et al (43) reported somatic frameshift mutations in the *MBD4* gene of sporadic colon cancers with mismatch repair deficiency.

It is notable that the present patient survived 10 yr after the first glioblastoma. In Turcot syndrome type 1, which

is characterized by colorectal carcinoma and glioblastoma caused by germline mutations of DNA mismatch repair genes, the survival after surgical resection of the glioblastoma is often remarkably long when compared to patients with sporadic glioblastoma, suggesting that deregulation of mismatch repair is associated with a better prognosis (44). Sporadic colorectal and gastric carcinomas with microsatellite instability also carry a somewhat better prognosis (45, 46), possibly because the accumulated DNA damage rapidly rises above the threshold that activates apoptosis signaling pathways (47). Furthermore, mutations in certain genes may lead to the presentation of new surface molecules and thereby trigger a host immune response (46, 48).

Second primary tumors are considered to be rare, except in familial cancer syndromes and at organ sites with significant exposure to environmental carcinogens, e.g. esophagus and oropharynx following many years of excessive tobacco and alcohol consumption (49). There is currently no evidence of the causation of human brain tumors by exogenous carcinogens, with the exception of therapeutic X-irradiation (2). The patient described in this report did not receive radiotherapy before the development of the first glioblastoma.

In conclusion, this study provides genetic evidence of a second primary glioblastoma, both tumors being characterized by multiple mutations in tumor suppressor genes. The underlying disease mechanism may be novel and remains to be elucidated but the type of mutations identified points to an endogenous formation involving deamination of 5-methylcytosine rather than to causation by exogenous mutagenic agents.

ACKNOWLEDGMENTS

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Short Communication

Genetic Profile of Gliosarcomas

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There are distinct genetic pathways leading to the
glioblastoma, the most malignant astrocytic brain tu-
mor. Primary (*de novo*) glioblastomas develop in
older patients and are characterized by epidermal
growth factor (*EGF*) receptor amplification/overex-
pression, *p16* deletion, and *PTEN* mutations,
whereas secondary glioblastomas that progressed
from low-grade or anaplastic astrocytoma develop in
younger patients and frequently contain *p53* muta-
tions. In this study, we assessed the genetic profile of
gliosarcoma, a rare glioblastoma variant character-
ized by a biphasic tissue pattern with alternating ar-
eas displaying glial and mesenchymal differentiation.
Single-strand conformation polymorphism followed
by direct DNA sequencing revealed *p53* mutations in
five of 19 gliosarcomas (26%) and *PTEN* mutations in
seven cases (37%). Homozygous *p16* deletion was de-
tected by differential polymerase chain reaction in
seven (37%) gliosarcomas. The overall incidence of
alterations in the Rb pathway (*p16* deletion, *CDK4*
amplification, or loss of pRb immunoreactivity) was
53%, and these changes were mutually exclusive. Co-
amplification of *CDK4* and *MDM2* was detected in one
gliosarcoma. None of the gliosarcomas showed am-
plification or overexpression of the *EGF* receptor.
Thus gliosarcomas exhibit a genetic profile similar to
that of primary (*de novo*) glioblastomas, except for
the absence of *EGFR* amplification/overexpression.
Identical *PTEN* mutations in the gliomatous and sar-
comatous tumor components were found in two
cases. Other biopsies contained *p16* deletions, an
identical *p53* mutation, or coamplification of *MDM2*
and *CDK4* in both tumor areas. This strongly supports
the concept of a monoclonal origin of gliosarcomas
and an evolution of the sarcomatous component due
to aberrant mesenchymal differentiation in a highly
malignant astrocytic neoplasm. *(Am J Pathol 2000,
156:425–432)*

Glioblastoma multiforme (WHO Grade IV), the most fre-
quent and malignant brain tumor, may arise *de novo* after
a short clinical history without an identifiable less malig-
nant precursor lesion. This type of glioblastoma has been
designated primary glioblastoma. Secondary glioblas-
tomas develop more slowly through progression from
low-grade (WHO Grade II) or anaplastic astrocytoma
(WHO Grade III).[1,2] Primary glioblastomas typically arise
in older patients (mean, 55 years), whereas secondary
glioblastomas develop in younger patients (mean, 39
years).[3] There is increasing evidence that these two sub-
types develop through different genetic pathways. Pri-
mary glioblastomas are characterized by epidermal
growth factor receptor (*EGFR*) amplification/overexpres-
sion, *p16* deletion, and *PTEN (MMAC1)* mutations. Sec-
ondary glioblastomas typically contain *p53* mutations but
rarely have *EGFR* amplification/overexpression, *p16* de-
letion, or *PTEN* mutation.[2–5]

We recently reported that the giant cell glioblastoma, a
rare glioblastoma variant characterized by the presence
of large, bizarre, multinucleated cells, occupies a hybrid
position, sharing with primary (*de novo*) glioblastomas a
short clinical history, the absence of a less malignant
precursor lesion, and a 30% frequency of *PTEN* muta-
tions. They have in common with secondary glioblas-
tomas a younger patient age at manifestation and a high
frequency of *p53* mutations.[6]

The gliosarcoma is another morphologically defined
glioblastoma variant, originally described in 1895 by Stro-
ebe et al.[7] Gliosarcomas comprise approximately 2% of
all glioblastomas[8,9] and are characterized by a biphasic
tissue pattern, with areas displaying glial and mesenchy-
mal differentiation.[10,11] Whereas morphological studies
suggested an evolution of the sarcomatous component
from microvascular proliferations within a highly malig-
nant glioblastoma, two recent genetic studies revealed

the presence of identical *p53* mutations[12] and similar chromosomal imbalances and cytogenetic alterations[13] in both tumor areas, suggesting a monoclonal origin. In this study, we screened 19 well-documented cases of gliosarcoma for a variety of genetic alterations in an attempt to identify the genetic profile of gliosarcoma as compared to other glioblastoma subtypes and to elucidate the histogenesis of the sarcomatous component present in this neoplasm.

Materials and Methods

Tumor Samples

The surgical specimens were obtained from a total of 19 patients diagnosed in the University Hospitals of Zürich (Switzerland), Porto (Portugal), and Ribeirão Preto, São Paulo (Brazil). Gliosarcomas were diagnosed according to the WHO classification of brain tumors.[10] Care was taken to include only classical cases, showing the typical biphasic pattern with alternating areas of glial and mesenchymal differentiation. High-grade gliomas with a mesenchymal component that might have resulted from infiltration of the dura were excluded. The areas with glial differentiation usually expressed glial fibrillary acidic protein (GFAP) and showed necrosis and/or vascular endothelial proliferation. The sarcomatous portions showed strong reticulin staining as well as signs of malignant transformation (eg, nuclear atypia, mitotic activity, and necrosis; Figure 1). Glioblastomas with focal sarcomatous appearance but without reticulin staining were not included. The age and sex of patients are shown in Table 1. Eleven patients were male and eight were female (M/F ratio, 1.4). The mean age of patients at first diagnosis of gliosarcoma was 56 ± 12 years (range, 32–76 years).

In seven cases, we were able to microdissect the glial and mesenchymal areas (cases 210, 213, 214, 215, 218, 220, and 223), and DNA was extracted separately from these two portions. Areas were sufficiently large for manual dissection after microscopic identification and labeling of the respective tumor component.

Of 12 patients for whom we could obtain detailed clinical data, 10 were diagnosed with gliosarcoma at the first biopsy; the mean preoperative clinical history was 12 ± 16 weeks. In one case (case 221), the first biopsy was histologically classified as glioblastoma (preoperative history, 4 months), and the second biopsy as gliosarcoma. In another case (case 216), the first biopsy showed an anaplastic astrocytoma, the second biopsy a glioblastoma, and only the third biopsy showed the typical features of gliosarcoma.

Polymerase Chain Reaction-Single-Strand Conformational Polymorphism Analysis and Direct Sequencing for p53 Mutations

DNA was extracted as previously described.[14] Mutations in exon 5–8 of the *p53* gene were screened using polymerase chain reaction-single-strand conformational poly-
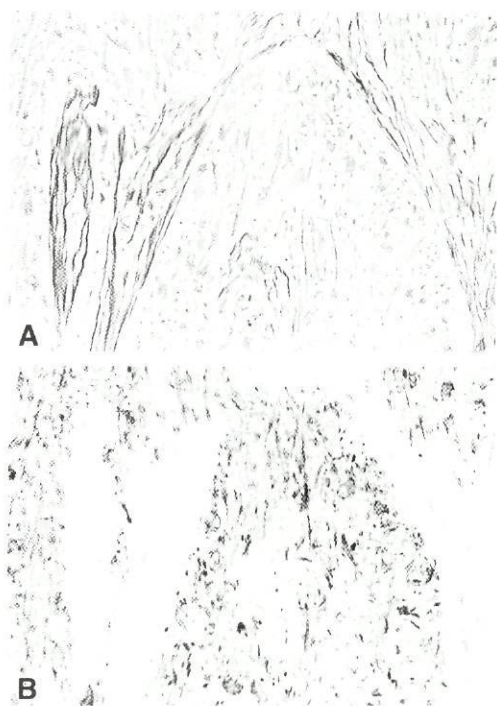

Figure 1. Histological features of gliosarcoma. The sarcomatous component shows a dense reticulin network (A) but lacks GFAP immunoreactivity, which is strongly expressed in the gliomatous component (B). Magnification, ×150.

morphism (PCR-SSCP) as previously described.[3] Samples that showed a mobility shift in the PCR-SSCP analysis were further analyzed by direct DNA sequencing. Primer sequences for PCR and DNA sequencing were described previously.[3]

PCR-SSCP Analysis and Direct DNA Sequencing for PTEN Mutations

Prescreening for mutations in exons 1–9 of the *PTEN* gene was carried out by PCR-SSCP as previously described.[6] Samples that showed a mobility shift in the SSCP analysis were further analyzed by direct DNA sequencing as previously described.[6] In some cases, individual abnormally shifted SSCP bands were cut directly from the dried gels, placed in 100 μl of distilled water, incubated at 80°C for 15 minutes and centrifuged briefly; 1 μl of the supernatant was used for PCR. Sequencing primers used were as follows: 5'-CTC TCC TCC TTT TTC TTC A-3' (sense) and 5'-AGA AAG GTA AAG AGG AGC AG-3' (antisense) for exon 1; 5'-TTT CAG ATA TTT CTT TCC TTA-3' (sense) and 5'-TGA AAT AGA AAA TCA AAG CAT-3' (antisense) for exon 2; 5'-TAA AGC TGG AAA GGG ACG AA-3' (sense) and 5'-TAT CAT TAC ACC AGT TCG TC-3' (antisense) for exon 5; 5'-TTT TTT TTT AGG

Table 1. Genetic Profile of Gliosarcomas

Patient no.	Age/sex	Biopsy/ diagnosis	Location	Area	p53 miscoding mutation	PTEN miscoding mutation	p16/sts ratio	CDK4/ IFGN ratio	MDM2/ DR ratio	EGFR/ CF ratio	p53	MDM2	EGFR	RB
200	72/M	1st/GS	F	S	—	—	0.38	0.95	1.17	1.25	—	+ +	—	+ +
206	72/M	1st/GS	T	S	Exon 7-codon 245 (GGC→GAC, Gly→Asp)	—	0.04	0.63	0.79	0.8	+ +	—	—	+ +
207	55/F	1st/GS	T,P	S	Exon 6-codon 197 (GTG→GTGTG; 2bp insertion, stop at codon 246)	Exon 5-codon 111 (TGG→TAG, Trp→stop)	0.44	1.06	1.02	0.99	—	—	—	+ +
208	76/F	1st/GS	O	S	—*	—	0.16	2.33	1.04	1.25	—	+	—	+ +
209	32/M	1st/GS	B,S	S	—*	Exon 5-codon 88 (TAT→TCT, Tyr→Ser)	0.51	1.30	1.03	0.94	—	+ +	—	+ + +
210	51/M	1st/GS	T	S	Exon 6-codon 190 (CCT→TCT, Pro→Ser)	—	1.10	0.92	1.40	0.96	+ +	+ +	—	—
	52/M	2nd/GS	T	S	Exon 5-codon 151 (CCC→TCC, Pro→Ser)	—	0.32	0.83	1.22	1.08	+ + +	—	—	+ +
				G	Exon 5-codon 151 (CCC→TCC, Pro→Ser)	—	0.50	0.44	0.78	0.85	+ + +	—	—	—
211	51/M	1st/GS	P	S	—*	—	0.17	1.34	1.49	1.67	+ + +	+	—	+ +
212	51/F	1st/GS	T,O	S	nd	Exon 1-codon 17(CAA→CCA, Gln→Pro)	nd	nd	nd	nd	nd	nd	nd	nd
	52/F	2nd/GS	T,O	S	—*	Exon 1-codon 17(CAA→CCA, Gln→Pro)	0.31	0.95	0.88	0.76	+ +	+	+	+ +
213	61/M	1st/GS	—	S	—	*—	0.08	0.61	0.79	0.75	nd	nd	nd	nd
				G	—	—	0.31	0.56	1.06	0.81	nd	nd	nd	nd
214	37/F	1st/GS	T	S	—	—	0.04	0.74	0.59	1.23	nd	nd	nd	nd
				G	—	—	0.01	0.79	0.60	1.18	nd	nd	nd	nd
215	43/F	1st/GS	C	S	—	Exon 5-codon 107 (GAT→GCT, Asp→Ala)	0.75	1.19	1.09	1.35	+ +	+	—	+ +
				G	—	Exon 5-codon 107 (GAT→GCT, Asp→Ala)	0.65	1.08	1.02	0.83	+ + +	+	—	+ +
216	41/M	1st/AA	T,P	G	—	nd	nd	nd	nd	nd	nd	nd	nd	nd
	42/M	2nd/GBM	T	G	—	nd	nd	nd	nd	nd	nd	nd	nd	nd
	42/M	3rd/GS	B,S	S	Exon 8-codon 274 (GTT→GCT, Val→Ala)	—	0.59	0.96	1.90	0.73	+ + +	+ +	—	+ +
217	52/M	1st/GS	T	S	—	—	0.33	2.3	1.43	0.83	—	+	—	+ + +
218	57/M	1st/GS	T	S	—	Exon 8-codon 274 (TGG→TGA, Trp→stop)	0.60	1.23	1.29	0.88	+ +	+	—	+ +
				G	—	Exon 8-codon 274 (TGG→TGA, Trp→stop)	0.78	1.24	1.47	1.28	+ +	—	—	+ +
219	67/M	1st/GS	C	S	—	Exon 8-codon 274 (TGC→TAC, Trp→stop)	0.17	0.34	0.49	0.58	+ +	—	—	+ + +
220	63/F	1st/GS	T	S	—	—	0.62	3.08	5.78	1.15	—	+ + +	—	+ + +
				G	—	—	0.45	3.44	9.24	1.18	—	+ + +	—	+ + +
221	56/M	1st/GBM	P,O	G	nd	nd	0.14	nd	nd	nd	nd	nd	nd	nd
		2nd/GS	P,O	S	—	—	0.02	0.98	0.96	0.83	+	+ +	—	+ + +
222	68/F	1st/GS	T	S	Exon 7-codon 237 (ATG→ATA, Met→Ile)	Exon 2-codon 48 (AAC→GAC, Asn→Asp)	0.8	3.10	0.97	1.08	+ +	—	—	+ + +
223	58/F	1st/GS	T	S	—	—	0.48	0.71	0.74	1.13	—	—	—	+ +
				G	—	—	0.58	0.80	0.68	1.06	—	—	—	+ +

*Previously reported by Biernat et al [12] nd, not determined; —, negative; GS, gliosarcoma; GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; S, sarcomatous component; G, gliomatous component; O, occipital; P, parietal; F, frontal; BS, brainstem; C, cerebrum. The results of immunohistochemistry were recorded as negative (—), positive in <5% of cells (+), in 5-50% of cells (+ +), and in >50% of cells (+ + +). Differential PCR data (in bold) indicate amplification or deletion (see Materials and Methods).

ACA AAA TGT TT-3' (sense) and 5'-TCA CAT ACA TAC AAG TCA CCA AC-3' (antisense) for exon 8.

Differential PCR for p16 Homozygous Deletion and EGFR, CDK4, and MDM2 Amplification

To assay *p16* homozygous deletions in gliosarcomas, differential PCR was carried out, using the *STS* reference sequence, as reported by Ueki et al,[15] with some modifications.[4] The average *p16/STS* ratio, using normal blood DNA, was 1.04, with a standard variation of 0.15. Values of less than 0.2 for the *p16/STS* ratio indicated deletions of the *p16* gene.[4] Two primary glioblastomas, which showed a ratio of less than 0.2 in the previous study,[4] served as positive controls for *p16* deletion.

To detect *CDK4* amplification, differential PCR was carried out as described previously.[4] Interferon γ (IFNγ) was used as a reference gene. The value for normal blood DNA was 1.07, with a standard variation of 0.19. A value of more than 2.7 for the *CDK4/IFNG* ratio was regarded as positive for *CDK4* amplification. This value was calculated according to the method of Rollbrocker et al.[16] One primary glioblastoma, which showed a ratio higher than 2.7 in a previous study,[4] served as the positive control for *CDK4* amplification.

MDM2 amplification was detected by differential PCR analysis as previously described.[17] Dopamine receptor (*DR*) was used as the reference gene. The *MDM2/DR* ratio from normal blood DNA was 0.91, with a standard variation of 0.4. A value of more than 3.02 for the *MDM2/DR* ratio was regarded as positive for *MDM2* amplification. Two primary glioblastomas, which showed a ratio higher than 3.02 in a previous study,[17] served as positive controls for *MDM2* amplification.

To detect *EGFR* amplification, differential PCR with the cystic fibrosis (*CF*) reference gene was carried out as described previously,[18] with some modifications.[5] The mean *EGFR/CF* ratio, from DNA from the peripheral blood of healthy adults, was 1.2, with a standard variation of 0.20. The threshold value 2.94 was regarded as evidence of *EGFR* amplification, according to the method of Rollbrocker et al.[16] One primary glioblastoma, which showed *EGFR* amplification in a previous study,[5] was used as a positive control.

Immunohistochemistry

The sections were deparaffinized in xylene and rehydrated in graded ethanol. The endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 solution in methanol for 30 minutes.

For p53 immunohistochemistry, the sections were boiled three times for 5 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven. The incubations of anti-human p53 monoclonal antibody (PAb 1801; Genosys Biotechnologies, Cambridge, UK; diluted 1:1000), were carried out overnight at 4°C after blocking of nonspecific binding with 5% skimmed milk for 60 minutes.

For MDM2 immunohistochemistry, the sections were boiled in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 minutes in a steam cooker, subsequently incubated in 5% skimmed milk for 1 hour at room temperature, then incubated overnight at 4°C with the monoclonal antibody to MDM2 (clone IF2; Oncogene Research Products, Cambridge, MA; diluted at 1:2000).

For EGFR immunohistochemistry, sections were pretreated with 0.1% trypsin in 0.1% $CaCl_2$ (pH 7.8) for 15 minutes at 37°C and then incubated in 5% skimmed milk for 60 minutes. Sections were then reacted overnight at 4°C with EGFR monoclonal antibody (NCL-EGFR; Novocastra Laboratories, Newcastle, UK), which recognizes the EGFR ligand binding domain (dilution 1:100).

For RB immunohistochemistry, the sections were boiled three times for 5 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven. The sections were allowed to cool at room temperature. Sections were incubated overnight at 4°C with the RB monoclonal antibody (clone G3-245; PharMingen, San Diego, CA; diluted 1:100), which recognizes an epitope between amino acids 300 and 380 and both phosphorylated and underphosphorylated RB protein.

For c-MET immunohistochemistry, the sections were boiled for 10 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) in a steam cooker and subsequently incubated in 5% skimmed milk overnight at room temperature. Sections were then incubated for 1 hour at room temperature with the c-MET monoclonal antibody (NCL-cMET, diluted 1:100; Novocastra Laboratories).

The reaction was visualized using the Vectastain ABC Kit and diaminobenzidine (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. Fractions of positive cells were recorded as follows: −, negative; +, <5%; ++, 5–50%; +++, >50%.

Results

p53 Mutations and p53 Protein Accumulation

Miscoding *p53* mutations were found in five of 19 (26%) gliosarcomas analyzed (Table 1). One tumor (case 211) contained a silent mutation (GTG→GTA, Val→Val) in codon 173. In case 210 (Table 1), the first biopsy showed the missense mutation CCC→TCT in codon 190; the second biopsy contained a different mutation, CCC→TCC, in codon 151, which was present in both the gliomatous and sarcomatous components (Table 2). In all cases, the wild-type base was present along with the mutated base. In case 216, the mutation was present only in the third biopsy but not in the first and second biopsies. Of five gliosarcomas with a *p53* mutation, four showed nuclear accumulation of p53 protein in a variable fraction of neoplastic glial and mesenchymal cells (Table 1). One gliosarcoma (case 207) contained a 2-bp insertion mutation, resulting in a stop codon, and did not show p53 immunoreactivity (Table 1).

PTEN Mutations

SSCP followed by direct DNA sequencing revealed that seven of 19 (37%) gliosarcomas contained a *PTEN* mu-

Table 2. Genetic Alterations in Sarcomatous and Gliomatous Components of Gliosarcomas

Patient no.	Biopsy	Area	Genetic alteration
210	2nd	S	p53 mutation (codon 151, CCC→TCC)
		G	p53 mutation (codon 151, CCC→TCC)
213	1st	S	p16 deletion
		G	—
214	1st	S	p16 deletion
		G	p16 deletion
215	1st	S	PTEN mutation (codon 107, GAT→GCT)
		G	PTEN mutation (codon 107, GAT→GCT)
218	1st	S	PTEN mutation (codon 274, TGG→TGA)
		G	PTEN mutation (codon 274, TGG→TGA)
220	1st	S	CDK4 amplification, MDM2 amplification
		G	CDK4 amplification, MDM2 amplification
223	1st	S	—
		G	—

S, sarcomatous component; G, gliomatous component; —, no genetic alterations.

tation (Table 1). Of these, three mutations were in exon 5 (phosphatase domain), two were in exon 8, and one each were in exons 1 and 2. Three were nonsense mutations leading to a truncated protein, and four were missense mutations. In all cases, the wild-type base was also detectable. In case 212, the same mutation was present in both primary and second biopsies. In cases 215 and 218, identical mutations were detected in gliomatous and sarcomatous areas (Tables 1 and 2 and Figure 2).

p16 Deletion, CDK4 Amplification, and RB Expression

In seven cases (37%), differential PCR revealed a homozygous p16 deletion (Table 1). In one tumor (case

214), the p16 deletion was detected in both gliomatous and sarcomatous areas (Tables 1 and 2 and Figure 3). In another case (case 221), a p16 deletion was detected in the first (glioblastoma) and second (gliosarcoma) biopsies. Differential PCR further revealed amplification of CDK4 in one gliosarcoma (case 220, Table 1), again in both gliomatous and sarcomatous areas (Figure 3).

Strong immunoreactivity to RB was observed in 5–50% of neoplastic cells in 10 cases and in more than 50% of neoplastic cells in six cases. In one case (case 210, Table 1) RB expression was not detectable.

A cell cycle-related gene alteration (p16 deletion, CDK4 amplification, or loss of RB expression) was found in 10 of 19 (53%) gliosarcomas, but these were mutually exclusive, ie, no biopsy contained more than one of these alterations.
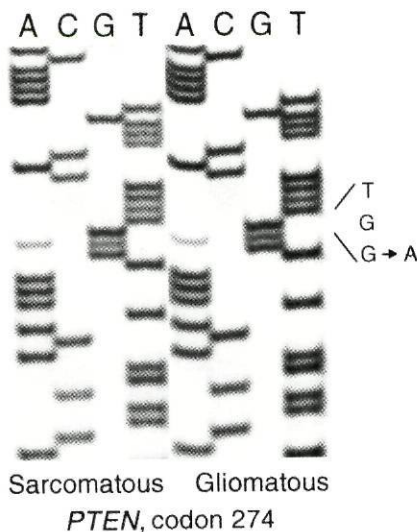

Figure 2. Sequencing gels showing an identical PTEN mutation in codon 274 (TGG→TGA, Trp→Stop) in microdissected sarcomatous and gliomatous areas of a gliosarcoma (case 218).
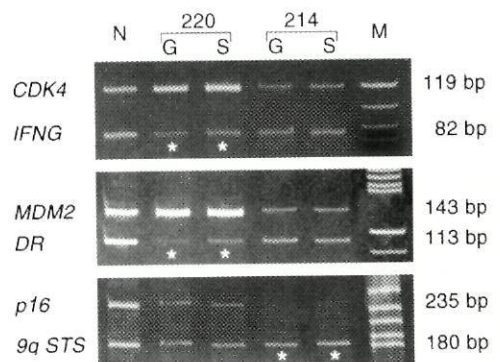

Figure 3. Differential PCR assay for p16 homozygous deletion and CDK4 and MDM2 amplification. Both gliomatous (G) and sarcomatous (S) areas in case 220 show a significantly increased signal intensity of the CDK4 and MDM2 bands (*) when compared to the respective reference sequence (IFNG, DR), suggesting a coamplification of these genes. In case 214 (bottom), both tumor components show a reduced p16 signal (*) when compared to the reference sequence (9q STS), suggesting a homozygous p16 deletion. N, normal DNA; M, molecular size marker.

Table 3. Clinical and Genetic Data of Glioblastoma Subtypes

	Primary glioblastoma	Gliosarcoma	Giant cell glioblastoma	Secondary glioblastoma
Clinical onset	De novo	De novo	De novo	Secondary
Preoperative clinical history	1.7 months[3]	3 months*	1.6 months[5]	53 m. from low-grade astrocytoma[3]
				25 m. from anaplastic astrocytoma[3]
Sex ratio (M/F)	1.4[3]	1.4*	1.2[6,44]	0.8[3]
		1.8[11]		
Age of diagnosis	56[3,46]	56*	44[0,44]	40[3,46]
		53[11]		
p53 mutation	2/19 (11%)[3]	8/35 (23%)*[12,43]	31/37 (84%)[6,44]	20/30 (67%)[3]
PTEN mutation	9/28 (32%)[5]	8/21 (38%)*[45]	6/22 (27%)[6,45]	1/25 (4%)[5]
p16 deletion	10/28 (36%)[4]	7/19 (37%)*	1/37 (3%)[6,44]	1/23 (4%)[4]
MDM2 amplification	2/29 (7%)[17]	1/19 (5%)*	0/18 (0%)[6]	0/27 (0%)[17]
EGFR amplification	11/28 (39%)[5]	1/22 (4%)*[42]	2/37 (5%)[6,44]	0/22 (0%)[5]
CDK4 amplification	1/28 (4%)[4]	2/19 (10%)*	1/19 (5%)[44]	3/23 (13%)[4]

*This study.
Superscripts are numbers from the list of references at the end of this paper.

Amplification and Overexpression of the MDM2, EGFR, and c-MET Genes

MDM2 amplification was detected by differential PCR in one biopsy (case 220), which also showed CDK4 amplification (Tables 1 and 2 and Figure 3). In this tumor, MDM2 overexpression was detected immunohistochemically in more than 50% of neoplastic cell nuclei of both gliomatous and sarcomatous areas.

Differential PCR did not reveal EGFR amplification in any of the 19 cases, and EGFR overexpression was also absent immunohistochemically (Table 1).

c-MET immunoreactivity presented as strong cytoplasmic and plasma membrane staining in glioma cells in gliomatous areas, but not in sarcomatous areas in all gliosarcomas analyzed.

Discussion

The term gliosarcoma was introduced in 1898 by Stroebe.[7] Fifty years later, Feigin et al[19] defined it as a glioblastoma subtype in which proliferating vessels had acquired the features of a sarcoma. Some studies showed expression of monohistiocytic markers, suggesting that gliosarcomas develop from histiocytes, whereas others suggested an origin from fibroblasts, pluripotent mesenchymal cells of the perivascular adventitia or perivascular spaces.[20,21] The expression of α-smooth muscle actin in sarcomatous portions pointed to vascular smooth muscle as the potential origin of the mesenchymal tissue component.[22] More recent investigations suggested a common origin of for the two tissue components; the sarcomatous areas result from advanced glioma progression with acquisition of a mesenchymal phenotype.[23,24] This view is strongly supported by genetic analyses, including the present study. Using interphase cytogenetic analysis, Paulus et al[25] detected similar cytogenetical abnormalities in the gliomatous and sarcomatous components of two gliosarcomas, but Biernat et al[12] were the first to prove a monoclonal origin by demonstrating the presence of identical p53 mutations in the two tumor areas. Similar genetic alterations in both tumor components

were subsequently reported by Boerman et al,[13] using comparative genomic hybridization (CGH), cytogenetic analysis, fluorescence in situ hybridization, and microsatellite analysis.

The present study extends these findings to a variety of other gene alterations (Table 2). We detected in gliomatous and sarcomatous tumor areas identical PTEN mutations (two cases), a p53 mutation (one case), homozygous p16 deletion (one case), and coamplification of MDM2 and CDK4 (one case). In one biopsy (case 213), only the sarcomatous area showed an unequivocal p16 deletion, whereas in the gliomatous portion the p16/sts ratio was 0.31 and thus did not reach the criterion of p16 deletion. This may be due to an admixture of DNA from nonneoplastic neural tissue. Taken together, these data firmly establish the gliosarcoma as a monoclonal tumor with focal aberrant mesenchymal differentiation. Identical genetic alterations have also been detected in both the carcinomatous and sarcomatous components of uterine carcinosarcomas,[26] and in epithelial and stromal components of pulmonary carcinosarcomas.[27]

In this as well as in previous studies,[11] gliosarcoma typically developed in older patients (Table 3) and was diagnosed at first biopsy after a short clinical history, suggesting that these tumors developed de novo, ie. without an identifiable, less malignant precursor lesion. Occasionally (case 221) the histological features of gliosarcoma appeared in the second biopsy of a primary glioblastoma. Perry et al[28] reported that 25 of 32 cases (78%) were diagnosed as gliosarcoma in the first biopsy, whereas seven (22%) developed after irradiation for glioblastoma. Rarely, gliosarcomas develop through progression from low-grade[25] or anaplastic astrocytoma (Ref. 29 and case 216 of this study).

The present study shows that gliosarcomas contain some genetic alterations similar to those typically encountered in primary glioblastomas, ie. frequent p16 deletions (37%) and PTEN mutations (37%) (Table 3). The unexpected and most striking difference is the complete absence of amplification or overexpression of the EGFR gene, a hallmark of primary (de novo) glioblastomas.[2,3,5] The absence of EGFR amplification in gliosarcomas may

affect their biological behavior, but large clinical trials showed no significant difference in prognosis between gliosarcomas and ordinary glioblastomas.[9,28] The reason for selective aberrant mesenchymal differentiation in a subset of glioblastomas without EGFR amplification remains to be elucidated.

The most common changes in gliosarcomas detected in cytogenetic studies included gains of chromosome 7 (EGFR gene locus) and loss of chromosome 10, followed by deletions of the long arm of chromosomes 13 and 9.[13,30-32] The absence of EGFR amplification in this study suggests that other protooncogenes on chromosome 7 may be involved in the evolution of this glioblastoma variant. CDK6, PDGF-A and c-MET genes on chromosome 7 have been reported to be amplified or overexpressed in malignant gliomas.[33-36] c-MET immunohistochemistry in these study shows that c-MET is overexpressed in gliomatous but not in sarcomatous components in gliosarcomas. It remains to be clarified whether amplification of CDK6, PDGF-A genes is involved in the development of gliosarcomas.

The frequency of p53 mutations in gliosarcomas was 26% and thus was significantly lower than in secondary glioblastomas (67%, $P = 0.0086$) but somewhat higher than in our cohort of patients with primary glioblastomas, but the difference was not significant (26 versus 11%, $P = 0.405$).[3]

The 12q13-14 chromosomal region contains several genes (MDM2, CDK4, sarcoma amplified sequence SAS and GLI) that have been reported to be coamplified in sarcomas[37] and glioblastomas.[38] In this study, one gliosarcoma (case 220) showed coamplification of MDM2 and CDK4 in gliomatous and sarcomatous tumor areas.

The progression of cells from G1 to S phase is regulated by cyclin-dependent kinases (CDKs), their inhibitors, and the retinoblastoma protein (pRB). In a simplified model, p16 protein binds to CDK4 and inhibits the formation of CDK4/cyclin D complex. When activated, this complex phosphorylates the RB protein, thereby inducing the release of the E2F transcription factor, which in turn activates genes involved in the late G1 and S phases.[39,40] Homozygous p16 deletion, CDK4 amplification, and loss of RB expression are frequent in glioblastomas.[4,41,42] In this study, approximately one-half of gliosarcomas showed aberrant expression in one of these genes (Table 1). The frequency of p16 homozygous deletion in seven (37%) gliosarcomas is similar to that in primary glioblastomas.[4] Our finding that in gliosarcomas homozygous p16 deletion, CDK4 amplification, and loss of RB expression were mutually exclusive corresponds to similar observations in other glioblastomas[4,15,42] and indicates that altered expression of any of these genes may lead to loss of cell cycle control.

In conclusion, gliosarcomas exhibit clinical features and a genetic profile similar to those of primary (de novo) glioblastomas, ie, advanced patient age, short clinical history, and frequent p16 deletions and PTEN mutations. The unexpected and most striking difference is the absence of amplification/overexpression of the EGFR gene, a genetic hallmark of primary glioblastomas. The presence of identical genetic alterations in both gliomatous and sarcomatous components strongly supports the concept of a monoclonal origin of gliosarcomas.

Acknowledgments

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