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Advances in Brief

Frequent Alterations of the *p14^{ARF}* and *p16^{INK4a}* Genes in Primary Central Nervous System Lymphomas

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Abstract

To elucidate the role of *p53*/*p16^{INK4a}*/*RB1* pathways in the tumorigenesis of primary central nervous system lymphomas (PCNSLs), we have analyzed *p14^{ARF}*, *p16^{INK4a}*, *RB1*, *p21^{Waf1}*, and *p27^{Kip1}* status in a series of their 18 sporadic cases of diffuse large B-cell lymphoma, using methylation-specific PCR, differential PCR, and immunohistochemistry. Homozygous deletion or methylation of *p14^{ARF}* was detected in 10 (56%) PCNSLs, and they were almost entirely deletions (except 1 case). A total of 11 (61%) PCNSLs demonstrated homozygous deletion (6 cases) or methylation (5 cases) of *p16^{INK4a}*. Six tumors showed both *p14^{ARF}* and *p16^{INK4a}* homozygous deletions. Hypermethylation of the *RB1* and the *p27^{Kip1}* promoter region was detected in 2 (11%) cases, whereas *p21^{Waf1}* methylation was not detected in any. Immunohistochemistry revealed loss of p14^{ARF} and p16^{INK4a} expression in 10 (56%) samples, correlating with the gene status. Four cases showed independent negative immunoreactivity for pRB and p27^{Kip1}, and nearly one-half of cases (8 of 18; 44%) were characterized by lack of p21^{Waf1} expression. These results indicate that inactivation of *p14^{ARF}* and *p16^{INK4a}* by either homozygous deletion or promoter hypermethylation represents an important molecular pathogenesis in PCNSLs. Hypermethylation of *RB1*, *p21^{Waf1}*, and *p27^{Kip1}* appears to be of minor significance, these genes being independently methylated in PCNSLs.

Introduction

The incidence of PCNSLs[2] in nonimmunodeficient patients has been markedly increasing over the past decades, and currently they are estimated to account for >6% of all primary brain tumors (1). Morphologically, the vast majority of PCNSLs are high-grade non-Hodgkin's lymphomas of diffuse large B-cell type, according to the revised European American Lymphoma classification (2). There are no lymph nodes or lymphatics within the nervous system, and therefore the pathogenesis and histogenetic origin of PCNSLs in immunocompetent patients are still poorly understood.

Molecular genetic studies have been conducted that revealed the *p16^{INK4a}* gene to be frequently inactivated by either homozygous deletion (40–50%) or 5′-CpG hypermethylation (15–30%; Refs. 3, 4). Mutations in the *p53* gene were observed in a small fraction of PCNSLs, whereas genetic alterations such as *MDM2*, *CDK4*, *CCND1*, *MYC*, and *REL* were not detected (3).

p14^{ARF} interacts physically with MDM2 and stabilizes p53 protein in the nucleus by blocking its cytoplasmic transport and MDM2-mediated degradation (5, 6) so that it may act as an upstream regulator of p53 function. Homozygous deletion of *p14^{ARF}* has been reported in 25–60% of glioblastomas (7, 8) and 8% of systemic non-Hodgkin's lymphomas (9). The human *p14^{ARF}* promoter has also been shown to be aberrantly methylated in gliomas, colorectal adenomas and carcinomas (7, 10), and esophageal carcinomas (11) but was not detected in one series of systemic non-Hodgkin's lymphomas (12).

Inactivation of the retinoblastoma gene (*RB1*) product by mutation, deletion, and/or promoter hypermethylation has been reported as an alternative molecular mechanism leading to *p16^{INK4a}* inactivation, *CDK4* amplification, and *CCND1* amplification/rearrangement in human tumors, including gliomas (8). Several previous studies have implicated alterations of *RB1* in systemic non-Hodgkin's lymphomas (13). Thus, genetic alterations of the *INK4a/ARF* locus may cause impairment in both *p14^{ARF}*/*p53* and *p16^{INK4a}*/*RB1* pathways in the development and progression of human non-Hodgkin's lymphomas.

To cast light on the presence of *p14^{ARF}* alterations in human PCNSLs and their possible alternative or coordinate inactivation with *p53*/*p16^{INK4a}*/*RB1* pathways, we have studied a series of 18 PCNSLs for genetic aberrations and expression of several genes shown previously to be altered and/or aberrantly expressed to some extent in systemic non-Hodgkin's lymphomas.

Materials and Methods

Tumor Samples and DNA Extraction. Eighteen primary malignant non-Hodgkin's lymphomas of the central nervous system were obtained from immunocompetent patients treated between 1984 and 2000 in the Department of Neurosurgery, Nara Medical University. Tumor samples were fixed in buffered formalin and embedded in paraffin. Pathological diagnosis was made according to the revised European American Lymphoma classification of lymphoid neoplasms (2). DNA was extracted from paraffin sections as described previously (7, 14). In one tumor (case 11), *p14^{ARF}* and *p16^{INK4a}* immunopositive and negative tumor areas could be clearly recognized. These areas were carefully microdissected and analyzed separately.

MSP. DNA methylation patterns in the CpG islands of the *p14^{ARF}*, *p16^{INK4a}*, *RB1*, *p21^{Waf1}*, and *p27^{Kip1}* genes were determined by MSP (15). Sodium bisulfite modification was performed using a CpGenome DNA Modification kit (Intergen, Oxford, United Kingdom) according to the manufacturer's protocol with minor modifications (7, 14). The primer sequences for *p14^{ARF}*, *p16^{INK4a}*, and *RB1* with methylated and unmethylated PCR have been reported previously (10, 15, 16). Other primer sequences were as follows: 5′TTG GGC GCG GAT TCG TC-3′ (sense) and 5′-CTA AAC CGC CGA CCC GA-3′ (antisense) for the *p21^{Waf1}* methylated reaction; 5′TTA GTT TTT TGT GGA GTT G-3′ (sense) and 5′-CTC AAC TCT AAA CCA CCA A-3′ (antisense) for the *p21^{Waf1}* unmethylated reaction, and 5′-AAG AGG CGA GTT AGC GT-3′ (sense) and 5′-AAA ACG CCG CCG AAC GA-3′ (antisense) for the *p27^{Kip1}* methylated reaction; 5′-ATG GAA GAG GTG AGT TAG T-3′ (sense) and 5′-AAA ACC CCA ATT AAA AAC A-3′ (antisense) for the *p27^{Kip1}* unmethylated reaction. MSP conditions for *p14^{ARF}*, *p16^{INK4a}*, and *RB1* were described in detail previously (7, 14). The annealing temperature for *p21^{Waf1}* methylated and unmethylated reactions was 62°C, and for *p27^{Kip1}* methylated and unmethylated reactions was 66°C. Amplified products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Differential PCR for *p14^{ARF}* and *p16^{INK4a}* Deletions. To assess homozygous deletions, we carried out differential PCR with primers covering exon

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[2] The abbreviations used are: PCNSL, primary central nervous system lymphoma; MSP, methylation-specific PCR.

1β of the *p14^{ARF}* gene, using the *GAPDH* gene as a reference. Differential PCR for homozygous deletion of *p16^{INK4a}* (exon 1α) was carried out using the *β-actin* gene as a reference. The primer sequences and PCR conditions were as described previously (7), and PCR products were analyzed in 8% acrylamide gels, photographed using a DC290 Zoom Digital Camera (Eastman Kodak, Rochester, NY). Densitometry of the PCR fragments was performed using Kodak Digital Science ID Image Analysis Software (Ver. 3.5.2; Eastman Kodak). Samples presenting <20% of the control signal were considered homozygously deleted (7).

PCR-Single Strand Conformation Polymorphism Analyses for *p53* Mutations. PCR amplification of exons 5, 6, 7, 8, and 9 of the *p53* gene, single-strand conformation polymorphism analysis, and DNA sequencing was conducted as described previously (17).

Immunohistochemistry. Expression was assessed immunohistochemically, using a polyclonal antihuman p14^{ARF} antibody (FL-132: SC1661; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibodies to p16^{INK4a} (F-12: SC1661; Santa Cruz Biotechnology), pRB (clone G3–245; PharMingen, San Diego, CA), p21^{Waf1} (F-5: SC-6246; Santa Cruz Biotechnology), p27^{Kip1} (clone 57; Transduction Laboratories, Lexington, KY), and MDM2 (clone IF2; Oncogene, Boston, MA). After deparaffinization, sections were heated to boiling for 5 min in 10 mM sodium citrate (pH 6.0) buffer in a pressure cooker. They were then incubated for overnight at 4°C with antibodies for p14^{ARF}, pRB, and p27^{Kip1} at a dilution of 1:500, p16^{INK4a} at a dilution of 1:1000, p21^{Waf1}, and MDM2 at a dilution of 1:100. Binding reactions were visualized using a Histofine SAB-PO kit and diaminobenzidine (Nichirei, Tokyo, Japan), and sections were counterstained with hematoxylin.

Statistical Analysis. The Fisher's exact test was used to examine possible associations between *p14^{ARF}* and other genetic alterations.

Results

***p14^{ARF}* and *p16^{INK4a}* Alterations.** Homozygous deletion of the *p14^{ARF}* gene was detected by differential PCR in 9 of 18 (50%) PCNSLs examined (Table 1 and Fig. 1) and hypermethylation of the *p14^{ARF}* promoter in 1 of 18 (6%). Methylated and unmethylated control DNAs showed the expected fragment sizes of 122 and 132 bp, respectively (Fig. 2). Homozygous *p16^{INK4a}* deletion was detected in 6 (33%) PCNSLs (Table 1 and Fig. 1) and promoter hypermethylation of the *p16^{INK4a}* gene in 5 (28%) cases. Methylated and unmethylated control DNAs showed the expected fragment sizes of 150 and 151 bp, respectively (Fig. 2).

Simultaneous homozygous deletion of *p14^{ARF}* and *p16^{INK4a}* was detected in 6 cases, whereas 3 showed *p14^{ARF}* deletion alone. No case showed *p16^{INK4a}* deletion alone without *p14^{ARF}* deletion. Overall, 8 cases showed both alterations (homozygous deletion or methylation) in *p14^{ARF}* and *p16^{INK4a}*. Except for one case (case 11), hypermethylation of *p16^{INK4a}* promoter did not correlate with *p14^{ARF}* methylation.

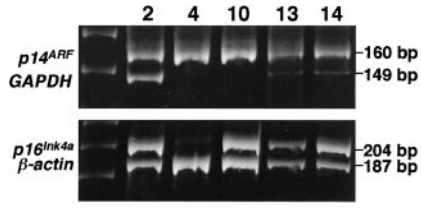

Fig. 1. Differential PCR to assess *p14^{ARF}* and *p16^{INK4a}* homozygous deletions in PCNSLs. Case 2 has a normal gene status. Case 4 shows *p14^{ARF}* and *p16^{INK4a}* deletion. Cases 10, 13, and 14 have *p14^{ARF}* homozygous deletions without *p16^{INK4a}* deletion.

***RB1, p21^{Waf1}, and p27^{Kip1}* Alterations.** *RB1* and *p27^{Kip1}* promoter hypermethylation was detected in 2 of 18 (11%) PCNSLs (Table 1 and Fig. 2). Methylated and unmethylated control DNAs showed the expected fragment sizes of 163 bp for *RB1* and 195 and 212 bp for *p27^{Kip1}*, respectively (Fig. 2). *p21^{Waf1}* methylation was not detected in any PCNSL (Table 1 and data not shown). Methylation patterns of these genes appeared independent of each other.

Immunohistochemistry and Correlation with Genetic Analyses. Nuclear immunoreactivity to p14^{ARF} was observed in neurons and glial cells in peritumoral brain tissues. Ten cases (56%) of PCNSLs showed loss of p14^{ARF} expression, and of the remaining cases, 4 showed immunoreactivity in >20% of the neoplastic cells (Table 1). Loss of p16^{INK4a} expression was observed in 10 of 18 cases (56%; Table 1). There was a close correlation between loss of p14^{ARF}/p16^{INK4a} expression, as detected by immunohistochemistry and homozygous deletion/promoter methylation. All 8 cases with p14^{ARF} expression showed a normal *p14^{ARF}* gene status, whereas one case (case 6) with *p16^{INK4a}* promoter methylation showed p16^{INK4a} expression (Table 1). In one case (case 11), some tumor areas showed p14^{ARF}/p16^{INK4a} expression, but there were also focal areas with neoplastic cells that lacked p14^{ARF}/p16^{INK4a} expression. In this case, only microdissected areas with loss of p14^{ARF}/p16^{INK4a} expression

Table 1 *Alterations of INK4A/ARF locus and the status of RB1, p21^{Waf1}, p27^{Kip1}, p53, and MDM2 in PCNSLs*

The percentage of neoplastic cells with nuclear immunoreactivity for p14^{ARF}, p16^{INK4a}, p21^{Waf1}, and p27^{Kip1} was recorded as − when only occasional (<5%) tumor cells were positive; +, with 5–20% positive; and ++, with >20% positive. For pRB staining, immunoreactivity in <5% of tumor cells was −; 5–25% tumor cells, +; 25–50% tumor cells, ++; and >50% tumor cells, +++. For MDM2 staining, samples were graded into an overexpression group when 5% or more of the tumor cells were stained. −*, case 11 exhibits extensive areas with marked immunoreactivity for p14^{ARF} and p16^{INK4a} but focal tumor cell clusters with loss of p14^{ARF} and p16^{INK4a}.

Patient ID no.	p14^{ARF} expression (IHC)[a]	*p14^{ARF}* methylation or deletion	*p16^{INK4A}* expression (IHC)	*p16^{INK4A}* methylation or deletion	pRB expression (IHC)	*RB1* methylation	p21^{Waf1} expression (IHC)	*p21^{Waf1}* methylation	p27^{Kip1} expression (IHC)	*p27^{Kip1}* methylation	*p53* mutation	MDM2 overexpression (IHC)
1	+	−	++	−	++	−	+	−	++	−	−	−
2	++	−	++	−	++	−	+	−	−	Methylation	−	−
3	++	−	++	−	+++	−	−	−	+	−	−	−
4	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−
5	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−
6	+	−	+	Methylation	−	Methylation	−	−	+	−	−	−
7	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−
8	++	−	++	−	++	−	−	−	−	−	−	−
9	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−
10	−	Deletion	−	Methylation	+++	−	−	−	+	−	−	−
11	−*	Methylation	−*	Methylation	+++	−	+	−	−	−	−	−
12	++	−	++	−	−	Methylation	−	−	+	−	−	−
13	−	Deletion	++	−	−	−	−	−	++	−	−	−
14	−	Deletion	++	−	−	−	−	−	−	Methylation	−	−
15	+	−	−	Methylation	+++	−	++	−	++	−	−	−
16	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−
17	+	−	−	Methylation	+++	−	−	−	+	−	−	−
18	−	Deletion	−	Deletion	+++	−	+	−	++	−	−	−

[a] IHC, immunohistochemistry.

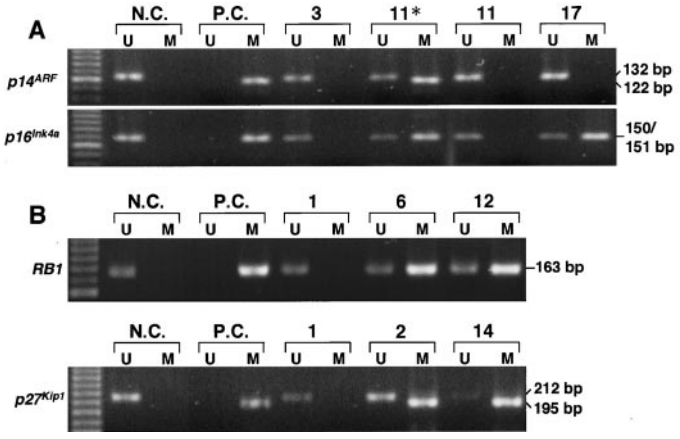

Fig. 2. Methylation specific PCR of CpG islands of the $p14^{ARF}$ and $p16^{INK4a}$ promoter in PCNSL. In case 3, there was only unmethylated DNA (U). In case 11, $p14^{ARF}$ and $p14^{INK4a}$ methylation (M) was restricted to areas (shown as *) lacked p14ARF and p16INK4a immunoreactivity. In patient 17, the biopsy sample showed only unmethylated status for $p14^{ARF}$, whereas the tumor revealed methylated DNA for $p16^{INK4a}$.

showed promoter hypermethylation of both genes (Table 1; Figs. 2 and 3).

Four cases demonstrated independent negative immunoreactivity for pRB and $p27^{Kip1}$. Promoter hypermethylation of these genes was detected in 2 cases without pRB/$p27^{Kip1}$ expression. A large fraction of PCNSLs (8 of 18; 44%) demonstrated loss of $p21^{Waf1}$ expression, but *p21*^{Waf1} hypermethylation was not detected in any case (Table 1). No overexpression of MDM2 protein was evident.

***p53* Gene Mutations and Correlation between *p14*^{ARF} and *p53* Status.** We failed to find any *p53* mutations in any of the 18 cases examined in this study. There was no significant correlation between $p14^{ARF}$ alterations, *p53* mutations, and MDM2 overexpression.

Discussion

The present study showed the most frequent abnormality in our series of PCNSLs to be homozygous deletion or promoter hypermethylation of the $p14^{ARF}$ (10 of 18; 56%) and $p16^{INK4a}$ (11 of 18; 61%) genes. We detected frequent $p14^{ARF}$ homozygous deletion (9 of 18; 50%), whereas the $p14^{ARF}$ promoter was unmethylated, even when the $p16^{INK4a}$ gene was methylated, except in 1 case (case 11). In this context, it is of interest that Baur *et al.* (12) found methylation silencing of $p15^{INK4b}$ and $p16^{INK4a}$ in human B-cell and T-cell lymphomas to be frequent, whereas methylation silencing of $p14^{ARF}$ was extremely rare. Meléndez *et al.* (18) also pointed out that $p19^{ARF}$ (the murine homologue of $p14^{ARF}$) expression was lost or reduced in a significant percentage of murine primary lymphomas, whereas the $p19^{ARF}$ CpG island was infrequently methylated. Thus, $p14^{ARF}$ homozygous deletion, rather than promoter hypermethylation, is the most likely to be the essential event for $p14^{ARF}$ inactivation in PCNSLs.

The $p16^{INK4a}$ and $p14^{ARF}$ genes are frequently co-deleted in human neoplasms, and this was also the case for 6 PCNSLs in our series. However, cases with $p14^{ARF}$ deletion alone were also encountered, and a higher frequency of $p14^{ARF}$ than $p16^{INK4a}$ deletions has been reported for other human neoplasms (7, 11), suggesting that $p14^{ARF}$ is the primary target with 9p21 deletions. This conclusion is supported by studies of mice, lacking $p19^{ARF}$ (the murine homologue of $p14^{ARF}$) expression alone through selective disruption of exon 1β, which develop tumors at several sites, including lymphomas, sarcomas, and gliomas (19).

$p14^{ARF}$ plays a major role in the p53 pathway by binding specifically to MDM2, resulting in stabilization of both p53 and MDM2 (5, 6). With regard to MDM2, PCNSLs appear similar to systemic lymphomas, which only exceptionally show amplification (3, 20). Growth arrest induced by $p14^{ARF}$ is therefore *p53* dependent. Recent studies of the *INK4a/ARF* locus as a regulatory region for both $p16^{INK4a}$/*RB1* and $p14^{ARF}$/*p53* pathways indicated that *p53* mutations may be more rare in tumors with inactivation of this locus than in those with wild-type *INK4a/ARF* genes (5). In the present series, $p14^{ARF}$ alterations and *p53* mutations appeared to be unrelated, and an inverse correlation between $p14^{ARF}$ and *p53* is not always detected, *e.g.*, in leukemia-lymphoma cell lines and large B-cell lymphomas (21). Although inactivation of the *p53* gene is a relatively common phenomenon (20–40%) in lymphomas outside central nervous system (22), *p53* mutations appear extremely rare in PCNSLs (3, 4). It has been suggested that the

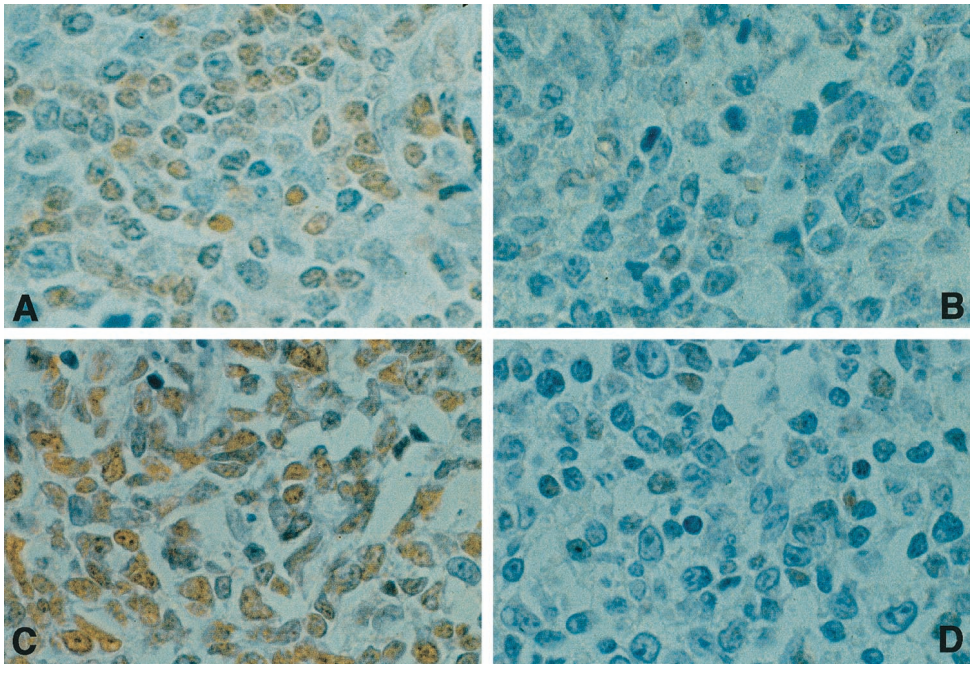

Fig. 3. *A*, $p14^{ARF}$ immunohistochemistry showing nuclear immunoreactivity in the majority of tumor cells (case 11). *B*, the same case with focal loss of $p14^{ARF}$ expression. *C* and *D*, case 11 exhibits extensive areas (*C*) with marked immunoreactivity for $p16^{INK4a}$ but focal tumor cell clusters (*D*) with loss of $p16^{INK4a}$, counterstained with hematoxylin. ×300.

pattern of these alterations in tumors may depend on the order of events (6). When $p14^{ARF}$ alterations occur early in the development of the PCNSLs, the tumors may retain wild-type *p53* genes.

For the majority of human neoplasms, a clear correlation has been reported between deletion/promoter methylation and loss of gene expression detected by immunohistochemistry (7, 14, 16). The present study also revealed a close link between gene inactivation and expression. All 8 PCNSLs with $p14^{ARF}$ expression showed a normal $p14^{ARF}$ gene status, whereas all 10 PCNSLs with $p14^{ARF}$ deletion or promoter methylation showed loss. There was also 1 case (case 6) with $p16^{INK4a}$ promoter methylation that expressed $p16^{INK4a}$, but this may be explained by incomplete gene silencing because of insufficient density and extent of methylation (23). The relation was also exemplified by the finding that in 1 case, promoter hypermethylation was detected only in areas lacking $p14^{ARF}$ and $p16^{INK4a}$ immunoreactivity but not in the areas with expression. Regarding alternative explanations, one common feature of $p16^{INK4a}$ regulation is that tumors with increased levels have *RB1* alterations. Although there was no inverse correlation between $p16^{INK4a}$ and *RB1* in our series, the $p16^{INK4a}$ immunopositive case (case 6) with $p16^{INK4a}$ promoter hypermethylation showed *RB1* alteration.

More than 50% of high-grade systemic non-Hodgkin's lymphomas lack pRB expression (13), whereas Cobbers *et al.* (3) reported that PCNSLs showed strong nuclear immunoreactivity in all of their 20 samples. We showed that loss of pRB expression was found in 4 (22%) cases examined. This is agreement with the former report on systemic lymphomas but at variance with the latter one on PCNSLs, although our cases are central nervous lymphomas. In glioblastomas (14) and pituitary adenomas (16), a clear correlation has been reported between *RB1* homozygous deletion and/or promoter hypermethylation and loss of pRB expression detected by immunohistochemistry. The present study suggests that this might also be the case for PCNSLs as the underlying cause of promoter methylation with loss of *RB1* expression.

Regarding lymphomas, $p21^{Waf1}$ alterations have been analyzed in only a few studies. A role for deletions and loss of expression of $p21^{Waf1}$ in aggressive lymphomas has been proposed (24), but others failed to identify mutations of this gene in a large series of lymphoid neoplasms (25). Our findings for PCNSLs are concordant with Cobbers' immunohistochemical results (3). $p21^{Waf1}$ expression was relatively low in many tumors (44%). However, promoter hypermethylation of $p21^{Waf1}$ was not detected, even in those without $p21^{Waf1}$ expression, and $p21^{Waf1}$ point mutations seem to be very rare in human tumors (26). $p21^{Waf1}$ expression might be regulated at the transcriptional level, although any significance of $p21^{Waf1}$ in the development of lymphomas is still unclear.

$p27^{Kip1}$ is also important as a cyclin-dependent kinase inhibitor impacting on passage through the G_1 as well as G_2 restriction points. Specific alterations of the $p27^{Kip1}$ gene, including mutations and homozygous deletions, are exceedingly rare in human cancers, including systemic non-Hodgkin's lymphomas (27), and Worm *et al.* (28) indicated that $p27^{Kip1}$ methylation may be a cause of monoallelic $p27^{Kip1}$ silencing in malignant melanomas. We found 4 cases (22%) to be negative for $p27^{Kip1}$ expression by immunohistochemistry, and $p27^{Kip1}$ hypermethylation was detected in 2 cases. However, regulation of $p27^{Kip1}$ expression may occur at various stages. Some osteosarcomas and breast and colon cancers may express $p27^{Kip1}$ mRNA, but $p27^{Kip1}$ protein is absent because of rapid proteasome-mediated degradation in the tumor cells (29). These findings suggest that aberrant $p27^{Kip1}$ methylation is not the only mechanism causing reduced $p27^{Kip1}$ levels, and epigenetic changes could also play a role in PCNSL pathogenesis.

In summary, the present study showed that alterations of $p14^{ARF}$ and $p16^{INK4a}$ genes are frequent in PCNSLs. Other related genes (*RB1* and $p27^{Kip1}$) were also found to be independently methylated, although infrequently, suggesting that *de novo* methylation during PCNSL tumorigenesis results from independent and sequence-specific mechanisms.

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