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

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Using reverse transcription-polymerase chain reaction (RT-PCR), six primary brain lymphomas, pathologically diagnosed as diffuse large B-cell lymphoma, were examined for rearranged VH-D-JH 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 VH 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 (VH), diversity (D) and joining (JH) gene segments are chosen and assembled, resulting in a considerable variety of rearranged VH-D-JH gene sequences among B cells.9, 10) In addition, further variability is introduced by random deletions and insertions of nucleotides at VH-D and D-JH junctions11, 12) as well as by somatic mutations in the entire VH-D-JH sequence.13–15) While VH-D-JH gene rearrangements, which are associated with random nucleotide deletions and insertions at the VH-D and D-JH junctions, occur at an earlier stage of B-cell differentiation, somatic mutations in the entire VH-D-JH 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 lymphomas18, 20, 21) and a fraction of diffuse large B-cell lymphomas.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,
although Thompsett et al.\textsuperscript{24} and Montesinos-Rongen et al.\textsuperscript{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 $V_{\text{H}}$D-J$\text{H}$ sequences from 6 brain lymphomas, confirmed their patient- or specimen-specificity by \textit{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.

**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\textsuperscript{26–28} (Fig. 1). In Table 1, 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 $\mu$l 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 $C_\mu$ antisense primer (5$'$-GGTTGGGGCGGAT-C

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Table 1. Summary of Mutation Analysis of $V_{\text{H}}$ Genes in Brain Lymphomas

| Case No. | Age (yr) | Tumor | $V_{\text{H}}$ gene usage | Site\footnote{a} | Diag\footnote{a} | No. (%) | Region\footnote{b} | R\footnote{c} | S\footnote{c} | P value\footnote{d} | $J_{\mu}$ gene usage\footnote{e} |
|----------|----------|-------|--------------------------|----------------|----------------|---------|----------------|------|------|----------------|-------------------|
| 1        | 65/M     | F     | DL/B($\mu$)              | V4-34          |                | 60 (20.6) | FR             | 17   | 22   | 3.3x10^{-6}   | $J_{\mu}$^4       |
| 2        | 61/F     | F     | DL/B($\mu$)              | V4-34          |                | 62 (21.3) | CDR            | 10   | 11   | NS             | $J_{\mu}$^4       |
| 3        | 58/M     | F     | DL/B($\mu$)              | V3-15          |                | 82 (27.3) | FR             | 34   | 26   | 3.3x10^{-3}   | $J_{\mu}$^5       |
| 4        | 54/F     | P     | DL/B($\mu$)              | V4-59          |                | 47 (16.2) | FR             | 16   | 14   | 1.1x10^{-3}   | $J_{\mu}$^3       |
| 5        | 78/M     | C     | DL/B($\mu$)              | V3-23          |                | 26 (8.8)  | FR             | 6    | 5    | 2.3x10^{-4}   | $J_{\mu}$^4       |
| 6        | 73/M     | P     | DL/B($\mu$)              | V4-34          |                | 43 (14.8) | FR             | 9    | 20   | 7.2x10^{-7}   | $J_{\mu}$^5       |

\footnotetext{a} F, frontal; P, parietal; C, cerebellar; DL, diffuse large cell lymphoma; B, B-cell type; $\mu$, IgM expression detected by northern blot analysis (data not shown).

\footnotetext{b} FR, framework regions 1–3; CDR, complementarity-determining regions 1 and 2.

\footnotetext{c} R, numbers of replacement mutations; S, numbers of silent mutations.

\footnotetext{d} Probability of scarcity (for FR) or excess (for CDR) of mutations occurring by chance. The $P$ values were calculated by the multinomial distribution model as described by Lossos et al.\textsuperscript{19} using the Mathema\textit{tica} software package (Wolfram Research, Inc.). NS, nonsignificant values. The $P$ values were also calculated by the binomial distribution model described by Chang and Casali.\textsuperscript{33} By this model, the $P$ values for FR and CDR in cases 1 to 6 were shown to be $3.7x10^{-6}$ and NS, $1.3x10^{-5}$ and NS, $2.1x10^{-3}$ and NS, $9.9x10^{-4}$ and NS, $3.0x10^{-3}$ and $5.4x10^{-4}$ and $9.8x10^{-7}$ and NS, respectively.

\footnotetext{e} The germline $J_{\mu}$ genes\textsuperscript{32} to which lymphoma $J_{\mu}$ genes showed the highest homology.
GC ACT-3) corresponding to the 5' end of the C 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 VH leader primers which corresponded to VH family-specific leader sequences,29) and that in another was the FR2A primer (5'-TGG(AG)TCCG(AC)CAG(GC)(CT)(CT)(CT)CNGG-3') corresponding to the framework (FR) 2 region of the VH gene. The target sequence for the former primer pair was a full-length VH-D-JH (nucleotide position (n.p.) 1-ca. 300), while that of the latter was a partial VH-D-JH (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 AmpliTaq 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 EcoRV cloning site of the pBlue-script-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 method31) 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 VH-D-JH 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 database32) 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 VH 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.,39) 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.39)

**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 VH sequences and, in 1 case (case 4), that of a JH 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 5×10^7 cpm/µl of the heat-denatured 35S-labeled CDR3 oligonucleotide probe described above in a hybridization solution34) 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 methyl-green-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, preparation of CDR3 clone sequences (underlined) in cases 1–6 (1–6) are shown together with the entire CDR3 sequences with or without adjacent FR3 and JH sequences. Codon numbers in the CDR3 region are indicated.

|     | FR | CDR3 | JH |
|-----|----|------|----|
| 1   | TCC AAA ATA GTA ACT TCG GAC CAA ACT GCC GGC GGC GGC GAT TAT GAC TCC | | |
| 2   | TGT GGC AGA CGA ATC GAT TTT GAC AAT GCT TTT TCA TAT TAT | GAC TAT | |
| 3   | TAT TGT TGT ATA GAC AAA TCA GTC TCT TCG GCA GCT GTC | GAT TCC | |
| 4   | TGT GGC AGC GCC GGT GGA TAC ACT AAT GCT CTT | GCA TTG TGC CG | |
| 5   | GGC AAA TGG GCA AGA CGC TCT TCA AGC ACT TCC GAG | GAC TA C | |
| 6   | GT GCC ACC TCT TCT TCT ACT ATG GCA CTT ACC GGC TGG TCC | GAC CCC | |

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 JH 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 VH-D-JH sequences. The monoclonality of neoplastic cells was also indicated in each lymphoma.

In the control competition experiment in which sections were hybridized with the 35S-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 VH-D-JH sequences The cloned VH-D-JH sequences in the brain lymphomas are shown in Figs. 5, 6 and 2. In each lymphoma, the abundant full-length VH-D-JH 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 VH gene segments to the germline VH genes was analyzed. The lymphoma VH gene segments showed the highest homology (72.7–91.2%) to the germline genes of the VH4 and VH3 gene families (Fig. 5 and Table I). While the V4-34 (also called VH4-21 or DP-63) gene of the VH4 gene family was used in 3 cases, the V4-59 gene of the VH4 gene family and the V3-15 gene and V3-23 gene of the VH3 gene family were used in 1 case each (Table I).

Analysis of somatic mutation From the percentage homology to the germline VH genes, the percentage of somatic mutations in the lymphoma VH 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-
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 Vδ genes contained a significantly lower number of R mutations than the expected values in the FRs 1 to 3 (Table I), suggesting the presence of a mutational hot spot.

Fig. 5. Vδ gene sequences in brain lymphomas. Vδ gene sequences of the abundant clones in brain lymphomas (cases 1–6) are shown along with the germline Vδ 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.
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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 \( \text{V}_{\mu} \text{-D-J}_{\mu} \) 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 \( \text{V}_{\mu} \text{-D-J}_{\mu} \) 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 \( \text{V}_{\mu} \) and \( \text{J}_{\mu} \) 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 \( \text{V}_{\mu} \text{-D-J}_{\mu} \) sequences, in which \( \text{V}_{\mu} \) gene segments belonged to either the \( \text{V}_{\mu} \text{4} \) or \( \text{V}_{\mu} \text{3} \) gene family, in particular to the \( \text{V4-34} \) gene of the \( \text{V}_{\mu} \text{4} \) gene family. Mutation analyses revealed frequent somatic mutations in their \( \text{V}_{\mu} \) 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 \( \text{V}_{\mu} \) 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 \( \text{V4-34} \) 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. In the report of Lossos et al., 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.

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