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Primary Central Nervous System Lymphomas. A validation study of array-based Comparative Genomic Hybridization in Formalin-Fixed Paraffin-Embedded Tumor Specimens

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Abstract

Purpose—Only a limited number of genetic studies have been performed in primary central nervous system lymphomas (PCNSL), partly due to the rarity of the tumors and the very limited amount of available tissue. In this report, we present the first molecular characterization of copy-number abnormalities (CNA) of newly diagnosed PCNSL by array-based comparative genomic hybridization in formalin fixed paraffin embedded (FFPE) specimens and compare the results with matched frozen tumor specimens.

Experimental design—We performed array-based comparative genomic hybridization (aCGH) in FFPE tissues from PCNSL. Results were compared with matched paired frozen tumors.

Results—Our analysis confirmed the good to fair quality and reliability of the data generated from limited amounts of tumoral FFPE tissue. Overall, all PCNSL cases were characterized by highly complex karyotypes with a median of 23 copy-number abnormalities (CNA) per patient (range 17–47). Overall, 20 chromosomal regions were recurrently found in more than 40% of cases. Deletions of 6p21, 6q, 9p21.3 and gain of 12q12-q24.33 were the commonest CNA. Other minimal affected regions were defined, and novel recurrent CNAs affecting single genes were identified in 3q26.32 (*TBLIXR1*) and 8q12.1 (*TOX*).

Conclusions—The results obtained are encouraging. Larger archival tissue collections can now be analyzed in order to complement the still fragmented knowledge we have of the genetic basis of the PCNSL.

Keywords

PCNSL; aCGH; FFPE; *TOX*; *TBLIXR1*

INTRODUCTION

Primary central nervous system lymphoma (PCNSL) is an aggressive primary brain tumor characterized by a perivascular accumulation of malignant cells that have lymphoid

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The Mayo SPORE in Brain Cancer (Drs. O'Neill and Giannini), and the Iowa/Mayo Lymphoma SPORE (Drs. McPhail, Macon and Dogan)

characteristics. Its cell of origin and tumorigenesis are unknown. PCNSL is typically lethal without treatment, is increasing in incidence, and targets vulnerable populations(1, 2). PCNSL is therapy responsive and aggressive management may lead to remission(3, 4). However, current treatments are not increasing cure rates(5) and the quality of such survival is often poor(6, 7). PCNSL has a disproportionate effect on quality of life because of its disabling impact on cognition, language, mobility, and adaptive skills. The advanced age of the average patient and neurotoxicity of standard therapy further amplify this morbidity.

The 2000 NCI-NINDS PRG report stated that “*Molecular characterization of PCNSL tumorigenesis is now needed to inform pathogenesis-based treatment and prevention strategies*” (8). To that end, a series of gene function-oriented publications including our own have identified chromosomal abnormalities that may have pathogenetic relevance(9). For example, deletions of 6q are a frequent observation in systemic DLBCL and are typically associated with a worse prognosis(10, 11). Cady et al. described a similar association of 6q22-23 deletions and prognosis in PCNSL patients with no apparent immunodeficiency and stated that the deletion implied a loss or modification of a tumor suppressor gene, *PTPRK*(11). In contrast, post-transplant lymphoproliferative disorder of the CNS (PTLD-CNS), the EBV-driven PCNSL-like disease seen in organ transplant recipients and other immunosuppressed states, is not associated with deletions of 6q or abnormalities of *c-MYC* and *BCL-6* suggesting a distinct pathogenesis(12).

Unlike systemic DLBCL and other forms of non-Hodgkin lymphomas (NHL), relatively little is known about the biology of PCNSL. Only a limited number of genetic studies have been performed, partly due to the rarity of the tumors (3% of all primary brain tumors)(13) and the very limited amount of available tissue. PCNSL lesions are typically deep-seated and usually best approached by stereotactic techniques(14). Aggressive debulking does not improve prognosis(13, 14), thus limiting the amount of tissue that can be safely removed, and specimens are typically exhausted by the diagnostic workup. In addition, no cell lines have been established in PCNSL, thus making the selection of appropriate *in vitro* systems for genomic validations and functional analysis more difficult. In PCNSL, the source of biological samples is often limited to formalin fixed and paraffin-embedded (FFPE) specimens. These specimens represent valuable materials for cancer research, especially in retrospective studies with long follow-up data, but its use in systematic studies has been challenging because the yield DNA obtained from this source is often very degraded. However, in recent years there has been progress in the use of FFPE specimens for whole genome array-based studies in solid tumors(15–17).

In this report we describe the molecular characterization of copy number abnormalities by array-based comparative genomic hybridization (aCGH) in matched FFPE and frozen tumor specimens taken from newly diagnosed PCNSL patients without apparent immunodeficiency. The aim of this study was, first, to better characterize the disease at chromosomal and gene level and to correlate these features with clinical and pathologic features; and, second, to validate the use of aCGH in FFPE tumor specimens as an alternative to frozen specimens.

PATIENTS AND METHODS

Tumor samples

Frozen PCNSL tumor samples in pellets and FFPE blocks were retrieved from the Mayo Clinic Tumor Registry (Mayo Foundation IRB approval 08-001933) and the University of Virginia (University of Virginia IRB approval 14225). A review of clinical histories confirmed that each case was newly diagnosed, was confined to the CNS, had no occult disease by standard staging, and that each patient had no apparent immunodeficiency. Given

the limited number of patients and the pilot nature of the study, statistical analyses were not performed.

Three 3 um sections were cut from each pellet and placed on clean glass slides. One slide was stained with H&E to confirm sufficient tumor rich tissue without significant hemorrhage, necrosis or artifact. Confirmatory immunohistochemistry (IHC) using antibodies directed against CD20 and CD3 was performed on the second and third slide of each patient set. All confirmed cases were then screened for Epstein-Barr virus (EBV) utilizing *in situ* hybridization probes that recognize EBV-encoded RNA (EBER). Any nuclear staining in tumor cells was viewed as a positive result. The specimens were subgrouped as germinal center (GC) or non-GC by IHC for CD10, MUM-1, and BCL6, according to the Hans algorithm(18).

Immunostaining was performed in one batch in order to maximize laboratory efficiency. Each batch contained positive and negative controls, and replicates from each case. Lymphomas were considered positive if 30% or more of the cells stained with antibody. Data on the intensity of staining was not used due to potential variations in tissue fixation and processing.

DNA isolation

Genomic DNA was obtained from frozen tumors using the Puregene Core A kit (Qiagen) according to manufacturer's recommendations. In the FFPE specimens, a slide was cut and stained as aforementioned to confirm the tumor-richness. In cases where tumor burden was less than 70%, tumor areas were identified on the H&E stained section. These areas were macro-dissected to minimize normal tissue content. Samples were deparaffinized using heptane at room temperature for 1 hour. Methanol was added and the sample was pelleted by centrifugation. DNA was obtained using the AllPrep DNA/RNA FFPE kit (Qiagen) according to manufacturer's recommendations. DNA concentration and purity was measured by spectrophotometry and DNA integrity was assessed on a 1% agarose gel.

aCGH

In order to assess the reliability of FFPE samples, we performed a comparison of assay performance between snap-frozen and FFPE samples in six PCNSL specimens. In an additional case, only the frozen tumor sample was analyzed.

Snap-Frozen samples—aCGH was performed in 3 samples with the Human Genome 244A microarray and in the remaining 4 with the Human Sureprint G3 microarray (Agilent Technologies). The digestion, labeling and hybridization steps were done as previously described(19). Microarrays were scanned in a DNA Microarray Scanner (Agilent). Feature extraction was performed with Feature extraction Software, version 9.5 (Agilent Technologies). Extracted data was imported and analyzed using Genomic Workbench version 5.0.14 (Agilent) and Nexus 5.1 (Biodiscovery).

FFPE samples—aCGH was performed on six FFPE specimens using the Human Genome 244A microarray. Briefly, 2 ug of reference DNA was fragmented by heating at 95°C for 10 minutes. Fragmentation was not necessary for the FFPE samples. Samples were labeled with the ULS kit (Agilent) for FFPE tissues according to manufacturer's recommendation. Labeled genomic reactions were cleaned-up with KREApure columns (Agilent) and hybridized at 65° C for 40 hours. Scanning and data analysis were the same as for the snap-frozen samples.

Data analysis—Copy-number abnormalities (CNA) were calculated using aberration detection module (ADM)-1 and RANK segmentation algorithms in Genomic Workbench and Nexus software, respectively(20). The derivative of the log ratio spread (DLRS) across the entire genome was calculated and used as a surrogate of assay quality. Assays with DLRS values lower than 0.2 are considered excellent; values between 0.2–0.3 are considered good; and values higher than 0.3 are considered marginal. A 2 probe, 0.25_log2 (244K array format) and a 3 probe, 0.25_log2 (Sureprint G3 format) filter were used in the aberration detection of fresh samples, obtaining an average genomic resolution of 17Kb and 4.5Kb, respectively. Copy number variations (CNV) were identified and excluded from the analysis as previously described(19).

FISH

Interphase FISH for 6q22 and 9p21 loci was performed using custom DNA probes as previously reported(11) or, if one existed, a commercially available probe was used according to the manufacturer's instructions. A minimum of 50 tumor cells was scored. A cohesive group of at least 20 cells, of which at least 80% were abnormal, was required for that sample to be considered abnormal.

RESULTS

Clinical characteristics

The cohort comprised 7 patients, 2 men and 5 women, with median age of 60 years (range of 52 to 77 years). All cases were histologically reconfirmed as CD20-positive DLBCL. Each specimen had diagnostic tissue without hemorrhage or necrosis; and each specimen was EBER negative. Three patients were identified at Mayo Clinic and 4 at the University of Virginia. No patient was HIV positive, had received a solid organ transplant, or had an active autoimmune disorder. Clinical details are shown in Table 1. Performance score could be ascertained in 5 patients. Survival data was available on all seven patients (cases A-G) and treatment information on five (cases A-D and G). Treatment varied from patient to patient. Median survival of the five treated patients was 12 months. Two patients received no treatment and survived 2.5 months and 3 weeks, respectively (cases B and D).

Of the 7 PCNSL patients, 3 were classified as having a GC phenotype and the remaining 4 as non-GC phenotype (ABC). In 6 out of these 7 PCNSL patients (cases B – G), aCGH experiments were performed in DNA samples obtained from both snap-frozen and FFPE tissues for assay quality comparative purposes. In the remaining sample (case A), only frozen sample was analyzed.

Performance of aCGH experiments in samples from FFPE and snap-frozen specimens

To measure the assay quality of the aCGH experiments we used the DLRS_{spread} value as a surrogate of signal noise. All aCGH experiments run from snap-frozen tumors showed DLRS_{spread} values in the range of good or excellent based on QC thresholds obtained from Genomic Workbench software. On the other hand, experiments run from FFPE showed higher DLRS_{spread} compared with paired frozen samples, with 3 assays considered in the good range and another 3 in the marginal range (Figure 1A).

Next, we analyzed the reliability of the CNA detection in assays run from FFPE specimens compared with the paired frozen samples. The data showed an inverse correlation between DLRS_{spread} values and the concordance of aberration calls between pairs of samples (Figure 1B). In FFPE cases with low DLRS_{spread} values (<0.3) there was an excellent equivalence in aberration calls with nearly identical results to frozen samples (92.3 to 95.2%). The FFPE cases with higher DLRS_{spread} values (>0.3) showed lower but still significant equivalence

with values ranging from 62% to 80%. Discrepancies in the aberration calls were mainly observed in CNA smaller than 50 Kb (50%) but became very infrequent in CNA larger than 100 Kb (Figure 1C).

Finally, the main limitation associated with the use of FFPE specimens was the inability to identify the precise location of the CNA breakpoints, with low concordance values ranging from 17.1% to 38.1% in assays with good DLRSreads and from 3.8% to 20% in poor quality assays (Figure 1D).

Characterization of CNA in PCNSL

All PCNSL cases were characterized by highly complex genomes with a median of 23 CNA per patient (total of 210 CNA, range 17–47). Deletions were more common than gains, comprising almost 70% of the CNAs (61.5% of monoallelic and 7.1% of biallelic deletions). The remaining 30% were one copy gain with the exception of 1 CNA characterized by the acquisition of 2 extra copies.

Overall, 20 chromosomal regions were recurrently affected in 3 or more cases, 9 of those being copy-number losses and the remaining 11 comprising copy-number gains. Deletion of 9p21.3 and gain of 12q12-q24.33 were the most common CNAs, observed in 5 out of 7 cases each (Figure 2 and Table 2), as previously found in other reports(21, 22). The minimal deleted region (MDR) at 9p21.3 targets the *CDKN2A* locus, being biallelically affected in 2 out of 5 cases. These deletions were further confirmed using FISH. The minimal amplified region (MAR) on chromosome 12 includes almost the whole q arm (95.2 Mb). Chromosome arms 6p and 6q were also deleted in 5 cases each but there was not a unique deleted region common to all cases. In 6p arm, several CNA were found in cytoband 6p21 affecting HLA genes and in two cases the deletion was biallelic. In cases with 6q deletions, cytobands q12-q14.3, q16.3-q22.2, q22.31 and 6q25.1 were lost in 4 out of 5 cases each (Figure 3). *PTPRK*, previously analyzed by Cady et al.(11) was only found deleted in 3 patients by aCGH.

Other frequent alterations were gains of 5p15.33-q23.3, 7p14.2-p22.3, 12p13.31, 16p12.3-p13.3, 18p11.31, 19q13.43, 21q22.11-q22.3, Xq22.1 and Xq28 and losses of 3p21.1 and 3q26.32 (Table 2). On cytoband 3q26.32, a recurrent focal deletion was found in 3 cases encompassing one gene, the transducin (beta)-like 1 X-linked receptor 1 (*TBLIXR1*).

A total of 15 regions were biallelically deleted, affecting 18 genes (Table 3). Besides the aforementioned losses on 6p21 and 9p21, a recurrent biallelic deletion was found in 6q14.1 including the sole gene *TMEM30A*. Other genes of interest targeted by biallelic deletions were thymocyte selection-associated high mobility group box (*TOX*) and the *ETV6* (TEL) on 8q12.1 and 12p13, respectively. On the other hand, two extra copies of 7p22.1-p22.3 were found in one case, targeting *CARD11* and another 42 genes (Table 3).

Finally, 144 genes were located in chromosomal breakpoints (Supplementary Table S1). Several of these genes were previously identified as being part of fusion protein in other hematological diseases such as *ETV6*, *NCOA2* and *FOXP1*.

DISCUSSION

In PCNSL, the source of biological samples is often limited to FFPE specimens. Only recently, aCGH has become a feasible methodology to be used in FFPE specimens(15–17). In this study, we present the first data obtained from FFPE specimens of PCNSL cases. Our analysis confirmed the good to fair quality and reliability of the data generated from a very limited amount of FFPE tissue. The results are encouraging us to analyze larger archival

tissue collections in order to complement the still fragmented knowledge we have of the genetics basis of PCNSL.

Based on our earlier observation that deletion of chromosomal region 6q22-23 by FISH correlated with shorter survival of PCNSL patients(14) one of the main goals of this study was to study this chromosomal region of interest in more detail by aCGH. Early studies in PCNSL demonstrated loss of one or two copies of 6q22-23 in 66% of cases in a small series(23). More precise mapping implicated the *PTPRK* gene within the 140 kb common minimally deleted region in these cases, and deletion of this region was often associated with loss of expression of *PTPRK*. Furthermore, this small series also demonstrated that loss of heterozygosity (LOH) at the *PTPRK* locus, as well as lack of PTPR-K protein expression, was associated with a poorer prognosis(24). Of note, 6q22 was only deleted in 3 out of 5 cases with 6q losses. Our aCGH findings are in agreement with previous studies in DLBCL and other B-cell malignancies showing multiple affected regions on 6q without a unique MDR to all cases(25, 26).

Previous comprehensive copy-number analysis in PCNSL was performed with spatial resolutions ranging from 200Kb to several Megabases (21, 22, 27). In this study we improved the resolution of the analysis, reaching an average of 6Kb and 17Kb by using Agilent G3 and 244A platforms, respectively. As a result, we were able to refine previously identified MDR/MAR as well as identify several novel abnormalities affecting tumor suppressor genes and oncogenes potentially implicated in the pathogenesis of PCNSL.

Although including few cases, our preliminary analysis identified potential hits to be followed in larger analysis. Genetic and epigenetic alterations affecting *CDKN2A* (9p21.3) have been previously identified in PCNSL(21, 22, 28). Here we found CNA in 5 out of 7 cases, with biallelic deletions observed in two cases. The resolution of the approach used provides a better appreciation of the precise prevalence of abnormalities affecting this and other critical genes.

A recurrent focal deletion was found on 3q26.32 targeting exclusively *TBLIXR1*. This gene is a transcriptional regulator that interacts with the co-repressors of nuclear hormone receptor (NHR)(29). Monoallelic deletions and the significant associated underexpression of *TBLIXR1* have been recently reported in 12–15% of *ETV6-RUNX1* positive acute lymphoblastic leukemias(30, 31). It has been hypothesized that loss of *TBLIXR1* would compromise the ability of co-repressor complexes to inhibit receptor activity, leading to the activation of receptor target genes in the presence of *TBLIXR1* deletions(31). In fact, experiments knocking-down *TBLIXR1* have removed the capacity of RA to induce gene expression(32). Of interest, *TBLIXR1* is widely expressed in hematopoietic tissues and may have a key regulatory role in the NF- κ B pathway(32) and Wnt-mediated transcription(33), thus suggesting its potential biological role in PCNSL pathogenesis.

Recurrent loss of 8q12.1, including biallelic loss in one case, has allowed us to refine previously described MDR(22) and to identify *TOX* as the target gene. *TOX* has been associated with CD4 T lineages development(34). Furthermore, a reduction of the spleen IgG B-cell population in a *TOX*-deficient mouse may be suggestive of the *TOX* involvement in the B-cell differentiation arrest(34).

Additional focal monoallelic deletions affecting negative regulators of the NF- κ B signaling pathway (*MAP4K1*, *TANK*, *TAX1BP1*, *TRIB3*), cell cycle (*RBI*) and immune-cell regulation (*SIRPB1*, *CBLB*, *NFATC2*) were also identified. Further analyses are needed in order to study in more detail these genes and their potential involvement in PCNSL pathogenesis.

In summary, the study reported here expanded the spectrum of chromosomal regions of interest; identified several highly prevalent regions that are thought to be biologically important, and demonstrated that FFPE-based aCGH was feasible and reliable in PCNSL cases, thus expanding the repertoire of investigative tools in this tumor. A better understanding of the underlying mechanisms leading to PCNSL development could result in the identification of prognostic markers and therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Translational Relevance

Genomic analysis has been challenging in primary central nervous system lymphomas (PCNSL), mainly due to the rarity of the tumors and the very limited amount of available tissue. In this manuscript we used tissue extracted from formalin-fixed paraffin-embedded (FFPE) blocks to perform array-based comparative genomic hybridization (aCGH) assays in PCNSL. The good quality of the results is encouraging. Larger archival tissue collections can now be analyzed in order to complement the still fragmented knowledge we have of the genetic basis of PCNSL, which is based on very few studies performed in small cohorts. In addition, we used an aCGH platform that provided us with the highest resolution analysis of the PCNSL genome performed to date and it enabled us to identify novel recurrent abnormalities affecting potential key genes in PCNSL pathogenesis.

Figure 1.

A) Assay quality comparison between matched paired FFPE and snap-frozen samples. Case A was only analyzed from snap-frozen sample. **B)** Correlation between assay quality (DLRSread used as a surrogate) and the CNA concordance level between matched paired FFPE-snap frozen samples. DLRSread values below 0.2 correspond to excellent quality assays; values between 0.2 and 0.3 correspond to good quality; and values above 0.3 are considered marginal. **C)** The concordance of CNA detection between paired samples increases with the size of the abnormalities; **D)** Graph showing that higher quality assay was associated with higher concordance of CNA breakpoint location between paired samples.

Figure 2.

Overview of copy-number abnormalities identified in this study. Bars at the left of the chromosomes represent losses and bars at the right represent gains. The amplitude of the bars denotes the frequency in which each region was affected.

Figure 3. Overview of 6q status. The 3 most commonly deleted regions (MDR1-MDR3) are highlighted. None of them includes *PTPRK* (solid line).

Table 1

Clinical characteristics of the patients included in this study. NA: not available

	Case A	Case B	Case C	Case D	Case E	Case F	Case G
Gender	F	M	F	F	F	M	F
Age at Dx	63	77	52	59	69	60	54
PS	1	N/A	N/A	3	2	2	2
Pathology	DLBC	DLBC	DLBC	DLBC	DLBC	DLBC	DLBC
Subtype	Germinal center	Germinal center	Germinal center	Non-GC	Non-GC	Non-GC	Non-GC
Treatment	CHOD-BLEO+RT	None	HDMTX	None	N/A*	N/A*	R-MPV
Survival (mo)	105	2.5	8+ (alive)	< 1	10	12	26+ (alive)

PS: performance status; DLBCL: diffuse large B-cell lymphoma; GC: germinal center; Non-GC: Non-germinal center; CHOD-BLEO: cyclophosphamide, vincristine, adriamycin, dexamethasone and bleomycin; HDMTX: high-dose methotrexate; R-MPV: Rituximab, methotrexate, procarbazine, vincristine; NA: not available; *=patients treated but details not known.

Table 2

Summary of the most commonly involved regions in CNA. In all regions smaller than 5Mb, the genes included are summarized.

Chromosome Region	Cytoband	Length (Mb)	Event	Percent	# Genes	Genes
chr3:53,188,281-53,220,792	p21.1	0.03	Loss	42.86	1	<i>PRKCD</i>
chr3:178,243,320-178,531,406	q26.32	0.29	Loss	42.86	1	<i>TBL1XR1</i>
chr5:4,025,353-127,435,612	p15.33 - q23.3	123.41	Gain	42.86	460	
chr6:32,560,987-32,591,889	p21.32	0.03	Loss	57.14	3	<i>HLA-DRA, HLA-DRB1, HLA-DRB5</i>
chr6:68,245,865-87,035,064	q12 - q14.3	18.79	Loss	57.14	64	
chr6:102,568,948-117,578,748	q16.3 - q22.2	15.01	Loss	57.14	79	
chr6:121,651,884-121,998,751	q22.31	0.35	Loss	57.14	2	<i>c6orf170, GJA1</i>
chr6:151,726,681-152,128,179	q25.1	0.4	Loss	57.14	5	<i>ZBTB2, RMND1, c6orf211, c6orf97, ESRI</i>
chr6:160,706,067-163,042,970	q25.3 - q26	2.34	Loss	57.14	8	<i>SLC22A3, LPAL2, LPA, PLG, MAP3K4, MTK1, AGPAT4, PARK2</i>
chr7:0-37,224,176	p22.3 - p14.2	37.22	Gain	42.86	230	
chr9:21,952,671-21,976,825	p21.3	0.02	Loss	71.43	1	<i>CDKN2A</i>
chr12:0-5,440,239	p13.33 - p13.31	5.44	Gain	57.14	47	
chr12:37,153,346-132,349,534	q12 - q24.33	95.2	Gain	71.43	796	
chr16:0-19,949,848	p13.3 - p12.3	19.95	Gain	42.86	312	
chr16:79,529,380-88,827,254	q23.2 - q24.3	9.3	Gain	42.86	109	
chr18:3,006,092-3,586,303	p11.31	0.58	Gain	42.86	5	<i>MYO1, MRCL3, MRLC2, TGIF1, DLGAP1</i>
chr19:52,173,665-63,811,651	q13.32 - q13.43	11.64	Gain	42.86	618	
chr21:33,423,626-46,862,008	q22.11 - q22.3	13.44	Gain	42.86	159	
chrX:100,348,062-100,668,066	q22.1	0.32	Gain	42.86	8	<i>DRP2, TAF7L, TIMM8A, BTK, RPL36A, GLA, HNRNPHA2, ARMCX4</i>
chrX:152,713,724-152,889,525	q28	0.18	Gain	42.86	9	<i>SSR4, PDZD4, LICAM, LCAP, AVPR2, ARHGAP4, ARD1A, RENBP, HCFC1</i>

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