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Amplification and Overexpression of MDM2 in Primary (de novo) Glioblastomas

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Abstract. Glioblastoma multiforme (WHO Grade IV), the most malignant neoplasm of the human nervous system, develops rapidly de novo (primary glioblastoma) or through progression from low-grade or anaplastic astrocytoma (secondary glioblastoma). We recently reported that mutations of the p53 gene are present in more than two-thirds of secondary glioblastomas but rarely occur in primary glioblastomas, suggesting the presence of different genetic pathways (Watanabe et al, Brain Pathol 1996;6:217–24). In the present study, primary and secondary glioblastomas were screened by immunohistochemistry for MDM2 overexpression and by differential PCR for gene amplification. Tumor cells immunoreactive to MDM2 were found in 15 of 29 primary glioblastomas (52%), but in only 3 of 27 secondary glioblastomas (11%; P=0.0015). MDM2 amplification occurred in 2 primary (7%) glioblastomas but in none of the secondary glioblastomas. Only one out of 15 primary glioblastomas overexpressing MDM2 contained a p53 mutation. These results suggest that MDM2 overexpression with or without gene amplification constitutes a molecular mechanism of escape from p53-regulated growth control, operative in the evolution of primary glioblastomas that typically lack p53 mutations.

Key Words: Amplification; MDM2; Overexpression; Primary glioblastoma; Secondary glioblastoma.

INTRODUCTION

Glioblastoma multiforme (WHO Grade IV) is the most frequent and malignant neoplasm of the human nervous system, with a mean survival time of usually less than 1 year after clinical diagnosis. Glioblastomas may arise rapidly de novo, i.e. without clinical or histologic evidence of a less malignant precursor lesion, and they have been designated as primary glioblastomas. In contrast, secondary glioblastomas develop more slowly by malignant progression from low-grade (WHO Grade II) or anaplastic astrocytomas (WHO Grade III) (1). Primary and secondary glioblastomas are regarded as histologically indistinguishable but occur in different age groups, the mean age at diagnosis being approximately 55 years for primary and 40 years for secondary glioblastomas (2).

The progression of low-grade astrocytomas is associated with the sequential acquisition of genetic alterations. Loss of heterozygosity (LOH) on chromosome 17p, p53 mutations and overexpression of the PDGF receptor are the earliest detectable changes (3–5). In addition, anaplastic astrocytomas contain LOH on chromosome 19q, homozygous deletion of the p16 tumor suppressor gene, amplification of the CDK4 gene, and alteration of the Rb gene (3, 6). Loss of heterozygosity on chromosome 10 is largely restricted to glioblastomas (3, 7). In contrast, amplification and/or overexpression of EGFR is a hallmark of the primary (de novo) glioblastoma that develops through a different molecular pathway (2, 7–9). In 2 groups of patients with primary or secondary glioblastoma selected under stringent clinical and histologic criteria, primary glioblastomas had a high frequency (63%) of EGFR overexpression but rarely had p53 mutations (11%), whereas secondary glioblastomas showed a high incidence of p53 mutations (67%) but infrequently showed EGFR overexpression (10%). The occurrence of p53 mutations and EGFR overexpression appeared to be mutually exclusive (2).

MDM2 is located on chromosomal region 12q13–14 (10) and binds to p53 protein, thereby eliminating its ability to function as a transcription factor (10, 11). Up to 10% of glioblastomas showed MDM2 amplification and mRNA overexpression (12, 13), and these neoplasms lacked p53 mutations (12). In the present study, we analyzed MDM2 gene amplification and immunoreactivity to MDM2 in biopsies from patients classified on clinical and histologic criteria as primary or secondary glioblastomas. The results obtained show that the incidence of MDM2 overexpression at the protein level was significantly higher than MDM2 amplification and that overexpression occurs almost exclusively in primary glioblastomas.

MATERIALS AND METHODS

Tumor Samples

Surgical specimens were from patients treated in the Department of Neurosurgery, University Hospital, Zürich, Switzerland, between 1977 and 1994. Twenty-nine patients with primary glioblastoma had a preoperative clinical history of less than 3 months (mean, 1.8 months) and histologic diagnosis of a glioblastoma at the first biopsy, without any evidence of a less malignant precursor lesion. Twenty-seven patients with secondary glioblastomas had at least 2 biopsies, with clinical and histologic evidence of progression from low-grade (20 cases) or anaplastic astrocytomas (7 cases). The age and sex of the

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patients and the location of tumors are shown in Table 1. For most of these tumors (except for cases 5, 10, 11, 15, 16, 18, 20, 21, 25, and 28), data on p53 mutations and p53 immunohistochemistry have been previously communicated (2). In 3 patients with secondary glioblastoma (Table 1, cases 35, 37, and 51), 2 glioblastomas obtained at separate surgical interventions were analyzed.

MDM2 Immunohistochemistry

Histologic sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in graded ethanol. The endogenous peroxidase was blocked by the incubation in 5% H_2O_2 solution in methanol for 20 minutes (min). The sections were boiled in 10 mM sodium citrate (pH 6.0) 3 times for 4 min in a microwave oven and subsequently incubated in 5% skimmed milk for 2 hours (h) at 4°C. After being rinsed once in distilled water and twice in PBS for 5 min, sections were incubated overnight at 4°C with the monoclonal antibody MDM2 (clone IF2, Oncogene Science, Uniondale, NY, USA) at a concentration of 0.01 mg/ml. The reaction was visualized using Vectastain elite ABC kit and a 3,3′-diaminobenzidine solution (Vector Laboratories, USA). Sections were slightly counterstained with hematoxylin. Staining without primary antibody served as a negative control, while formalin-fixed, paraffin-embedded sections of a liposarcoma with MDM2 amplification were used as a positive control.

Only unequivocal nuclear immunoreactivity was counted as positive while cytoplasmic staining was regarded as nonspecific. Fractions of positive cells were recorded as + (less than 5%), ++ (5–50%), and +++ (more than 50%).

p53 Alterations

p53 protein accumulation was assessed using the monoclonal antibody PAb 1801, as previously described (2). Mutations of the p53 gene were identified by SSCP and DNA sequencing, as previously reported (2).

Differential PCR

DNA was extracted from paraffin sections as described previously (14). Areas of the tumors with the highest immunoreactivity were selected for DNA extraction. In MDM2-negative tumors the area with clear histologic appearance of glioblastoma was chosen for the analysis.

PCR was carried out as described previously with some modifications (15). Briefly, 3 μl of template was amplified in 25 μl of a reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM primers for MDM2 and 0.5 μM primers for dopamine receptor reference gene, and 0.625 U Taq DNA polymerase (Boeringher Mannheim, Germany). After denaturing DNA at 95°C for 3 min, 25 cycles of PCR (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) were carried out in an automated DNA Thermal Cycler (Perkin Elmer Cetus) with a final extension at 72°C for 5 min. The primer sequences for differential PCR were as follows (15): 5′-GAGGGCTTTGATGTTCCTGA (sense) and 5′-GCTACTAGAAGTTGATGGC (anti-sense) for MDM2 and primers 5′-CCACTGAATCTGTCCTGGTATG (sense) and 5′-GTGTGGCATAGTAGTTGTAGTGG (antisense) for the dopamine receptor gene. After PCR, the entire reaction product was electrophoresed on an 8% polyacrylamide gel and stained by ethidium bromide. Gels were photographed using Polaroid film and the intensity of MDM2 fragment (143bp) and dopamine receptor reference sequence (113bp) was measured by means of a densitometer GS-670 (Bio-Rad). More than 4 of MDM2/dopamine receptor ratio was regarded as positive for MDM2 amplification.

Statistical Analyses

Fisher's exact test was carried out to compare the incidence of MDM2 overexpression between primary and secondary glioblastomas.

RESULTS

MDM2 immunoreactivity presented as clear nuclear staining in neoplastic cells (Fig. 1). Of 29 primary glioblastomas, 15 (52%) contained cells immunoreactive to MDM2, whereas in secondary glioblastomas, the incidence was much lower (3 of 27; 11%, P=0.0015, Tables 1 and 2). In the majority of primary glioblastomas with MDM2 overexpression, a significant fraction of tumor cells were positive (Fig. 1A), while less than 5% of neoplastic cells were immunoreactive in the 3 secondary glioblastomas with MDM2 overexpression (Fig. 1B, Table 2).

Differential PCR showed a similar intensity of 143bp MDM2 fragment and 113bp reference dopamine receptor fragment in all cases except for 2 primary glioblastomas (cases 8 and 12; Table 1 and Fig. 2). These 2 primary glioblastomas showed overexpression of MDM2 in more than 50% of neoplastic cells (Table 1 and Fig. 1A). MDM2 amplification was not detected in any of the secondary glioblastomas (Table 1). Neither of the primary glioblastomas with MDM2 amplification contained a p53 mutation (Table 1). There was no correlation between MDM2 overexpression and p53 mutation or p53 protein accumulation.

DISCUSSION

The p53 protein is a sequence-specific transcription factor that activates genes containing p53-response elements (16–18). The MDM2 gene contains a p53 DNA-binding site and it has been shown that in a variety of conditions the transcription of MDM2 is strongly induced by wild-type p53 (19–24). The MDM2 protein, in turn, forms a complex with p53, thereby abolishing its transcriptional activity (11, 20, 21, 24). Thus, in normal cells this autoregulatory feedback loop regulates both the activity of the p53 protein and the expression of the MDM2 (21, 25–27). An increase in p53 levels would block the

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TABLE 1
Amplification and Overexpression of MDM2 in Primary and Secondary Glioblastomas

Case no.	Patient no.	Age/sex	Location	MDM2 IHC	MDM2/ DR ratio	*p53* IHC	*p53* mutation		
							Exon	Codon	Nucleotide
Primary Glioblastomas									
1.	N94-604	34/M	T	++	0.69	+	7	239	AAC → TAC
2.	N94-498	35/M	FrT	−	0.7	−			
3.	N94-962	39/F	T	−	1.7	−			
4.	N94-789	46/F	TO	−	2.30	−			
5.	N86-31822	47/M	P	−	1.32	++			
6.	N94-855	48/M	P	−	3.9	−			
7.	N94-30	50/M	T	−	1.25	+			
8.	N94-1794	51/M	FrT	+++	17.1	+			
9.	N94-819	54/M	TPO	++	1.88	−			
10.	N85-28007	55/F	T	++	2.45	+			
11.	N86-32391	58/F	Fr	++	1.44	+			
12.	N94-229	58/M	O	+++	11.7	+			
13.	N94-1908	58/M	FrT	−	ND	−			
14.	N94-1916	58/M	T	+	0.69	−			
15.	N85-28006	58/M	FrP	−	2.37	++	5	169	1 bp del.
16.	N86-3843	59/F	TPO	−	0.87	−			
17.	N94-356	61/F	TO	+	0.5	−			
18	N86-26812	63/F	T	++	1.22	−			
19.	N94-609	63/M		++	0.6	+			
20.	N86-17679	63/M	T	+	0.58	++			
21.	N86-19214	63/M	Fr	+	0.73	++	8	273	CGT → TGT
22.	N94-9	65/F	T	−	0.61	+			
23.	N94-519	66/F	F	−	1.24	++			
24.	N94-1665	69/F	TO	+	0.9	−			
25.	N86-22011	69/M	FrP	++	1.25	+			
26.	N94-1103	70/F	FrT	−	1.85	−			
27.	N94-1402	73/M	FrT	−	2.29	++	7	248	CGG → TGG
28.	N86-28504	73/M	PO	+	1.45	+			
29.	N94-1526	78/F	P	−	2.33	−			
Secondary Glioblastomas									
30.	N92-200	23/F	Fr	−	0.82	+	8	301	2 bp del.
31.	B80-16597	23/F	T	−	1.91	−			
32.	N91-514	24/M	Fr	−	0.74	−			
33.	N91-354	25/F	T	−	0.48	−			
34.	N90-72	25/F	TP	+	2.7	−			
35.	B82-25100	25/M	Fr	−	1.14	++	5	175	CGC → CAC
	B83-116			−	ND	++	5	175	CGC → CAC
36.	B83-4259	29/F	Fr	+	0.63	++	5	163	TAC → TGC
37.	N92-414	29/F	Fr	−	2.41	++	8	273	CGT → TGT
	N94-1055			−	0.61	++	8	273	CGT → TGT
38.	B81-2019	29/F	T	−	ND	++	8	278	CCT → CTT
39.	B82-26732	30/F	FrP	+	0.82	++	8	273	CGT → TGT
40.	B87-22194	30/M	Fr	−	1.47	+	8	300	90 bp del.
41.	B83-28811	32/F	BG	−	2.41	++	5	163	TAC → TGC
42.	B85-23214	34/M	FrP	−	1.39	++	5	175	CGC → CAC
43.	N90-361	35/M	FrT	−	1.45	+	5	175	CGC → CAC
44.	B83-21529	39/F	T	−	1.2	++	8	275	TGT → TTT
45.	B90-7648	39/M	Fr	−	2.12	++	8	275	15 bp del.
46.	N90-144	40/F	T	−	0.69	+	7	256	1 bp del.
47.	B84-27872	40/M	Fr	−	1.25	−			
48.	B77-3229	44/M	TP	−	0.38	+	8	280	AGA → AAA
49.	B85-29270	45/F	T	−	1.29	−			
50.	B88-33616	49/F	Fr	−	1.31	++	8	278	CCT → ACT
51.	B84-33417	51/M	T	−	0.64	++	8	273	CGT → TGT
	B87-9111			−	1.29	++	8	273	CGT → TGT
52.	N90-232	52/M	BG	−	0.71	−			
53.	B86-33873	74/M	T	−	0.68	−			

The results of MDM2 immunohistochemistry were recorded as negative (−), less than 5% immunoreactive tumor cells (+), 5 to 50% immunoreactive tumor cells (++), or more than 50% immunoractive tumor cells (+++). MDM2/DR, MDM2/dopamine receptor gene ratio. The results of *p53* immunohistochemistry and *p53* mutations have been previously reported (except for cases, 5, 10, 11, 15, 16, 20, 21, 25, and 28) (2). T, temporal; O, occipital; P, parietal; Fr, frontal; BG, basal ganglia; IHC, immunohistochemistry.

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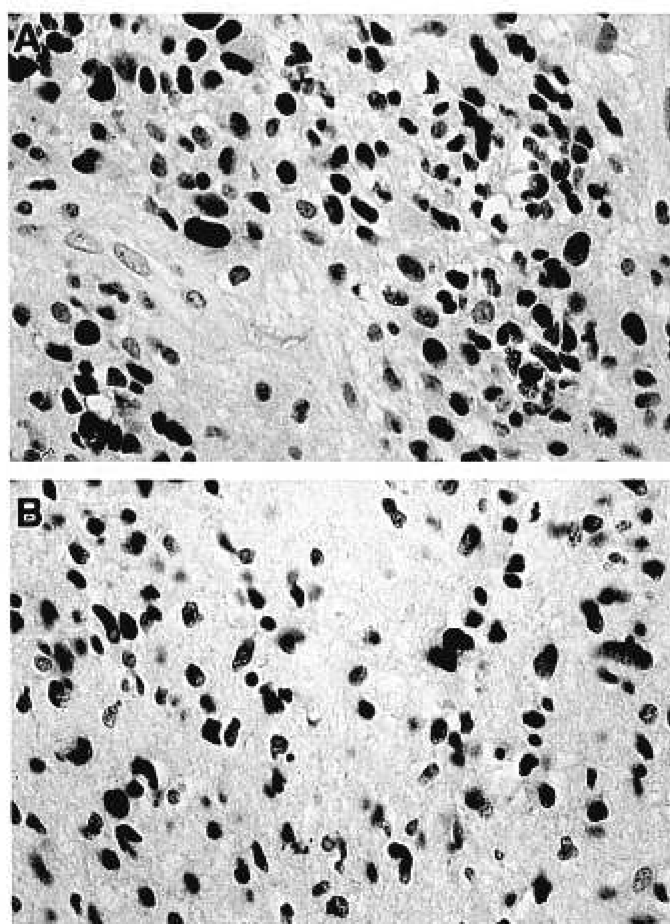

Fig. 1. MDM2 immunohistochemistry. Note the expression of MDM2 in the majority of tumor cell nuclei of a primary glioblastoma (A, case 8). Only a few cells are immunoreactive in the secondary glioblastoma (B, case 36).

TABLE 2
MDM2 Immunoreactivity in Primary and Secondary Glioblastomas

Glioblastoma subtype	Number of cases	Fraction of immunoreactive cells		
		<5%	5 to 50%	>50%
Primary glioblastoma	n = 29	6 cases (21%)	7 cases (24%)	2 cases[a] (7%)
Secondary glioblastoma	n = 27	3 cases (11%)	— (0%)	— (0%)

[a] Both cases showed MDM2 gene amplification.

entry into cycle at the late G_1 checkpoint; at the same time, p53 would induce the expression of MDM2, resulting in p53-MDM2 complex formation that may overcome the G_1 checkpoint and allow entry into the S-phase of the cell cycle (28).

In the present study, we present evidence that overexpression of MDM2 with or without gene amplification

Fig. 2. Differential PCR of primary and secondary glioblastoma DNA for gene amplification of MDM2 (143 bp fragment). The dopamine receptor gene was used as a reference (DR, 113 bp fragment). Two primary glioblastomas (cases 8 and 12) showed MDM2 amplification.

is a genetic hallmark of the rapidly evolving primary (de novo) glioblastoma that typically does not contain a p53 mutation (2). This is consistent with previous studies showing that the gliomas and sarcomas with MDM2 amplification typically lack p53 mutations (12, 29). It has been reported that MDM2 gene amplification is a rare event, occurring in 5 to 10% of glioblastomas (12, 13); the present study (7% incidence) corroborates this experience. Our results differ from earlier reports with respect to the fraction of glioblastomas with MDM2 overexpression. Reifenberger et al (12) found that mRNA overexpression as determined by Northern blots occurs only in glioblastomas with gene amplification. In contrast, our study using immunohistochemistry suggests that more than 50% of primary glioblastomas contain cells overexpressing MDM2, although the fraction of immunoreactive cells varied considerably (Table 2).

MDM2 amplification and overexpression at the mRNA and/or protein level have been found in a variety of human neoplasms including soft tissue sarcoma (29–31), osteosarcoma (30), testicular germ cell tumor (32), as well as breast (33, 34), urothelial (35, 36), and lung carcinoma (37). However, the relation between MDM2 amplification and overexpression is complex, since tumors may show simultaneous amplification and overexpression, amplification without overexpression, or overexpression without amplification (31). It has been reported that in human bladder (36) and breast cancer (33, 34), MDM2 overexpression detectable by immunohistochemistry is significantly more frequent than MDM2 gene amplification. In contrast, Marchetti et al (37) reported a similar incidence of MDM2 amplification estimated by Southern blot and overexpression assessed by Northern blot and immunohistochemistry in human lung cancer. In the present study, MDM2 overexpression in primary glioblastoma was significantly more frequent than gene amplification. Only 2 primary glioblastomas showed gene amplification, and these were the cases which showed the strongest immunoreactivity to MDM2, with more than 50% of tumor cell nuclei staining (Fig. 1A). The absence of MDM2 amplification in tumors with MDM2 overexpression may be due in part to a dilution effect, since some biopsies (marked + in Table 1) contained less than 5% immunoreactive cells, and selective

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gene amplification in this minority of tumor cells may not have been detectable by differential PCR. Alternatively, MDM2 overexpression may be due to enhanced translation as previously observed in a choriocarcinoma cell line (38).

Although the present study clearly shows that MDM2 overexpression occurs in a significant fraction of primary glioblastomas, its role in the evolution of this highly malignant glioma remains to be explained. Our immunohistochemical analysis suggests that only a fraction of tumor cells are affected. Inactivation of p53 by complex formation may lead to escape of these cells from cell cycle control, but it remains unclear whether this effect contributed significantly to the overall malignancy of this neoplasm. Concurrent overexpression of EGFR may occur, but co-expression by the same tumor cells remains to be shown. Recent studies indicate that MDM2 may physically and functionally interact with the retinoblastoma gene product protein pRB and thereby inhibit its key regulatory role in the cell cycle (39). Furthermore, overexpression of MDM2 resulted in protection from apoptosis, and this effect was seen only under conditions allowing the formation of p53-MDM2 complexes, suggesting that one of the roles of MDM2 may be to modulate the apoptotic activity of p53 (40).

While the functional significance of MDM2 overexpression in malignant gliomas remains to be explained, the present study clearly demonstrates that this change, if present, is associated with the development of the most malignant human brain tumor, the primary glioblastoma.

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