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Short Communication

Chromosomal Imbalances in Primary Lymphomas of the Central Nervous System

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Twenty-two primary central nervous system lymphomas of immunocompetent adults were studied by comparative genomic hybridization. All were high-grade diffuse large B cell lymphomas. Comparative genomic hybridization revealed an average of 5.5 chromosomal changes per tumor, with gains being more common than losses (3.5 *vs.* 2.0). The most frequent DNA copy number changes were gains on chromosomes 1, 12, 18 (41% each), 7 (23%), and 11 (18%) and losses involving chromosomes 6 (59%), 18, and 20 (18% each). Commonly involved regions were +12q (41%), +18q (36%), +1q (32%), and +7q (23%), as well as −6q (50%), −6p (18%), −17p, and −18p (14% each). High-level gains were found on 7 chromosomes, mainly involving chromosomes 18q (23%), 12q (18%), and 1q (14%). Minimal common regions of over- and underrepresentation were found on +1q25–31, −6q16–21, +7q11.2, +12p11.2–13, +12q12–14, +12q22–24.1, and +18q12.2–21.3. A significant correlation between loss of DNA copy numbers on chromosome 6q and shorter survival could be established (10.2 *vs.* 22.3 months; $P < 0.05$). Our findings suggest that chromosomal imbalances of primary central nervous system lymphomas are similar to those of diffuse large B cell lymphomas at other locations and are probably not related to cerebral presentation; however, they may be prognostically relevant. *(Am J Pathol 1999, 155:1445–1451)*

Primary central nervous system lymphomas (PCNSL) are defined as extranodal malignant lymphomas presenting in the central nervous system (CNS) in the absence of obvious lymphoma outside the nervous system at the time of diagnosis. Approximately 98% of them are B cell lymphomas with immunohistochemical expression of pan-B markers such as CD20; diffuse large cell lymphomas are the most common subgroup.[1,2] The incidence of PCNSL has been increasing recently in both immunosuppressed and immunocompetent patients from 0.8–1.5% up to 6.6% of primary intracranial neoplasms in some studies.[3–5] The peculiar clinicopathological setting of PCNSL suggests the presence of distinct molecular aberrations underlying their pathogenesis. However, the cytogenetic and molecular genetic profile of PCNSL is still virtually unknown.[6]

Comparative genomic hybridization (CGH) is a recently developed technique that identifies imbalances of the entire genome in terms of DNA copy number changes. Its main advantage is that it bypasses the need for cell culture to harvest metaphase spreads. CGH has previously been applied to nodal and other extracerebral lymphomas[7–15] and B-cell leukemias.[14,16,17] However, it has hitherto not been used for the assessment of PCNSL.

To screen PCNSL for DNA copy number changes that may show the location of relevant oncogenes and tumor suppressor genes, to compare our findings with the data gained from extracerebral lymphomas, and to correlate chromosomal gains and losses with clinical features, we applied CGH on primary high-grade non-Hodgkin's diffuse large B-cell lymphomas (DLCL) of the brain obtained from 22 immunocompetent patients.

Materials and Methods

Patients and Tumors

Formalin-fixed, paraffin wax-embedded biopsy specimens from 22 patients (6 men, 16 women; mean age 63.5 ± 13.2 years; range, 33–84 years) suffering from previously untreated primary CNS non-Hodgkin's lymphomas (NHL) were analyzed (Table 1). None of the patients suffered from apparent immunodeficiency. The diagnosis of PCNSL was established according to the revised European-American classification of lymphoid

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Table 1. Summary of Findings

Case No.	Age (yrs.)	Sex	MIB	Gains	Losses	Survival (months)
1	77	M	61.3	**18p+q**	3p,6q,10p	21
2	72	F	62.2	6p,**7q**,9q,12q,**18q**	1q,4q,6q,18p	>13
3	33	F	51.6	**1q**,11q,**18q**,20q	1p+q,6p,8p,11p,13q,17p,20p	>24
4	84	F	53.3	**1q**,**12q**,22q	5q,6q	nd
5	47	M	62.2	12p+q,21q	15q	>55
6	51	F	77.0	**18p+q**	6q,9q	3
7	41	F	59.8	–	–	>23
8	65	F	62.5	**3q**,**17q**,18p+q	6p+q,17p	nd
9	65	F	56.5	2q,7q,10q	20p	22
10	68	F	49.4	1p,6p,11p+q,12p+**q**,16p,17p	6q	6
11	63	F	40.7	–	18q,20q	nd
12	70	F	54.0	1q,18q,22q	6q	nd
13	75	F	38.5	**3q**,7q,**12q**,18q	6p,14q,17p,18p	6
14	55	M	72.6	1q,16p+q	8p	>19
15	68	M	66.9	1q,**7q**,13q,**18q**	2p,6q,18p	16
16	51	F	50.4	11q,12p+q	–	nd
17	65	M	58.4	3p+q,7p+q,**11q**,12p+q,14q,18p	6p+q	nd
18	80	F	49.1	**1q**,4q,6p,7p+q,12p+q	6q,15q	2
19	74	F	56.2	1q,20p+q,21q	1p,3q	nd
20	54	M	50.4	–	–	12
21	78	F	57.5	1p,**12q**	6q,11q	nd
22	62	F	43.2	–	20q	17

Bold Face type denotes high-level gains. MIB, MIB-1 proliferation index (per cent); >, still alive at time of investigation, nd, no data available.

neoplasms (REAL classification).[18] All 22 cases were DLCL. Only tumor samples that had been shown histologically to contain more than 50% tumor cells were included. Routine hematoxylin and eosin staining and immunohistochemistry using an avidin-biotin complex (ABC) technique and monoclonal antibodies against CD20 (clone L26) as well as the proliferation antigen Ki-67 (clone MIB-1) were performed. All lymphomas showed a positive immunoreaction for B-cell antigen CD20. Furthermore, all available clinical data were reviewed.

CGH Analysis

DNA was isolated by phenol-chloroform extraction according to standard protocols. With minor modifications, CGH analysis was performed as described by du Manoir et al.[19] Briefly, tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) and reference DNA from a healthy male donor with digoxigenin-11-dUTP (Boehringer Mannheim) in a standard nick translation reaction. The DNase concentration in the labeling reaction was adjusted to reveal an average fragment size of 200 to 500 bp. The labeled DNA fragments were purified from remaining nucleotides by column chromatography.

For CGH, 500 ng of tumor DNA, 300 ng of reference DNA, and 30 μg of human Cot1 DNA (Gibco, Karlsruhe, Germany) were coprecipitated and redissolved in 10 μl of hybridization buffer. Denaturation of DNA at 75°C for 5 minutes was followed by a preannealing time of 45 minutes at 37°C. Target metaphase spreads (46,XY), which had been prepared following standard procedures, were denatured separately in 70% formamide/2× SSC for 2 minutes at 72°C. Hybridization was allowed to proceed for 3 to 4 days. Posthybridization washes were carried out to a stringency of 50% formamide/2× SSC at 45°C and 0.1× SSC at 60°C. Biotinylated and digoxiginated sequences were detected simultaneously, using avidin-fluorescein isothiocyanate (FITC; Boehringer Mannheim, 1:200) and anti-digoxigenin-rhodamine (Boehringer Mannheim, 1:40). The slides were counterstained with diauridino-phenylindol (DAPI) and mounted in an antifade solution (Vectashield, Vector Laboratories).

Microscopy and Digital Image Analysis

Separate digitized gray level images of DAPI, FITC, and rhodamine fluorescence were taken with a CCD camera connected to a Leica DMRBE microscope (Leica, Wetzler, Germany). The image processing was carried out by use of Applied Imaging software (Applied Imaging, Sunderland, UK). Average green-red ratios were calculated for each chromosome in at least 10 metaphases.

Statistical Thresholds and Controls

Chromosomal regions with CGH ratio profiles surpassing the 50% CGH thresholds (upper threshold 1.25, lower threshold 0.75) were defined as loci with copy number gains or losses. Based on experiments with normal control DNA, these thresholds have been shown to eliminate false positive results. These values have been used in several studies comparing CGH data with results obtained by other cytogenetic methods and have proven to provide robust criteria for the diagnosis of chromosomal gains and losses. Overrepresentations were diagnosed as high-level gains or amplifications when the fluorescence intensity levels exceeded 1.5[15] or when the FITC

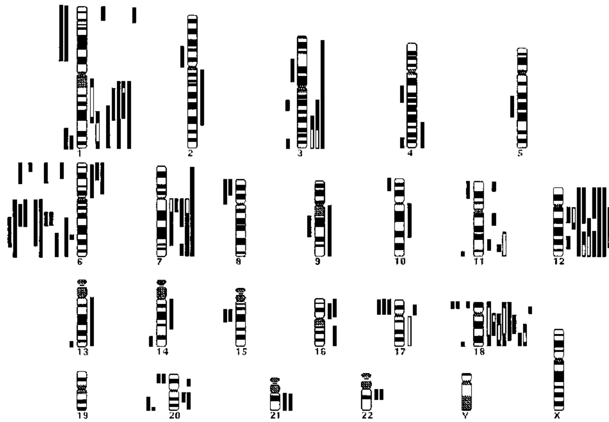

Figure 1. Summary of gains and losses of DNA sequences identified by CGH. Gains are shown as black bars on the right side of the chromosome ideogram and losses on the left. High-level amplifications are marked as white inlays within the black bars. Each vertical represents the affected chromosomal region seen in a single tumor specimen in case number order.

fluorescence showed strong focal signals. For the assignment of these high-level amplifications to chromosomal bands, the signal intensities were compared to the DAPI banding on individual chromosomes. As tumor specimens and normal DNA were not sex-matched, X and Y chromosomes were excluded. Also excluded were centromeric and satellite regions of the acrocentric chromosomes and chromosome 19, because of the abundance of highly repetetive DNA sequences and the frequent occurrence of false positive CGH results as shown by interphase fluorescence *in situ* hybridization using suitable DNA probes. Student's *t*-test was used to prove significance.

Results

Our CGH investigation of primary DLCL of the brain revealed DNA copy number changes in 20 of 22 patients (91%; Table 1). In two cases (7 and 20) no chromosomal gains or losses were found by CGH analysis, whereas one case (3) showed a maximum of 12 changes in total. An average of 5.5 chromosomal changes per tumor was found, consisting of more gains (mean, 3.5) than losses (mean, 2.0) of genetic material. The most frequent changes were gains of DNA copies on chromosomes 1, 12, and 18 in 41% each as well as on chromosomes 7 (23%) and 11 (18%; Figure 1). Losses most commonly involved chromosomes 6 (59%), 18, and 20 (18% each). The most frequently affected chromosomal regions were +12q (41%), +18q (36%), +1q (32%), and 7q (23%), as well as −6q (50%), −6p (18%), −17p, and −18p (14% each); a typical CGH image and profile is shown for case 15 in Figure 2. High-level gains were found on 7 chromosomes, 1, 3, 7, 11, 12, 17, and 18, mainly involving chromosomes 18q (5 high-level gains), 12q (4), and 1q (3). On each of the frequently affected chromosomes, CGH analysis allowed us to delineate the following minimal common regions of over- and under-representation: +1q25–31, −6q16–21, +7q11.2, +12p11.2–13, +12q12–14, +12q22–24.1, and +18q12.2–21.3.

The respective MIB-1 proliferation indices were 56.1 ± 9.7%, ranging from 38.5 to 77.0%. The average survival time for patients suffering from primary cerebral DLCL was 17.1 months. At the time of the survey, nine patients had died of the disease after 2 to 22 months, whereas five were still alive after 13 to 55 months (4 free of disease, 1 (case 7) with residual tumor). Eight other patients had been lost to follow-up and no clinical data could be obtained for them. Recurrences occurred in five patients (cases 9, 13, 15, 20, and 22), all of whom died after 6 to 22 months.

The only statistically significant correlation between shorter survival and loss of DNA could be found for chromome −6q ($P = 0.045$). Loss on chromosome 6p and gains on chromosomes 1, 7, 12, and 18 or their respective long arms as well as combinations of the above imbalances did not significantly correlate with survival, nor did the sum of chromosomal imbalances (gains plus losses), the number of gains alone, or the number of losses alone. Furthermore, no correlations could be found between any of the chromosomal changes and tumor proliferation, gender, or age of the patient, or between Ki-67/MIB-1 proliferation index and survival. Moreover, neither clusters of specific gains or losses nor specific combinations of chromosomal changes could be identified.

Discussion

The rapidly growing number of studies applying CGH to different entities impressively demonstrates the potential of this approach to detect chromosomal gains and losses in tumor genomes. No CGH study on PCNSL has been reported in the literature, and conventional and interphase cytogenetic as well as molecular data on PCNSL have also been scarce. Cytogenetic analyses of single cases of PCNSL in non-AIDS patients revealed gains of 6q, 7p, and chromosome 12 and losses of chromosomes 6, 7, 8, 14q, and 19 as well as translocations (1;14), (6;14), (13;18), and (14;21), findings similar to those observed in nodal B cell lymphoma.[6,20] In comparison with systemic DLCL where amplifications for *CDK4*, *BCL2*, *MDM2*, *MYC* and *REL* have been demonstrated,[8,9,13,14,21,22] the mutational spectrum of oncogenes and tumor suppressor genes in PCNSL is still largely unknown.[6] *CDKN2A* and *CDKN2B* mutations were found in 4 of 5 PCNSL[23] whereas *TP53* mutations were found in 2 of 5 sporadic PCNSL.[24] Cobbers et al,[25] in their analysis of 20 PCNSL of immunocompetent patients, found *CDKN2A* deletions in 50% but detected no amplifications for *CDK4*, *CCND1*, *BCL2*, *MDM2*, *MYC* or *REL*, and only one case showed a *TP53* mutation. Larocca et al[26] found a frequent association of PCNSL with *BCL6* mutations but no alterations for *C-MYC* or *BCL2*. Immunohistochemical studies on PCNSL of non-AIDS cases reported varying overexpression of p53 (50%,[25] 30%,[27] and none[28]), bcl-2,[25–29] and bcl-6[26,27] whereas c-*myc*,[27] mdm2,[25,27] p16,[25] and cyclin D1[25] were not expressed.

One of the mechanisms for activating proto-oncogenes is gene amplification resulting in an enhanced

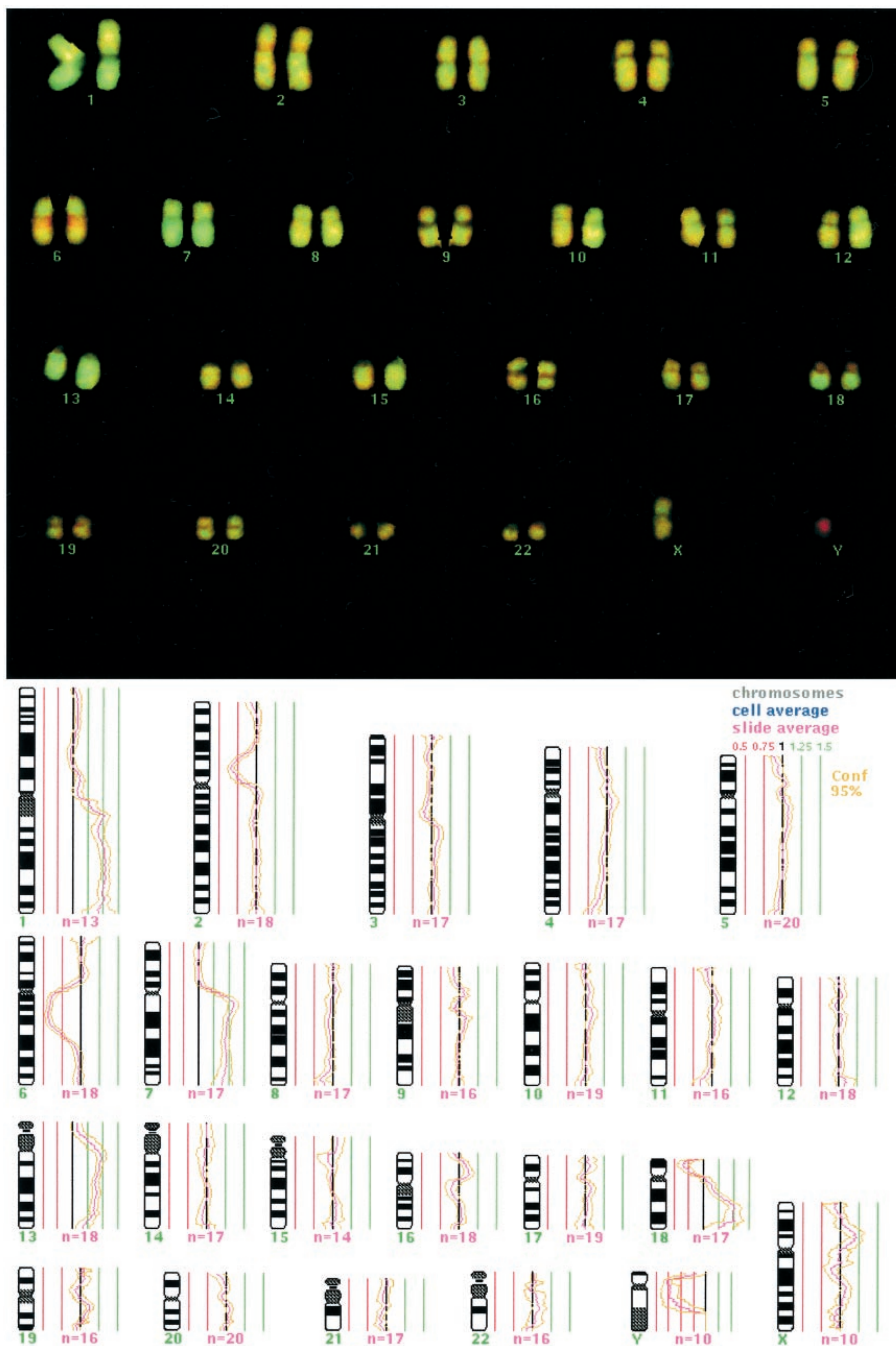

Figure 2. Two-color CGH image of hybridized chromosomes of case 15 with computer-generated CGH ratio superimposed. Green regions represent gains, red regions losses (**top**). Calculated CGH profile shows gains on 1q, 7q, 13q, and 18q as well as losses on 2p, 6q, und 18p. Average ratio profile of autosomal chromosomes is depicted with a 95% confidence interval. The ratios are plotted alongside the chromosome ideogram. A balanced copy number has a baseline ratio of 1, represented by the central black line; thresholds of copy number gains (1.25) and losses (0.75) are shown (**bottom**).

expression of the corresponding gene product. In non-Hodgkin's lymphomas such gene amplifications have rarely been identified. Using CGH, a technique that has not only proven to be very sensitive for the detection of high-level DNA amplifications of units as small as 90 kb[30] but also points to the chromosomal localization of the amplified sequences, we analyzed 22 primary CNS non-Hodgkin's lymphomas of DLCL histological subtype. As shown in previous studies on systemic lymphomas, chromosomal gains were more frequent than losses.[9,10,12,15] The most common imbalances involved gains on chromosomes 1, 12, 18, and 7 (high-level gains mainly affected 18q, 12q, and 1q) as well as losses on chromosomes 6, 18, and 20. The most frequently involved chromosomal regions were +12q, +18q, +1q, and +7q, as well as −6q, −6p, −17p, and −18p. Similar changes were observed in a series of 32 DLCL, albeit extracerebral, in which CGH revealed DNA copy number gains on chromosomes 1q, 3, 6p, 7, 11, 12, and 18, as well as losses on 6q, 1p, and 8p.[10]

Amplifications of 18q and *BCL2* (18q21) are commonly found in nodular DLCL and suggest that, in addition to 14/18 translocation, *BCL2* amplification might be another mechanism for BCL2 protein overexpression. *BCL2* is a proto-oncogene that is known to inhibit apoptosis and deregulation plays an important role in many cases of DLCL[10] as well as most cases of nodular follicle center lymphoma[7,11] and marginal zone B cell lymphoma.[12,31] However, two recent studies on PCNSL in immunocompetent patients found no amplifications, mutations, or other alterations of *BCL2*,[25,26] whereas overexpression of bcl-2 protein was shown to be consistently present by several authors.[25–29]

Findings similar to ours were also reported for DLCL of the gastrointestinal tract, where CGH and fluorescence *in situ* hybridization revealed gains on 1q and 12 and losses on 6q and 17p[15,21]; gains on chromosome 12 were found in 10/10[15] and 9/31[21] cases, respectively. Our identification of amplified genes on chromosome 12, in particular 12q, is in accordance with previous studies, as amplification on chromosome 12 was found to be a common cytogenetic finding in nodal B cell neoplasms; these changes have also been documented by CGH investigations performed on B cell chronic lymphatic leukemia,[16,17] follicular lymphomas,[7] and mediastinal thymic B cell lymphomas.[9] Several candidate genes are located on chromosome 12 and have been proposed to play a role in tumorigenesis: *CCND2* (12p13), *FGF6* (12p13), *KRAS2* (12p12.1), *CDK4* (12q13), *MDM2* (12q13–14), and *GLI* (12q13–14)[14,16,22]; however, no CDK4 amplification was found in PCNSL by Cobbers and coworkers.[25] Thus, the relevant genes on 12q involved in PCNSL pathogenesis remain to be determined.

Gains on 1q were among the most common changes and have also been found by CGH to be present in nodular DLCL[10] and DLCL of the gastrointestinal tract[15,21] as well as marginal zone B cell lymphomas[12,31] and follicular lymphomas.[32] A candidate gene on 1q23–31, a frequently highly amplified region, has not been put forward; however, it corresponds to the location of the proto-oncogene *TRK/TRKC*. The next most frequent gains after chromosomes 1, 12, and 18 affected amplification on 7q, which has also been found on a number of B cell neoplasms in general[14] as well as extranodal systemic DLCL,[8,10] follicular lymphomas,[11] high-grade MALT,[15] and mediastinal thymic B cell lymphomas.[9] Here, too, candidate proto-oncogenes and tumor suppressor genes have not yet been located.

Gains of 3q in our study have been found in only 3 of 22 patients; all of them, however, showed copy number changes in the region of *BCL6* (3q27), 2 as high-level amplifications. Similar findings have been reported for PCNSL[26] as well as for non-Hodgkin's lymphomas in general,[33,34] systemic and gastrointestinal DLCL,[10,21] and marginal zone B cell lymphomas.[12,31]

Deletion of 6q was found to be the single most common chromosomal change among our patients and has so far also frequently been discovered in other hematological malignancies and solid tumors.[10] It is a recurrent cytogenetic event in many B cell neoplasms and three regions have been isolated, possibly containing different genes involved in lymphoma development[35]; 27% of NHL had structural abnormalities of chromosome 6, which are among the most common recurring karyotypic abnormalities in NHL, most of them 6q deletions the frequency of which ranged from 14 to 31% in six large series of NHL.[36] Deletions of 6q have been found almost consistently in nodal and extranodal DLCL,[8,10,15,21] follicular lymphomas,[7,11] and chronic B cell leukemias.[16] These structural aberrations have occasionally been correlated with clinical features of non-Hodgkin's lymphoma like tumor progression, transformation,and survival.[32,35] In our study, loss of chromosomal material on 6q was significantly correlated with shorter survival compared to patients without loss of 6q (10.2 *vs.*22.3 months; $P < 0.05$).

Frequent chromosomal gains of chromosome 2p have been reported in several studies on nodal and extranodal DLCL,[8] primary mediastinal thymic B-cell lymphomas,[9] and follicular lymphomas[11] and were identified to correspond to amplifications of *REL* (2p12–13)[8,21,22] and *N-MYC* (2p24.1).[14,21,22] However, no case of DNA copy number gains on 2p were found in our series, so that amplifications of *REL* and *N-MYC* do not seem to play a role in PCNSL, a finding that corroborates data recently published by Cobbers et al[25] and Larocca et al.[26] Similarly, gains on chromosome 8 identified in several studies on follicular lymphomas,[7,11] systemic DLCL,[22] and chronic B cell lymphomas[16] could not be found among our patients.

Current therapy regimens consisting of radiotherapy and/or chemotherapy in non-AIDS-associated PCNSL show response rates of 85% with a median survival of 17 to 44 months[1] and 2- and 5-year survival rates of 40 to 70% and 25 to 45%, respectively.[37,38] Clinically, gene amplifications often have been associated with a more aggressive tumor phenotype and shorter overall survival in several tumor types.[39] Three cytogenetic studies on systemic nodal and extranodal non-Hodgkin's lymphomas found that in follicular lymphomas, six or more chromosomal breaks and structural abnormalities of chromosomal regions 1p, 6q, or 17p,[32] as well as gains on chromosomes 5, 6, 17, and 18,[33] were associated with a

poorer prognosis, whereas a significantly shorter survival in high-grade large B cell lymphomas of the gastrointestinal tract was reported to be associated with two or more chromosomal aberrations.[21] We found that among our patients with PCNSL losses of chromosome 6q were significantly correlated with a shorter median survival of 10.2 months, compared to 22.3 months in patients with unaffected chromosome 6q. However, prognosis did not depend on proliferation index, age, or gender. Furthermore, we could not identify clusters of specific gains or losses, specific combinations of chromosomal changes, or a correlation between chromosomal changes and proliferation. Clearly, possible correlations among these parameters should be reassessed in a larger series of PCNSL.

In conclusion, our results suggest that most of the chromosomal regions affected in PCNSL are similar to those found in systemic extracerebral DLCL, whereas other regions implicated in several investigations on nodal non-Hodgkin lymphoma, eg, gains on chromosomes 2 and 8, do not seem to play a major role in PCNSL. Chromosomal imbalances of PCNSL do not seem to account for cerebral location; however, they may be prognostically relevant.

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