Jpn. J. Cancer Res. **93**, 1308–1316, December 2002

Primary Malignant Lymphoma of the Brain: Mutation Pattern of Rearranged Immunoglobulin Heavy Chain Gene

Sumio Endo,[1] Shu-Jing Zhang,[2] Takafumi Saito,[3] Mitsuo Kouno,[4] Toshihiko Kuroiwa,[5] Kazuo Washiyama[1] and Toshiro Kumanishi[1, 6, 7]

[1]Molecular Neuropathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata 951-8585, [2]Biotechnology, Biosciences at Novum, Karolinska Institute, Hudding, S-141 57 Sweden, [3]Neurosurgery, Nagano Red Cross Hospital, 5-22-1 Wakasato, Nagano 380-8582, [4]Neurosurgery, Toyama Prefectural Central Hospital, 2-2-78 Nishinagae, Toyama 930-8550, [5]Neurosurgery, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686 and [6]Niigata Longevity Research Institute, 766 Shimoishikawa, Shibata, Niigata 959-2516

Using reverse transcription-polymerase chain reaction (RT-PCR), six primary brain lymphomas, pathologically diagnosed as diffuse large B-cell lymphoma, were examined for rearranged V_H-D-J_H sequences of the immunoglobulin heavy chain gene, focusing on somatic mutations and intraclonal heterogeneity. The reliability of the isolated PCR clones was confirmed by *in situ* hybridization (ISH) with complementarity-determining region (CDR) 3 oligonucleotide probes. Sequence analysis of the PCR clones revealed a high frequency of somatic mutation, ranging from 8.8 to 27.3% (mean 18.2%) in the V_H gene segments in all the lymphomas. A significantly lower frequency of replacement (R) mutations than expected was also seen in their frameworks (FRs) in all cases. These findings suggested that the precursor cells were germinal center (GC)-related cells in these lymphomas. However, despite extensive cloning experiments, intraclonal heterogeneity was not detected in any case except for one in which it could not be ruled out. Thus, it seemed likely that all of our brain lymphomas were derived from GC-related cells and that at least most of them were from post-GC cells.

Key words: Lymphoma — Brain lymphoma — B-cell lymphoma — Immunoglobulin gene — *In situ* hybridization

Primary brain lymphoma usually occurs in the brain parenchyma of aged patients whose general organs show no lymphoproliferative focus.[1, 2] Pathologically, most brain lymphomas belong to the category of diffuse large B-cell lymphoma.[3–5] The incidence of this tumor is low, accounting for about 1–3% of all intracranial tumors.[2] However, a high incidence has been noted in immunodeficient patients.[2] In addition, an increased incidence in non-immunodeficient patients has recently been reported in several countries.[2] Although the clinical, pathological, and immunological characteristics of this tumor have been adequately described,[1–8] there still remain a number of unsolved problems, including the histogenesis of the neoplastic B lymphocytes.

In B lymphocytes in general, immunoglobulin heavy and light chain genes are rearranged.[9, 10] In the heavy chain gene, 1 each of heavy chain variable (V_H), diversity (D) and joining (J_H) gene segments are chosen and assembled, resulting in a considerable variety of rearranged V_H-D-J_H gene sequences among B cells.[9, 10] In addition, further variability is introduced by random deletions and insertions of nucleotides at V_H-D and D-J_H junctions[11, 12] as well as by somatic mutations in the entire V_H-D-J_H sequence.[13–15] While V_H-D-J_H gene rearrangements, which are associated with random nucleotide deletions and insertions at the V_H-D and D-J_H junctions, occur at an earlier stage of B-cell differentiation, somatic mutations in the entire V_H-D-J_H sequences occur at a later stage when B cells are in the germinal center (GC) microenvironment of the lymphatic tissues.[13–15]

Using polymerase chain reaction (PCR), recent studies of B-cell lymphomas have analyzed the rearranged immunoglobulin genes for somatic mutations to gain an insight into the histogenesis of neoplastic cells.[16–19] In systemic lymphomas, somatic mutations have been detected in a variety of B-cell tumors,[16–19] suggesting that the neoplastic cells were derived from GC-related cells, namely GC cells or post-GC cells, but not pre-GC cells. In addition, intraclonal heterogeneity has been detected by cloning experiments in some of these lymphomas including follicular lymphomas[16, 20, 21] and a fraction of diffuse large B-cell lymphomas.[19, 22, 23] These findings suggested that the precursor cells were GC cells, but not post-GC cells, since the ability to induce somatic mutations is a hallmark of GC cells.

In brain lymphomas, mutation patterns of the rearranged immunoglobulin genes have not been fully detailed,

[7] To whom correspondence should be addressed.
E-mail: kumanisi@pop.shibata.ne.jp

although Thompsett *et al.*[24] and Montesinos-Rongen *et al.*[25] have examined 5 and 10 cases, respectively. Thus, further studies on the mutation patterns of rearranged immunoglobulin genes are required for a better understanding of the histogenesis of this tumor.

In this study, we isolated reverse transcription (RT)-PCR clones of the rearranged V_H-D-J_H sequences from 6 brain lymphomas, confirmed their patient- or specimen-specificity by *in situ* hybridization (ISH) and analyzed their nucleotide sequences focusing on somatic mutations and intraclonal heterogeneity. From the findings obtained, it was suggested that all of these brain lymphomas were derived from GC-related cells and that at least most of them were from post-GC cells.

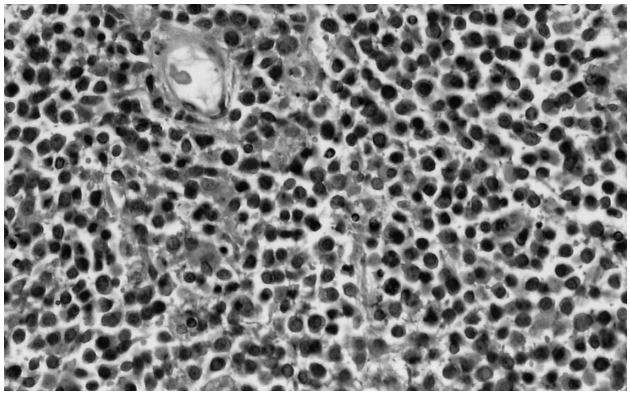

Fig. 1. Histology of a brain lymphoma case (case 1, primary tumor). Formalin-fixed paraffin section. Hematoxylin-eosin stain. ×275.

MATERIALS AND METHODS

Specimens Six primary brain lymphoma tissues from 6 untreated non-immunodeficient patients were used. An additional 1 lymphoma tissue was from the secondary tumor of 1 patient (case 1); this had recurred in a different brain region after a 9-month interval. The pathological diagnosis made in all cases was diffuse large B-cell lymphoma[26–28] (Fig. 1). In Table I, the clinical, pathological and immunological data of the patients are summarized.

Isolation of RT-PCR clones Five micrograms of DNase I-treated total RNA was reverse-transcribed with an oligo(dT) primer in 21 μl of reaction mixture using a commercial kit, SuperScript Preamplification System (Gibco BRL, Grand Island, NY). Subsequent PCR amplification was performed with two primer pairs. Each primer pair contained Cμ antisense primer (5′-GGTTGGGGCGGAT-

Table I. Summary of Mutation Analysis of V_H Genes in Brain Lymphomas

Case No.	Age (yr) /Sex	Tumor		V_H gene usage	Somatic mutations in V_H gene					J_H gene usage[e]
		Site[a]	Diag[a]		No. (%)	Region[b]	R[c]	S[c]	*P* value[d]	
1	65/M	F	DL/B(μ)	*V4-34*	60 (20.6)	FR	17	22	3.3×10^{-6}	*J_H4*
						CDR	10	11	NS	
2	61/F	F	DL/B(μ)	*V4-34*	62 (21.3)	FR	19	20	1.3×10^{-5}	*J_H4*
						CDR	11	12	NS	
3	58/M	F	DL/B(μ)	*V3-15*	82 (27.3)	FR	34	26	3.3×10^{-3}	*J_H5*
						CDR	13	9	NS	
4	54/F	P	DL/B(μ)	*V4-59*	47 (16.2)	FR	16	14	1.1×10^{-3}	*J_H3*
						CDR	9	8	NS	
5	78/M	C	DL/B(μ)	*V3-23*	26 (8.8)	FR	6	5	2.3×10^{-4}	*J_H4*
						CDR	12	3	4.2×10^{-4}	
6	73/M	P	DL/B(μ)	*V4-34*	43 (14.8)	FR	9	20	7.2×10^{-7}	*J_H5*
						CDR	11	3	NS	

a) F, frontal; P, parietal; C, cerebellar; DL, diffuse large cell lymphoma; B, B-cell type; μ, IgM expression detected by northern blot analysis (data not shown).
b) FR, framework regions 1–3; CDR, complementarity-determining regions 1 and 2.
c) R, numbers of replacement mutations; S, numbers of silent mutations.
d) Probability of scarcity (for FR) or excess (for CDR) of mutations occurring by chance. The *P* values were calculated by the multinominal distribution model as described by Lossos *et al.*[19] using the Mathematica software package (Wolfram Research, Inc.). NS, nonsignificant values. The *P* values were also calculated by the binominal distribution model described by Chang and Casali.[33] By this model, the *P* values for FR and CDR in cases 1 to 6 were shown to be 3.7×10^{-6} and NS, 1.3×10^{-5} and NS, 2.1×10^{-3} and NS, 9.9×10^{-4} and NS, 3.0×10^{-4} and 5.4×10^{-4} and 9.8×10^{-7} and NS, respectively.
e) The germline J_H genes[32] to which lymphoma J_H genes showed the highest homology.

GCACT-3′) corresponding to the 5′ end of the Cμ constant region, since all the lymphomas revealed IgM mRNA in northern blot analysis (Table I). The sense primer in one pair was either of the 7 V_H leader primers which corresponded to V_H family-specific leader sequences,[29] and that in another was the FR2A primer (5′-TGG(AG)TCCG (AC)CAG(GC)C(CT)(CT)CNGG-3′) corresponding to the framework (FR) 2 region of the V_H gene. The target sequence for the former primer pair was a full-length V_H-D-J_H (nucleotide position (n.p.) 1-ca. 300), while that of the latter was a partial V_H-D-J_H (n.p. 120-ca. 300), each containing the CDR3 region. The PCR mixture, 50 μl in volume, contained 1 μl of the 1st-strand cDNA solution described above, 100 ng each of primers, 0.25 U of Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA) and other standard reagents.[30] The PCR temperature profile was as previously described.[30] The PCR products obtained were purified by agarose gel electrophoresis, ligated into the *Eco*RV cloning site of the pBluescript-SK(+) plasmid vector, transfected into *Escherichia coli* JM109 cells and propagated.

Sequencing of RT-PCR clones RT-PCR clones were sequenced on both strands by the dideoxy chain termination method[31] using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed with an Applied Biosystems 373S DNA sequencer. In each lymphoma, 22–24 full-length and 10–16 partial V_H-D-J_H clones from different bacterial colonies were examined.

Analysis of somatic mutations and intraclonal heterogeneity The sequences of the most abundant clones in individual lymphomas were compared with the germline gene sequences in the IMGT database[32] and the most closely related germline genes were considered as the used genes. The number of somatic mutations and the mutation frequency were determined by comparison of lymphoma sequences with the used germline V_H gene sequences. The numbers of replacement (R) and silent (S) mutations in FRs 1 to 3 and in complementarity-determining regions (CDRs) 1 and 2 were also determined and the frequencies of mutations in FRs and CDRs were analyzed using the equation of Lossos *et al.*,[19] as well as that of Chang and Casali.[33] Calculation with the former equation used the Mathematica software package (Wolfram Research, Inc., Champaign, IL). In analysis of intraclonal heterogeneity, a nucleotide substitution in more than 1 clone was considered as a mutation according to Lossos *et al.*[19]

Preparation of CDR3 oligonucleotide probe Based on the CDR3 sequences in the PCR clones, antisense oligonucleotide probes, 41- to 48mer, were constructed and used for ISH as described below. The probe sequences are shown in Fig. 2. In most cases, the probe sequences contained not only CDR3 sequences, but also short stretches of adjacent V_H sequences and, in 1 case (case 4), that of a J_H sequence as well. These short stretches which were mutated or not mutated accounted for about 15–30% of the total probe length in these cases. The short stretches without mutations accounted for about 15–20% of the total probe length.

ISH analysis Ten-micrometer-thick cryostat sections were attached to 0.01% poly L-lysine-coated glass slides and fixed in 4% paraformaldehyde/0.1 *M* sodium phosphate-buffered saline (PBS), pH 7.2, for 30 min. The sections were treated with 2 mg glycine/ml PBS for 20 min and with 0.25% acetate/0.1 *M* Tris-HCl, pH 8.0, for 10 min. The sections were then prehybridized at 42°C for 3 h in a prehybridization solution,[34] and hybridized with 5×10^3 cpm/μl of the heat-denatured ^{35}S-labeled CDR3 oligonucleotide probe described above in a hybridization solution[34] at 42°C for 16 h. The sections were washed three times in 0.1× SSC (salt sodium citrate)/0.1% Sarkosyl at 42°C for 40 min, dehydrated, and autoradiographed using NTB2 nuclear track emulsion (Kodak, Rochester, NY) at 4°C for 4–8 weeks. After development, the sections were counterstained with methylgreen-pyronin and examined with a light microscope. The hybridized sections were also examined by a Fujix BAS5000 image analyzer (Fuji Photo Film, Tokyo) for rapid detection of overall signal distributions. In the control competition experiment,

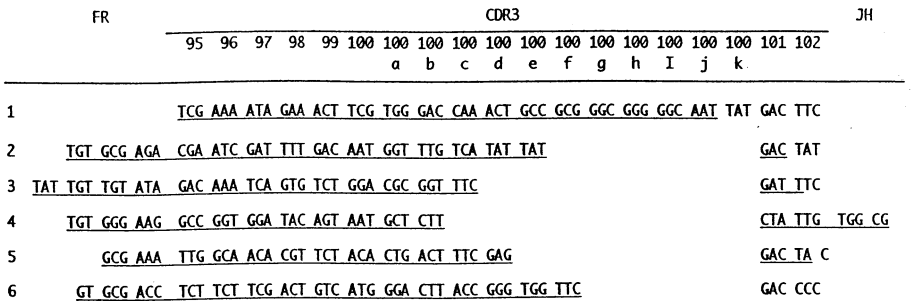

Fig. 2. CDR3 probe sequences. CDR3 probe sequences (underlined) in cases 1–6 (1–6) are shown together with the entire CDR3 sequences with or without adjacent FR3 and J_H sequences. Codon numbers in the CDR3 region are indicated.

the sections were hybridized with a mixture of the radiolabeled oligonucleotide probe and a 25-fold excess of unlabeled probe.

RESULTS

ISH analysis As described later, the CDR3 sequences were identical in all the PCR clones in individual lymphomas. In ISH with the use of CDR3 oligonucleotide probes, all 6 primary lymphomas revealed distinct autoradiographic signals in almost all the constituent neoplastic cells. The signals were seen only when the CDR3 probe sequences and the tissue sections were derived from the same patient's lymphomas (Figs. 3 and 4). No distinct signals were seen when the CDR3 probe sequences and the tissue sections were from different patients' lymphomas (Fig. 3). In 1 case (case 1), distinct signals were seen in both the primary and the secondary lymphoma sections with the CDR3 probe derived from the primary lymphoma (data not shown). These ISH findings indicated the patient- or specimen-specificity of the CDR3 sequences and, therefore, the specificity of the cloned V_H-D-J_H sequences. The monoclonality of neoplastic cells was also indicated in each lymphoma.

In the control competition experiment in which sections were hybridized with the ^{35}S-labeled specific CDR3 probe in the presence of a 25-fold excess of unlabeled probe, a remarkable decrease in signals was seen, supporting the reaction specificity of the probes (Fig. 4).

Analysis of V_H-D-J_H sequences The cloned V_H-D-J_H sequences in the brain lymphomas are shown in Figs. 5, 6 and 2. In each lymphoma, the abundant full-length V_H-D-J_H clones revealed an in-frame nucleotide sequence and were identical to the abundant partial clones in the corresponding sequences including CDR3, strongly suggesting that these abundant clones were representative ones. In addition, in both the full-length and the partial clones, the abundant clones were almost identical to the minor clones in sequence, except for a few nucleotide substitutions (Fig. 6). Using the abundant full-length clones, the homology of the V_H gene segments to the germline V_H genes[32] was analyzed. The lymphoma V_H gene segments showed the highest homology (72.7–91.2%) to the germline genes of the V_H4 and V_H3 gene families (Fig. 5 and Table I). While the *V4-34* (also called V_H4-*21* or *DP-63*) gene of the V_H4 gene family was used in 3 cases, the *V4-59* gene of the V_H4 gene family and the *V3-15* gene and *V3-23* gene of the V_H3 gene family were used in 1 case each (Table I).

Analysis of somatic mutation From the percentage homology to the germline V_H genes, the percentage of somatic mutations in the lymphoma V_H gene segments was calculated. A high frequency of somatic mutation, ranging from 8.8 to 27.3% (mean 18.2%), was seen in all the lymphomas (Table I), indicating that they were derived from GC-related cells. The locations of the somatic mutations in individual lymphomas are shown in Fig. 5. As seen in Fig. 5, the somatic mutations were exclusively R and S muta-

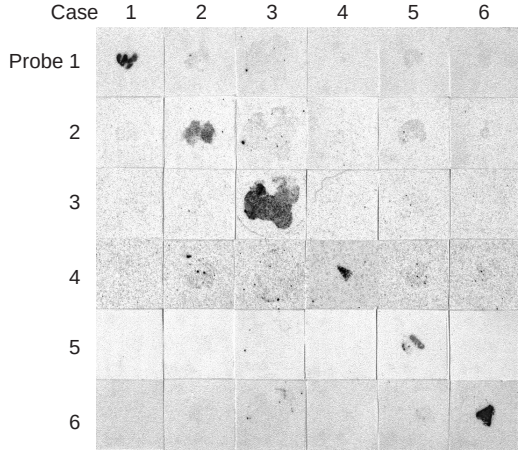

Fig. 3. Demonstration of the patient- or specimen-specificity of isolated RT-PCR clones for V_H-D-J_H sequences by ISH analyses. Images of ISH signals obtained by an image analyzer are shown. 1–6, numbers of cases from which tissue sections and CDR3 probe sequences were derived. Positive hybridization signals are seen only when the lymphoma tissues and probe sequences are derived from the same cases. No distinct signals are seen when the lymphoma tissues and probe sequences are from different cases.

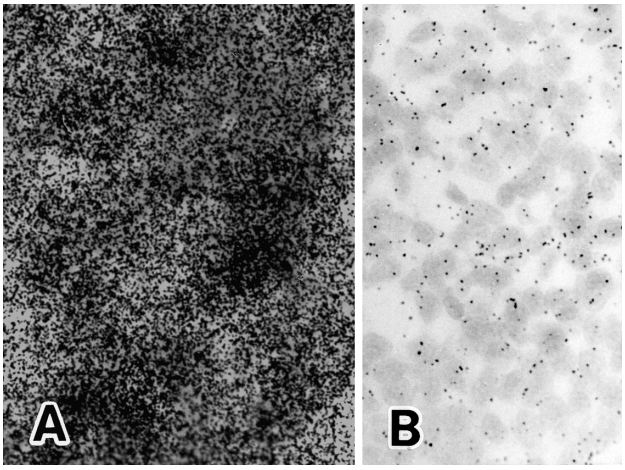

Fig. 4. Positive ISH signals in a representative brain lymphoma. Positive ISH signals are seen in almost all the lymphoma cells. A. Positive signals in case 6 are shown. B. No distinct signals are seen in the control section hybridized with a mixture of the radiolabeled CDR3 probe and a 25-fold excess of unlabeled probe. Light counterstaining with methylgreen-pyronin. ×445.

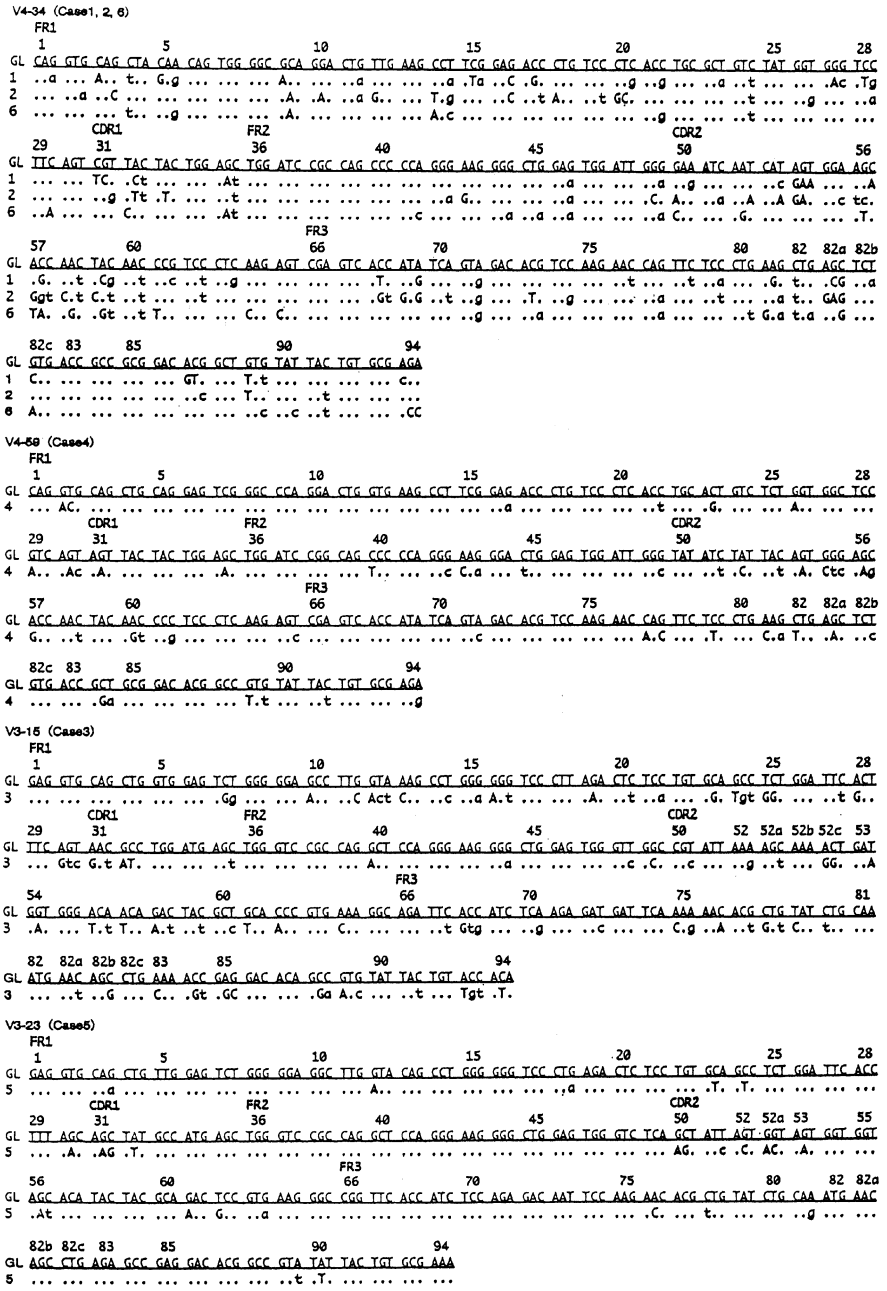

Fig. 5. V_H gene sequences in brain lymphomas. V_H gene sequences of the abundant clones in brain lymphomas (cases 1–6) are shown along with the germline V_H gene sequences (GL). Codon numbers are indicated. The positions of the beginning of FRs 1–3 and CDRs 1 and 2 are also indicated. Dots, same nucleotides as those in the germline sequences; capital letters, replacement mutations; small letters, silent mutations.

tions. There was no deletion or insertion. In the 3 lymphomas with *V4-34* gene usage, no mutation was seen in the first codon of CDR1, which was previously proposed to be a mutational hot spot.[25)]

Analysis of the distributions of R and S mutations revealed that all the lymphoma V_H genes contained a significantly lower number of R mutations than the expected values in the FRs 1 to 3 (Table I), suggesting the presence

Case 1	/	FR1		/	CDR1	/	FR2	/		CDR2		/			FR3				/
		18	19		35		37	48	50	52	54	63	71	73	77	78	80	94	
15/24	5′-	CTG	TCC		AAT		ATC	ATT	GAG	AAT	GAA	CTG	GTG	ACG	CAG	TTT	AGG	CGA	-3′
1/24	5′-	..T	...		...		...	...	...	...	...	...	...	...	...	..C	...	...	-3′
1/24	5′-	...	..T		...		...	...	...	...	...	...	...	...	T..	...	...	...	-3′
1/24	5′-	...	...		..G		...	...	...	...	...	...	...	...	...	...	...	...	-3′
1/24	5′-	...	...		...		G..	...	...	...	...	...	...	...	...	...	...	...	-3′
1/24	5′-	...	...		...		...	.C.	...	...	...	...	...	...	...	...	...	T..	-3′
1/24	5′-	...	...		...		...	...	.T.	...	...	...	...	...	...	...	...	...	-3′
1/24	5′-	...	...		...		...	...	...	...	.G.	...	...	...	...	...	...	...	-3′
1/24	5′-	...	...		...		...	...	...	...	...	...	..A	...	...	...	...	...	-3′
1/24	5′-	...	...		...		...	...	...	...	...	...	...	C..	...	...	...	...	-3′
10/11						5′-	...	...	...	...	...	...	...	...	...	...	...	...	-3′
1/11						5′-	...	...	...	G..	...	...	...	...	...	...	...	...	-3′
Case 1′																			
6/ 7	5′-	...	...		...		...	...	...	...	...	...	...	...	...	...	.C.	...	-3′
1/ 7	5′-	...	...		...		...	...	...	...	...	T..	...	...	...	...	.C.	...	-3′

Case 2	/	FR1		/	CDR1		/	FR2	/		CDR2			/		FR3			/
		29																	
23/24	5′-	TTC																	-3′
1/24	5′-	C..																	-3′
10/10							5′-												-3′

Case 3	/	FR1		/	CDR1		/	FR2	/		CDR2			/		FR3			/
		20			32						54					83			
17/22	5′-	CTT			GTC						GAT					CAA			-3′
1/22	5′-	..C			...						...					...			-3′
1/22	5′-	...			A..						...					...			-3′
1/22	5′-	...			.A.						...					...			-3′
1/22	5′-	...			...						.G.					...			-3′
1/22	5′-	...			...						...					.G.			-3′
10/10							5′-				...					...			-3′

Case 4	/	FR1		/	CDR1		/	FR2	/		CDR2			/		FR3			/
	4	7	12		32	35		41							68	88	89	90	
15/24	5′- CTG	TCG	GTG		TAC	TGG		CCA							CCA	GCC	TTT	TAT	-3′
2/24	5′- ...	...	...		...	...		...							..G	...	...	...	-3′
1/24	5′- .C.	...	...		...	...		...							...	...	...	...	-3′
1/24	5′- ...	..A	...		...	...		...							...	...	...	...	-3′
1/24	5′- ...	...	.C.		...	...		...							...	...	...	...	-3′
1/24	5′- ...	...	...		G..	...		...							...	...	...	...	-3′
1/24	5′- ...	...	...		...	..A		...							...	...	C..	...	-3′
1/24	5′- ...	...	...		...	...		.G.							...	...	...	...	-3′
1/24	5′- ...	...	...		...	...		...							...	.T.	...	...	-3′
14/16							5′-	...							...	...	...	...	-3′
1/16							5′-	...							...	...	..C	...	-3′
1/16							5′-	...							...	...	...	..C	-3′

Case 5	/	FR1		/	CDR1		/	FR2	/		CDR2			/		FR3			/
		18			35						62					82a	86		
18/22	5′-	CTA			AGC						GCC					AAC	GAC		-3′
1/22	5′-	..G			...						...					...	...		-3′
1/22	5′-	...			..T						...					...	...		-3′
1/22	5′-	...			...						...					.G.	...		-3′
1/22	5′-	...			...						...					...	A..		-3′
11/12							5′-				...					...	...		-3′
1/12							5′-				.A.					...	...		-3′

Case 6	/	FR1		/	CDR1		/	FR2	/		CDR2			/		FR3			/
	13	19	23		31			42								73			
18/23	5′- AAG	TCC	GCT		GGT			GGG								ACA			-3′
1/23	5′- T..	...	..C		...			...								...			-3′
1/23	5′- ...	A..	...		...			...								...			-3′
1/23	5′- ...	...	...		.C.			...								...			-3′
1/23	5′- ...	...	...		...			.A.								...			-3′
1/23	5′- ...	...	...		...			...								G..			-3′
12/12							5′-	...								...			-3′

Fig. 6. Analysis of intraclonal heterogeneity in brain lymphomas. Cloning experiments revealed no distinct intraclonal heterogeneity in most cases. Codons which showed nucleotide variations among clones are shown. Codon numbers and V_H regions are indicated above. The left-most fractions indicate numbers of clones with same sequences/numbers of clones examined. Nucleotide triplets in the abundant full-length clones are shown. The same nucleotides as those of the abundant clones are indicated by dots. The different nucleotides from those of the abundant clones are indicated by letters. Note that most nucleotide substitutions are seen only in 1 clone except for the one in 2 clones in case 4.

of a force to maintain antibody structure in the precursor cells. In the CDRs 1 and 2, however, the V_H genes did not show a significantly higher number of R mutations than expected in all but 1 case, suggesting the absence of a strong force to enhance antibody affinity in the precursor cells, in most cases.

Analysis of intraclonal heterogeneity Intraclonal heterogeneity, which suggests ongoing mutation characteristic of

GC cells, was analyzed in 22 to 24 full-length and 10 to 16 partial V_H-D-J_H clones in individual lymphomas (Fig. 6). When the abundant clones were compared with the minor clones, 1 or 2 nucleotide substitutions were seen in each of the minor clones. Among them, one nucleotide substitution was found in 2 clones in 1 case (case 4) (Fig. 6). The other nucleotide substitutions were found only in 1 clone in the other cases. Thus, according to the definition[19] that a nucleotide substitution in more than 1 clone is a mutation, intraclonal heterogeneity was not suggested in any cases, except for one in which intraclonal heterogeneity was a possibility.

In 1 case (case 1), both the primary and the secondary lymphomas were examined. Two nucleotide substitutions were found at codons 80 (Fig. 6) and 100c (not shown) in all the clones of the secondary tumor, suggesting intraclonal heterogeneity. However, it seemed difficult to rule out the possibility that these nucleotide substitutions were due to the effect of irradiation and chemotherapy on the secondary tumor.

DISCUSSION

In this study, we isolated RT-PCR clones for the rearranged V_H-D-J_H sequences from 6 brain lymphomas. Since it is known that false-positive amplification is not uncommon in PCR of rearranged immunoglobulin genes,[35–39] we first examined the reliability of the isolated clones by ISH using CDR3 oligonucleotide probes. Subsequently, we analyzed the cloned sequences, focusing on their somatic mutations and intraclonal heterogeneity.

In ISH analysis, it was shown that the isolated clones were derived from the individual lymphomas, but not from false-positive DNA amplification due to coexisting reactive lymphocytes in the tumor tissues, DNA contamination during the experimental procedure or PCR errors. From the distribution of the hybridization signals, it was also clear that the constituent neoplastic cells in each lymphoma were monoclonal in origin. In 1 case (case 1), the neoplastic cells in the primary and the secondary tumors were shown to be monoclonal. Although PCR has often been used in analyses of immunoglobulin gene rearrangements in various lymphoproliferative diseases, the reliability of the PCR clones has not been fully confirmed in most previous studies. The present study showed that ISH was very useful in confirmation of the patient- or specimen-specificity of the PCR clones and in the determination of the clonality of neoplastic cells in lymphoma tissues as well. It was also shown that the CDR3 probes were specific in ISH, even if they contained adjacent germline V_H and J_H sequences at about 15–30% of the total probe length. This finding indicated that ISH analysis is applicable to almost all B-cell lymphomas including those with a short CDR3 sequence.

In subsequent nucleotide analysis of the PCR clones, all the brain lymphomas revealed in-frame rearranged V_H-D-J_H sequences, in which V_H gene segments belonged to either the V_H4 or V_H3 gene family, in particular to the *V4-34* gene of the V_H4 gene family. Mutation analyses revealed frequent somatic mutations in their V_H gene segments (8.8 to 27.3%, mean=18.2%) with a significantly lower frequency of R mutations in the FR regions than expected. These findings indicated that all of our brain lymphomas were GC-related cells in origin. Similar findings have also been obtained by Thompsett *et al.*[24] and Montesinos-Rongen *et al.*,[25] who observed frequent somatic mutations (mean=18.4% and 13.2%, respectively) in the V_H gene segments in all[24] or most cases.[25] A lower-than-expected frequency of R mutations in FRs was observed in all examined cases[24] or presumed from the R/S ratio in most cases.[25] Frequent usage of the *V4-34* gene family was also observed in these studies.

However, regarding the intraclonal heterogeneity, which suggested ongoing mutation in the lymphoma cells, there seems to be a discrepancy between the present and the previous studies. In the present study, we examined more than 30 clones in each case using the definition that a nucleotide substitution in more than 1 clone was a mutation. We found no additional mutation in any case except for one in which a mutation could not be ruled out. Since a nucleotide substitution was seen only in 2 clones in the latter case, the finding could have been due to an experimental artifact. In the previous studies, despite the fact that the cloning experiments were not so extensive and only 6–13[24] or 8[25] clones were analyzed, clones with additional mutations were suggested in 3 of 5 cases[24] or in at least 1 of 3 examined cases.[25] When the data were revised using the same definition of the mutation as in this study, clones with additional mutations were suggested in 3 of 5 cases[24] and in 2 of 3 examined cases.[25] Thus, although intraclonal heterogeneity seemed rare in our cases, it was more frequent in previous studies. The reason for this discrepancy remains unknown, but nevertheless, the present study indicated that brain lymphomas without intraclonal heterogeneity, or ongoing mutation, were more commonly present than previously expected. From the findings of somatic mutations without ongoing mutation, it seemed likely that at least most of our lymphomas were derived from post-GC cells, in particular from memory cells. Consistent with this consideration, some previous studies[40, 41] have shown a high level of soluble CD27, a memory cell marker,[16] in the cerebrospinal fluid of all[40] or most[41] brain lymphoma patients examined. In a few brain lymphoma cases, however, a flow cytometry study[42] failed to detect lymphoma cells positive for CD10, a GC cell marker.[16]

In systemic diffuse large B-cell lymphoma, the same histological subtype as the brain lymphoma, the frequency

of somatic mutations in the V_H gene segments, as well as the frequency of R mutations in their FRs, was variable among lymphomas.[19] In the report of Lossos *et al.*,[19] who examined more than 50 systemic lymphomas, the frequency of somatic mutations was more than 8% in only half of the cases. It ranged from 0 to 8% in the remainder of the cases. The frequency of R mutations in FRs was significantly lower than expected in about 60% of cases, but not in the remaining cases. Thus, the frequency of somatic mutations and the frequency of R mutations in FRs seemed more variable in systemic lymphomas compared with those in brain lymphomas. The reasons for these differences between systemic and brain lymphomas remain unknown. It seems possible that, even if the pathological subtype is the same, systemic lymphomas consist of more heterogeneous tumors compared with brain lymphomas, although further studies on larger numbers of cases are required.

ACKNOWLEDGMENTS

We are grateful to Drs. H. Matsuzawa, K. Suzuki and T. Kimura of the Dept. of Integrated Neuroscience of our Institute for very useful suggestions regarding statistical analysis. We also thank Drs. I. Takeshita, M. Fumita, Y. Kotani, K. Yamada, H. Usui, and the late Drs. H. Kosaka and K. Nishida of our Department for their continuous interest and support. Special thanks are due to Messrs. T. Ichikawa and K. Kobayashi, and Mss. K. Honma, K. Sekikawa and K. Furui for technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Association of International Education, Japan, the Inoue Foundation for Science, the Yoneyama Foundation, the Fujisawa Foundation, the Fumita Medical Association, the Kosaka Medical Association, the Kotani Medical Association and the Takeuchi Medical Association.

(Received August 19, 2002/Revised October 7, 2002/Accepted October 9, 2002)

REFERENCES

1) Russell, D. S. and Rubinstein, L. J. "Pathology of the Tumours of the Nervous System," 5th Ed., pp. 590–638 (1989). Edward Arnold, London.
2) Hayakawa, T., Takakura, K., Abe, H., Yoshimoto, T., Tanaka, R., Sugita, K., Kikuchi, H., Uozumi, T., Hori, T., Fukui, H., Ushio, Y., Nomura, K., Matsutani, M., Mohri, N., Kumanishi, T., Aozasa, K. and Nagashima, K. Primary central nervous system lymphoma in Japan—a retrospective, co-operative study by CNS-Lymphoma Study Group in Japan. *J. Neurooncol.*, **19**, 197–215 (1994).
3) Kumanishi, T., Washiyama, K., Saito, T., Nishiyama, A., Abe, S. and Tanaka, R. Primary malignant lymphoma of the brain: an immunohistochemical study of eight cases using a panel of monoclonal and heterologous antibodies. *Acta Neuropathol.*, **71**, 190–196 (1986).
4) Kumanishi, T., Washiyama, K., Nishiyama, A., Abe, S., Saito, T. and Ichikawa, T. Primary malignant lymphoma of the brain: demonstration of immunoglobulin gene rearrangements in four cases by the Southern blot hybridization technique. *Acta Neuropathol.*, **79**, 23–26 (1989).
5) Kleihues, P. and Cavenee, W. K. "Pathology and Genetics of Tumours of the Nervous System," pp. 198–203 (2000). IARC Press, Lyon.
6) Zhang, S.-J., Endo, S., Ichikawa, T., Washiyama, K. and Kumanishi, T. Frequent deletion and 5′ CpG island methylation of the p16 gene in primary malignant lymphoma of the brain. *Cancer Res.*, **58**, 1231–1237 (1998).
7) Koga, H., Zhang, S.-J., Ichikawa, T., Washiyama, K., Kuroiwa, T., Tanaka, R. and Kumanishi, T. Primary malignant lymphoma of the brain: demonstration of the p53 gene mutation by PCR-SSCP analysis and immunohistochemistry. *Brain Tumor Pathol.*, **11**, 151–155 (1994).
8) Nishiyama, A., Saito, T., Abe, S. and Kumanishi, T. An immunohistochemical analysis of T cells in primary B cell malignant lymphoma of the brain. *Acta Neuropathol.*, **79**, 27–29 (1989).
9) Tonegawa, S. Somatic generation of antibody diversity. *Nature*, **302**, 575–581 (1983).
10) Rajewsky, K. Clonal selection and learning in the antibody system. *Nature*, **381**, 751–758 (1996).
11) Alt, F. W. and Baltimore, D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. *Proc. Natl. Acad. Sci. USA*, **79**, 4118–4122 (1982).
12) Alt, F. W., Blackwell, T. K. and Yancopouisos, G. D. Development of the primary antibody repertoire. *Science*, **238**, 1079–1087 (1987).
13) Jacob, J., Kelsoe, G., Rajewsky, K. and Weiss, U. Intraclonal generation of antibody mutants in germinal centres. *Nature*, **354**, 389–392 (1991).
14) Berek, C., Berger, A. and Apel, M. Maturation of the immune response in germinal centers. *Cell*, **67**, 1121–1129 (1991).
15) Küppers, R., Zhao, M., Hansmann, M.-L. and Rajewsky, K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J.*, **12**, 4955–4967 (1993).
16) Klein, U., Goossens, T., Fischer, M., Kanzler, H., Braeuninger, A., Rajewsky, K. and Küppers, R. Somatic hypermutation in normal and transformed human B cells. *Immunol. Rev.*, **162**, 261–280 (1998).
17) Hsu, F. J. and Levy, R. Preferential use of the V_H4 Ig gene family by diffuse large-cell lymphoma. *Blood*, **86**, 3072–3082 (1995).
18) Küppers, R., Rajewsky, K. and Hansmann, M.-L. Diffuse large cell lymphomas are derived from mature B cells carrying V region genes with a high load of somatic mutation and evidence of selection for antibody expression. *Eur. J.*

Immunol., **27**, 1398–1405 (1997).
19) Lossos, I. S., Okada, C. Y., Tibshirani, R., Warnke, R., Vose, J. M., Greiner, T. C. and Levy, R. Molecular analysis of immunoglobulin genes in diffuse large B-cell lymphomas. *Blood*, **95**, 1797–1803 (2000).
20) Bahler, D. W. and Levy, R. Clonal evolution of a follicular lymphoma: evidence for antigen selection. *Proc. Natl. Acad. Sci. USA*, **89**, 6770–6774 (1992).
21) Matolcsy, A., Schattner, E. J., Knowles, D. M. and Casali, P. Clonal evolution of B cells in transformation from low- to high-grade lymphoma. *Eur. J. Immunol.*, **29**, 1253–1264 (1999).
22) Lossos, I. S., Alizadeh, A. A., Eisen, M. B., Chan, W. C., Brown, P. O., Botstein, D., Staudt, L. M. and Levy, R. Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas. *Proc. Natl. Acad. Sci. USA*, **97**, 10209–10213 (2000).
23) Ottensmeier, C. H., Thompsett, A. R., Zhu, D., Wilkins, B. S., Sweetenham, J. W. and Stevenson, F. K. Analysis of V_H genes in follicular and diffuse lymphoma shows ongoing somatic mutation and multiple isotype transcripts in early disease with changes during disease progression. *Blood*, **91**, 4292–4299 (1998).
24) Thompsett, A. R., Ellison, D. W., Stevenson, F. K. and Zhu, D. V_H gene sequences from primary central nervous system lymphomas indicate derivation from highly mutated germinal center B cells with ongoing mutational activity. *Blood*, **94**, 1738–1746 (1999).
25) Montesinos-Rongen, M., Küppers, R., Schlüter, D., Spieker, T., Roost, D. V., Schaller, C., Reifenberger, G., Wiestler, O. D. and Deckert-Schlüter, M. Primary central nervous system lymphomas are derived from germinal-center B cells and show a preferential usage of the V4-34 gene segment. *Am. J. Pathol.*, **155**, 2077–2086 (1999).
26) Suci, T., Tajima, K., Nanba, K., Wakasa, H., Mikata, A., Kikuchi, M., Mori, S., Watanabe, S., Mohri, N., Shamoto, M., Harigaya, K., Itagaki, T., Matsuda, M., Kirino, Y., Takagi, K. and Fukunaga, S. Some problems on the histopathological diagnosis of non-Hodgkin's malignant lymphoma—a proposal of a new type. *Acta Pathol. Jpn.*, **29**, 755–776 (1979).
27) The non-Hodgkin's lymphoma pathologic classification project. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. *Cancer*, **49**, 2112–2135 (1982).
28) Harris, N. L., Jaffe, E. S., Stein, H., Banks, P. M., Chan, J. K. C., Cleary, M. L., Delsol, G., De Wolf-Peeters, C., Falini, B., Gatter, K. C., Grogan, T. M., Isaacson, P. G., Knowles, D. M., Mason, D. Y., Muller-Hermelink, H.-K., Pileri, S. A., Piris, M. A., Ralfkiaer, E. and Warnke, R. A. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood*, **84**, 1361–1392 (1994).
29) Rettig, M. B., Vescio, R. A., Cao, J., Wu, C. H., Lee, J. C., Han, E., DerDanielian, M., Newman, R., Hong, C., Lichtenstein, A. K. and Berenson, J. R. V_H gene usage in multiple myeloma: complete absence of the V_H4.21 (V_H4-34) gene. *Blood*, **87**, 2846–2852 (1996).
30) Kumanishi, T., Zhang, S., Ichikawa, T., Endo, S. and Washiyama, K. Primary malignant lymphoma of the brain: demonstration of frequent p16 and p15 gene deletions. *Jpn. J. Cancer Res.*, **87**, 691–695 (1996).
31) Sanger, F., Nicklen, S. and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467 (1977).
32) Lefranc, M.-P. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res.*, **29**, 207–209 (2001).
33) Chang, B. and Casali, P. The CDR1 sequences of a major proportion of human germline Ig V_H genes are inherently susceptible to amino acid replacement. *Immunol. Today*, **15**, 367–373 (1994).
34) Katagiri, T., Feng, X.-L., Ichikawa, T., Usui, H., Takahashi, Y. and Kumanishi, T. Neuron-specific enolase (NSE) and non-neuronal enolase (NNE) mRNAs are co-expressed in neurons of the rat cerebellum: *in situ* hybridization histochemistry. *Brain Res. Mol. Brain Res.*, **19**, 1–8 (1993).
35) Taylor, J. M. E., Spagnolo, D. V. and Kay, P. H. B-Cell target DNA quantity is a critical factor in the interpretation of B-cell clonality by PCR. *Pathology*, **29**, 309–312 (1997).
36) Diaz-Cano, S. PCR-based alternative for diagnosis of immunoglobulin heavy chain gene rearrangement. *Diagn. Mol. Pathol.*, **5**, 3–9 (1996).
37) Rockman, S. P. Determination of clonality in patients who present with diagnostic dilemmas: a laboratory experience and review of the literature. *Leukemia*, **11**, 852–862 (1997).
38) Macintyre, E. A. and Delabesse, E. Molecular approaches to the diagnosis and evaluation of lymphoid malignancies. *Semin. Hematol.*, **36**, 373–389 (1999).
39) Diss, T. C. and Pan, L. Polymerase chain reaction in the assessment of lymphomas. *Cancer Surv.*, **30**, 21–44 (1997).
40) Kersten, M. J., Evers, L. M., Dellemijn, P. L., van den Berg, H., Portegies, P., Hintzen, R. Q., van Lier, R. A., von dem Borne, A. E. and van Oers, R. H. Elevation of cerebrospinal fluid soluble CD27 levels in patients with meningeal localization of lymphoid malignancies. *Blood*, **87**, 1985–1989 (1996).
41) Murase, S., Saio, M., Andoh, H., Takenaka, K., Shinoda, J., Nishimura, Y., Sakai, N. and Takami, T. Diagnostic utility of CSF soluble CD27 for primary central nervous system lymphoma in immunocompetent patients. *Neurol. Res.*, **22**, 434–442 (2000).
42) Arai, T., Nagai, M. and Okuhata, S. Immunophenotypic analysis of primary malignant lymphoma of the brain. *Neurol. Med. Chir. (Tokyo)*, **34**, 136–142 (1994).