p14^{ARF} nuclear overexpression in aggressive B-cell lymphomas is a sensor of malfunction of the common tumor suppressor pathways

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p14^{ARF}, the alternative product from the human *INK4a/ARF* locus, antagonizes Hdm2 and mediates p53 activation in response to oncogenic stimuli. An immunohistochemical study of p14^{ARF} expression in 74 samples of aggressive B-cell lymphomas was performed, demonstrating an array of different abnormalities. A distinct nucleolar expression pattern was detected in nontumoral tissue and a subset of lymphomas (50/74). In contrast, a group of cases (8/74) showed absence of p14^{ARF} expression, dependent either on promoter hypermethylation or gene loss. Additionally, 16 out of 74 cases displayed an abnormal nuclear p14^{ARF} overexpression not confined to the nucleoli, as confirmed by confocal microscopy, and that was associated with high levels of p53 and Hdm2. A genetic study of these cases failed to show any alteration in the p14^{ARF} gene, but revealed the presence of p53 mutations in over 50% of these cases. An increased growth fraction and a more aggressive clinical course, with a shortened survival time, also characterized the group of tumors with p14^{ARF} nuclear overexpression. Moreover, this p14^{ARF} expression pattern was more frequent in tumors displaying accumulated alterations in the p53, p16^{INK4a}, and p27^{KIP1} tumor supressors. These observations, together with the consideration of the central role of p14^{ARF} in cell cycle control, suggest that p14^{ARF} abnormal nuclear overexpression is a sensor of malfunction of the major cell cycle regulatory pathways, and consequently a marker of a high tumor aggressivity. (Blood. 2002;99:1411-1418)

Introduction

The 2 major tumor suppressor pathways, represented by the proteins p16^{INK4a}-CDK4/6-Rb and p14^{ARF}-Hdm2-p53,[1] are inactivated in most human cancers. p53 is a transcription factor that induces cell cycle arrest and/or apoptosis in response to a variety of stimuli (eg, DNA damage, hyperproliferative signals).[2] p53 is negatively regulated by Hdm2 through a multiple mechanism: Hdm2 binds to the transactivation domain of the p53 tetramer, inhibiting p53 transcriptional activity[3,4]; in addition, Hdm2 functions as an E3-ubiquitin ligase which targets p53 for nuclear export and proteasomal degradation.[5-8] *Hdm2* is itself a p53-responsive gene, thus establishing a feedback loop through which p53 regulates its own activity and turnover.[9]

The atypical structure of the *INK4a/ARF* locus in 9p21 encodes 2 unrelated tumor suppressor proteins, p16^{INK4a} and p14^{ARF} (the human counterpart of murine p19^{ARF}).[10,11] These are specified by different first exons that are spliced to a common second exon translated in alternative reading frames, their expression being controlled by independent promoters. p16^{INK4a}, a specific inhibitor of cyclin D–dependent kinases, contributes to G1 arrest by blocking Rb phosphorylation.[1] On the other hand, p14^{ARF} interferes with all of the known functions of Hdm2 (eg, direct inhibition of p53-mediated transactivation,[12,13] ubiquitin ligase activity,[14] and nuclear export of p53[15,16]), possibly through induction of Hdm2 degradation,[17] indirectly leading to an increase in the activity and stability of p53. p14^{ARF} is induced by inappropriate hyperproliferative signals (such as *myc*,[18] E2F-1,[19] *ras*,[20] E1A,[21] v-*abl*[22]) and mediates p53 activation in response to oncogenic stimuli. Specifically, responsiveness of the p14^{ARF} promoter to E2F-1 makes p14^{ARF} a nexus between the Rb and p53 pathways. p53 suppresses p14^{ARF} expression through a poorly understood mechanism, which generates an additional regulatory circuitry.[13,23]

p14^{ARF} is a highly basic protein that localizes to the nucleolus.[10,12,24] When induced, p14^{ARF} binds to Hdm2, thereby allowing p53 to stabilize and accumulate in the nucleoplasm. Classically, this p14^{ARF}-Hdm2 binding has been assumed to take place in the nucleolus,[16] although antagonization of Hdm2 by p14^{ARF} independently of nucleolar localization has recently been reported.[25] In addition, it has been suggested that p14^{ARF}-p53 direct binding without requirement for Hdm2 as a bridging molecule is also possible,[23] although the functional implications of this interaction remain unknown.

Both human and murine p14^{ARF} contact the central acidic domain of Hdm2 through 2 independent binding sites.[26-28] Additionally, nucleolar localization sequences (NrLS) have been mapped in exons 1β and 2 of p14^{ARF}. These motifs are required for the localization of p14^{ARF} to the nucleolus; mutations in the p14^{ARF} NrLS have been described as impeding the correct localization of this protein, resulting in its nucleoplasmic accumulation and the consequent loss of its ability to stabilize p53.[29]

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In human tumors, the p53 gene is inactivated by mutation in more than 50% of cases; in a high proportion of the rest, the p53 pathway would be expected to be disrupted by *Hdm2* amplification or $p14^{ARF}$ loss. In some cancers, the frequency of $p14^{ARF}$ alteration is remarkably high; deletions affecting the 9p21 region (eg, in the cases of glioblastoma and astrocytoma[30,31]) and hypermethylation of CpG islands in the $p14^{ARF}$ promoter (eg, in the case of gastric cancer[32]) are the main inactivation mechanisms. Point mutations are infrequent and usually also affect $p16^{INK4a}$. In other neoplasias, $p14^{ARF}$ loss appears to be a rarer event. Nevertheless, most of the information available is derived from analysis at the gene level, whereas little is known about the expression level and distribution of the protein in different tumors.

In aggressive B-cell non-Hodgkin lymphomas (NHLs), the frequency of p53 mutation is modest (~20%),[33,34] whereas amplification of the 12q14 region (where the *Hdm2* gene is located) has not been detected.[35] Therefore, our purpose was to investigate the expression pattern of the $p14^{ARF}$ protein and its subcellular localization, the possible presence of genetic or epigenetic alterations in $p14^{ARF}$, and the relationship between the status of $p14^{ARF}$ and the major tumor suppressor pathways in a group of large B-cell lymphoma and Burkitt lymphoma.

Materials and methods

Tissue samples

We obtained 74 samples of tumor specimens from aggressive NHLs from the routine files of the Virgen de la Salud Hospital (Toledo, Spain) and the Spanish National Cancer Center (CNIO) Tumor Bank (Madrid, Spain) and diagnosed using the criteria of the Revised European-American Lymphoma (REAL) classification.[36] The samples included 55 cases of diffuse large B-cell lymphoma (DLBCL), 7 cases of follicular lymphoma grade 3 (FL-3), and 12 cases of Burkitt lymphoma (BL). Frozen tissue (for performing molecular studies) and clinical follow-up information were available for 51 of these samples (36 DLBCL, 3 FL-3, and 12 BL).

Cell lines

The human lymphoid cell lines RAJI, NAMALWA, MOLT-4, GRANTA-519, KARPAS-422, WSU-NHL, RPMI 8226, and HuT 78 were obtained from ATCC (Manassas, VA). Cells were cultured in Dulbecco modified Eagle medium (DMEM) (GRANTA-519) or RPMI-1640 medium (all other cell lines) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. Cells were kept in cell culture flasks in a humidified incubator at 37°C and 5% CO_2.

For immunostaining, cells were harvested by centrifugation, washed with cold phosphate buffered saline (PBS), cytospun onto poly-L-lysine–coated slides, and fixed in ethanol/acetone 1:1.

Mutational analysis

Exons 5 to 8 of the p53 gene and exons 1β and 2 of the $p14^{ARF}$ gene were amplified from genomic DNA extracted from tissue samples and cell lines, using previously described primers and conditions.[31,34,37] Polymerase chain reaction (PCR) products were purified using the Microcon PCR system (Millipore, Bedford, MA). Direct sequencing of purified PCR products was performed with an automated DNA Sequencer ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). Results for p53 and $p14^{ARF}$ exon 2 mutations in tumor samples have been previously published.[33,38]

Allelic loss

Homozygous deletions affecting $p14^{ARF}$ in cell lines were confirmed by simultaneous amplification of $p14^{ARF}$ exon 2 and p53 exon 8 (multiplex PCR). Results for homozygous or hemizygous deletions in the 9p21 region in the series of NHLs have been published as part of previous studies concerning $p16^{INK4a}$ status.[33,38]

Analysis of $p14^{ARF}$ promoter hypermethylation

Methylation-specific PCR (MSP)[39] assays were performed to determine the methylation status of the CpG islands of the $p14^{ARF}$ promoter in tissue samples and cell lines. Briefly, 1 μg of denatured genomic DNA was modified by reaction with sodium bisulfite under conditions that convert unmethylated cytosines to uracils. Modified DNA was purified using the Wizard DNA Clean-Up system (Promega, Madison, WI). Modification was completed by NaOH 0.3 M treatment for 5 minutes at room temperature, followed by ethanol precipitation.

A quantity of 50 ng bisulfite modified DNA was amplified using $p14^{ARF}$ unmethylated-specific and methylated-specific primers.[40] The Hodgkin disease–derived L-540 cell line was used as a positive control of $p14^{ARF}$ methylation[41]; DNA from nontumoral samples was included as a negative control. Methylation of the $p14^{ARF}$ promoter was detected by the amplification of a 122-bp fragment with the methylated-specific primers.

Antibodies

The following primary antibodies were used for immunohistochemistry: goat polyclonal anti-$p14^{ARF}$ C-18 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-Hdm2 Ab-1 2IF-2 (Oncogene, Darmstadt, Germany); mouse monoclonal anti-p53 DO-7 (Novocastra, Newcastle upon Tyne, United Kingdom). Proliferation index was evaluated by the expression of the nuclear antigen Ki67, detected with the MIB1 monoclonal antibody (Novocastra).

Immunofluorescence was performed with the same antibodies, with the exception of Hdm2, which was detected with the SMP14 monoclonal antibody (Santa Cruz Biotechnology). Mouse monoclonal anti-C23 MS-3 (Santa Cruz Biotechnology) was used as a nucleolar marker.[42,43]

Immunohistochemistry

Immunohistochemical techniques were performed on paraffin-embedded tissue sections or cytospin preparations of the cell lines using an initial heat-induced antigen retrieval step (slides were heated in a pressure cooker for 3 minutes in a 0.01 M solution of sodium citrate prior to incubation with the antibodies).

After incubation with the primary antibodies, immunodetection was performed with biotinylated antimouse or antigoat secondary antibodies as appropriate, followed by peroxidase-conjugated streptavidine (DAKO, Carpinteria, CA) with diaminobenzidine chromogen as substrate. Immunostaining was performed with the Techmate 500 (DAKO) automatic immunostaining device. Incubation omitting the specific antibody was used as a control of the technique.

p53 and Hdm2 expression were scored semiquantitatively and expressed as the nearest tenth percentile. Ki67 expression was quantified by scoring up to 200 tumoral cells in representative areas.

Immunofluorescence and confocal microscopy

Double fluorescent immunolabeling was performed on 3-μm–thick tissue sections mounted on poly-L-lysine–coated slides. After antigen retrieval and simultaneous incubation with the 2 primary antibodies, slides were washed and incubated with the secondary antibodies Cy3-conjugated donkey anti–goat (Jackson Immunoresearch, Baltimore, MD) and Alexa 488–conjugated rabbit anti–mouse (Molecular Probes, Eugene, OR). Nuclei were stained with TO-PRO-3 (Molecular Probes). The slides were mounted with VectaShield (Vector, Burlingame, CA) and examined with a TCS NT laser scanning confocal microscopy system (Leica Microsystems, Wetzlar, Germany). The series of images was processed with the software package provided (Leica Microsystems) and Adobe Photoshop 5.5.

Statistical analysis

The relationship between $p14^{ARF}$ and other variables (p53, Hdm2, Ki67) was examined using the Kruskal-Wallis test. In order to assess the specific

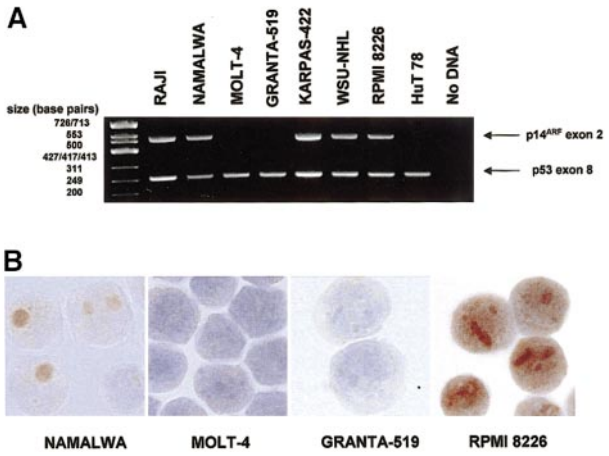

Figure 1. p14ARF expression in lymphoid cell lines. (A) Multiplex PCR demonstrating deletion of p14ARF exon 2 in some of the cell lines. p53 exon 8 is simultaneously amplified as a control. (B) Images showing absence of p14ARF staining in 2 cell lines with exon 2 deletion (MOLT-4, GRANTA-519) and nucleolar p14ARF expression in 2 nondeleted cell lines (NAMALWA, RPMI 8226). Note the nucleoplasmic staining for RPMI 8226 observed in addition to the nucleoli. Original magnification × 1000.

association between p14ARF and Ki67, a multivariate analysis was performed including those markers significantly related to Ki67 levels as revealed by the univariate analysis. Since neither Ki67 nor its log-transformed values were normally distributed, as assumed by standard multiple regression techniques, a median regression analysis[44] was performed. This method compares the median instead of the mean of each category.

The prognostic value of p14ARF and all other markers was evaluated by standard survival analysis, using Kaplan-Meier and Cox regression. Hazard ratios were computed for each marker, adjusting for International Prognostic Index (IPI) and histology.

Statistical analyses were performed using the STATA and SPSS software packages.

Results

Analysis of p14ARF, Hdm2, and p53 expression

Cell lines. p14ARF expression was analyzed by immunostaining in several human lymphoid cell lines in which p53 and p14ARF gene status were also determined (Figure 1; Table 1).

Inactivation of the p53 pathway was observed in the vast majority (7/8) of cell lines: 3 out of 8 lines had only p53 mutation; 1 out of 8 had only p14ARF deletion; 2 out of 8 featured both alterations simultaneously; 1 out of 8 had both p53 mutation and a heterozygous mutation within p14ARF exon 2 (R120L). Only in one of the cell lines were no alterations detected. None of the cell lines analyzed showed p14ARF promoter hypermethylation.

Loss of p14ARF expression was observed exclusively in the cell lines displaying p14ARF deletion. In the cell lines expressing p14ARF the signal was predominantly nucleolar, although additional nucleoplasmic staining was evident in the RPMI 8226 cell line (Figure 1).

Normal human tissues. In nontumoral lymphoid tissue (tonsils and reactive lymphadenitis), reactive cells showed distinct nucleolar p14ARF immunostaining (Figure 2A). Staining for Hdm2 (Figure 2B) and p53 (Figure 2C) revealed weak nucleoplasmic expression of both proteins in proliferating cells within the germinal centers. No Hdm2 nucleolar staining was observed in normal cells.

Aggressive B-cell lymphomas. p14ARF expression was analyzed in a series of 74 cases of NHLs including 55 DLBCL, 7 FL-3, and 12 BL. In these samples, an internal control of the technique was provided by the nucleolar staining of small lymphocytes, macrophages, and some endothelial cells.

Concerning the p14ARF expression pattern in tumoral cells, the following situations were observed: (1) p14ARF loss in a subset of NHLs. A small number of cases (8/74; Table 2) showed no appreciable p14ARF staining in large cells (Figure 2D), whereas nucleolar staining was observed in benign lymphocytes. The predominant phenotype in this subset of cases was characterized by a low level of expression of both Hdm2 (Figure 2E) and p53 (Figure 2F). Of these cases, 3 could be sequenced for p14ARF, but no mutations were found. In 1 of 3 cases (case 19), silencing of the

Table 1. p14ARF and p53 status in lymphoid cell lines

Cell line	p53 gene	p14ARF gene	p14ARF expression
RAJI	R213Q (het) + Y234H (het)	WT	+
NAMALWA	R248Q	R120L (het)	+
MOLT-4	R306STOP (het)	Del	−
GRANTA-519	WT	Del	−
KARPAS-422	WT	WT	+
WSU-NHL	R248Q	WT	+
RPMI 8226	E285K	WT	+
HuT 78	R196STOP	Del	−

Del indicates homozygous deletion; WT, wild-type; het, heterozygous; −, negative; +, positive.

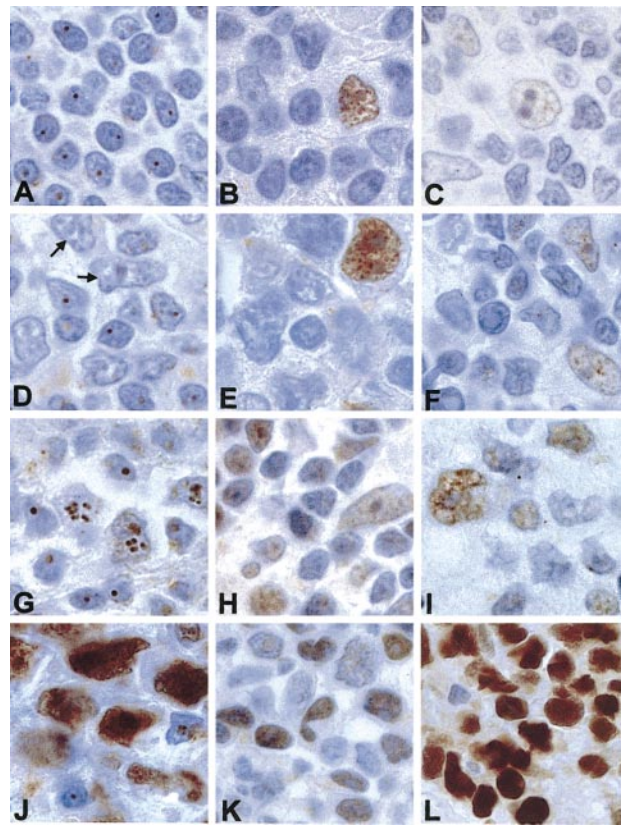

Figure 2. Immunohistochemical study of p14ARF, Hdm2, and p53 in normal and tumoral lymphoid tissue. (A) Distinct nucleolar p14ARF staining, in nontumoral lymphoid tissue (a reactive lymphadenitis). (B) Hdm2 and (C) p53 in the same sample. (D) An NHL case with loss of p14ARF in tumoral cells (indicated by arrows); normal lymphocytes retain nucleolar p14ARF expression. Hdm2 (E) and p53 (F) in this case are also shown. (G) An NHL case showing nucleolar p14ARF in tumoral cells, with intermediate Hdm2 (H) and p53 (I) expression. (J) Nuclear p14ARF overexpression (note the nucleoli in small lymphocytes) associated with high levels of Hdm2 (K) and p53 overexpression (L). Original magnification × 1000.

Table 2. Cases with loss of p14^{ARF} expression

Case no.	Diagnosis	p53 (%)	Hdm2 (%)	Ki67 (%)
19	DLBCL	50	50	60
40	DLBCL	20	0	75
47	BL	< 10	< 10	90
51	DLBCL	10	NA	90
53	DLBCL	< 10	< 10	NA
59	FL-3	30	40	70
60	DLBCL	10	10	65
64	DLBCL	0	0	30

DLBCL indicates diffuse large B-cell lymphoma; BL, Burkitt lymphoma; FL-3, follicular lymphoma grade 3; NA, not available.

p14^{ARF} gene was associated with promoter hypermethylation coupled with an LOH (loss of heterozygosity), which could affect the unmethylated allele. A homozygous deletion within the 9p21 region was detected in another case (case 47). The p53 gene was wild-type in the 4 cases sequenced. (2) Nucleolar p14^{ARF} expression. Nucleolar p14^{ARF} staining in tumoral cells was the most frequently observed expression pattern (50/74 cases). The intensity of staining was variable, ranging from weak to strong nucleolar staining in most large cells (Figure 2G). The staining for Hdm2 (Figure 2H) and p53 (Figure 2I) was always nucleoplasmic and of variable intensity, often with distinct nucleolar exclusion. Nucleolar accumulation of Hdm2 was never observed. No alterations in the p14^{ARF} sequence (a nucleotide change in exon 2, detected in one case,[33] does not affect the p14^{ARF} protein) or promoter hypermethylation were detected in any of the 42 cases analyzed in this group; 4 cases showed LOH in 9p21. p53 mutations were detected in a minority of cases (1 nonsense and 3 missense mutations out of 42 samples). (3) p14^{ARF} nuclear overexpression. A subset of NHLs (16/74) showed an atypical p14^{ARF} expression pattern characterized by intense nuclear staining that was not confined to the nucleolus (Figure 2J). Since mutations affecting the NrLS of p14^{ARF} have been described as partially or totally impeding the correct localization of this protein,[29] both exons of the p14^{ARF} gene were sequenced in 9 of 16 cases. However, no alterations were detected. Allelic loss and promoter hypermethylation analyses, performed in the same samples, also yielded negative results. This group of cases (Table 3) was characterized by high p53 and Hdm2 levels of expression (Figure 2K-L). When compared with the rest of the series, a statistically significant relationship was found between p14^{ARF} nuclear overexpression and higher expression of both p53 and Hdm2 (Kruskal-Wallis test, $P = .001$ for p53 and $P = .010$ for Hdm2). p53 was either wild-type or mutated in this subset of cases, but the frequency of p53 mutation was significantly higher (5/9 sequenced cases) when compared with the rest of the series (Fisher exact test, $P = .005$). There were no significant differences in the distribution of the cases with p14^{ARF} nuclear overexpression between the categories defined by histologic diagnosis (BL vs non-BL; Fisher exact test, $P = .715$) or IPI (Pearson chi square: 0.360, $P = .948$).

Analysis of subcellular localization

In order to characterize more precisely the subcellular localization of the p14^{ARF}, Hdm2, and p53 proteins, 5 tumor samples with different patterns of expression of p14^{ARF} were analyzed by confocal microscopy. Double fluorescent immunolabeling was performed for p14^{ARF} and either C23 (nucleolar marker), p53, or Hdm2.

Existence of a nucleoplasmic fraction of p14^{ARF} was effectively observed in cases with abnormal p14^{ARF} nuclear overexpression, as demonstrated by double labeling with C23 (Figure 3A). Although nucleolar exclusion was not observed, part of the signal was clearly extranucleolar. Nucleoplasmic p14^{ARF} was restricted to the large tumoral cells, whereas small lymphocytes showed distinct nucleolar p14^{ARF} staining. This pattern contrasts with the more typically observed situation in both normal and tumoral cells, in which p14^{ARF} was confined to the nucleolus (Figure 3B). C23 characteristic nucleolar staining demonstrates that the integrity of the nucleolus was always preserved, and anomalous localization of p14^{ARF} was not a consequence of structural alterations of the nucleolus.

Double labeling for p14^{ARF}-p53 revealed a partial colocalization of these proteins in the nucleoplasm, suggesting the existence of a fraction of nucleoplasmic p14^{ARF} not bound to p53 (Figure 3C). This colocalization was absent in samples exhibiting p14^{ARF} nucleolar localization (Figure 3D).

A striking pattern was observed for Hdm2, in that it was not found to colocalize with nucleoplasmic or nucleolar p14^{ARF}; in fact, these signals usually appeared in alternative cells, and Hdm2 never accumulated in the nucleolus (Figure 3E-F).

Clinical and biologic significance of p14^{ARF} nuclear overexpression

Since p14^{ARF} has been reported as accumulating in response to oncogenic stimuli,[18-22] we examined whether p14^{ARF} nuclear overexpression had any correspondence with the biology of the tumor or clinical outcome of the patients.

To this end, Ki67 expression was quantified in 70 of 74 cases (range: 32%-100%, median: 82%) as a measure of proliferation index. Univariate analysis showed a strong association between p14^{ARF} nuclear overexpression and higher Ki67 levels (Kruskal-Wallis test, $P = .002$). Histology (BL vs non-BL) and p53 and Hdm2 expression were also significantly related to Ki67 (Table 4). However, after performing a multivariate analysis that included the variables found to be significant in the univariate analysis, the only variable that retained statistical significance, in addition to histology, was p14^{ARF} nuclear overexpression ($P = .035$).

To test this possible association between p14^{ARF} nuclear overexpression and tumor aggressivity further, an overall survival analysis was performed for the subset of cases (51/74) for which clinical follow-up information during at least 60 months was available. A

Table 3. Cases with nuclear p14^{ARF} overexpression

Case no.	Diagnosis	p53 gene	p53 (%)	Hdm2 (%)	Ki67 (%)
3	DLBCL	C135F	100	10	100
6	DLBCL	R158H	60	30	80
13	BL	WT	NA	50	100
21	BL	WT	100	30	95
26	DLBCL	G245S	100	0	90
34	BL	WT	60	80	95
36	DLBCL	G244D	80	10	95
46	DLBCL	WT	60	80	95
48	DLBCL	V157F	100	80	70
52	DLBCL	NA	NA	NA	NA
54	DLBCL	NA	20	20	NA
63	DLBCL	NA	60	40	40
67	DLBCL	NA	20	30	85
69	DLBCL	NA	40	100	75
72	DLBCL	NA	60	100	85
73	DLBCL	NA	10	50	85

DLBCL indicates diffuse large B-cell lymphoma; BL, Burkitt lymphoma; FL-3, follicular lymphoma grade 3; WT, wild type; NA, not available.

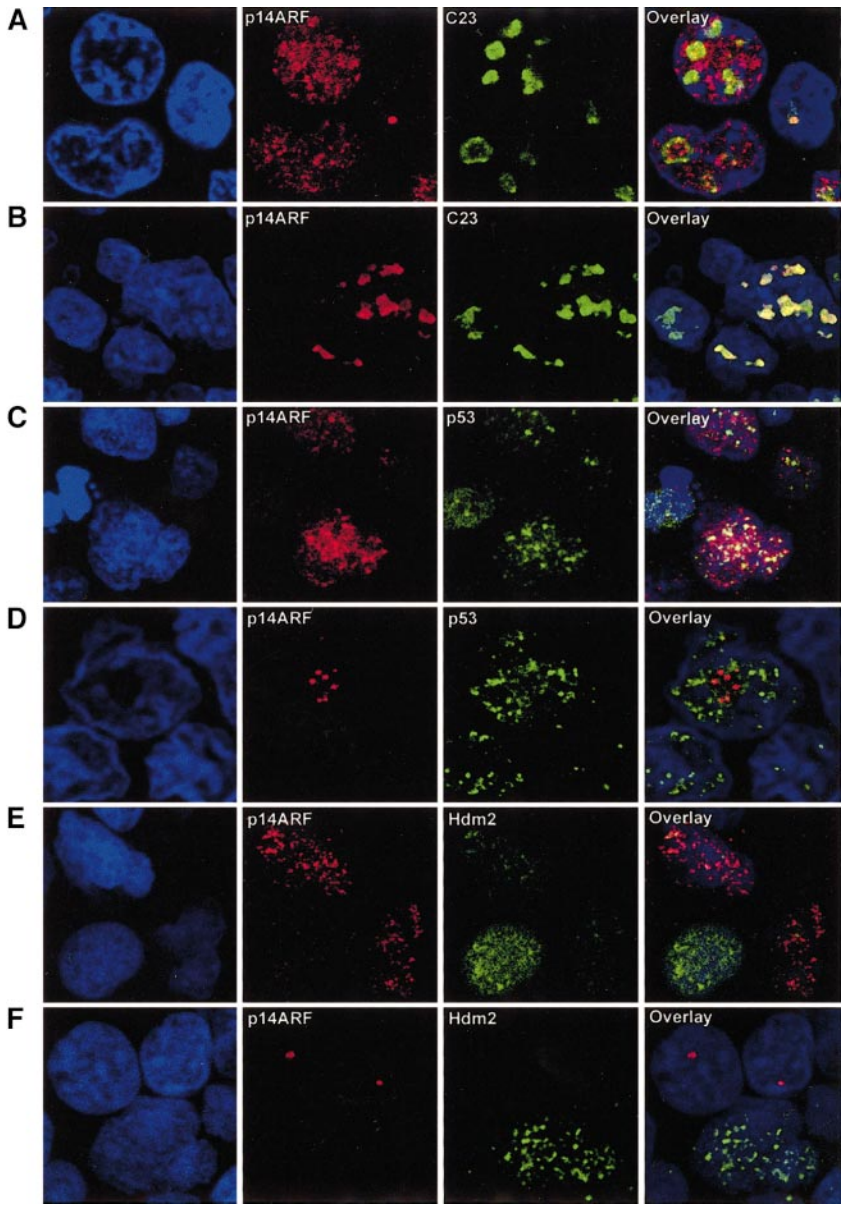

Figure 3. Confocal microscopy analysis of NHL samples. (A) Double immunolabeling for $p14^{ARF}$ and the nucleolar marker C23 in a sample with $p14^{ARF}$ nuclear overexpression, showing that a significant fraction of the $p14^{ARF}$ signal is extranucleolar. Note the nucleolar $p14^{ARF}$ signal in the normal lymphocyte (right). (B) A sample with intense nucleolar accumulation of $p14^{ARF}$, coincident with the C23 signal, is shown for comparison. (C) Double immunolabeling for $p14^{ARF}$ and p53 shows a partial colocalization of nucleoplasmic $p14^{ARF}$ and p53. (D) Lack of colocalization in a sample with nucleolar $p14^{ARF}$, where the discrete granular $p14^{ARF}$ signal contrasts with the nucleoplasmic distribution of p53. (E) Lack of colocalization between nucleoplasmic $p14^{ARF}$ and Hdm2. (F) Hdm2 expression in a tumoral cell where $p14^{ARF}$ is absent; however, nucleolar $p14^{ARF}$ is expressed in adjacent lymphocytes lacking detectable Hdm2. Original magnification × 2000-3500.

worse prognosis was observed for the cases with nuclear $p14^{ARF}$ when compared with the rest of the series ($P = .060$, crude analysis) (Table 5); this relationship proved to be statistically significant after adjusting the results by IPI and histology ($P = .043$; Figure 4A). Since the small group of cases in which $p14^{ARF}$ expression was not detected may include aggressive tumors in which $p14^{ARF}$ overexpression is impeded by alterations at the gene level, the cases lacking $p14^{ARF}$ expression were excluded from the analysis and overall survival of the patients overexpressing $p14^{ARF}$ was compared with that of the cases showing a nucleolar expression of the protein; again, differences were found to be significant ($P = .037$).

$p14^{ARF}$ overexpression was also a negative predictor of survival when only DLBCLs were considered ($P = .047$, crude analysis); however, the reduced number of BL cases prevented us from performing informative statistical analyses for this histologic class as a separate group. When cases were grouped according to IPI and overall survival was analyzed separately for each group, a similar association between $p14^{ARF}$ nuclear overexpression and prognosis was found for the category of medium IPI (2-3) ($P = .055$); analyses were not considered informative for the groups of low (0-1) or high (4-5) IPI due to the reduced number of events in the first case and the small number of patients with $p14^{ARF}$ nuclear overexpression (2 patients) in the second.

In a previous report[33] we analyzed different alterations in tumor suppressor pathways in a group of NHLs that included 51 of the samples described here. For these cases, information was available concerning p53 mutations, $p16^{INK4a}$ inactivation by promoter hypermethylation, deletion, or mutation, and $p27^{KIP1}$ overexpression, presumably reflecting its inactivation by CDK4-cyclin D3 binding.[45]

First, the relationship between $p14^{ARF}$ nuclear overexpression and each of these individual alterations was analyzed. As mentioned above, there was a strong association between p53 mutation and $p14^{ARF}$ nuclear overexpression ($P = .005$). Presence of nuclear $p14^{ARF}$ was also more frequent in tumors overexpressing $p27^{KIP1}$, although this did not reach statistical significance (Fisher exact test, $P = .118$).

An association between $p16^{INK4a}$ alterations and $p14^{ARF}$ overexpression was not found when all the possible mechanisms of inactivation of $p16^{INK4a}$ were considered as a whole. However, this

Table 4. Relationship between proliferation index provided by Ki67 expression and other variables

Variable	N	Median of Ki67 expression (%)	Univariate analysis		Median regression		
			P25-P75 (%)	*P*	Beta (%)	95% CI (%)	*P*
p14^{ARF}							
Nonnucleoplasmic	56	77	60-90				
Nucleoplasmic	14	91	86-95	.002	12	0.9-23.1	.035
IPI							
0 to 1	15	85	65-95				
2	14	73	60-95				
3	10	83	70-95				
4 to 5	11	80	70-90	.955			
Histology							
Non-Burkitt	58	76	30-87				
Burkitt	12	95	90-99	< .001	23	11.9-34.1	< .001
p53 expression							
Less than 10%	17	60	50-90				
10% to 50%	26	80	70-90				
At least 50%	23	90	75-95	.022	10	−1.4-21.4	.086
p53 mutation							
No	42	80	60-95				
Yes	9	90	85-90	.226			
Hdm2							
Less than 10%	18	65	60-75				
10% to 40%	21	87	75-95				
At least 40%	24	86	70-92	.007	5	−6.2-16.2	.374

N indicates number of patients; CI, confidence interval; Beta, change in the median value of Ki67 compared with the reference category.

may be due to the fact that LOHs or homozygous deletions at 9p21 are likely to affect both genes simultaneously, preventing p14^{ARF} from being overexpressed. Effectively, when LOHs were excluded and only p16^{INK4a} promoter hypermethylation—plus a single mutation that did not affect p14^{ARF}—was considered, an almost significant association was obtained between these alterations and higher frequency of p14^{ARF} nuclear overexpression (Fisher exact test, $P = .053$). It was also observed that hypermethylation of the p14^{ARF} and p16^{INK4a} promoters were independent events, as could be inferred from their remarkably different incidence in this group of lymphomas (17/51 cases displayed p16^{INK4a} hypermethylation whereas only 1/51 cases had p14^{ARF} hypermethylation).

Finally, we analyzed the frequency of p14^{ARF} abnormal overexpression as a function of the number of accumulated alterations in p53, p16^{INK4a}, and p27^{KIP1}. As shown in Figure 4B, the proportion of cases with anomalous p14^{ARF} expression increases with the number of cell cycle defects, this relationship being statistically significant (Pearson chi square: 10.233, $P = .017$). Interestingly, p14^{ARF} nuclear overexpression was not observed in any of the 19 cases where no alterations in the p53 and Rb pathways were found.

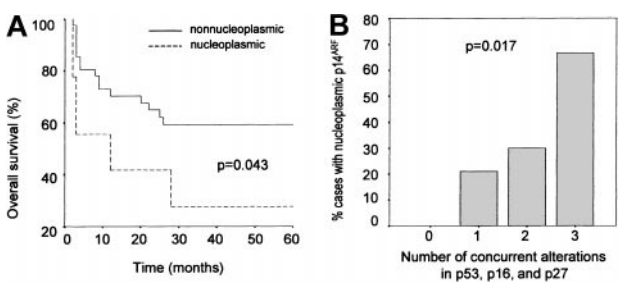

Figure 4. p14^{ARF} overexpression as a marker of aggresivity. (A) Kaplan-Meier analysis: overall survival curves showing a shorter survival in cases with p14^{ARF} nuclear overexpression compared with the rest of the series. (B) Relative frequency of abnormal p14^{ARF} overexpression as a function of cell cycle status. Cases are grouped according to the number of simultaneous alterations in major tumor suppressors (p53, p16^{INK4a}, p27^{KIP1}). Nuclear p14^{ARF} overexpression becomes more frequent as the number of alterations increases.

Discussion

Information concerning regulation of the p14^{ARF}-Hdm2-p53 pathway is mainly derived from in vitro studies and animal models, whereas a comprehensive analysis of its significance in human tumors has been hampered by technical considerations such as the lack of appropriate antibodies for detecting the p14^{ARF} protein. We have chosen NHL as a model, since it features tumors with a high growth fraction in which p53 mutations are present in only a small proportion of cases, and therefore may be useful for revealing the complexity of molecular alterations taking place in this pathway.

Unlike what has been found in other neoplasias, molecular alterations resulting in loss of p14^{ARF} expression are rare in NHLs. Hypermethylation of p14^{ARF} promoter and deletions within the 9p21 region, the 2 main mechanisms for p14^{ARF} silencing, have been detected in our series of cases only exceptionally. This situation sharply contrasts with that observed in lymphoid cell lines, in which cancellation of the p53 pathway by alternative—and occasionally simultaneous—inactivation of p14^{ARF} or p53 is a very frequent finding (Table 1). Tumors lacking detectable p14^{ARF} are characterized by low levels of p53 and Hdm2 proteins, which is consistent with the proposed role of p14^{ARF} as an inhibitor of Hdm2; thus, in the absence of p14^{ARF}, active Hdm2 promotes a rapid p53 degradation, which in turn leads to a low level of p53-induced Hdm2 expression.

In contrast with the exclusively nucleolar localization of p14^{ARF} observed in normal cells and most in vitro models, abnormal p14^{ARF} nucleoplasmic accumulation has been found in a significant number of aggressive NHLs. It does not seem likely that this is a consequence of nonspecific staining, as a distinct nucleolar signal has been obtained with the same antibody and under the same conditions in nontumoral tissue, many NHL samples, and several lymphoid cell lines. Moreover, no staining (either nucleolar or

Table 5. Relationship between p14ARF nuclear overexpression and overall survival

Variable	N	Deaths	% Survival (60 months)	Crude analysis			Adjusted by IPI and histology		
				HR	95% CI	*P*	HR	95% CI	*P*
p14ARF									
Nonnucleoplasmic	41	16	59	1.00			1.00		
Nucleoplasmic	9	6	28	2.47	0.96-6.32	.060	2.65	1.03-6.83	.043
IPI									
0 to 1	15	1	93	1.00			1.00		
2	14	7	43	12.17	1.49-99.3	.020	11.65	1.43-95.1	.022
3	10	6	34	13.88	1.67-116	.015	12.69	1.51-106	.019
4 to 5	11	8	27	20.40	2.53-164	.005	19.12	2.36-155	.006
Histology									
Non-Burkitt	38	19	48	1.00			1.00		
Burkitt	12	3	73	0.43	0.13-1.47	.180	0.56	0.17-1.90	.352

N indicates number of patients; HR, hazard ratio; CI, confidence interval.

nucleoplasmic) has been detected in cell lines with p14ARF silencing by homozygous deletion (Figure 1). p14ARF-C23 double immunolabeling has demonstrated that our observations are not an artifact related to loss of nucleolar integrity. We have also ruled out the possibility that nucleolar distribution is impeded by mutations affecting the p14ARF NrLS, since the p14ARF gene has been found to be wild-type in all the cases that have been sequenced.

The significance of this findings is enhanced by the observation that p14ARF atypical overexpression defines a group of lymphomas characterized by higher aggresivity. A strong association exists between p14ARF nuclear overexpression and higher proliferation index, which remains significant even in a multivariate analysis including relevant variables such as p53, Hdm2, and histologic diagnosis. A similar correlation existed at the prognostic level, since cases with abnormal p14ARF overexpression were also characterized by a shorter overall survival.

An explanation for these findings can be found in the consideration of the central role of p14ARF in cell cycle control, as a nexus between the major tumor suppressor pathways. Thus, the p14ARF promoter has an E2F-1 binding site which "senses" oncogenic stimuli transduced through the Rb pathway[19]; p14ARF is also induced by typical oncogenes (*myc*, *ras*, viral genes), and negatively regulated by p53 (Figure 5A). With the exception of alterations affecting p14ARF itself, which we have shown to be very rare in NHLs, virtually every cancer-related defect in these pathways (Figure 5B) should result in p14ARF upregulation: alterations of the Rb pathway such as p16INK4a inactivation or cyclin D overexpression, deregulation of oncogenes (eg, *myc* in BL), and p53 inactivation by mutation or by Hdm2 overexpression (resulting in disruption of the p53-p14ARF negative feedback loop). Therefore p14ARF should integrate all these stimuli, its level of expression being a measure of the accumulation of alterations in different points of the cell cycle, and consequently a marker of tumor aggressivity. Consistent with this hypothesis, p14ARF nuclear overexpression is a more frequent finding in tumors displaying simultaneous inactivation of several major tumor suppressors (p53, p16INK4a, p27KIP1), in addition to correlate with some of these alterations, taken individually. The postulation of an overexpressed nuclear p14ARF as a surrogate of a highly deregulated cell cycle is consistent with the higher aggressivity (as measured by proliferation index and worse prognosis) observed in this group of lymphomas.

Even if p14ARF overexpression is a consequence of cell cycle malfunction, the atypical nuclear localization of the protein remains an intringuing finding. Results of confocal microscopy suggest that p14ARF nucleoplasmic accumulation is only partially dependent on p53 binding and probably independent of Hdm2 binding. A recent report has suggested that the predominant p14ARF nucleolar accumulation is accompanied by an usually undetectable nucleoplasmic fraction which could be responsible for p53 activation.[25] Consistent with this model, it would be expected that under conditions of massive p14ARF induction the nucleoplasmic fraction would become detectable, as could be the case for a group of aggressive lymphomas.

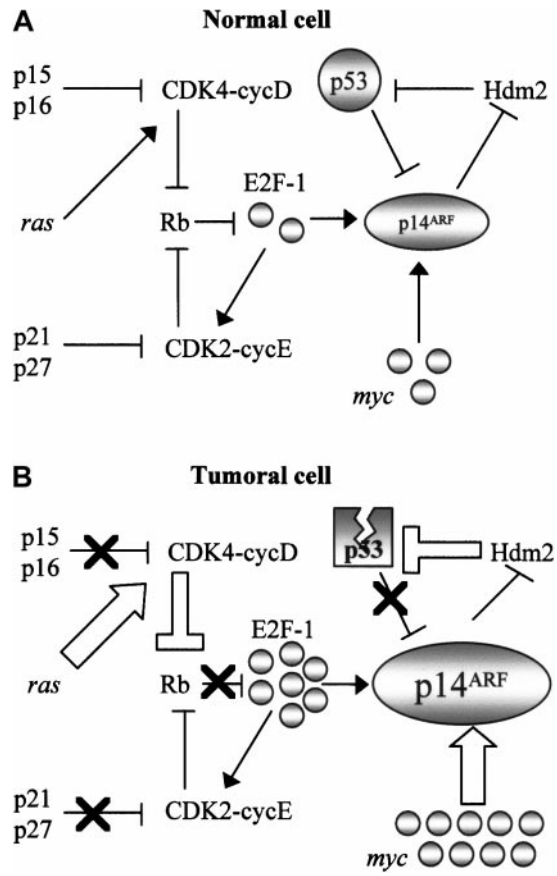

Figure 5. Central role of p14ARF in cell cycle regulation. The central role of p14ARF in the control of cell cycle in normal cells (A) converts it into a marker of inactivation of multiple cell cycle regulatory pathways (B). Nuclear overexpression as a consequence of multiple molecular alterations involving Rb pathway (inactivation of the Rb pathway generates active E2F-1, which induces transcription of the p14ARF gene); *myc*, and viral oncogenes such as E1A or v-*abl*, which also induce p14ARF; and alterations in the p53-Hdm2 pathway resulting in disruption of the p53-mediated negative regulation of p14ARF. Thick arrows indicate events derived from oncogene activation; black crosses represent inactivation of tumor suppressor genes.

Our findings may help to resolve the controversy concerning Hdm2 and $p14^{ARF}$ subcellular localization, both in normal and tumoral cells. Thus, the presence of Hdm2 and $p14^{ARF}$ seems to be mutually exclusive, as shown by the lack of nucleolar Hdm2 staining in cells expressing nucleolar $p14^{ARF}$, and the absence of Hdm2 in cells overexpressing nuclear $p14^{ARF}$. This suggests that $p14^{ARF}$-Hdm2 complexes, if they exist, should have a short half-life, dependent on a rapid Hdm2 degradation induced after $p14^{ARF}$ binding.

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