Short Communication

Genetic Profile of Gliosarcomas

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There are distinct genetic pathways leading to the glioblastoma, the most malignant astrocytic brain tumor. Primary (de novo) glioblastomas develop in older patients and are characterized by epidermal growth factor (EGF) receptor amplification/overexpression, p16 deletion, and PTEN mutations, whereas secondary glioblastomas that progressed from low-grade or anaplastic astrocytoma develop in younger patients and frequently contain p53 mutations. In this study, we assessed the genetic profile of gliosarcoma, a rare glioblastoma variant characterized by a biphasic tissue pattern with alternating areas 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 detected 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. Coamplification of CDK4 and MDM2 was detected in one gliosarcoma. None of the gliosarcomas showed amplification 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 EGF amplification/overexpression. Identical PTEN mutations in the gliomatous and sarcomatous 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 frequent and malignant brain tumor, may arise de novo after a short clinical history without an identifiable less malignant precursor lesion. This type of glioblastoma has been designated primary glioblastoma. Secondary glioblastomas 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 subtypes develop through different genetic pathways. Primary glioblastomas are characterized by epidermal growth factor receptor (EGFR) amplification/overexpression, p16 deletion, and PTEN (MMAC1) mutations. Secondary glioblastomas typically contain p53 mutations but rarely have EGFR amplification/overexpression, p16 deletion, 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 mutations. They have in common with secondary glioblastomas 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 Strobe et al.7 Gliosarcomas comprise approximately 2% of all glioblastomas8,9 and are characterized by a biphasic tissue pattern, with areas displaying glial and mesenchymal differentiation.10,11 Whereas morphological studies suggested an evolution of the sarcomatous component from microvascular proliferations within a highly malignant glioblastoma, two recent genetic studies revealed
the presence of identical p53 mutations and similar chromosomal imbalances and cytogenetic alterations 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. 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. Mutations in exon 5–8 of the p53 gene were screened using polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) as previously described. 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.

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. Samples that showed a mobility shift in the SSCP analysis were further analyzed by direct DNA sequencing as previously described. 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 AGA GTA AAG AGG AGC AGG-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 ACQ AA-3’ (sense) and 5’-TAT CAT TAC ACC AGT TCG TC-3’ (antisense) for exon 5; 5’-TTT TTT TTT TGT AGG
PCR data (in bold) indicate amplification or deletion (see Materials and Methods).

<table>
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<th>Patient no.</th>
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<th>Biopsy/ diagnosis</th>
<th>Location</th>
<th>Area</th>
<th>p53 miscoding mutation</th>
<th>PTEN miscoding mutation</th>
<th>p16/145 ratio</th>
<th>CDK4/ IFNG ratio</th>
<th>MDM2/ DR ratio</th>
<th>EGFR/ CF ratio</th>
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<td>—</td>
<td>0.38</td>
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<td>S</td>
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<td>Exon 5-codon 111 (TGG→TAG, Trp→stop)</td>
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<td>S</td>
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<td>0.75</td>
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<td>—</td>
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<td>1st/GS</td>
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<td>S</td>
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<td>Exon 2-codon 48 (AAC→GAC, Asn→Asp)</td>
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<td>3.10</td>
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<td>1.08</td>
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<tr>
<td>223</td>
<td>58/F</td>
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<td>S</td>
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<td>—</td>
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<td>0.71</td>
<td>0.74</td>
<td>1.13</td>
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</table>

| *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). |
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. 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. Two primary glioblastomas, which showed a ratio of less than 0.2 in the previous study, served as positive controls for p16 deletion.

To detect CDK4 amplification, differential PCR was carried out as described previously. Interferon γ (IFN-γ) 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/IFN-γ ratio was regarded as positive for CDK4 amplification. This value was calculated according to the method of Rollbrocker et al. One primary glioblastoma, which showed a ratio higher than 2.7 in a previous study, served as the positive control for CDK4 amplification.

MDM2 amplification was detected by differential PCR analysis as previously described. 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, 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, with some modifications. 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. One primary glioblastoma, which showed EGFR amplification in a previous study, 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 3% H2O2 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% CaCl2 (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 (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 (clone G3-245; Pharmingen, San Diego, CA; 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; +, 5–50%; ++, 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 immunoactivity (Table 1).

PTEN Mutations

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Biopsy Area</th>
<th>Genetic alteration</th>
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<tbody>
<tr>
<td>210</td>
<td>2nd S</td>
<td>p53 mutation (codon 151, CCC→TCC)</td>
</tr>
<tr>
<td>213</td>
<td>1st G</td>
<td>p53 mutation (codon 151, CCC→TCC)</td>
</tr>
<tr>
<td>214</td>
<td>1st G</td>
<td>p16 deletion</td>
</tr>
<tr>
<td>215</td>
<td>1st S</td>
<td>p16 deletion</td>
</tr>
<tr>
<td>218</td>
<td>1st G</td>
<td>PTEN mutation (codon 107, GAT→GCT)</td>
</tr>
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<td>1st S</td>
<td>PTEN mutation (codon 274, TGG→TGA)</td>
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<td>1st G</td>
<td>PTEN mutation (codon 274, TGG→TGA)</td>
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<tr>
<td></td>
<td></td>
<td>CDK4 amplification, MDM2 amplification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDK4 amplification, MDM2 amplification</td>
</tr>
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S, sarcomatous component; G, gliomatous component; —, no genetic alterations.

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

![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).](image)

**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 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.
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 Stroebel. Seventy years later, Feigin et al defined it as a glioblastoma subtype in which proliferating vessels had acquired the features of a sarcoma. Some studies showed expression of mononuclear markers, suggesting that gliosarcomas develop from histiocytes, whereas others suggested an origin from fibroblasts, pluripotent mesenchymal cells of the perivascular adventitia or perivascular spaces.

The expression of α-smooth muscle actin in sarcomatous portions pointed to vascular smooth muscle as the potential origin of the mesenchymal tissue component. More recent investigations suggested a common origin for the two tissue components; the sarcomatous areas result from advanced glioma progression with acquisition of a mesenchymal phenotype. This view is strongly supported by genetic analyses, including the present study. Using interphase cytogenetic analysis, Paulus et al detected similar cytogenetical abnormalities in the gliomatous and sarcomatous components of two gliosarcomas, but Biernat et al 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, 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 p16/65 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 and in epithelial and stromal components of pulmonary carcinosarcomas.

In this as well as in previous studies, 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 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 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. 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.\textsuperscript{9,28} The reason for selective aberrant mesenchymal differentiation in a subset of glioblastomas without \textit{EGFR} amplification remains to be elucidated.

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

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

The 12q13–14 chromosomal region contains several genes (\textit{MDM2, CDK4, sarcoma amplified sequence SAS, and GLI}) that have been reported to be coamplified in sarcomas\textsuperscript{37} and glioblastomas.\textsuperscript{38} In this study, one gliosarcoma (case 220) showed coamplification of \textit{MDM2} and \textit{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.\textsuperscript{39,40} Homozygous \textit{p16} deletion, \textit{CDK4} amplification, and loss of RB expression are frequent in glioblastomas.\textsuperscript{4,11,42} In this study, approximately one-half of gliosarcomas showed aberrant expression in one of these genes (Table 1). The frequency of \textit{p16} homozygous deletion in seven (37\%) gliosarcomas is similar to that in primary glioblastomas.\textsuperscript{4} Our finding that in gliosarcomas homozygous \textit{p16} deletion, \textit{CDK4} amplification, and loss of RB expression were mutually exclusive corresponds to similar observations in other glioblastomas\textsuperscript{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 \textit{p16} deletions and \textit{PTEN} mutations. The unexpected and most striking difference is the absence of amplification/overexpression of the \textit{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.

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