RESEARCH ARTICLE

Molecular Characterization of *BCL6* Breakpoints in Primary Diffuse Large B-cell Lymphomas of the Central Nervous System Identifies *GAPD* as Novel Translocation Partner

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Primary central nervous system lymphomas (PCNSL) constitute diffuse large B-cell lymphomas arising in and remaining confined to the brain. Little information is available on cytogenetic changes in PCNSL, and recurrent chromosomal translocations have not yet been identified. Fluorescence in situ hybridization (FISH) of a series of 13 PCNSL from immunocompetent patients revealed 3 cases with signal patterns of a *BCL6*-specific probe suggesting a breakpoint in this oncogene locus in chromosome band 3q27. Here, we describe cloning of the translocation breakpoints by long-distance inverse polymerase chain reaction (LDI-PCR) in 2 of these tumors. Both breakpoints affected the first intron of *BCL6*. In one PCNSL, the *HSPCA* (*HSP90A*) gene in 14q32.31 was identified as *BCL6* partner. In the second lymphoma, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) on 12p13.31 was detected as a hitherto unknown partner of *BCL6*. Our results suggest translocation-mediated *BCL6* oncogene activation as a so far unknown pathogenetically relevant mechanism in PCNSL.

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Introduction

Primary central nervous system lymphomas (PCNSL) are extranodal non Hodgkin's lymphomas (NHL) arising in and remaining confined to the central nervous system (CNS). Morphologically, they are classified as diffuse large B-cell lymphomas (DLBCL) according to the REAL and WHO classifications (7, 8). They are histogenetically derived from germinal center B cells (10, 12) as demonstrated by an extremely high load of somatic mutations in immunoglobulin (*IG*) loci with evidence for ongoing mutation (12, 16). In addition to *IG*, the *BCL6* locus is somatically mutated in these tumors (10).

PCNSL in immunocompromised patients have been associated with Epstein-Barr-Virus (EBV), whereas pathogenetic events involved in the development of PCNSL in immunocompetent individuals are still largely unknown. In contrast to PCNSL, nodal and extranodal DLBCL originating outside the CNS are well known to frequently harbor chromosomal translocations leading to activation of oncogenes involved in B-cell lymphomagenesis. The detection of similar genetic changes in PCNSL has been hampered by the scarcity of viable tumor cells from stereotactically derived minute tissue fragments for conventional chromosome banding analyses. Using interphase fluorescence in situ hybridization (FISH) on a series of 13 PCNSL from immunocompetent patients, we have recently demonstrated recurrent chromosomal breakpoints in immunoglobulin loci as well as in the *BCL6* gene in chromosome band 3q27 in PCNSL (13). Interphase FISH suggested breakpoints within the *IGH* and *IGK* locus in 5 and 1 of the 13 PCNSL, respectively. Signal constellations indicating breakpoints in the *BCL6* loci were observed in 3 of the 13 cases. Here, we describe cloning of the *BCL6* breakpoints in 2 of the 3 latter tumors by means of long-distance inverse polymerase chain reaction (LDI-PCR) for the major translocation cluster (MTC). Our results molecularly prove the presence of *BCL6* translocations in PCNSL and, thus, suggest *BCL6* oncogene activation as a pathogenetic mechanism in this unusual DLBCL entity occurring in an immune-privileged organ. Moreover, the *GAPD* gene was identified as a hitherto unknown translocation partner of *BCL6*.

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Material and Methods

Tumor samples. Clinical, morphological, immunohistochemical and interphase cytogenetic features of the three PCNSL from HIV-negative patients analysed have been described in detail previously (cases 2, 8 and 13; for reference, see 13). In brief, PCNSL #2 was a right frontal lymphoma from a 61-year-old male, PCNSL #8 a multifocal lymphoma from a 78-year-old female, and PCNSL #13 a left parieto-occipital lymphoma from a 74-year-old female. There was no evidence for extracerebral lymphoma manifestation in any of these patients. Neuropathologically, all tumors were classified as CD20+ DLBCL with predominance of centroblasts (7, 8) and a proliferative activity as determined by MIB-1 positivity of at least 50%. Interphase FISH with the LSI *BCL6* break-apart probe (Vysis, Downers Grove, Ill) revealed signal constellations indicating a breakpoint in the *BCL6* locus in 54%, 48%, and 66% of nuclei from PCNSL #2, #8 and #13, respectively, with an atypical signal pattern in the latter case.

DNA extraction and LDI-PCR. DNA was extracted from frozen tissue as described previously (12). DNA was dissolved in 40 μl of TE low buffer (10 mM Tris, 1 mM EDTA, pH 7.6). LDI-PCR to amplify translocation junctions affecting the major translocation cluster of *BCL6* was applied as previously described with minor modification (1). Briefly, 100 to 500 ng of genomic DNA was digested with *Bam*HI or *Xba*I and purified. The DNA was diluted to a concentration of 1 μg/ml and incubated in the presence of *T4* DNA ligase to facilitate intramolecular ligation at 4°C overnight. The self-ligated circular DNA was used as a template for a nested PCR (3). Aliquots of the PCR products were analyzed by agarose gel electrophoresis and visualized under UV illumination after ethidium bromide staining. Reproducibility of non-germline bands was demonstrated by a second independent nested PCR using the same self-ligated circular DNA template.

Molecular cloning and nucleotide sequencing. All PCR products, which showed a non-germline band in the gel electrophoresis, were purified by gel extraction (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). Nucleotide sequencing of the PCR products was performed with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif), and the sequencing reactions were resolved on an ABI 377 automated sequencer (Applied Biosystems). Sequence comparisons were performed using the BLAST program (*http//www.ncbi.nlm.nih.gov/blast/*) and the UCSC Genome Browser (Human June 2002 Freeze at *http://genome.ucsc.edu/*).

Breakpoint spanning PCR. To prove the specificity of the LDI-PCR fragments, direct PCR crossing the breakpoint was performed on genomic tumor DNA using primers PCNSL2_chr3F AGGGG GCGGC CGGAG CAGAG A and PCNSL2_chr14R CCAAC ACCGA ACTGG CCAAT C in PCNSL #2 and primers PCNSL8_chr3F CCGTG ATCTT CCTAA TGAGA G and PCNSL8_chr12R TGCGG TGGGA GATCT GGTTT C in PCNSL #8. The resulting PCR products of 766bp and 748bp, respectively, were purified and sequenced.

Results and Discussion

Interphase FISH analysis of 13 cryopreserved PCNSL samples obtained by stereotactic biopsy enabled us to demonstrate for the first time that breakpoints in the *BCL6* locus do occur recurrently in PCNSL of immunocompetent patients (13). In order to prove involvement of this oncogene locus in the pathogenesis of PCNSL, we decided to clone the *BCL6* breakpoints in all PCNSL with FISH signal patterns indicating structural chromosomal changes of the *BCL6* locus in chromosome band 3q27. By means of an LDI-PCR approach for the major translocation cluster (MTC) of *BCL6*, we were able to amplify specific junctional fragments in 2 of the 3 PCNSL. In PCNSL #13, which shows a strong expression of the BCL6 protein as evidenced by immunohistochemistry, we failed to amplify a non-germline product although reliable amplification of up to 11 kb *BCL6* germline fragments showed a DNA quality sufficient for LDI-PCR. Nevertheless, it cannot be ruled out that somatic mutations prevented proper binding of the primers on the affected chromosome in this case. Moreover, a complex FISH pattern was seen in PCNSL #13, which may hint to a breakpoint outside the MTC. In this regard it is remarkable that a recent Southern blot study using a probe for the 5′ major cluster region (alternatively called MTC) failed to detect *BCL6* breakpoints in any of nine PCNSL (10). Thus, some *BCL6* breakpoints in PCNSL may be located in other parts of the *BCL6* locus such as the alternative breakpoint region (ABR) mapping 200 to 270 kb telomeric to the MTC (6).

In cases PCNSL #2 and #8 specific junctional sequences were reproducibly amplified from the tumor DNA by LDI-PCR and confirmed by direct breakpoint-spanning PCR. Sequence analysis revealed the break-

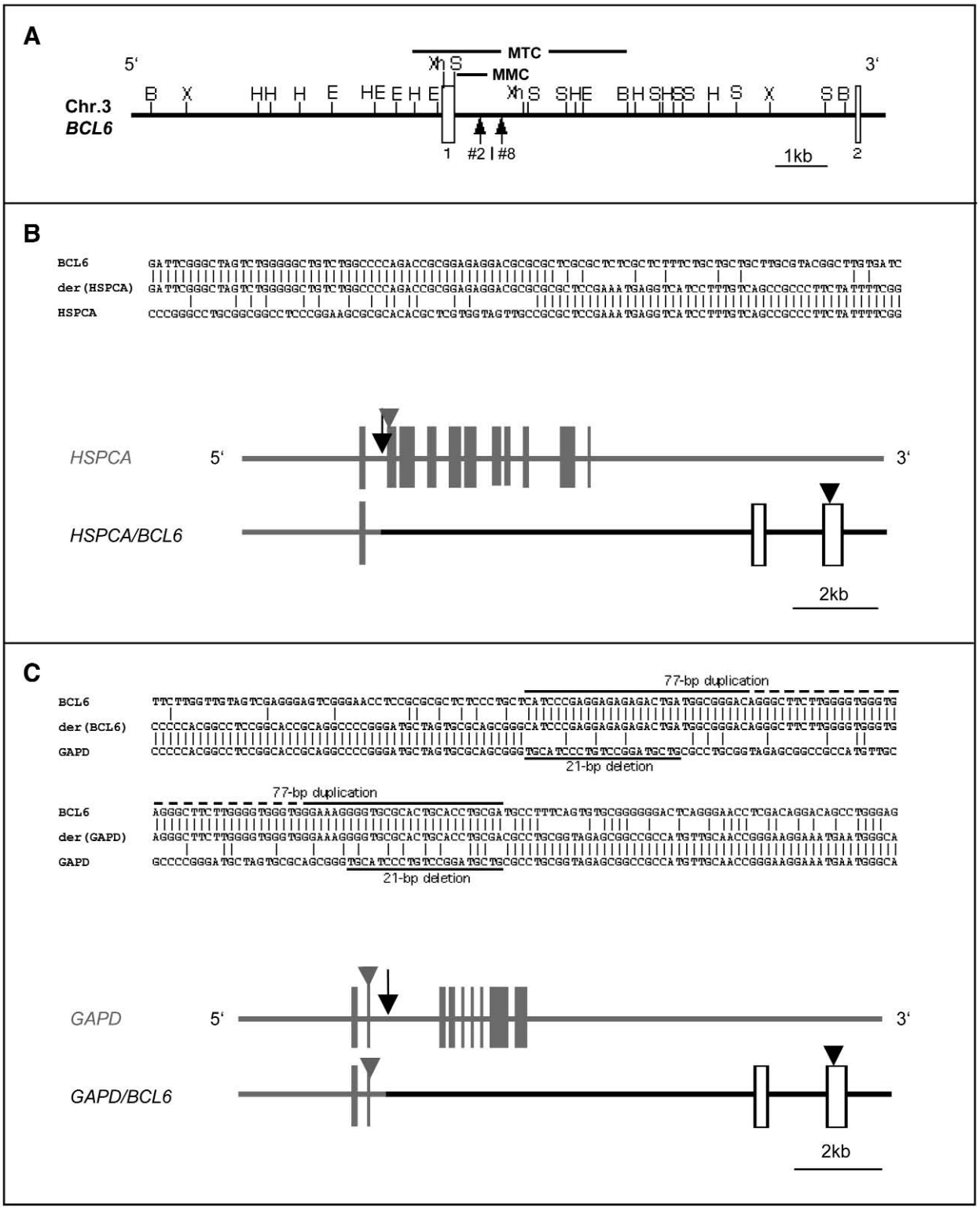

Figure 1. Schematic representation of the *BCL6*-breakpoints cloned from PCNSL. **A.** Organization of the normal *BCL6* locus. **B.** Cloned breakpoints of PCNSL #2 demonstrating translocation breakpoints in the first introns of *BCL6* and *HSPCA*. **C.** Cloned breakpoints of PCNSL #8 shows a 77-bp duplication of *BCL6* sequences in association with a 21-bp deletion. Arrows indicate breakpoints of each translocation. Boxes indicate the exons, and arrowheads indicate the translation initiation sites of each gene. MMC, major initiation cluster.

points in both PCNSL to be located within the MTC in the first intron of the *BCL6* gene (Figure 1A-C). A 77-bp duplication of *BCL6* sequences was observed at the breakpoint in case PCNSL #8 along with a 21-bp deletion. Duplications and deletions have been shown to be one mechanism of somatic hypermutation and to mediate chromosomal translocations of the *IGH* locus (Figure 1C). The presence of these genetic changes at the junctional sequence of PCNSL #8 may, thus, suggest that this non-*IGH* translocation also resulted from a misdirected somatic hypermutation.

In case PCNSL #2, LDI-PCR amplified a junction on the derivative partner chromosome, which was identified as chromosome band 14q32.31 by sequence analysis. The breakpoint targeted the first intron of the gene for heat-shock 90-kD protein alpha, *HSPCA*, which gives rise to 2 different mRNA transcripts encoding 2 members of the HSP90 family, HSP89-alpha and HSP89-beta. *BCL6*-translocations affecting the very same gene (previously called *HSP89A*) have so far been described in 2 primary gastric high-grade B-cell lymphomas (1, 2, 18). In PCNSL #2 as well as in the published cases, the translocation breakpoints occurred in the first introns of *BCL6* and *HSPCA* resulting in an exchange of the first non-coding exons of both genes (Figure 1B).

In PCNSL #8 both junctions on the der(3) and the derivative partner chromosome could be amplified by LDI-PCR. Remarkably, the *GAPD* gene encoding glyceraldehyde-3-phosphate dehydrogenase was affected by the translocation. It has, so far, not been reported as *BCL6* partner gene. *GAPD* is located in chromosomal band 12p13.31. The breakpoint in PCNSL #8 occurred in the second intron of *GAPD* and leads to juxtaposition of the complete coding region of *BCL6* next to the first 2 exons of *GAPD*. *GAPD* is considered a ubiquitously expressed housekeeping gene in mammalian cells as its product catalyzes an important energy-yielding step in the carbohydrate metabolism. Similar to other non-*IG* translocations involving *BCL6*, the *GAPD*/*BCL6* translocation leads to juxtaposition of both genes in the same transcriptional orientation and fusion of the complete set of the *GAPD* promotor upstream of the coding region of *BCL6* on the der(3) chromosome. *BCL6* promotor substitution is a well-known mechanism resulting in deregulated expression of the BCL6 protein in lymphomas. Recent work suggests that this deregulation may be caused by the loss of BCL6-protein binding sequences from the der(3) chromosome, which are located in the first non-coding exon of *BCL6*. This leads to disruption of the autoregulatory loop of BCL6 and its increased expression from the allele affected by the translocation (4, 11). In this regard it is of clinical interest to note that BCL6 expression in PCNSL was associated with longer overall survival and, thus, may serve as a prognostic marker (5). Unfortunately, immunohistochemistry for determination of potential translocation-mediated BCL6 protein overexpression could neither be performed in PCNSL #8 nor in PCNSL #2 due to the scarcity of material. Follow-up in our patients disclosed that patient with PCNSL #2 had a favorable clinical course with complete remission and is still alive two years after treatment, whereas therapy in patient with PCNSL #8 failed leading to death due to lymphoma progression. Nevertheless, it has repeatedly been shown in systemic lymphomas that the relation between genomic *BCL6* changes and BCL6 expression levels is poor. Chromosomal translocations affecting the *BCL6* gene do not regularly result in overall increased expression of the BCL6 transcript or protein which may be explained by the *BCL6*-mediated transcriptional suppression of the intact *BCL6* allele (15, 17).

In conclusion, the molecular characterization of chromosomal breakpoints affecting the *BCL6* locus presented here sheds new light on the pathogenesis of PCNSL in immunocompetent patients and unravels *BCL6* promotor substitution as a recurrent genetic aberration in these DLBCL confined to an immuno-privileged organ. With respect to the frequency of *BCL6* aberrations, PCNSL may resemble systemic DLBCL, in which translocations involving the *BCL6* gene locus have been described in 30 to 40%, and moreover, have been reported to be associated with extranodal involvement (9, 14). Nevertheless, the finding that both PCNSL characterized here carried *BCL6* fusions, which in systemic DLBCL have either not or only extremely rarely been reported, may be suggestive for distinct pathogenetic mechanisms for the development of primary extranodal lymphomas in the CNS.

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