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RESEARCH ARTICLE

Frequent Inactivation of *CDKN2A* and Rare Mutation of *TP53* in PCNSL

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Twenty primary central nervous system lymphomas (PCNSL) from immunocompetent patients (nineteen B-cell lymphomas and one T-cell lymphoma) were investigated for genetic alterations and/or expression of the genes *BCL2, CCND1, CDK4, CDKN1A, CDKN2A, MDM2, MYC, RB1, REL,* and *TP53*. The gene found to be altered most frequently was *CDKN2A*. Eight tumors (40%) showed homozygous and two tumors (10%) hemizygous *CDKN2A* deletions. Furthermore, methylation analysis of six PCNSL without homozygous *CDKN2A* loss revealed methylation of the CpG island within exon 1 of *CDKN2A* in three instances. Reverse transcription PCR analysis of *CDKN2A* mRNA expression was performed for 11 tumors and showed either no or weak signals. Similarly, immunocytochemistry for the *CDKN2A* gene product (p16) remained either completely negative or showed expression restricted to single tumor cells. None of the PCNSL showed amplification of *CDK4.* Similarly, investigation of *CCND1* revealed no amplification, rearrangement or overexpression. The retinoblastoma protein was strongly expressed in all tumors. Only one PCNSL showed a mutation of the *TP53* gene, i.e., a missense mutation at codon 248 (CGG to TGG: Arg to Trp). No evidence of *BCL2* gene rearrangement was found in 11 tumors investigated. The bcl-2 protein, however, was strongly expressed in most tumors. None of the 20 PCNSL demonstrated gene amplification of *MDM2, MYC or REL*. In summary, inactivation of *CDKN2A* by either homozygous deletion or DNA methylation represents an important molecular mechanism in PCNSL. Mutation of the *TP53* gene and alterations of the other genes investigated appear to be of minor significance in these tumors.

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Introduction

Primary central nervous system lymphomas (PCNSL) are rare tumors estimated to account for less than 2% of all primary brain tumors and 1 - 2% of malignant lymphomas from all sites (21, 34, 50). PCNSL may develop either in immunocompetent patients or in immunosuppressed patients suffering from congenital immunodeficiency disorders (e.g. Wiskott-Aldrich syndrome) or acquired conditions of immunosuppression such as the acquired immunodeficiency syndrome (AIDS), immunosuppression for organ transplantation, and various autoimmune diseases (21). Several studies have reported a significant increase in the incidence of PCNSL during recent years in both the immunocompetent and immunosuppressed populations (13, 14, 54). Morphologically, the vast majority of PCNSL are high-grade diffuse B-cell non-Hodgkin's lymphomas. Primary T-cell lymphomas of the central nervous system are exceedingly rare (21, 34, 50).

In immunocompromised patients, PCNSL are associated with Epstein-Barr virus (EBV) infection. EBV may activate proto-oncogenes or inactivate tumor suppressor

genes through a variety of mechanisms (27, 37, 50). In contrast, only a minority (< 20%) of PCNSL from immunocompetent patients are associated with EBV infection. Furthermore, molecular genetic studies of PCNSL from immunocompetent patients have revealed few consistent abnormalities (34, 50). Thus, the pathogenesis and molecular genetic basis of PCNSL from immunocompetent patients are essentially unknown.

In the present study, we have therefore investigated a series of twenty PCNSL from immunocompetent patients for genetic alterations and/or expression of several genes that have been previously shown to be genetically altered and/or aberrantly expressed in subsets of non-Hodgkin's lymphomas outside the central nervous system. Here we report that PCNSL frequently have inactivated the tumor suppressor gene *CDKN2A* (also known as *MTS1* or $p16^{INK4A}$) either by homozygous deletion or transcriptional silencing due to DNA methylation. Mutation of the *TP53* tumor suppressor gene was found in only one PCNSL. Neither genomic rearrangements of the *CCND1* (*BCL1*) and *BCL2* genes nor amplification of the proto-oncogenes *CCND1*, *MDM2*, *CDK4*, *MYC*, *and REL* were detected in any of the PCNSL investigated.

Materials and Methods

Tumor Material. Twenty primary malignant non-Hodgkin's lymphomas of the central nervous system were selected from the files of the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf. The tumors were from twenty adult immunocompetent patients operated between 1981 and 1995 at the Department of Neurosurgery of the Heinrich-Heine-University or the Neurosurgical Clinic of the Evangelische-und-Johanniter-Krankenanstalten in Duisburg-Nord/Oberhausen. All cases were primary tumors treated by neurosurgical tumor resection (stereotactic biopsy specimens were deliberately excluded from the study because of the limited tumor material). None of the patients had received irradiation or chemotherapy prior to the operation. In 11 cases, parts of the tumor tissue had been frozen immediately after operation and stored at - 80°C. From the remaining 9 tumors no frozen tumor tissue was available and DNA was extracted from formalin-fixed paraffin blocks. To assure that the tumor pieces (either frozen or paraffin tissue) taken for molecular genetic analysis contained a sufficient proportion of tumor cells, histological evaluation of each of these pieces was performed.

Immunocytochemistry. All tumors were immunocytochemically evaluated on formalin-fixed paraffin sections for the expression of leucocyte common antigen (LCA), B- and T-cell specific antigens, proliferation-associated nuclear antigen Ki-67, p53 tumor suppressor protein, mdm2 oncoprotein, and the Epstein-Barr virus (EBV) associated antigens LMP (latent membrane protein) and ZEBRA (Bam HI Z fragment, Epstein-Barr replication activator). Eighteen tumors were additionally stained for p16 (*CDKN2A* gene product), cyclin D1 (bcl-1), pRB1 (retinoblastoma gene product), p21 (*CDKN1A* gene prduct), and bcl-2. For detection, we used the indirect avidin-biotin peroxidase method for monoclonal mouse antibodies and the peroxidase-anti-peroxidase method for polyclonal rabbit antibodies as described (58). To enhance immunoreactivity for Ki-67, p53, p16, p21, mdm2, cyclin D1, bcl-2, and ZEBRA, sections were pretreated by microwave heating in 10 mM citrate buffer pH 6.0 for 3 x 10 minutes. The following mouse monoclonal antibodies were used as primary antibodies: LCA (CD45), clones PD7/26 and 2B11 (Dako, Copenhagen, Denmark); T-cells (CD45RO), clone UCHL1 (Dako); B-cells (CD45RA), clone 4KB5 (Dako); Ki-67, clone MIB-1 (Dianova, Hamburg, Germany); p53, clone DO-7 (Dako); p16, clone JC8 (9); p21, clone EA10 (Dianova); mdm2, clone IF2 (Dianova); cyclin D1, clone DCS-6 (Medac, Hamburg, Germany); bcl-2, clone 124 (Dako); pRB1, clone G3-245 (Pharmingen); p21, clone EA10 (Dianova); EBV-LMP, clone CS1-4 (Dako); EBV-ZEBRA, clone BZ.1 (Dako). All monoclonal antibodies were used at final concentrations of 1 - 2 μg/ml for an incubation period of 16 hours at room temperature. As additional T-cell marker we used a rabbit polyclonal anti-CD3 antiserum (Dako) at a dilution of 1 : 50 after pretreatment of the sections with pronase E (0.1% for 15 min at 37°C). Negative controls were performed by omission of the primary antibody and its substitution with either an irrelevant mouse monoclonal IgG antibody or a rabbit polyclonal antibody. Expression of Ki-67, p53, pRB1, p21, and mdm2 was quantified by counting positive and negative tumor cells in 5 representative microscopical fields at high-power magnification (400x). The results were calculated as percentage of positive cells (Table 1).

DNA and RNA Extraction, Single Strand Conformational Polymorphism (SSCP) Analysis, and DNA Sequencing. The preparation of high molecular weight DNA and RNA from frozen tumor tissue and cell lines was carried out by ultracentrifugation as described (32). The method used for DNA extraction from formalin-

fixed and paraffin-embedded tumor tissue has also been reported previously (60).

PCR amplification of exon 5, 6, 7, and 8 of the *TP53* gene, SSCP analysis, and DNA sequencing was done as described (60). Exons 1, 2, and 3 of *CDKN2A* were amplified using primers and conditions as reported (63). SSCP analysis of the PCR products from CDKN2A exons 1 - 3 was performed as described (60). The PCR product obtained from exon 1 was cut with *Sma* I prior to SSCP analysis.

Fluorescent Differential PCR. The gene dosages of *MDM2, CDK4, MYC, RET, and CDKN2A* were analyzed by differential PCR with fluorescein-labeled primers (3, 51). Three different reference loci were used: *IFNG* (12q15), *GAPDH* (12p), and a sequence tagged site (STS) on 9q. Primer sequences for the target genes and the control loci were as follows (primers prefixed by 5'-F- carried a 5'-fluorescein label): 5'-F-AACGTGTCAGTGGTGGACCTG-3' and 5'-AGTGGGTGTCGCTGTTGAAGT-3' for *GAPDH* generating a 160-bp PCR product, 5'-F-CATGTAGACCAGGACCTAAGG-3' and 5'-AACTGGCGCATCAGATCCTAG-3' for *CDK4* generating a 205-bp PCR product, 5'-F-CTCGGAAGGACTATCCTGCTGCCAA-3' and 5'-GGCGCTCCAAGACGTTGTGTGTTCG-3' for *MYC* generating a 150-bp PCR product, 5'-F-GAACGATTGGGAAGCAAAAGGC-3' and 5'-CTGTTACGGGTTCTGTGATAGC-3' for *REL* generating a 109-bp PCR product, and 5'-F-CCAACGCACCGAATAGTTACGG-3' and 5'-AAACTTCGTCCTCCAGAGT-CGC-3') generating a 97-bp fragment from exon 1 of *CDKN2A.* Primer sequences for *IFNG* were taken from Neubauer *et al*. (51), for *9qSTS* from Ueki *et al*. (75), and for *MDM2* from Hunter *et al*. (30).

The various primer pairs were used to differentially amplify target and reference genes in the following combinations: *MDM2* and *GAPDH, MDM2* and *IFNG, CDK4* and *GAPDH, MYC* and *IFNG, REL* and *IFNG, CDKN2A* and *9qSTS*. PCR was performed with 100 ng of genomic DNA as template. An initial denaturation of 4 min at 94°C was followed by 25 cycles of 1 min at 94 oC, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C. For differential amplification of *CDKN2A* and *9qSTS* 5% formamide was added to the reaction mix and the annealing temperature was increased to 58°C.

Controls included DNA from the glioblastoma cell line TP365MG, previously shown to have amplification of *MDM2* and *CDK4* (25), the colon carcinoma cell line COLO320DM, known to have amplified *MYC* (65), and the glioblastoma cell line U118MG, which has homozygously deleted *CDKN2A* (63). Peripheral leukocyte DNA was used as reference template with normal gene copy number.

The fluorescein-labeled PCR products were analysed with an automated fluorescent DNA sequencer (A.L.F., Pharmacia) on 6% denaturing polyacrylamide gels. Quantitative analysis of the peak areas obtained for each target gene and the respective reference locus was performed with the Fragment Manager (FM1.1) software (Pharmacia) and target gene dose was calculated relative to control blood as described (3, 60). Only increases in the target gene/reference locus quotients of more than 3-times relative to control blood were considered as evidence of gene amplification. *CDKN2A/9qSTS* quotients of less than 0.3 relative to control blood were considered as homozygous deletion, while quotients between 0.3 and 0.7 were regarded as hemizygous deletion.

PCR-based methylation analysis of CDKN2A. The methylation status of the 5' CpG island of *CDKN2A* was analysed for six tumors without homozygous *CDKN2A* loss (only cases from which high molecular weight DNA was available were analyzed). A PCR-based methylation assay was used as described (63). In brief, genomic DNA from T4, T5, T6, T9, T10, T12, non-neoplastic brain tissue (cortex and white matter from the temporal lobe of a patient operated for epilepsy), and peripheral leukocytes was digested with methylation-sensitive restriction enzymes (*Sma* I, *Hpa* II, *Ksp* I), followed by ethanol precipitation. The genomic segment of *CDKN2A* containing the CpG island (exon 1) was then amplified with primers 2F and 1108R (36) using either digested DNA or, as control, non-digested DNA as templates. As control for template recovery after ethanol precipitation, a genomic fragment of *REL* containing no *Sma* I, *Hpa* II, and *Ksp* I restriction sites was PCR amplified using the primers described above.

Analysis of CDKN2A mRNA Expression by Reverse Transcription PCR. For 11 PCNSL, the expression of *CDKN2A* was investigated at the mRNA level by reverse transcription PCR using primers and conditions as described (63). For the assessment of the quantity and quality of the cDNA used as template in the PCR reactions, β-actin cDNA was amplified as control. PCR products were separated on 1% agarose gels and the ethidium bromide stained bands were recorded by the Gel-Doc 1000 system (Bio-Rad Laboratories). Control samples included cDNA from a histopathologically normal axillary lymph node, cDNA from non-neoplastic

Case No.	Type	Sex	Age (years)	Localization	Survival (days)	Ki-67 (%)	CDKN2A copy number	CDKN2A 5' CpG methylat.	CDKN2A mRNA	p16 protein (%)	CCND1 amp	CCND1 rearrang.	cyclin D1 protein (%)
T1	B-cell	f	72	occipital left	>258	80	0	n.d.	+	0	-	-	0
T2	B-cell	m	50	pineal region	17	79	2	n.d.	n.d.	0	n.d.	n.d.	0
T3	T-cell	m	50	parietal left	64	76	0	n.d.	n.d.	0	n.d.	n.d.	<1
T4	B-cell	f	75	cerebellar hemisphere left	n.d.	64	2	-	+	0	-	-	<1
T5	B-cell	f	60	bifrontal	>118	63	2	+	-	n.d.	n.d.	-	n.d.
T6	B-cell	f	47	temporal right	>1915	44	2	+	-	<1	-	-	0
T7	B-cell	f	57	fronto-temporo-parietal right*	5	78	0	n.d.	+	<1	-	-	<1
T8	B-cell	f	73	parietal right*	n.d.	25	0	n.d.	-	0	-	-	0
T9#	B-cell	f	75	frontal right	579	35	1	-	-	n.d.	n.d.	-	n.d.
T10	B-cell	f	50	cerebellum*	n.d.	48	2	+	-	0	-	-	0
T11	B-cell	f	62	frontal right	>800	57	0	n.d.	+	0	-	-	<1
T12#	B-cell	m	49	frontal left	266	21	2	-	-	0	-	-	0
T13	B-cell	f	64	frontal left*	78	22	0	n.d.	-	0	n.d.	-	0
T14	B-cell	f	61	bifrontal	6	92	0	n.d.	n.d.	0	n.d.	n.d.	0
T15	B-cell	f	74	frontal left	120	59	2	n.d.	n.d.	<1	n.d.	n.d.	<1
T16	B-cell	m	65	frontal right*	67	21	0	n.d.	n.d.	0	n.d.	n.d.	0
T17	B-cell	m	54	frontal right*	n.d.	51	2	n.d.	n.d.	<1	n.d.	n.d.	0
T18	B-cell	m	48	basal ganglia right*	3256	87	2	n.d.	n.d.	<1	n.d.	n.d.	0
T19	B-cell	f	58	temporal right	623	30	1	n.d.	n.d.	0	n.d.	n.d.	0
T20	B-cell	m	62	temporo-occipital left	43	34	2	n.d.	n.d.	0	n.d.	n.d.	0

Case No.	CDK4 amp	pRB1 protein (%)	TP53 mutation	p53) protein (%)	MDM2 amp	mdm2 protein (%)	p21 protein (%)	BCL2 rearrang.	bcl-2 protein	MYC amp	REL amp
T1	-	60	-	0	-	0	<1	-	+++	-	-
T2	-	67	-	15	-	0	<1	n.d.	+++	-	-
T3	-	84	-	8	-	2.5	57	n.d.	++	-	-
T4	-	82	-	<1	-	0	2	-	+++	-	-
T5	-	n.d.	-	0	-	0	n.d.	-	n.d.	-	-
T6	-	85	-	5	-	0	13	-	+++	-	-
T7	-	76	-	20	-	16	10	-	+++	-	-
T8	-	58	-	8	-	<1	2	-	++	-	-
T9#	-	n.d.	-	<1	-	0	n.d.	-	n.d.	-	-
T10	-	50	-	<1	-	<1	<1	-	+	-	-
T11	-	59	-	20	-	0	<1	-	+++	-	-
T12#	-	77	-	<1	-	0	<1	-	++	-	-
T13	-	58	-	3	-	<1	2	-	++	-	-
T14	-	87	-	1	-	<1	2	n.d.	+++	-	-
T15	-	87	-	1	-	<1	3	n.d.	+++	-	-
T16	-	62	-	0	-	0	<1	n.d.	++	-	-
T17	-	69	Codon 248 CGG - TGG (Arg - Trp)	32	-	0	<1	n.d.	++	-	-
T18	-	57	-	5	-	<1	2	n.d.	+	-	-
T19	-	65	-	<1	-	0	2	n.d.	++	-	-
T20	-	78	-	2	-	0	2	n.d.	++	-	-

Table 1. Summary of patient data as well as histopathological, immunocytochemical and molecular genetic findings in the 20 PCNSL investigated. Abbreviation used: f, female; m, male; amp, amplification; rearrang, rearrangement; n.d., not determined. #Low-grade tumors. *Cases with multifocal intracerebral lesions; only the location of the mass operated on is given. the CDKN2A copy numbers indicate: 2, retention of both alleles; 1, loss of one allele; 0, loss of both alleles. Staining intensity for bcl-2 was semiquantitatively graded: +++, strong expression (>50% positive tumor cells); ++, moderate expression (10-50% positive tumor cells); +, weak expression (<10% positive tumor cells).

human brain, cDNAs from the glioblastoma cell lines U118MG and TP365MG, and cDNA from selected primary gliomas (OA1, AO11) without loss of *CDKN2A* or amplification of *CDK4*.

PCR-based Analysis for the Presence of EBV sequences. The 11 tumors from which high-molecular weight DNA was available (T1, T4 - T13) were investigated by PCR for the presence of EBV sequences using primers for the BamHI W repeat region of EBV as described by Miyashita *et al*. (49). PCR was performed for 35 cycles (60°C annealing temperature, 2.5% formamide) with 200 ng of genomic DNA as template. As positive control, PCR was performed under the same conditions with 10 ng DNA from an EBV-transformed lymphoblastoid cell line as template.

PCR-based Analysis of CCND1 (BCL1) and BCL2 Gene Rearrangements. Tumors T1 and T4 - T13 were investigated by PCR-based assays for *CCND1* and *BCL2* rearrangements. Rearrangement of *CCND1* was analyzed with primers MCL-1 (for the major translocation cluster of *CCND1*) and JH (for the IgH chain joining region) as described by Segal *et al*. (66). As positive controls, we used DNA from two mantle cell lymphomas shown to carry t(11;14) translocations by interphase cytogenetics. For the analysis of *BCL2* rearrangements, PCR was performed with primers MBR (for the major breakpoint region of *BCL2*) and JH as described by Liu *et al*. (45). DNA from a follicular lymphoma with a known t(14;18) translocation served as positive control.

Southern Blot Analysis. From eight tumors sufficient amounts of high molecular weight DNA could be extracted from frozen tumor tissue to perform Southern blot analyses. For Southern blotting, 2.5μg DNA of each tumor and of normal leukocyte DNA were digested with a restriction enzyme (*Taq* I or *Hind* III), electrophoresed on 0.8% agarose gels, and alkali blotted to Hybond-N^+-membranes (Amersham-Buchler, Braunschweig, Germany). The membranes were sequentially hybridized with [^{32}P]dCTP labeled probes for *MDM2*, *CDK4*, *CDKN2A*, *CCND1*, and, as control locus, *CCNA*. The probes for *MDM2*, *CDK4*, *CCND1*, and for exon 1 and exon 2 of *CDKN2A* have been published previously (59, 64). The probe for *CCNA* was a 528 bp fragment corresponding to nucleotides 352 - 879 of the *CCNA* cDNA sequence (EMBL accession No. X51688). This probe was generated by reverse transcription PCR from non-neoplastic brain cDNA and cloned into the TA-cloning vector pCR^{II} (Invitrogen). Quantitative densitometric evaluation of the target gene dose relative to control (leukocyte) DNA was performed with the Molecular Analyst™ version 2.1 software after scanning the autoradiograms with a GS-700 imaging densitometer (Bio-Rad Laboratories).

Results

Clinical and Morphological Findings. Table 1 summarizes selected clinical data of the 20 patients. Seven patients were male and 13 female. The mean age at operation was 60 years (range: 47 - 75 years). All patients were treated by operation. Twelve patients received post-operative radiotherapy. Four patients were treated by adjuvant chemotherapy. Two patients had cerebellar tumors while all other patients had supratentorial neoplasms. Evidence of multifocal brain lesions was revealed by computerized tomography and/or magnetic resonance imaging in seven patients. None of the 20 patients showed lymphoma manifestations outside the central nervous system at the time of operation. Furthermore, for 16 patients the follow-up data showed no evidence of systemic spread during the post-operative course of the disease. Complete follow-up was available from 12 patients and revealed a mean post-operative survival time of 427 days. For 3 patients, a postoperative survival of more than 2 years could be documented (Table 1).

Histopathologically, all tumors were subclassified according to both the revised Kiel classification of non-Hodgkin's lymphomas (44) and the revised European-American Lymphoma (REAL) classification (23). According to the Kiel classification, two of the nineteen B-cell tumors (T9, T12) were centroblastic-centrocytic non-Hodgkin's lymphomas of the diffuse subtype. The remaining 17 B-cell lymphomas were all high-grade tumors corresponding to: centroblastic, monomorphic subtype (5 tumors); centroblastic, polymorphic subtype (7 tumors) (Fig. 1a); centroblastic, multilobulated subtype (1 tumor); immunoblastic (3 tumors); Burkitt-like with plasmablastic differentiation (1 tumor). According to REAL classification, all the 17 high-grade tumors corresponded to the diffuse large B-cell lymphoma type. One exceptional tumor (T3) was diagnosed as a high-grade malignant T-cell lymphoma (Fig. 1h-l).

Immunocytochemical Findings. All tumors strongly expressed leukocyte common antigen. Nineteen tumors were additionally positive for the B-cell associated antigen CD45RA (Table 1; Fig. 1b). One tumor (T3) was

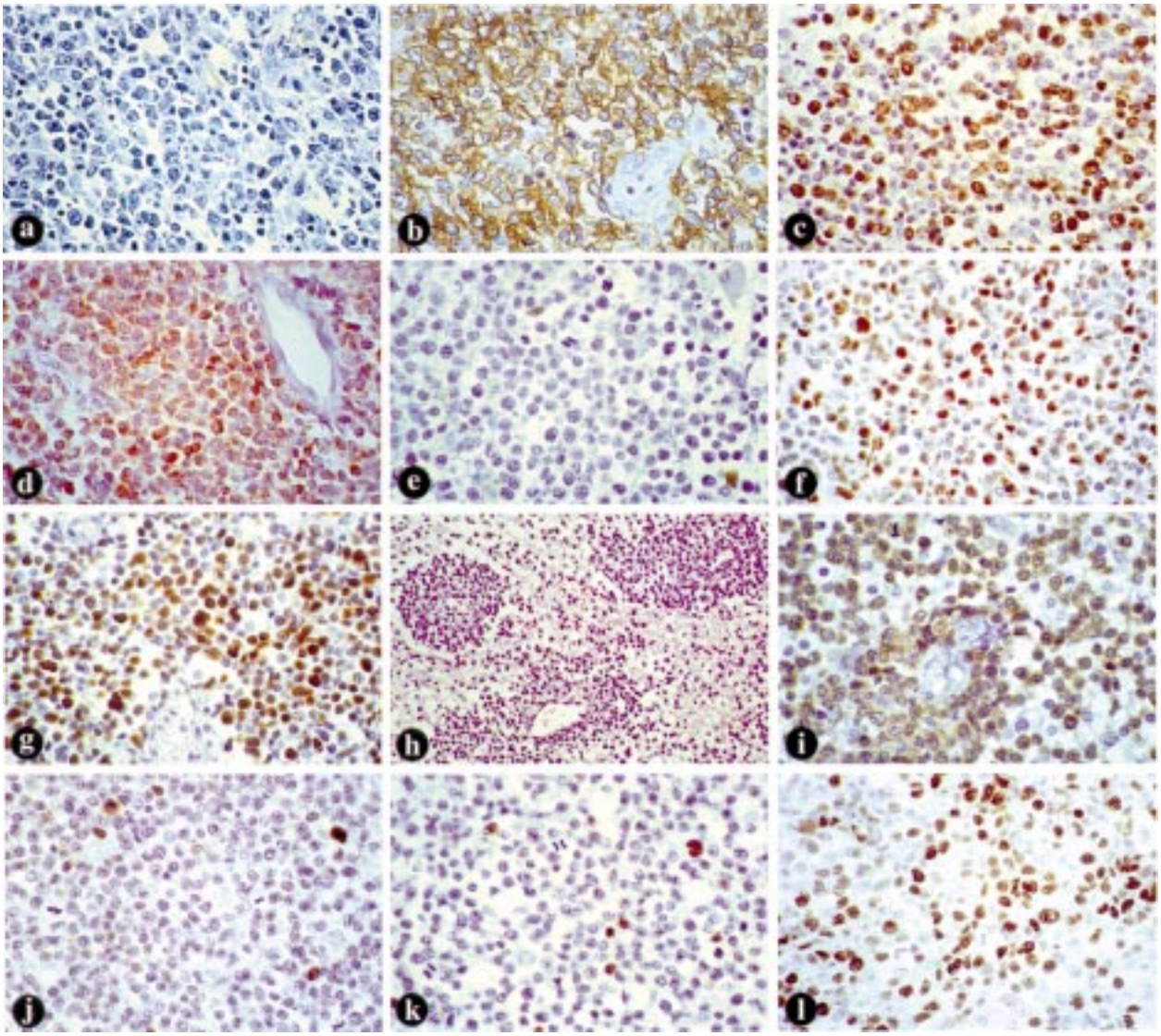

Figure 1. Histopathology and immuncytochemical results of selected PCNSL. **a-f.** High-grade B-cell PCNSL (T6). **a.** Giemsa stain shows a cellular tumor of large cells, many of which resemble centroblasts. **b.** The tumor cells strongly express the B-cell associated antigen CD45RA. **c.** Staining for Ki-67 (MIB1) reveals a high labelling index. **d.** Strong expression of bcl-2 is seen in the tumor cells. **e.** Immunoreactivity for p16 is restricted to single cells. **f.** The majority of tumor cells shows nuclear expression of pRb1. **g.** High-grade B-cell PCNSL (T17). Demonstration of nuclear accumulation of p53 in most tumor cells. T17 carried a TP53 point mutation at codon 248 (see Fig. 5). **h-l.** High-grade T-cell PCNSL (T3). **h.** H&E stain shows the typical angiocentric growth pattern of PCNSL. **i.** The tumor cells of T3 strongly expressed the T-cell associated antigen CD3. **j.** Only a small fraction (< 10%) of tumor cells showed nuclear immunoreactivity for p53. **k.** Similarly mdm2 immunoreactivity is confined to a few tumor cell nuclei. **l.** T3 was the only PCNSL investigated that demonstrated nuclear immunoreactivity for p21 in the majority of tumor cells. Microscopical magnifications are: 200x for a-g and i-l, 80x for h.

negative for CD45RA but strongly positive for CD3 (Fig. 1i). None of the tumors was immunopositive for the EBV-associated antigens LMP and ZEBRA. This immunocytochemical result was confirmed by PCR for 11 tumors, none of which carried EBV sequences at detectable levels (data not shown). In most tumors, the Ki-67 antigen was strongly expressed (Table 1; Fig. 1c). The mean Ki-67 labeling index for all 20 tumors was 55% (range: 21%-92%). Nuclear immunoreactivity for p53 in more than 10% of the tumor cells was found in 4 tumors (T2, T7, T11, T17), all of which were high-grade B-cell lymphomas. The p53 immunopositive lym-

phomas included one tumor (T17) with a TP53 gene mutation (Fig. 1g; Fig. 5). Immunoreactivity for mdm2 in >10% of the tumor cells was detected in only one PCNSL (T7). This tumor demonstrated immunoreactivity for p53 protein in an approximately equal percentage of tumor cells (Table 1). Only the T-cell lymphoma (T3) showed a widespread immunopositivity for p21 in more than 50% of the tumor cells (Fig. 1l). Two further tumors (T6, T7) exhibited fractions of 10% or more p21 positive tumor cells, while the remaining cases had p21 indices of less than 5%. The bcl-2 protein was found to be widely expressed in the majority of cases (Table 1; Fig. 1d). Immunoreactivity for p16 was either absent or restricted to single tumor cells intermingled among negative tumor cells (Fig. 1e). Similarly, staining for cyclin D1 was either completely absent or restricted to occasional tumor cells. In contrast, all PCNSL investigated demonstrated nuclear pRB1 expression in at least 50% of the tumor cells (Table 1; Fig. 1f).

Molecular Genetic Findings. Analysis of the *CDKN2A* gene by Southern blotting and/or differential PCR revealed homozygous deletion in 8 of 20 PCNSL (40%) (Table 1; Figs. 2a,b and 3a). Two additional tumors (T9, T19) showed evidence of loss of one *CDKN2A* allele. Among the tumors that could be evaluated by Southern blotting, all cases having homozygously lost *CDKN2A* had also deleted both copies of the neighbouring and closely related *CDKN2B* (MTS2/p15^{INK4B}) gene (data not shown). Six tumors without homozygous *CDKN2A* loss (T4, T5, T6, T9, T10, and T12) were investigated for methylation of the 5' CpG island of *CDKN2A.* Evidence of *CDKN2A* methylation was found in three tumors (T5, T6, and T10) (Fig. 4). Unfortunately, the remaining six tumors with retention of one or both *CDKN2A* copies (T2, T15, T17-20) could not be studied for methylation because of DNA degradation. SSCP analysis of *CDKN2A* exons 1 - 3 in tumors T4, T9, and T12 revealed no evidence for mutations.

Analysis of *CDKN2A* mRNA expression by reverse transcription PCR was performed for 11 PCNSL and revealed no signals in 7 tumors, including 2 tumors with homozygous *CDKN2A* loss (T8, T13), one tumor with hemizygous *CDKN2A* loss (T9), all 3 tumors with 5'-CpG methylation (T5, T6, and T10), and one tumor without a detected *CDKN2A* aberration (T12). In 4 tumors (T1, T4, T7 and T11) weak reverse transcription PCR signals were obtained (Fig. 5). Among these latter tumors, T4 had no detectable *CDKN2A* alteration while T1, T7 and T11 demonstrated homozygous *CDKN2A* deletion.

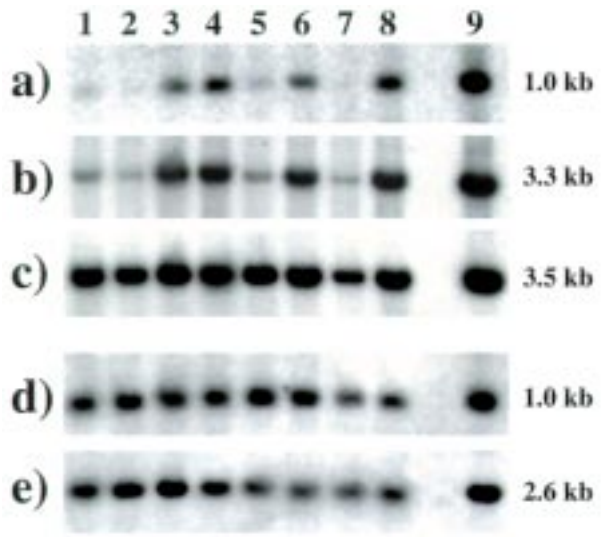

Figure 2. Southern blot analysis of *CDKN2A, CDK4* and *CCND1* gene dose in 8 PCNSL. The same blot was sequentially hybridized with probes for **a.** exon 1 and **b.** exon 2 of *CDKN2A,* the reference locus **c.** *CCNA*, **d.** *CDK4* and **e.** *CCND1*. Lanes 1 - 9 correspond to: 1, T1; 2, T7; 3, T6; 4, T4; 5, T8; 6, T10; 7, T11; 8, T12; 9, normal leukocyte DNA. a-b) Note homozygous deletion of *CDKN2A* in tumors T1 (lane 1), T7 (lane 2), T8 (lane 5), and T11 (lane 7). Quantitative densitometric analysis of *CDKN2A* gene dose using *CCNA* (**c**) as reference locus revealed values between 0.1 and 0.2 for these four tumors. In contrast, tumors T4 (lane 4), T6 (lane 3), T10 (lane 6), and T12 (lane 8) showed values between 0.8 and 1.0 indicating retention of both alleles. **d-e.** None of the 8 tumors showed evidence of *CDK4* or *CCND1* gene amplification.

Screening of the 20 PCNSL for *TP53* gene mutations by SSCP analysis of exons 5 - 8 showed aberrant SSCP bands (exon 6) in only one tumor (T17) (Fig. 6a). DNA sequencing of the aberrant PCR product revealed a point mutation that changed the sequence of codon 248 from CGG to TGG (Fig. 6b). This mutation translates into an amino acid exchange from arginine to tryptophan.

All 11 tumors from which high-molecular weight DNA was available were screened for genomic rearrangements of the *CCND1* and *BCL2* genes. In contrast to the respective control cases, none of the PCNSL revealed a PCR signal that would indicate genomic rearrangements of these genes (data not shown). In addition, no amplification or gross rearrangement of *CCND1* (BCL1) was found by Southern analysis of 8 PCNSL (Fig. 2e).

All 20 tumors were screened by fluorescent differential PCR for amplification of the proto-oncogenes *MDM2, CDK4, MYC, and REL.* None of tumors showed evidence of amplification of one of these genes. The

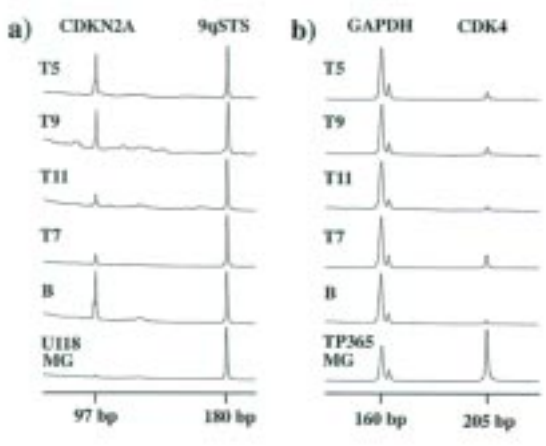

Figure 3. Fluorescent differential PCR analysis of PCNSL for deletion of exon 1 of **a.** *CDKN2A* and amplification of **b.** *CDK4.* Shown are electrophoretograms obtained for selected tumors (T5, T6, T7, and T11), peripheral blood (leukocyte) DNA (B), and the cell lines U118MG (homozygous *CDKN2A* loss), and TP365MG *(CDK4* amplification). The data were obtained with an automated fluorescent DNA sequencer (A.L.F., Pharmacia) and analyzed with the Fragment Manager™ software. In the abscissa of the electrophoretogram the fragment sizes in base pairs (bp) of the respective PCR products are indicated. **a.** Note homozygous loss of exon 1 of *CDKN2A* in tumors T7 and T11 as well as in the U118MG cell line. **b.** *CDK4* amplification is seen only in TP365MG but not in any of the PCNSL.

absence of *CDK4* amplification determined by fluorescent differential PCR (Fig. 3b) was corroborated for 8 tumors by Southern analysis (Fig. 2d). Similarly, probing of the same blot for *MDM2* revealed no amplification (data not shown).

Discussion

The pathogenesis and molecular genetics of PCNSL occuring sporadically in immunocompetent individuals are poorly understood at present. We have therefore studied a series of twenty such tumors for genetic aberrations and expression of several genes previously shown to be altered and/or aberrantly expressed in variable fractions of non-Hodgkin's lymphomas outside the central nervous system. The most frequent abnormality detected in our series of PCNSL was homozygous deletion of the *CDKN2A* tumor suppressor gene. *CDKN2A* maps to the short arm of chromosome 9 (9p21) and has been found to be inactivated in many human cancers (36, 52). The gene product (p16) is a potent inhibitor of cell cycle progression from G1 to S phase (67). It acts by binding to complexes of cyclin dependent kinases (Cdk4 and Cdk6) with D-type cyclins and thereby inhibits the Cdk activation required for cell cycle progression (67, 69). Thus, loss of functional p16 may result in uncontrolled cell cycle progression and promote neoplastic growth. Experimental support for the function of p16 in tumor suppression has been provided by the generation of *CDKN2A* knock-out mice, which were viable but developed spontaneous tumors (mostly lymphomas and sarcomas) at an early age (68). In addition, transfection of *CDKN2A* cDNA into human tumor cell lines lacking *CDKN2A* has been shown to result in growth inhibition (4, 70).

We found homozygous loss of *CDKN2A* in 8 of 20 PCNSL (40%) while two additional tumors showed evidence of loss of one allele. This result is in line with a recent study which had investigated 5 PCNSL and found that *CDKN2A* was deleted homozygously in three tumors and hemizygously in one tumor (41). Taken together, these data indicate that about half of all PCNSL have homozygously lost *CDKN2A.* This incidence is higher than that reported for B-cell lymphomas outside the central nervous system, which have been found to carry homozygous *CDKN2A* deletions in between 10% and 20% of the cases (18, 22, 28, 39, 53, 55). The reason for this difference is unclear at present. However, it cannot be exluded that it simply reflects the larger fraction of high-grade tumors among the PCNSL investigated compared to the other series of B-cell lymphomas outside the central nervous system.

The significance of *CDKN2A* inactivation in PCNSL is further stressed by the results of our *CDKN2A* expression analyses. The majority of PCNSL did not express *CDKN2A* transcripts at detectable levels and immunocytochemistry showed no protein expression in the tumor cells. Some cases, including three tumors with homozygous *CDKN2A* loss, however, showed weak signals for *CDKN2A* mRNA by reverse transcription PCR. This finding might be due to low level expression by contaminating non-neoplastic cells such as reactive astrocytes, endothelial cells, and infiltrating non-neoplastic lymphocytes. Our control experiments showed that *CDKN2A* transcripts are expressed in non-neoplastic cells from a normal lymph node (Fig. 5). Furthermore Tulchinsky *et al*. (73) have shown that human peripheral blood cells, including macrophages, neutrophils and lymphocytes, express *CDKN2A*. Our immunocytochemical analyses did not identify clear expression of p16 protein in non-neoplastic cells in PCNSL. However, the sensitivity of the immunocytochemical method may be limited and therefore lack of immunostaining is not necessarily indicative of complete absence of expression. In some PCNSL of our series, individual p16 positive cells were observed in the tumor tissue. This suggests that a small subpopulation of

tumor cells had retained expression of p16.

The absence of detectable *CDKN2A* mRNA in 5 of 6 tumors with retention of at least one gene copy indicated that other mechanisms than homozygous deletion may lead to inactivation of this tumor suppressor gene in PCNSL. Recently, several studies have reported that methylation of a CpG island within exon 1 of *CDKN2A* is associated with transcriptional silencing of the gene in tumor cells (20, 26, 48). We therefore analysed six PCNSL without homozygous *CDKN2A* deletion by a PCR-based methylation assay and could thereby demonstrate methylation at the CpG island in exon 1 in three of these tumors. All three tumors did not express *CDKN2A* mRNA and protein at detectable levels. In line with our results, a study on lymphoma cell lines has shown that DNA methylation may regulate *CDKN2A* transcription in lymphoma cells *in vitro* (73). Furthermore, a recent study reported hypermethylation of *CDKN2A* in about 20% of B-cell lymphomas outside the nervous system (47). Thus, it appears that DNA methylation represents an alternative molecular mechanism of *CDKN2A* inactivation in a subset of non-Hodgkin's lymphomas including some PCNSL.

In contrast to homozygous deletion and DNA methylation, mutation in the *CDKN2A* gene appears to be rare in malignant lymphomas. Occasional cases with *CDKN2A* point mutations have been reported (74), however, other authors did not detect mutations of this gene even in lymphomas with hemizygous *CDKN2A* loss (18, 39, 41, 53, 55). We have investigated three PCNSL of our series that had retained at least one *CDKN2A* copy and had not methylated the 5' CpG island. This analysis, however, revealed no evidence for mutation. Nevertheless, two of these tumors lacked detectable expression of *CDKN2A* mRNA and protein, a finding that might be explained by the presence of mutations outside the coding regions analyzed, e.g. in promotor sequences.

Amplification of *CDK4* has been found as an alternative genetic alteration to *CDKN2A* inactivation in certain types of tumors such as malignant gliomas (24, 64). In contrast, none of our 20 cases had *CDK4* amplification, suggesting that *CDK4* alterations may not be involved in PCNSL. The *CCND1* (BCL1) gene, which encodes the cyclin D1 regulatory subunit of Cdk4, is rearranged and overexpressed in centrocytic (mantle cell) lymphomas (8, 62, 66, 71), but only rarely in high-grade non-Hodgkin's lymphomas (8, 71). We and others (34) did not detect rearrangements or amplification of *CCND1* in PCNSL, nor was cyclin D1 overexpressed in our series. These results suggest that *CCND1* alterations are not involved in the formation of PCNSL.

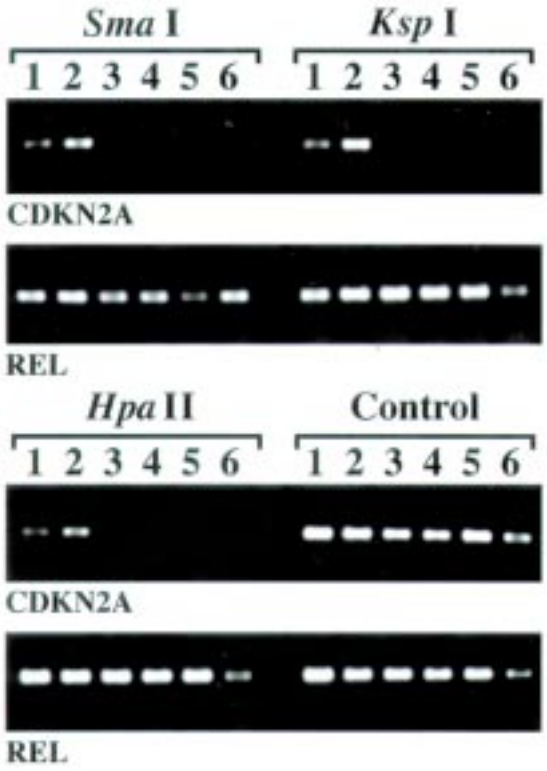

Figure 4. Results of PCR-based methylation analysis of the 5' CpG island of *CDKN2A*. DNA from T5 (lane 1), T6 (lane 2), T12 (lane 3), T4 (lane 4), normal leukocytes (lane 5), and non-neoplastic brain tissue (lane 6) was digested with the methylation-sensitive restriction enzymes *Sma* I, *Ksp* I, and *Hpa* II. One or more restriction sites for each of these enzymes are present within this CpG island (64). A genomic segment of *CDKN2A* spanning the 5' CpG island was then PCR amplified using either digested DNA or undigested DNA from the same cases (control) as template. As control for the amount of template DNA used, a segment of the *REL* gene containing no *Sma* I, *Ksp* I, and *Hpa* II restriction sites was PCR amplified. Note signals for *CDKN2A* in lanes 1 and 2 but not in lanes 3 - 6 after restriction digestion. This finding indicates methylation of the respective restriction sites within the 5' CpG island of *CDKN2A* in tumors T5 and T6. In contrast, the other tumors (T4, T12) and reference samples reveal no amplification products for *CDKN2A* after restriction digestion due to complete template DNA digestion in the absence of restriction site methylation.

Inactivation of the retinoblastoma gene product by mutation and/or deletion has been reported as a further alternative molecular mechanism to *CDKN2A* inactivation or *CDK4* amplification or *CCND1* amplification/ rearrangement in human tumors (32). Several previous studies have implicated alterations of RB1 in non-Hodgkin's lymphomas (17, 22, 76). According to Weide *et al*. (76) more than 50% of high-grade non-Hodgkin's

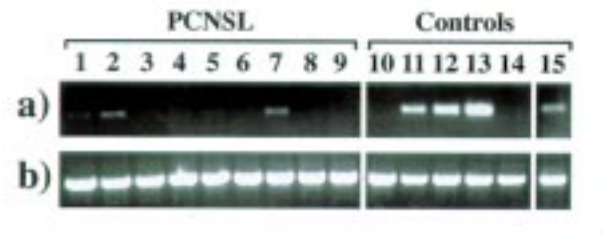

Figure 5. a. Analysis of *CDKN2A* mRNA expression by reverse transcription PCR in 9 PCNSL (lanes 1-9) and various control samples (lanes 10-16). Lanes 1 - 16 correspond to: 1, T1; 2, T4; 3, T5; 4, T6; 5, T7; 6, T8; 7, T11; 8, T12; 9, T13; 10, U118MG; 11, TP365MG; 12, oligoastrocytoma (OA1); 13, anaplastic oligodendroglioma (AO11); 14, non-neoplastic brain tissue, 15, normal lymph node. No signals for *CDKN2A* mRNA are present in tumors T5 and T6 (lanes 3-4), T8 (lane 6), T12 - T13 (lanes 8-9), U118MG (lane 10), and non-neoplastic brain tissue (lane 14). Weak signals for *CDKN2A* mRNA are visible in tumors T1 and T4 (lanes 1-2), T7 (lane 5), and T11 (lane 7). Among the control samples, strong signals are present in TP365MG, OA1, and AO11 (lanes 11-13) while the normal lymph node shows a weak to moderate signal (lane 16). **b.** As control for the assessment of the cDNA quality and quantity used as template in the PCR reactions, *β-actin* was amplified from the same cases.

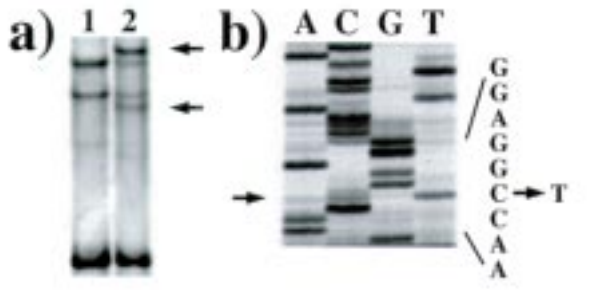

Figure 6. Demonstration of *TP53* gene mutation in tumor T17. **a.** Single strand conformational polymorphism analysis of PCR products amplified with primers for exon 6 of the *TP53* gene shows aberrant bands (arrows) in the PCR product amplified from T17 (lane 2) compared to normal leukocyte DNA (lane 1). **b.** DNA sequencing of the aberrant PCR product from T17 reveals a point mutation (arrow) at codon 248 (CGG→TGG) resulting in an aminoacid exchange of arginine to tryptophan. Part of the sequence depicted, including the mutation site, is printed on the right-hand side.

lymphomas, particularly centroblastic and Burkitt lymphomas, lack pRB1 expression. In contrast, immunocytochemical expression analysis of our series of PCNSL showed strong nuclear pRB1 immunoreactivity in all tumors investigated. Although this type of analysis does not compensate for detailed mutational investigation of the *RB1* gene, the results obtained indicate that the gene is neither homozygously lost nor transcriptionally silenced in PCNSL.

Mutation of the *TP53* tumor suppressor gene has been reported in about 30% of high-grade B-cell lymphomas and 40% of Burkitt's lymphomas (15, 31, 33, 38). In addition, a subset of high-grade B-cell lymphomas may show overexpression/accumulation of p53 protein in the absence of a demonstrated *TP53* gene mutation (1, 33, 38, 43). A single previous study on *TP53* alterations in PCNSL reported *TP53* mutations in 2 of 5 tumors investigated (40%) (40). In contrast, our data indicate that the incidence of *TP53* mutations in PCNSL is lower than reported for systemic high-grade B-cell lymphomas. We identified only one tumor among 20 PCNSL with a *TP53* point mutation and 4 tumors (including the one with *TP53* mutation) with p53 immunoreactivity in >10% of the tumor cells. One p53 positive tumor (T7) also showed immunoreactivity for mdm2 protein in an approximately equal percentage of tumor cells. However, neither this tumor nor any other of the PCNSL investigated showed *MDM2* amplification. In this regard, PCNSL appear similar to systemic lymphomas, which only exceptionally show *MDM2* amplification (10, 57, 61).

Immunocytochemical assessment of p21 expression in our series of PCNSL revealed only low fractions of positive tumor cells in most tumors. Only the single T-cell tumor (T3) strongly expressed p21. Although some authors suggested a role for deletions and loss of expression of *CDKN1A* in the progression of mantle cell lymphomas (56), others failed to identify mutations of this putative tumor suppressor gene in a large series of lymphoid neoplasms (19). Thus, the significance of p21 in the development of lymphomas is still unclear. Chilosi *et al*. (11) reported that p21 immunopositive lymphomas usually show concomitant expression of wild-type p53. Tumor T3 of our series demonstrated no detectable *TP53* mutation, however, expression of p53 was confined to a low fraction of tumor cells.

The *BCL2* gene is commonly rearranged by t(14;18) translocations in follicular lymphomas (72). In addition, *BCL2* rearrangements have been found in a fraction of diffuse B-cell lymphomas (2, 77). However, we found no evidence for *BCL2* rearrangement in our series of PCNSL and thus corroborate the data reported by Jellinger and Paulus (34) for 5 PCNSL. Immunocytochemically, the majority of PCNSL investigated here were strongly positive for bcl-2 protein. Similar findings were reported by others (7). The molecular mechanisms underlying the strong expression of bcl-2 in PCNSL as well as the functional consequences of this overexpression remain to be elucidated.

The *MYC* proto-oncogene has been shown to be rearranged and overexpressed in most Burkitt's lymphomas due to chromosomal translocations (12). In

addition, individual cases of B-cell lymphomas with gene amplification of *MYC* have been reported (6, 42). In our study, none of the twenty PCNSL showed evidence of *MYC* amplification. It remains to be shown, however, whether PCNSL carry rearrangements of the *MYC* gene locus.

A recent study reported amplification of the *REL* proto-oncogene from 2p13-p15 in 23% of extranodal diffuse large cell lymphomas (29). In addition, *REL* amplification was detected in 2 of 26 mediastinal high-grade B-cell lymphomas (35). The *REL* gene product belongs to the NF-κB/REL family of transcription factors, the members of which are involved in lymphoid cell differentiation as well as in growth stimulation of a variety of cell types (for recent reviews see Refs. 5, 16). Investigation of *REL* gene dose by fluorescent differential PCR revealed no evidence of amplification in the 20 PCNSL of our series. This finding does not exclude, however, the possibility of structural rearrangements of the *REL* locus, which have been found in individual lymphomas outside the nervous system (46).

In summary, the present study shows that the *CDKN2A* tumor suppressor gene is homozygously deleted or transcriptionally silenced by hypermethylation in the majority of PCNSL from immunocompetent patients. Mutation of the *TP53* gene and nuclear immunoreactivity for p53 protein are restricted to a low fraction of PCNSL while rearrangements of *CCND1* and *BCL2* as well as amplification of *CCND1*, *CDK4*, *MDM2*, *MYC* and *REL* appear not to be involved in the pathogenesis of these tumors. However, since it is very likely that PCNSL have multiple genetic abnormalities in addition to inactivation of *CDKN2A* and occasional mutation of *TP53*, further studies aiming at the identification of new chromosomal regions and genes involved in these tumors are necessary.

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