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Advances in Brief

Amplification and Overexpression of the *MDM2* Gene in a Subset of Human Malignant Gliomas without *p53* Mutations[1]

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Abstract

The *MDM2* (murine double minute 2) gene has recently been shown to code for a cellular protein that can complex the *p53* tumor suppressor gene product and inhibit its function. We studied a series of 157 primary brain tumors and report here that the *MDM2* gene is amplified and overexpressed in 8–10% of glioblastomas and anaplastic astrocytomas. Thus, *MDM2* represents the second most frequently amplified gene after the epidermal growth factor receptor gene in these tumor types. Sequencing of the *p53* transcripts in the cases with *MDM2* amplification revealed no mutations and restriction fragment length polymorphism analysis showed, with one exception, no losses of alleles on chromosome 17. Our results indicate that amplification and overexpression of *MDM2* may be an alternative molecular mechanism by which a subset of human malignant gliomas escapes from *p53*-regulated growth control.

Introduction

Different molecular mechanisms have been implicated in the malignant transformation of glial cells and the progression of gliomas. These include amplification and enhanced expression of certain cellular oncogenes as well as inactivation of tumor suppressor genes by mutation or deletion. The gene most frequently amplified in human gliomas is the *EGFR*[4] gene, amplification occurring with an incidence of about 40% in glioblastomas and less than 10% in anaplastic astrocytomas (1). Other genes reported to be occasionally amplified in malignant gliomas include the *MYCN* gene, the *GLI* gene, and the platelet derived growth factor receptor A gene (1). In addition to gene amplification, malignant gliomas are characterized by specific chromosomal deletions which are believed to involve tumor suppressor genes. In gliomas, the best studied tumor suppressor gene is the *p53* gene which has been found to be mutated in a considerable fraction of astrocytomas and glioblastomas (2–4), as well as in occasional cases of oligodendrogliomas (5) and primitive neuroectodermal tumors (6). As in other types of neoplasm, the *p53* mutations in gliomas are frequently associated with loss of one allele (4). However, there are gliomas in which LOH on 17p occurs but which have no *p53* mutations (2, 7).

Another mechanisms of *p53* inactivation is exemplified by certain virally induced tumors in which products of viral transforming genes, such as the adenovirus protein E1B, the SV40 T-antigen, or the human papilloma virus protein E6, bind to the p53 protein and inactivate it presumably through complex formation and/or enhanced degradation (8). Recently, the product of a cellular gene called *MDM2* was shown to be a potential cellular regulator of p53 protein activity (9). The *MDM2* gene was originally cloned as a highly amplified gene from a spontaneously transformed BALB/c 3T3 cell line containing double minutes (10). The *MDM2* gene product is able to form oligomeric complexes with the p53 protein and, when experimentally overexpressed, can abolish its *trans*-activating capability (9). Furthermore, transfection studies have revealed that overexpression of *MDM2* can increase the tumorigenic potential of NIH3T3 cells (11) and overcome wild-type p53 suppression of transformed cell growth (12). Oliner *et al.* (13) recently cloned the human *MDM2* gene, localized it to chromosome 12q13–14, and reported its amplification in a significant percentage of sarcomas of bone and soft tissue.

In the present study we show that the *MDM2* gene is amplified and overexpressed in 8–10% of glioblastomas and anaplastic astrocytomas. None of the tumors with *MDM2* amplification had mutations of the *p53* gene and deletion mapping of chromosome 17 showed LOH only in one case. Our results suggest that amplification and overexpression of *MDM2* may represent an alternative molecular mechanism by which malignant glioma cells escape from *p53*-regulated growth control.

Materials and Methods

Tumor Material and Cell Lines. Paired tumor and blood samples were collected from 157 brain tumor patients, frozen immediately, and stored at −135°C for up to 4 years. To assure that the tumor pieces taken for molecular analysis contained a sufficient proportion of tumor cells, histological evaluation of a part of each piece taken for freezing was performed. All tumors were classified on the basis of the WHO classification of tumors of the central nervous system (14). As control tissue for the expression studies we used nonneoplastic adult human brain tissue (cortex and white matter) from the temporal lobe of a patient given an operation for epilepsy. The human glioblastoma cell line 86HG39 was a kind gift of Dr. T. Bilzer, Department of Neuropathology, University of Düsseldorf.

DNA and RNA Extraction and Analysis. High molecular weight DNA from tumor pieces and blood as well as total RNA from tumor pieces and cultured cells were extracted as described previously (15, 16). For Southern blotting DNA was digested with restriction enzymes, electrophoretically separated in 0.8% agarose gels, and alkali blotted to Hybond-N$^+$ membranes (Amersham). Total RNA was available from 119 of the 157 tumors. For Northern blotting 20 μg of total RNA were electrophoresed over a denaturing 1% agarose gel and blotted to Hybond-N$^+$ membranes. The membranes were hybridized with DNA probes labeled with [^{32}P]dCTP by random priming. Hybridized membranes were exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) which were than analyzed using the Molecular Dynamics PhosphorImager. Densitometric analysis of gene dosage and mRNA expression was performed with the PhosphorImager using ImageQuant. The variable number of tandem repeats probe pYNH24 detecting the anonymous locus *D2S44* was used as reference for the assessment of gene copy number (Fig. 1*b*). A relative increase in signal intensity of more than 5 times that of the normalized constitutional DNA signal was considered as gene amplification. A synthetic 50-base oligonucleotide probe complementary to bases 101–150 in the glyceraldehyde-3-phosphate dehydrogenase mRNA (EMBL Accession No. XO1677) was used as a standard to assess variations in RNA loading of the

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[4] The abbreviations used are: EGFR, epidermal growth factor receptor; *MDM2*, murine double minute 2; LOH, loss of heterozygosity; PCR, polymerase chain reaction; cDNA, complementary DNA.

Northern blots (Fig. 2*c*). A more than 5-fold relative increase in signal intensity than that obtained from the normalized control brain was regarded as significant overexpression.

Probes. Three different probes for *MDM2* were synthesized by reverse transcriptase-PCR amplification. The cDNA sequence for *MDM2* was obtained from Oliner *et al.* (13). The following primer and template combinations were used for probe production: (*a*) glioblastoma cDNA (K6668-90) and PC249 (5′-AAGATGGAGCAAGAAGCCGAGC-3′) and PC250 (5′-TTCCTGCTGATTGACTACTACC-3′) resulting in a 600-base pair fragment from bases 53 to 653 of the human *MDM2* cDNA sequence (probe MDM2a), (*b*) 86HG39 cDNA and PC225 (5′-TAACCACCTCACAGATTCCAGC-3′) and PC226 (5′-CTTCATCTGAGAGTTCTTGTCC-3′) resulting in a 773-base pair fragment from bases 352–1125 of *MDM2* (probe MDM2b), (*c*) control brain cDNA and primers PC251 (5′-TTGGTAGTAGTCAATCAGCAGG-3′) and PC252 (5′-CTTCATCTGAGATTCTTGTCC-3′) resulting in a 495-base pair fragment from bases 630 to 1125 of *MDM2* (probe MDM2c). The identity of the generated probes was corroborated by partial sequencing of approximately 200 bases at each end of each probe, by their correct size, and by their hybridization patterns in Northern blots demonstrating the expected transcript size. Plasmid probes were used for *p53* (php53B, pR4-2), *EGFR* (pE7), as well as a panel of polymorphic DNA markers covering the whole chromosome 17 (see Fig. 3). All probes were purchased from the American Type Culture Collection.

cDNA Sequencing. The p53 transcripts were sequenced after RT-PCR amplification. The sequence starting from within exon 1 to exon 4 was amplified using primers PC277 (5′-CCCTGGAAGATGGAATAAACC-3′) and PC279 (5′-AGACTTGGCTGTCCCAGAATGC-3′) resulting in a 560-base pair fragment. Exons 4 to 10 were amplified with PC180 (5′-TGGTTCACTGAAGACCCAGGTC-3′) and PC181 (5′-AAGGCCTCATTCAGCTCTCGGA-3′) yielding a 860-base pair fragment. The resulting PCR products were reamplified in nested PCR reactions using the biotinylated primer PC 282b (5′-biotin-TGCCATGGAGGAGCCGCAGT-3′) together with PC279 for exons 2–4 and the biotinylated primer PC50b (5′-biotin-GGAATTCTCCATCCAGTGGTTTC) together with PC49 (5′-GGAATTCCCAGAATGCCAGAGGC-3′). Single stranded DNA was then isolated by denaturing and separating the biotinylated from the nonbiotinylated strand using streptavidin-coated magnetic beads (Dynabeads; Dynal AS, Oslo, Norway). Both strands were sequenced using the Sanger dideoxy method with the USB Sequenase Version 2.0 kit (United States Biochemicals, OH).

Immunohistochemistry. Paraffin sections were immunohistochemically stained for p53 protein using the monoclonal antibody DO7 (Dakopatts, Copenhagen, Denmark) in an avidin-biotin-peroxidase method as described previously (17). As positive control we used paraffin sections of a glioblastoma with known immunopositivity for p53 protein. Negative controls were performed by omitting the primary antibody and its substitution with an irrelevant mouse monoclonal antibody.

Results

Among 75 glioblastomas (WHO grade IV) and 27 anaplastic astrocytomas (WHO grade III) we identified 6 glioblastomas and 3 anaplastic astrocytomas that had *MDM2* amplification (Fig. 1, *a*, *c*). The extent of amplification varied from case to case ranging from about 8 to more than 70 times. In one of the cases with *MDM2* amplification we were able to study the first recurrence (GB20) in addition to the primary tumor (GB7). Interestingly, the extent of amplification and overexpression had approximately doubled in the recurrent tumor (Table 1). One glioblastoma with *MDM2* amplification (GB26) showed an amplified rearranged *Eco*RI restriction fragment of approximately 3.0 kilobases in addition to the normal 2.1-kilobase *Eco*RI fragment (Fig. 1*c*). This band was detected only with probes MDM2a and MDM2b but not with MDM2c, which implies that the rearranged band includes bases between 352 and 630 of the *MDM2* cDNA. However, no rearrangement was seen when GB26 DNA was analyzed after digestion with *Taq*I (Fig. 1*a*), *Msp*I, or *Pvu*II. On Northern blots GB26 showed no aberrant *MDM2* transcript and the expression level was increased by a factor equivalent to the amplification level (Fig. 2; Table 1). Amplification of the *EGFR* gene was found in two glioblastomas with *MDM2* amplification (Fig. 1*d*). In

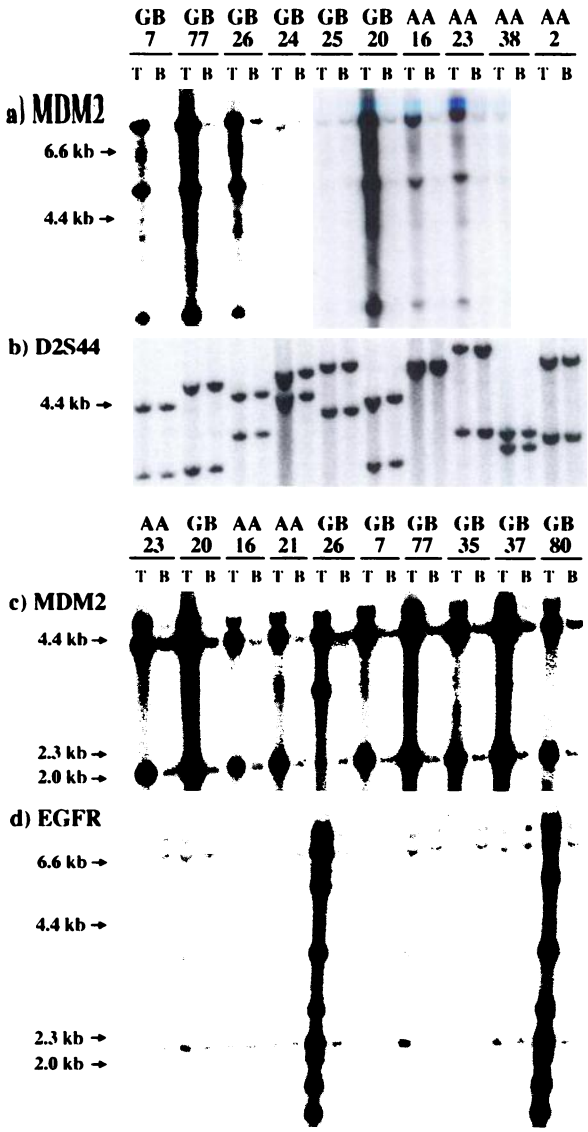

Fig. 1. Southern blot analysis of *MDM2* amplification in malignant gliomas. The case numbers are given on top of the blots (*GB*, glioblastoma; *AA*, anaplastic astrocytoma; GB7 and GB20, the primary and recurrent glioblastoma of the same patient). *Ordinates*, DNA molecular weight marker positions in kilobases (*kb*). Tumor (*T*) and peripheral blood (*B*) DNA of malignant glioma patients was digested with *Taq*I (*a*, *b*) or *Eco*RI (*c*, *d*), electrophoresed, Southern blotted, and subsequently probed with radiolabeled probes. *a*, analysis of *MDM2* amplification in different randomly arranged malignant gliomas using the reverse transcriptase-PCR generated probe MDM2a (bases 53 to 653 of the MDM2 cDNA). The *MDM2* gene is amplified in the tumor DNA of patients GB7, GB77, GB26, GB20, AA16, and AA23. In contrast, the tumors of patients GB24, GB25, AA38, and AA2 have no *MDM2* amplification. *b*, control probing of the same blot as in *a* with the VNTR probe pYNH24 (*D2S44*) to demonstrate approximately equal DNA loading of the lanes. *c*, another Southern blot representing all malignant gliomas with *MDM2* amplification. Probing with MDM2a reveals gene amplification in the tumor DNA of all cases. Note the rearranged amplified band in the tumor DNA of case GB26. *d*, the same blot as in *c* reprobed with the *EGFR* specific probe pE7 demonstrating additional amplification of *EGFR* in the tumors GB26 and GB80. Exposure times and PhosphorImager color ranges are, *a*, 48 h, 10–2500; *b*, 48 h, 10–250; *c*, 24 h, 25–2500; *d*, 24 h, 25–2500.

both tumors the amplification level of *EGFR* was more than twice that of *MDM2* (Table 1). In addition, *EGFR* amplification was detected in 22 of 69 glioblastomas and 5 of 24 anaplastic astrocytomas without *MDM2* amplification. No amplification of *MDM2* or *EGFR* was seen in 16 astrocytomas (WHO grade II), 6 pilocytic astrocytomas (WHO grade I), 8 anaplastic oligodendrogliomas (WHO grade III), 10 oligodendrogliomas (WHO grade II), 5 anaplastic ependymomas (WHO grade III), 3 ependymomas (WHO grade II), and 7 primitive neuroectodermal tumors (WHO grade IV).

On Northern blots a single *MDM2* transcript of approximately 5.5 kilobases, corresponding in size to that reported previously (13), was

Table 1 *Results of densitometric analyses of MDM2 amplification (MDM2 amp), EGFR amplification (EGFR amp), MDM2 mRNA expression (MDM2 mRNA), and p53 mRNA expression (p53 mRNA) in malignant gliomas with MDM2 amplification*

The case numbers are given on top of the table (AA, anaplastic astrocytoma; GB, glioblastoma; CB, control brain). Densitometry was performed with the MolecularDynamics PhosphorImager using the ImageQuant software. Integrated signal intensities obtained for *MDM2* and EGFR on blood and tumor DNA were normalized against the reference probe pYNH24. The quotient between the normalized tumor and blood signal intensity is given as amplification level. A quotient of more than 5 was regarded as gene amplification. On Northern blots integrated signal intensities for *MDM2* or TP53 obtained for each tumor and for control brain were normalized against the reference probe glyceraldehyde-3-phosphate dehydrogenase. The quotient between normalized tumor and control brain signal intensity is given as mRNA expression level. A quotient of more than 5 was regarded as significant overexpression. ND, not done; –, not significantly increased gene copy number. The results of immunohistochemistry for p53 protein (p53 IMH) are represented as semiquantitative estimations: ++, majority of tumor cells immunopositive; +, individual tumor cells immunopositive; –, no immunopositive tumor cells.

	AA23	AA16	AA21	GB26	GB77	GB35	GB37	GB7	GB20	GB80	CB
MDM2 amp	37	16	18	21	41	41	63	41	77	8	–
MDM2 mRNA	7	6	46	18	89	52	83	52	114	6	1
p53 mRNA	2	1	14	4	7	1	3	2	4	ND	1
p53 IMH	–	–	++	+	–	–	–	+	–	–	–	–
EGFR amp	–	–	–	44	–	–	–	–	–	27	–

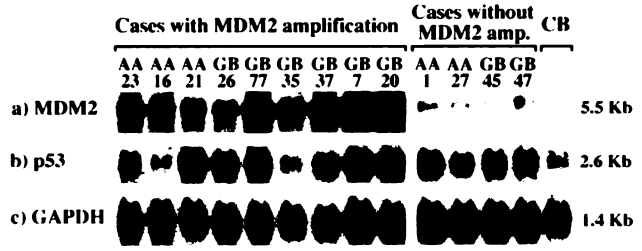

Fig. 2. Northern blot analysis of *MDM2* (*a*) and *p53* (*b*) mRNA expression in malignant gliomas with *MDM2* amplification, without *MDM2* amplification, and in control brain (*CB*). Case numbers are given on top of the blots. *c*, the same blot probed for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as control for RNA loading. *Right ordinate*, approximative transcript sizes in kilobases (*kb*). Exposure times and PhosphorImager color ranges are; *a*, 5 days, 50–2000; *b*, 5 days, 25–200; *c*, 24 h, 25–400.

found in control brain as well as in all gliomas investigated. Significant overexpression as compared to control brain was restricted to the tumors with *MDM2* amplification, which demonstrated an increase in signal intensity of between 6 and more than 100 times the normal (Fig. 2*a*). There was, however, no close correlation between the levels of *MDM2* amplification and transcript overexpression (Table 1). Furthermore, there was no apparent relationship between the levels of *MDM2* and *p53* mRNA expression and *p53* protein abundancy as determined by immunohistochemistry (Table 1).

The tumors with *MDM2* amplification and ten randomly chosen malignant gliomas without *MDM2* amplification were subjected to a detailed deletion mapping of chromosome 17. Only one anaplastic astrocytoma with *MDM2* amplification (AA21) revealed LOH on chromosome 17. This tumor showed loss at all informative loci on both arms of chromosome 17, a finding which is in accord with monosomy 17. In contrast, four of the ten tumors without *MDM2* amplification revealed LOH of at least one informative locus on chromosome 17 and in three of these the LOH was restricted to 17p (Fig. 3). Sequencing of the reverse transcriptase-PCR amplified *p53* transcripts from exons 2 to 10 (includes the highly conserved regions I to V) revealed no mutations in any of the tumors with *MDM2* amplification, including the anaplastic astrocytoma with monosomy 17.

Analysis of *p53* mRNA expression by Northern blotting and p53 protein abundancy by immunohistochemistry showed heterogeneous results in the tumors with *MDM2* amplification (Fig. 2*b*; Table 1). Among these, the anaplastic astrocytoma with monosomy 17 and about 18 times *MDM2* amplification (AA21) demonstrated an increase in *p53* mRNA expression of about 14 times compared to control brain. Immunohistochemically, this tumor showed strong nuclear *p53* immunoreactivity in the majority of its tumor cells. One glioblastoma (GB77) had a *p53* transcript signal intensity 7 times that of control brain but no immunohistochemically detectable *p53*. In the other cases with *MDM2* amplification *p53* mRNA levels were not significantly elevated. Two of these tumors (GB26 and GB37) contained scattered individual p53 protein immunopositive tumor cells, while 6 tumors were completely negative.

Discussion

Mutations in the *p53* gene are a common genetic alteration detected in a variety of human tumors including gliomas (18). Loss of function of this tumor suppressor gene thus appears to be an important step in the complex process of oncogenesis. However, it is very likely that not only the *p53* gene itself but also genes involved in the regulation of its function are potential targets in neoplastic transformation and/or progression. The *MDM2* gene product is believed to act as a cellular regulator of the p53 protein since it can form oligomeric complexes with p53 protein and thereby inhibit at least one of its important functions, *i.e.*, trans-activation of other genes (9). Furthermore, experimental overexpression of *MDM2* can overcome the growth sup-

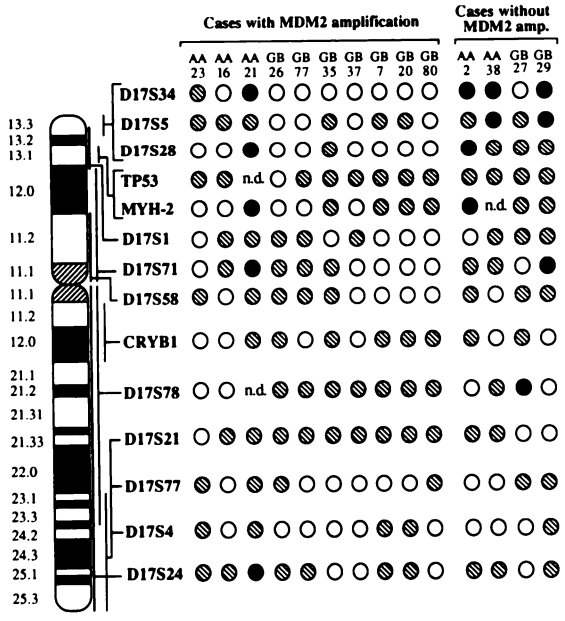

Fig. 3. Schematic representation of chromosome 17 deletion mapping in the malignant gliomas with *MDM2* amplification and in four of ten randomly chosen malignant gliomas without *MDM2* amplification. Case numbers are given on top of each *lane*. *Ordinate*, approximative location of the loci studied. The following probe and restriction enzyme combinations were used to detect restriction fragment length polymorphisms: *D17S34* (p144D6, *MspI*), *D17S5* (pYNZ22.1, *TaqI*); *D17S28* (pYNH37.3, *TaqI*); *p53* (pR4-2, *BglII*); *D17S1* (pHF12-1, *MspI*); *D17S71* (pH10-41, *MspI*); *D17S58* (pEW301, *TaqI*); *CRYB1* (pbeta8-2, *MspI*); *D17S78* (p131-A8, *MspI*); *D17S21* (pC63, *TaqI*); *D17S77* (p128-E1, *TaqI*); *D17S4* (pTHH59; *TaqI*); and *D17S24* (pRMU3, *TaqI*). ○, retention of both alleles in tumor DNA; ⊘, somatic homozygosity (marker not informative); ●, loss of one allele in tumor DNA (LOH). *n.d.*, not determined. Note that only one anaplastic astrocytoma with *MDM2* amplification (AA21) showed LOH in a pattern indicating monosomy 17 while four of the ten malignant gliomas studied without *MDM2* amplification showed LOH on chromosome 17 (GB29, GB27, AA38, AA2).

pressive properties of wild-type p53 protein and may result in neoplastic transformation (11, 12).

Amplification of *MDM2* was first found in certain types of bone and soft tissue sarcomas (13, 19), a tumor group in which cytogenetical abnormalities of chromosome 12 are well documented (20). In malignant gliomas, however, cytogenetic studies have not revealed alterations of this chromosome at more than random frequencies (21, 22). Nevertheless, the *MDM2* gene is amplified and overexpressed in 8–10% of human glioblastomas and anaplastic astrocytomas. Occasionally, amplification of MDM2 may be accompanied by additional genomic rearrangements. This is exemplified by one of our cases, which demonstrated an aberrant amplified *Eco*RI restriction fragment on Southern blots.

At present, *MDM2* represents the gene with the second highest incidence of amplification in malignant gliomas after the *EGFR* gene (1). Interestingly, two of our glioblastomas with *MDM2* amplification had also amplified the *EGFR* gene. Since *MDM2* and *EGFR* are located on separate chromosomes, *i.e.*, 12q13–14 and 7p13–12, one would not expect them to be included in the same amplicon. This is supported by our finding that the amplification level of *EGFR* was different from that of *MDM2* in both tumors (Table 1). The presence of two different amplicons in individual gliomas has not been reported previously; however, it is not unlikely considering recent studies of gene amplification in tumors (23).

Except for the single anaplastic astrocytoma with monosomy 17 we found no LOH on chromosome 17 in our glioma cases with *MDM2* amplification. Even more important, none of these tumors had mutations of the *p53* gene. Our results parallel the findings of Oliner *et al.* (13) in sarcomas and substantiate the hypothesis that tumors with *MDM2* amplification and overexpression need not to have mutations or losses of the *p53* gene for functional inactivation of this tumor suppressor gene. Thus it is likely that a subset of human malignant gliomas escapes from *p53*-regulated growth control by amplification and overexpression of *MDM2*. However, as yet, little is known about the precise functional capabilities of *MDM2* and one cannot exclude the possibility that its overexpression might also promote neoplastic growth by other mechanisms than the inactivation of the p53 protein.

In spite of the fact that none of the tumors with *MDM2* amplification had *p53* gene mutations, analysis of *p53* mRNA expression and immunohistochemistry for p53 protein revealed unexpected heterogeneous results. Two tumors demonstrated significantly elevated *p53* mRNA levels. In one of them this did not result in increased p53 protein levels, whereas the other showed strong nuclear immunostaining of most tumor cells. Furthermore, although the remaining tumors with *MDM2* amplification did not show significantly elevated *p53* mRNA expression, two of them contained individual p53 protein positive tumor cells. At present, we do not know whether the p53 protein detected in these cases has retained its normal function or has been inactivated by complexing with MDM2 protein or by other mechanisms. In any case, our results underline the complexity of the disturbances of p53 protein homeostasis in some malignant gliomas and suggest that, in addition to *p53* gene mutations and *MDM2* overexpression, further factors may disturb the normal metabolism of *p53* in these cases. We conclude that a significant subset of malignant gliomas has amplification and overexpression of *MDM2* and may thereby escape from *p53*-regulated growth control.

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