Loss of p16/INK4A Protein Expression in Non-Hodgkin's Lymphomas Is a Frequent Finding Associated with Tumor Progression

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The *CDKN2A* gene located on chromosome region 9p21 encodes the cyclin-dependent kinase-4 inhibitor p16/INK4A, a negative cell cycle regulator. We analyzed p16/INK4A expression in different types of non-Hodgkin's lymphoma to determine whether the absence of this protein is involved in lymphomagenesis, while also trying to characterize the genetic events underlying this p16/INK4A loss. To this end, we investigated the levels of p16/INK4A protein using immunohistochemical techniques in 153 cases of non-Hodgkin's lymphoma, using as reference the levels found in reactive lymphoid tissue. The existence of gene mutation, CpG island methylation, and allelic loss were investigated in a subset of 26 cases, using single-strand conformational polymorphism and direct sequencing, Southern Blot, polymerase chain reaction, and microsatellite analysis, respectively. Loss of p16/INK4A expression was detected in 41 of the 112 non-Hodgkin's lymphomas studied (37%), all of which corresponded to high-grade tumors. This loss of p16/INK4A was found more frequently in cases showing tumor progression from mucosa-associated lymphoid tissue low-grade lymphomas (31 of 37) or follicular lymphomas (4 of 4) into diffuse large B-cell lymphomas. Analysis of the status of the *p16/INK4A* gene showed different genetic alterations (methylation of the 5'-CpG island of the *p16/INK4A* gene, 6 of 23 cases; allelic loss at 9p21, 3 of 16 cases; and non-sense mutation, 1 of 26 cases). In all cases, these events were associated with loss of the p16/INK4A protein. No case that preserved protein expression contained any genetic change. Our results demonstrate that p16/INK4A loss of expression contributes to tumor progression in lymphomas. The most frequent genetic alterations found were 5'-CpG island

methylation and allelic loss. (Am J Pathol 1998; 153:887-897)

Progression through the cell cycle is regulated by complexes formed of cyclins and their associated catalytic subunits, cyclin-dependent kinases (CDKs). CDKs bind to G1 cyclins and control G1/S transition by the phosphorylation of pRb and other proteins. These complexes have recently been shown to interact with a group of small molecules known as CDK inhibitors, which inhibit cell cycle progression. These CDK inhibitors act by inhibiting CDK-mediated phosphorylation and subsequent functional inactivation of the Rb protein, preventing the release of E2F, DP1, and other transcription factors.^{1,2} The CDK4 inhibitor p16/INK4A is encoded by the *CDKN2A* gene (also known as *MTS1*, ie, multiple tumor suppressor 1) located on chromosome 9p21. The *p16/INK4A* sequence has been shown to encode a 156-amino acid protein that contains four ankyrin repeats, motifs that are recognized in protein-protein interaction.^{3,4} p16/INK4A exerts its function by competing with cyclin D in binding to CDK4 and preventing the activation of this kinase.^{3,4} It has been demonstrated that p16/INK4A prevents cellular transformation by H-Ras, acting as a tumor suppressor gene.⁵

p16/INK4A is frequently inactivated by biallelic deletion, although hemizygous deletion associated with mutation or allelic rearrangement has also been described in different types of malignancies. These genetic alterations at the 9p21 region have been found in a high percentage of tumor cell lines (70 to 80%),⁶⁻⁸ whereas in primary human tumors they have been identified with a slightly lower frequency (10 to 70% of cases).⁹⁻¹³ Previous studies in non-Hodgkin's lymphomas (NHL) have detected deletions and/or mutations at the *p16/INK4A* gene in a relatively low proportion of cases (0 to 14%); this proportion was higher in T-acute lymphoblastic leukemia.¹⁴⁻¹⁸

RV and MS-B contributed equally to this work.

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Inactivation of the *p16/INK4A* gene has also been described as arising due to *de novo* methylation at the 5'-CpG island, leading to transcriptional blockage of full-length *p16/INK4A*,¹⁹ while permitting the expression of a shorter transcript with a different exon 1 (*p16-β*).²⁰

The aim of this study was to determine whether or not *p16/INK4A* inactivation is involved in the development of NHLs. Because *p16/INK4A* inactivation has already been found to be the final consequence of several genetic alterations, we decided to complement molecular study with immunohistochemical analysis of *p16/INK4A* expression, as a majority of the *p16/INK4A* gene alterations characterized to date eventually give rise to the absence or reduction of the *p16/INK4A* protein. To this end, we selected a series of NHLs with various histological types, also including selected specimens from cases showing tumor progression from low- to high-grade lymphoma. Reactive lymphoid tissue samples (tonsil and lymph node) were included to define references of *p16/INK4A* staining in nontumor lymphoid cells. *In vitro* studies on resting and mitogenically stimulated peripheral blood lymphocytes (PBLs) were also performed to characterize changes in *p16/INK4A* protein expression along the cell cycle.

To determine the underlying genetic mechanism associated with loss of expression, a group of 26 cases was analyzed for loss of heterozygosity (LOH), mutational spectrum, and *de novo* methylation pattern.

Materials and Methods

In Vitro Studies

Normal peripheral blood was obtained by venipuncture from voluntary healthy donors. PBLs were isolated by Histopaque (Sigma Diagnostics, St. Louis, MO) density gradient centrifugation and washed in RPMI 1640. Cells were kept at 37°C in a 5% CO₂ humidified incubator in cell culture flasks at 2×10^6 cells/ml of RPMI 1640 supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, and 2% phytohemagglutinin (PHA) (Life Technologies, Inc., Grand Island, NY). Aliquots of the activated cells were harvested every 24 hours and prepared for analysis.

Control cell lines included in the study were Molt-4 (a T-acute lymphoblastic leukemia cell line defective in the *p16/INK4A* gene, with deletion of one allele and rearrangement of the other²¹) and Saos-2 (an osteosarcoma cell line defective in *p53* and *RB* genes). These cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Western Blot

Aliquots from cell lines were collected at confluence. Aliquots from Molt-4 and Saos-2 cell lines, PBLs, and PHA-stimulated PBLs were washed twice with cold phosphate-buffered saline (PBS) before protein extraction procedure. Protein was extracted with a triple-detergent lysis buffer (50 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl,

0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin) for 30 minutes at 4°C. Extracts were cleared by centrifugation. Fifty μg of extracted protein was resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose-ECL (Amersham, Poole, UK) according to the manufacturer's instructions. The blots were blocked overnight with 5% bovine serum albumin in PBS at 37°C. *p16/INK4A* protein was detected by using the monoclonal antibody DCS-50.1 (Calbiochem, Oncogene Research Products, La Jolla, CA), followed by incubation with goat anti-mouse antibody coupled to horseradish peroxidase (Amersham). Blots were developed using the enhanced chemiluminescence ECL detection kit (Amersham).

Flow Cytometry

The expression of *p16/INK4A* was also studied by flow cytometry (FCM) analysis of PHA-activated lymphocytes. To make it possible to detect this protein by immunofluorescence, fixation/permeabilization for optimal immunodetection was performed with paraformaldehyde/Triton X-100, according to the method of Aiello et al.²² The monoclonal antibody used was the same as in the Western blot (see above). An isotype-matched negative control was used to estimate the quantity of nonspecific binding.

Before labeling, the cells were incubated for 30 minutes with 20% goat serum. They were then incubated for 30 minutes at 4°C with antibodies, followed by two consecutive washes in 0.1% bovine serum albumin diluted in PBS. For *p16/INK4A* detection, cells were incubated once again (30 minutes at 4°C) with goat anti-mouse monoclonal antibody (F(ab)'2 fragments) conjugated with fluorescein isothiocyanate (Dakopatts, Glostrup, Denmark). After two more washes, the cells were analyzed.

Parallel samples of PHA-activated lymphocytes were prepared for FCM analysis of their nuclear DNA content using the CycleTEST DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Double staining for *p16/INK4A* and DNA was performed in PHA-activated lymphocytes that had previously been fixed, permeabilized, and indirectly labeled for *p16/INK4A* using a fluorescein isothiocyanate-tagged secondary antibody. The lymphocytes were then incubated (30 minutes at room temperature) with 100 μl of RNase (2 mg/ml) and 200 μl of propidium iodide (2 mg/ml).

FCM was performed using a FACSORT instrument (Becton Dickinson, San Jose, CA), equipped with a double discrimination module. Cell cycle analysis was carried out using the CELLFIT software package for data acquisition and analysis. Protein analysis was undertaken using the LYSIS II software program.

In Vivo Studies

Formalin-fixed paraffin embedded-tissue sections from 153 NHLs samples, 4 reactive tonsils, and 4 cases of

lymphadenitis were selected from the routine files of the Pathology Department of the "Virgen de la Salud" Hospital, Toledo, Spain.

Lymphomas were classified according to the REAL classification,²³ including 9 cases of B cell chronic lymphocytic leukemia, 6 cases of splenic marginal zone lymphoma, 8 cases of mantle cell lymphoma, 8 cases of low-grade (small cell) mucosa-associated lymphoid tissue (MALT) lymphoma, 10 cases of follicular lymphoma (FL), 23 cases of diffuse large B cell lymphoma of nodal origin (nodal DLBCL), 20 cases of DLBCL arising in mucosa (mucosa DLBCL), 14 cases of Burkitt's lymphoma, 12 cases of peripheral T-cell lymphoma, and 2 cases of anaplastic large cell lymphoma.

For practical purposes, we denominated those composed mainly of the small cell component as "low-grade MALT lymphoma," whereas large cell tumors located in gastric or other mucosa without an adjacent low-grade component were termed "mucosa DLBCL."

To address the possible role of p16/INK4A in tumor progression, we included samples of MALT lymphoma and FL showing aggressive transformation. Thus, samples from 37 cases of MALT lymphoma including simultaneously separate areas of small cells (low grade) and large cells (high grade) were included. These composite tumors in which both components (separate areas of small and large cells) could be discerned were denominated "MALT + DLBCL." A series of 4 cases of FL that contained diffuse areas of the large cell component was included as well with the same objective.

Immunohistochemistry Techniques

Immunohistochemistry detection of p16/INK4A was performed by incubating tissue sections overnight at 4°C with p16/INK4A monoclonal antibody (Calbiochem, Oncogene Research Products) diluted at 1:3000. After incubation with the primary antibody, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidin (Histostain Plus; Zymed, San Francisco, CA) with diaminobenzidine chromogen as substrate. Quick washes with Chemmate washing buffers (Dakopatts) between successive incubations were included to obtain a clean background.

For double-labeling experiments, after p16 immunodetection, an incubation with antibodies for CD20 (L26, Dakopatts) and CD3 (Polyclonal, Dakopatts) was performed, followed by incubation with the EnVision (Dakopatts) system, alkaline phosphatase, and Fast Red chromogen as substrate.

Incubation omitting the specific antibody, as well as with unrelated antibodies, was used as a control of the technique.

Results were interpreted according to established criteria.^{24,25} Briefly, p16/INK4A protein expression was considered normal when p16/INK4A nuclear immunostaining was present in all areas. The absence of nuclear staining in part or all of a tissue section was only considered to be a loss of p16/INK4A protein expression when the interspersed nuclei of reactive cells (lymphocytes, endothelial

cells) displaying p16/INK4A expression were observed and used as an internal positive control. A partial loss of p16/INK4A staining was considered to have occurred when areas of preserved staining alternated with others showing regional loss of staining by the tumor cells.

Double Immunolabeling and Laser Scanning Confocal Microscopy

Tissue sections were also stained using double labeling with both mouse monoclonal antibody anti-p16 DCS-50.1 (Calbiochem, Oncogene Research Products) diluted 1:3000 and rabbit polyclonal anti-CD3 diluted 1:50 (Dakopatts). After simultaneous overnight incubation at 4°C with primary antibodies, sections were incubated with biotin-conjugated donkey anti-rabbit immunoglobulin G (1:50, Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-mouse immunoglobulin G (1:50, Jackson ImmunoResearch Laboratories). After washing in Tris-buffered saline, sections were incubated with streptavidin Cy2 (1:50, Amersham) and Cy3-conjugated donkey anti-goat immunoglobulin G (1:50, Jackson ImmunoResearch Laboratories). Sections were mounted with glycerol and examined with an MRC 1024 Bio-Rad confocal system (Bio-Rad, Richmond, CA) mounted on a Zeiss Axiovert 135 microscope (Zeiss, Oberkochen, Germany). A 25-mw multilinea argon laser producing two major lines at 488 and 514 nm, was used. Images were stored on magnetic optical disks. Series of images were processed with the Lasersharp software package (Bio-Rad).

Molecular Studies

A total of 26 lymphoma DNA samples were included in the study, including cases with normal and absent p16/INK4A protein expression. Cases were included at random on the basis of the availability of frozen material for DNA extraction. Each tissue fragment used for molecular study was paralleled by another equivalent fragment that was processed for routine histology. DNA from reactive tonsils and PBLs from healthy donors were also included in the study.

Polymerase Chain Reaction-Single-Strand Conformational Polymorphism

Single-strand conformational polymorphism (SSCP) analysis was used to screen for p16/INK4A gene mutations, according to a slightly modified protocol of the type previously reported by Orita et al.²⁶ We analyzed exons 1 and 2 of the CDKN2A gene, comprising 97% of the coding sequence. One hundred ng was amplified with 30 pmol of each primer, 200 μ mol/L deoxynucleotide triphosphates, 5% dimethylsulfoxide, 1 μ Ci of ³²P (Amersham), and 0.5 U of Taq polymerase with the primers previously described by Kamb et al.⁶ Twenty-five cycles of the polymerase chain reaction (PCR) at 94°C, 60°C, and 72°C for 20 seconds, 30 seconds, and 1 minute,

respectively, with five previous cycles at a 68°C annealing temperature were performed in a Thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The products were diluted in formamide buffer and electrophoresed at room temperature and at 10°C, with and without 10% of glycerol, for 16 to 18 hours at 7 to 12 W.

Direct Sequencing

Sequences of samples showing mobility shifts by PCR-SSCP analysis were confirmed by direct sequencing with an Automated DNA Sequencer ABI PRISM 310 Genetic Analyzer (Perkin Elmer) according to the manufacturer's instructions.

Methylation

PCR was used to determine the methylation status of the first exon of *p16/INK4A*, as described by Martinez-Delgado et al.²⁷ Briefly, 100 ng of DNA digested by *EagI* enzyme was amplified in a multiple PCR for exons 1 and 2 of the *p16/INK4A* gene. Products were resolved on a 2% Nusieve agarose gel, blotted onto nylon membranes, and hybridized to digoxigenin-labeled internal oligonucleotide for exons 1 and 2. Oligonucleotide sequences were as follows: 5'-CCAGGCATCGCGCACGTCCAG-3' for exon 1 and 5'-AGGAGGTGCGGGCGCTGCTG-3' for exon 2. After digestion, only methylated tumors showed amplification of exon 1. Southern blot analysis was used to confirm the methylation status of the *p16/INK4A*, using restriction enzymes *EcoRI* and *SacII* in a double digestion. *SacII* does not cut DNA when there is methylation of the restriction site. Five μ g of DNA was digested using 40 units of *EcoRI* and 60 units of *SacII* enzyme electrophoresed in 0.8% agarose gel and hybridized with a PCR-generated probe corresponding to exon 1 of *p16/INK4A*.

Analysis of Allelic Loss on Chromosome 9p21

DNA from lymphoid tumors and reactive lymphoid tissue was analyzed for LOH or homozygous deletion by amplification of dinucleotide repeat-containing sequences using PCR.¹⁶ Primers D9S171, D9S1747, D9S1748, D9S1749, and interferon α (IFN α) were obtained from Research Genetics (Huntsville, AL). The amplification conditions were: 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 45 seconds, for 25 cycles. Radiolabeled, amplified products were separated by electrophoresis in denaturing 8 mol/L urea-6% acrylamide gels, followed by autoradiography. Homozygous deletion was scored when one or more closely spaced markers demonstrated apparent retention of heterozygosity when flanked by markers demonstrating clear LOH.¹⁶ The signal intensity of fragments was determined by densitometry (one-dimensional analysis and hand scanner settings; Biomed Instruments, Zeineh Programs Fullerton, CA), scoring LOH if an entire band was missing or the intensity of the band was reduced below >40% of the normal band^{27,28}.

Results

In Vitro Studies

PBLs had an undetectable *p16/INK4A* protein signal when studied by Western blot. After PHA stimulation, a 16-kd band corresponding to *p16/INK4A* protein was detected at 24 hours, with the signal attaining a moderate intensity at 72 hours.

The FCM expression of *p16/INK4A* in PHA-stimulated PBLs is illustrated in Figure 1A. The expression of *p16/INK4A* on unstimulated PBLs was restricted to 11.26% of

Figure 1. A: One-color (left) and two-color (right) FCM analysis of *p16* and cell cycle in PHA-stimulated PBLs; double labeling for propidium iodide and *p16/INK4A*, showing a progressive increase in *p16/INK4A* staining along the progression of the cell cycle. B: Diagram illustrating *p16/INK4A* changes along the cell cycle phases (upper line) and cells in the S and G2-M phases (lower line).

Figure 2. A and B: Low and high power views of a reactive lymphoid follicle showing p16/INK4A staining of both mantle and germinal center cells. C: p16/INK4A staining of a MALT lymphoma, in which a large percentage of tumor cells show preserved staining. Notice the staining of some epithelial cells. D: Focal p16/INK4A loss in a case of large cell transformation of MALT lymphoma. Small tumor cells show the usual pattern of p16/INK4A staining, whereas large cells are negative. E: p16/INK4A-positive large B-cell lymphoma. F: Low power view of a p16/INK4A-negative Burkitt lymphoma. A positive internal control is provided by the endothelial and epithelial cells. G: Double staining of p16/INK4A and CD20 in an FL; most of the p16-positive cells are CD20. H: Double staining of p16/INK4A and CD3, in which CD3 staining of p16-positive cells is more rare.

Table 1. p16/INK4A Expression Detected by Immunohistochemistry Techniques in NHLs, According to Histological Type

	Positive	Negative		Total
		Focal	Diffuse	
B-CLL	9	0	0	9
MCL	8	0	0	8
FL	10	0	0	10
MALT, low grade	8	0	0	8
SMZL	6	0	0	6
Nodal DLBCL	11	2	10	23
Mucosa DLBCL	6	0	14	20
Burkitt's lymphoma	6	5	3	14
PTCL and ALCL	7	7	0	14
Total	71 (63%)	14 (12%)	27 (24%)	112

B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle cell lymphoma; SMZL, splenic marginal cell lymphoma; PTCL, peripheral T-cell lymphoma; ALCL, anaplastic large cell lymphoma.

Figure 3. Double immunostaining by confocal microscopy for CD3 (green) and p16 (red) in both an FL (A) and a reactive germinal center (B), showing that some scattered T cells are p16 positive, but most of the p16-positive cells are B cells.

cells. However, after 24 hours of PHA stimulation, PBLs displayed a marked increase in p16/INK4A reactivity, amounting to 39.53% of positive cells at 96 hours after PHA stimulation.

A two-color immunofluorescence analysis confirmed this increase in the expression of p16/INK4A protein, when all of the phases of the cell cycle are considered (Figure 1B). This increase was also found when only the S and G2/M phases were analyzed.

In Vivo Studies

Reactive Lymphoid Tissue

A diffuse mosaic pattern of p16/INK4A nuclear immunoreactivity was observed in all of the different lymphoid compartments (germinal centers, mantle cells, and interfollicular space), in different proportions of cells. A proportion of roughly one-third of positive cells was found in the different compartments, with a slight increase in the germinal centers (Figure 2). Similar results were obtained in the eight different samples analyzed.

Tumors

All 41 cases of NHL with a low growth fraction (chronic lymphocytic leukemia, mantle cell lymphoma, FL, MALT low grade, and splenic marginal zone lymphoma) showed preserved normal p16/INK4A protein expression, characterized by a diffuse mosaic pattern of p16/INK4A-positive nuclear immunoreactivity of the neoplastic cells. Of the 71 NHLs with a high-growth fraction, p16/INK4A protein expression was maintained in 30 cases (42%) and had undergone alteration in the remaining 41 cases (58%) because of partial (14 cases, 20%) or total loss (27 cases, 38%) (Table 1 and Figure 2).

For the purposes of this analysis, only those areas of tumor showing p16/INK4A staining of reactive lymphocytes and endothelial cells were considered, thus providing an internal positive control that prevented potential misinterpretation due to staining defects. In all of the low-grade tumors analyzed, p16/INK4A expression was mainly present in small- or medium-sized neoplastic nu-

Table 2. p16/INK4A Expression in Cases Presenting Tumor Progression, with Low- and High-Grade Components

	p16 positive	p16 negative	
		Diffuse	Focal
MALT low grade + DLBCL			
Small cells	37	0	0
Large cells	6	23	8
FL + DLBCL			
Small cells	4	0	0
Large cells	0	4	0

MALT low grade + DLBCL, composite tumors showing areas of MALT low grade evolving into DLBCL; FL + DLBCL, composite tumors showing areas of FLs evolving into DLBCL.

clei, and it was unusual to find p16/INK4A-positive large cells (Figure 2). To confirm that positive cells were tumor cells and not bystander T cells, a double-immunostaining procedure was performed that showed that most p16-positive cells were B cells, whereas it was possible to find only occasional p16-positive T cells (Figure 2). These results were confirmed using double immunostaining by laser scanning confocal microscopy with both CD3 and p16 antibodies, in both an FL and a reactive germinal center, which detected p16 expression in both cell types, although most p16-positive cells in the FL case were B cells (Figure 3).

DLBCLs of nodal and mucosal origins presented the highest incidence of total loss of p16/INK4A expression, which took place in 10 of 23 cases (43%) of nodal DLBCL and 14 of 20 (70%) of mucosa DLBCL. However, this complete absence of p16/INK4A protein was only seen in 3 of 14 Burkitt cases (21%) and in none of the 15 peripheral T-cell lymphoma and anaplastic large cell lymphoma cases (Figure 2). Macrophage staining was usually weak or null, thus not precluding the recognition of p16/INK4A expression in aggressive lymphomas.

For the analysis of an eventual p16/INK4A loss in tumor progression, a large group of cases with both low- and high-grade components were included (Table 2). All 37 MALT + DLBCL cases showing transformation from the small cell/low growth component into an aggressive variant displayed normal diffuse p16/INK4A protein expression by the small cell areas. However, p16/INK4A expres-

sion by the large cell/high growth component was normal in only 6 cases (16%) and was found to be altered in the remaining 31 cases (84%), either by focal ($n = 8$, 22%) or total loss ($n = 23$, 62%) (Figure 2). Likewise, the 4 FL cases showing areas of transformation into DLBCL presented normal p16/INK4A expression by the low-grade component, whereas in the same specimens, a p16/INK4A loss in the large cell component was found in all 4 cases.

P16/INK4A Gene Study

Analysis of p16/INK4A Gene Mutation

Only one case was found to be mutated when 26 samples were screened for p16/INK4A mutations (4%) (Table 3 and Figure 4). Nucleotide sequences of the *CDKN2A* gene with mobility shifts shown by PCR-SSCP analysis were determined by direct sequencing. Four nucleotide changes were identified in three different cases in exon 2. Three of the changes correspond to a G→A transition in codon 148 that substitutes ACG^{Thr} for GCG^{Ala}. This missense mutation has previously been reported as a polymorphism.⁶ Cases L170 and L169 show heterozygous polymorphism, whereas case BK20 shows hemi- or homozygous polymorphism. The fourth change, found in a Burkitt lymphoma (BK20), is a C→G transversion at codon 129 (Figure 4). This transversion produces a nonsense mutation TAC^{Tyr}→TAG^{Stop} at the fourth ankyrin repeat domain of *CDKN2A*. The wild-type allele can be observed (Figure 4B).

Analysis of Methylation at CpG Islands of the p16/INK4A Gene

The methylation status of two CpG islands in exon 1 of p16/INK4A was analyzed by PCR and Southern blot using two methylation-sensitive enzymes, *EagI* and *SacII*. We examined a group of 23 cases with low and high proliferation indexes. An abnormal pattern of methylation was detected in 6 of the 23 cases studied (26%), all of which showed a high proliferation index. As is shown in Figure 5, exon 1 of p16/INK4A was amplified only in cases methylated after digestion with *EagI*. Nevertheless, exon

Table 3. Frequency of Genetic Alteration of p16/INK4A Related to Histological Diagnosis

	p16 protein loss	Allelic loss	Mutation	Methylation
CLL	0/9	0/1	0/2	0/2
MCL	0/8	0/1	0/1	0/1
FL	0/10	0/1	0/2	0/2
MALT low grade	0/8	ND	ND	ND
SMZL	0/6	0/4	0/4	0/2
Nodal DLBCL	12/23 (52%)	0/1	0/4	2/4
Mucosa DLBCL	14/20 (70%)	ND	ND	ND
FL + DLBCL	4/4 (100%)	1/2	0/3	0/3
MALT + DLBCL	31/37 (84%)	2/4	0/5	3/5
Burkitt's lymphoma	8/14 (57%)	0/3	1/6	1/5
PTCL and ALCL	7/14 (50%)	ND	ND	ND
Total	76/153 (49%)	3 / 16 (19%)	1 / 26 (4%)	6 / 23 (26%)

CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; SMZL, splenic marginal zone lymphoma; PTCL, peripheral T-cell lymphoma; ALCL, anaplastic large cell lymphoma; ND, not done.

Figure 4. Mutational analysis by PCR-SSCP and sequencing of the exon 2 from *p16/INK4A* gene in tumor BK20. **A:** SSCP exhibits anomalous emigration of BK20. **B:** DNA sequencing of the antisense chain showing a single-base transition in codon 129 of tumor DNA that produces a nonsense mutation (TAC^{Tyr}→TAG^{Stop}). The normal allele can also be detected, suggesting the existence of heterozygous mutation in the tumor or admixed reactive cells around the tumor.

2, which lacks an *EagI* site, was amplified in all cases. These experiments were performed three times, and the same results were obtained in all of them. For all of the cases except one (LL173), hypermethylation status was confirmed with another methylation-sensitive enzyme (*SacII*) detected by Southern blot. The diagnoses corresponding to the methylated cases were MALT + DLBCL

Figure 5. Methylation status of the *p16/INK4A* exon 1 detected by PCR. Products of exons 1 (320 bp) and 2 (510 bp) are indicated. The presence of an amplified exon 1, after digestion with *EagI*, indicates that the *EagI* site was methylated. Digested DNA from PBLs showed absence of methylation at *EagI* site. Methylation in cases BK21, LL139, and LL173 is revealed by exon 1 amplification.

(three cases), nodal DLBCL (two cases), and Burkitt's lymphoma (one case) (Table 3).

Allelotype Analysis with Microsatellite Markers

Sixteen cases of NHL for which DNA from noninfiltrate tissue was available were studied using markers for the microsatellites surrounding the *CDKN2A* locus on 9p21. Allelic loss was found in 3 of 16 cases (19%), all of them high-grade tumors (Table 3 and Figure 6). The genetic change detected by microsatellite analysis was LOH in the three cases, with no cases showing homozygous deletion. In the case MT51, it is not possible to assume that the LOH detected at *IFNa* locus implicates the *p16* gene.

Relationship between Changes in Protein Expression and Genetic Findings

No genetic alteration was found in the group of 13 cases with preserved *p16/INK4A* expression (Table 4). Consequently, no genetic alteration was identified in low-grade lymphomas. In contrast with this finding, 9 of 13 cases with abnormal protein expression, with either partial or total loss, showed different genetic changes (Tables 4 and 5). The most frequent alteration identified in the cases with *p16/INK4A* loss of expression was CpG island methylation (6 of 12 cases), followed by allelic loss (3 of 9 cases) and, more rarely, nonsense mutation (1 of 13 cases).

Table 4. Relationship between Changes in Protein Expression and Genetic Findings in *p16/INK4A* in NHLs

	Cases	Mutation	Methylation	Allelic loss
p16 loss	13	1/13	6/12	3/9
p16 preservation	13	0/13	0/11	0/7

Figure 6. A: Allelic loss at IFNa, D9S1749, D9S1747, D9S1748, and D9S171 markers in nine cases of NHLs that have lost p16/INK4A protein. NI, noninformative; ND, not done. **B:** Demonstration of allelic loss at IFNa and D9S171 markers. N and T, normal and tumor paired samples, respectively.

There are four p16/INK4A-negative cases in which genetic study did not disclose underlying genetic alterations. Nevertheless, we cannot discard the existence of these genetic alterations in this group of cases, because their study has been restricted due to the existence of noninformative microsatellites (LL198 and LL182), lack of

normal tissue for analysis of allelic loss (LL168), or the absence of DNA suitable for Southern blot study (BK13).

Analysis of the cases did not show any specific relationship between individual genetic changes and histological diagnosis.

The partial expression of p16 protein in case BK20, with the detection of apparent hemizygous polymorphism and heterozygous mutation (Figure 4B), suggests that this case lost one allele in the tumor and later in evolution acquired the mutation in the left allele in only a part of the tumor.

Discussion

This study examined p16/INK4A protein expression in normal and mitogenically stimulated PBLs and reactive and neoplastic lymphoid tissue.

The expression of p16/INK4A was weak in PBLs as detected by FCM. However, after PHA stimulation, these cells display a rise in p16/INK4A expression, which is more accentuated in the S and G2-M phases of the cell cycle. This is consistent with previous studies showing that the level of p16/INK4A protein varies along the cell cycle.²⁹ In concordance with these data, the samples from reactive lymphoid tissue (tonsils and lymph nodes) showed a variable intensity of staining in the different cellular microenvironments present in lymphoid tissue. Thus, although cells in all compartments express p16/INK4A, higher levels are found in those compartments where cell proliferation is increased (the germinal center and interfollicular area), in comparison with the weaker levels found in the mantle zone, where mainly resting lymphocytes are found.

Here we analyzed p16/INK4A protein expression in a series of 153 NHLs: 41 low-grade and 71 high-grade tumors, also including 41 cases with aggressive transformation from low to high grade. The pattern found in NHLs differs from that identified in reactive lymphoid tissue. Thus, although the expression of p16/INK4A is a constant feature in all 41 cases of low-grade NHL, it seems to be

Table 5. Summary of p16/INK4A Genetic Alteration Findings in the Group with Loss of p16/INK4A Protein Expression

Number	Diagnosis	p16 protein expression*	Gene status [†]	Allelic loss at 9p21	Methylation in exon 1 [§]
L174	MALT + DLBCL	-/+	wt		met +
L139	MALT + DLBCL	-	wt	ND	met +
MT51	MALT + DLBCL	-	wt	LOH	met +
MT53	MALT + DLBCL	-	wt	LOH	
L198	MALT + DLBCL	-	wt		
L182	FL + DLBCL	-	wt		
LF4	FL + DLBCL	-	wt	LOH	
L168	FL + DLBCL	-	wt	ND	
L173	Nodal DLBCL	-	wt		met +
L169	Nodal DLBCL	-	wt	ND	met +
BK13	Burkitt's lymphoma	-/+	wt		ND
BK20	Burkitt's lymphoma	-/+	Mut	ND	
BK21	Burkitt's lymphoma	-/+	wt		met +

*p16 protein expression: -/+, partial loss; -, diffuse loss.

[†]wt, wild type; Mut, mutation.

[§]met +, existence of CpG methylation; ND, not done.

lacking in a significant proportion (41 of 71) of high-grade tumors due to total (27 cases) or partial loss (14 cases).

Our results show that this loss of p16/INK4A, besides being more frequent in aggressive cases, is strongly associated with tumor progression from low-grade to high-grade neoplasms. Thus, when the group of 41 cases showing areas of aggressive transformation from low- to high-grade component was analyzed, the low-grade component displayed normal p16/INK4A protein expression, which was confirmed by using double immunostaining for CD20 or CD3 and p16/INK4A, whereas the expression of p16/INK4A protein appears to be altered in the high-grade component in a majority of cases (35 of 41) either by partial (8 cases) or total (27 cases) loss. These data are roughly in agreement with those obtained for other types of tumors, in which the loss of p16/INK4A expression has been found to be associated with tumor progression.^{30–32} Indeed, the largest group in which p16/INK4A loss was found to be associated with tumor progression, large B-cell lymphoma presenting in mucosa, is claimed to frequently arise superimposed on low-grade MALT lymphomas, as an aggressive transformation of the same.³⁴

Several previous studies have addressed the frequency of *p16/INK4A* molecular alterations in lymphoid malignancies. Frequent loss of p16/INK4A in T-acute lymphoblastic leukemia has been confirmed by different authors.^{21,34,35} Although the incidence of *p16/INK4A* homozygous deletion in NHL found to date ranged from 0 to 12%,^{15,17,18,35–37} it was significantly more frequent in high-grade neoplasms^{18,37} and lymphoid cell lines.⁸

The results shown here take advantage of the high sensitivity of immunohistochemical techniques for the detection of p16/INK4A loss, increasing the frequency of the finding of p16/INK4A loss in high-grade neoplasms while confirming its specific distribution in the subset of cases characterized by higher aggressiveness and showing that p16/INK4A loss is characteristically associated with tumor progression in NHLs.

Study of the genetic basis for this p16/INK4A loss disclosed some noticeable findings, mainly the existence of a relatively good correlation between immunohistochemistry and genetic studies and the high frequency of 5'-CpG island methylation. These observations are consistent with previous studies on the same subject. Thus, *p16/INK4A* mutation in NHLs has only rarely been detected,¹⁷ and the most frequent molecular alteration identified to date is homozygous deletion,^{17,21,34–38} although recent studies provide data that suggest that 5'-CpG methylation of *p16/INK4A* could play a significant role in p16/INK4A inactivation in lymphomas^{28,39} and myelomas,⁴⁰ as has also been seen in other tumors.^{19,30}

Thus, p16/INK4A inactivation seems to play a major role in tumor progression in lymphomas, in parallel with p53 mutations.³⁷ Further efforts are, however, necessary to identify additional genetic changes in cell cycle-negative regulators that complement these findings and allow a more comprehensive explanation for NHL pathogenesis.

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