Cytoplasmic localization of wild-type p53 in glioblastomas correlates with expression of vimentin and glial fibrillary acidic protein¹

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Cytoplasmic accumulation of wild-type p53 in tumor cells indicates that the tumor suppressor is inactive with regard to growth suppressive functions. Whether this occurs randomly during tumor development or characterizes a certain tumor cell subset is not known. Here we assayed primary glioblastomas for expression and subcellular localization of p53 and determined a correlation with expression of intermediate filament proteins characterizing glial cell development. Sixty-nine percent of the tumors were p53 positive in immunohistochemistry. A significant number of tumors (23%) accumulated wild-type p53 in the cytoplasm, which correlated with the presence of vimentin and glial fibrillary acidic protein, except for 1 case. Tumors with exclusive nuclear p53 contained none or only one of these intermediate filament proteins. In an alternative approach, tumors positive for glial fibrillary acidic protein were screened for expression of p53 and vimentin. Thirty-eight percent of these tumors showed cytoplasmic p53, and all of those also expressed vimentin. Tumors with only nuclear p53 were vimentin negative,

except for 1 case. No mutation was detected in p53 exons 5 to 8 in tumors with cytoplasmic p53, suggesting that they express wild-type p53. The data indicate that a cytoplasmic accumulation of wild-type p53 in human primary glioblastomas correlates with a certain intermediate filament protein expression, suggesting that it identifies a certain subset of tumors. *Neuro-Oncology* 4, 171–178, 2002 (Posted to *Neuro-Oncology* [serial online], Doc. 01-055, April 10, 2002. URL <neuro-oncology.mc.duke.edu>)

Inactivation of the tumor suppressor protein p53 is considered a major risk for acquiring multiple gene lesions, thereby promoting tumor development (Janus et al., 1999; Levine, 1997). This risk is indicated by the high number (50%) of human tumors associated with mutations in the p53 gene. Most of these mutations (80%) are missense mutations of the central-sequence-specific DNA-binding domain (exons 5–8), impairing contact formation with consensus DNA and thereby preventing transactivation of p53 target genes (Hussain et al., 2000).

Loss of p53-specific functions due to mutation seems also to be involved in the development of astrocytic tumors, the most frequent neoplasms of the human CNS (Collins, 1999; Fulci et al., 1998). Diffusely infiltrating astrocytomas account for more than 60% of all primary brain tumors, and glioblastomas represent the most malignant tumor type within this group (Kleihues and Cavenee, 2000). p53 mutations are predominantly associated with secondary glioblastomas, tumors that have developed from low-grade astrocytomas (Sidransky et al., 1992). The mutation in the secondary glioblastoma normally is already present in the primary lesion, indicating

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³Abbreviations used are as follows: GFAP, glial fibrillary acidic protein; IFP, intermediate filament protein.

that it is acquired early in tumor development. This initial loss of wild-type p53 functions is supposed to be a decisive step that favors the accumulation of deletions and gene amplifications typical of secondary glioblastomas. In primary (de novo) glioblastoma, the most frequent astrocytic tumor seen in adults, the frequency of p53 mutations is low (10%) (Watanabe et al., 1996). Nevertheless, primary glioblastomas often react positively with p53 antibodies (Von Eckardstein et al., 1997). This indicates that most of these tumors express wild-type p53, which accumulates to high levels without suppressing growth or inducing apoptosis. An accumulation of wild-type p53 has also been found in breast carcinomas and in undifferentiated neuroblastomas (Moll et al., 1992) where p53 was localized in the cytoplasm. All these observations suggest that wild-type p53 can be inactivated by a mechanism that is associated with cytoplasmic sequestration of the protein. A cytoplasmic localization of p53 was also reported for glioblastomas, in which it is assumed that certain intracellular conditions rather than the type of p53 mutation may be decisive for the localization (Ali et al., 1994).

Here we studied the subcellular localization of p53 in primary glioblastomas and its correlation with the expression of astrocyte-specific IFPs.³ The results show that a significant number of primary glioblastomas exhibit a cytoplasmic p53 staining reaction. These tumors consistently express the IFP vimentin in addition to GFAP, whereas tumors with nuclear p53 are almost exclusively vimentin negative. The data indicate that the presence of cytoplasmic wild-type p53 in primary glioblastomas correlates with a certain IFP expression pattern typical of an early stage in astrocyte differentiation. The correlation suggests that cytoplasmic wild-type p53 in primary glioblastomas does not occur randomly, but identifies a tumor subset with certain phenotypic markers.

Materials and Methods

Immunohistochemistry

Tumor biopsies were fixed in 3% formaldehyde, dehydrated with ethanol, and processed for embedding in paraffin. The tumors selected for this study had been classified as glioblastomas according to the World Health Organization classification standards (Kleihues and Cavenee, 2000). Paraffin sections were dewaxed and rehydrated. Endogenous peroxidase reaction was blocked with 1% H₂O₂ in ethanol. The sections were coated with 10% normal goat serum (Dako, Hamburg, Germany) and subsequently overlaid with the primary antibody for 60 min at room temperature. Bound primary antibodies were detected with biotinylated goat antimouse or goat antirabbit antibodies (Dianova, Hamburg, Germany) (as bridge antibodies) and soluble complexes of streptavidin and biotinylated horseradish peroxidase (Dako). Diaminobenzidine was used as chromogen. The sections were counterstained with hemalum. p53 was localized with a polyclonal rabbit antiserum developed against a recombinant human wild-type p53 (SAPU, Scotland, U.K.) diluted 1:1000. Vimentin was

detected with the monoclonal antibody V9 (Boehringer, Mannheim, Germany); GFAP was traced with the monoclonal antibody 6F2 (Dako). A monoclonal antibody produced against a measles virus structural protein was used as control.

Determination of the Proliferation Index

The Ki-67 proliferation index was determined as described previously (Hagel et al., 1995). Sections were labeled with the monoclonal antibody MIB-1 (Dako); 200 to 800 cells were counted in 3 to 19 high-power fields (0.0153 mm²/field) in the tumor area showing the highest proliferative activity. The proliferation index was calculated as number of labeled tumor cells multiplied by 100 divided by the total number of counted nuclei.

Polymerase Chain Reaction Amplification and Sequencing of p53 Exons

DNA was extracted from 8-µm-thick sections of paraffin-embedded tumor tissue using the DNeasy tissue kit (Qiagen, Hilden, Germany). Three to 5 sections of a tumor were deparaffinized with 1 ml n-octane at 65°C for 10 min, centrifuged at 14,000 rpm for 5 min, and repeatedly washed with 100% ethanol. The sections were dried briefly at 37°C, resuspended in 180 µl ATL digestion buffer provided with the kit, and incubated in the presence of Proteinase K at 55°C for 18 h. Subsequently, 200 µl AL buffer from the kit was added, and the probes were further incubated at 70°C for 10 min, then 200 µl absolute ethanol was added, the probes were vortexed, and the DNA was isolated by use of a DNeasy minicolumn. For polymerase chain reaction amplification of exons 5-9 of the p53 gene, 3 to 10 µl of the final DNA preparation was added to a total volume of 100 µl reaction mixture as described previously (Hagel et al., 1996). The following primer pairs (Mashiyama et al., 1991) were used: for exon 5/6, 5'-ACCATGAGCGCTGCTAGAT-3' and 5'-AGTTGCAAACCAGACCT-3'; for exon 7, 5'-GTGTTATCTCCTAGGTTGGC-3' and 5'-CAAGTGCTCCTGACCTGGA-3'; and for exon 8/9, 5'-CCTATCCTGAGTAGTGGTAA-3' and 5'-CCAAGACTTAGTACCTGAAG-3'. The polymerase chain reaction product was subjected to agarose gel electrophoresis and automatic sequencing.

Statistical Analysis

An extended Fisher's exact test (Hartung et al., 1999) was used to analyze possible correlations between p53 and vimentin expression or p53 and GFAP expression in sample groups A and B given in Tables 1 and 2, respectively. The one-sided Fisher's exact test was used to analyze correlations between subcellular localization of p53 and expression of vimentin or GFAP. Statistical analysis with regard to Ki-67 index and patient age was performed with the Kruskal-Wallis test. The calculations were done with SAS V81 software.

Table 1. Detection of p53, vimentin, and GFAP in randomly selected glioblastomas

Case no.	Sex	Age (yrs)	p53 mutation in exons 5-8 (codon)	IFP staining		Cytomorphology	Ki-67 proliferation index (%)	Survival time (mo)
				Vimentin	GFAP			
Tumors with p53 in the cytoplasm								
1	M	60	ND	+	+	Fibrillary	17	U
2	M	56	None	+	+	Fibrillary/gemistocytic	24	92
3	M	58	None	+	+	Fibrillary	16	U
4	M	57	None	+	+	Gemistocytic	21	25
5	M	41	None	+	+	Fibrillary/gemistocytic	27	52
6	M	59	None	+	+	Fibrillary	29	U
Tumors with only nuclear p53								
7	F	43	ND	+	–	Fibrillary	ND	220
8	M	57	None	–	+	Fibrillary	16	113
9	F	61	None	+	+	Fibrillary	18	122
10	M	35	201 and 204	–	+	Small cell	66	55
11	M	60	213	–	–	Small cell	44	119
12	M	56	ND	+	–	Fibrillary	37	U
13	M	68	ND	–	+	Fibrillary	20	83
14	M	56	175	–	–	Fibrillary/gemistocytic	31	61
15	F	59	ND	–	+	Fibrillary	64	32
16	M	57	None	–	+	Fibrillary	30	104
17	F	48	None	+	–	Fibrillary	27	54
18	F	53	None	–	+	Fibrillary/gemistocytic	7	208
Tumors without p53 staining reaction								
19	M	70	ND	+	–	Gemistocytic	ND	U
20	M	59	ND	–	+	Fibrillary	20	U
21	M	46	None	+	–	Fibrillary/gemistocytic	18	39
22	F	63	ND	+	+	Gemistocytic	26	59
23	M	64	ND	–	–	Gemistocytic	18	70
24	F	55	ND	+	+	Fibrillary	26	78
25	F	37	ND	–	+	Fibrillary	17	52
26	F	54	ND	–	+	Fibrillary	15	30

Immunohistochemical staining on paraffin-embedded tissue sections.

Abbreviations: ND, not done; U, unknown.

Results

p53 Staining Patterns in Randomly Selected Primary Glioblastomas

Immunohistochemical studies were performed on formalin-fixed and paraffin-embedded biopsies of tumors identified as glioblastomas according to World Health Organization classification standards (Kleihues and Cavenee, 2000). In the first investigation, 26 samples were randomly selected from the archive (Table 1). All of these tumors showed an astrocytic morphology. In some parts, the growth pattern of the small-cell glioblastomas resembled that of oligodendrogliomas. Sections from solid, nonhemorrhagic areas of the tumor were chosen to evaluate the p53 staining pattern. Endothelial cells in areas of vascularization usually were p53 negative and were used as an internal reference for the specificity of the

staining. Of the 26 tumors, 18 (69%) reacted positively with the p53 antiserum. p53 was exclusively nuclear in 12 (46%) of the 26 cases (Fig. 1A), whereas in 6 cases (23%), a cytoplasmic staining was detected (Fig. 1C); this was partially associated with nuclear staining (Fig. 1E). Eight cases (31%) showed no p53 staining at all. The staining patterns were typical for the entire solid tumor area and not confined to specific regions such as the infiltrating edges or areas of vascularization. No certain correlation was found between the staining pattern and the intratumoral level of anaplasia.

Vimentin and GFAP Expression Pattern in Randomly Selected Tumors

Early precursor cells of the glial cell lineage express vimentin. In differentiating astrocytes, this IFP is gradually replaced by GFAP (Lee et al., 2000). Glioblastomas

Table 2. Detection of p53 and vimentin in GFAP-positive glioblastomas

Case no.	Sex	Age (yrs)	p53 mutation in exons 5-8 (codon)	IFP staining		Cytomorphology	Ki-67 proliferation index (%)	Survival time (mo)
				Vimentin	GFAP			
Tumors with p53 in the cytoplasm								
27	M	53	None	+	+	Gemistocytic	29	U
28	F	55	None	+	+	Fibrillary	57	U
29	M	53	ND	+	+	Fibrillary	40	U
30	M	36	None	+	+	Fibrillary	33	U
31	M	58	ND	+	+	Fibrillary/gemistocytic	34	U
32	F	48	None	+	+	Fibrillary/gemistocytic	11	U
33	M	41	None	+	+	Fibrillary	53	U
Tumors with only nuclear p53								
34	M	59	ND	+	+	Fibrillary/gemistocytic	44	U
35	M	58	ND	–	+	Fibrillary	13	U
36	F	50	ND	–	+	Fibrillary	29	U
37	F	53	ND	–	+	Fibrillary	61	U
38	F	53	ND	–	+	Gemistocytic	46	U
39	F	59	ND	–	+	Small cell	44	U
Tumors without p53 staining reaction								
47	F	48	ND	+	+	Fibrillary/gemistocytic	45	U
48	F	52	ND	–	+	Fibrillary	49	U
49	F	55	ND	+	+	Fibrillary/gemistocytic	8	U
50	M	54	ND	–	+	Fibrillary/gemistocytic	22	U
51	M	42	ND	–	+	Gemistocytic	31	U

Immunohistochemical staining on paraffin-embedded tissue sections.

Abbreviations: ND, not done; U, unknown.

can express both IFP types (Herpers et al., 1986). When the tumor samples were probed immunohistochemically for expression of these proteins, a clear correlation was found between p53 localization and the IFP expression pattern (Table 1). In tumors with only nuclear p53 staining, the presence of these IFPs varied strongly. Most of the cases (6 cases) were positive only for GFAP. Three of them expressed vimentin, and 1 case was positive for both IFPs. Two cases did not show either of these IFPs. Similarly, a heterogeneous expression of these IFPs was found in p53-negative tumors. Three tumors showed GFAP only; 2 cases expressed vimentin; another 2 cases showed both IFPs; and 1 case was negative for both IFPs. In contrast, all tumors exhibiting cytoplasmic p53 staining were found to be positive for both IFPs.

A statistical analysis performed with an extended Fisher's exact test indicated that the probability of vimentin expression was different in the 3 groups ($P = 0.0294$). As determined with the one-sided Fisher's exact test, samples with cytoplasmic p53 showed a significantly higher probability ($P = 0.013$) of vimentin expression than did samples with only nuclear p53 or samples lacking p53. In contrast, the probability of expressing GFAP was not different in these groups ($P = 0.216$). Tumors with cytoplasmic p53 did not show a significantly higher probability ($P = 0.0806$) of GFAP expression than did those with nuclear p53 or those lacking p53.

Vimentin and p53 Expression Patterns in GFAP-Positive Tumors

The statistical analysis suggested that vimentin is a strong indicator for a cytoplasmic accumulation of p53 in tumors that also express GFAP. To test this assumption, we selected 18 additional paraffin-embedded tumor biopsies that had been identified as GFAP positive in immunohistochemistry. Sections of these tumors were subsequently probed for the presence of p53 and vimentin. As shown in Table 2, 13 of these cases (72%) reacted positively with the polyclonal p53 antibody. Seven of the 18 cases (38%) showed the presence of p53 in the cytoplasm. Six tumors (33%) revealed an exclusive nuclear p53 staining, whereas 5 cases (28%) did not react at all with p53 antibodies. Except for 1 case, all of the tumors exhibiting only nuclear p53 did not react with vimentin-specific antibodies. p53-negative tumors separated at nearly equal numbers into those positive for GFAP and those positive for both IFPs. However, all of the tumors with cytoplasmic p53 also contained vimentin. Statistical analysis with the modified Fisher's exact test indicated that the probability of expressing vimentin was different in the 3 groups ($P = 0.0044$). As assayed subsequently with the one-sided Fisher's exact test, the probability of vimentin expression was significantly higher ($P = 0.0038$) in the sample group with

Fig. 1. Immunostains of p53 and vimentin on tissue sections from 3 cases of human primary glioblastoma, counterstained with hemalum. Staining of p53 in case no. 11 is exclusively nuclear (A), and labeling of vimentin is confined to endothelial cells lining the vessels (B, arrows). Tumor cells are vimentin negative. Localization of p53 in multinucleated cells from case no. 27 is exclusively cytoplasmic (C, arrows); labeling of vimentin is also shown (D). Cytoplasmic localization of p53 is partially combined with a nuclear staining reaction (E, arrows); vimentin staining is also shown (F). Scale bar = 30 µm.

cytoplasmic p53 than in the 2 other groups. No statistically significant differences were found between these groups with regard to proliferation index as determined by Ki-67 labeling, cytomorphology, age in years, and survival time.

Mutational Analysis

Although secondary glioblastomas show a high incidence of *p53* mutations in exons 5 to 8, mutations are rare in primary glioblastomas. We examined whether the different staining patterns of p53 in relation to that of IFP expression showed any preference for the presence of either wild-type or mutant p53. Genomic DNA was extracted from tumor sections, and the region

encoding the DNA-binding domain of p53 was sequenced using primers specific for exons 5 to 8 of the *p53* gene. In some cases, sequencing was extended to exon 9 by using primer pairs covering the 5'-end of exon 8 and the 3'-end of exon 9. Exon 9 encompasses the sequence that codes for the major nuclear localization signal (NLS I). As shown in Tables 1 and 2, none of the tumors with cytoplasmic p53 tested so far exhibited a mutation in these exons. In 1 case (Table 2, case 27), a single base substitution was found at position 3092, located within intron 8. Mutations were found in some of the primary tumors with exclusive nuclear p53 (Table 1, cases 10, 11, and 14). The data suggest that tumors with cytoplasmic p53 preferentially express wild-type p53.

Discussion

We screened human primary glioblastomas for expression of tumor suppressor protein p53 and IFPs vimentin and GFAP. Most cases (~70%) within both sample groups proved to be p53 positive in immunohistochemistry, but only 3 of 19 tumors harbored a mutation in the central DNA binding domain of p53. Our findings correspond to observations of others showing that p53 mutations are rare in human primary glioblastomas and that the number of cases that are p53 positive in immunohistochemistry exceeds those having a p53 mutation (Louis et al., 1993; Rubio et al., 1993; Vital et al., 1998; Von Eckardstein et al., 1997). So far we cannot preclude the existence of mutations in exons coding for the amino (exons 2-4) and carboxy (exons 10-11) terminal domains of p53, but these occur at a low frequency (1%-3%) in human tumors (Hussain et al., 2000).

p53 mutations were absent not only from primary glioblastomas exhibiting cytoplasmic staining but also from tumors with an exclusive nuclear p53 staining reaction. As pointed out by others, not only wild-type but also mutant p53 can be cytoplasmic in human glioblastomas (Ali et al., 1994). This suggested that cell-specific features and not the p53 genotype determine the localization of this protein. Our findings, showing that a cytoplasmic accumulation of wild-type p53 correlates with a certain IFP expression pattern, substantiate this assumption. All of the tumors with cytoplasmic p53 also expressed vimentin and GFAP. In contrast, glioblastomas with an exclusive nuclear p53 were either GFAP or vimentin positive. Thus, when considering IFP expression and subcellular localization of p53 as independent parameters, primary glioblastomas are heterogeneous. When correlated with each other, however, a subpopulation of tumors could be identified where the presence of p53 in the cytoplasm was consistently associated with expression of both IFPs. It is not known whether a certain correlation between subcellular localization of p53 and IFP expression is also found with secondary glioblastomas, tumors which have evolved from low-grade astrocytomas and reveal a higher incidence of p53 mutations. At least, secondary glioblastomas with mutated p53 preferentially seem to accumulate the protein in the nucleus (Watanabe et al., 1996).

Recently we showed that C6 rat glioma cells transfected with a temperature-sensitive mutant p53 (p53^{val-135}) could anchor p53 in the cytoplasm, provided that these cells contained vimentin IFPs (Klotzsche et al., 1998). A C6 subclone completely lacking cytoplasmic intermediate filaments (Röser et al., 1991; Bohn et al., 1993) constantly harbored p53^{val-135} in the nucleus. Vimentin-deficient cells that were retransfected with an exogenous mouse vimentin protein were again capable of anchoring p53^{val-135} to the cytoplasm. The data indicated that the presence of vimentin is decisive for a cytoplasmic localization of p53 in these cells. Furthermore, only vimentin-positive C6 cells can overcome growth suppression by p53^{val-135} (unpublished data, W.B., 2001). In these cells, the wild-type form of p53^{val-135} accumulates only transiently in the nucleus after the shift to the permissive temperature and concomitantly induces a transient G₁ arrest. The cells rapidly exclude the wild-type form of

p53^{val-135} from the nucleus, bind it to the vimentin filament system, and subsequently start to proliferate. In contrast, vimentin-deficient cells constantly harbor p53^{val-135} in the nucleus and stay in a permanent G₁ arrest. Thus, binding of p53 to the vimentin filament system seems to be tightly connected with an inactivation of its growth regulatory functions. p53 that is cytoplasmic in glioblastomas may also be directly bound to this filament system, which may be essential to escaping a p53-mediated growth arrest. The results obtained with the second GFAP-positive tumor sample group at least give evidence that GFAP alone cannot promote a cytoplasmic accumulation of p53 but demands the presence of vimentin. In the absence of vimentin, p53 was exclusively nuclear.

Cytoplasmic accumulation of wild-type p53 is not a tumor-specific event but is also found in normal tissues, such as in embryonic stem cells of mice (Sabapathy et al., 1997), epithelial cells of the basal cell layer in human skin (Spandau, 1994), and cells lining the mammary duct in lactating tissue (Moll et al., 1992). In the embryonic brain tissue of rats, p53 mRNA and protein accumulate to high levels in proliferating cells without any indication of apoptosis (Van Lookeren Campagne and Gill, 1998). Oligodendrocytes explanted from embryonic rat brain exhibit wild-type p53 in the cytoplasm as long as the cells stay in an undifferentiated state. When they achieve the proper stimulus, p53 translocates into the nucleus and the cells complete differentiation and cease proliferation, suggesting that cytoplasmic wild-type p53 participates in pathways of differentiation (Eizenberg et al., 1996). Thus, the presence of cytoplasmic wild-type p53 does not necessarily define an aberrant situation, but is a normal event early in differentiation and development—stages that are also defined by certain IFP expression patterns (Franke et al., 1982). Precursor cells of the glial cell lineage express only vimentin. Cells that differentiate into oligodendrocytes cease expression of this IFP, whereas cells following the astrocytic pathway switch on GFAP expression. In an intermediate stage, both IFPs are present in astrocytic cells (Ho and Liem, 1996; Lee et al., 2000).

Astrocytomas frequently coexpress vimentin and GFAP, a pattern that does not change with increasing anaplasia (Herpers et al., 1986). In addition, they express nonglial IFPs (Hirato et al., 1994). In a recent DNA microarray and tissue chip technique analysis, overexpression of vimentin was found in both low-grade astrocytomas (grade II diffuse) and high-malignancy tumors (grades III-IV), except for some glioblastomas (Sallinen et al., 2000). However, astrocytomas generally seem to be less positive for vimentin than for GFAP (Schiffer et al., 1986). Well-differentiated human astrocytomas stain less frequently with vimentin antibodies than do anaplastic forms or glioblastomas multiforme (Cosgrove et al., 1989). In contrast, GFAP was present in all forms in this study and proved to be a regular constituent. Human astrocytomas also express nestin (Dahlstrand et al., 1992), a class VI IFP. Nestin is characteristic of CNS progenitor cells. It is also present in cells that are in transition from a progenitor stage to glial differentiation, and it costains with vimentin (Messam et al., 2000). Glioblastomas (grade IV) exhibit a strong expression of nestin, whereas grade II astrocytomas lack this protein, suggest-

ing that nestin expression varies with the degree of malignancy (Dahlstrand et al., 1992). The data indicate that glioblastomas exhibit an IFP expression pattern that is typical of undifferentiated glial cells. Therefore, primary glioblastomas showing wild-type p53 in the cytoplasm and expressing vimentin and GFAP may have developed from cells arrested in an early stage of astrocyte differentiation. Whether or not inactivation of wild-type p53 associated with cytoplasmic sequestration of the protein is an initial event in tumor development is still unknown.

Normal tissues and tumors that accumulate large amounts of wild-type p53 both have in common the fact

that p53 is inactive with regard to growth-suppressive functions. Correspondingly, we observed no differences with regard to the proliferation index between tumors overexpressing wild-type p53 and those that were p53 negative. Also, the presence of cytoplasmic p53 did not correlate with certain clinical features. However, as shown for neuroblastoma cell lines, p53-dependent signaling pathways probably are intact in tumor cells accumulating wild-type p53 in the cytoplasm (Goldman et al., 1996). Thus, reactivation of cytoplasmic wild-type p53 in primary glioblastomas could be a favorable approach for specific treatment of this tumor subset.

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