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 $p16^{INK4a}$, 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 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 either by homologous 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 alternate 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 $p14^{ARF}$ 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.

MSCP. 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 CpGnome 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 GCC GCG CAT TCG TC-3′ (sense) and 5′-GTT TTT TGT GGA GTT G-3′ (antisense) for the $p21^{Waf1}$ methylated reaction; 5′-ATG GAA GAG CGG GTT AGC GT-3′ (sense) and 5′-ATT ATT ATT ACC AAA AAC A-3′ (antisense) for the $p21^{Waf1}$ unmethylated reaction; 5′-TAG AAG CGG CC GC TTT GTT AAT CGC GTG-3′ (sense) and 5′-ATA ACC CAA AAA CAA A-3′ (antisense) for the $p27^{Kip1}$ methylated reaction; 5′-CCA GTG GAT CCA TAC A-3′ (sense) and 5′-AAA ACG GCC CGG AAC GA-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 in 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 p14ARF gene, using the GAPDH gene as a reference. Differential PCR for homozygous deletion of p16INK4a (exon 1a) 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).

**PCRSingle 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 p14ARF antibody (FL-132: SC166; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal antibodies to p16INK4a (F-12: SC166; Santa Cruz Biotechnology), pRB (clone G3–245; PharMingen, San Diego, CA), p21Waf1 (F-5; SC-6246; Santa Cruz Biotechnology), p27Kip1 (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 p14ARF, pRB, and p27Kip1 at a dilution of 1:500, p16INK4a at a dilution of 1:1000, p21Waf1, and MDM2 at a dilution of 1:200. Binding reactions were visualized using a Histofine SAB-PO kit and diaminobenzidine (Nichirei, Tokyo, Japan), and sections were counterstained with hematoxylin.

**Results**

**p14ARF and p16INK4a Alterations.** Homozygous deletion of the p14ARF gene was detected by differential PCR in 9 of 18 (50%) PCNSLs examined (Table 1 and Fig. 1) and hypermethylation of the p14ARF promoter in 1 of 18 (6%). Methylation and unmethylated control DNAs showed the expected fragment sizes of 122 and 132 bp, respectively (Fig. 2). Homozygous p16INK4a deletion was detected in 6 (33%) PCNSLs (Table 1 and Fig. 1) and promoter hypermethylation of the p16INK4a gene in 5 (28%) cases. Methylation and unmethylated control DNAs showed the expected fragment sizes of 150 and 151 bp, respectively (Fig. 2).

Simultaneous homozygous deletion of p14ARF and p16INK4a was detected in 6 cases, whereas 3 showed p14ARF deletion alone. No case showed p16INK4a deletion alone without p14ARF deletion. Overall, 8 cases showed both alterations (homozygous deletion or methylation) in p14ARF and p16INK4a. Except for one case (case 11), hypermethylation of p16INK4a promoter did not correlate with p14ARF methylation.

**RB1, p21Waf1, and p27Kip1 Alterations.** RB1 and p27Kip1 promoter hypermethylation was detected in 2 of 18 (11%) PCNSLs (Table 1 and Fig. 2). Methylation and unmethylated control DNAs showed the expected fragment sizes of 163 bp for RB1 and 195 and 212 bp for p27Kip1, respectively (Fig. 2). p21Waf1 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 p14ARF was observed in neurons and glial cells in peritumoral brain tissues. Ten cases (56%) of PCNSLs showed loss of p14ARF expression, and of the remaining cases, 4 showed immunoreactivity in >20% of the neoplastic cells (Table 1). Loss of p16INK4a expression was observed in 10 of 18 cases (56%; Table 1). There was a close correlation between loss of p14ARF/p16INK4a expression, as detected by immunohistochemistry and homozygous deletion/promoter methylation. All 8 cases with p14ARF expression showed a normal p14ARF gene status, whereas one case (case 6) with p16INK4a promoter methylation showed p16INK4a expression (Table 1). In one case (case 11), some tumor areas showed p14ARF/p16INK4a expression, but there were also focal areas with neoplastic cells that lacked p14ARF/p16INK4a expression. In this case, only microdissected areas with loss of p14ARF/p16INK4a expression showed nuclear immunoreactivity for p14ARF and p16INK4a but focal tumor cell clusters with loss of p14ARF and p16INK4a.

**Table 1.** Alterations of INK4A/ARF locus and the status of RB1, p21Waf1, p27Kip1, p53, and MDM2 in PCNSLs

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

Four cases demonstrated independent negative immunoreactivity for pRB and p27Kip1. Promoter hypermethylation of these genes was detected in 2 cases without pRB/p27Kip1 expression. A large fraction of PCNSLs (8 of 18; 44%) demonstrated loss of p21Waf1 expression, but p21Waf1 hypermethylation was not detected in any case (Table 1). No overexpression of MDM2 protein was evident.

**p53 Gene Mutations and Correlation between p14ARF 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 p14ARF 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 p14ARF (10 of 18; 56%) and p16INK4a (11 of 18; 61%) genes. We detected frequent p14ARF homozygous deletion (9 of 18; 50%), whereas the p14ARF promoter was unmethylated, even when the p16INK4a 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 p15INK4B and p16INK4a in human B-cell and T-cell lymphomas to be frequent, whereas methylation silencing of p14ARF was extremely rare. Meléndez et al. (18) also pointed out that p19ARF (the murine homologue of p14ARF) expression was lost or reduced in a significant percentage of murine primary lymphomas, whereas the p19ARF CpG island was infrequently methylated. Thus, p14ARF homozygous deletion, rather than promoter hypermethylation, is the most likely to be the essential event for p14ARF inactivation in PCNSLs.

The p16INK4a and p14ARF genes are frequently co-deleted in human neoplasms, and this was also the case for 6 PCNSLs in our series. However, cases with p14ARF deletion alone were also encountered, and a higher frequency of p14ARF than p16INK4a deletions has been reported for other human neoplasms (7, 11), suggesting that p14ARF is the primary target with 9p21 deletions. This conclusion is supported by studies of mice, lacking p19ARF (the murine homologue of p14ARF) expression alone through selective disruption of exon 1f, which develop tumors at several sites, including lymphomas, sarcomas, and gliomas (19).

p14ARF 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 p14ARF is therefore p53 dependent. Recent studies of the INK4a/ARF locus as a regulatory region for both p16INK4a/RBI and p14ARF/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, p14ARF alterations and p53 mutations appeared to be unrelated, and an inverse correlation between p14ARF 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

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

**Fig. 3.** A, p14ARF immunohistochemistry showing nuclear immunoreactivity in the majority of tumor cells (case 11). B, the same case with focal loss of p14ARF expression. C and D, case 11 exhibits extensive areas (C) with marked immunoreactivity for p16INK4a but focal tumor cell clusters (D) with loss of p16INK4a, counterstained with hematoxylin. ×300.
pattern of these alterations in tumors may depend on the order of events (6). When p14ARF 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 p14ARF deletion or promoter methylation showed loss. There was also 1 case (case 6) with p16INK4a promoter methylation that expressed p16INK4a, but this may be explained by incomplete gene silencing because of insufficient density and extent of methylation (23). The correlation was also exemplified by the finding that in 1 case, promoter hypermethylation was detected only in areas lacking p14ARF and p16INK4a immunoreactivity but not in the areas with expression. Regarding alternative explanations, one common feature of p16INK4a regulation is that tumors with increased levels have RBL1 alterations. Although there was no inverse correlation between p16INK4a and RBL1 in our series, the p16INK4a immunopositive case (case 6) with p16INK4a promoter hypermethylation showed RBL1 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 (22%) cases examined. This is agreement with the former report on glioblastomas (25). Our findings for PCNSLs are concordant with Cobbers et al. (13)

References


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