INK4a/ARF Locus Alterations in Human Non-Hodgkin's Lymphomas Mainly Occur in Tumors with Wild-Type *p53* Gene

Magda Pinyol,* Luis Hernández,*
Antonio Martínez,* Francesc Cobo,[†]
Silvia Hernández,* Silvia Beà,*
Armando López-Guillermo,[†] Iracema Nayach,*
Antonio Palacín,* Alfons Nadal,*
Pedro L. Fernández,* Emilio Montserrat,[†]
Antonio Cardesa,* and Elías Campo*

From the Laboratory of Pathology* and Department of Hematology,[†] Hospital Clínic, Institut d'Investigacions Biomediques August Pi i Sunyer, University of Barcelona, Barcelona, Spain

***INK4a/ARF* locus codes for two different proteins, *p16^{INK4a}* and *p14^{ARF}*, involved in cell cycle regulation. *p14^{ARF}* is considered an upstream regulator of *p53* function. To determine the role of these genes in the pathogenesis of human non-Hodgkin's lymphomas we have analyzed exon 1 β , 1 α , and 2 of the *INK4a/ARF* locus and *p53* gene aberrations in 97 tumors previously characterized for *p16^{INK4a}* alterations. *p53* alterations were detected in four of 51 (8%) indolent lymphomas but in 15 of 46 (33%) aggressive tumors. Inactivation of *p14^{ARF}* was always associated with *p16^{INK4a}* alterations. Exon 1 β was concomitantly deleted with exon 1 α and 2 in eight tumors. One additional lymphoblastic lymphoma showed deletion of exon 1 α and 2 but retained exon 1 β . No mutations were detected in exon 1 α and 1 β in any case. Two of the three mutations detected in exon 2 caused a non-sense mutation in the *p16^{INK4a}* reading frame and a missense mutation in the ARF reading frame involving the nucleolar transport domain of the protein. The third mutation was a missense mutation in the *p16^{INK4a}* reading frame, but it was outside the coding region of *p14^{ARF}*. Aggressive lymphomas with *p14^{ARF}* inactivation and *p53* wild type showed a significantly lower *p53* protein expression than tumors with no alteration in any of these genes. In this series of tumors, inactivation of the *INK4a/ARF* locus mainly occurred in tumors with a wild-type *p53* gene because only two lymphomas showed simultaneous aberrations in these genes. Tumors with concomitant alterations of *p16^{INK4a}* and *p14^{ARF}/p53* genes seem to exhibit a worse clinical behavior than lymphomas with no alterations or isolated inactivation of any of these**

genes. These findings indicate that *p14^{ARF}* genetic alterations occur in a subset of aggressive NHLs, but they are always associated with *p16^{INK4a}* aberrations. Concomitant disruption of *p16^{INK4a}* and *p14^{ARF}/p53* regulatory pathways may have a cooperative effect in the progression of these tumors. (Am J Pathol 2000, 156:1987–1996)

Cytogenetic and molecular studies have identified a series of gene alterations associated with particular types of non-Hodgkin's lymphomas (NHLs).¹ Most of these alterations are chromosomal translocations involving specific oncogenes, the activation of which plays an important role in the pathogenesis of the tumor. On the other hand, inactivation of the *p53* and *p16^{INK4a}* tumor suppressor genes is commonly found in different types of lymphomas mainly associated with primary aggressive and transformed variants.^{2,3} The *p53* and *p16^{INK4a}* genes are key elements in two different cell cycle regulatory pathways frequently disrupted in a broad range of human tumors. The *p53* pathway induces growth arrest or apoptosis in response to DNA damage and other stimuli of cellular distress,⁴ whereas *p16^{INK4a}* participates in the control of the G₁ phase by inhibiting the kinase activity of CDK4/6 and, consequently, the phosphorylation status of retinoblastoma protein (Rb).⁵

The recent understanding of the peculiar genomic organization of the *INK4a* locus has provided strong evidence for a common genetic link between the *p53* and *p16^{INK4a}/Rb* pathways. In addition to *p16^{INK4a}*, this locus codifies for a second transcript, called *p14^{ARF}* in humans and *p19^{ARF}* in mice (ARF for alternative reading frame), which is derived from a different exon 1 (exon 1 β) centromeric to the first exon of *p16^{INK4a}* (exon 1 α).^{6,7} Exon 1 β is spliced to the same exon 2 used by exon 1 α but produces a different open reading frame coding for a protein that is structurally different from *p16^{INK4a}*. *p14/*

Supported by the Comisión Interministerial de Ciencia y Tecnología (CICYT) (SAF 99/20), Asociación Española Contra el Cáncer, and CIRIT, Generalitat de Catalunya (98SGR21).

Accepted for publication February 16, 2000.

The first two authors contributed equally to this study.

Address reprint requests to Dr. Elías Campo, Laboratory of Pathology, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain. E-mail: campo@medicina.ub.es.

Table 1. *INK4a/ARF* and *p53* Genetic Alterations in Human NHL

Diagnosis	N	<i>p53</i>	<i>p16^{INK4a}</i>			<i>p14^{ARF}</i>	
			Exon 1α/2 deletion	Mutation	Methylation	Exon 1β/2 deletion	Mutation
Indolent	51	4 (8%)	2 (4%)	1 (2%)	0	2 (4%)	0
CLL	18	3	0	1*	0	0	0*
HCL	2	0	0	0	0	0	0
FL	14	0	1	0	0	1	0
MCL	17	1	1	0	0	1	0
Aggressive	46	15 (33%)	7 (15%)	2 (4%)	5 (11%)	7 (15%)	2 (4%)
RS	7	2	2	0	0	2	0
FL-T	10	3	1	2†	0	1	2†
MCL-B	9	5‡	2	0	0	2	0
LCL	15	3	1	0	4	1	0
Other	5	2	1§	0	1	1§	0
Total	97	19 (20%)	9 (9%)	3 (3%)	5 (5%)	9 (9%)	2 (2%)

CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; FL, follicular lymphoma; MCL, typical mantle cell lymphoma; RS, large cell lymphoma transformed from a CLL (Richter's syndrome); FL-T, large cell lymphoma transformed from follicular lymphoma; MCL-B, blastoid mantle cell lymphoma; LCL, B-cell large cell lymphoma; Other, includes one Burkitt's lymphoma, two lymphoblastic lymphomas, and two anaplastic large cell lymphomas.

*This mutation in exon 2 is located outside the *p14^{ARF}* open reading frame.

†These two mutations in exon 2 produced a stop codon in *p16^{INK4a}* and a missense mutation in the *p14^{ARF}* open reading frame.

‡Two cases had homozygous deletions of the *p53* gene.

§One case had exon 1α/2 deletion, but exon 1β was retained.

p19^{ARF} overexpression induces a cell cycle arrest in both G₁ and G₂ without direct interaction with cyclin-dependent kinases (CDKs).⁷ However, its inhibitory effect is dependent on *p53*, indicating that *p14/p19^{ARF}* may act as an upstream regulator of *p53* function.^{7,8} Recent studies have shown that *p14/p19^{ARF}* interacts physically with MDM2 and stabilizes *p53* protein in the nucleus of the cell by blocking its cytoplasmic transport and MDM2-mediated degradation.^{9–14} These observations have suggested that inactivation of *p14/p19^{ARF}* may lead to an increased degradation of *p53*, and, consequently, it may function as an alternative mechanism to *p53* mutations in the pathogenesis of tumors. Therefore, genetic alterations of the *INK4a/ARF* locus may impair both *p14^{ARF}/p53* and *p16^{INK4a}/Rb* pathways, providing a selective advantage in the development and progression of tumors.

Different studies have suggested a possible role of *p14/p19^{ARF}* in tumorigenesis, independently of the inactivation of *p16^{INK4a}*. Knockout mice lacking exon 1β develop a spectrum of malignant neoplasms similar to those described in animals deficient for *INK4a* exon 2,^{8,15,16} as well as to the tumors described in *p53* null mice.¹⁷ In addition, selective losses of exon 1β have been observed in other experimental murine lymphomas,¹⁸ occasional human melanomas,¹⁹ and acute lymphoblastic leukemias.^{20,21} However, the presence of *p14^{ARF}* alterations in human NHLs and their possible alternative or coordinate inactivation with *p16^{INK4a}* and *p53* genes are not well known. To determine the potential role of *p14^{ARF}* in the pathogenesis of NHLs and its relationship with *p16^{INK4a}* and *p53* inactivation, we have investigated the possible *p14^{ARF}* and *p53* gene alterations in a large series of NHLs previously characterized for *p16^{INK4a}* abnormalities.²² Our results indicate that *p14^{ARF}* alterations in human NHLs are always associated with *p16^{INK4a}* aberrations and occur mainly in tumors with wild-type *p53* gene.

Materials and Methods

Case Selection

Tumor specimens from 97 NHLs were obtained from the Department of Pathology of the Hospital Clinic, University of Barcelona, on the basis of the availability of frozen samples for molecular studies. These cases were classified according to the Revised European-American Classification of Lymphoid Neoplasms.²³ The tumors were grouped into indolent and aggressive categories for statistical purposes. Indolent tumors included 18 chronic lymphocytic leukemias (CLLs), two hairy cell leukemias (HCLs), 14 follicular lymphomas (FLs), and 17 typical mantle cell lymphomas (MCLs). Aggressive NHLs comprised seven large cell lymphomas evolved from CLLs (Richter's Syndrome), 10 diffuse large-cell lymphomas (LCLs) transformed from FLs, nine blastoid variants of MCLs, 15 *de novo* diffuse B-cell LCLs, two lymphoblastic lymphomas (LBLs), one Burkitt's lymphoma (BL), and two anaplastic large cell lymphomas (ALCLs). All of these tumors had previously been analyzed for *p16^{INK4a}* exon 2 deletions, exon 1α and 2 mutations, and hypermethylation of the gene.²² Sixteen MCLs had been included in a previous study of exon 5–9 mutations of the *p53* gene.²⁴

p53 Gene Analysis

Mutational analysis of exons 5–9 was performed using single-stranded conformation polymorphism (SSCP) and direct sequencing as previously described.²⁴ In addition, exon 4 was amplified by polymerase chain reaction (PCR), using the primers 5'-TTTTCACCCATCTACAGTCCC-3' and 5'-CTCAGGGCAACTGACCGTG-3'. Amplifications were performed with 0.5 μg of genomic DNA, 1.75 U of *Taq* polymerase (Boehringer-Mannheim, Mannheim, Germany),

Table 2. Genetic Alterations of the *INK4a/ARF* Locus and *p53* in 34 NHLs

Case	Diagnosis	<i>INK4a</i> locus		<i>p53</i>		Altered pathway	
		Exon 1 β	Exon 1 α /2	Gene	IHQ	p16	p53/p14
7003	CLL	GL	GL	Δ 151–154 (121 stop)*	0.1	–	+
8406	CLL	GL	GL	R175G cgc-ggc	76	–	+
1764	CLL	GL	GL	R174S agg-agt	28	–	+
2101	CLL	GL	MUT [†]	WT	0	–	–
6016	RS	GL	GL	H178D cac-gac	ND	–	+
321	RS	DEL	DEL	Δ 902–906 (303 stop)*	91	+	+
8324	RS	DEL	DEL	WT	2	+	+
291	FL	DEL	DEL	WT	3	+	+
1072	FL-T	GL	GL	A76G gca-gga	95	–	+
3834	FL-T	GL	MUT [†]	WT	2	+	+
17000	FL-T	GL	MUT [†]	WT	4	+	+
14760	FL-T	GL	GL	K139Q aag-cag	81	–	+
1106	FL-T	DEL	DEL	WT	20	+	+
14763	FL-T	GL	GL	D281Y gac-tac	32	–	+
6473	MCL	GL	GL	T155N acc-aac	60	–	+
346	MCL	DEL	DEL	WT	2	+	+
3858	MCL-B	DEL	DEL	WT	1	+	+
441	MCL-B	GL	GL	DEL	0	–	+
4958	MCL-B	GL	GL	P278L cct-ctt	76	–	+
1104	MCL-B	GL	GL	R273H cgt-cat	98	–	+
11218	MCL-B	GL	GL	G243S ggc-agc	76	–	+
15855	MCL-B	GL	GL	DEL	2	–	+
15566	MCL-B	DEL	DEL	WT	1	+	+
9630	LCL	DEL	DEL	WT	0	+	+
13576	LCL	GL	M	WT	8	+	–
15136	LCL	GL	M	WT	6	+	–
2502	LCL	GL	GL	Y236C tac-tcg	69	–	+
3591	LCL	GL	GL	A83V gcg-gtg	75	–	+
14796	LCL	GL	M	WT	4	+	–
12866	LCL	GL	M	WT	16	+	–
2584	LCL	GL	GL	R248Q cgg-cag	82	–	+
1765	BL	GL	GL	R248W cgg-tgg	24	–	+
2286	LBL	GL	DEL	R282W cgg-tgg	41	+	+
15345	ALCL	GL	M	WT	11	+	–

*Deletions corresponding to the mentioned nucleotides. The number between parentheses represents the corresponding amino acid of the generated stop codon.

[†]This mutation in exon 2 (A143T) is outside the ARF reading frame.

[‡]This mutation in exon 2 produces the changes R80ter in *INK4a* and P94L in ARF reading frames, respectively.

ND, not done; DEL, homozygous deletion; GL, germ line; IHQ, immunohistochemistry, percentage of p53-positive cells; M, methylated; MUT, mutated; WT, wild type.

0.5 μ mol/L of each primer, 100 μ mol/L of deoxynucleoside triphosphate, and PCR buffer in a final volume of 50 μ L. The reaction was performed for 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Two microliters of amplified products were diluted sixfold in formamide dye loading buffer, incubated for 3 minutes at 95°C, and immediately cooled on ice. Twenty microliters was electrophoresed at 130 V for 20 hours at room temperature on a 12% nondenaturing polyacrylamide gel and at 4°C on a 10% nondenaturing polyacrylamide gel. The gels were developed using a previously described silver staining procedure.²⁵

To confirm the possible *p53* gene mutations, the samples with altered migration in the SSCP analysis were sequenced using a commercial cycle sequencing kit (Perkin Elmer, Branchburg, NY) and [α -³²P]deoxyadenosine triphosphate. A total of 0.5 μ L of the *p53* gene PCR product was used as a template for sequencing. The primers and conditions used for exon 5–9 sequencing were described elsewhere.²⁴ The primers for exon 4 were the same as those used in the PCR reaction described

above. Amplification conditions included an annealing temperature of 68°C.

Southern blot analysis was performed with genomic DNA from frozen material in 55 cases, using proteinase K/RNase treatment and phenol-chloroform extraction. DNA from each case (10 μ g) was digested with *Eco*RI and *Hind*III, separated on 0.8% agarose gels, and transferred to QuiaBrane Nylon Plus (Qiagen, Hilden, Germany). The membranes were prehybridized, hybridized with a cDNA *p53* probe, and washed as previously described.²⁶

INK4a Locus Analysis

Deletions of exon 1 α , exon 1 β , and exon 2 of the *INK4a/ARF* locus were analyzed by multiplex PCR, using the amplification of the β -actin gene as an internal control. The primers used for β -actin were 5'-TCCTTAATGT-CACGCACGATTTC-3' and 5'-GTCACCCACACTGTGCCATCTA-3', the primers for exon 1 β were 5'-GGAG-GCGGCGAGAACAT-3' and 5'-GGGCCTTTCCTACCTG-

Figure 1. A: *INK4a/ARF* locus map. B: Analysis of *p16^{INK4a}* and *p14^{ARF}* gene deletions by PCR. *p16^{INK4a}* exon 1α and *p14^{ARF}* exon 1β deletions were always associated with exon 2 losses. However, exon 1β was retained in one lymphoblastic lymphoma in which exon 1α and exon 2 were both deleted (case 2286). This case showed a *p53* mutation. Case (321) showed concomitant *p16^{INK4a}* and *p14^{ARF}* gene deletion associated with *p53* mutation.

GTCTT-3', the primers for exon 1α were 5'-GAAGAAAG-AGGAGGGGCTG-3' and 5'-GCGCTACCTGATTCC-AATTC-3', and the primers for exon 2 were 5'-CTCTACA-CAAGCTTCCTTTC-3' and 5'-CGGGCTGAACCTTCTGT-GCTGG-3'. We used a "touchdown" PCR strategy for the amplification. Conditions were one cycle at 95°C for 5 minutes; four cycles at 94°C for 45 seconds, at 68°C for 1 minute, and at 72°C for 1 minute; four cycles with an annealing temperature of 67°C; and 30 cycles with an annealing temperature of 66°C. PCR products were resolved on a 2% agarose gel. DNA from laryngeal tumors previously analyzed for homozygous deletions by micro-satellite amplification were used as positive and negative controls.²⁷ Homozygous deletions were considered in our samples when the normalized intensity was less than 60% of the intensity of a normal control case.

Exon 1β mutational analysis was also performed by PCR-SSCP, using the same primers as described above. The PCR products were diluted in formamide-dye loading buffer and electrophoresed on a 15% nondenaturing polyacrylamide gel with or without 10% glycerol at 150 V for 14 hours at room temperature. The gels were devel-

oped with a previously described silver staining procedure.²⁵

p14^{ARF} mRNA Expression

Total RNA was obtained from 20 tumors by guanidine isothiocyanate extraction and cesium chloride gradient centrifugation. The reverse transcription reaction was performed with the SuperScript preamplification kit (Gibco BRL, Gaithersburg, MD) from 1 μg of total RNA, following the manufacturer specifications for a cDNA synthesis using an oligo (dT) primer. A 259-bp fragment of *p14^{ARF}* was amplified by PCR, using the primers 5'-AGTGGCGCTGCTCACCTC-3' and 5'-CTAGGAAGCG-GCTGCTGC-3'. In each case the RPS14 ribosomal mRNA (143 bp) was amplified as positive control, using the primers 5'-GGCAGACCGAGATGAATCCTCA-3' and 5'-CAGGTCCAGGGGTCTTGGTCC-3'. The amplification conditions were four cycles at 95°C for 45 seconds, 68°C for 1 minute, and 72°C for 1 minute, and 30 cycles with an annealing temperature of 66°C, with an additional elongation step at 72°C for 10 minutes. The amplified products were separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

p53 Immunohistochemical Analysis

p53 protein expression was assessed immunohistochemically on fixed and paraffin-embedded material, using the anti-*p53* clone BP53-12 monoclonal antibody (Novocastra, Newcastle on Tyne, UK) at a final dilution of 1:50. The deparaffinized and rehydrated slides were placed in Dako ChemMate citrate solution (Dako, Glostrup, Denmark) in a pressure cooker before incubation with the primary antibody. *p53* antibody was incubated for 1 hour at room temperature. The immunoreaction was developed in an automated TechMate 500 (Dako), using the Dako EnVision+ System, peroxidase (DAB) technique, with diaminobenzidine as the chromogen. The slides were counterstained with Mayer's hematoxylin. Quantification of positive cells was performed with a computerized digital analyzer (Microlmage; Olympus Europe, Hamburg, Germany). To score the positive

Table 3. Correlation between *p16^{INK4a}* and *p14^{ARF}* Alteration and *p53* Gene Status in NHLs

<i>INK4a/ARF</i>	n	<i>p53</i> gene	
		Wild type	Mutated/deleted
Wild type	80	63	17
Exon 1α/2 deletion	9	7	2*
Exon 1β alteration	8	7	1
Exon 2 mutation	3†	3	0
<i>p16^{INK4a}</i> hypermethylation	5	5	0
Total	97	78	19

*One lymphoblastic lymphoma with homozygous deletion of exons 1α/2 but retaining exon 1β had a *p53* mutation.

†One mutation in exon 2 was outside the *p14^{ARF}* open reading frame.

Table 4. p53 Expression Levels and p14^{ARF} Gene Status in 44 Aggressive NHLs*

p53 and p14 ^{ARF} gene status		N	p53 protein expression			
			mean (%) ± SD	<5%	5–30%	>30%
p53 wild type	p14 ^{ARF} wild type	22	17 ± 25 [†]	9	8	5
p53 mutated	p14 ^{ARF} wild type	11	68 ± 25	0	1	10
p53 deleted	p14 ^{ARF} wild type	2	1 ± 2	2	0	0
p53 wild type	p14 ^{ARF} altered	7	4 ± 7 [†]	6	1	0
p53 mutated	p14 ^{ARF} altered	2	66 ± 36	0	0	2

*In two cases, immunohistochemistry could not be evaluated.

[†]p53/p14^{ARF} wild type versus p53 wild type/p14^{ARF} altered (P = 0.04).

nuclei, the percentage of the immunohistochemically stained area was measured against the total nuclear area. Five or more fields of each tumor were analyzed, until a minimum of 1000 cells had been examined. The fields in each slide were selected from the areas with greatest number of stained cells. p53 expression was considered as negative, low, or high when less than 5%, 5–30%, or more than 30% of the cells showed nuclear immunostaining.

Statistical Analysis

Categorical data were compared using the Fisher's exact test (two-sided P value), whereas for ordinal data non-parametric tests were used. The actuarial survival analysis was performed according to the method described by Kaplan and Meier, and the curves were compared by the log-rank test.

Results

Analysis of the p53 Gene

p53 gene mutations in exons 4–9 were analyzed by PCR-SSCP, and cases with anomalous migrating bands were sequenced. Gene alterations were found in four (8%) indolent lymphomas and 15 (33%) aggressive tumors (indolent versus aggressive; P = 0.01) (Table 1). The mutated indolent lymphomas were three CLLs and one typical MCL. The CLLs showed two missense mutations at exon 5 and one microdeletion of four nucleotides at exon 4, with a frameshift in the sequence leading to a stop codon (Table 2). The typical MCL had a missense mutation in exon 5 (Table 2). The 15 aggressive lymphomas with p53 alterations were two LCLs transformed from CLL (Richter's syndrome), three LCLs transformed from FL, five blastoid MCLs, three *de novo* diffuse B-cell LCLs, one Burkitt's lymphoma, and one T-cell lymphoblastic lymphoma (Tables 1 and 2). Twelve of these tumors showed missense mutations, two at exon 4, two at exon 5, four at exon 7, and four at exon 8 (Table 2). A LCL transformed from a CLL showed a deletion of five nucleotides at exon 8 with a frameshift in the sequence causing a subsequent stop codon. The PCR amplification signal of the p53 gene was very low in two blastoid MCLs (cases 441 and 15855). Southern blot analysis of these two cases showed a homozygous deletion of the p53 gene (Table 2). The very faint band obtained in the PCR analysis could be due to amplification of the gene from

the scarce normal or reactive cells present in the sample. No p53 gene alterations were detected in 53 additional tumors that were also examined by Southern blot.

Analysis of p16^{INK4a} and p14^{ARF} Gene Deletions

Deletions of exons 1α, 1β, and 2 of the INK4a/ARF locus were analyzed by multiplex PCR. Exon 2 deletions were

Figure 2. p53 immunohistochemical analysis in three representative cases of aggressive NHLs. **A:** High p53 protein expression in a tumor with p53 missense mutation and p14^{ARF} wild-type gene. **B:** Moderate p53 protein expression in a tumor with p53 and p14^{ARF} wild-type genes. **C:** Negative p53 protein expression in a tumor with p14^{ARF} deletion and p53 wild-type gene.

Figure 3. RT-PCR analysis of *p14^{ARF}* mRNA. *p14^{ARF}* expression was detected in cases with the *INK4a/ARF* locus in germ line (GL) (cases 4966, 1072, 5377, 2101) and cases with *p16^{INK4a}* hypermethylation (met) (cases 15136, 13576). No signal was observed in cases with homozygous deletions of the *INK4a/ARF* locus (del) (cases 15566, 321, 3858, 346, 8324).

detected in nine cases, two indolent and seven aggressive lymphomas. The two indolent tumors were a typical MCL and a follicular lymphoma. The seven aggressive lymphomas were two LCLs transformed from CLL (Richter's syndrome), one LCL transformed from FL, two blastoid MCLs, one *de novo* diffuse B-cell LCL, and one lymphoblastic lymphoma of T-cell phenotype (Table 1). The results obtained in this PCR analysis of exon 2 were concordant with those obtained in the previous analysis of exon 2 by Southern blot.²²

p16^{INK4a} exon 1 α and *p14^{ARF}* exon 1 β deletions were always associated with exon 2 losses. However, exon 1 β was retained in one lymphoblastic lymphoma in which exon 1 α and exon 2 were both deleted (case 2286) (Figure 1). Interestingly, the Southern blot analysis of this case showed a deletion of *p16^{INK4a}* exon 2, but *p15^{INK4b}* was in germ line configuration.²² *p15^{INK4b}* was also deleted in other seven tumors with exon 1 β , 1 α , and 2 deletions but was in germ line configuration in one Richter's syndrome with deletion of exons 1 β , 1 α and 2.²² No isolated deletions of exon 1 β were detected in any case.

Mutational Analysis of the *p14^{ARF}* Gene

Mutations of exons 1 β and 2 were analyzed by PCR-SSCP followed by sequencing of cases with anomalous migrating bands. No mutations were detected in exon 1 β in any case (Table 1). Exon 2 mutations had been detected in one CLL and two transformed follicular lymphomas.²² In the open reading frame of *p16^{INK4a}*, the CLL mutation was at codon 143 (GCC $\rightarrow$ ACC), resulting in a change of alanine to threonine. This mutation was located outside the open reading frame of *p14^{ARF}*. The two transformed FLs showed the same mutation. In the open reading frame of *p16^{INK4a}*, this change was a nonsense mu-

tation at codon 80 CGA (Arg) $\rightarrow$ TGA (Stop). The same change in the *p14^{ARF}* open reading frame was at codon 94, producing a missense mutation, CCG (Pro) $\rightarrow$ CTG (Leu). Western blot analysis of these two cases had shown a lack of p16 protein expression.²²

Correlation between *p53*, *p14^{ARF}*, and *p16^{INK4a}* Gene Alterations

The correlation between *p53*, *p14^{ARF}*, and *p16^{INK4a}* gene alterations is shown in Table 3. *p53* inactivation was detected in 19 (20%) tumors: 15 missense mutations, two microdeletions leading to stop codons, and two homozygous deletions. *p14^{ARF}* gene alterations were detected in 11 (11%) tumors: eight showed deletions of exon 1 β and exon 2, one case showed deletion of exon 2 but retained exon 1 β , and two tumors had point mutations in exon 2 involving the nucleolar transport domain of the protein. *p16^{INK4a}* alterations were detected in 17 (18%) tumors, including nine homozygous deletions involving both exons 1 α and 2, five hypermethylations, and three point mutations.²²

Genetic alterations of the *p53* gene and the *INK4a/ARF* locus mainly occurred in different tumors (Table 3). Seventeen cases with *p53* gene alterations had a wild-type *INK4a/ARF* locus, whereas 15 tumors with genetic alterations in this locus had a wild-type *p53* gene. Concomitant alterations in the *p53* gene and the *INK4a/ARF* locus were detected in only two cases (Table 2). Interestingly, one of these cases was the lymphoblastic lymphoma in which the *p14^{ARF}* exon 1 β was retained, whereas *p16^{INK4a}* exons 1 α and 2 were deleted.

p53 and *p14^{ARF}* Gene Expression

Experimental studies have shown that *p14^{ARF}* stabilizes *p53* protein in the nucleus of the cell by inhibiting its cytoplasmic transport and the MDM2-mediated degradation.⁹⁻¹⁴ To determine whether *p14^{ARF}* gene alterations may be associated with lower *p53* levels in human tumors, *p53* protein expression was examined by immunohistochemistry, and its expression levels were compared to the status of the *p53* and *p14^{ARF}* genes.

p53 protein expression in indolent lymphomas was usually negative (<5%). Similarly, the CLL with a nonsense mutation in exon 4 was negative for *p53* protein. The two CLLs and the typical MCL with *p53* gene missense mutations showed a high *p53* protein expression. The typical MCL and the FL with *p14^{ARF}* deletion showed

Table 5. Inactivation of *p53/p14^{ARF}* and *p16^{INK4a}* Pathways in NHLs

Pathway status	Indolent (n = 51)	Aggressive (n = 46)
<i>p53/p14^{ARF}</i> and <i>p16^{INK4a}</i> wild type	88%	41%
<i>p53/p14^{ARF}</i> or <i>p16^{INK4a}</i> pathways impaired	8%	39%
<i>p53/p14^{ARF}</i> and <i>p16^{INK4a}</i> pathways impaired	4%	20%

P < 0.001.

negative levels similar to those of the remaining tumors with wild-type $p14^{ARF}$ gene.

Contrary to the negative immunostaining in indolent lymphomas, aggressive tumors with wild-type $p53$ and $p14^{ARF}$ genes showed a broad range of p53 protein expression (mean 17% SD $\pm$ 25) (Table 4). Tumors with $p53$ missense mutations, including the two cases with concomitant *INK4a/ARF* exon 2 deletions, showed high levels of p53 protein expression, whereas the two tumors with homozygous deletions of $p53$ were negative (Tables 2 and 4). Interestingly, protein expression in aggressive tumors with $p14^{ARF}$ altered and wild-type $p53$ was significantly lower than in tumors with no alteration in any of these genes (mean 4%, SD $\pm$ 7 versus mean 17%, SD $\pm$ 25; $P = 0.04$) (Table 4 and Figure 2).

$p14^{ARF}$ mRNA expression was analyzed in 20 tumors, including five cases with homozygous deletions of the gene, two cases with $p16^{INK4a}$ hypermethylation but wild-type $p14^{ARF}$, and 13 with no alterations of the *INK4a/ARF* locus. $p14^{ARF}$ mRNA was present in all cases with wild-type *INK4a/ARF* locus and in the two cases with $p16^{INK4a}$ hypermethylation. No expression was detected in the cases with deletion of the gene (Figure 3).

Correlation between $p53/p14^{ARF}$ and $p16^{INK4a}$ Gene Alterations and the Outcome of the Patients

The $p53/p14^{ARF}$ and $p16^{INK4a}$ gene alterations according to the histological subgroup (indolent versus aggressive) are detailed in Table 5. $p53$ and $p14^{ARF}$ genes are considered to be in the same regulatory pathway. Therefore, patients with alterations in either of these genes were included in the same group. Patients with aggressive lymphomas showed one impaired pathway (either $p53/p14^{ARF}$ or $p16^{INK4a}$) (39% versus 8%) and two impaired pathways (both $p53/p14^{ARF}$ and $p16^{INK4a}$) more frequently (20% versus 4%; $P < 0.001$) than indolent lymphomas.

To determine the possible correlation between the gene alterations and the outcome of the patients, 54 patients with appropriate clinical data and all of the molecular studies performed at diagnosis were included in the analysis. For this purpose, patients with no alterations in $p53/p14^{ARF}$ or $p16^{INK4a}$ (group A) were compared with patients with alterations in either $p53/p14^{ARF}$ or $p16^{INK4a}$ (group B), and patients with concomitant alterations in both $p53/p14^{ARF}$ and $p16^{INK4a}$ (group C). In the group of MCLs, whose clinical characteristics have previously been published,²⁸ patients with inactivation of both pathways had a significantly worse prognosis than patients with alterations in only one gene or no alterations in any of them. Thus, in these patients, the 36-month survival rates of groups A, B, and C were 87%, 34%, and 0%, respectively ($P = 0.007$) (Figure 4). In the whole series, patients of group A showed a trend for better survival than those of groups B and C (median survival: 60, 24, and 17 months, respectively; $P = 0.08$).

Figure 4. Overall survival of 24 patients with mantle cell lymphoma according to the gene alterations at diagnosis. **A:** $p53/p14^{ARF}$ and $p16^{INK4a}$ pathways nonaltered ($n = 15$; dead: 9). **B:** Alterations in the $p53$ pathway only ($n = 6$; dead: 5). **C:** Concomitant alterations in both $p53/p14^{ARF}$ and $p16^{INK4a}$ pathways ($n = 3$; dead: 3) ($P = 0.007$).

Discussion

Inactivation of the two major human tumor suppressor genes $p53$ and $p16^{INK4a}$ is a relatively common phenomenon in primary and transformed high-grade lymphomas, occurring in 20–50% of these tumors.^{2,22,24,29} Most of the previous studies have evaluated the two genes individually, and, therefore, it is not known whether these alterations may function as cooperative or alternative mechanisms in the pathogenesis of aggressive lymphomas. The recent understanding of the peculiar genomic structure of the *INK4a/ARF* locus coding for two overlapping genes, $p16^{INK4a}$ and $p14^{ARF}$, and the function of $p14^{ARF}$ as an upstream regulator of $p53$ activity, has raised the question of the relative importance of these genes in the development of human tumors.

In this study we have examined the status of $p53$ and $p14^{ARF}$ genes simultaneously in a large series of human NHLs previously analyzed for $p16^{INK4a}$. The frequency of alterations in the $p53$ and $p16^{INK4a}$ genes was similar to that observed in other series of lymphomas.^{29–33} Thus $p53$ aberrations were detected in 8% of indolent lymphomas but in 33% of aggressive tumors, whereas $p16^{INK4a}$ alterations were found in 6% and 30% of low- and high-grade lymphomas, respectively. Inactivation of $p14^{ARF}$ was always associated with $p16^{INK4a}$ alterations. Exon 1 β was concomitantly deleted with exon 1 α and 2 in almost all cases. Only one lymphoblastic lymphoma, which harbored exon 1 α and 2 deletions, retained exon 1 β . In addition, no mutations were detected in exon 1 β in any case. Although some studies in murine models^{8,16,18} and human acute lymphoblastic leukemias^{20,21} have suggested that $p14^{ARF}$ may be selectively inactivated in lymphoproliferative disorders, our findings suggest that independent inactivation of $p14^{ARF}$ in human NHLs is a rare phenomenon.

$p14/19^{ARF}$ is considered to be an upstream regulator of $p53$ function. Experimental studies have shown that $p14/19^{ARF}$ interacts physically with MDM2, thus preventing $p53$ degradation and promoting $p53$ stabilization and accumulation.^{9,14,34,35} These observations suggest that tumors with inactivation of the *ARF* locus may have an increased degradation of $p53$ protein and, consequently,

a defective regulation of the *p53* regulatory pathway. Consistent with these experimental studies, we observed that *p53* protein expression in aggressive lymphomas with *p14^{ARF}* exon 1 β deletions was significantly lower than in other aggressive tumors with a wild-type *INK4a/ARF* locus.

Interestingly, two transformed FLs with mutations in exon 2 were also negative for *p53* protein. Initial studies of the functional domains of *p19^{ARF}* showed that exon 1 β was necessary and sufficient to produce cell-cycle arrest, whereas exon 2 mutations seemed to specifically inactivate the *p16^{INK4a}* gene but not *p19^{ARF}*.^{9,36} However, recent studies on human *p14^{ARF}* have identified a C-terminal domain between residues 83 and 101 of exon 2 that are required for its localization in the nucleolus and for the formation of stable *p14^{ARF}*-MDM2-*p53* nuclear complexes.¹³ Mutations in these residues disrupt the nucleolar localization of *p14^{ARF}*, leading to a cytoplasmic diffusion of the protein and a reduced stabilization of *p53* protein.¹³ Interestingly, in contrast to human *p14^{ARF}*, the nucleolar localization domain of murine *p19^{ARF}* seems to reside in a region coded by exon 1 β .³⁴ These observations suggest that mutations in the human exon 2 involving this nucleolar domain may target both the *p14^{ARF}* and *p16^{INK4a}* genes. The exon 2 mutation detected in our two transformed FLs was found in codon 80 of *INK4a* and codon 94 of *ARF* reading frames, leading to a stop codon in *p16^{INK4a}* and a missense mutation within the nucleolar localization domain of *p14^{ARF}*. In fact, these two tumors showed a lack of expression of both *p16^{INK4a}* and *p53* protein, suggesting that this mutation may target *p14^{ARF}* and *p16^{INK4a}* genes simultaneously.

We have also detected one exon 2 mutation in an indolent CLL. This mutation was identified in *p16^{INK4a}* codon 143, which is outside the ankyrin repeat motifs in a region that is not required for the protein to bind and inhibit CDK4,³⁷ and it is outside the open reading frame of *p14^{ARF}*. Therefore, it is possible that this mutation was not interfering with the function of either of these proteins. Concordantly, the clinical behavior of this patient was similar to that of other indolent CLL patients with an *INK4a/ARF* wild-type locus. In a review of the literature of the *INK4a/ARF* alterations in lymphoproliferative disorders, we have identified 21 mutations involving the *p16^{INK4a}* gene, with eight occurring in indolent disorders (six CLLs and two FLs) and 13 in aggressive tumors (nine acute lymphoblastic leukemias, three LCLs, and one Burkitt's lymphoma).^{20,22,29} Six of the 13 mutations in aggressive tumors, but only one of the eight indolent disorders, led to a change in the *ARF* open reading frame. The remaining changes were downstream of the *ARF* stop codon or did not cause an amino acid change in the sequence. These observations suggest that exon 2 mutations involving both genes may play a role in the development of aggressive tumors.

The recent understanding of the *INK4a/ARF* locus as a regulatory region of both *p16^{INK4a}/Rb* and *p14^{ARF}/p53* pathways has suggested that tumors with inactivation of this locus may harbor fewer *p53* mutations than tumors with wild-type *INK4a/ARF* genes.¹⁰ In our study *p53* mutations and *INK4a/ARF* alterations appeared to occur in

different tumors because only two aggressive lymphomas, one lymphoblastic lymphoma and one Richter's syndrome, harbored simultaneous *p53* mutation and homozygous deletion of the *INK4a/ARF* locus. Reciprocal alterations between *p53* mutations and *p16^{INK4a}* alterations have been observed in some tumors.^{25,30,38} However, mutual exclusion between these alterations has not been detected in leukemia-lymphoma cell lines³⁹ and other human tumors, including large B-cell lymphomas.^{40,41} It has been suggested that the pattern of these alterations in tumors may depend on the order of events.¹² If deletion of the *INK4a/ARF* locus occurs early in the development of the tumor, the neoplastic cells may remain wild type for *p53*. In contrast, an initial mutation in the *p53* gene may be followed by a strong pressure against *p16^{INK4a}* that would lead to a concomitant deletion of *p14^{ARF}*. However, tumors with inactivation of both *p53* and *p14^{ARF}* may have a selective growth advantage because there is no complete overlap between the *ARF* and *p53* functions.⁴⁰ In fact, *p14^{ARF}* does not seem to participate in the apoptotic response to DNA damage, and, consequently, cells with *p53* loss may tolerate higher levels of genomic damage than tumor cells with wild-type *p53*.^{8,12}

Different studies have demonstrated the prognostic value of *p53* mutations in NHLs. However, the prognostic significance of *INK4a/ARF* alterations in these tumors is not well known. Garcia-Sanz et al have indicated that *p16^{INK4a}* deletions and rearrangements are associated with poor prognosis in B-cell NHLs, but this study did not include *p14^{ARF}* and *p53* analysis.⁴² Similarly, we have recently shown that DNA losses in 9pter are associated with a poor prognosis in MCL patients.²⁶ Genetic alterations in the *INK4a/ARF* locus target both *p14^{ARF}/p53* and *p16^{INK4a}/Rb* pathways and, therefore, may provide a selective growth advantage in the progression of tumors. In our study, although the series was heterogeneous and the number of patients relatively small, there was a trend indicating a poor prognosis for patients with inactivation of both *p16^{INK4a}* and *p14^{ARF}/p53* genes. In the homogeneous subset of MCLs, patients with genetic alterations in the *INK4a/ARF* locus had a significantly worse prognosis than patients with tumors with only *p53* mutations or with no aberrations in any of these genes.

In this study, independent inactivation of *p16^{INK4a}* only occurred by hypermethylation. Similar to observations of other tumors, in our lymphomas hypermethylation of the *p16^{INK4a}* gene does not affect the expression of *p14^{ARF}*.^{43,44} On the other hand, different studies have already shown that inactivation of *p14^{ARF}* by hypermethylation is extremely rare.^{44,45} The number of patients with isolated inactivation of *p16^{INK4a}* in our series was too small to discern whether hypermethylation of *p16^{INK4a}* may have any particular influence on the survival of the patients.

In conclusion, our findings indicate that genetic alterations in *p14^{ARF}* occur in a subset of aggressive NHLs, but they are always associated with *p16^{INK4a}* aberrations. Alterations in the *INK4a/ARF* locus seem to develop frequently in tumors with a wild-type *p53* gene. The shorter survival of patients with lymphomas harboring concomi-

tant alterations of the *p16^{INK4a}* and *p14^{ARF}/p53* genes suggests that simultaneous inactivation of these two regulatory pathways may have a cooperative effect on the progression of the tumors.

Acknowledgments

We thank Dr. Manuel Serrano for his helpful comments on the manuscript.

References

- Nowell PC: Genetic alterations in leukemia and lymphomas: impressive progress and continuing complexity. *Cancer Genet Cytogenet* 1997, 94:13–19
- Imamura J, Miyoshi I, Koeffler HP: p53 in hematologic malignancies. *Blood* 1994, 84:2412–2421
- Hirama T, Koeffler HP: Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 1995, 86:841–854
- Levine AJ: p53, the cellular gatekeeper for growth and division. *Cell* 1997, 88:323–331
- Serrano M, Hannon GJ, Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993, 366:704–707
- Mao L, Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ, Sidransky D: A novel p16INK4a transcript. *Cancer Res* 1995, 55:2995–2997
- Quelle DE, Zindy F, Ashmun RA, Sherr CJ: Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995, 83:993–1000
- Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, Sherr CJ: Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997, 91:649–659
- Zhang Y, Xiong Y, Yarbrough WG: ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998, 92:725–734
- Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C, De Pinho RA: The INK4a tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998, 92:713–723
- Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, Sherr CJ: Functional and physical interactions of the ARF tumor suppressor with p53 and MDM2. *Proc Natl Acad Sci USA* 1998, 95:8292–8297
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G: The alternative product from the human CDKN2a locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* 1998, 17:5001–5014
- Zhang Y, Xiong Y: Mutations in human ARF exon 2 disrupt its nuclear localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell* 1999, 3:579–591
- Tao W, Levine AJ: P19(ARF) stabilizes p53 by blocking nucleocytoplasmic shuttling of MDM2. *Proc Natl Acad Sci USA* 1999, 96:6937–6941
- Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, De Pinho RA: Role of the INK4a locus in tumor suppression and cell mortality. *Cell* 1996, 85:27–37
- Kamijo T, Bodner S, van de Kamp E, Randle DH, Sherr CJ: Tumor spectrum in ARF-deficient mice. *Cancer Res* 1999, 59:2217–2222
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr, Butel JS, Bradley A: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992, 356:215–221
- Zhuang SM, Schippert A, Haugen-Strano A, Wiseman RW, Soderkvist P: Inactivations of p16INK4a-alpha, p16INK4a-beta and p15INK4b genes in 2',3'-dideoxycytidine- and 1,3-butadiene-induced murine lymphomas. *Oncogene* 1998, 16:803–808
- Kumar R, Lundh Rozell B, Louhelainen J, Hemminki K: Mutations in the CDKN2a (p16INK4a) gene in microdissected sporadic primary melanomas. *Int J Cancer* 1998, 75:193–198
- Heyman M, Rasool O, Brandtner LB, Liu Y, Grandt D, Einhorn S, Soderhall S: Exclusive p15INK4B gene deletions in acute lymphocytic leukemia include the E1 beta exon of the p16INK4 gene. *Blood* 1996, 87:1657–1658
- Gardie B, Cayuela JM, Martini S, Sigaux F: Genomic alterations of the p19ARF encoding exons in T-cell acute lymphoblastic leukemia. *Blood* 1998, 91:1016–1020
- Pinyol M, Cobo F, Beà S, Jares P, Nayach I, Fernández PL, Montserrat E, Cardesa A, Campo E: p16INK4a gene inactivation by deletions, mutations and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. *Blood* 1998, 91:2977–2984
- Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peters C, Falini B, Gatter KC, Grogan TM, Isaacson P, Knowles DM, Mason DY, Muller-Hermelink HK, Pileri S, Piris MA, Raffkiaer E, Warnke RA: A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 1994, 84:1361–1392
- Hernandez L, Fest T, Cazorla M, Teruya-Feldstein J, Bosch F, Peinado MA, Piris MA, Montserrat E, Cardesa A, Jaffe ES, Campo E, Raffold M: p53 gene mutations and protein overexpression are associated with aggressive variants of mantle cell lymphomas. *Blood* 1996, 87:3351–3359
- Pinyol M, Hernandez L, Cazorla M, Balbin M, Jares P, Fernandez PL, Montserrat E, Cardesa A, Lopez-Otin C, Campo E: Deletions and loss of expression of p16INK4a and p21Waf1 genes are associated with aggressive variants of mantle cell lymphomas. *Blood* 1997, 89:272–280
- Beà S, Ribas M, Hernández JM, Bosch F, Pinyol M, Hernández L, García JL, Flores T, Gonzalez M, Lopez-Guillermo A, Piris MA, Cardesa A, Montserrat E, Miró R, Campo E: Increased number of chromosomal imbalances and high-level DNA amplifications in mantle cell lymphoma are associated with blastoid variants. *Blood* 1999, 93:4365–4374
- Jares P, Nadal A, Fernandez PL, Pinyol M, Hernandez L, Cazorla M, Hernandez S, Bea S, Cardesa A, Campo E: Disregulation of p16MTS1/CDK4i protein and mRNA expression is associated with gene alterations in squamous-cell carcinoma of the larynx. *Int J Cancer* 1999, 81:705–711
- Bosch F, Lopez-Guillermo A, Campo E, Ribera JM, Conde E, Piris MA, Vallespi T, Woessner S, Montserrat E: Mantle cell lymphoma: presenting features, response to therapy, and prognostic factors. *Cancer* 1998, 82:567–575
- Ruas M, Peters G: The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998, 1378:F115–F177
- Hangaishi A, Ogawa S, Imamura N, Miyawaki S, Miura Y, Uike N, Shimazaki C, Emi N, Takeyama K, Hirotsawa S, Kamada N, Kobayashi Y, Takemoto Y, Kitani T, Toyama K, Ohtake S, Yazaki Y, Ueda R, Hirai H: Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4a/CDKN2, MTS2/p15INK4b, p53, and Rb genes in primary lymphoid malignancies. *Blood* 1996, 87:4949–4958
- Ogawa S, Hangaishi A, Miyawaki S, Hirotsawa S, Miura Y, Takeyama K, Kamada N, Ohtake S, Uike N, Shimazaki C: Loss of the cyclin-dependent kinase 4-inhibitor (p16; MTS1) gene is frequent in and highly specific to lymphoid tumors in primary human hematopoietic malignancies. *Blood* 1995, 86:1548–1556
- Fizzotti M, Cimino G, Pisegna S, Alimena G, Quartarone C, Mandelli F, Pelicci PG, Lo Coco F: Detection of homozygous deletions of the cyclin-dependent kinase 4 inhibitor (p16) gene in acute lymphoblastic leukemia and association with adverse prognostic features. *Blood* 1995, 85:2685–2690
- Stranks G, Height SE, Mitchell P, Jadavay D, Yuille MA, De Lord C, Clutterbuck RD, Treleaven JG, Powles RL, Nacheva E: Deletions and rearrangement of CDKN2 in lymphoid malignancy. *Blood* 1995, 85:893–901
- Weber JD, Taylor LJ, Roussel MF, Sherr CJ, Bar-Sagi D: Nucleolar ARF sequesters Mdm2 and activates p53. *Nat Cell Biol* 1999, 1:20–26
- Honda R, Yasuda H: Association of p19ARF with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J* 1999, 18:22–27

36. Quelle DE, Cheng M, Ashmun RA, Sherr CJ: Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. *Proc Natl Acad Sci USA* 1997, 94:669–673
37. Yang R, Gombart AF, Serrano M, Koeffler HP: Mutational effects on the p16INK4a tumor suppressor protein. *Cancer Res* 1995, 55:2503–2506
38. Newcomb EW, Rao LS, Giknavorian SS, Lee SY: Alterations of multiple tumor suppressor genes (p53 (17p13), p16INK4 (9p21), and DBM (13q14)) in B-cell chronic lymphocytic leukemia. *Mol Carcinog* 1995, 14:141–146
39. Drexler HG: Review of alterations of the cyclin-dependent kinase inhibitor ink4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. *Leukemia* 1998, 12:845–859
40. Sharpless NE, DePinho RA: The INK4a/ARF locus and its two gene products. *Curr Opin Genet Dev* 1999, 9:22–30
41. Moller MB, Ino Y, Gerdes AM, Skdjodt K, Louis DN, Pedersen NT: Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma. *Leukemia* 1999, 13:453–459
42. Garcia-Sanz R, Gonzalez M, Vargas M, Chillon MC, Balanzategui A, Barbon M, Flores MT, San Miguel JF: Deletions and rearrangements of cyclin-dependent kinase 4 inhibitor gene p16 are associated with poor prognosis in B cell non-Hodgkin's lymphomas. *Leukemia* 1997, 11:1915–1920
43. Della Valle V, Duro D, Bernard O, Larsen CJ: The human protein p19ARF is not detected in hemopoietic human cell lines that abundantly express the alternative beta transcript of the p16INK4a/MTS1 gene. *Oncogene* 1997, 15:2475–2481
44. Baur AS, Shaw P, Burri N, Delacretaz F, Bosman FT, Chaubert P: Frequent methylation silencing of p15(INK4b) (MTS2) and p16(INK4a) (MTS1) in B-cell and T-cell lymphomas. *Blood* 1999, 94:1773–1781
45. Robertson KD, Jones PA: The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol Cell Biol* 1998, 18:6457–6473