Methylation of $p14^{ARF}$ gene in meningiomas and its correlation to the p53 expression and mutation

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We have previously reported the statistically significant correlation of immunohistochemical expression of MIB-1 and p53 proteins among benign, atypical, and anaplastic meningiomas and p53 protein expression was high in atypical and anaplastic meningiomas. In the present study, we analyzed 22 cases of meningiomas for mutation of $p53$ gene in its spectrum of exon 5 to 8 using automated genetic analyzer. We did not find any mutation of $p53$ in any of these cases, thus suggesting the p53 protein expression is wild type. We analyzed 72 cases of meningiomas for determining the methylation status of $p14^{ARF}$ gene and the immunohistochemical expression of MDM2 protein to explain p53 protein expression in these meningiomas. We found methylation of $p14^{ARF}$ gene in five of 58 cases of benign meningiomas (8.6%), two of 10 cases of atypical meningiomas (20%), and two of four cases of anaplastic meningiomas (50%). In absence of $p53$ gene mutation, the high percentage of $p14^{ARF}$ gene methylation in high-grade meningioma may have been responsible for accumulation of wild-type p53 protein. In addition, we also found the loss of MDM2 protein in high-grade meningiomas. These deregulations of p14–MDM2–p53 pathway may contribute to the malignant progression of meningioma.

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Meningiomas are common among intracranial tumors, estimated to account for 13–19% of all primary brain tumors.¹ In general, most of them are slow-growing tumors with benign biological behavior. The different histological types of meningiomas have different risk of recurrence and their aggressiveness for histological grades is described in the classification of tumors of the nervous system adopted by World Health Organization (WHO).² We have reported the statistically significant correlation of immunohistochemical expression of MIB-1, p53, p21, p27 proteins among benign, atypical, and anaplastic meningiomas and found high MIB-1 and p53 protein immunoexpression in atypical and anaplastic meningiomas.³ Here, we investigated the mutational status of $p53$ gene in 22 meningioma cases, including 10 anaplastic meningiomas and 12 other meningiomas from our previous study.

The p53 is a key regulator of cell cycle checkpoints. It binds to DNA in a sequence-specific manner and functions as a transcription factor. It induces either G1 arrest or apoptosis in response to various forms of cell stresses.⁴ The expression of p53 protein is, mainly, regulated at the post-transcription stage and maintained at a very low level in normal cells. MDM2 is an important regulator of p53. MDM2 binds to p53 and inhibits its function by concealing the activation domain of $p53^{5,6}$ and by promoting degradation of p53 protein.^{7,8} In response to DNA damage, the MDM2 binding site of p53 is phosphorylated and the p53–MDM2 interaction is attenuated and p53 accumulates rapidly, relieved from MDM2-mediated suppression.⁹

Recent investigations have identified another important regulator in this pathway, $p14^{ARF}$ (a human homologue of mouse $p19^{ARF}$). CDKN2A/INK4A locus encodes the $p14^{ARF}$ protein but distinct from the p16INK4A protein.¹⁰ The $p14^{ARF}$ is encoded by the unique exon 1 β , and exons 2 and 3 of CDKN2A, using an alternative reading frame. Exon 1 β is located between exons 1 α and 2 of CDKN2A on 9p21.^{11,12} The previous reports showed that the $p14^{ARF}$ protein binds to the p53/MDM2 complex and inhibits MDM2-mediated degradation of p53, which indicates that $p14^{ARF}$ is an upstream regulator of p53 via MDM2.^{13–16} There is evidence suggesting that

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p53 downregulates expression of p14^{ARF} and MDM2, which would establish an autoregulatory feedback loop between p53, MDM2, and p14^{ARF}.¹⁷ These reports prompted us to examine the status of genes involved in the p14^{ARF}/MDM2/p53 pathway in our series of meningiomas. So far, no mutation of p14^{ARF} gene is identified in the human malignancies. After the inactivation of gene by the methylation of CpG islands was reported,¹⁸ p14^{ARF} hypermethylation was also proposed as one of the factors for tumorigenesis in the human malignancies like lung, breast, and glioma.^{19–21}

In the present study, we studied CpG island promoter methylation of the human p14^{ARF} gene in meningiomas, and correlated with the status of p53 gene mutation and the immunohistochemical expression of MIB-1, p53, and MDM2 proteins.

Materials and methods

Methylation-Specific PCR

DNA methylation patterns in the CpG islands of p14^{ARF} genes were determined by methylation-specific PCR (MSP). MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA.²²

In all, 72 cases of gross total resected meningiomas were obtained from the archives of Department of Pathology of Hiroshima University Hospital from 1996 to 2002. This included 58 cases of WHO grade I (benign) meningiomas, 10 cases of WHO grade II (atypical) meningiomas, and four cases of WHO grade III (anaplastic) meningiomas according to the criteria of the World Health Organization. We included only four cases of anaplastic meningiomas out of 10 anaplastic cases of previous study for the unavailability of good-quality DNA to undergo methylation study. DNA was isolated from tumor tissue microdissected from paraffin-embedded material using QIAamp DNA Mini-kit (Qiagen KK, Japan). The bisulfite modification of the extracted DNA was carried out using CpGenomeTM DNA Modification Kit (Intergen, USA). Primer sequences of p14^{ARF} gene for the unmethylated reaction were 5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3' (sense) and 5'-CACAAAACCCCTCACTCACAACAA-3' (antisense), which amplify a 132-bp product, and primer sequences for the methylated reaction were 5'-GTGTTAAAGGGCGGCGTAGC-3' (sense) and 5'-AAAACCCCTCACTCGCGACGA-3' (antisense), which amplify a 122-bp product. The 5' position of the sense unmethylated and methylated primers corresponds to bp 195 and 201 of GeneBank sequence number L41934. Both antisense primers originate from bp 303 of this sequence. The annealing temperature for both the unmethylated and methylated

reactions was 60°C. In all, 10 µl of each PCR reaction were loaded directly onto 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination. The detection of 132 and 122 bp DNA bands in the agarose gel was considered for the unmethylated and methylated case, respectively.

Mutational Analysis of p53 Gene

The mutation analysis of p53 gene was performed in 22 cases of meningiomas. It included all of 10 cases of anaplastic meningiomas of our previous study and other seven cases of meningiomas with high immunohistochemical expression of p53 protein and five benign cases without p53 expression. The tumor tissues were microdissected using a sterile syringe with 23G needle, observing the tissue direct under a dissecting microscope from formalin-fixed paraffin-embedded tissue. DNA was extracted of microdissected tumor tissue using standard phenol–chloroform extraction procedure as previously described method.²³ The PCR product of exons 5–8 of p53 gene, after purification, were sequenced in both direction using BigDye cycle sequencing kit run in ABI Prism 310 genetic analyzerTM (Perkin-Elmer Japan Co, Chiba, Japan) according to the manufacturer's protocol.

Immunohistochemistry

The immunohistochemical study of meningiomas with anti-Ki-67, anti-p53 protein was carried out as described previously.³ The anti-MDM2 antibody (monoclonal, clone Ab1, Oncogene, Cambridge, MA, USA; dilution 1:30) is used for the study of immunohistochemical expression of MDM 2 in 72 cases of meningiomas. The antigen was retrieved by autoclaving the paraffin section at 121°C for 10 min in 0.01 mol/l citrate buffer, pH 6.0. The immunohistochemical expression of MIB-1, p53, and MDM 2 was labeled from – to + + + + as described in Table 1.

Statistical Analysis

The statistical analysis on correlation of the methylation status of benign, atypical and anaplastic meningiomas were performed by Fisher's exact test using GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego CA, USA, www.graphpad.com.

Result

Clinicopathological Features

The clinicopathological features of 72 cases of meningiomas were analyzed and histologically categorized according to WHO classification of tumors of the nervous system. In total, 58 cases were benign meningiomas (Figure 1a), 10 cases were

Table 1 *p14^{ARF}* gene methylation status of meningiomas and its relation with *p53* gene mutation and MIB-1, p53, MDM2 expression

Cases	Histology	<i>p14^{ARF}</i> methylation	<i>p53</i> gene mutation	MIB-1 expression	<i>p53</i> expression	MDM2 expression
MN 1	Benign	Methylated	No	++	+	+++
MN 4	Benign	Methylated	No	++	+	+
MN 18	Benign	Methylated	No	++	++	++
MN 23	Benign	Methylated	ND	+	—	++
MN 25	Benign	Unmethylated	ND	++	—	++
MN 39	Benign	Unmethylated	ND	+	—	++
MN 45	Benign	Unmethylated	ND	++	—	++
MN 47	Benign	Methylated	ND	++	—	++
MN 63	Benign	Unmethylated	No	++++	+++	+
MN 22	Atypical	Methylated	No	++++	++	+
MN 9	Atypical	Methylated	No	+++	++	+
MN 5	Anaplastic	Methylated	No	++++	+++	+
MN 59	Anaplastic	Methylated	No	++++	+++	+

Note: —, negative; +, positive less than 5%; ++, positive 5–10%; +++ positive 10–20%; +++++, positive more than 20%; ND, not done.

Figure 1 Histology and immunohistochemistry: The MIB-1 immunoexpression (d, e, f) and p53 protein expression (g, h, i) are directly proportional to meningioma grades, increasing from benign to atypical and anaplastic meningiomas. The MDM2 protein immunoexpression (j, k, l) was maintained in most of benign meningioma, but were decreased in atypical and anaplastic meningiomas. (a, d, g, and j) Benign meningioma (b, e, h, and k) atypical meningioma (c, f, i, and l) anaplastic meningioma.

atypical meningiomas (Figure 1b), and four cases were anaplastic meningiomas (Figure 1c). The total number of cases of this study were reduced from 146 of previous study³ to 72 cases due to poor preservation of the tissue for undergoing genetic analysis.

Promoter Hypermethylation of $p14^{ARF}$ Gene

Among all of the meningioma studied, nine cases out of 72 cases (12.5%) showed methylation of $p14^{ARF}$ gene (Figure 2). The $p14^{ARF}$ promoter hypermethylation was present in five of 58 benign meningiomas (8.6%), two of 10 atypical meningiomas (20%), and two of four anaplastic meningiomas (50%) ($P=0.31$). Abnormal methylation of the $p14^{ARF}$ promoter region in meningiomas was not associated with significant differences of gender, age of onset, and location of tumor (data not shown). All of the methylated cases of atypical and anaplastic meningiomas have increased expression of p53 protein and three of five benign meningiomas with hypermethylation showed moderate degree of p53 expression. All of the methylated meningiomas, in spite of atypical or anaplastic nature, showed increased expression of MIB-1, directly correlating with p53 protein. A case of benign meningioma, which showed increased expression of p53 protein and MIB-1, was not methylated for $p14^{ARF}$ gene. In Table 1, the summarized result of $p14^{ARF}$ gene methylation, p53 gene mutation, and their correlation with immunohistochemical expression of MIB-1, p53, and MDM2 protein were shown. In this table, of 13 cases of meningiomas, eight cases of meningioma were expressing the p53 protein, five cases were not expressing, and all of these cases included were studied for $p14^{ARF}$ methylation.

MDM 2 Immunohistochemistry and p53 Gene Mutation

In all, 22 cases of meningiomas in our previously reported study³ were analyzed for the detection of p53 gene mutation. All of 10 anaplastic meningiomas and 12 other atypical and benign meningiomas

of the previous study were included in the present study. No mutation was detected in exons 5–8 of p53 gene in any of these cases. The MDM2 protein expression was preserved in most of the benign meningiomas (Figure 1j) with mean labeling index of 34.5% and decreased in the atypical (Figure 1k), mean labeling index of 4.5% and lost in the anaplastic meningiomas (Figure 1l) as mean labeling index was 2.3%. The benign meningioma with methylation of $p14^{ARF}$ gene had decreased expression of MDM2 protein as in atypical and anaplastic meningiomas. However, a single case of benign meningioma had preserved expression of MDM2 protein, despite methylation of its $p14^{ARF}$ gene.

Discussion

Meningiomas account for 13–19% of primary intracranial tumors and most of them are benign (WHO grade I). The atypical (WHO grade II) and anaplastic (WHO grade III) meningiomas have behavior that is more aggressive and are supposed to have increased genetic abnormalities. Our immunohistochemical study of meningiomas showed the statistically significant difference of MIB-1 expression and p53 protein expression among these anaplastic, atypical, and benign meningiomas, with increased expression of MIB-1, p53, p21 protein in high-grade meningiomas.³ The MIB-1 immunoreactivity in benign meningiomas (Figure 1d) was considerably lower than that in atypical (Figure 1e) and anaplastic meningiomas (Figure 1f). There was increased expression of p53 protein in atypical (Figure 1h) and anaplastic meningioma (Figure 1i) compared to negligible p53 expression in benign meningiomas (Figure 1g). Overexpression of p53 protein in meningiomas was associated with increased MIB-1 labeling index and with recurrence in some studies,^{24,25} and aggressive histology²⁶ in others.

Mutation of the p53 gene has been reported to be extremely rare in meningiomas and is mainly associated with malignant histology.²⁷ However, approximately 26% of meningiomas express the accumulation of wild-type p53 protein, suggesting the possibility of another mechanism of p53 protein expression.²⁸ In the present study, we could not detect any mutation in exons 5–8 of these meningioma cases. These findings of absence of p53 gene mutations suggest the p53 protein expression in meningiomas are wild type, and this does not have effect on suppressing the p21 protein in these cases. Thus, p53 protein expression in these meningiomas is not due to p53 gene mutation and other cause for these results should be considered. The p21 expression may be regulated by p53-independent pathway, like TGF- β and other growth factors.

The MDM2 is an important regulator of p53. MDM2 binds to p53 and inhibits its function by concealing the activation domain of p53^{5,6} and by

Figure 2 $p14^{ARF}$ gene methylation: The agarose gel electrophoresis shows methylation of $p14^{ARF}$ gene in five of 58 cases of benign meningiomas (cases 1, 4, 18, 25, 45), two of 10 cases of atypical meningioma (cases 9, 22), and two of four cases of anaplastic meningiomas (cases 5, 59). Many cases showed unmethylated DNA also. The amplification of unmethylated DNA was probably due to contamination of non-neoplastic tissue. Arrow = DNA ladder, white triangle = atypical meningioma cases, black triangle = anaplastic meningioma cases.

promoting degradation of p53.^{7,8} MDM2 protein expression in meningiomas was described in some of the recently published reports and showed correlation with the p53 protein and MIB-1 expression.²⁹ In the present study, we also found the loss of the expression of MDM2 protein in high-grade meningiomas.

Recent investigations have identified another important regulator in p53 expression, p14^{ARF}. It has been shown that the p14^{ARF} protein binds to the p53/MDM2 complex and inhibits MDM2-mediated degradation of p53, which indicates that p14^{ARF} is an upstream regulator of p53 through MDM2.^{13–16} The methylation of the p14^{ARF} gene is responsible for its inactivation, as no mutation of this gene has so far identified in human malignancies. Higher percentage of the p14^{ARF} gene methylation in anaplastic meningioma and atypical meningiomas resembles with the higher incidence of methylation glioblastoma compared to low-grade astrocytoma. The methylation of this gene in high-grade meningiomas may have associated with the expression of wild-type p53 protein probably by the inhibition of the MDM2-mediated degradation of p53.

In present study, we found methylation of p14^{ARF} gene is more common to atypical and anaplastic meningioma than benign meningiomas. There is no mutation of p53 gene in these meningioma cases. The expression of MDM 2 protein is higher in the cases with methylation of p14^{ARF} gene. All of these findings may suggest the explanation of increased expression of p53 protein in high-grade meningiomas and possible explanation of tumorigenesis of meningiomas.

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