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Primary Malignant Lymphoma of the Brain: Demonstration of Frequent p16 and p15 Gene Deletions

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The p16 (MTS-1) gene, a candidate tumor suppressor gene, was examined by means of Southern blot, PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism) and nucleotide analyses in 5 cases of primary malignant lymphoma of the brain. By Southern blot analysis, the p16 gene was found to be deleted in at least 4 cases, homozygously (3 cases) or hemizygously (1 case). The p15 (MTS-2) gene, another candidate tumor suppressor gene located in the vicinity of the p16 gene, to which it shows structural and functional similarity, was also found to be deleted in 4 cases. Our frequent detection (80%) of p16 and p15 gene deletions might suggest that these deletions are closely related to carcinogenesis in primary malignant lymphoma of the brain. SSCP and nucleotide analyses revealed no mutations of the p16 gene in any of the cases.

Key words: Lymphoma — Brain tumor — Tumor suppressor gene — CDK inhibitor gene — p16 gene deletion

The p16 (MTS-1) gene is a candidate tumor suppressor gene which is located on chromosome 9p; it consists of 3 exons and encodes 156 amino acids.[1–4] Functionally, the gene product, p16 protein, binds to and inactivates CDK4 and CDK6 (cyclin-dependent kinases 4 and 6) in the late G1 phase of the cell cycle and ultimately suppresses cell division.[4,5] This gene was found to be deleted at high frequency in cultured cell lines as well as primary tumor tissues of a wide variety of human malignancies.[1,2,6–9] With respect to brain tumors, deletion of the p16 gene has been detected frequently in astrocytoma cell lines[1,2] and in primary tumor tissues of high-grade astrocytoma,[10–13] but not or only rarely in low-grade astrocytomas, medulloblastomas or ependymomas.[10] However, the state of the p16 gene has not been detailed in other brain tumors of various histological types and origins.

Primary malignant lymphoma of the brain usually occurs in the brain parenchyma of aged patients whose organs show no lymphoproliferative focus.[14] The incidence of this tumor is low, and it accounts for about 1–3% of all intracranial tumors.[15] However, a high incidence has been noted in immunodeficient patients.[15] In addition, its incidence in non-immunodeficient patients has been increasing in recent years in several countries, including Japan.[15] Although the clinical, pathological and immunological characteristics of this tumor have been adequately described,[14–16] the genetic alteration(s) involved in this tumor remain largely unknown.

In this study, we examined five cases of primary malignant lymphoma of the brain for p16 gene alteration by means of Southern blot, PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism) and nucleotide analyses. The p15 (MTS-2) gene,[4,5,7,8] which is another candidate tumor suppressor gene with structural and functional similarity to the p16 gene, was also examined.

MATERIALS AND METHODS

Specimens Five biopsied tissues of primary malignant lymphoma of the brain from non-immunodeficient patients were examined. The pathological diagnosis made in all cases was diffuse large cell lymphoma, based on the LSG[17] and WF[18] criteria. Immunological analyses revealed B cell nature in 4 cases examined. In Table I, the clinical, pathological and immunological data of the patients are briefly shown.

DNA isolation High-molecular-weight DNA was isolated from frozen lymphoma tissues according to the method of Blin and Stafford.[19]

Southern blot analysis Seven micrograms of *Eco*RI-digested DNA was electrophoresed on horizontal 0.7% agarose gel and transferred to nitrocellulose membranes by the method of Southern.[20] The membranes were baked *in vacuo* at 80°C for 2 h and prehybridized at 42°C for 3 h in a prehybridization solution.[16] Then, they were hybridized with ^{32}P-labeled DNA probe at about 3×10^6 cpm/ml at 42°C for 16 h in a solution containing 4 vol of prehybridization solution and 1 vol of 50% sodium dextran sulfate. After hybridization, membranes were washed to a final stringency of 0.1×SSC and 0.1% sodium dodecyl sulfate for 40 min at 37°C and exposed to X-ray film or to an imaging plate for the image analysis

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Table I. Summary of p16 and p15 Gene Alterations in Primary Malignant Lymphomas of the Brain

Case	Age/ sex[a]	Site of tumor[b]	Type of tumor[c]	p53 mutation[d]	p16					p15 Deletion[i]
					Signal density[e]		p16/ S-100 (%)[f]	Deletion[g]	Mutation[h]	
					p16	S-100				
Control					17.8	31.7	0.562 (100)	−	−	−
1	42/M	F	DL/B	−	11.2	28.2	0.397 (70.7)	(+/−)	−	(+/−)
2	74/M	P	DL/B	−	3.9	28.3	0.138 (24.5)	+	−	+
3	54/F	P	DL/B	−	4.9	33.4	0.147 (26.1)	+	−	+
4	59/F	Th	DL/B	+	8.0	30.3	0.264 (47.0)	+/−	−	+/−
5	71/F	O	DL	+	1.9	17.8	0.107 (19.0)	+	−	+

a) Age, years; M, male; F, female.
b) F, frontal; P, parietal; Th, thalamus; O, occipital.
c) DL, diffuse large cell lymphoma; B, B cell type.[16]
d) +, missense (Cys to Phe in codon 176 in case 4) or nonsense (Trp to Stop in codon 52 in case 5) mutation[24]; −, no mutation.
e) Signal densities of the bands for exon 1 of the p16 gene in Southern blot were estimated using an image analyzer. count/ mm^2.
f) Signal densities for the p16 gene were normalized with respect to that for the S-100β gene and represented as % of the control.
g) +, homozygous deletion; +/−, hemizygous deletion presumed; (+/−), either no deletion or hemizygous deletion; −, no deletion.
h) −, no mutation in exons 1 and 2 of p16 gene in SSCP analysis and direct sequencing.
i) Same as in g.

described below. The probes were PCR-amplified exon 1 and exon 2 of the p16 gene prepared from placental DNA using the primers described by Kamb *et al.* (2F and 1108R)[1] and Mori *et al.*,[21] respectively. S-100β cDNA[22] was kindly supplied by Drs. R. Kuwano and K. Morii of the Research Laboratory for Molecular Genetics of our University and was used as a control probe to estimate the amount of sample DNAs applied in the Southern blots. The signal density of hybridized bands was quantitatively estimated using a Fujix BAS 2000 image analyzer (Fuji Photo Film Co., Tokyo). Hybridized membranes were exposed to an imaging plate for ~3 h and the signal density of bands was read as the intensity of fluorescence by the image analyzer.

PCR-SSCP analysis PCR-SSCP analysis[23] was performed on exons 1 and 2 of the p16 gene, which contain about 97% of the coding sequence. The PCR mixture, 50 μl in volume, containing 7 pmol each of paired primers, 10 nmol each of dNTPs, 100 ng of genomic DNA, 2.5 U of Taq polymerase (Takara, Tokyo), 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 0.01% gelatin and 2% dimethyl sulfoxide was subjected to amplification for 35 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C, except for the first and the last cycles, for which 5 min denaturation time and 5 min extension time were used, respectively. In the examination of exon 1 of the p16 gene, the primers used were 5′-GAAGAAAGAGGAGGGGCTG-3′ (sense) and 5′-GCGCTACCTGATTCCAATTC-3′ (antisense).[1] In the examination of exon 2, regions corresponding approximately to the 5′ half and the 3′ half were separately amplified to enhance sensitivity in SSCP. The primers for the former were 5′-GGCTCTACACAAGCTTCCTT-3′ (sense) and 5′-ACCACCAGCGTGTCCAGGAA-3′ (antisense) and those for the latter were 5′-ACTCTCACCCGACCCGTGCA-3′ (sense) and 5′-TGAGCTTTGGAAGCTCTCAG-3′ (antisense). The annealing site of the sense primer for the 3′ half was located upstream of the site of the antisense primer for the 5′ half.

The PCR products obtained were subjected to a second PCR with the same temperature profile as described above. The PCR mixture, 10 μl in volume, contained the same concentrations of reagents as used in the first PCR, except that 7.4 kBq of [α-^{32}P]dATP was added and the amount of unlabeled dATP was decreased to 0.4 nmol. For SSCP analysis, the second PCR products were diluted 1:20–50 with a diluent solution,[24] denatured at 95°C for 5 min, electrophoresed in 5% non-denaturing polyacrylamide gel containing 5% glycerol at 4°C and exposed to X-ray film.

Nucleotide analysis of PCR products The PCR products were prepared by the same method as described for the first PCR in PCR-SSCP. Direct sequencing was performed by the dideoxy termination method[25] using an fmol DNA Sequencing System sequencing kit (Promega, Madison, WI). Briefly, an extension/termination reaction mixture, 6 μl in volume, containing 25 ng of the

PCR products, 25 ng of one of two primers described, 1.25 U of Taq polymerase, 46 kBq of [α-^{32}P]dATP and dNTP/ddNTP mix was subjected to 30 cycles of PCR amplification using the temperature profile recommended by the manufacturer. The ^{32}P-incorporated PCR products were electrophoresed in a sequencing gel and autoradiographed.

RESULTS

Southern blot analysis In Southern blot analysis, the p16 exon 1 probe revealed a single band of about 4.3 kbp after *Eco*RI digestion of both the control and the lymphoma samples (Fig. 1). While the signal density of the band was intense in the control, it was less intense or faint in all of the lymphomas, especially in 4 cases (cases 2–5). Quantitative analysis by an image analyzer revealed that the value of the signal density normalized to that for the internal control, S-100β, was only about 20–25% of the control value in 3 cases (cases 2, 3 and 5), about 50% in 1 case (case 4) and about 70% in 1 case (case 1) (Table I). Considering these findings in the light of possible contamination of normal cell elements in tumor tissues, it seemed likely that the p16 gene was deleted in at least 4 cases (cases 2–5), homozygously in 3 cases (cases 2, 3 and 5) and probably hemizygously in 1 case (case 4).

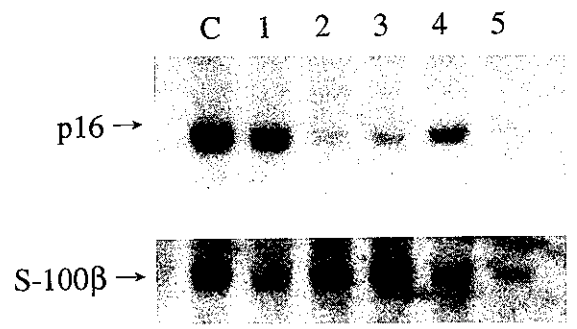

Fig. 1. Southern blot analysis with the p16 gene exon 1 probe. The band for the p16 gene is faint in brain lymphomas (1–5), especially in 4 cases (2–5), as compared with that in control placenta (C). S-100β gene used as an internal control was hybridized in the same membrane.

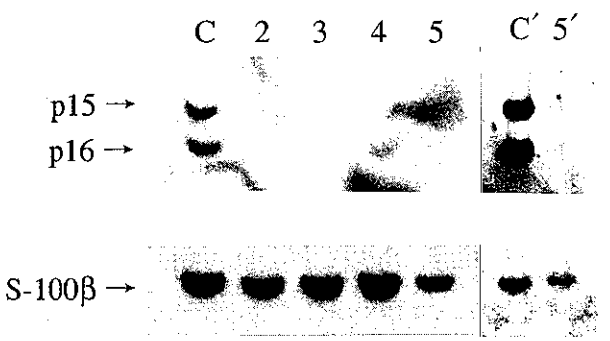

Fig. 2. Southern blot analysis with the p16 gene exon 2 probe. The band for the p16 gene is faint in brain lymphomas (2–5) as compared with that in control placenta (C). The band for the p15 gene, which resulted from cross-hybridization with the p16 gene exon 2 probe, is also faint in these lymphomas. S-100β gene used as an internal control was hybridized in the same membrane. Two lanes on the far right, reexamination of case 5 (5′) together with control (C′).

In 4 cases (cases 2–5), analysis with the p16 exon 2 probe was also performed. As seen in Fig. 2, the bands of about 4 kbp which represented the p16 gene were apparently faint in these cases. Their signal densities were comparable to those of the bands for exon 1 described above, suggesting that exons 1 and 2 were similarly deleted in each case.

Moreover, using the p16 exon 2 probe we also detected the p15 gene as a band of about 4.6 kbp. This was considered to be a result of cross-hybridization due to structural similarity of the exons 2 of the two genes, as suggested by the previous work[26] and our competition experiment using the p15 and the p16 exon 2 probes (data not shown). As seen in Fig. 2, the bands for the p15 gene were also faint in the 4 lymphoma cases, suggesting that the p15 gene was also deleted in these cases.

PCR-SSCP and nucleotide analyses SSCP and direct sequencing analyses were performed on PCR products from exons 1 and 2 of the p16 gene. However, no aberrant electrophoretic mobility shift or intragenic mutation was detected in any of the 5 cases, including the 2 cases in which homozygous deletion was not suggested (Table I). The nucleotide sequences were identical with the wild-type nucleotide sequence as revised by Okamoto *et al.*[27]

DISCUSSION

In this study, we examined p16 gene alteration in 5 cases of primary malignant lymphoma of the brain by Southern blot, SSCP and nucleotide analyses. In the Southern blot analysis with the p16 exon 1 probe, we observed that the bands for the p16 gene were fainter in all 5 of the lymphomas than in the control. Similar results were obtained with the p16 exon 2 probe in 4 cases examined. From the values of the signal density of the bands estimated quantitatively using the image analyzer, homozygous (cases 2, 3 and 5) or hemizygous (case 4) deletion was suggested in at least 4 lymphoma cases. In addition, during examination of exon 2 of the p16 gene, the p15 gene, another candidate tumor suppressor gene,[4,5] was also shown to be deleted in these cases. Our

observation of frequent deletions (80%) of the p16 and p15 genes might suggest that deletions of these genes are closely related to carcinogenesis in primary malignant lymphoma of the brain.

In the SSCP and direct sequencing analyses, no mutations were detected in exons 1 and 2 of the p16 gene in any of the 5 cases, including the case (case 4) in which hemizygous deletion was suggested. Similar negative findings have been reported for other tumors with hemizygous p16 gene deletion.[12] Dysfunction of both alleles is usually considered to be necessary in carcinogenesis by alteration of a tumor suppressor gene. Thus, in such cases without mutation, it is possible that a mutation might be located outside the region examined. Dysfunction due to methylation at the 5′ CpG island of the p16 gene is also possible, as suggested by a recent study.[28] In addition, in our case 4, we detected a coincidental mutation of the p53 tumor suppressor gene (Table I), suggesting that dysfunction of the p16 gene was not necessarily required in carcinogenesis. Thus, it remains possible that normal p16 function was maintained even in the hemizygous state. Further studies focussing on these possibilities might be of value in reaching a better understanding of the hemizygous state of this gene.

Regarding generalized lymphomas, only a few studies[8, 29–32] have analyzed p16 and p15 gene alterations. Among them, Uchida *et al.*[29] and Gombart *et al.*[30] examined B cell type diffuse large cell lymphomas and detected p16 gene deletion in 2 (10%) of 19 cases and p16 and p15 gene deletions in 1 (17%) of 6 cases, respectively. Other authors[8, 31, 32] have examined diffuse large cell lymphomas and non-Hodgkin lymphomas and also observed genetic alteration of these genes only at low frequencies, ranging from 0 to 15% of cases. These findings are inconsistent with our frequent detection of p16 and p15 gene deletions in brain lymphomas, most of which were classified as B cell type diffuse large cell lymphomas. The reason for this discrepancy between generalized and brain lymphomas with the same histological and immunological features remains unknown. It seems possible that such a discrepancy indicates a difference in the mechanism of carcinogenesis between these lymphomas of different localizations. To investigate this possibility, however, further studies with larger sample sizes are required in both brain and generalized lymphomas.

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