Primary malignant lymphoma of the brain: Frequent abnormalities and inactivation of p14 tumor suppressor gene

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Ten primary central nervous system lymphomas (PCNSL, brain lymphomas) were examined for p14 gene exon 1β deletion, mutation and methylation by Southern blot analysis, nucleotide analysis of polymerase chain reaction clones and Southern blot-based methylation assay. In Southern blot analysis, from the signal densities of the hybridized bands and their similarities to those of exons 2 and 3 in our previous quantitative study, we found that exon 1β was homozygously deleted in four cases, hemizygously deleted in five cases and not deleted in one case. Thus, the same deletion patterns covered the entire p14 gene for all cases except for one case, which suggested the hemizygous deletion of exons 1β and 2 and homozygous deletion of exon 3. In addition, although exon 1β mutation is rare in various tumors, we detected a missense mutation (L50R) in one case with a hemizygous deletion. Methylation of the 5′CpG island of the p14 gene was not suggested for any case without homozygous deletion. Our observation of frequent p14 gene abnormalities (90%) and inactivation (40–60%) was in striking contrast to the same pathological subtype of systemic lymphoma in which p14 gene abnormalities and inactivation were infrequent, suggesting a difference in carcinogenesis between PCNSL and systemic lymphoma. (*Cancer Sci* 2005; 96: 38–41)

In the INK4a/ARF locus of the chromosome 9p21 region are located two different tumor suppressor genes, p16 ($p16^{INK4a}$, MTS-1, CDKN2A) and p14 ($p14^{ARF}$).[1–3] These genes have a separate exon 1, exon 1α for p16 and exon 1β for p14 genes, and share exons 2 and 3.[1–3] p16 and p14 mRNAs use different reading frames for exons 2 and 3, and during translation generate different proteins.[1–3] Functionally, p16 protein inactivates cyclin-dependent kinases (CDK) 4 and 6 and thereby activates Rb protein, which suppresses cell growth.[4] In contrast, p14 protein inactivates MDM2 protein and thereby stabilizes p53 protein, which suppresses cell growth.[5,6] Additional functions related to cell growth suppression have also been found for p14 protein. In mice, p19 ($p19^{ARF}$) protein, a mouse homolog of p14 protein, directly interacts with p53 protein and stabilizes it.[7] In the absence of p53 protein, p19 protein inactivates E2F transcription factors which promote cell cycle progression and cell growth.[8] p14 protein also suppresses DNA synthesis by DNA replication protein A in the absence of p53 protein.[9] Considering these cell growth suppressive functions in the presence and absence of p53 protein, the p14 gene plays an important role in suppressing the development of various tumors.

Abnormalities in the p16 gene have been extensively analyzed for a variety of tumors in previous studies.[10] From the abnormalities detected in these studies in exons 2 and 3, which are shared with the p14 gene, some but not all abnormalities in the p14 gene have also been implicated. However, abnormalities in the p14 gene-specific exon 1β have not been extensively analyzed for a range of tumors. In addition, most previous studies have not simultaneously analyzed all p14 exons (exons 1β, 2 and 3) for the same tumor. Thus, abnormalities associated with the p14 gene have not been fully detailed for many tumors including primary central nervous system lymphomas (PCNSL).

In the present study, we examined the p14 gene for exon 1β deletion, mutation and methylation in 10 PCNSL. We found frequent homozygous (40%) and presumed hemizygous (50%) deletions of exon 1β. Together with our previous findings for other exons, deletion patterns of the entire p14 gene were established.[11,12] In addition, despite exon 1β mutation being a rare phenomenon in various tumors,[13] we found a missense mutation in exon 1β for one case with a presumed hemizygous p14 gene deletion.

Materials and Methods

Specimens. Ten biopsied tissues of PCNSL from untreated non-immunodeficient patients were used. The pathological diagnosis made for all cases was diffuse large B-cell lymphoma.[14–16] An additional tumor (Case 8) was diagnosed as brain localization (metastasis) of a systemic diffuse large B-cell lymphoma. The state of the p16 gene (exons 1α, 2 and 3) and p15 gene (exon 2) in all cases had already been analyzed in previous studies published by the authors.[11,12] The clinical, pathological and immunological data are briefly shown in Table 1.

Southern blot analysis of p14 exon 1β. The method of Southern blot analysis was as previously described.[11,17] Briefly, 10 μg of EcoRI-digested DNA was electrophoresed in 0.7% agarose gel and transferred to a nitrocellulose membrane. The membrane was prehybridized and then hybridized with a ^{32}P-labeled probe, washed to a final stringency of 0.1 × SSC/0.1% sodium dodecyl sulfate (SDS) at 37°C for 60 min and autoradiographed. For some experiments, the same membrane that was used for the previous analysis of the p16 and p15 genes was reprobed after treatment with hot 2% SDS solution for 60 min.[12] The probe was PCR-amplified p14 exon 1β prepared from wild-type DNA using the primers used for the mutation analysis described below. S-100β cDNA was used as a control probe.

Assessment of the deletion patterns was performed by comparing the images of exon 1β bands with those of either the exon 3 bands (cases other than Case 6) or exon 2 bands (Case 6) from the previous study, in which quantitative analysis was carried out using an image analyzer.[12] In that study, when the value of the signal density, normalized against that of the

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Table 1. Summary of p14 gene abnormalities in primary central nervous system lymphomas

Case	Age (y)/sex	Site of tumor	Type of tumor	p15 exon 2†		p14 exon 1β			p16 exon 1α†			exon 2†		exon 3†	p53‡
				Me§	D	Me§	D	Mu¶	Me§	D	Mu¶	D	Mu¶	D	Mu¶
1	42/M	F	DL/B	–	+/+	–	+/+	–	Me	+/+	–	+/+	–	+/+	–
2	74/M	P	DL/B		D/D		D/D			D/D	–	D/D	–	D/D	–
3	54/F	P	DL/B		D/D		D/D			D/D	–	D/D	–	D/D	–
4	59/F	Th	DL/B	–	D/+	–	D/+	–	Me	D/+	–	D/+	–	D/+	C176F
5	71/F	O	DL/B		D/D		D/D			D/D	–	D/D	–	D/D	W52Stop
6	78/M	Cbll	DL/B	Me	d/+	–	D/+	–	–	D/+	–	D/+	–	D/D	–
7	73/M	P	DL/B	–	d/+	–	D/+	L50R	Me	D/+	–	D/+	–	D/+	–
9	59/F	T	DL/B	–	d/+	–	D/+	–	–	D/+	–	D/+	–	D/+	–
10	62/M	P	DL/B		D/D		D/D			D/D	–	D/D	–	D/D	–
11	39/M	Th	DL/B	–	d/+	–	D/+		–	D/+	–	D/+	–	D/+	–
8††	60/M	P	DL/B	–	+/+	–	+/+	–	–	+/+	–	+/+	–	+/+	

B, B cell type [determined by analysis of immunoglobulin gene rearrangements (Cases 6–11), immunostaining (Case 5) or both (Cases 1–4)[12]]; Cbll, cerebellum; D, deletion; D/D, homozygous deletion; D/+, presumed hemizygous deletion; DL, diffuse large cell lymphoma; F, frontal; Me, methylation; Mu, mutation; O, occipital; P, parietal; T, temporal; Th, thalamus; +/+, no deletion.
§–, No methylation. ¶–, no mutation. †The states of the p15 and p16 genes obtained from our previous work.[12] ‡The states of the p53 gene for Cases 1–5 obtained from our previous work[27] and those for Cases 6, 7 and 9–11 obtained by PCR-SSCP (not shown). ††Brain localization of a systemic lymphoma.

internal control (S-100β), was 0–25% of the value of the normal control we presumed it to be a homozygous deletion; when it was 50–60% of the value of the normal control we presumed it to be a hemizygous deletion; and when it was approximately 100% of the value of the the normal control, we presumed there was no deletion.

Mutation analysis. Mutation analysis was performed on PCR clones from PCNSL cases (Cases 1, 4, 6, 7 and 9), which revealed no homozygous deletion of exon 1β as seen by Southern blot analysis. In PCR, the primers were 5′-TGCCGAGCTCGG-CCCTGGAG-3′ (sense), corresponding to part of the 5′ non-coding region and 5′-CCAAACAAAACAAGTGCGCCCCGGAC-3′ (antisense), corresponding to part of the 5′ end of Intron I. Thus, the target sequence contained the coding region of exon 1β spanning nucleotide positions (nps) 1–193 (codons 1–64 1/3), where the first nucleotide of the coding region was taken to be np 1. The PCR products were ligated into the SmaI cloning site of the pUC118 plasmid vector, transfected into Escherichia coli JM109, propagated, sequenced on both strands by the dideoxy chain termination method using a Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an Applied Biosystems 373S DNA sequencer (Applied Biosystems).[18] The findings obtained were confirmed by autoradiographic analysis of the sequencing gels (data not shown).

Methylation analysis. Methylation of the 5′CpG island of the p14 gene was examined by a Southern blot-based assay.[19] Genomic DNA doubly digested with EcoRI and the methylation-sensitive restriction enzyme SacII was electrophoresed, blotted, and hybridized with a ^{32}P-labeled p14 exon 1β probe. It was then examined to see whether small DNA fragments (3.4 and 0.5 kbp bands) were generated by SacII digestion of the EcoRI fragment of size 7.5 kbp.

Results

(1) Analysis of p14 exon 1β deletion. On Southern blot analysis, the p14 exon 1β probe demonstrated variable densities of hybridized bands, depending on the case. The hybridized bands were not distinct in four brain lymphoma cases (Cases 2, 3, 5 and 10), weak in five cases (Cases 4, 6, 7, 9 and 11) and intense in one case (Case 1). Brain localization of a systemic lymphoma (Case 8) also demonstrated an intense band. In Fig. 1, representative exon 1β bands are shown.

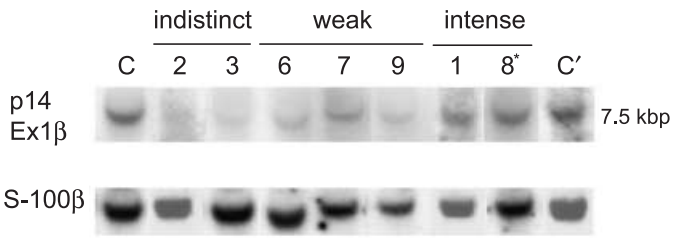

Fig. 1. Southern blot analysis of the p14 exon 1β deletion in PCNSLs. Representative p14 exon 1β bands of various densities are shown. An internal control (S-100β gene) was hybridized on the same membranes. Numbers, case numbers; 8*, brain localization of a systemic lymphoma; C, control (lymph node) for Cases 3, 6, 7, 9 and 8*; C′, control (placenta) for Cases 1 and 2. Signal densities of the hybridized bands were indistinct (Cases 2 and 3), weak (Cases 6, 7 and 9) and intense (Cases 1 and 8*), suggesting homozygous deletion, hemizygous deletion and no deletion, respectively.

When the images of the exon 1β bands were compared with those of the exon 3 (cases other than Case 6) or exon 2 (Case 6) bands, which were quantified in our previous analysis,[12] both images were shown to be highly comparable in individual lymphomas, suggesting that, like exon 2 or 3, exon 1β was homozygously deleted in 4 PCNSL cases (Cases 2, 3, 5 and 10) and hemizygously deleted in five cases (Cases 4, 6, 7, 9 and 11), and not deleted in one case (Case 1) (Table 1). Brain localization of a systemic lymphoma (Case 8) suggested no deletion of exon 1β.

In addition, for all cases except one, the images of exon 1β bands were also highly comparable to those of other exons in the INK4a/ARF locus including exon 1α and p15 exon 2 in our previous observations.[11,12] For a single case (Case 6), the image of the exon 1β band was comparable with that of the exon 2 band,[12] and with those of the exon 1α and p15 exon 2 bands, but not with that of the exon 3 band,[12] which was invisible. This indicates that the same deletion patterns occurred across the entire p14 gene, as well as the p16 gene and p15 gene (exon 2), in all cases except one (Case 6) (Table 1). In one case (Case 7) in which a hemizygous deletion of the p14 gene was suggested, a missense mutation was detected in exon 1β as described below.

(2) Mutation analysis. Mutation analysis was carried out on PCR clones of exon 1β from five PCNSL (Cases 1, 4, 6, 7 and

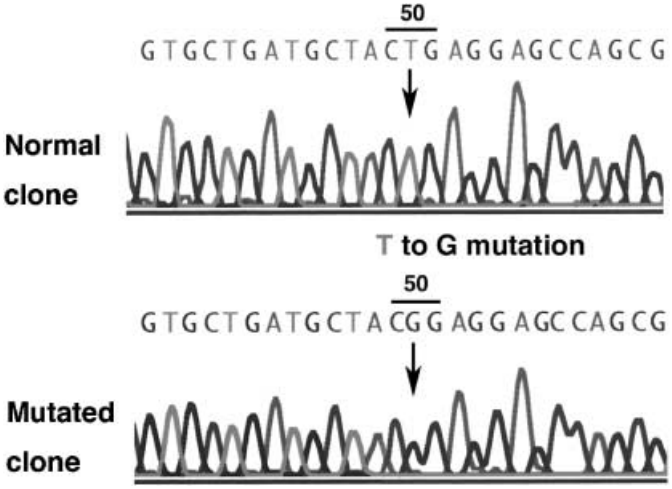

Fig. 2. Detection of a mutation in p14 gene exon 1β for a primary central nervous system lymphoma (PCNSL) (Case 7). Sequence change of p14 gene exon 1β in a PCR clone of a PCNSL (Case 7). The arrows point to the T to G substitution at codon 50, changing leucine to arginine (L50R) in a polymerase chain reaction (PCR) clone.

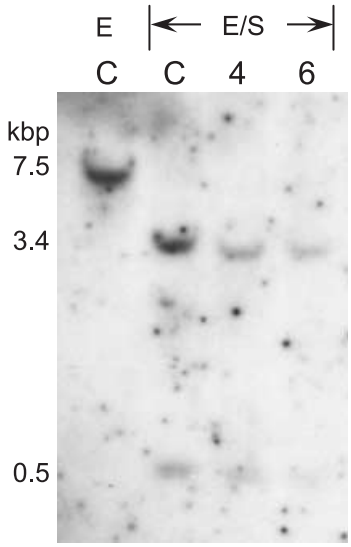

Fig. 3. Southern blot-based assay of 5′-CpG island methylation. Representative cases are shown. Southern blots of DNAs doubly digested with EcoRI and the methylation-sensitive enzyme SacII were hybridized with the p14 exon 1β probe. C, control lymph node; E, digested with EcoRI alone; E/S, digested with EcoRI and SacII; numbers, case numbers. A 7.5 kbp EcoRI-digested DNA fragment was further digested for Cases 4 and 6, for which a hemizygous p14 exon 1β deletion was suggested.

9) which did not suggest a homozygous deletion in the Southern blot analysis. In four of the five cases no mutation was detected in any of several clones examined. In the remaining case (Case 7), however, a missense mutation (CTG-CGG, L50R) was detected in four of five clones examined (Fig. 2 and Table 1). Detection of the mutation in the majority of the clones examined suggested the hemizygous deletion of exon 1β, consistent with the above-described findings of the Southern blot analysis. For our PCNSL cases including this case, the other exons revealed no mutation as previously described.[12] The brain localization of a systemic lymphoma (Case 8) also revealed no mutation. These findings were confirmed by autoradiographic analysis of the sequencing gels (data not shown).

(3) Methylation analysis. Six PCNSL (Cases 1, 4, 6, 7, 9 and 11) which did not suggest the homozygous deletion of p14 exon 1β as seen by the Southern blot analysis were examined for methylation of the 5′CpG island of the p14 gene by a Southern blot-based assay. It was discovered that a 7.5 kbp EcoRI-digested DNA fragment was further digested by the methylation-sensitive enzyme SacII for all of the brain lymphomas. Representative findings are shown in Figure 3. An additional case of brain localization of a systemic lymphoma (Case 8) also revealed further digestion of the EcoRI fragment by SacII. These findings indicate that the 5′CpG island was not methylated in these lymphomas, suggesting that the p14 gene promoter was not inactivated (Table 1).

Discussion

In this study, we analyzed 10 PCNSL for p14 exon 1β deletion, mutation and 5′CpG island methylation. From the findings obtained and those of our previous studies of other exons, we determined the profile of p14 gene abnormalities for individual tumors (Table 1).

On the Southern blot analysis of p14 exon 1β, we found frequent homozygous (40%) and presumed hemizygous (50%) deletions. The same deletion patterns covered the entire p14 gene, as well as the p16 gene and p15 gene, for all cases except one (Case 6), which suggested hemizygous deletion in exons 1β and 2 and homozygous deletion in exon 3 (Table 1).[12] Previously, Nakamura *et al.* observed the homozygous deletion of p14 exon 1β in 50% (9 of 18 cases) of PCNSL studied by differential PCR.[20] However, they did not examine exons 2 and 3, although they examined exon 1α. In addition, hemizygous deletions were not examined in their analysis. Our observations suggested that, in addition to homozygous deletion, hemizygous deletion also frequently occurs in exon 1β, and in the entire p14 gene as well, in PCNSL.

Mutations in p14 exon 1β are rare in various tumors.[13] Burri *et al.* reported a missense mutation at codon 12 (CGG-TGG, R12W) in a colon cancer, and a frameshift mutation at codon 33 (1 bp deletion) in a colon cancer cell line,[13] while Rizos *et al.* reported a germ line frameshift mutation at codon 21 (16 bp insertion) in a melanoma family.[21] The N-terminal region (codons 1–20) contains a nucleolar localization signal and MDM2 conjugation signal,[22,23] which are important for normal p14 gene function in terms of stabilizing the p53 tumor suppressor protein. Thus, these mutations, which are located in or near the N-terminal region, suggest a functional effect. In cases of frameshift mutations, the severe sequence changes themselves suggested severe dysfunction. In the present study, we detected a missense mutaton (L50R) in exon 1β in one PCNSL case with presumed hemizygous deletion (Case 7). However, its functional effect remains unknown, because the function of the p14 gene region in which this mutation was located is unknown. As described in the Introduction, p14 gene functions include not only interaction with the MDM2-p53 pathway, but with other molecular pathways as well. Considering these various functions, further studies on the effect of this mutation will be of value.

In this study, we also carried out methylation analysis on six cases with no homozygous deletion of p14 exon 1β. However, methylation of the 5′CpG island of the p14 gene was not suggested for any case. This finding is consistent with the previous work of Nakamura *et al.* and Gonzalez-Gomez *et al.* who detected methylation of the p14 gene only in one of nine and none of three cases examined, respectively.[20,24] Thus, in PCNSL p14 gene inactivation due to methylation is rare, unlike for the neighboring p16 gene in which methylation is frequent, next to deletion.[12]

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Regarding systemic lymphomas, Pinyol *et al*. and Baur *et al*. examined 15 and 13 cases of diffuse large B-cell lymphomas, respectively, the same pathological subtype as PCNSL, and found infrequent abnormalities in the p14 gene.[25,26] Pinyol *et al*. detected homozygous deletion of the p14 gene in only one case, while Baur *et al*. detected no methylation of the p14 gene.[25,26] Both authors detected no mutation in p14 exons 1β and 2, or exon 3.[25,26] We detected no abnormality in any exons of the p14 gene, or the p16 and p15 genes, in the brain localization (Case 8) of a systemic lymphoma.

In summary, we detected frequent homozygous and presumed hemizygous deletions in p14 gene exon 1β in nine of 10 PCNSL. The same deletion patterns covered the entire p14 gene for all of these cases except one. In addition, a missense mutation was detected in p14 exon 1β in one case with a presumed hemizygous deletion. Our observation of frequent p14 gene abnormalities (90%) (cases other than Case 1) and inactivation (at least 40% and at most 60%) (Cases 2, 3, 5 and 10 with or without Cases 6 and 7) is in striking contrast to its infrequent abnormality in systemic lymphoma of the same pathological subtype, suggesting a difference in carcinogenesis between PCNSL and systemic lymphoma.

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