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Comparative Assessment of the Functional p53 Status in Glioma Cells

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Abstract. *Background: p53 is the most frequently mutated gene in human cancers and its functional integrity is an important predictor of treatment response and clinical outcome. The majority of mutations found in different types of cancer cluster within the DNA binding domain encoded by exons 5-8. In clinical specimens the functional status of p53 is, therefore, often evaluated by direct mutation analysis of exons 5-8 or indirectly by immunostaining and evaluation of the subcellular localization pattern or protein accumulation. Materials and Methods: In a panel of glioma cell lines, the status of the P53 gene was analyzed by temperature gradient gel electrophoresis (TGGE) of exons 5-8 and direct sequencing of all p53 exons. The nuclear accumulation of p53 in unstressed cells was assessed by immunostaining. These data were correlated with stress induction of the p53 protein, nuclear translocation and a direct determination of the transcriptional activity of endogenous p53 protein and induction of p53 target genes. Results: Our analysis demonstrated that a p53 gene mutation analysis limited to exons 5-8 and analysis of immunostaining patterns can not serve as reliable predictors of functional p53 in tumor cells. Conversely, in some presumably rare cases, the transcriptional activity of p53 may be retained in tumor cells in the presence of a mutation and a pathological immunostaining pattern. In our analysis, the constitutive dephosphorylation at Ser 376 correlated with the nuclear accumulation of p53, but not with the transcriptional activity of the protein. This suggests that constitutive dephosphorylation at Ser376 may be one of the factors determining stabilization of mutant and wild-type p53, which is frequently observed in glial tumors. Conclusion: The incidence of a dysfunctional p53 protein in gliomas may be higher than expected, based on a single parameter evaluation by mutation analysis of exons 5-8 or assessment of p53 accumulation and subcellular localization by immunostaining.*

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The tumor suppressor p53 is a transcription factor, which is found mutated in more than half of human cancers (1, 2). In normal cells, p53 is a very short-lived protein with a half-life of approximately 20 minutes. Low concentrations of the protein in normal cells are sustained by proteasome-mediated degradation of p53 *via* an ubiquitine-dependent pathway (3, 4). Under cellular stress conditions, such as UV- or gamma-irradiation, hypoxia, nucleotide depletion or viral infection, wt p53 protein becomes stabilized and translocates into the nucleus where it can regulate transcription of a number of genes (5, 6). Growth arrest, apoptosis and repression of invasion genes are major functional outcomes mediated by activated p53 protein (7-10). p53-dependent transient arrest of the cell cycle allows the repair of DNA damage and is predominantly a consequence of p53-dependent transcriptional activation of the cyclin-dependent kinase inhibitor p21 (11, 12). When irreversible damage occurs, p53 can initiate a cell suicide program to eliminate the affected cell through an apoptotic pathway (reviewed in 13 and 14).

Functional inactivation of p53 in cells abrogates the ability to maintain the integrity of the genome, resulting in accumulation of mutations. Furthermore, p53 inactivation in tumor cells leads to a growth advantage, which ultimately leads to cancer progression. Determination of the functional p53 status in tumor specimens commonly serves as a prognostic marker of clinical outcome and as a predictor of response to chemotherapy and radiation therapy, highlighting the importance of a reliable evaluation of the function of p53. Based on known mechanisms inactivating p53 in tumor cells, several predictive parameters have been established to evaluate the functional status of p53 in biopsy specimens. The vast majority of p53 mutations in human cancers cluster within the central DNA binding domain

(DBD) (15, 16) and affect the sequence-specific DNA binding activity, which is indispensable for transcriptional regulation of target genes by p53 (17). As a consequence, mutations within the DBD, including the six most frequently occurring hot-spot mutations, impair the transcriptional activity of the protein. Therefore, the presence of mutations in exons 5-8 is highly indicative of a transcriptionally inactive p53 protein and is commonly used in clinical correlative studies as a predictive parameter for a non-functional p53 in tumor specimens.

The prolonged half-life is a distinct feature of mutant p53 proteins, which is a consequence of their inability to activate the transcription of a major regulator of p53's stability, the E3 ubiquitine ligase MDM2 (3). The MDM2 protein targets nuclear p53 to proteasomal degradation in the cytosol (18, 3, 19, 4). The presence of large amounts of nuclear p53 in cells of biopsy specimens detected by immunohistochemistry is, therefore, interpreted as an indirect marker of a transcriptionally inactive p53 protein (20-25).

Post-translational modifications play an important role in the regulation of the activity of p53 and several sites at the N- and the C-terminal domains are modified upon activation of p53 (14). Stress-induced changes in the modification profile of the p53 protein appear to control specific properties of p53. For example, γ-irradiation or UV-irradiation-induced phosphorylation at Ser15 by ATM (26) and Ser20 by Chk2 (27) prevent binding and repression of p53 by MDM2 and thereby increase p53's transcriptional activity. On the other hand, γ-irradiation also induces ATM-dependent dephosphorylation at Ser376, which leads to the association of p53 with the 14-3-3 protein and, subsequently, to enhanced DNA binding activity of p53 (28). Therefore, aberrations in stress-induced transient changes in the modification profile of the p53 protein may also be indicative of an altered functional p53 pathway.

It is emerging that detection of p53 mutations in human cancers may not always adequately reflect the impact of p53 in the biology of these neoplasms, due to a bias that may be introduced by the evaluation strategy itself (16, 14). For example, many earlier studies, including ours (29), have limited their mutation analysis to exons 5-8, assuming that mutations outside exons 5-8 occur extremely rarely. However, more recent investigations analyzing all exons of the *P53* gene demonstrated that such a view is not correct and that a substantial number of mutations locate outside exons 5-8 (30, 31). Therefore, it is important to carefully re-evaluate the results from earlier studies that have limited their analysis to exons 5-8 or that have used single parameter approaches for evaluation of the p53 status.

The analysis of the p53 status in central nervous system tumors has demonstrated frequent alterations of the *P53* gene as well as stabilization of mutant and wt p53 proteins in tumor cells. Glioblastoma multiforme represents the most frequent and most malignant tumor of glial origin. Although 40% of all glioblastomas show mutations in the *P53* gene, earlier studies suggest that about 60% of these tumors carry no p53 mutation (reviewed in 32). This would suggest that a significant subset of these tumors arise and progress independently of a deregulated p53 pathway. In this study, we directly assessed the transcriptional activity of p53 in a range of glioma cell lines. The functional status of p53 was correlated with the predicted status, based on evaluations by three independent approaches commonly used in clinical practice: TGGE analysis of exons 5-8, direct sequencing of the *P53* gene, and immunostaining analysis of subcellular localization patterns. Our analysis showed that the functional impairment caused by some mutations may not always be anticipated based on mutation analysis of exons 5-8 or analysis of the subcellular distribution alone. Some mutations in our panel of glioma cell lines did not impair the transcriptional activity of p53 in the absence of

Table I. *Mutation analysis, subcellular p53 localization and transcriptional activity of endogenous p53 protein.*

cells	TGGE (exons 5-8)	Sequencing (exons 2-11)	subcellular localization*	transcriptional activity
normal astrocytes	no mutation	no mutation	cytoplasmatic	+
GBM - NCE G 168	no mutation	no mutation	cytoplasmatic	+
GBM - NCE G 63	no mutation	no mutation	cytoplasmatic	+
GBM - NCE G 120	no mutation	no mutation	cytoplasmatic	+
GBM - NCE G 22	exon 5; exon 7	R158 ⇒ H; G245 ⇒ S	nuclear	-
GBM - NCE G 112	exon 7	R248 ⇒ W	nuclear	-
GBM - NCE G 123	exon 5; exon 7	R175 ⇒ H; R248 ⇒ W	nuclear	-
GBM - NCE G 44	no mutation	E224 ⇒ stop	cytoplasmatic	-
GBM - NCE G 84	no mutation	A79 ⇒ V	cytoplasmatic	-
GBM - NCE G 62	no mutation	D49 ⇒ Y	nuclear	+
GBM - NCE G 130	gross rearrangements	gross rearrangements	no signal	-

*The subcellular localization in unstressed cells was analyzed by immunostaining with DO1 antibodies.

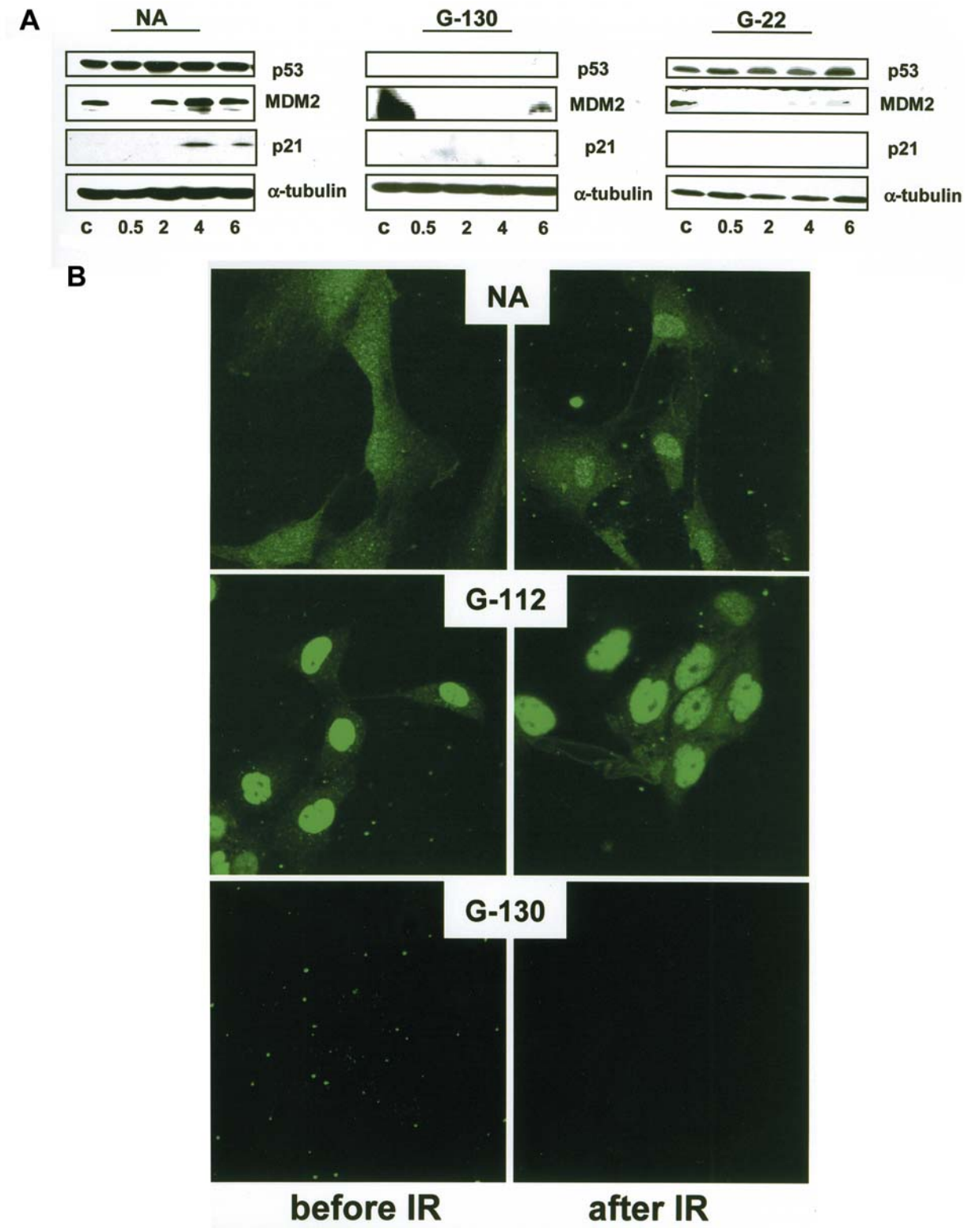
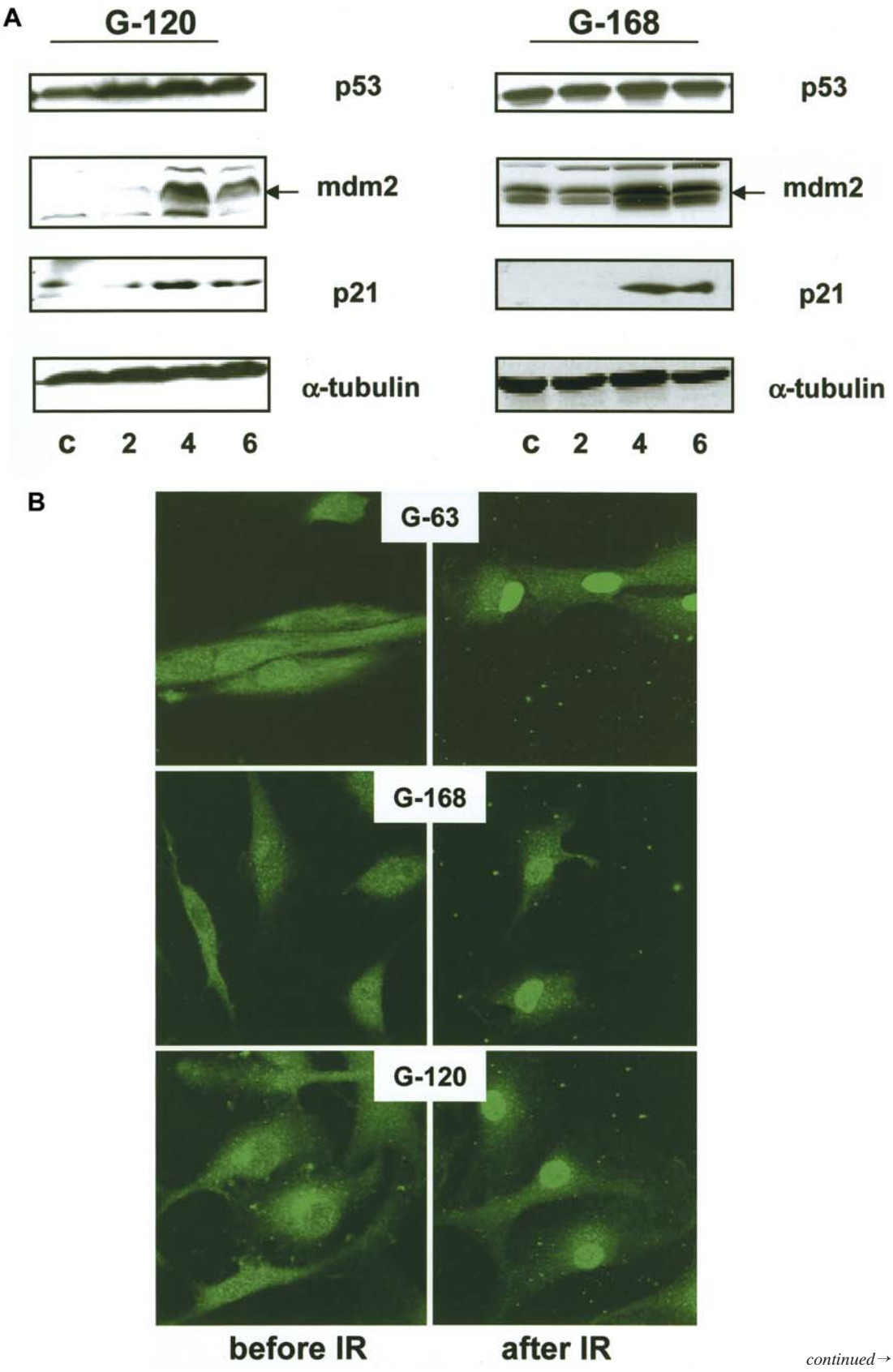


Figure 1. *A. Activation of p53 in response to γ-irradiation. Western blot analysis of p21 and MDM2 proteins in normal astrocytes (NA), a p53 null glioma cell line (G-130) and a mutant cell line (G-22, R158 ⇒ H; G245 ⇒ S). The cellular lysates were prepared from untreated cells (c) or from cells exposed to γ-irradiation and harvested at 0.5, 2, 4, and 6 hours post γ-irradiation treatment. α-tubulin was used as internal control for equal loading. B. Nuclear translocation of functional p53 induced by γ-irradiation. γ-irradiation induces rapid accumulation of p53 in the nucleus of normal astrocytes (NA) but not in glioma cells with a transcriptionally inactive p53 (G-112, R248 ⇒ W). p53 null G-130 glioma cells were used as a negative control.*

Figure 2.



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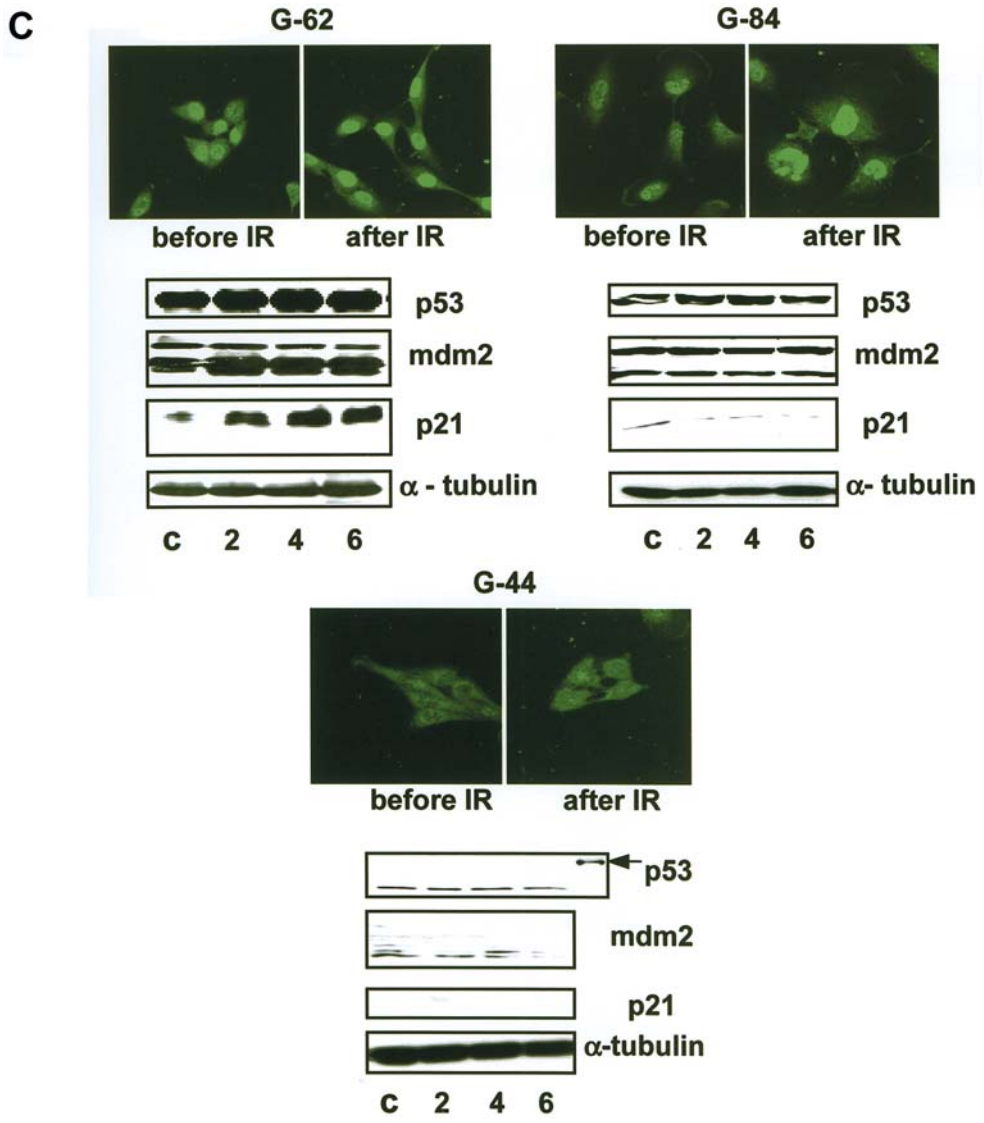

Figure 2. *A. Assessment of the transcriptional activity of p53 in glioma cell lines with functional p53. Western blot analysis of p21 and MDM2 proteins in untreated and γ-irradiated cells. B. γ-irradiation induced nuclear accumulation of p53 in cell lines with functional p53 protein. C. Glioma cell lines with subcellular localization pattern not matching the transcriptional activity of the p53 protein. Immunostaining analysis of untreated cells or cells exposed to γ-irradiation was performed with the antibody DO-1 (upper panels). Western blot analysis of cellular lysates from untreated (c) or γ-irradiated cells (lower panel). In G-44 cells, a short form of p53 represented the only form of p53 migrating as a 35KDa protein (the arrow indicates a full length recombinant p53 protein used as a control).*

LOH. Furthermore, the analysis of posttranslational modifications showed that p53 was constitutively dephosphorylated at Ser376 in glioma cell lines with mutant p53. A lack of phosphorylation at Ser376 in this panel of cell lines correlated with nuclear accumulation of the protein but not with its transcriptional activity, suggesting that deregulated phosphorylation at Ser376 may be important for p53 stabilization in gliomas. These data emphasize that the role of p53 in the biology of gliomas may be underestimated and argue for the necessity of a multiple parameter evaluation to predict the functional status of the p53 protein in brain tumors.

Materials and Methods

Cell culture. The human glioma cell lines (NCE-G22, G44, G62, G63, G84, G112, G120, G121, G130, G168, G260) were propagated in MEM (Biochem, Berlin, Germany) with 10% FCS in a humidified incubator containing 5% CO_2 and were passaged using trypsinization at regular intervals, depending on growth

characteristics (29, 7). Human astrocytes were established from normal human brain (33) and cultured in Ham's F12/Dulbecco's MEM (1:2) (Biochem) containing 20% FCS.

Western blot analysis. Subconfluent cultures of glioma cells and normal astrocytes were irradiated with 7.5 Gy and harvested at 0.5, 2, 4 and 6 hours. The cells were washed with cold PBS twice, then scraped in cold PBS and pelleted by centrifugation at 3,000 g at 4˚C for 5 minutes. The cells were lysed immediately in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% NP40 in the presence of 5μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A and 5 μg/ml pefabloc). After 30 minutes' incubation on ice, the samples were centrifuged for 25 minutes at 13,000g, the supernatants were collected and the protein concentration was measured using a BCA protein assay Kit (Pierce, Rockford, IL, USA). The lysates were adjusted to the protein concentration, and boiled at 95˚C for 5 minutes in loading SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromphenol blue). Fifty to 100 μg of cell lysate were electrophoresed on SDS-PAGE (7.5% to 15%), and transferred to a polyvinylidene fluoride membrane (Millipore, Eschborn, Germany). The membrane was treated with anti-p53 antibodies [Ab421 and DO-1 (Oncogene, San Diego, USA), an anti-p21 antibody (Santa Cruz Biotechnology, Santa Cruz, USA), or the anti-mdm2 antibody 2A10 (Oncogene)]. The immunoreactive bands were visualized with an ECL detection system (Amersham, Freiburg, Germany).

DNA preparation. DNA from frozen tissue was prepared from cryostat sections using the QIAamp tissue kit (Qiagen GmbH, Hilden, Germany).

PCR/TGGE. Mutations were screened by PCR and temperature gradient gel electrophoresis (TGGE) as described by Kappes *et al.* (34). All exons were separately amplified. PCR reactions were carried out in 50 μl containing 500 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each of the deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 1 U Taq DNA polymerase (Perkin-Elmer, Oak Brook, IL, USA) and 12 pmol of each primer containing a 40 bp GC clamp (29, 34). PCR was performed at 94˚C/3 minutes (initial denaturation), followed by 35 cycles at 94˚C /1 minute, 60˚C /30 seconds and 72˚C /1 minute. The PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany) and separated on a denaturing polyacrylamide gel with increasing temperature gradients. TGGE was carried out using a TGGE system (Qiagen) according to the manufacturer's instructions. Briefly, the PCR products were mixed with an equal volume of denaturing buffer (8 M urea, 40 mM MOPS, 2 mM EDTA, pH 8.0, 0.01% each bromophenol blue and xylane cyanol FF), heated for 5 minutes at 95˚C and further incubated for 15 minutes at 50˚C to promote heteroduplex formation. The samples were loaded on gels consisting of 8% polyacrylamide, 8 M urea, 20 mM MOPS (pH 8.0), 1 mM EDTA and 2% glycerol and separated by a temperature gradient. Optimal temperature gradients and run times were determined for each exon by perpendicular TGGE (TGGE Handbook, Qiagen). For each exon analyzed, at least one sample with a known point mutation was used as a positive control.

DNA sequencing. PCR products, demonstrating formation of homo-/heteroduplexes on TGGE indicative of mutations, were subjected to cycle sequencing using the Ready Reaction Big Dye Terminator Cycle Sequencing Kit (Biosystems, Foster City, CA, USA). The PCR products were purified and separated using an automated sequencing system (ABI-Prism 310 Genetic Analyzer, Applied Biosystems). All mutations were confirmed by sequencing both DNA strands. Direct sequencing of exons 2-11 was performed as described by Reles *et al.* (35).

Immunofluorescence microscopy. Cells grown on glass cover slips were irradiated with 7.5 Gy and incubated for 2-4 hours, then fixed in 95% ethanol with 5% glacial acetic acid for 15 minutes at -20˚C and permeabilized with 3 % Triton X-100 in PBS for 15 minutes at room temperature. The cover slips were washed in culture medium containing 10% FCS and incubated for 30 minutes at room temperature with the anti-p53 antibody DO-1. The primary antibodies were detected with fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse antibodies (DAKO, Copenhagen, Denmark). After incubation, preparations were washed, mounted on microscope slides and examined with a Zeiss Axiscope fluorescence microscope.

Results

Correlation of the functional status of p53 with TGGE mutation analysis of exons 5-8 and the subcellular localization of the p53 protein. Mutation analysis of exons 5-8 of the *P53* gene is commonly used in clinical practice to predict the functional status of p53 in tumor cells and biopsy specimens, because the majority of p53 mutations found in tumors localizes to this region. We analyzed exons 5-8 in a panel of glioma cell lines by TGGE and the subcellular localization of p53 protein was assessed by immunostaining with the DO-1 antibody directed to the N-terminus of p53. These data were correlated to a direct assessment of the transcriptional activity of p53.

The ability of cellular p53 to induce its target genes, p21 (11) and MDM2 (36), in response to ionizing radiation was examined by Western blot analysis. Cultured human astrocytes were used as a cellular system with a presumably intact p53 signaling pathway. As expected for a normal cellular system, in astrocytes γ-irradiation resulted in an increase of p53 protein and induction of both p21 and MDM2 (Figure 1A). The induction of these target genes after γ-irradiation can be assumed to be p53-dependent, because no increase in p21 or MDM2 levels was observed in a glioblastoma-derived cell line that lacks p53 (G-130) or in a mutant p53 cell line (G-22). A characteristic decrease of the MDM2 immunoreactivity was observed with the phosphorylation-sensitive antibody 2A10 30 minutes after γ-irradiation. This phenomenon is a consequence of MDM2 phosphorylation by ATM kinase, which phosphorylates both p53 and MDM2 proteins in response to DNA damage (37).

Rapid translocation of the p53 protein from the cytoplasm to the nucleus upon activation is characteristic of a functional p53 pathway and may therefore serve as a parameter to predict the functional integrity of p53.

Figure 3. *TGGE analysis of exon 6 in glioma cell lines. An abnormal heteroduplex (asterisk) in G-60 and G-140 cell lines indicates the presence of mutations that were further verified by a direct sequencing. Samples prepared from G-44 cells showed the same migration pattern compared to the corresponding wild-type genotype (positive control wtp53 G-168 cells).*

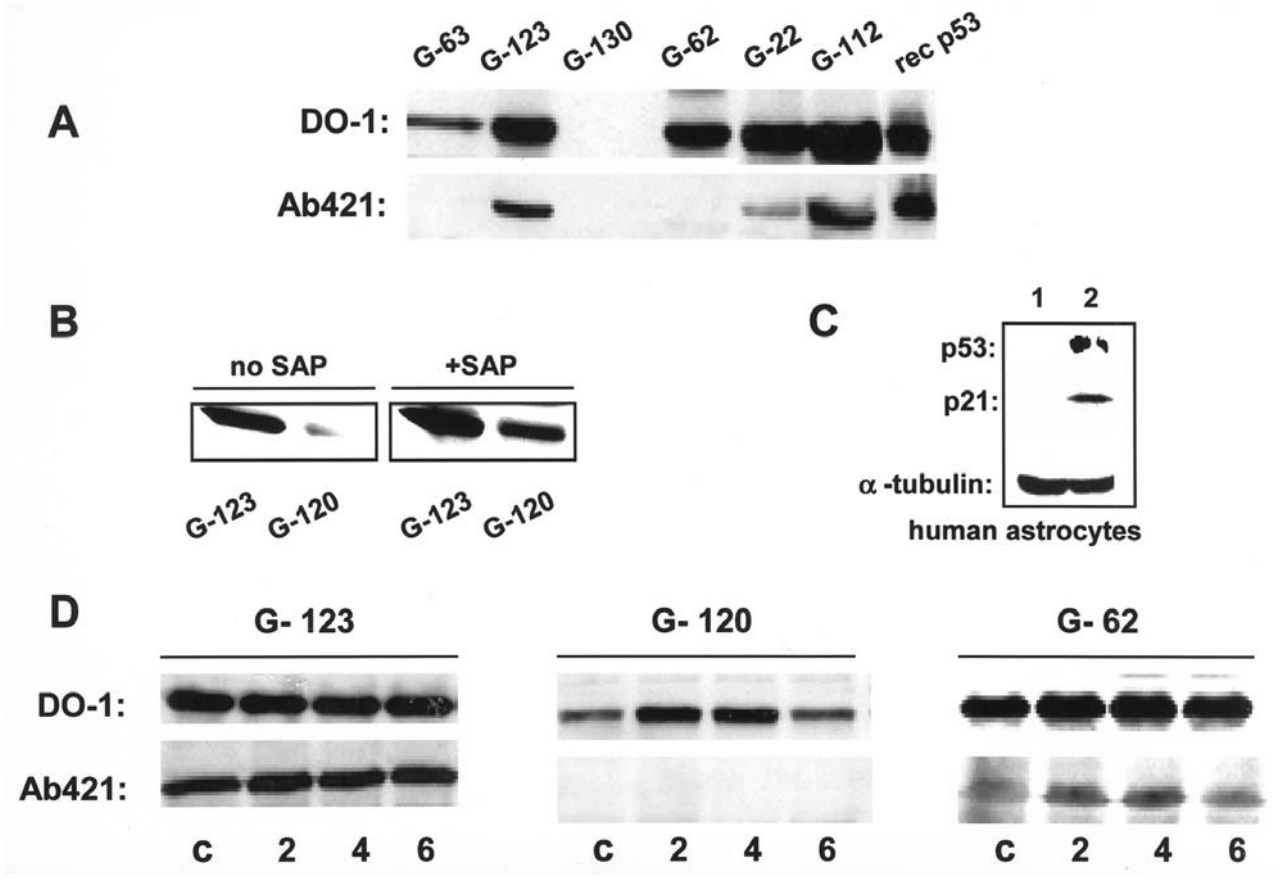

Figure 4. *The phosphorylation pattern detected by PAb421 correlates with the constitutive nuclear localization of p53 in gliomas.*
A: Cell lysates from untreated glioma cells were probed with DO-1 or PAb421 antibodies. Recombinant wt p53 protein is shown in the last lane as a control.
B: Cell lysates from G-123 and G-120 lines were probed with PAb421 directly or following in vitro phosphatase treatment of the membranes with shrimp alkaline phosphatase (SAP).
C: The appearance of a PAb421-reactive p53 form correlates with the p53-dependent induction of p21 by γ-irradiation in human astrocytes. Cell lysates were prepared from untreated (lane 1) or γ-irradiated (lane 2) cells and were analyzed by Western blot.
D: Differential PAb421 reactivity observed in gliomas. Cellular lysates were prepared from untreated cells (c) or cells exposed to γ-irradiation (at hours indicated after treatment) and analyzed by Western blot with DO-1 or PAb421 antibodies.

Table II. *Phosphorylation at Ser376 correlates with the nuclear localization of p53 in glioma cell lines.*

Cells	Transcriptional activity	Accumulation in unstressed cells*	Stress-induced nuclear translocation**	PAb421 reactive p53***
normal astrocytes	+	-	+	+
GBM - NCE G 63	+	-	+	-
GBM - NCE G 168	+	-	+	-
GBM - NCE G 120	+	-	+	-
GBM - NCE G 62	+	nuclear	-	+
GBM - NCE G 112	-	nuclear	-	+
GBM - NCE G 84	-	nuclear	-	+
GBM - NCE G 123	-	nuclear	-	+
GBM - NCE G 22	-	nuclear	-	+

Transcriptional activity was assessed by induction of p53 target genes after γ-irradiation. *p53 accumulation in unstressed cells was analyzed by immunostaining with DO1 antibodies. ** Changes in the subcellular distribution were assessed following γ-irradiation by immunostaining with DO1 antibodies. *** The phosphorylation status at Ser376 was determined by immunoreactivity with the phosphorylation-sensitive antibody PAb421

Immunostaining demonstrated that, after γ-irradiation, p53 protein accumulated in the nucleus of normal astrocytes but not in the nuclei of G-112, which showed strong nuclear staining independent of γ-irradiation treatment, a characteristic feature of mutant p53 (Figure 1B). Therefore, our experimental conditions demonstrate the monitoring of two important aspects of a functionally active p53 protein, the transcriptional activity and the nuclear translocation of p53 following the activation of a DNA-damage signaling pathway by γ-irradiation. An abnormal subcellular localization pattern with nuclear accumulation and a failure to translocate to the nucleus after γ-irradiation was consistently found in all cell lines with mutations detected by TGGE in exons 5-8. Constitutive nuclear staining correlated with an impaired transactivation of p21 and MDM2 in response to γ-irradiation in all cases (summarized in Table I). However, in three out of six cell lines with no mutations detected by TGGE of exons 5-8, the immunostaining pattern did not correlate with the transcriptional activity of p53. A normal immunostaining pattern corresponded to a transcriptionally active p53 protein in the cell lines G-63, G-120 and G-168 (Figure 2A and B). In G-44, G-62 and G-84, the data obtained by TGGE and immunostaining were not predictive of a functional p53 protein. Unstressed G-62 cells showed a predominant localization of p53 in the nucleus that did not change after γ-irradiation treatment (Figure 2C, upper panel), a pattern characteristic of mutant p53. However, the transcriptional activity of p53 was undisturbed in this cell line, which was demonstrated by the induction of p21 and MDM2 proteins after γ-irradiation treatment (Figure 2C, lower panel). In G-84 cells, a characteristic translocation of p53 to the nucleus following γ-irradiation treatment was indicative of a functional p53 pathway suggesting that, in the absence of mutations detected by TGGE in exons 5-8, p53 may be fully functional in this cell line. However, the analysis of p53 target genes expression revealed that p53 was transcriptionally inactive in G-84 cells (Figure 2C). In unstressed G-44 cells, a diffuse cytoplasmic staining reminiscent of the pattern seen in cells with functional wild-type p53 was observed (Figure 2C, upper panel). However, the transcriptional activity of p53 was impaired in G-44 and no nuclear accumulation of p53 occurred after γ-irradiation (Figure 2C, lower panel). Western blot analysis revealed that in G-44 cells the p53 protein is shorter than the full length protein and migrates as a 35 KDa protein (Figure 2C, lower panel).

Mutation analysis by sequencing exons 2-11 of the P53 gene. The inconsistency of the predicted p53 status based on TGGE and immunostaining and the functional status of p53 in G-44, G-84 and G-62 lines suggested that these lines may carry mutations outside of exons 5-8 missed by the TGGE analysis. Therefore, the coding exons 2-11 of the p53 gene were sequenced and for G-84 and G-62 a mutation was detected in exon 4 (G-84: A79⇒V; G-62: D49⇒Y; Table I). The A79⇒Val mutation in G-84 cells can explain the lack of p53-dependent transactivation and the pathological immunostaining pattern in this cell line. The identification of a heterozygous D49⇒Y mutation in G-62 line explained the constitutive nuclear accumulation of p53 and supported the prediction of a non-functional p53 based on the immunostaining analysis. However, the induction of p21 and MDM2 after γ-irradiation treatment showed that the

transcriptional activity of p53 was undisturbed in the G-62 cells, which indicated that a heterozygous D49⇒Y mutation may be silent and does not have a dominant-negative effect on p53 expressed from the wild-type allele in G-62 cells (Figure 2C). The sequencing analysis of G-44 cells detected no mutations outside exons 5-8 but, surprisingly, it identified a homozygous mutation (E224⇒stop) in exon 6 that was not detected by the TGGE analysis (Figure 3). The mutation E224⇒stop identified in G-44 causes a premature termination of the translation leading to a truncated p53 protein lacking 171 C-terminal amino acid residues. In fact, the only detectable form of p53 in G-44 cells was shorter than full length p53 and migrated as a 35 KDa protein (Figure 2C). The 35KDa p53 could only be detected by antibodies specific to the N-terminal (DO-1) and to the central domain (PAb240), but not by the C-terminally directed antibodies PAb421 or PAb122, confirming that 35KDa p53 lacks the C-terminal domain and represents a mutant E224⇒stop p53 protein. Translocation of p53 to the nucleus is essentially dependent on three nuclear localization signals (NLS), which are located beyond codon 224 in the C-terminal region of p53. Therefore, the inability of p53 to translocate to the nucleus in γ-irradiated G-44 cells is consistent with the absence of NLS due to the termination of translation at codon 224 (Figure 2C).

These results demonstrate that the absence of mutations in exons 5-8 (for example in G-84) and the cytoplasmic localization of p53 (in G-44) can not be considered valid criteria for the prediction of a functionally active p53. On the other hand, the presence of certain types of mutations and the constitutive localization of p53 in the nucleus (in G-62) are not always indicative of an inactive p53 protein.

Unphosphorylated Ser376 is a characteristic feature of nuclear p53 in gliomas. In normal cells, the p53 protein is unstable and is present in very low amounts. In gliomas, not only mutant but also wild-type p53 is stabilized by unknown mechanisms. One of the proposed mechanisms for stabilization of mutant p53 proteins in tumor cells is a disrupted p53 - MDM2 feedback loop due to the inability of mutant p53 proteins to transcriptionally activate the *mdm2* gene. The mechanisms that lead to stabilization of wild-type p53 are less clear. In our panel of glioma cell lines, differential reactivity with the phosphorylation-sensitive antibody PAb421 was observed (Figure 4A). This could not be attributed to varying steady-state levels of p53 in different cell lines because the DO-1 antibody detected similar amounts of the protein in PAb421-reactive and non-reactive cell lysates. PAb421 is a phosphorylation-sensitive antibody, which does not bind p53 protein when Ser376 is phosphorylated (38). To test whether the differential reactivity with PAb421 reflected a different phosphorylation status at Ser376, cellular lysates from a PAb421 non-reactive cell line (G-120) were subjected to *in vitro* phosphatase treatment prior to probing with PAb421 in a phosphatase Western blot assay (39). The results showed that the PAb421 reactivity was significantly increased after treatment with shrimp alkaline phosphatase, which confirmed that the lack of reactivity with PAb421 is due to phosphorylation at Ser376 (Figure 4B). Dephosphorylation of Ser376 increases the stability and transcriptional activity of p53 protein in cells with an intact p53 signaling pathway (28). In agreement with these previously reported findings, our experiments demonstrated the detection of a PAb421-reactive form of p53 in normal astrocytes, which was paralleled by the induction of transcriptional activity of endogenous p53 in response to γ-irradiation (Figure 4C). To correlate the phosphorylation status at Ser376 with the transcriptional activity of p53 in glioma cell lines, cell lysates were prepared from γ-irradiated or mock-treated cell lines and probed with PAb421. PAb421 reactive protein was detected in six out of nine cell lines. Three different patterns were observed in this panel of cell lines: a) strong and constitutive PAb421 reactivity, b) transient and weak reactivity induced by γ-irradiation or c) the absence of a PAb421 reactive protein form (Figure 4D and Table II). In three out of four lines in which p53 was transcriptionally active, only one cell line (G-62) showed the presence of a PAb421-reactive protein after γ-irradiation. In G-63, G-120 and G-168, γ-irradiation induced a p53-dependent transcriptional response but no PAb421-reactive p53 protein was detected. In all cell lines with transcriptionally inactivating p53 mutations, a constitutive and strong PAb421 reactivity was observed (Table II). While the constitutive presence of PAb421-reactive p53 did not correlate with the transcriptional activity of the protein, there was a positive correlation with nuclear localization of p53 (Table II). These results suggest that a dephosphorylated status at Ser 376 may be important for nuclear retention and the frequently observed stabilization of p53 in gliomas.

Discussion

In several cancers including breast (40, 41), lung (42), gastric (43) and colorectal tumors, (44) mutations of the *P53* gene are associated with a more aggressive clinical course and a worse prognosis. The significance of *P53* mutations as a prognostic marker in human gliomas is less well documented. Different clinical studies, aiming to correlate mutations in the *P53* gene with the course of the disease, have led to conflicting results. Some authors have suggested that p53 mutations show no association with the overall survival in astrocytic tumors (45, 46). Others have proposed that p53 mutations may be associated with progression in gliomas (47) and poor outcome, for example in pediatric brain tumors (48) and adult gemistocytic

astrocytomas (49). Surprisingly, in a small series of malignant gliomas, the presence of p53 mutations was found to be a positive predictor for response to radiation therapy (50). These conflicting data may well be a result of the problems associated with determination of the functional status of p53 in clinical specimens. Commonly, the functional status of p53 is indirectly concluded from mutation analysis of exons 5-8, representing the region of hot-spot mutations and analysis of the subcellular localization pattern of the p53 protein by immunostaining. Neither of the two approaches allows the drawing of firm conclusions on the functional integrity of the p53 protein or on a functional p53 signaling pathway. This, however, is possible by assessment of the transcriptional activity of p53 detected by the induction of p53-responsive genes upon activation of DNA damage signaling pathways (7). In this study, we analyzed the transcriptional activity of p53 in a set of glioma cell lines and we correlated the results with the p53 status assessed by TGGE analysis of exons 5-8 and the immunostaining pattern of p53. Regarding the prediction of a functional p53 status, TGGE and immunostaining showed high reliability for cell lines in which mutations were detected in exons 5-8. However, in the absence of mutations detected by TGGE or in the presence of normal immunostaining patterns, these analyses were less reliable. Even in this small set of cell lines, we found significant differences between the deferred p53 status based on mutation analysis of exons 5-8 and the functional p53 status. This was explained by the presence of mutations outside of exons 5-8 (G-84 and G-62 lines) and by false-negative results of the TGGE analysis (G-44).

Protein accumulation in a high percentage of nuclei in specimens is characteristic of mutant p53 proteins and, therefore, is considered a marker for mutant and inactive p53 forms. However, our analysis of the transcriptional activity showed that immunostaining patterns do not always reflect the functional status of p53. For example, transcriptionally active wild-type p53 may show constitutive nuclear accumulation characteristic of mutant p53 proteins (G-62) and, *vice versa*, transcriptionally impaired p53 may show an immunostaining pattern characteristic of wild-type protein (G-44 and G-84).

Post-translational modifications play an important role in the regulation of the biochemical activities of p53. Previous studies have established that nuclear accumulation and stability of wild-type p53 protein is regulated by transient post-translational modifications (reviewed in 51, 52). For example, phosphorylation at several sites in both the N-and the C-terminal domains of p53 leads to nuclear retention of the protein (37, 53-57). In gliomas, not only mutant but also wild-type p53 proteins are often stabilized by mechanisms not entirely understood. Our analysis showed that constitutive accumulation of p53 in the nucleus correlates with a dephosphorylated status at Ser376 in glioma cell lines, suggesting that deregulation of mechanisms that control the phosphorylation at Ser376 may be the underlying reason for the stabilization of mutant and wt p53 in gliomas.

Our analysis of the functional status of p53 in gliomas highlights that single parameters such as mutation analysis limited to exons 5-8 or analysis of the subcellular distribution of the protein may not allow reliable conclusions on the functional status of p53. This may explain why the correlation of the p53 status with response to therapy and clinical outcome in these tumors has led to controversial and often inconclusive results. It is possible that the incidence of dysfunctional p53 in glial tumors is much higher then expected, based on the current evaluation of the p53 status in tumor specimens and this may have led to an underestimation of the impact of p53 in the biology of brain tumors.

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