Nucleolar p14^{ARF} Overexpression in Reed-Sternberg Cells in Hodgkin's Lymphoma

Absence of p14^{ARF}/Hdm2 Complexes Is Associated with Expression of Alternatively Spliced Hdm2 Transcripts

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The development of human cancers is frequently associated with the silencing of the two major tumor suppressor pathways represented by retinoblastoma protein and p53. As the incidence of p53 mutations is significantly lower in Hodgkin's lymphoma than in other neoplasias, we investigated whether the malfunction of other proteins in this pathway could be responsible for its inactivation. Because the existence of nucleolar complexes between p14^{ARF} and Hdm2 has been described as having a critical effect on p53 function by inhibiting its degradation, we analyzed the expression and subcellular localization of these proteins in 52 cases and in Hodgkin's cell lines. Two of four cell lines revealed loss of p14^{ARF} expression secondary to gene promoter methylation, this being mutually exclusive with p53 mutations (1 of 4), illustrating the existence of selective pressure to inactivate the p53 pathway. The majority of Hodgkin's samples showed a strong nucleolar expression of p14^{ARF} that was not associated with Hdm2. They also showed the existence of Hdm2/p53 complexes, and the absence of complexes containing either p14^{ARF}/Hdm2 or p14^{ARF}/p53. The different localization of Hdm2 (nucleoplasm) and p14^{ARF} (nucleoli) observed in Hodgkin's tumors and cell lines is associated with the presence of short alternatively spliced transcripts of Hdm2 lacking the ARF-binding region and the nuclear export signal. The absence of these p14^{ARF}/Hdm2 nucleolar complexes could be sufficient to inactivate the pathway and may explain the low frequency of p53

mutations in this tumor. (*Am J Pathol* 2002, 160:569–578)

Most common neoplasias accumulate inactivation of both the p53 and Rb pathways, these tumor suppressor genes being silenced by a diverse repertoire of genetic and epigenetic mechanisms.¹ The INK4a/ARF locus encodes two tumor suppressor proteins specified by the use of alternative reading frames within a common second exon of both genes. p16^{INK4a} is an inhibitor of cyclin D-dependent kinases that prevents the phosphorylation of Rb.² In contrast, murine p19^{ARF} (and p14^{ARF} in humans) increases the stability and expression of p53.^{3–6} Although in Hodgkin's lymphoma (HL) inactivation of the p16-Rb pathway has been shown to take place in the majority of cases as a consequence of p16^{INK4a} gene methylation,⁷ there has been no unequivocal demonstration of the silencing of the p53 pathway. Thus, the frequency of p53 mutation is distinctly lower in HL than it is in other tumoral conditions, as has been shown by different research groups.^{8–10}

The p53 pathway is critically dependent on the status of p53 and its interactions with Hdm2 (the human counterpart of murine Mdm2) and p14^{ARF}.¹¹ p53 accumulates in response to DNA damage or oncogenic signaling, primarily through protein stabilization after disruption of its interaction with its negative regulator, Hdm2.^{5,12} Hdm2 opposes p53 function at several levels. It can bind to the N-terminal transcriptional activation domain of p53 to block the expression of p53-responsive genes.^{13–15} Additionally, Hdm2 has an intrinsic E3 ligase activity that conjugates ubiquitin to p53,^{16,17} playing a role in shuttling p53 from the nucleus to the cytoplasm, where p53 is degraded in the proteasomes.^{18–20}

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In murine models, the interaction of p53 with Mdm2 is dependent on the interaction of both proteins with p19^{ARF}. It has been shown under different experimental conditions that the nucleolar sequestration of Mdm2 by p19^{ARF} antagonizes the ubiquitination of p53 and its transport to the cytoplasm, inducing p53 stabilization and activation in the nucleoplasm, and leads to the induction of a battery of p53-responsive genes.^{18,19,21} According to this model, p14^{ARF} inactivation or Hdm2 overexpression occurs more commonly in human tumor cells that retain wild-type p53, consistent with the hypothesis that disruption of the ARF-Hdm2-p53 pathway is important in the commonest types of cancer.²²⁻²⁷

Previous studies have shown that the Hdm2 protein has a nucleolar localization signal (NrLS) contained within the C-terminus RING-finger domain,^{28,29} and also contains a central ARF-binding domain.^{4-6,30} The NrLS in Hdm2 seems normally to be concealed and is unmasked by conformational changes after p14^{ARF} binding.²⁸ Therefore, cell-cycle arrest by human p14^{ARF} requires both binding and nucleolar importation of Hdm2. This nucleolar compartmentalization of the p14^{ARF}/Hdm2 complexes is critical for the capacity of p14^{ARF} to inhibit cell-cycle progression.

The interaction between p14^{ARF} and Hdm2 is bi-directional, each protein being capable of regulating the sub-nuclear localization of the other.²⁹ Murine p19^{ARF} mutants that bind Mdm2 but fail to move it into the nucleolus do not trigger p53-dependent responses.²¹ The existence of complexes containing both Hdm2 and p14^{ARF} that lead to inactivation of Hdm2 has also been demonstrated in human tumoral cells.^{31,32} However, there is controversy about the exact localization of these complexes, which under some experimental conditions has been found to be mainly nucleoplasmic.³²

The interaction of Hdm2 with p14^{ARF} and p53 is critically dependent on the integrity of the Hdm2 molecule, and specifically on the NrLS, p14^{ARF}-binding domain, p53-binding domain, and nuclear-export signal (NES). The analysis of Hdm2 transcripts and protein, both in human tumors and cell lines have revealed the presence of various alternatively spliced Hdm2 transcripts,³³⁻³⁵ whose functional significance remains to be examined. It is of particular note that many of these Hdm2-spliced forms have lost the central region of the protein, including the p14^{ARF}-binding region, and the NES.

In HL, the presumed inactivation of the p53 pathway seems to be achieved mainly through an alternative mechanism to p53 mutations. This prompted us to study the expression and subcellular localization of p14^{ARF} and its relationship with other proteins in the p53 pathway, in particular Hdm2, in this disease. The results revealed an increased nucleolar expression of p14^{ARF} and an absence of nucleolar or nucleoplasmic p14^{ARF}/Hdm2 complexes. Moreover, overexpression of Hdm2 protein in Reed-Sternberg (RS) cells is associated with the expression of several alternatively spliced transcripts that lack the p14^{ARF}-binding domain, which could be the basis of the malfunction of the p53 pathway in this tumor.

Materials and Methods

Tissue Samples

Twenty samples of reactive lymphoid tissue (tonsils and reactive lymphadenitis) and 52 tumor specimens from HL cases were obtained from the tissue archives of the CNIO tumor bank. All specimens were obtained from previously untreated cases of HL, and were diagnosed according to the criteria used in the Revised European-American Lymphoma classification,³⁶ and the World Health Organization classification.³⁷ They included 23 cases of nodular sclerosis HL, 17 cases of mixed cellularity HL, 1 case of lymphocyte-rich classical HL, 3 cases of lymphocyte depletion HL, and 7 cases of nodular lymphocyte-predominant HL.

Cell Lines

Four HL-derived cell lines (HDLM2, L428, L540, and KMH2) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Normal peripheral blood lymphocytes (PBLs) were obtained from voluntary healthy donors. All cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10 or 20% heat-inactivated fetal calf serum (Life Technologies, Inc.), glutamine, penicillin, and streptomycin. Cells were grown at 37°C in 5% CO₂. Peripheral blood lymphocytes were additionally supplemented with 2% phytohemagglutinin (Life Technologies, Inc.). For immunostaining, cells were harvested by centrifugation, washed with cold phosphate-buffered saline (PBS), cytospun onto poly-L-lysine-coated slides, and fixed in 50% ethanol/50% acetone.

Antibodies (Abs)

p14^{ARF} protein was detected with a goat polyclonal Ab (C-18; Santa Cruz Biotechnology, Santa Cruz, CA).^{31,38} The staining of nucleoli in some endothelial cells, macrophages, and small lymphocytes provided an internal control. Nucleolin-C23 was detected with a monoclonal Ab (MS-3, Santa Cruz). Nucleolin expression was used as a nucleolar marker.^{39,40} Human Hdm2 protein was detected with the monoclonal Ab MDM2 Ab-1 (IF-2; Oncogene Research, Cambridge, MA). IF-2 recognizes an epitope within amino acid residues 26 to 169 of human Hdm2 protein. We used the monoclonal Ab DO7 (Novocastra, Newcastle on Tyne, UK) for p53 protein analyses, and rabbit polyclonal Ab CM1 (Novocastra) for double immunolabeling Hdm2-p53.

Immunostaining Techniques

All immunostaining techniques were performed in paraffin-embedded tissue sections and cytospin preparations of the different HL-derived cell lines, using an initial heat-induced antigen retrieval step (slides were heated in a

pressure cooker for 3 minutes in a 0.01 mol/L solution of sodium citrate before incubation with the Abs).

After incubation with the primary Ab, immunodetection was performed with biotinylated anti-mouse or anti-goat immunoglobulins as appropriate, followed by peroxidase-labeled streptavidin (LSAB-DAKO; Glostrup, Denmark) and diaminobenzidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device. Incubations either omitting the specific Ab or containing unrelated Abs were used as a control of the technique.

For double-immunolabeling and laser confocal analyses, 3- μ m-thick paraffin-embedded tissue sections from reactive lymphoid tissues (tonsils) and eight neoplastic specimens with different levels of p14^{ARF} expression, were cut and mounted on poly-L-lysine-coated slides, and stained with polyclonal anti-p14^{ARF} and either monoclonal anti-Hdm2, or monoclonal anti-nucleolin, or monoclonal anti-p53. After simultaneous overnight incubation at 4°C with the primary Abs, sections were washed in PBS and incubated with secondary Abs: Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR), Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes), and Cy3-conjugated goat anti-mouse IgG, (Jackson ImmunoResearch, Baltimore, MD). For immunofluorescence analyses, tissue sections were counterstained using 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) and directly visualized under a DMRA microscope (Leica Microsystems, Wetzlar, Germany) fitted with appropriate fluorescence filters. For laser confocal analyses in cell lines, nuclei were stained with TO-PRO3 (Molecular Probes), mounted with glycerol and examined with a laser-scanning confocal microscopy system TCS NT (Leica Microsystems). Series of images were processed and analyzed with the accompanying software package (Leica Microsystems) and Adobe Photoshop 5.5 image-processing software.

Immunoprecipitation

For p53 and Hdm2 immunoprecipitation, the L428 cell line was harvested by centrifugation, washed with cold PBS, and lysed with buffer (50 mmol/L Tris-HCl, pH 8, 1% Triton X-100, 150 mmol/L NaCl, and protease inhibitors). Lysed cells (1 mg of protein) were incubated with primary Abs and protein A/G, and filtered using the IMMUNOCATCHER kit (CytoSignal, Irvine, CA) following the manufacturer's instructions. Immunoprecipitates were electrophoresed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes (Hybond ECL, Amersham, UK). Membranes were serially incubated with anti-Hdm2 or anti-p53 primary Abs and anti-mouse (Amersham) horseradish peroxidase-labeled secondary Abs, followed by detection with the enhanced chemiluminescence system (Amersham). The anti-p14^{ARF} Ab used for these experiments is not suitable for detecting endogenous protein and complexes in this model.

Methylation Study and 5-Aza-2'-Deoxycytidine Treatment in Hodgkin's-Derived Cell Lines

Methylation-specific polymerase chain reaction (PCR) assays were performed to analyze the methylation status of CpG islands of the promoter region of p14^{ARF} gene in DNA extracted from the different HL-derived cell lines used in this study, as previously described.²⁶ Briefly, 1 μ g of denatured genomic DNA was modified by reaction with sodium bisulfite under conditions that convert all unmethylated cytosines to uracils. Modification was completed by NaOH, 0.3 mol/L, treatment for 5 minutes at room temperature, followed by ethanol precipitation.

Fifty ng of bisulfite-modified DNA was amplified using previously described p14^{ARF} unmethylated-specific primers (U)⁴¹ and methylated-specific primers (M),²⁶ with 1 U of AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany) under the following conditions: 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, for 35 cycles. Controls without DNA and positive controls for U and M reactions were performed for each set of PCRs. The PCR product was visualized under UV illumination in agarose gels stained with ethidium bromide.

Additionally, to confirm that loss of p14^{ARF} protein expression is the result of gene promoter hypermethylation, we subjected the methylated cell lines to different doses (1 to 3 μ mol) of the demethylating agent 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) for 3 days.

Mutation Study in the Hodgkin's-Derived Cell Lines

DNA from the four cell lines was analyzed for mutations in exons 5 to 8 of the p53 gene using previously described primers and conditions.⁴² The HDLM2 cell line was additionally amplified for exons 9 and 10.

The mutational study of p14^{ARF} gene comprised exon 1 β and exon 2. For amplification of p14^{ARF} exon 1 β the primers used were as follows: GCCTGCGGGGCGGAGAT (sense) and AGGGCTGTGTGAAGGGAGGTC (antisense). Briefly, 200 ng of DNA were amplified with 25 pmol of each primer, 200 μ mol/L of each dNTP, and 1 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The conditions were 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, for 35 cycles. Primers and conditions for p14^{ARF} exon 2 amplification have been previously described.⁴³

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and directly sequenced with an automated DNA Sequencer ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, CA) in the DNA Sequencing Core Service of the CNIO.

Nested Reverse Transcriptase (RT)-PCR for HDM2 Transcripts

Total RNA was extracted from cultured cells with the RNeasy MiniKit (Qiagen). cDNA synthesis was performed with random primers and AMV reverse transcrip-

Figure 1. A: p14^{ARF} nucleolar expression in the HL cell lines. Lines L540 and KMH2 are p14^{ARF}-negative. B: Methylation-specific PCR study of the cell lines showing that p14^{ARF} methylation blocks p14^{ARF} expression. C: Re-expression of transcriptionally silenced p14^{ARF} after treatment with 5-aza-2'-deoxycytidine. Partial demethylation (top) in cell lines KMH2 and L540 is associated with p14^{ARF} expression (bottom).

tase at 42°C for 1 hour. A 1526-bp fragment (for the full-length Hdm2 transcript) was amplified using nested PCR primers and conditions as previously described.³⁵ To corroborate the identity of the different PCR products, each band was purified from the gel and directly sequenced.

Results

p14^{ARF} Is Expressed in the Nucleoli of Tumoral Cells in Some HL-Derived Cell Lines

L428 and HDLM2 HL-derived cell lines displayed strong p14^{ARF} expression (Figure 1A). Localization of the protein was always nucleolar, with variable numbers of positive cells (greater in the HDLM2 than in the L428 cell line). Double-immunolabeling and confocal analysis were performed for p14^{ARF} and nucleolin in both cell lines, and confirmed that the entire p14^{ARF} signal was confined within the nucleolus (Figure 2).

Nucleolar Expression of p14^{ARF} in HL Cell Lines Is Abrogated by p14^{ARF} Gene Promoter Hypermethylation

Additionally, p14^{ARF} immunostaining of the different HL-derived cell lines demonstrated that p14^{ARF} immunostaining was absent in cell lines in which the gene was hypermethylated, as revealed by use of a methylation-specific PCR assay: KMH2 and L540 cell lines showed

Figure 2. Confocal study of p14^{ARF}, subcellular distribution in the L428 cell line. p14^{ARF} protein co-localizes with C23 (nucleolin).

absence of p14^{ARF}-positive cells and hypermethylation of the p14^{ARF} gene (Figure 1B and Table 1). Nucleolar p14^{ARF} was only present in cell lines lacking p14^{ARF} gene promoter hypermethylation.

Treatment with 5-aza-2'-deoxycytidine led to partial demethylation of the CpG sites precisely associated with reversal of the transcriptional block of p14^{ARF} (Figure 1C), thus confirming the specificity of the p14^{ARF} antibody and the direct association between gene promoter hypermethylation and loss of protein expression.

Both p14^{ARF} and p53 genes were sequenced in all four cell lines to exclude the possibility that sequence alterations were responsible for changes in the expression of these genes. The only anomaly observed was the deletion of the p53 exons 8 to 10 in the HDLM2 cell line (Table 1).

Tissue Sections of HL Show Nucleolar High Expression of p14^{ARF} in RS Cells

In normal lymphoid tissue (tonsils and reactive lymphadenitis), reactive cells showed distinct weak nucleolar staining when using p14^{ARF} Ab (Figure 3I). Hdm2 staining in these reactive cells was mostly absent and was only detected in scattered macrophages or endothelial cells. However, Hdm2 immunostaining was almost undetectable in normal lymphocytes, apart from a very small proportion of centroblasts that stained faintly in germinal centers. p53 protein was only weakly expressed by benign B cells within the germinal centers.

In most HL cases (44 of 52, Table 2), a large proportion of RS cells usually displayed characteristic strong nucleolar staining with p14^{ARF} Ab (Figure 3; A to D). This intense staining was mainly restricted to the large atypical cells and was present in both classical and lymphocyte predominance forms. Reactive small lymphocytes, histiocytes, and endothelial cells in the background usually displayed distinct nucleolar labeling (Figure 3), providing an internal positive control of the technique. The

Table 1. Analysis of p53 and p14^{ARF} in HL-Derived Cell Lines

	KMH2	L428	L540	HDLM2
p53 Gene status	wt	wt	wt	Deletion
p14 ^{ARF} Gene status	wt	wt	wt	wt
p14 ^{ARF} Expression	—	+	—	+
p14 ^{ARF} Promoter methylation	+	—	+	—

wt, wild type

Figure 3. Immunostaining for p14^{ARF}, Hdm2, and p53 in HL tissue sections and reactive lymphoid tissue. **A to D:** p14^{ARF} staining by RS cells in HL samples. p14^{ARF}-positive cases. Notice the nucleolar-positive signal also in the small reactive cells. **E:** p14^{ARF}-negative case. **F to H:** Hdm2 nucleoplasmic (extranucleolar) expression. **I:** Reactive lymphoid tissue: p14^{ARF} nucleolar expression by small reactive lymphocytes. **J to L:** p53 nucleoplasmic (extranucleolar) expression in the RS cells.

Table 2. HL Tissue Sample Immunostaining for p14^{ARF}, p53, and Hdm2

HL cases	p14 ^{ARF}	p53	Hdm2
39	++	++	++
1	++	++	—
2	++	—	+
2	+	—	—
7	—	+	+
1	—	—	+

Protein expression in RS cells: —, <5% positive RS cells; +, 5 to 50% positive RS cells; ++, >50% positive RS cells.

number of positive cases in classical (33 of 44, 75%) and lymphocyte predominance (5 of 8, 63%) HL samples were also similar, not showing significant differences neither in the pattern nor the intensity of p14^{ARF} expression.

In the great majority of HL samples, the RS cells also stained positively when using Abs for p53 and Hdm2 (Table 2). The pattern of staining for both Abs was always nucleoplasmic, with apparent nucleolar exclusion (Figure 3). Again, there were no significant differences in the expression of these proteins between classical and lymphocyte predominance HL.

Thus, the distinct and repeated pattern of staining of RS cells in the majority (39 of 52, 75%) of cases was of nucleolar p14^{ARF} strong staining, associated with nucleoplasmic (with nucleolar exclusion) Hdm2 and p53 staining. p14^{ARF} was absent in a small group of cases also associated with nucleoplasmic Hdm2 and p53. Additionally, p53 and Hdm2 immunostaining were also performed in cytospin preparations of the different HL-derived cell lines, displaying the same pattern of expression.

Absence of Nucleolar p14^{ARF}-Hdm2 Complexes

Double-immunofluorescence analyses for p14^{ARF}/p53 and p14^{ARF}/Hdm2 proteins were performed in representative HL tissue samples (eight cases). These cases were selected on the basis of their strong positive immunostaining for these three proteins, all of which had a large number of p14^{ARF}-, p53-, and Hdm2-positive cells. Simultaneous analysis of p14^{ARF} and Hdm2 showed that, despite a significant proportion of cells being simultaneously labeled for the two proteins in all cases studied, there were no cases showing nucleolar complexes between p14^{ARF} and Hdm2 proteins. Instead, Hdm2-positive cells showed only nucleoplasmic reactivity, whereas p14^{ARF} was always confined to the nucleolus (Figure 4).

To confirm these findings, we also performed double-immunofluorescence and confocal analyses in the HL-derived cell line L428, in which expression of the three proteins can be seen. (Cell lines L540 and KMH2 have a p14^{ARF} gene abrogated by promoter hypermethylation, whereas cell line HDLM2 has a p53 gene abolished by exon 8 deletion.) In these cells, we found the same pattern of expression and nuclear compartmentalization of these proteins as in the HL tissue sections: p14^{ARF} protein was always expressed in the nucleoli, whereas p53

and Hdm2 proteins were only expressed in the nucleoplasm (Figure 5).

We have demonstrated that, in RS cells in HL, the p14^{ARF} protein has a characteristic nucleolar localization, whereas the p53 and Hdm2 proteins are located in the nucleoplasm. Therefore, p14^{ARF} does not seem to co-localize with Hdm2 and/or p53 proteins. Additionally, taking into account the possible existence of complexes containing p53 and Hdm2 proteins, double Hdm2/p53 immunofluorescence was also performed (Figure 6A). This revealed simultaneous nucleoplasmic expression in the majority of RS cells, although occasional cells exclusively expressed p53 or Hdm2. Consistently with these observations, Hdm2/p53 complexes could be detected when using immunoprecipitation assays (Figure 6B), confirming that both proteins are bound in this cell line.

RS Cells Show Different Alternatively Spliced Hdm2 Transcripts

RT-PCR amplification of the complete coding region using nested primers for Hdm2 yielded multiple PCR products (Figure 7A). Normal peripheral blood lymphocytes only showed the Hdm2 full-length transcript (1526 bp), whereas alternatively spliced Hdm2 mRNAs were also detected in all of the HL-derived cell lines, L428, L540, HDLM2, and KMH2.

The full-length and additional bands identified in this experiment were confirmed by sequence analysis to correspond to the previously reported Hdm2 isoforms:^{34,35} Hdm2-a (941 bp), Hdm2-b (707 bp), Hdm2-c (1016 bp), and Hdm2-d (449 bp). The previously published shortest Hdm2 transcript (Hdm2-e, 303 bp) was not identified in any of these cell lines. It is of particular note that the KMH2 cell line displayed a new transcript, which we have designated Hdm2-f, that lacks bases 291 to 666 (1150 bp; Figure 7, A and B) and is different from those previously reported.

All these different isoforms have lost the central portion of the protein, including the NES and partially (Hdm2-a and -c) or totally (Hdm2-b, -d, and -f) the p14^{ARF}-binding region. It is worth pointing out that these isoforms retain the NrLS, except the transcript Hdm2-f, lacking all subsequent domains contained in the full-length transcript as a result of a premature stop codon. This new transcript, Hdm2-f, also retains most of the p53-binding region.

Discussion

HL is characterized by the presence of hyperproliferative tumoral RS cells, in which the p53 gene has been found to be wild-type in most cases.^{8–10} The existence of a very high growth fraction in the tumoral cells in this neoplasm supports the assumption that there must be inactivation of the p14^{ARF}/Hdm2/p53 pathway, presumably by a mechanism other than p53 mutation. Otherwise, p14^{ARF} activated by inappropriate hyperproliferative signals should in turn activate a p53-dependent stress response,

Figure 4. Double-immunofluorescence study in paraffin-embedded tissue sections. Samples of HL showing Hdm2 *versus* p14^{ARF} (A) and p53 *versus* p14^{ARF} (B). Cases showing nucleolar p14^{ARF} and extranucleolar Hdm2 and p53.

as has been shown in other tumoral models, finally inducing apoptosis.^{6,44}

The results obtained from the analysis of the cell lines reveal that expression of p14^{ARF} protein is abrogated by hypermethylation of the p14^{ARF} gene in two of the cell lines. Moreover, protein expression is reinduced by demethylation of the promoter in these cell lines. This confirms that the Ab used does indeed recognize the product of the p14^{ARF} gene, and shows that there is selective pressure to inactivate the p14^{ARF}/Hdm2/p53 pathway in these cell lines. This is accomplished either by p53 exon 8 deletion (HDLM2) or p14^{ARF} methylation (L540, KMH2), no genetic alterations being observed in the L428 cell line.

This study demonstrates p14^{ARF} expression in a human neoplasm for the first time, and illustrates the usefulness of the demonstration of this protein in tissue sections. This p14^{ARF} expression has been observed in human tumors and cell lines, both paraffin-embedded and cytoprep slides. The absence of p14^{ARF} protein in cell lines with p14^{ARF} methylation and re-expression of transcriptionally silenced p14^{ARF} after treatment with 5-aza-2'-deoxycytidine confirms that the signal is attributable to the protein and cannot be explained as an artifact derived from the antigen-unmasking procedure. In these HL samples and the L428 cell line, nucleolar p14^{ARF} expression was associated with nucleoplasmic overexpression of p53 and Hdm2 proteins. Experiments

Figure 5. Confocal analysis in the L428 cell line, showing absence of co-localization of Hdm2/p14^{ARF} (A) and p53/p14^{ARF} (B).

Figure 6. A: Double-immunofluorescence analyses for p53 and Hdm2. Binucleate cell (L428 cell line). One of the nuclei exhibits exclusively p53 staining. **B:** Co-immunoprecipitation of Hdm2 and p53 in this cell line.

using double-immunofluorescence and laser-scanning confocal microscopy failed to show either p14^{ARF}/Hdm2 or p14^{ARF}/p53 complexes in these tissue samples and cell lines. p14^{ARF} precipitation assays were not success-

fully performed, because the anti-p14^{ARF} Ab used for these experiments is not suitable for immunoprecipitation in these conditions, probably because of the small quantity of endogenous protein physiologically present.

In contrast to these observations in cell lines, in this study we found consistent p14^{ARF} nucleolar overexpression in RS cells in tissue samples. These findings in tissue samples exemplify how the selective pressure to inactivate the p53 pathway takes a different form in most tissue samples in comparison with most of the cell lines, as shown by the strong nucleolar expression of the p14^{ARF} protein, which clearly indicates that there was no p14^{ARF} methylation in these cases. The absence of nucleolar p14^{ARF}/Hdm2 complexes could represent an alternative way of inactivating the p14^{ARF}/Hdm2/p53 pathway, as has been demonstrated in cell lines in which the absence of these complexes blocks the capacity of p53 to stop the cell cycle after DNA damage.^{21,29} The observations from this study of HL tissue samples and cell lines show that, in parallel with the absence of these Hdm2/p14^{ARF} complexes, p53 is bound by Hdm2, which under different circumstances has been shown to inactivate the ability of p53 to induce the expression of p53-transactivated genes.^{14,15} It has recently been suggested that complexes containing Hdm2/p14^{ARF} differ from their murine counterpart,^{32,45} Mdm2/p19^{ARF}, in their nucleoplasmic rather than nucleolar localization. Our findings in HL do not confirm these observations, and they clearly show p14^{ARF} to be preferentially located in the nucleolus in normal and RS cells.

Deletion mapping and sequence analysis studies have shown that the Hdm2 protein contains a NrlS within the highly conserved C-terminus RING-finger domain, and also a central p14^{ARF}-binding domain^{4-6,28} that is prob-

Figure 7. A: Results of nested RT-PCR amplification for Hdm2 transcripts in the different HL-derived cell lines and peripheral blood lymphocytes showing multiple-sized products. **B:** Hdm2 alternatively spliced transcripts are shown in comparison with the coding region of the full-length transcript with the functional domains of the Hdm2 protein indicated. Partial sequence and structure of the novel Hdm2-f variant are also shown. Numbers indicate the position of the splicing sites, referred to the nucleotide sequence of the full-length Hdm2 (see text).

ably located between residues 140 to 350²⁹ (Figure 7). The NrlS in Hdm2 is apparently hidden under normal circumstances and is unmasked by conformational changes after p14^{ARF} binding. Therefore, cell-cycle arrest by human p14^{ARF} requires both binding and nucleolar import of Hdm2.^{28,29}

Overexpression of various alternatively spliced Hdm2 transcripts has been described in human tumors and cell lines.^{33–35} The presence of these spliced Hdm2 fragments now provides an explanation for the absence of these p14^{ARF}/Hdm2 complexes. Because the short Hdm2 variants promote cell growth when transduced in cultured cells, it is considered that these alternatively spliced forms confer a gain in their function as an oncogene. The study of HL cell lines has revealed the presence of Hdm2 transcripts that lack the NES (all of them), the p14^{ARF}-binding region (Hdm2-b, Hdm2-d, and Hdm2-f), but are at least partially capable of binding p53 in some cases (Hdm2-f). Strikingly, with the exception of Hdm2-f, none of these transcripts lacks the NrlS present in the RING-finger domain, thus confirming previous observations that the nucleolar localization of Hdm2 requires previous binding to p14^{ARF}.²⁹

Consistently with these results, p14^{ARF} was not found in complexes containing Hdm2 (which lack the p14^{ARF}-binding region), and overexpressed as a consequence of the disruption of the autoregulatory feedback loop with functional p53.^{3,30} The partial preservation of the p53-binding region and the lack of the NES in some of these transcripts can explain the presence of nucleoplasmic p53-Hdm2 complexes, where Hdm2 seems to be unable to induce the cytoplasmic shuttling of p53, as demonstrated by confocal and co-immunoprecipitation studies.

The presumed cancellation of the p53 pathway because of the absence of these Hdm2/p14^{ARF} complexes also offers an explanation for the relatively high level of p14^{ARF} expression by RS cells. Thus, conclusions from different observations coincide in suggesting that, as an autoregulatory mechanism, p53 negatively regulates the level of expression of p14^{ARF}, and that the lack of a functional p53 is associated with increased p14^{ARF} expression^{30,31,44} through loss of feedback regulation.

Nevertheless, alternative explanations exist for this absence of p14^{ARF}/Hdm2 complexes. An alteration of the p14^{ARF} gene affecting the Hdm2-binding domain of the protein could block both the nucleolar localization of the Hdm2 protein and the p14^{ARF}-Hdm2 binding,⁴⁶ but this study revealed the absence of mutations in the p14^{ARF} gene exons 1 β and 2 in the cell lines analyzed here. Furthermore, we cannot exclude the presence of other proteins that interact with either p14^{ARF} or Hdm2, and block their binding and functions. In addition to p53 and p14^{ARF}, Hdm2 can also bind to other p53 family members,⁴⁷ and to other significant proteins involved in cell-cycle control, such as E2F-1,⁴⁸ p300,⁴⁹ and Rb,⁵⁰ underscoring its potential for interaction with other targets.

Finally, there is a small subgroup of cases in which p14^{ARF} is not expressed. The existence in these cases of distinct p14^{ARF} expression by small lymphocytes suggests that the silencing of the gene could be secondary

to genetic loss or hypermethylation, as observed in the cell lines studied.

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