Amplification and Overexpression of *mdm2* Gene in Ependymomas

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Ependymomas rarely show *p53* gene alteration, and the tumorigenic mechanism of ependymomas still remains to be elucidated. We investigated the amplification and overexpression of *mdm2* gene, whose product (MDM2) is considered to be one of the major cellular regulators of *p53*-mediated growth control, in 26 specimens of ependymomas obtained from 20 patients. The majority of the ependymomatous samples (96%) showed at least focal immunopositivity for MDM2; however, only 8% of the samples were immunopositive for *p53*. *mdm2* gene amplification was detected in 35% of the samples by differential polymerase chain reaction, all of which overexpressed MDM2. These results suggest that the amplification and/or overexpression of *mdm2* may be one of the major molecular events occurring in the tumorigenesis of ependymomas.

KEY WORDS: Ependymoma, Gene amplification, Immunohistochemistry, *mdm2*, Oncogene, *p53*.

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Relatively few studies have focused on the molecular biology of ependymomas in comparison to astrocytic tumors (1). In contrast to astrocytomas, *p53* gene aberration is rarely detected in ependymomas (2–4), although a germline mutation has been described in one patient with anaplastic ependymoma (5, 6). The findings therefore indicate the existence of tumorigenic mechanisms of ependymomas that escape from normal *p53*-related cell cycle regulation.

Oncogene *mdm2*, localized to chromosome 12q13–14 (7), was originally cloned as a highly amplified gene from spontaneously transformed

BALB/c 3T3 cell lines (8). The *mdm2* gene product (MDM2) binds to *p53* protein and inactivates its transactivating capability and is thus believed to act as a cellular regulator of the *p53* protein. When overexpressed, MDM2 is capable of increasing the tumorigenic potential of NIH3T3 cells (9). Furthermore, *mdm2* has been reported to be amplified and/or overexpressed in a variety of human neoplasms, such as sarcomas (7, 10–13), lymphomas (14, 15), breast cancers (16, 17), lung cancers (18), and testicular germ cell tumors (19).

Regarding neuroepithelial tumors, Newcomb and colleagues (20, 21) failed to detect *mdm2* amplification in any grade of astrocytomas. However, Reifenberger *et al.* (22, 23) reported that up to 10% of glioblastomas showed *mdm2* amplification, and these samples lacked *p53* mutations. On the other hand, MDM2 overexpression in astrocytic tumors has been detected in 29% of low-grade astrocytomas, 13 to 43% of anaplastic astrocytoma, and 46 to 59% of glioblastomas (21, 24, 25). Recently, Biernat *et al.* (26) described that *mdm2* was amplified and/or overexpressed preferentially in primary glioblastomas, which typically lack *p53* mutations. Reifenberger *et al.* (22) and Tong *et al.* (27) also tested ependymal tumors, and both of them mentioned that almost no *mdm2* amplification was detected. They did not, however, study the MDM2 protein expression. As discussed in the previous studies of glioblastomas (26), as well as those of lymphomas (14, 15), MDM2 was found to be overexpressed in some types of neoplasms without *mdm2* gene amplification.

These previous findings prompted us to investigate the amplification and expression of the *mdm2* gene in various subtypes of ependymomas in an extended series. In the current study, we screened biopsied ependymomatous samples for *mdm2* gene amplification by differential polymerase chain reaction (PCR) and for its overexpression by an immunohistochemical analysis. We discuss the implications of our findings in the tumorigenic mechanisms of ependymomas.

MATERIALS AND METHODS

Ependymomatous Samples

Twenty-six ependymomatous specimens from 20 patients were obtained at surgery. Of these, six specimens were recurrent tumors sampled from four patients. The specimens were immediately immersed in 10% buffered formalin and were fixed overnight. They were then embedded in paraffin. The sections were routinely stained with hematoxylin and eosin. According to the revised World Health Organization classification (28), the specimens included 4 samples of myxopapillary variant taken from 2 patients, 13 samples of common ependymoma from 11 patients, and 9 samples of anaplastic variant from 7 patients. The patients' age and sex, the location of the tumor, and the histology are summarized in Table 1.

Immunohistochemistry

Anti-MDM2 monoclonal antibody (mAb) (clone Ab1) and anti-p53 mAb (clone Pab1801) were purchased from Calbiochem (La Jolla, CA) and Novocastra (Newcastle, UK), respectively. Immunohistochemical analysis for MDM2 and p53 were carried out by biotin-streptavidin method as described elsewhere (29). Briefly, the sections were dewaxed and incubated in 0.3% H₂O₂/methanol for 30 min, followed by antigen retrieval by autoclaving in 10 mM

citrate buffer pH 6.0 at 120° C for 10 min. After being washed in Tris-HCl buffer (TB) pH 7.6, the sections were incubated with Ab1 (1:10) or Pab1801 (1:20) at 4° C overnight. The sections were then washed in TB and incubated with biotinylated anti-mouse immunoglobulin (Amersham, Buckinghamshire, UK; 1:200) for 60 min. After being washed in TB, the sections were incubated with horseradish peroxidase-conjugated streptavidin (Amersham; 1:300) for 30 min. The sections were washed in TB, and the reaction products were visualized with diaminobenzidine. In both MDM2 and p53 immunostaining, nuclear staining was judged as positive. We regarded weak staining as positive and indicative of an overexpression of proteins, because both MDM2 and p53 are hardly detected immunohistochemically in mature tissue specimens under normal cell cycle regulation because of their short half-life. We classified the immunopositivity into 2+ (more than 50% of cells are positive), + (fewer than 50% of cells are positive), ± (only a few positive cells), and – (no positive cells). In cases of a heterogeneous distribution of the labeled nuclei, we assessed the immunopositivity in the area with the highest density of stained nuclei.

Differential PCR

DNA was extracted as previously described (30) from the histologically verified, paraffin-embedded

TABLE 1. Case Profiles of *mdm2* Gene Amplification and Overexpression in Ependymomas

Case No ^a	Age/Sex	Location	MDM2 IHC	MDM2/DR2 Ratio	MDM2 DPCR	p53 IHC
Common type						
C1	1/M	4th ventricle	2+	1.48	–	–
C2	5/M	CP angle	±	0.96	–	–
C3	6/M	4th ventricle	2+	2.01	–	–
C4	26/F	4th ventricle	2+	11.39	+	–
C5	29/M	Supratentorial	2+	7.66	+	–
C5'	35/M	Supratentorial	+	3.20	–	–
C6	35/M	Spinal cord	2+	4.09	+	±
C7	36/M	Spinal cord	2+	2.75	–	+
C8	43/M	4th ventricle	2+	8.34	+	–
C9	44/F	4th ventricle	2+	15.59	+	–
C9'	44/F	4th ventricle	2+	5.48	+	±
C10	45/M	Supratentorial	2+	1.35	–	–
C11	62/M	Spinal cord	+	11.24	+	–
Anaplastic type						
A1	2/M	4th ventricle	2+	0.78	–	±
A2	2/M	Supratentorial	+	0.92	–	–
A3	2/M	Supratentorial	2+	1.88	–	±
A3'	2/M	Supratentorial	2+	8.07	+	±
A3''	12/M	Supratentorial	2+	9.37	+	–
A4	5/F	4th ventricle	2+	2.91	–	±
A5	14/F	4th ventricle	+	1.50	–	±
A6	42/M	Supratentorial	2+	1.43	–	2+
A7	68/M	Supratentorial	2+	1.15	–	±
Myxopapillary type						
M1	16/M	Spinal cord	2+	1.40	–	–
M2	28/M	4th ventricle	2+	0.75	–	±
M2'	28/M	Cauda equina	2+	1.67	–	±
M2''	32/M	4th ventricle	2+	2.21	–	–

IHC, immunohistochemistry; DPCR, differential polymerase chain reaction; M, male; F, female; CP angle, cerebellopontine angle; MDM2/DR2 ratio, ratio of the intensity of *mdm2* band to dopamine receptor 2 band.

^a A numeral with prime mark means a recurrence of the tumor.

FIGURE 1. Immunohistochemistry for MDM2. **A**, sample C9', common ependymoma. **B**, sample M1, myxopapillary type. In both cases, more than half of the tumor cells show nuclear staining with varying intensities. These samples were evaluated as 2+. **C**, sample A5, anaplastic ependymoma. Fewer than half of the tumor cells show nuclear staining. This sample was evaluated as + (bar = 50 μ m).

ependymoma specimens. Differential PCR was performed to investigate the amplification of *mdm2* gene according to the protocols reported previously (26, 31), with some modifications. Briefly, 5 μ l of a template was amplified in 50 μ l reaction mixture containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M primers for *mdm2*, and 0.5 μ M primers for dopamine receptor 2 gene (*DR2*) as reference (26) and 1.5 U *Taq* DNA polymerase (Promega, Madison, WI). After denaturation 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 performed with a final extension at 72° C for 5 min. The PCR products were electrophoresed in 8% polyacrylamide gel and then stained with ethidium bromide. The intensity of the fluorescence of each band was estimated semiquantitatively with Fluoroimager 595 and ImageQuant version 4.2-J (Molecular Dynamics, Sunnyvale, CA). Any samples showing an intensity ratio of more than 4 for *mdm2* and *DR2* were considered to be positive for *mdm2* amplification. Nontumoral DNA extracted from peripheral white blood cells of 21 healthy volunteers was also tested as controls.

RESULTS

Immunohistochemistry

By MDM2 immunohistochemistry, 21 specimens (81%) showed 2+, 4 (15%) showed +, and 1 (4%) showed $\pm$. Thus, the majority of specimens showed at least focal immunopositivity for MDM2 (Fig. 1). Positive nuclei showed variable staining intensity.

The immunopositivity did not correlate with the anaplastic features described in the World Health Organization classification (28); however, it was interesting to note that both cases of myxopapillary ependymoma showed immunopositivity to MDM2 (Fig. 1B). One exception was a unique case of anaplastic ependymoma, Case A2, which showed the features of clear cell ependymoma intermingled with foci with increased cellularity, prominent mitotic figures, and apoptosis, thus indicating focal anaplastic transformation (Fig. 2C). This case demonstrated MDM2 immunopositivity preferentially in the anaplastic foci (Fig. 2D), whereas only a small population of the tumor cells were stained in the less cellular, clear cell ependymoma sites (Fig. 2A, B).

Only two specimens (8%, corresponding to 10% of the cases) showed immunopositivity for p53 (one 2+ sample and one + sample). Both p53-positive specimens were also MDM2 positive, whereas most of the MDM2-positive specimens (96%, corresponding to 89% of the MDM2-positive cases) were negative for p53 (Table 1). Both MDM2 and p53 immunostaining tended to show a relatively intense positivity in cells adjoining necrotic foci, probably secondary to ischemia (32). We therefore evaluated the overall positivity of each section to the exclusion of such epigenetic events in the perinecrotic areas.

Differential PCR

None of the 21 nontumoral DNA samples showed *mdm2* amplification (*mdm2/DR2* ratio ranging

FIGURE 2. Clear cell ependymoma showing focal anaplastic transformation (Sample A2). **A** and **B**, clear cell ependymoma site. Hematoxylin-eosin staining (HE) (**A**) shows diffuse proliferation of clear cell type ependymoma. MDM2 immunostaining (**B**) reveals that the majority of tumor cells in the clear cell ependymoma area are immunonegative for MDM2. **C** and **D**, anaplastic site. Dense proliferation of anaplastic tumor cells forming ependymal rosettes is evident (**C**, HE). Both mitotic figures and apoptotic bodies are frequently seen. MDM2 immunostaining of the anaplastic foci (**D**) shows immunopositivity in the nuclei of the tumor cells in more cellular, anaplastic tissue, whereas much less staining was seen in the intervening clear cell ependymoma tissue (bar = 50 μ m).

FIGURE 3. *mdm2* amplification demonstrated by differential polymerase chain reaction. C6 and A3'' are considered to be positive for *mdm2* amplification. N1-N6 shows results in nontumoral DNA. *mdm2*/DR2 ratio of each lane is given below the gel image.

from 0.84 to 1.06). In ependymomatous samples, *mdm2* amplification was detected in nine specimens (35%), seven of which were common ependymomas from six cases and two of which were anaplastic ones from the recurrent samples of the same case (sample A3', A3''). The myxopapillary type did not show *mdm2* amplification. Except for case A3, all patients were adults and had common ependymomas. A representative gel image is shown in Figure 3. All nine specimens showed immunopositivity for MDM2 (Table 1).

DISCUSSION

The *mdm2* gene is transactivated by p53 protein, and, conversely, MDM2 binds to the p53 protein and inhibits its transactivating capability, thus

forming a negative-feedback loop between the molecules (33, 34). In addition, recent reports have demonstrated that MDM2 promotes the rapid degradation of p53 protein (35, 36). MDM2 is therefore believed to act as a cellular regulator of p53-mediated growth control.

In our study, the majority of the ependymomatous specimens showed at least focal immunopositivity for MDM2, whereas only two specimens (8%) showed immunopositivity for p53. Furthermore, *mdm2* gene amplification was detected in up to 35% of the 26 surgical specimens. These findings are consistent with previous reports describing that ependymomas rarely show *p53* gene mutations (2-4) and that neoplasms with *mdm2* amplification typically lack *p53* mutations (12, 22). The immunohistochemical findings are in contrast to those reported in glioblastomas by Newcomb *et al.* (21) in that up to 20 of the 80 glioblastomatous samples overexpressed both MDM2 and p53. The interaction of MDM2 and p53 may be differently altered in the oncogenesis of different types of tumor.

The frequency of *mdm2* amplification was much higher in ependymomas than for that reported in

glioblastomas (22, 23, 26), as well as our personal experience regarding glioblastomas (unpublished data); it was approximately 10%. In addition, *mdm2* amplification tended to be detected in adult patients, whereas only one childhood case (Case A3) showed *mdm2* amplification in recurrent samples. Whether this observation is due to a different cause between pediatric and adult ependymoma awaits further investigation.

Two cases, C5 and A3, showed different results in differential PCR between the primary samples and the recurrent ones. Case C5 initially showed *mdm2* amplification; however, the recurrent sample did not. Case A3 showed *mdm2* amplification only in the two recurrent samples. Although we cannot exclude the possibility of biologic changes occurring in the tumors after the initial operation, other possible explanations could be proposed. The behavior of MDM2 is not equal in individual tumor cells even in the same section, and immunopositivity does not always correlate with gene amplification. Sample C5' showed less population of the stained nuclei than C5 did (Table 1). Thus, the intensity of nuclear staining for MDM2 seems to reflect indirectly the degree of gene amplification, because in Cases C5 and A3, the samples that were negative for *mdm2* amplification were obviously more weakly stained than those positive for the gene amplification. The results of differential PCR should therefore be interpreted as a characteristic of the predominant population of tumor cells in each sample examined.

The immunohistochemical findings in one anaplastic ependymoma (A2) seem to suggest a potential role of MDM2 in the anaplastic transformation; however, the overall results indicate that in ependymomas, *mdm2* amplification and/or overexpression is not generally implicated in the development of anaplastic histologic features described in the World Health Organization classification (28).

In the present study, MDM2 immunopositivity was detected more frequently than *mdm2* amplification (96% versus 35%). This finding suggests the existence of a mechanism of MDM2 overexpression other than gene amplification in ependymomas, as has been suggested in astrocytomas (21) and other neoplasms, such as enhanced transcription (37) and enhanced translation (38). A dilution effect caused by the heterogeneity of the tumor tissue may also result in an underestimation of *mdm2/DR2* ratio (Table 1).

Our results contrast with those described by Reifenberger *et al.* (22) as well as Tong *et al.* (27) in which almost no *mdm2* amplification was detected in ependymomas. The former used Southern hybridization, whereas the latter performed differential PCR using DNA extracted from paraffin sections, as we did in the present study. Although we

chose a cutoff value of *mdm2/DR2* intensity ratio more strictly than Tong *et al.* did, we detected *mdm2* amplification more frequently among our cases. The reason for this discrepancy is uncertain. However, we assume that high frequency in MDM2 overexpression may, at least in part, result from the gene amplification.

p73 tumor suppressor protein, a p53 homologue that shares structural and functional homology with p53 protein (39, 40), also binds MDM2 (41–44). Loiseau *et al.* (45) recently stated that p73 was transcriptionally overexpressed in ependymomas. Although MDM2 does not promote the degradation of p73 (41–44), the functional significance of MDM2–p73 interaction in the tumorigenesis has yet to be determined (42–44).

Further investigation is therefore necessary to elucidate the relevance of *mdm2* gene amplification and overexpression to the oncogenesis of ependymomas; however, this current study demonstrates that a considerable number of ependymomas are clearly associated with this genotypic and/or phenotypic change.

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