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Expression of *p16/INK4a* in Posttransplantation Lymphoproliferative Disorders

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It was recently demonstrated that classification of posttransplantation lymphoproliferative disorders (PT-LPDs) into morphological and molecular categories is clinically relevant. It was also reported that PT-LPD not associated with Epstein-Barr virus (EBV) had a more aggressive course than most lesions associated with EBV. Because the cyclin-dependent kinase inhibitor *p16/INK4a* has been reported to be frequently inactivated in high-grade lymphomas, we evaluated 17 PT-LPD to determine whether *p16/INK4a* expression could be correlated to morphology, EBV detection, and a Ki-67 labeling index. We demonstrated that tumors with no *p16/INK4a* expression ($n = 8$) had a predominantly monomorphic appearance, and most were EBV negative (respectively, 7/8 and 5/8), whereas lesions with *p16/INK4a* expression ($n = 9$) were mostly polymorphic PT-LPD (6/9) ($P = 0.049$) and associated with EBV (9/9) ($P = 0.015$). In particular, strong *p16/INK4a* expression was observed in atypical immunoblasts and Reed-Sternberg-like cells. Furthermore, the proliferation index was significantly higher in tumors lacking *p16/INK4a* expression than in other lesions ($P = 0.0008$). In conclusion, down-regulation of *p16/INK4a* was mostly observed in PT-LPD lesions known to follow more aggressive courses: monomorphic tumors and EBV-negative PT-neoplasms. Conversely, overexpression of *p16/INK4a* was associated with EBV-positive PT-LPD. While p16/INK4a might play a role in the proliferative rate of LP-LPD, further investigations are needed to assess the clinical relevance of *p16/INK4a* expression in predicting the evolution of tumors and to explain how EBV could favor p16/INK4a protein accumulation in lesions. ***(Am J Pathol 2000, 156:1573–1579)***

Lymphoproliferative disorders (LPDs) are a major cause of morbidity in organ transplant recipients receiving immunosuppression,[1,2] varying between 1 and 12% of the graftees, for the most part according to the dose and duration of immunosuppressive therapy and the organ transplanted.[1,3–6] These tumors frequently involve extranodal sites and generally regress with reduction or withdrawal of immunosuppressive therapy, but some of them progress independently from the restoration of the immune system. It has been hypothesized that PT-LPDs arise from a polyclonal expansion of Epstein-Barr virus (EBV)-infected B cells and can evolve into a lymphoid tumor with the emergence of an oligoclonal or monoclonal dominant population.[7] PT-LPDs are usually classified into two subgroups, according to their appearance: polymorphic and monomorphic.[3] The latter closely resembles the diffuse large B-cell lymphoma (DLCL) of the Revised European American Lymphoma (REAL) classification.[8] Based on morphological and molecular analysis, Knowles et al[7] distinguished three main subgroups: 1) plasmacytic hyperplasia, 2) polymorphic B-cell hyperplasia and polymorphic B-cell lymphomas, and 3) malignant lymphoma or multiple myeloma, with the latter group being more frequently associated with genetic alterations, such as the inactivation of tumor suppressor gene *p53* and the activation of c-*myc* and N-*ras* oncogenes. Moreover, Chadburn et al[9] recently showed the clinical relevance of this classification, as no response to aggressive clinical intervention was more frequently seen in patients with the lesions from the last group than those with the other tumors. Some patients with PT-LPDs that are not associated with EBV have a shorter survival time than those with EBV-positive lesions.[10]

Expansion of B cells after organ transplantation should involve an excessive activation of the cell cycle, as in many tumors. The *p16/INK4a* gene is a major inhibitor of the cell cycle that is found to be inactivated in many cancers.[11] It prevents the association between cyclin D

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of the G_1 phase and cyclin-dependent kinases (CDK)4–6 and thus the inactivation of the retinoblastoma protein (pRb) through its phosphorylation by these complexes.[12] Homozygous deletions, hypermethylation of 5′ CpG islands, and mutations are the most common mechanisms of *p16/INK4a* gene inactivation that have been demonstrated in cancers.[13,14] An alternative p16/INK4a transcript was recently described that encodes a structurally and functionally different protein, p14ARF,[15] which is able to inhibit the cell cycle in G_1-S and G_2-M phases via a p53-dependent pathway by interacting with murine double minute 2 (MDM2) protein.[16] Frequent modifications of the *p16/INK4a* gene have been reported in lymphoid malignancies, such as acute T-cell lymphoblastic leukemia[17,18] high-grade lymphomas,[19,20] and Burkitt's lymphomas.[21] Furthermore, a high percentage of *p16/INK4a* knock-out mice develop B-cell lymphomas.

In *in vitro* experiments, EBV induced the phosphorylation of pRb in B cells through the up-regulation of cyclins D2 and E, and the down-regulation of p16/INK4a.[22,23] However, little is known about the regulation of p16/INK4a in EBV-driven lymphoid tumors.

Because inactivation of *p16/INK4a* is implicated in the transformation of indolent lymphomas,[19,20] it has been hypothesized that this gene could potentially play a role in the aggressiveness of PT-LPD. Our analysis of a series of PT-LPDs demonstrated that the expression of the p16/INK4a protein was associated with the morphological categories of tumors, EBV status, and Ki-67 proliferation index.

Materials and Methods

Materials

Formaldehyde-fixed PT-LPD biopsies from 17 adults were reviewed for the study. The following clinical data were collected for each patient: sex, age, tumor localization, and time since transplantation. Hematoxylin-eosin- and Giemsa-stained paraffin sections of all biopsies were reviewed by a panel of pathologists who classified them according to standard morphological criteria[3] as polymorphic or monomorphic PT-LPD. Immunophenotyping of the tumors by immunolabeling with B-cell and T-cell differentiation antigens, detection of EBV by *in situ* hybridization (ISH) and by Southern blotting, and immunoglobulin (Ig) gene heavy-chain and T-cell receptor rearrangements by Southern blotting had previously been conducted on these specimens.[10]

Immunohistochemical Analysis

The streptavidin biotin-peroxidase labeling method was applied to 4-μm-thick paraffin sections with the LSAB kit (Dakopatts, Trappes, France) and monoclonal antibodies to p16/INK4a (G1175-405; PharMingen, San Diego, CA), p53 (DO7; Dakopatts), and Ki-67 (MIB-1; Immunotech, Marseille, France). Briefly, dewaxed sections were incubated overnight at 4°C with the anti-p16/INK4a antibodies diluted by 1:200 and 30 minutes at room temperature, after a microwave pretreatment of sections in citrate buffer (10 mmol/L, pH 6), for p53 and Ki-67 detection. Endogenous peroxidase blocking, inhibition of specific antibody binding, and visualization were carried out as previously described.[24] As previously reported,[20] a tumor was considered to be p16/INK4a negative when no nuclear labeling was observed in the tumor other than that of interspersed small reactive lymphocytes and endothelial cells used as an internal positive control. Ki-67-positive cells were counted on the digitized images (Visu Dis Light system; DIS, Blanc-Mesnil, France) of four high-power fields (magnification ×400) without any knowledge of the clinical information and morphological findings. p53 labeling was quantified as previously reported,[24] and only tumors with more than 10% labeled nuclei were considered positive. Normal preimmune mouse serum (Dakopatts) was used to assess nonspecific labeling.

To assess the cell-cycle status of the p16/INK4a-positive cells, double labeling with anti-p16/INK4a and anti-Ki-67 antibodies was performed using an EnVision system (Dakopatts). Briefly, after microwave treatment in citrate buffer for 15 minutes and blocking of endogenous peroxidase, the dewaxed sections were incubated with 1:200-diluted Ki-67 antibody for 30 minutes at room temperature and washed in Tris-buffered saline. Then the peroxidase-labeled polymer conjugated to goat anti-mouse Ig was applied for 30 minutes at room temperature. After revelation with 3,3′-diaminobenzidine and substrate chromogen solution, the rinsed sections were incubated with polyclonal antibodies to p16/INK4a (PharMingen) (diluted 1:400), overnight at 4°C, then with the phosphatase-labeled polymer conjugated to goat anti-mouse Ig, and were visualized with fast red and substrate-chromogen solution.

Southern Blot Analysis of the p16/INK4a Gene

High-molecular-weight DNA was extracted from frozen material by standard procedures. Southern blot analysis could be performed on seven samples that yielded sufficient DNA. *Eco*RI digests (15 μg) were analyzed as previously described.[18] The p16/INK4a probe (a gift from J.-M. Cayuela, Hôpital Saint-Louis, Paris, France) consisted of the exon 2 sequence cloned in PCR-Script SK(+) plasmid (Stratagene, La Jolla, CA), which was radiolabeled using a random DNA labeling kit (Boehringer-Mannheim, Mannheim, Germany) with [α-^{32}P]dCTP. The hybridized membrane was exposed on autoradiographic film (Kodak, Rochester, NY).

Methylation-Specific Polymerase Chain Reaction

5′ CpG methylation was analyzed with the recently described methylation-specific polymerase chain reaction (PCR) (MSP),[25] using bisulfite-modified DNA, the unmethylated cytosines of which were converted into uracil. A 5′ CpG-rich site was amplified with pairs of primers

Table 1. Clinical and Demographic Characteristics, Pathologic Findings, Clonality, and EBV Detection for 17 Patients with Posttransplantation B-Cell Neoplasms

Cases	Age	Sex	Organ transplanted	Graft-PT-LPD interval PT-LPDs	Morphology	Light chain	Clonality	EBV	
								ISH	Southern
1	45	M	Heart	150	Polymorphic	M	ND	+	ND
2	50	M	Heart	90	Polymorphic	M	ND	+	ND
3	38	F	Heart	150	Polymorphic	M	JHR	+	+
4	68	F	Heart	210	Polymorphic	M	JHR	+	+
5	20	M	Heart	150	Polymorphic	NM	ND	+	ND
6	52	M	Heart	150	Polymorphic	NM	GL	+	+
7	51	M	Lung	180	Polymorphic	NM	GL	+	+
8	43	M	Heart	750	Monomorphic	M	JHR	+	+
9	56	F	Kidney	1200	Monomorphic	M	ND	+	ND
10	32	M	Kidney	450	Monomorphic	M	JHR	+	+
11	63	M	Kidney	680	Monomorphic	M	JHR	+	+
12	31	M	Heart	1660	Monomorphic	M	JHR	+	+
13	50	F	Heart	180	Monomorphic	M	JHR	−	−
14	43	F	Liver	900	Monomorphic	M	JHR	−	−
15	59	M	Kidney	3450	Monomorphic	M	JHR	−	−
16	57	M	Kidney	450	Monomorphic	M	JHR	−	−
17	56	F	Kidney	390	Monomorphic	M	ND	−	−

M, male; F, female; PT-LPD, posttransplantation lymphoproliferative disorders; M, monotypic light chain; NM, nonmonotypic; EBV, Epstein-Barr virus; ISH, in situ hybridization; ND, not done; Southern, Southern blot; JHR, rearrangement of Ig gene; GL, germline; +, positive; −, negative.

distinguishing between methylated and unmethylated DNA in bisulfite-modified DNA.[25] DNA extracted from frozen samples was treated with 3 mol/L sodium bisulfite and purified as previously described.[25] Amplification was carried out in a Hybaid OmniGene thermocycler with 50 μl of PCR mix containing 1× PCR buffer (16.6 mmol/L ammonium sulfate, 67 mmol/L Tris (pH 8.8), 1.5 mmol/L $MgCl_2$), deoxynucleoside triphosphates (each at 1.25 mmol/L), 300 ng of sense and antisense primers, and 50 ng of bisulfite-modified DNA. The p16 primers for methylated (p16-M) and unmethylated (p16-U) DNA were, respectively, 5′-TTATTAGAGGGTGGGGCGGATCGC (p16-M sense), 5′-GACCCCGAACCGCGACCGTAA (P16-M antisense), 5′-TTATTAGAGGGTGGGGTGGATTGT (p16-U sense), and 5′-CAACCCCAAACCACAACCATAA (p16-U antisense). Reactions without DNA or with untreated DNA from normal peripheral blood mononuclear cells and with treated DNA of the Jurkat lymphoblastic cell line with homozygous deletions of *p16/INK4a* gene were used as negative controls for MSP. A Burkitt cell line (Raji) served as the positive control for *p16/INK4a* methylation. PCR reaction mixture (10 μl) was electrophoresed in a nondenaturing 6% polyacrylamide gel and stained with ethidium bromide.

Statistical Analyses

Statistical analysis was performed with Stawiew software (Abacus Concepts, Berkeley, CA). A comparison between p16/INK4a status and the Ki-67 labeling index was made with the Mann-Whitney *U*-test, and the relationship between *p16/INK4a* expression and EBV detection or morphology was assessed using Fisher's exact test. A *P* value less than 0.05 was considered significant.

Results

Clinical and Pathological Findings, Clonality, and EBV Detection

Clinical and demographic characteristics of the 17 patients are presented in Table 1. Tumors involved extranodal sites in 15 of 17 cases. The time interval between organ allografting and tumor diagnosis ranged from 90 to 3450 days, with a mean of 658 ± 841 days and a median of 390 days.

Among the 17 PT-LPDs examined, 41.2% were polymorphic and all were immunophenotype B. These diffuse lesions were composed of a mixture of small lymphocytes and large cells. The large cells varied in number but were generally numerous, consisting of centroblast-like cells, atypical immunoblasts, plasmablasts, Reed-Sternberg-like cells, and large areas of necrosis. The clonality of four of the polymorphic PT-LPDs was determined: two had Ig gene rearrangement and two exhibited a germline pattern. Among the three remaining samples, immunohistochemistry detected two monotypic light chains and one polytypic Ig. EBV genome and/or Epstein-Barr-encoded RNAs (EBERs) were detected in all of these polymorphic proliferations. The 10 remaining cases were monomorphic, met the REAL classification morphological criteria of diffuse large B-cell lymphomas, and were composed of uniform sheets of large noncleaved cells. All of these lesions exhibited Ig gene rearrangements and/or monotypic light chains. EBV was not detected in half of them.

Relationship between p16/INK4a Expression and Morphology

According to the level of *p16/INK4a* expression, two groups were distinguished: group 1 (n = 8), with no

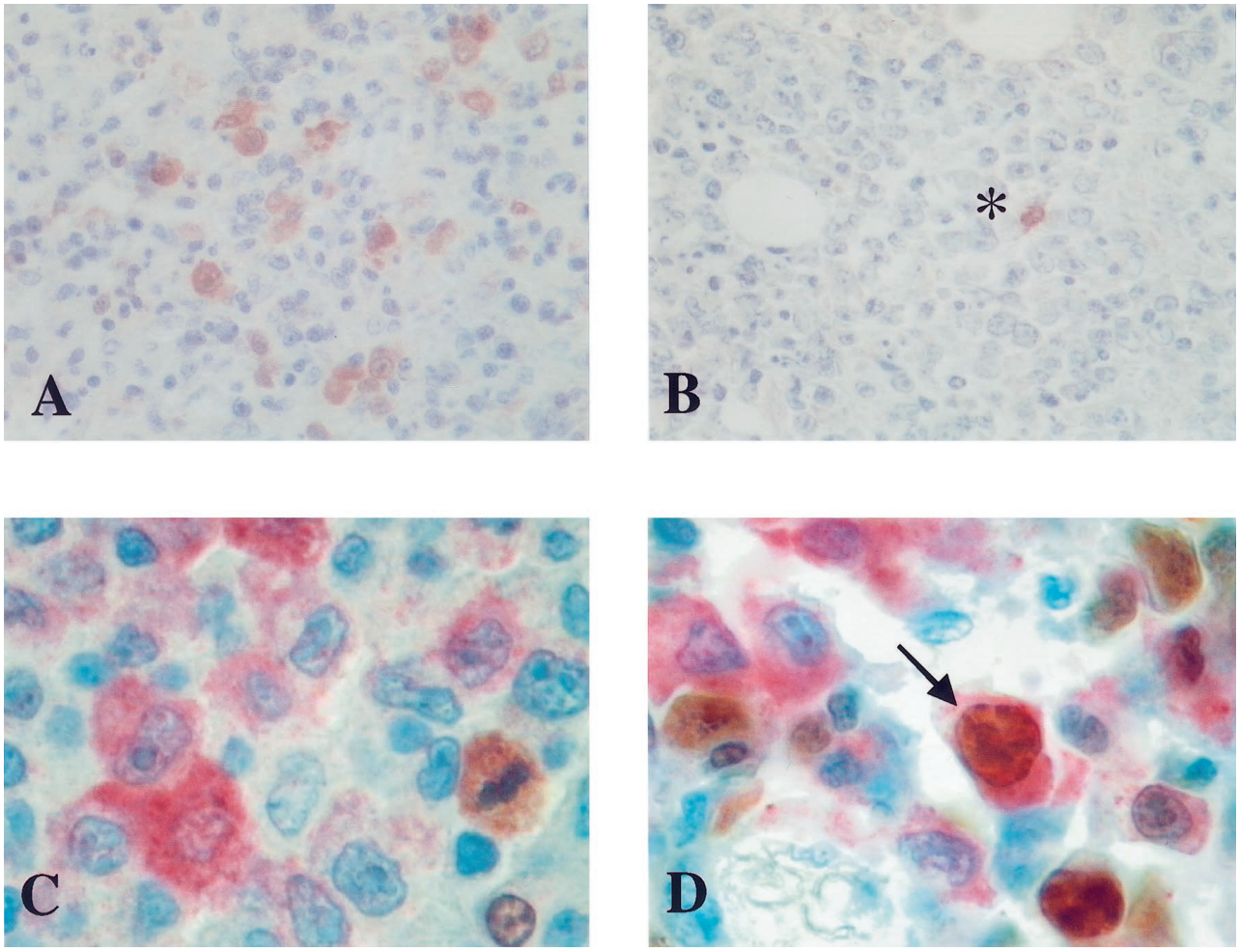

Figure 1. A and **B:** High-power (×400) field of p16/INK4a labeling in PT-LPD. **A:** Numerous atypical immunoblasts and Reed Sternberg-like cells in polymorphic lesion showing strong cytoplasmic and nuclear labeling. **B:** *p16/INKa* expression is detected in the tumor cells of a PT-LPD with the appearance of a large-cell lymphoma. In this case, a single reactive cell shows nuclear labeling (*). **C** and **D:** Double labeling for p16/INKa and Ki-67. **C:** The p16/INK4a-positive cells (red) are Ki-67 negative. Only one mitosis and one reactive cell are Ki-67 positive (brown). **D:** Note the double-labeled large cell (**arrow**).

significant expression, and group 2 ($n = 9$), with nuclear labeling in 10–80% of the tumor cells. Most *p16/INK4a*-positive cells exhibited diffuse cytoplasmic labeling and were large B cells, corresponding to atypical immunoblasts or Reed-Sternberg-like cells (Figure 1A). The majority of group 1 tumors had a monomorphic appearance (7/8). On the contrary, most in group 2 were polymorphic lesions (6/9) ($P = 0.049$). All tested tumors from group 1 (7/8) had monoclonal Ig gene rearrangement. In group 2, six tumors had Ig gene rearrangement and/or monotypic light-chain expression, and three lesions expressed the germline Ig gene pattern and/or had polytypic light chains (Tables 2 and 3).

Southern Blotting and MSP Analysis

Homozygous deletion was detected in patient 13's lesion, and methylation of a 5′ CpG was demonstrated in PT-LPD 14. p16/INK4a protein was not detected in either. The remaining tested cases, in which no deletion or methylation could be detected, always expressed p16/INK4a protein (Figure 2 and Table 2).

Relationship between p16/INK4a Expression and EBV

EBV-genome sequences and/or nuclear EBER was detected in three of eight (37%) cases from group 1 and in nine of nine from group 2 (100%) ($P = 0.015$) (Tables 2 and 3).

Ki-67 Labeling Index and Relationship with p16/INK4a Expression

The Ki-67 labeling was significantly higher in group 1 than in group 2 ($P = 0.0008$) (Tables 2 and 3). Because of the polymorphic appearance of the majority of tumors with *p16/INK4a* expression, we wanted to assess the proliferative status of the *p16/INK4a*-positive cells. Taking advantage of the diffuse cytoplasmic labeling of p16/INK4a in the majority of positive cells, we performed double labeling with anti-p16/INK4a and anti-Ki-67 antibodies. Most of the p16/INK4a-positive cells were Ki-67 negative, except for patient 12, who had numerous dou-

Table 2. Comparison between p16/INK4a Status and Ki-67 Labeling Index, p53 Expression, Morphology, EBV Detection, and Clonality

Cases	Marker expression			Morphology	EBV	Clonality	Light chain	p16/INK4a	
	p16/INK4a	Ki-67	p53					Deletion	Methylation
Group 1									
8	−	81%	−	Monomorphic	+	JHR	M	ND	ND
13	−	61%	−	Monomorphic	−	JHR	M	+	−
15	−	67%	−	Monomorphic	−	JHR	M	ND	ND
14	−	56%	−	Monomorphic	−	JHR	M	−	+
10	−	72%	−	Monomorphic	+	JHR	M	ND	−
4	−	63%	−	Polymorphic	+	JHR	M	−	−
16	−	54%	−	Monomorphic	−	JHR	M	ND	ND
17	−	90%	−	Monomorphic	−	ND	M	ND	ND
Group 2									
11	+	40%	−	Monomorphic	+	JHR	M	ND	ND
6	+	34%	−	Polymorphic	+	GL	NM	−	−
1	+	33%	−	Polymorphic	+	ND	M	ND	ND
5	+	28%	−	Polymorphic	+	ND	NM	ND	ND
3	+	37%	−	Polymorphic	+	JHR	M	ND	ND
2	+	29%	−	Polymorphic	+	ND	M	−	−
12	+	55%	+ (50%)*	Monomorphic	+	JHR	M	−	−
7	+	27%	−	Polymorphic	+	GL	NM	−	−
9	+	24%	−	Monomorphic	+	ND	M	ND	ND

+, positive; −, negative. *50% of the tumor cells were p53 positive; for other abbreviations see footnote to Table 1.

ble-labeled cells (Figure 1, C and D). This case was the only one with p53 accumulation in more than 50% of tumor cells.

Discussion

In this series of PT-LPDs, we found a relationship between morphology and *p16/INK4a* expression, inasmuch as most lesions with no protein were monomorphic proliferations, whereas most lesions with *p16/INK4a* expression were polymorphic. Among the positive lesions, the labeling of the large atypical immunoblasts and Reed-Sternberg-like cells was particularly strong, whereas scattered lymphocytes and endothelial reactive cells for p16/INK4a, used as positive internal control, were weakly labeled. In the literature, some conflicting results have been reported concerning the reactivity of normal lymphocytes with anti-p16/INK4a antibodies. Betticher et al[26] did not observe labeling in normal lymphocytes in lung specimens, whereas Villuendas et al[20] found labeled lymphocytes in different compartments of reactive lymph nodes. Perhaps this discrepancy reflects differences between methods, as it is known that fixatives, duration of fixation, or use of older slides can influence the labeling of the p16/INK4a protein. To minimize false negative results, we used only sections freshly cut from paraffin blocks. In our experience we were able to detect weak labeling in some connective tissue cells and lymphocytes in different organs. Furthermore, Villuendas et al[20] showed by double labeling that most p16/INK4a-positive lymphocytes in reactive lymph nodes or in low-grade lymphomas were B cells, whereas T cells with p16/INK4a label were rare and scattered. Most of the reactive lymphocytes observed in PT-LPDs (as in Figure 1A) were T cells, perhaps explaining why most small reactive cells were p16/INK4a negative.

The Ki-67 proliferation index was significantly higher in specimens with no *p16/INK4a* expression than in the

Table 3. Statistical Comparisons between p16/INKK4a and Morphology, EBV Detection, and Ki-67 Proliferation Index

Parameters	Group 1 (p16/INK4a−) (n = 8)	Group 2 (p16/INK4a+) (n = 9)	P values
Morphology			
Polymorphic LP-PLDs	1	6	0.049*
Monomorphic LP-PLDs	7	3	
EBV genome			
Absent	5	0	0.015*
Present	3	9	
Ki-67 labeling index			
Mean ± SD	68 ± 12.4%	34 ± 9.3%	0.0008†

SD: standard deviation.
*Fisher's exact test.
†Mann-Whitney *U*-test.

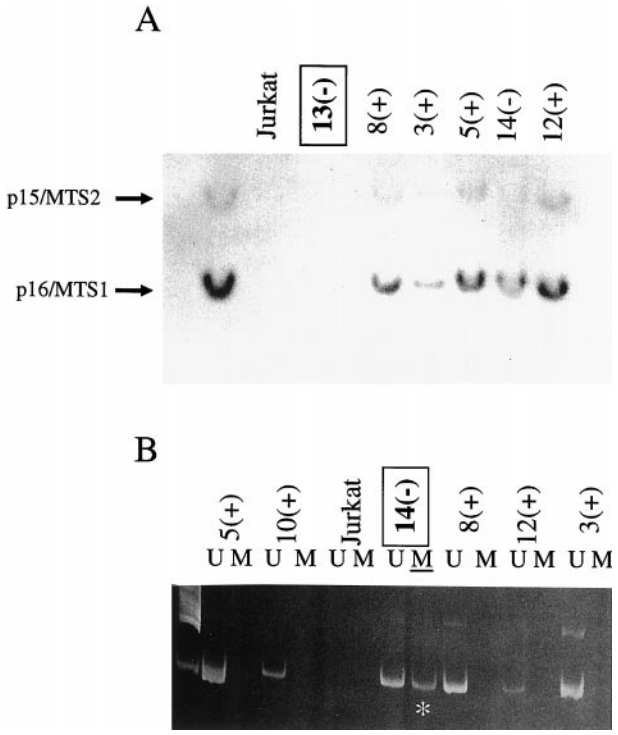

Figure 2. A: Southern blot with a p16/MTS1-exon-2 probe that also recognized p15/INK4b-exon2. PT-LPD 13 showed a pattern similar to that of Jurkat cells, which are known to have homozygous deletion of the p16/MTS1-p15/MTS2 locus. −, 16/INK4a protein is absent; +, p16/INK4a protein is present. **B:** Methylation-specific PCR. Methylated (M) DNA (*) was detected in PT-LPD 14, from a patient belonging to group 1 (no detection of p16/INK4a protein). U, unmethylated.

others. However, it is necessary to exercise caution in interpreting the Ki-67 labeling index, because proliferating lymphoid cells can be dispersed among reactive T cells. Therefore, we verified that the tumor areas evaluated were mainly composed of B cells (data not shown), and we performed double labeling with anti-p16/INK4a and anti-Ki-67 antibodies. Interpretation of these slides was facilitated here because of the diffuse cytoplasmic labeling of p16/INK4a in large atypical cells. Although we cannot completely exclude the possibility that the number of double-labeled cells could be underestimated because of nucleus-localized p16/INK4a or poorly diffused cytoplasmic labeling, we showed in our series that most p16/INK4a-labeled large cells were Ki-67 negative, indicating that they were in G_0. This observation is in agreement with the lower proliferation rate observed in PT-LPDs with *p16/INK4a* expression. Although diffuse cytoplasmic p16/INK4a labeling was previously reported for other tumors,[27,28] its significance remains uncertain. It might reflect artifacts or translocation of the protein into the cytoplasm by an unknown mechanism. p16/INK4a protein was reported to be present in quiescent B cells[22,] and in a small percentage of peripheral blood lymphocytes.[20] Results of *in vitro* experiments are controversial. Cannell et al[22] showed a down-regulation of *p16/INK4a* in B cells after EBV infection, whereas for Villuendas et al,[20] its expression increased in circulating lymphocytes after mitogen stimulation and was more accentuated in S and G_2/M phases. In reactive lymph nodes, *p16/INK4a* expression was observed in all compartments, but at higher levels in areas of proliferation.[20] However, it remains uncertain whether the positive cells in the proliferating compartments are effectively in cycle. In our specimens, p16/INK4a might have accumulated in cells to counteract excessive activation of the cell cycle. But the protein could be rendered ineffective by alterations of other genes implicated in the control of the cell cycle. Interestingly, one specimen with double labeling of numerous cells also had p53 protein accumulation, in contrast to the overwhelming majority of samples with only a few double-labeled cells.

A recent study showed that PT-LPDs with the large cell lymphoma pattern responded significantly less well to aggressive clinical intervention than did polymorphic lesions.[9] Furthermore, patients with EBV-negative PT-LPD seem to have a shorter survival time than patients with EBV-associated lesions.[10] Consequently, it is not surprising that a strong cell-cycle inhibitor like p16/INK4a was found to be down-regulated in lesions that are expected to have a more aggressive course.

Loss of *p16/INK4a* expression in tumors is strongly associated with gene deletions or 5′ CpG island methylation, and *p16/INK4a* inactivation is one of the most frequent genetic alterations seen in high-grade lymphomas and, particularly, in the progression of low-grade tumoral proliferations.[20] In PT-LPD, oncogene activation or tumor suppressor gene inactivation occurs in only a small percentage of lesions[7] and is associated with large-cell lymphomas or myelomas. Among the cases that we could test in this series, we found one case each of methylation and gene deletion in group 1 and no alterations in group 2.

We postulate that the integrity of *p16/INK4a*, in conjunction with cytotoxic cells and in the absence of genetic alterations of oncogenes or other tumor suppressor genes, could favor the rapid regression of PT-LPD frequently observed after the reduction of immunosuppressive therapy. Furthermore, it has been advanced that the loss of the *p16/INK4a* gene might play a role in the outgrowth of PT-LPD transplanted into mice with severe combined immunodeficiency.[29]

It has been shown that *in vitro* EBV infection of B cells down-regulates *p16/INK4a*.[22] However, all of our p16/INK4a-positive PT-LPDs also expressed EBV genome. Furthermore, more than half of the p16/INK4a-negative specimens were also EBV negative. In EBV-positive lymphoblastoid cell lines that we tested previously, we did not detect p16/INK4a protein expression or gene deletion and methylation (data not shown). The discrepancy between *p16/INK4a* expression in *in vitro* cells and *in vivo* tumors remains to be elucidated. Finally, the relationship between p16/INK4a accumulation and morphological features of atypical immunoblasts or Reed-Sternberg-like cells infected by EBV raises some questions about the function of p16/INK4a in these cells. Indeed, p16/INK4a accumulation could reflect functions other than the regulation of the cell cycle. For example, it could favor the senescence of cells, because it is known that p16/INK4a accumulation induces senescence in *in vitro* cells.[30,31]

Thus down-regulation of *p16/INK4a* is particularly observed in the lesions of PT-LPDs, which are known to

have a more aggressive course, have a large-cell lymphoma appearance, and are more frequently EBV-negative than the other lesions. On the contrary, the p16/INK4a protein is often present in EBV-positive polymorphic lesions. We tried to correlate *p16/INK4a* expression with clinical response in our population. Unfortunately, no significant correlation with patient outcome could be established, but the number of studied cases was relatively small, and several patients died rapidly because of infections or conditions other than their LPDs (data not shown). Further investigations are necessary to determine the clinical relevance of p16/INK4a status and to specify the role of EBV in the expression of this cell cycle inhibitor.

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