RESEARCH ARTICLE

Genetic Alterations in Gliosarcoma and Giant Cell Glioblastoma

Ji Eun Oh[1]; Takashi Ohta[1,2]; Naosuke Nonoguchi[1,3]; Kaishi Satomi[1]; David Capper[4];
Daniela Pierscianek[1,5]; Ulrich Sure[5]; Anne Vital[6]; Werner Paulus[7]; Michel Mittelbronn[8];
Manila Antonelli[9]; Paul Kleihues[10]; Felice Giangaspero[9,11]; Hiroko Ohgaki[1]

[1] Section of Molecular Pathology, International Agency for Research on Cancer, F-69372 Lyon, France.

[2] Department of Neurological Surgery, Nihon University School of Medicine, Tokyo, Japan.

[3] Department of Neurosurgery, Osaka Medical College, Takatsuki, Japan.

[4] Department of Neuropathology, Institute of Pathology, University of Heidelberg, and CCU Neuropathology, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany.

[5] Department of Neurosurgery, University Hospital Essen, Essen, Germany.

[6] Bordeaux Institute of Neuroscience, CNRS UMR 5293, F-33076 Bordeaux, France.

[7] Institute of Neuropathology, University Hospital Munster, D-48149 Munster, Germany.

[8] Institute of Neurology (Edinger Institute), Goethe-University, Frankfurt/Main, Germany.

[9] Department of Radiology, Oncology and Anatomic Pathology, Sapienza University of Rome, I-00161, Rome, Italy.

[10] Medical Faculty, University of Zurich, CH-8091 Zurich, Switzerland.

[11] IRCCS Neuromed, I-86077, Pozzilli, Italy.

Abstract

The majority of glioblastomas develop rapidly with a short clinical history (primary glioblastoma *IDH* wild-type), whereas secondary glioblastomas progress from diffuse astrocytoma or anaplastic astrocytoma. *IDH* mutations are the genetic hallmark of secondary glioblastomas. Gliosarcomas and giant cell glioblastomas are rare histological glioblastoma variants, which usually develop rapidly. We determined the genetic patterns of 36 gliosarcomas and 19 giant cell glioblastomas. *IDH1* and *IDH2* mutations were absent in all 36 gliosarcomas and in 18 of 19 giant cell glioblastomas analyzed, indicating that they are histological variants of primary glioblastoma. Furthermore, LOH 10q (88%) and *TERT* promoter mutations (83%) were frequent in gliosarcomas. Copy number profiling using the 450k methylome array in 5 gliosarcomas revealed *CDKN2A* homozygous deletion (3 cases), trisomy chromosome 7 (2 cases), and monosomy chromosome 10 (2 cases). Giant cell glioblastomas had LOH 10q in 50% and LOH 19q in 42% of cases. ATRX loss was detected immunohistochemically in 19% of giant cell glioblastomas, but absent in 17 gliosarcomas. These and previous results suggest that gliosarcomas are a variant of, and genetically similar to, primary glioblastomas, except for a lack of *EGFR* amplification, while giant cell glioblastoma occupies a hybrid position between primary and secondary glioblastomas.

INTRODUCTION

Glioblastoma (WHO grade IV) is the most frequent and malignant glioma. The majority of glioblastomas (>90%) develop rapidly after a short clinical history, without evidence of a less malignant precursor lesion (primary glioblastoma). In contrast, secondary glioblastomas develop through progression from diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III) (24, 27, 28). The decisive genetic alterations that reliably distinguish between primary and secondary glioblastoma are *IDH* mutations (3, 24, 28, 35, 37) which constitute a reliable genetic marker of secondary glioblastoma. In a large population-based study, *IDH1* mutation status corresponded to the respective clinical diagnosis in 95% of cases (24, 28).

Gliosarcoma and giant cell glioblastoma are rare histological variants of glioblastoma (16). On clinical grounds, they are considered variants of primary glioblastoma, but genetic data that would allow an unambiguous classification are still scant. Gliosarcomas constitute approximately 2% of all glioblastomas (16), and are characterized by a biphasic tissue pattern with alternating areas displaying glial or mesenchymal differentiation (16). Despite these two distinct histological components, gliosarcomas are considered monoclonal, since glial and mesenchymal tumor areas were usually found to be genetically similar (4, 31). It has been reported that *PTEN* mutations (37%–45%) and *p16^{INK4a}* homozygous deletion (37%) are common, *TP53* mutations (24%–26%) less frequent, and *EGFR* amplification rare or absent (0%–8%) (1, 31).

Giant cell glioblastomas constitute up to 5% of all glioblastomas, and are characterized by a predominance of bizarre, multinucleated giant cells with an occasionally abundant reticulin network (16). *TP53* mutations are frequent (78%–90%) and *PTEN* mutations are common (33%), but *EGFR* amplification and $p16^{INK4a}$ homozygous deletion have been reported to be rare (17, 18, 30).

The objective of the present study was to further genetically characterize these glioblastoma variants. We screened 36 gliosarcomas and 19 giant cell glioblastomas for *IDH1* and *IDH2* mutations (genetic hallmarks of secondary glioblastoma), and various additional genetic alterations known to be operative in diffuse gliomas.

MATERIALS AND METHODS

Tumor samples

Formalin-fixed and paraffin embedded tissue samples of 36 gliosarcomas were obtained from the Neurological Institute (Edinger Institute), University Hospital Frankfurt, Germany, the Institute of Neuropathology, University Hospital Munster, Germany, the Department of Neuropathology, University Hospital Rome, Italy, the Institute of Neuroscience, Bordeaux, France, and the Department of Neuropathology, University Hospital Zurich, Switzerland. Formalin-fixed tissue samples of 19 giant cell glioblastomas, as well as a frozen sample of a giant cell glioblastoma were obtained from the Department of Neuropathology, University Hospital Zurich, Switzerland, the Institute of Neuropathology, University Hospital Munster, Germany, and the Departments of Neuropathology and Neurosurgery, University Hospital Essen, Germany.

Gliosarcomas and giant cell glioblastomas were diagnosed according to the 2007 WHO Classification (16). Histologically, gliosarcomas showed the typical biphasic pattern with alternating areas of glial and mesenchymal differentiation (Figure 1A). The glial area was composed of anaplastic glial cells with GFAP expression, and the mesenchymal component demonstrated bundles of spindle cells with malignant transformation and abundant connective tissue stained by reticulin, without GFAP expression (Figure 1B). For selection of giant cell glioblastomas, care was taken to include only typical cases showing a predominance of multinucleated, GFAP positive tumor cells in at least one large area of the biopsy specimen. All gliosarcomas and 18 giant cell glioblastomas were located in cerebral hemispheres; one giant cell glioblastoma was in the thalamus. This study was approved by the International Agency for Research on Cancer Ethics Committee.

DNA extraction

Genomic DNA was extracted from typical tumor areas that were scraped from formalin-fixed and paraffin-embedded tissue slides or cryostat section from a frozen sample as previously described (23, 29). DNA concentration and purity were measured by a ND8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

IDH mutations

Screenings for the *IDH1* and *IDH2* mutations were performed in 36 gliosarcomas and 19 giant cell glioblastomas by Sanger sequencing as described previously (24, 35).

TERT promoter mutations

Sequences covering the mutational hotspots (C228T and C250T) in the *TERT* core promoter were amplified by nested PCR. Primer sequences and detailed protocols were reported previously (25). We considered as *TERT* mutations only cases when the height of the mutated allele was >20% of that of the wild-type allele on sequencing data, as previously described (25).

LOH 1p, 19q, 10q

Quantitative PCR was performed to assess LOH 1p, 19q, and 10q in 17 gliosarcomas and 12 giant cell glioblastomas. Microsatellite markers were D1S214, D1S468, and D1S2736 at 1p36.32-1p36.22, D19S408, D19S596, and D19S867 at 19q13.31-19q13.33, D10S536 at 10q23.3, and D10S1683 at 10q24.2 (22, 26). We interpreted as LOH when 2 or 3 markers for each chromosome suggested loss, as described previously (22, 26).

ATRX immunohistochemistry

ATRX immunohistochemistry was carried out in 17 gliosarcomas and in 16 giant cell glioblastomas. Paraffin sections were deparaffinized in xylene and 95% ethanol for 5 min. After inactivation of endogenous peroxidases with 3% of H_2O_2 in methanol for 30 min, sections were incubated in epitope retrieval solution (diluted 1:10, H-3300, Low pH 6.0, Vector Laboratories, inc. Burlingame, CA, USA) for 20 min at 95°C–99°C. Sections were cooled for 40 min at room temperature, and were incubated with anti-ATRX antibody (1:400, HPA001906, rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, USA), diluted in antibody diluent (S3022, Dako, Les Ulis, France) at 4°C overnight. Sections were then washed in phosphate buffered saline (PBS) and incubated with the second antibody (EnVision+Single Reagents HRP rabbit, K4002, Dako, Les Ulis, France) for 30 min. Visualization was performed using Vector DAB Substrate Kit (SK-4100; Vector Laboratories) for 4 min. After washing in PBS, sections were counterstained with hematoxylin.

For optimization of immunohistochemistry protocols, normal human brain tissue was used as a positive control, and human liver tissue was used as a negative control. For interpretation of ATRX immunoreactivity, vascular endothelial cells within tumor tissues on histological slides were used as internal positive controls. Cases with more than 10% immunoreactive tumor cells were considered as positive as previously described (36).

Methylome and copy number profiling

The Illumina Infinium HumanMethylation450 (450k) array was used in 5 gliosarcomas, for which sufficient DNA from paraffin sections were available, to obtain the DNA methylation status of 482,421 CpG sites (Illumina, San Diego, CA, USA), according to the manufacturer's instructions at the Core Facility of the DKFZ. The methylation level of each CpG site was represented by beta-values, which ranged from 0 (unmethylated) to 1 (methylated). Genome-wide copy-number profiles were calculated using the intensity measures of the aforementioned methylation probe loci throughout the genome. Chromosomal copy-number alterations were visualized by the IdeogramBrowser tool (19).
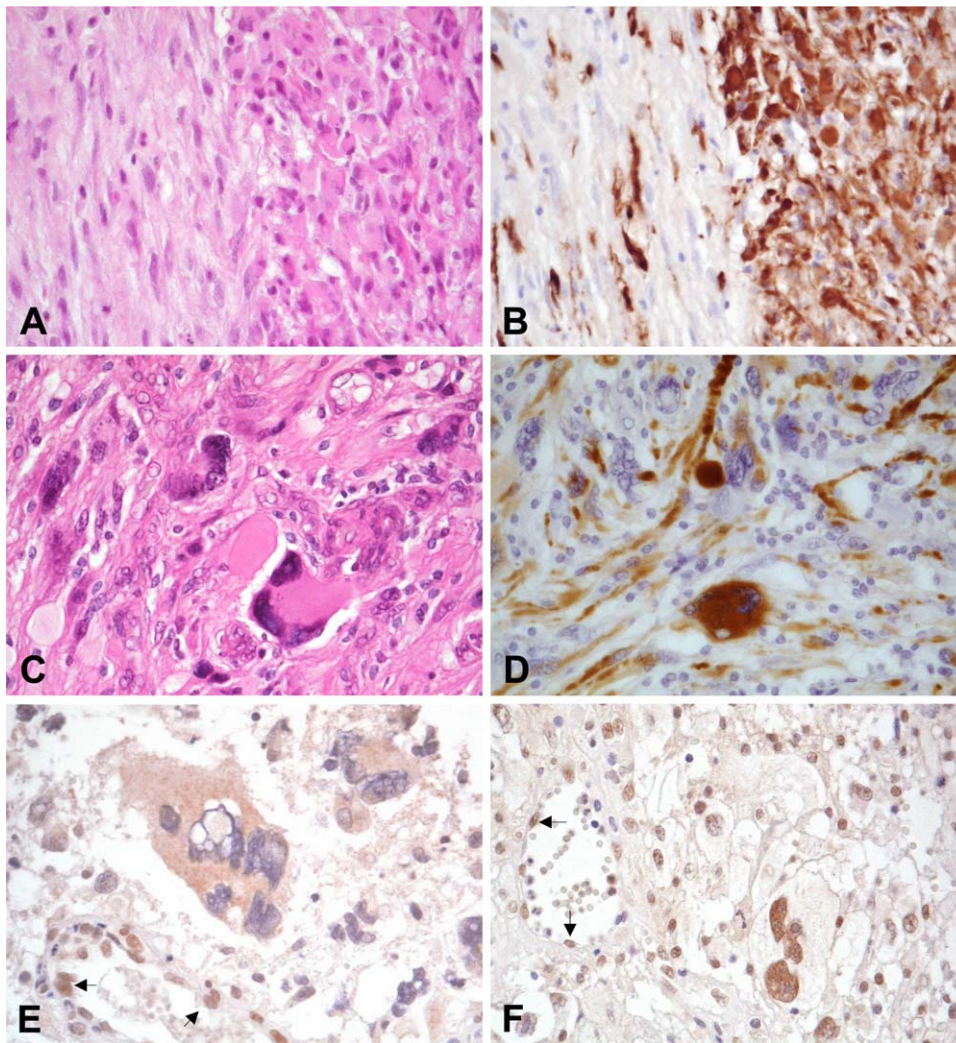
Figure 1. Gliosarcomas showing the typical biphasic pattern with alternating areas of glial and mesenchymal differentiation (**A**). Note that the glial area was composed of anaplastic glial cells with GFAP expression, while mesenchymal component lacks GFAP expression (**B**). Giant cell glioblastoma with multinucleated giant cells (**C**). GFAP is expressed at different levels in multinucleated giant cells in giant cell glioblastoma (**D**). Multinucleated giant cells with loss of nuclear ATRX expression in giant cell glioblastoma (**E**). Another giant cell glioblastoma showing nuclear immunoreactivity to ATRX (**F**). Arrows indicate nuclear ATRX expression in endothelium served as an internal positive control.

Statistical analysis

Fisher exact test was performed to evaluate the significance of difference in various genetic alterations. The significance level chosen was $P < 0.05$. Statistical analyses were carried out using Stat View J-5.0 software (Abacus Concepts, Berkeley, CA, USA).

RESULTS

IDH mutations

IDH1 mutations were absent in all 36 gliosarcomas and in 18 out of 19 giant cell glioblastomas analyzed. One giant cell glioblastoma with an *IDH1* (R132S) mutation was diagnosed in a 44 year-old male patient, who developed anaplastic astrocytoma one year ear-lier. *TP53* mutation (R280T) was also detected in both anaplastic astrocytoma and giant cell glioblastoma. *IDH2* mutations were absent in all gliosarcomas and giant cell glioblastomas analyzed.

TERT promoter mutations

TERT promoter mutations were detected in 30 of 36 (83%) gliosarcomas. Of these, 76% were C228T and 24% were C250T. This ratio was similar to that reported in primary glioblastomas (25).

For the 20 selected gliosarcomas, we carried out screening for *TERT* promoter mutations separately in glial and mesenchymal components, and found identical results in 19 cases. In one gliosarcoma, *TERT* mutation was observed in only the mesenchymal component, but not in the glial component.

Table 1. Clinical and genetic profile of the gliosarcoma and the giant cell glioblastoma, in comparison with primary and secondary glioblastoma.

	Primary glioblastoma (*IDH* wild-type)	Gliosarcoma	Giant cell glioblastoma	Secondary glioblastoma (*IDH* mutant)
Age at GBM diagnosis	59 years[1,2,3,4]	56 years[5]	44 years[6,7]	43 years[1,2,3,4]
Sex ratio M/F	1.4[1,2]	1.4[5]	1.6[6]	1.0[1,2]
Clinical history	3.9 months[1]	3.0 months[5]	1.6 months[6]	15.2 months[1]
IDH1/2 mutation	0%[1,8,9]	0%*	5%*	100%[1,8,9]
PTEN mutation	24%[1,8,9]	41%[5,10]	33%[6]	5%[1,8,9]
ATRX expression loss	0%[11]	0%*	19%*	100%[11]
TERT mutation	72%[12,13,14]	83%*	25%*	26%[12,13,14]
TP53 mutation	23%[1,8,9]	25%[5,10]	84%[6,7,15]	74%[1,8,9]
LOH 19q	4%[1]	18%*	42%*	32%[1]
EGFR amplification	42%[1,8,9]	5%[5,10]	6%[6,7,15]	4%[1,8,9]
p16INK4a deletion	45%[1,8,9]	37%[5]	3%[6,7]	30%[1,8,9]
LOH 1p	15%[1]	6%*	17%*	24%[1]
LOH 1p/19q	2%[1]	6%*	17%*	11%[1]
LOH 10q	67%[1]	88%*	50%*	73%[1]

*This study.
[1]Nobusawa *et al* (24).
[2]Bleeker *et al* (5).
[3]Ichimura *et al* (12).
[4]Reuss *et al* (32).
[5]Reis *et al* (31).
[6]Peraud *et al* (30).
[7]Meyer-Puttlitz *et al* (18).
[8]Gao *et al* (11).
[9]Cerami *et al* (9).
[10]Actor *et al* (1).
[11]Liu *et al* (14).
[12]Nonoguchi *et al* (25).
[13]Eckel-Passow *et al* (10).
[14]Brennan *et al* (8).
[15]Martinez *et al* (17).

    Typical for primary glioblastomas
    Typical for secondary glioblastomas

    TERT promoter mutations (C228T) were found in 4 of 16 (25%) giant cell glioblastomas.

LOH 1p, 19q, 10q

Quantitative PCR revealed LOH 1p in 1/17 (6%) gliosarcomas and in 2 of 12 (17%) giant cell glioblastomas. LOH 19q was detected in 3 gliosarcomas (3/17; 18%) and 5 giant cell glioblastomas (5/12; 42%). LOH 10q was detected in 15/17 gliosarcomas (88%) and in 6 of 12 (50%) giant cell glioblastomas. Co-deletion of 1p/19q was detected in 1/17 (6%) gliosarcomas and in 2/12 (17%) giant cell glioblastomas.

ATRX immunohistochemistry

Loss of nuclear ATRX expression was observed in 3 of 16 (19%) giant cell glioblastoma, but in none of the 17 gliosarcomas analyzed (Figure 1).

    ATRX was expressed in vascular endothelial cells (internal positive control) in all histological sections. Sections without ATRX antibodies showed no immunoreactivity.

Copy number profiling

Copy number plots generated from 450k methylation data in 5 gliosarcomas revealed *CDKN2A* homozygous deletion (3 cases), monosomy chromosome 10 (2 cases), trisomy chromosome 7 (2 cases), gain at chromosome 7 (one case), and *CDK4* amplification (one case).

Comparison of genetic alterations with primary and secondary glioblastomas

Genetic alterations of the gliosarcomas and giant cell glioblastomas which were obtained in the present study as well as those reported previously are summarized in Table 1. These data were compared with those of the primary glioblastomas (*IDH*-wild-type) and

secondary glioblastomas (*IDH* mut) reported previously (Table 1) (24, 25).

PTEN mutations in gliosarcomas (41%) were significantly more frequent than those in primary glioblastomas (24%; *P* = 0.0190) and in secondary glioblastomas (5%; *P* = 0.0001). Loss of ATRX expression was less frequent in gliosarcomas than secondary glioblastomas (*P* = 0.0009). *TERT* promoter mutations in gliosarcomas were more frequent (83%) than those in secondary glioblastomas (26%; *P* < 0.0001). LOH 19q was more frequent in gliosarcomas (18%) than those in primary glioblastomas (4%; *P* = 0.0406). LOH 10q was more frequent in gliosarcomas (88%) than those in primary glioblastomas (67%; *P* = 0.0452).

PTEN mutations in giant cell glioblastomas (33%) were significantly more frequent than those in secondary glioblastomas (5%; *P* = 0.0126). Loss of ATRX expression was less frequent in giant cell glioblastomas than in secondary glioblastomas (*P* = 0.0206). *TP53* mutations were more frequent in giant cell glioblastomas (84%) than in primary glioblastomas (23%; *P* < 0.0001). LOH 19q was more frequent in giant cell glioblastomas (42%) than in primary glioblastomas (4%; *P* = 0.0002; Table 1).

DISCUSSION

IDH1 mutations are the definitive molecular marker of secondary glioblastoma (24, 28). Gliosarcomas and giant cell glioblastomas usually develop rapidly without less malignant precursor lesions (31), and have thus been considered to be primary glioblastomas. There have been few studies on *IDH* mutations. Lee *et al* (13) screened 26 gliosarcomas (21 primary gliosarcomas, 2 progressed from grade II or grade III astrocytomas, 2 developed from glioblastomas; one developed 9 years after radiotherapy for meningioma) for *IDH1* mutations. *IDH1* mutations were detected in one primary gliosarcoma and in a secondary gliosarcoma that progressed from a grade III astrocytoma (13). Balss *et al* (3) reported that 2 of 8 giant cell glioblastomas but none of 5 gliosarcomas carried *IDH1* mutations. Lotsch *et al* (15) reported the absence of *IDH1* mutations in one gliosarcoma and one giant cell glioblastoma.

This study provides evidence that gliosarcoma is indeed a histological variant of primary glioblastoma *IDH* wild-type. *IDH1* and *IDH2* mutations were absent in all 36 gliosarcomas analyzed. *TERT* promoter mutations, which are frequent in primary glioblastomas (2, 25), were detected in 83% of gliosarcomas (Table 1). Furthermore, LOH 10q was observed in 15/17 (88%) gliosarcomas. Monosomy of chromosome 10 was also observed in 2/5 gliosarcomas in the analysis calculated from 450k methylome array. Immunohistochemistry showed that ATRX is expressed in all gliosarcomas, suggesting the absence of *ATRX* mutations (14, 36). Thus, gliosarcomas share clinical and genetic features with primary glioblastomas *IDH* wild-type. The only major difference was *EGFR* amplification, which is common in primary glioblastomas and was considered to be a rare event in gliosarcomas (1, 31) (Table 1).

Despite two distinct histological components, gliosarcomas are considered monoclonal, since glial and mesenchymal tumor areas were usually found to be genetically similar with respect to *TP53* mutations, *PTEN* mutations, *p16*^{INK4a} deletion, *EGFR* amplification, and 10q loss (4, 31). The present study shows that this is also the case for *TERT* promoter mutations, as they were present in both glial and mesenchymal tumor areas in 95% (19/20) of gliosarcomas.

The molecular mechanisms involved in the mesenchymal differentiation in gliosarcomas are not yet fully understood. Expression of several transcription factors (eg, Slug, Twist) associated with epithelial-mesenchymal transition (EMT), was found in mesenchymal tumor areas, suggesting that signalling pathways involved in EMT may play a role in the induction of mesenchymal differentiation (21). In studies using conventional CGH, patterns of chromosomal imbalance were largely similar in glial and mesenchymal tumor areas, but there were also gains and losses at several loci unique to either glial or mesenchymal tumor areas (1, 6). Using array CGH, we have shown that in a small fraction of gliosarcomas the gain at 13q13.3-q14.1 and expression of several genes (*STOML3, FREM2, LHFP*) at this locus was restricted to the mesenchymal tumor area of gliosarcomas (20).

Giant cell glioblastomas develop rapidly with a short clinical history, but they occur in young adults (mean, 44 years), similar to secondary glioblastomas *IDH* mut (18, 30). We here provide genetic evidence that giant cell glioblastoma is a histological variant of primary glioblastoma *IDH* wild-type as *IDH1/2* mutations were absent in 18 of 19 giant cell glioblastomas analyzed. However, frequencies of *TERT* promoter mutations, *TP53* mutations, LOH 19q, and *EGFR* amplification were similar to those of secondary *IDH1* mut glioblastomas (17, 18, 30) (Table 1). Thus, giant cell glioblastoma occupies a hybrid position, sharing with primary glioblastoma a short clinical history, the absence of less malignant lesions, absence of *IDH1/2* mutations, frequent *PTEN* mutations and infrequent ATRX loss. In common with secondary glioblastomas, they have a younger patient age at manifestation, infrequent *TERT* promoter mutations, frequent *TP53* mutations, frequent LOH 19q, and lack of *EGFR* amplification.

There was the exceptional case of a giant cell glioblastoma *IDH1* mut (R132S) with clinical evidence suggesting progression from an anaplastic astrocytoma diagnosed a year earlier. This suggests that rarely, a secondary glioblastoma may show the histologic features of a giant cell glioblastoma.

The molecular mechanisms involved in the giant cell phenotype are not yet fully understood. Temme *et al* (34) reported that Aurora B expression is significantly higher in giant cell glioblastomas than in other glioblastomas, and that ectopic overexpression of Aurora B induced a significant increase in the proportion of multinucleated giant cells in *TP53* mutant but not in *TP53* wild-type malignant glioma cells.

LOH 10q is a frequent genetic alteration in both primary and secondary glioblastomas, suggesting that this chromosomal region may contain tumor suppressor gene(s), essential for malignant glioblastoma phenotype (27, 28). LOH 10q is also associated with shorter survival of glioblastoma patients in both population based (24, 26) and hospital-based studies (7, 33). We here provide evidence that LOH 10q is the genetic hallmark of gliosarcomas (88%) and giant cell glioblastomas (50%), further suggesting that gene(s) at 10q play an important role in the pathogenesis of glioblastomas and their variants.

In summary, gliosarcomas and giant cell glioblastomas are both histological variants of primary glioblastoma *IDH1* wild-type. The genetic pattern of gliosarcoma is largely similar to that of primary glioblastoma except for a lack of *EGFR* amplification, while giant cell glioblastoma genetically occupies a hybrid position between primary and secondary glioblastomas.

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