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RESEARCH ARTICLE

Characteristic Chromosomal Imbalances in Primary Central Nervous System Lymphomas of the Diffuse Large B-Cell Type

Tillmann Weber[1,2], Ruthild G. Weber[1,4], Kerstin Kaulich[3], Bertrand Actor[1], Birgit Meyer-Puttlitz[3], Stefan Lampel[1], Rainer Büschges[3], Ralf Weigel[2], Martina Deckert-Schlüter[3], Peter Schmiedek[2], Guido Reifenberger[3], Peter Lichter[1]

[1] Abteilung "Organisation komplexer Genome", Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
[2] Neurochirurgische Klinik, Klinikum Mannheim, Theodor-Kutzer-Ufer 4-6, D-68167 Mannheim, Germany
[3] Institut für Neuropathologie, Rheinische Friedrich-Wilhelms-Universität, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany
[4] Present address: Institut für Humangenetik, Ruprecht-Karls-Universität, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany

We performed a genome wide screening for genomic alterations on a series of 19 sporadic primary central nervous system lymphomas (PCNSL) of the diffuse large B-cell type by comparative genomic hybridization (CGH). The tumors were additionally analyzed for amplification and rearrangement of the *BCL2* gene at 18q21 as well as for mutation of the recently cloned *BCL10* gene at 1p22. Eighteen tumors showed genomic imbalances on CGH analysis. On average, 2.1 losses and 4.7 gains were detected per tumor. The chromosome arm most frequently affected by losses of genomic material was 6q (47%) with a commonly deleted region mapping to 6q21-q22. The most frequent gains involved chromosome arms 12q (63%), 18q and 22q (37% each), as well as 1q, 9q, 11q, 12p, 16p and 17q (26% each). High-level amplifications were mapped to 9p23-p24 (1 tumor) and to 18q21-q23 (2 tumors). However, PCR-based analysis, Southern blot analysis and high-resolution matrix-CGH of the *BCL2* gene revealed neither evidence for amplification nor for genetic rearrangement. Mutational analysis of *BCL10* in 16 PCNSL identified four distinct sequence polymorphisms but no mutation. Taken together, our data do not support a role of *BCL2* rearrangement/amplification and *BCL10* mutation in PCNSL but indicate a number of novel chromosomal regions that likely carry yet unknown tumor suppressor genes or proto-oncogenes involved in the pathogenesis of these tumors.

Corresponding author:
Dr. Peter Lichter, Deutsches Krebsforschungszentrum, Abt. H0700, INF 280, 69120 Heidelberg, Germany. Phone: +49-6221-424619. Fax: +49-6221-424639. E-mail: p.lichter@dkfz-heidelberg.de

Introduction

The incidence of primary central nervous system lymphomas (PCNSL) has markedly increased over the past decades (9, 10, 15). Currently, these tumors are estimated to account for more than 6% of all primary brain tumors (33). Morphologically, the vast majority of PCNSL are high-grade non-Hodgkin lymphomas of the diffuse large B-cell type (DLBCL) according to the revised European American lymphoma (REAL) classification (17). The molecular pathogenesis and histogenetic origin of PCNSL is still poorly understood. Infection with Epstein-Barr virus (EBV) appears to be an important factor for the development in immunocompromised individuals (36). In contrast, EBV infection is rare in PCNSL from immunocompetent patients (20, 36). One study reported evidence that PCNSL are frequently positive for human herpesvirus 8 (HHV-8) sequences (8). However, this finding was not confirmed by others (1, 16, 37). Recently, several tumor suppressor genes and proto-oncogenes were investigated for their involvement in PCNSL from immunocompetent patients (7, 61). These studies revealed that the *CDKN2A* gene is frequently inactivated by either homozygous deletion or 5′-CpG hypermethylation. Mutations in the *TP53* gene were observed in a small fraction of PCNSL, while genetic aberrations of the proto-oncogenes *MDM2*, *CDK4*, *CCND1* (*BCL1*), *MYC* and *REL* were not found (7). With respect to the histogenesis of PCNSL, it has been suggested that the tumor cells are related to germinal center B-cells since they frequently carry mutations in the *BCL6* gene, an aberration regarded as a marker of B-cell transition through the germinal center of secondary lymphatic organs (28).

Case No.	Losses detected by CGH	Gains detected by CGH	*BCL2* amp.	*BCL2* rearrang.*	bcl-2 protein*	*BCL10* mutation
T1	4q12-q24	1q23-q44, 12, 18q11.2-q12, **18q21-q23**	-	-	+++	-
T4	-	-	-	-	+++	-
T5	-	1q23-q44, 13q33-q34	ns	-	ns	-
T6	6q12-q24, 13q21-q31	5q31-q35, 9p12-p13, 9q, 12q23-q24, 17q25, 22q	-	-	+++	-
T7	-	3, 11, 12q13-q14, 16	-	-	+++	-
T8	6q	2p16-p25, 5q35, 12p13, 12q12-q14, 12q22-q24, 13q12-q14, 13q33-q34, 16, 17q21-q25, 18q12-q23	-	-	++	-
T10	1p31, 3p12, 13q21-q31	12q24, 16, 21q22, 22q13	-	-	+	-
T11	4q, 4p12-p15, 6p11.2-p21.1, 6q12-q25, 13q21-q31, 17p	10p14-p15, 10q22-q26, 11q13, 11q22-q25, 12q24, 20, 21q22, 22q	-	-	+++	-
T13	6p22-p25, 11p13-p14, 17p13	1p12-p13, 1q, 9q22-q34, 10q21-q26, 11q12-q14, 11q23-q25, 12q24, 16p13, 17q25, 22q13	ns	-	++	-
T14	6q, Xp, Xq12-q26	1p12-p13, 3p21-p26, 4p, 4q12-q27, 7, 9q34, 17p13, 17q25, 18q12-q25, 22	ns	ns	+++	-
T17	3p24-p26, 4q12-q21, 6p22-p25, 6q, 7p15-p22, 8q, 17p12-p13, 20p, Y	1q21, 2p16-p25, 3p21, 4q24-q35, 5p, 9q22-q34, 11, 12q, 13q32-q34, 14q21-q32, Xp11, Xq	ns	ns	++	-
T18	-	1q32, 22q13, Xp, Xq22-q28	ns	ns	+	-
T19	6q, 8p22-p23	6p23-p25, 7, 8q24, 11q23, 12	ns	ns	++	-
T20	2p11.2-p13, 3p12-p13, 6q, 15q21, Y	5q35, 9q34, 14q32, 16p, 16q22-q24, 17q25, 22q13, Xp, Xq21-q28	ns	ns	++	-
T21	6p11.2-p21.1, 6q, 9q13-q22	12, 18q21, 21	ns	-	+	-
T22	Xp21-p22	5q23-q35, 12, 13q	ns	-	+	-
T23	15q15-q22	13q, 18q, 21q21-q22, X	ns	ns	++	ns
T24	6q21-q22	**9p23-p24**, 12q23-q24, 18q12-q21,	-	ns	+++	ns
T25	9, 14q, 22q, Y	2q31-q37, 3p24-p26, 4p, 4q12-q23, **18q21-q23**, 18p, 18q11.2-q12	-	ns	+++	ns

High copy number amplifications are printed with bold facing.

Abbreviations: amp., amplification; rearrang., rearrangement; - denotes absence of detectable *BCL2* rearrangement/amplification and *BCL10* mutation, respectively; ns, not studied.

In immunohistochemical staining, intensity for bcl-2 was semiquantitatively graded: +++, strong expression (> 50 % of positive tumor cells); ++, moderate expression (10-50 % positive tumor cells); + weak expression (< 10 % positive tumor cells).

* Data on *BCL2* gene rearrangement and bcl-2 protein expression includes findings previously published (7, 11).

Table 1. CGH karyotype, *BCL2* gene amplification, *BCL2* gene rearrangement, bcl-2 protein expression and *BCL10* mutations in 19 PCNSL.

Comparative genomic hybridization (CGH) is a powerful molecular cytogenetic technique (14, 24) that allows the detection of gains and losses of genetic material across the entire genome at a resolution of 10-20 Mbp for low copy number imbalances (4, 24). High copy number changes can be detected if the product of amplicon size and copy number amounts to at least 2 Mbp (23, 42). Furthermore, the use of degenerate

oligonucleotide primed polymerase chain reaction (DOP-PCR) for the universal amplification of tumor DNA (51) prior to CGH analysis allows the investigation of minute tumor specimens. This is particularly helpful for the study of PCNSL since these tumors are commonly diagnosed by stereotactic biopsy.

In the present study, we investigated 19 PCNSL from immunocompetent patients by CGH using either DNA isolated from surgical specimens or DOP-PCR amplified DNA prepared from stereotactic biopsy material. As amplified sequences were mapped to 18q21-q23 in 2 of the tumors, we additionally performed analyses concerning amplification and rearrangement of the *BCL2* gene at 18q21 along with expression of the bcl-2 protein. Furthermore, we searched for mutations in the recently cloned *BCL10* gene at 1p22, which was shown to be altered not only in B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) with t(1;14)(p22;q32), but also in other types of lymphoid neoplasms as well as in colon carcinomas, mesotheliomas and germ cell tumors (57).

Materials and Methods

Tumor Material. Tissue samples from 19 immunocompetent patients (14 female, 5 male; mean age at operation: 60 years) with PCNSL of the diffuse large B-cell type were obtained by either open resection (n = 17) or by stereotactically guided needle biopsy (n = 2). None of the patients had received corticosteroids, irradiation or chemotherapy prior to surgery. Seventeen of the nineteen tumors had been analyzed for the presence of EBV by either PCR amplification of EBV specific DNA sequences, RNA in situ hybridization and/or immunohistochemical detection of EBV associated antigens. None of these tumors was EBV positive. The tumors were classified according to the REAL classification of lymphoid neoplasms (17). To assure a sufficient proportion of neoplastic cells in the tumor samples investigated by CGH and molecular genetic analyses, each specimen was histologically evaluated.

DNA Extraction and Comparative Genomic Hybridization (CGH). From 14 tumors (12 open resections, 2 stereotactic biopsies), unfixed frozen tumor specimens were available for DNA extraction. In 5 cases, DNA was extracted from formalin-fixed paraffin-embedded tissue samples. The preparation of high molecular weight DNA from either frozen or paraffin-embedded open resection specimens was carried out as described (19, 44). For DNA preparation from frozen stereotactic biopsy material, the specimens were subjected to a proteinase K digest (3 mg/ml proteinase K, 125 mmol/L KCl, 25 mmol/L Tris-HCl, pH 8.3, in a total volume of 20 μl) at 55°C for 32 hours, followed by proteinase K inactivation at 90°C for 10 minutes. The tumor DNA was then universally amplified by DOP-PCR.

Preparation of reference metaphase chromosomes and of control DNA from peripheral blood leukocytes of a normal male subject, as well as labeling of probe DNA and CGH were performed as described (30). Briefly, 700 ng of biotin-labeled tumor DNA, 700 ng of digoxigenin-labeled control DNA, and 70 μg of unlabeled Cot-1-fraction from human DNA were cohybridized to reference metaphase spreads from a healthy male subject (46, XY) and incubated at 37°C for 4 days. After posthybridization washes to a stringency of 0.1 x SSC at 60°C, probe and control DNA were detected via avidin-conjugated fluorescein isothiocyanate (FITC) and anti-digoxigenin antibody-conjugated rhodamine, respectively. When DNA was prepared from formalin-fixed cells, FITC signals were amplified once according to the protocol by Pinkel and co-workers (41). Chromosomes were counterstained with 4,6 diamino-2-phenylindole (DAPI). The image acquisition and processing was performed by using CYTOVISION system version 3.1 (Applied Imaging, U.K.). Mean ratio profiles were determined from analysis of 12-14 metaphase spreads. The threshold values used to distinguish between the balanced and imbalanced state of the average ratio profiles were 0.75 (lower threshold) and 1.25 (upper threshold) in accordance with reported CGH analysis protocols (13). High-level amplifications were defined as gains of chromosomal material that led to either a strong focal fluorescent band in the hybridization pattern of the tumor DNA and/or a deviation of the average ratio profile to the right beyond the 2.0 threshold.

As negative controls for CGH experiments, differently labeled normal DNAs were hybridized to reference metaphase chromosomes. These experiments showed no diagnostic alterations. Nonetheless, chromosomal region 1p34-p36 and chromosome 19, which are regions known to occasionally demonstrate false-positive CGH results in our and other laboratories, were excluded from CGH evaluation (25, 30, 53).

Degenerate Oligonucleotide Primed-Polymerase Chain Reaction (DOP-PCR). Tumor DNA prepared from stereotactic biopsy specimens (see above) and control DNA were both universally amplified using a DOP-PCR protocol modified from Telenius *et al*. (51). PCR

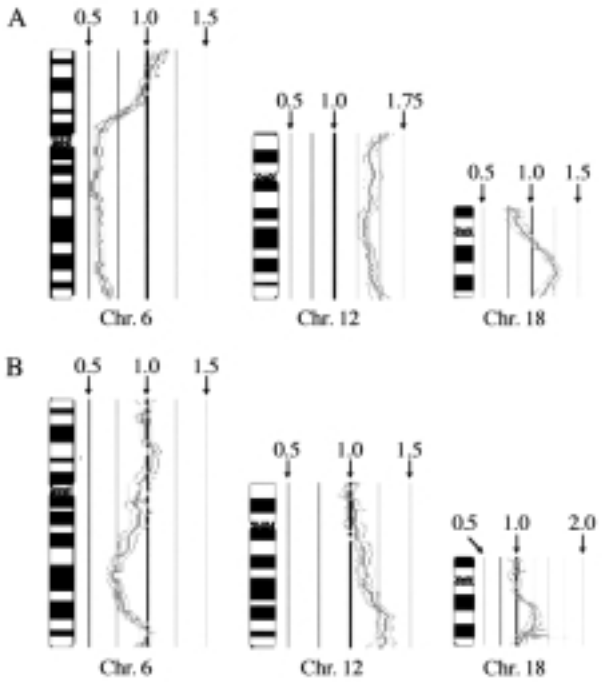

Figure 1. Selected CGH average ratio profiles of two PCNSL including the 95% confidence intervals. Tumor material was obtained by stereotactic biopsy (T24) or by open resection (T21). The three chromosomes most frequently affected by chromosomal imbalances in PCNSL are depicted (chromosomes 6, 12, and 18). (**A**) Results of CGH performed with high molecular weight DNA extracted from frozen tumor material acquired by open surgery. (**B**) CGH experiment performed with DOP-PCR amplified DNA prepared from small amounts of tumor material obtained by stereotactic biopsy.

was performed in a final volume of 50 μl (4 μl 25 mmol/L $MgCl_2$, 2 μl 5 mmol/L deoxynucleotide triphosphate, 5 μl 17 μM primer 6MW (5′-CCGACTCGAGNNNNNNATGTGG-3′), 0.5 μl (2.5 U) Taq polymerase, and tumor or control DNA in 20 μl 2.5 × PCR buffer from the proteinase K digest). The PCR conditions used were: 10 min at 93°C, followed by 10 cycles of 1 min at 94°C, 1.5 min at 30°C, 3 min transition from 30-72°C, and 3 min extension at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, 3 min at 72°C with an addition of 1 sec/cycle to the extension step, and a final extension of 10 min.

The use of protocols for universal DNA amplification followed by CGH was validated for material from stereotactic biopsies by hybridizing differently labeled DOP-PCR-amplified control DNAs to reference chromosomes. As a further control experiment, DNA from stereotactic biopsies rendering only normal brain tissue were amplified by DOP-PCR and hybridized together with differently labeled DOP-PCR-amplified control DNA to reference chromosomes. Each control experiment revealed a balanced state of all CGH average ratio profiles.

Analysis for amplification and genetic rearrangement of BCL2. Seven PCNSL of our series were investigated by Southern blot analysis for amplification of the *BCL2* gene at 18q21. For Southern blot analysis, 2.5 μg DNA from each case were digested with the restriction enzyme *Taq*I, separated by electrophoresis on 0.8% agarose gels, and alkali blotted to Hybond-N^+-membranes (Amersham-Buchler, Braunschweig, Germany). The blots were sequentially hybridized with [α-^{32}P]dCTP labeled probes for *BCL2* (Oncogene Research Products, Cambridge, MA, USA) and the control locus *CCNA* on 4q. The probe for *CCNA* was generated by PCR as described (7). Hybridized membranes were exposed to imaging plates (Fuji, Kanagawa, Japan) and analyzed using the FLA-2000 Phosphor and Fluorescent Image Analyzer (Fuji). A PCR-based assay employing primers MBR (for the major breakpoint region of *BCL2*) and JH (for the IgH chain joining region) was used for the detection of *BCL2* gene rearrangements as described (31). DNA from a follicular lymphoma with a known translocation t(14;18) served as positive control.

Matrix-CGH. Matrix-CGH was performed as previously described (48). Alu-PCR products from target YAC DNAs were arrayed using a robot device (Nanoplotter, GeSim, Germany). YAC clone 153a6 containing *BCL2* served as target to be tested. YAC clones 935a12, 809e5 and 933e9, mapping to chromosome bands 8q13-q14, 12q13-q15, and 13q14, respectively, served as references (balanced in the tumors). Tumor DNA and normal genomic DNA, both DOP-PCR amplified, were cohybridized to the slides followed by washing and probe detection via FITC and Cy5. The arrays were then subjected to automated scanning using a confocal laser scanning microscope. Read-out of fluorescence intensities, normalization, ratio calculation and statistical evaluation were performed using a computerized procedure dedicated to matrix-CGH analysis (Lampel *et al.*, in preparation).

Immunohistochemical analysis of bcl-2 expression. The experimental procedure as well as part of the results obtained for bcl-2 expression were reported on previously (7, 11).

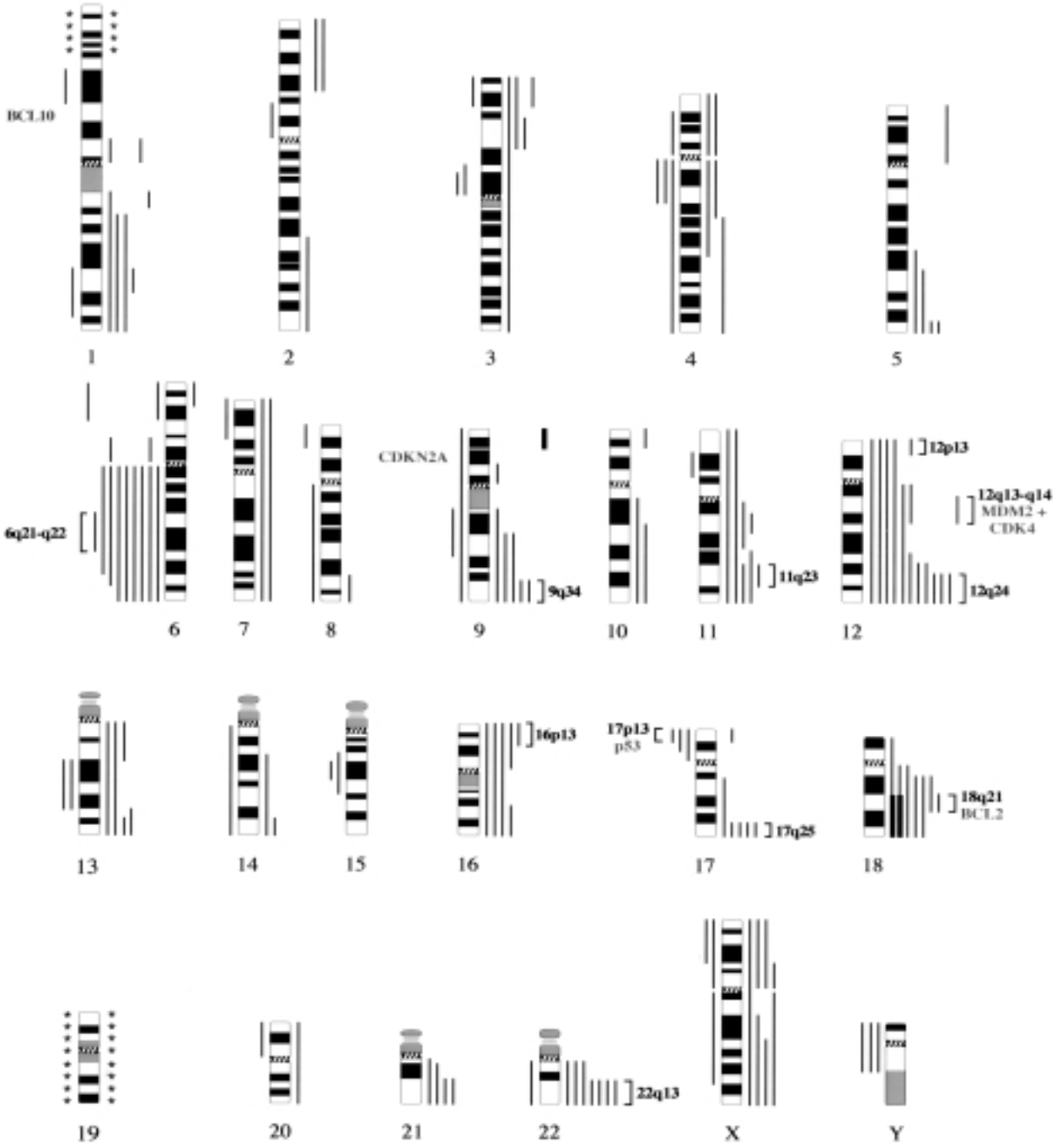

Figure 2. Location of chromosomal imbalances detected by CGH in 19 PCNSL. Vertical lines on the right side of each chromosome ideogram represent gains of genetic material, whereas vertical lines on the left side correspond to losses. High-level amplifications are indicated by bold bars. The chromosomal region 1p34-p36 and chromosome 19 are marked by asterisks because they were excluded from evaluation. Consensus regions of gains (on the right of the chromosome ideogram) and of losses (on the left of the chromosome ideogram) are indicated by parentheses together with their respective chromosomal bands. Localization of the proto-oncogenes and tumor suppressor genes discussed in the text are indicated by gray gene symbols.

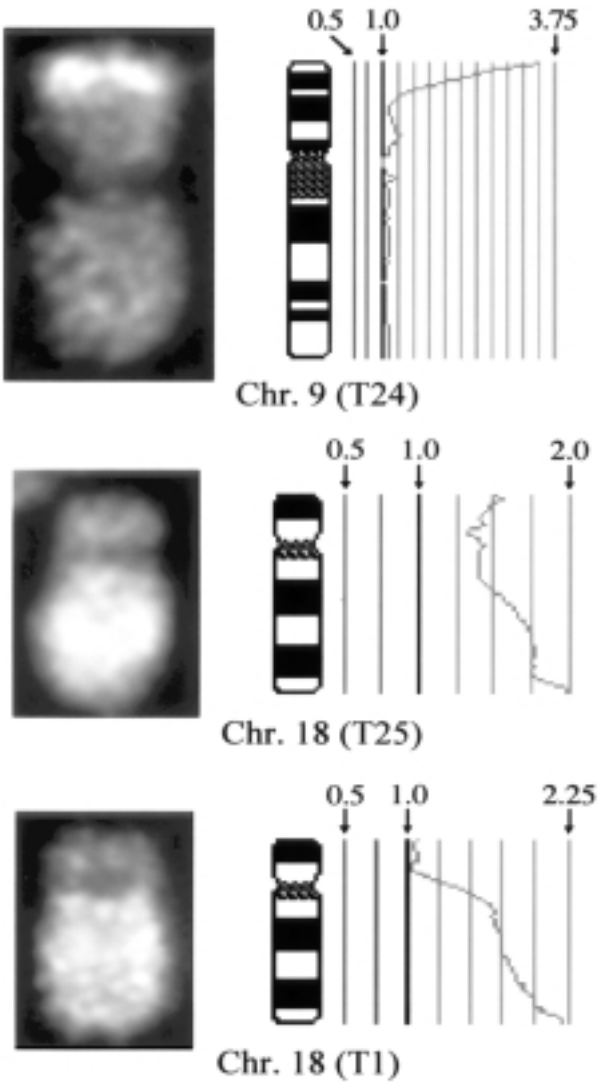

Figure 3. Amplified DNA sequences were identified in three PCNSL (T1, T24, T25) and mapped to two different chromosomes by CGH. Shown are images revealing the hybridization pattern of the tumor DNA (left) next to the average ratio profile (right). The value for the balanced threshold (1.0), the lowest (0.5) and the highest threshold depicted are given for each profile.

Mutation analysis of BCL10. Seventeen tumors of our series were analyzed for *BCL10* mutations (Table 1). The three coding exons of *BCL10* were amplified by PCR using a set of 5 primer pairs as described (57) except that a different forward primer (5'-GCATC-CACTGTCATGTACC-3') was used for amplification of fragment 3.2. The PCR products were screened for mutations after electrophoresis on 8% and 10% non-denaturating polyacrylamide gels at 120-180 V for 16 h. Each PCR product was analyzed at room temperature and at 4°C. The SSCP/heteroduplex band patterns were visualized by silver staining of the gels. Aberrant SSCP and/or heteroduplex bands were excised from the gel, reamplified using the same set of primers, and then sequenced in both directions on a semi-automated DNA sequencer (Applied Biosystems, model 373A) using a Taq cycle sequencing kit (Applied Biosystems).

Results

Eighteen of the nineteen primary PCNSL (95%) displayed chromosomal imbalances by CGH analysis (Table 1). Material from two of the eighteen tumors was acquired by stereotactic biopsy. The quality of the CGH ratio profiles obtained with the DOP-PCR amplified DNA from these biopsies was similar to that yielded by high-molecular weight DNA prepared from frozen surgical tumor samples, i.e. the 95% confidence intervals of the CGH-ratio profiles as a measure for experimental quality were comparably narrow (see Figure 1). Among the imbalances found, overrepresentations of chromosomal material were approximately twice as frequent as underrepresentations (90 gains vs. 40 losses). An average of 4.7 gains and 2.1 losses per case was calculated. Three chromosome arms showed gains or amplifications in more than one third of the cases: 12q (12 of 19, 63%), 18q and 22q (7 of 19, 37% each) (Figure 2). Two distinct regions of frequent gain could be distinguished on 12q: one at 12q13-q14 and the other at 12q24. On 18q and 22q the consensus regions were mapped to 18q21 and 22q13, respectively. Further chromosomal gains identified in 5 of 19 PCNSL each (26%) involved the chromosomal arms 1q, 9q (consensus region: 9q34), 11q (consensus regions: 11q13 and 11q23), 12p (consensus region: 12p13), 16p (consensus region: 16p13) and 17q (consensus region: 17q25). The second most common chromosomal imbalance identified in our tumor series was underrepresentation on 6q (9 of 19, 47%). The smallest region of common loss was delineated by tumor 24 which displayed a loss confined to 6q21-q22. Other chromosome arms showing losses in 3 tumors each (16%) were 17p (consensus region: 17p13), 4q (consensus region: 4q12-q21) and 3p.

Three tumors of our series demonstrated high-level amplifications by CGH. These were mapped to 18q21-q23 in two cases (T1, T25) and to 9p23-p24 in one case (T24) (Figure 3). Southern blot analysis of tumor T1 and six additional PCNSL (T4, T6-8, T10-11), however, revealed no *BCL2* gene amplification (Figure 4A). The two stereotactically biopsied tumors revealed a gain at 18q12-q21 (T24) as well as a high-level amplification at

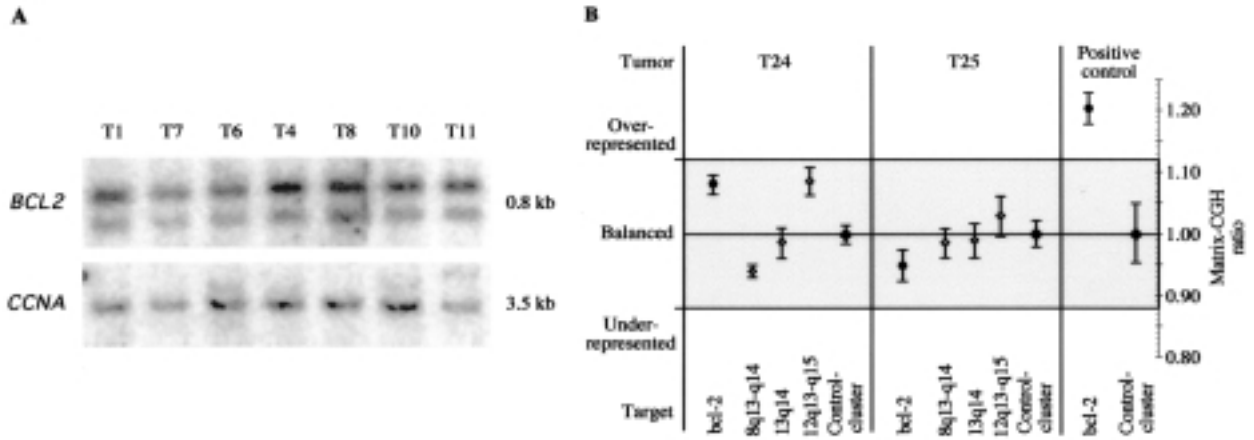

Figure 4. Molecular analysis of *BCL2* copy number. A) Southern blot analysis revealed no amplification of *BCL2* in 7 PCNSL, including one tumor with 18q21-q23 amplification (T1) and another tumor with gain of 18q12-q23 (T8) as detected by CGH. The filter was stripped and reprobed for *CCNA* as control for DNA loading. The case numbers are given on top of each lane. B) Stereotactic biopsies obtained from one tumor with 18q21-q23 amplification (T25) and another tumor with gain of 18q12-q21 (T24), were analyzed for *BCL2* amplification by matrix-CGH using DOP-PCR amplified tumor DNA. Target probes for *BCL2* as well as reference probes from 8q, 12q, 13q were arrayed and subjected to hybridization as described (see Material and Methods). The signal ratios on reference targets were subjected to a cluster analysis using two one-sided t-tests ($\alpha = 0.05$). This procedure defines the range of ratios representing equivalent (gray zone), i.e. balanced, regions versus non-equivalent white zones, i.e. unbalanced, regions in the tumor genome. In both biopsies, the matrix-CGH ratio of *BCL2* was found equivalent to the control cluster, i.e. there was no evidence for an increase of *BCL2* copy number. For the positive control, a follicular lymphoma with a known *BCL2* amplification, the matrix-CGH ratio indicated an overrepresentation of *BCL2* as expected.

18q21-q23 (T25), yet could not be subjected to Southern blot analysis due to the minute amounts of high molecular weight DNA available. We therefore employed matrix-CGH with DOP-PCR amplified DNA from T24 and T25. As presented in Figure4B, the matrix CGH ratios indicated a balanced status for the *BCL2* gene in both tumors.

SSCP/heteroduplex analysis of the BCL10 gene using the primer pair for exon 1 revealed evidence for two DNA polymorphisms, which on sequencing corresponded to single base pair substitutions at codon 8 (CTC $\rightarrow$ CTG: Leu $\rightarrow$ Leu) and within intron 1 (nucleotide 57+11: G $\leftrightarrow$ C, nucleotide numbering from translation start according to GenBank accession no. AJ006288), respectively. In addition to these frequent sequence variations, one tumor (T21) showed an aberrant SSCP pattern for exon 1 while two tumors (T7, T18) demonstrated identical SSCP shifts for exon 3 (fragment 3.2). DNA sequencing revealed a G-to-T transversion in codon 5 (GCA $\rightarrow$ TCA: Ala $\rightarrow$ Ser) in T21. The identical sequence change was found in the patient's constitutional DNA, indicating that it represents a rare polymorphism rather than a missense mutation. Tumors T7 and T19 showed a G-to-A transition in codon 213 (GGA $\rightarrow$ GAA: Gly $\rightarrow$ Glu). Since we had no corresponding constitutional DNA from these patients, we performed SSCP analysis of exon 3 (fragment 3.2) on peripheral blood samples from 50 unrelated individuals. We found 5 samples showing identical SSCP shifts as T7 and T19, indicating that this nucleotide exchange also represents a polymorphism.

Discussion

Our molecular cytogenetic analysis of 19 PCNSL of the diffuse large B-cell type revealed a set of characteristic chromosomal imbalances. The most common alteration, detected in 63% of the cases, was gain on 12q. Two commonly gained subregions were mapped to 12q13-q14 and 12q24. In line with our results, polysomy of chromosome 12 was detected as the most common abnormality in B-cell malignancies studied by fluorescence in situ hybridization (59). In addition, previous CGH studies reported gains and/or amplifications on 12q as recurrent aberrations in various types of non-Hodgkin lymphomas, including extracerebral DLBCL (3, 5, 6, 22, 35, 55). A molecular genetic study reported amplifications of the proto-oncogenes *GLI1*, *CDK4* and *MDM2* (all located at 12q13-q15) in 11-14% of extracerebral DLBCL (43). In contrast, Cobbers *et al*. (7) found neither amplification of *MDM2* and *CDK4* nor overexpression of Mdm-2 protein in 17 primary DLBCL of the CNS (14 of which were also included in the pres-

ent study). In line with these data, CGH showed no 12q13-q15 high copy number amplification in any of the 19 tumors investigated here. These findings suggest that the frequent gains at 12q13-q14 in PCNSL target growth-promoting genes distinct from *MDM2* and *CDK4*.

CGH demonstrated gains on 18q in 37% of PCNSL and amplified sequences from 18q21-q23 in 2 tumors. These aberrations are also frequent in extracerebral lymphomas of various types (2, 5, 12, 34, 35, 43, 56). All previously analyzed amplicons that were mapped to 18q21 by CGH were shown to include the *BCL2* gene by either Southern blot analysis or FISH (12, 34, 35, 56). Amplification of *BCL2* was also detected in cases of extracerebral DLBCL exhibiting a low copy number gain of 18q by CGH (34, 35). In contrast, we found no evidence of *BCL2* amplification in PCNSL, including four tumors with high or low copy number gains at 18q21 demonstrated by CGH. Thus, it appears that aberrations of the *BCL2* gene are of minor significance in the pathogenesis of PCNSL. This conclusion is further supported by the absence of *BCL2* rearrangements in 11 tumors from our series as well as in all PCNSL analyzed previously (7, 21, 26, 28). However, despite the lack of detectable *BCL2* aberrations at the genomic level, a fraction of PCNSL, including 8 tumors in the present study (see Table 1), shows strong expression of bcl-2 as determined immunohistochemically (7, 11, 28, this study). The molecular mechanisms underlying this finding are presently unclear. There may be point mutations in the coding region (50) or in regulatory elements of the *BCL2* gene. Alternatively, epigenetic events or post-translational changes could also result in enhanced bcl-2-protein expression. In addition to *BCL2*, several other candidate genes are located on 18q21 that could be targets for amplification events in PCNSL. These include *FVT1* (follicular lymphoma variant translocation 1 gene), and an as yet unidentified gene targeted by a t(11;18) (q21;q21) translocation event in some non-Hodgkin lymphomas (29, 39, 45).

We identified one tumor with a high-level amplification of sequences from 9p23-p24. Amplification on distal 9p was previously observed in primary mediastinal B-cell lymphomas (22). Further evidence for a lymphoma-associated proto-oncogene at distal 9p comes from cytogenetic studies, which reported translocation breakpoints at 9p23-p24 in malignant lymphomas (40). Candidate genes mapping to distal 9p include *MLLT3* at 9p22 as well as *JAK2* and *NF1B* at 9p24.

CGH demonstrated losses on the long arm of chromosome 6 in 47% of PCNSL from our series. A region of common deletion was mapped to 6q21-q22. Losses on 6q were described as recurrent alterations in malignant non-Hodgkin lymphomas and leukemias, (2, 5, 18, 32, 46) including extracerebral DLBCL (3, 6, 35, 52). In the latter, the same subregion of common loss, i.e. 6q21-6q22, was identified by CGH (3, 35). These findings are supported by conventional cytogenetic data on high-grade non-Hodgkin lymphomas, which also indicated a region of common deletion at 6q21 (38). Thus, it is likely that a tumor suppressor gene of importance for the development of high-grade lymphomas of the diffuse large B-cell type, including PCNSL, is located at 6q21-q22.

Chromosome arms other than 6q were infrequently affected by losses in PCNSL. Three tumors of our series showed underrepresentations on the short arm of chromosome 17 including 17p13 in all cases. One of these tumors (T17) carried a missense mutation in the *TP53* tumor suppressor gene located at 17p13. No *TP53* mutations were detected in the other two tumors (T11, T13), but one of them (T13) showed pathological accumulation of the p53 protein (7). Taken together, these data indicate a role for *TP53* alterations in a minor fraction of PCNSL.

The *CDKN2A* tumor suppressor gene at 9p21 was shown to be frequently inactivated in PCNSL due to homozygous deletion or 5′-CpG hypermethylation (7, 61). Fourteen tumors from our present series were previously analyzed for *CDKN2A* aberrations, and homozygous gene deletions were identified in 6 tumors (7). However, none of these tumors showed evidence of 9p deletions by CGH analysis. This indicates that the deletions involving the *CDKN2A* gene locus at 9p21 in PCNSL are rather small, i.e., below the resolution limit of CGH, which is around 10 Mbp for low-copy number changes (4, 24).

In order to elucidate the genetic relationship between DLBCLs of different anatomic localizations, we compared the genomic alterations detected in our tumor series with published data from extracerebral DLBCL including nodal and extranodal (35) as well as gastrointestinal DLBCL (3, 6). The available data indicate that losses of genetic information from 6q21-q22 and gains on 1q, 11q and 12q are common in all DLBCL, irrespective of the tumor site. In contrast, gains on chromosomes 3 and 7 are rare in DLBCL of extranodal origin (3, this study), but common in nodal DLBCL (35). Furthermore, gains on 18q were not found as recurrent aberrations in gastrointestinal DLBCL (3), but in DLBCL of other localizations (34, 35, this study). Notably, high-copy number amplifications on 18q were

observed in PCNSL and in extracerebral DLBCL outside the gastrointestinal tract (34, this study). Only in the latter type of tumors, however, *BCL2* was part of the amplicon (34). Nevertheless, DLBCL of different sites cannot easily be distinguished on the basis of their chromosomal imbalances because none of these aberrations is truly specific.

The *BCL10* gene was recently identified by cloning the breakpoint of the translocation t(1;14)(p22;q32) present in a subset of MALT lymphomas (57, 60). Genetic alterations of *BCL10* were also found in other types of B-cell lymphomas, as well as in colorectal carcinomas, mesotheliomas, and germ cell tumors (57). We investigated 16 PCNSL from our series for *BCL10* alterations by SSCP/heteroduplex analysis of all 3 coding exons. This analysis revealed 4 different sequence polymorphisms but no mutations, indicating that BCL10 alterations are of minor significance in the pathogenesis of PCNSL. Interestingly, two of the polymorphisms detected in our series (codon 5: GCA $\rightarrow$ TCA; codon 213: GGA $\rightarrow$ GAA) were originally reported as missense mutations in two MALT lymphomas (60).

The current routine clinical management of neuroradiologically suspected PCNSL consists of stereotactic biopsy for diagnosis, followed by treatment with radiation and/or chemotherapy. This procedure, however, may hamper molecular genetic analyses due to the minute amounts of tumor tissue available. To circumvent this problem, we employed universal DNA-amplification by DOP-PCR prior to CGH analysis. We and others had successfully used this approach for the analysis of small cell samples (3, 27, 47, 49, 54, 58). In this study, application of a modified DOP-PCR protocol enabled us to perform high-quality CGH from stereotactic biopsy samples. Since universally amplified template DNA, as obtained by DOP-PCR, cannot be used for Southern blot analysis, we employed the novel technique of matrix-CGH for further molecular evaluation of imbalanced regions identified by CGH (48). In matrix-CGH, the chromosome targets are substituted by an array consisting of specific DNA fragments which are hybridized according to the same principles as chromosomal CGH. In the present study, matrix-CGH was performed with universally amplified DNA, thus demonstrating the feasibility of this approach for the molecular analysis of small tumor tissue samples as obtained by stereotactic biopsy.

In conclusion, we identified a number of novel chromosomal regions and subregions showing frequent gains or losses in PCNSL, thereby facilitating the identification of yet unknown proto-oncogenes and tumor suppressor genes associated with the development of these tumors. The present study does not support a significant role of *BCL2* and *BCL10* gene alterations in PCNSL. The successful use of chromosomal CGH as well as matrix-CGH for the analysis of stereotactic brain biopsy specimens opens up promising perspectives with respect to scientific and diagnostic applications.

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