

# Overall Survival in Aggressive B-Cell Lymphomas Is Dependent on the Accumulation of Alterations in p53, p16, and p27

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Different studies have already shown that the isolated inactivation of p21, p16, or p27 cyclin-dependent kinase inhibitors (CKIs) is associated with increased growth fraction, tumor progression, or decreased overall survival in cases of non-Hodgkin's lymphoma. In this study we linked molecular study of the p53 and p16 genes with immunohistochemical analysis of p27 expression in a group of aggressive B-cell lymphomas [large B-cell lymphomas (LBCLs) and Burkitt's lymphomas]. This was done to analyze the relationship between p53 and p16 silencing, p27 anomalous overexpression, and clinical follow-up, testing the hypothesis that the accumulation of CKI alterations could confer to the tumors a higher aggressivity. In a group of 62 patients, p53 inactivation as a result of mutation was observed in 11 cases (18%) and p16 silencing was seen in 27 cases (43.5%) as a result of methylation (20 of 62), 9p21 deletion (7 of 44), or p16 mutation (2 of 62). The simultaneous inactivation of p53 and p16 was detected exclusively in five LBCL cases. Anomalous expression of p27, which has been proven to be associated with the absence of p27/CDK2 complexes and the formation of p27/cyclin D3 complexes where p27 is inactivated, was detected in 19 of 61 cases (31%). Cases characterized by p27 anomalous expression display concurrent inactivation of p21 (provided by p53 mutations) and/or p16 CKIs in 11 of 14 LBCL cases ( $P = 0.040$ ). When the relationship between the association of inactivated CKIs and overall survival was considered, a significant relationship was found between a lower overall survival probability and an increased number of inactivated CKIs in LBCL cases, with the worst prognosis for the cases displaying concurrent p53, p16, and p27 alterations. This proves that simultaneous inactivation of different tumor suppressor pathways does

indeed take place, and that tumor aggressiveness takes advantage of this CKI-concerted silencing. In this same series of data, Burkitt's lymphoma patients seem to behave in a different way than LBCLs, with p53 and p16 alteration being mutually exclusive and the association with p27 anomalous expression not being clinically significant. These facts seem to support that the additive effect of the inactivation of different CKIs could be dependent of the histological type. (*Am J Pathol* 2001, 159:205–213)

Different genes working in multiple pathways tightly regulate cell cycle control. Two of these genes, p53 and p16, are the most frequently altered genes in human tumors.<sup>1–4</sup> The p16 protein is codified by INK4A/ARF located at the 9p21 locus, which also codifies for the p14/ARF (p14) protein. This locus is the nexus between the two main pathways that control cell cycle progression. The first pathway involves p16 cyclin-dependent kinase inhibitor (CKI), Rb tumor suppressor gene, CDK4 kinase, and cyclin D, and regulates passage through the G<sub>1</sub> restriction point. p14 acts together with the p53 tumor suppressor gene, p21CKI, and MDM2, forming the pathway that regulates the G<sub>1</sub> and G<sub>2</sub> checkpoints or induces apoptosis in response to DNA damage or other cellular insults. MDM2 is also a nexus between both pathways, as p53 and p14 regulate it, and it inhibits p53 and regulates Rb, impeding its association with E2F transcription factors. Both pathways have been shown to be inactivated in a high percentage of human tumors, including lymphomas.<sup>1–7</sup> More frequently, alterations in the p53 and/or p16 genes are the cause of disruptions in both pathways, although anomalies in the other components have also been described.<sup>8,9</sup>

In lymphomas, the p53 gene has been found to be mutated in 19 to 35% of cases,<sup>10,11</sup> whereas p16 has

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been found silenced in from 14 to 85% of cases, usually because of promoter hypermethylation or allelic loss, although rare mutations have been observed.<sup>12-15</sup>

p27/KIP1 (p27) is another CKI, which also acts at the restriction point, blocking Rb phosphorylation by cyclin E/CDK2 inactivation. p27 overexpression in a group of aggressive B-cell lymphomas with a high proliferative index and adverse clinical outcome has been shown to occur in previous studies.<sup>16-18</sup> p27 overexpression has also been referred to in other types of tumors.<sup>19,20</sup> This anomalous expression of p27 was described in association with the absence of p27/CDK2 complexes and the formation of p27/cyclin D3 complexes where p27 is inactive, stabilized, and detectable by immunohistochemical techniques.<sup>21,22</sup>

*In vitro* studies have demonstrated the existence of p27-cyclin D/CDK6 or p27-cyclin D/CDK4 complexes with Rb-kinase activity in cell lines of different types,<sup>23-26</sup> suggesting that p27 is not active when joined to cyclin D/CDK4-6 complexes. A redistribution of p27 from cyclin E/CDK2 to cyclin D/CDK4 complexes, which precludes its negative regulator action on CDK2 activity, has also been described in growing cells, where p27 is associated with cyclin D/CDK4.<sup>21,22,27,28</sup> p15 induction by transforming growth factor- $\beta$  displaces p27 from these complexes to cyclin E/CDK2, co-operating in inducing cell cycle arrest.<sup>23</sup>

The co-operation of the p16/Rb and p53/ARF pathways is suggested by evidence of the synergy found in *in vitro* analysis and the concomitant alteration in both pathways in many tumor types.<sup>29-37</sup> Initial studies in non-Hodgkin's lymphomas have shown hints of concerted inactivation in both pathways, as has also been described in virally -induced cell transformation.<sup>34</sup> In mantle cell lymphomas it has recently been shown that simultaneous inactivation of the p16/Rb and p53/ARF pathways is associated with higher aggressivity,<sup>38</sup> but this has not yet been fully explored in more frequent types of lymphoma, such as large B-cell lymphoma (LBCL) and Burkitt's lymphoma (BL). We therefore explore the status and correlation of the p53 and p16 pathways and the possible relationship with anomalous p27 expression, to find if the accumulation of alterations in CKIs in the same tumor could give more aggressiveness to the tumor. To this end, we performed a molecular analysis for the p53 and p16 genes, together with an immunohistochemical study of p27 and Ki67 proliferation index, in a group of 62 cases of LBCLs and BLs.

## Materials and Methods

### Tissue Samples and Clinical Study

Sixty-two samples of tumor specimens from aggressive B-cell lymphoma, including 46 cases of LBCL and 16 BL, were obtained from the routine files of the Virgen de la Salud Hospital, and diagnosed using the criteria of the revised European-American Classification (REAL) classification.<sup>39</sup>

Cases were selected taking into account the existence of an adequate clinical follow-up and the availability of tissue for molecular studies. Some of the tumors in this series were included in previous studies of p53 mutations<sup>10,11</sup> and p16 alterations.<sup>12</sup>

## Molecular Studies

### Allelic Loss Assays at the 9p21 Locus

Tumor and normal DNA were analyzed for loss of heterozygosity (LOH) or homozygous deletion (HD) by amplification of dinucleotide repeats containing sequence microsatellite markers,<sup>40</sup> under conditions previously described by Villuendas and colleagues.<sup>12</sup> These markers are at the 9p21 region, surrounding the p16 gene (IFN- $\alpha$ , D9S736, D9S1749, D9S1747, D9S974, D9S1748, D9S1752, D9S1711). Briefly, 100 ng of DNA was amplified in a volume of 25  $\mu$ l with 1 $\times$  polymerase chain reaction (PCR) buffer, 200  $\mu$ mol/L dNTPs, 10 pmol of each primer, 1  $\mu$ Ci of <sup>32</sup>P-dCTP and 1 U of *Taq* polymerase. Amplified products were separated by electrophoresis in denaturing 7 mol/L urea-6% acrylamide gels, followed by autoradiography. LOH was considered to have occurred if an entire band was missing or was reduced by >40% of the normal band. If the band was reduced by <40% it was considered to be imbalanced.<sup>12,41</sup> The presence of HDs was assessed in all cases by comparative multiplex PCR assay, using primer sets from loci outside the 9p21 region (D9S934 and D7S1824, in chromosome 9q and 7q, respectively).

## Methylation Studies

Methylation-specific PCR (MSP) assays were performed to analyze the methylation status of CpG islands of the first exon of p16, as described by Herman and colleagues.<sup>42</sup> Briefly, 1  $\mu$ g of denatured genomic DNA was modified by reaction with sodium bisulfite under conditions that convert all 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 mol/L treatment for 5 minutes at room temperature, followed by ethanol precipitation.

Bisulfite-modified DNA was amplified using p16 unmethylated-specific primers (U), methylated-specific primers (M), and unmodified or wild-type primers (W).<sup>42</sup> Fifty ng of bisulfite-modified DNA was amplified using 1 U of AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany) under the following conditions: 30 seconds at 94°C, 30 seconds at 60°C (for U and W), or 65°C (for M) 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. If a methylation-specific PCR product was detected, the whole procedure using sodium bisulfite and MSP was performed again to minimize the possible influence of contamination or incomplete bisulfite treatment.

**Table 1.** Cases with p53 Mutations Including Type of Mutation

Case	Codon (exon)	Mutation	Change in amino acid
LB14	158 (5)	CGC→CAC	Arg→His
LB21	244 (7)	GGC→GAC	Gly→Asp
LB47	273 (8)	CGT→CAT	Arg→His
LB48	273 (8)	CGT→CAT	Arg→His
LB54	270 (8)	TTT→CTT	Phe→Leu
LB146	245 (7)	GGC→AGC	Gly→Ser
LB194	157 (5)	GTC→TTC	Val→Phe
LB271	135 (5)	TGC→TTC	Cys→Phe
LB322	213 (6)	CGA→CGG	Arg→Arg
LB415	213 (6)	CGA→CGG	Arg→Arg
BL13	196 (6)	CGA→TGA	Arg→STOP
BL172	248 (7)	CGG→CAG	Arg→Gln
BL324	215 (6)	AGT→CGT	Ser→Arg
	213 (6)	CGA→CGG	Arg→Arg

DNA methylation was determined by the presence of a 150-pb fragment in those samples amplified with the M primers. DNA from nontumor samples (including peripheral blood lymphocytes from eight healthy donors and three reactive lymphoid nodes) was analyzed as a negative control. DNA from the Raji Burkitt cell line was used as a positive control of p16 methylation.<sup>43</sup>

### Mutation Study

Mutations in exons 5 to 8 of p53<sup>10</sup> and exon 2 of p16<sup>44</sup> genes were analyzed by direct sequencing with an Automated DNA sequencer ABI PRISM 310 genetic analyzer (Applied Biosystems) according to the manufacturer's procedures. The primers and conditions for this sequencing have been previously described.<sup>10,44</sup>

### Immunostaining Techniques

All immunostaining techniques were performed in paraffin-embedded tissue sections, using a previous step of heat-induced antigen retrieval for all antibodies. Thus before incubation with the antibodies, the slides were heated in a pressure cooker for 3 minutes in a solution of 0.01 mol/L sodium citrate. After incubation with the primary antibodies, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidin (LSAB-DAKO, Glostrup, Denmark) with diaminobenzidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device. Incubation omitting the specific antibodies was used as negative control of the technique.

p27 protein was detected with a monoclonal antibody, K25020 (1:1000 dilution; Transduction Laboratories, Lexington, KY), generated against the full-length mouse KIP1 protein. Expression in small lymphocytes was used as an internal control of immunostaining. p27 was scored in the following way: the mean value of the series, (17%), was chosen as a threshold for p27 reactivity. This is approximately equivalent to the level of expression of p27 within the germinal centers.

The proliferation index was evaluated using nuclear antigen Ki67 expression detected with MIB1 antibody from Novocastra (Newcastle on Tyne, UK). Cells showing

nuclear staining were considered to be Ki67-positive. The Ki67 index was determined by scoring at least 300 tumor cells in well-preserved areas as a percentage of Ki67-positive cells. Ki67 reactivity was rounded to the nearest fifth percentile.

### Statistical Analysis

Statistical study of the correlation between distributions was performed using either the chi-square test for categorical variables (Fisher's exact test, Yates' correction, or the Pearson test as appropriate) and the Kruskal-Wallis test for single ordered data. Survival curves were calculated by the Kaplan-Meier method and compared by log rank. Differences were considered significant if  $P < 0.05$ . We did not have appropriate clinical data for only 2 of 62 cases (two LBCL cases). The SPSS software package for Windows (SPSS, Inc., Chicago, IL) was used for all statistical analysis.

## Results

### Analysis of p53 Mutation

We searched for mutations in exons 5 to 8 of the p53 gene. Fourteen mutations were identified in 13 cases (Table 1). Ten mutations were missense, and there was one nonsense mutation. Three silent mutations were identified in codon 213 (exon 6) in cases LB322, LB415, and BL324. In case BL324 two mutations were detected: one silent mutation in codon 213, and one missense mutation in codon 215 (Table 1). In both codons, as well as in the mutations detected in cases LB194 and LB271, the peak corresponding to mutated nucleotide is higher than that corresponding to wild-type (wt), suggesting that one allele is mutated and the other deleted. The wt peak can be interpreted as being derived from the nontumoral cells present in the sample.

Cases LB14, LB48, LB54, LB146, and BL172 showed both wt and mutant dinucleotides at similar intensity, suggesting that they also retained the normal allele. Another possibility is that one allele was deleted and the wt nucleotide detected was because of the nontumor cells



**Table 2.** Cases with p16 Mutations Showing Type of Mutation

Case	Codon	Mutation	Change in amino acid
LB20	129	TAC→TAG	Tyr→STOP
	148	ACG→GCG	Thr→Ala
LB47	73	GCC→GGC	Ala→Gly
LB66	148	ACG→GCG	Thr→Ala
LB169	148	ACG→GCG	Thr→Ala

tions with wild-type primers (W) were used as a control of the efficacy of chemical modification.

#### *p16 Mutation*

To determine whether mutations of the p16 gene were present, we analyzed exon 2 of this gene by direct sequencing in 62 cases. Five mutations in exon 2 were identified in four different cases. Three of them correspond to the change that substitutes ACG<sup>Thr</sup> for GCG<sup>Ala</sup>, which has previously been described as a polymorphism (Table 2).

#### *p27 Expression*

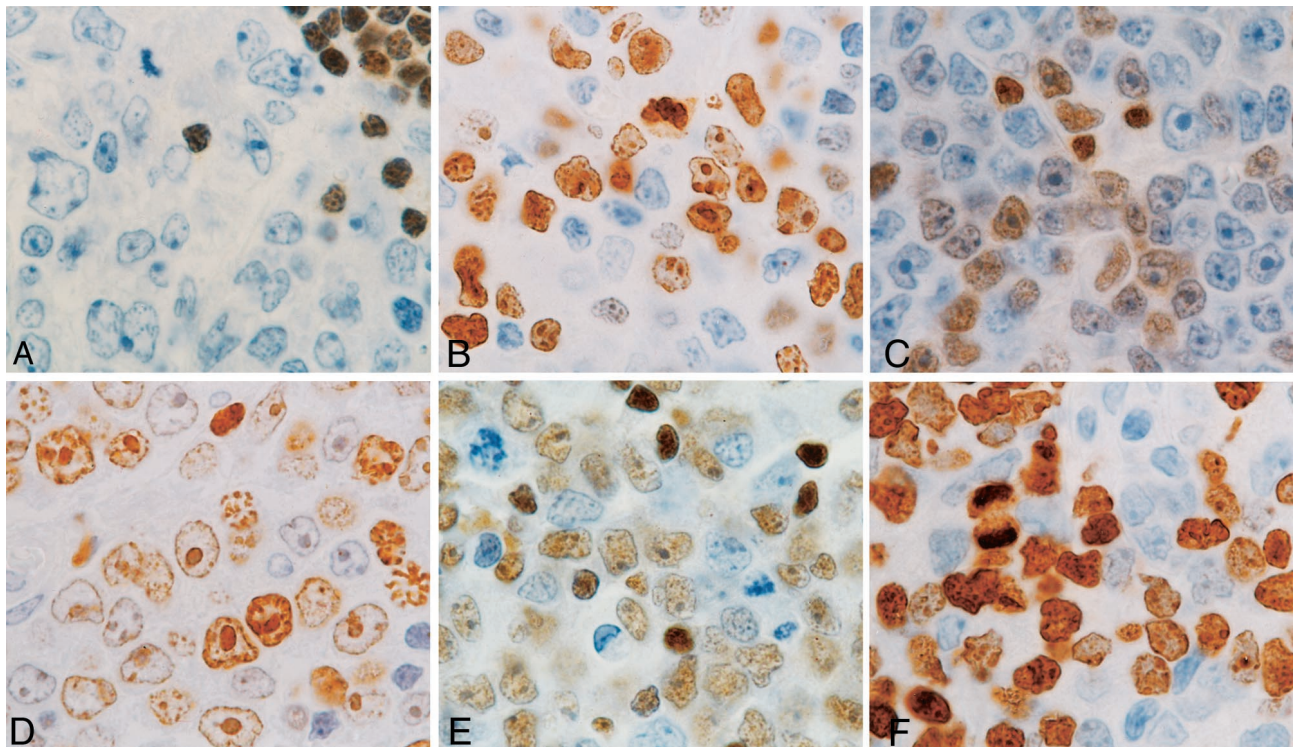
We investigated p27 expression by immunohistochemistry in 61 of 62 cases. Forty-two of 61 lymphoma cases showed absent or low reactivity, below the chosen

threshold. All other cases (19 of 61, 31%; 14 LBCLs and 5 BLs) were considered to be positive for p27 (Figure 4 and Table 3).

#### *Interrelations of p16, p53, and p27 Alterations*

A summary of alterations of p16 and p53 together with p27 immunohistochemistry is shown in Table 3. A total of 33 cases (24 of 46 LBCLs and 9 of 16 BLs) had inactivated p16 and/or p53 genes (53.2% of cases). Five cases (5 LBCLs) showed concurrent p53 mutation and p16 inactivation (8.1%), all of which had p16 inactivated by methylation, and one by mutation together with methylation. Remarkably, although it is not statistically significant, none of the seven cases showing 9p21 locus deletion (where p14, a member of the p53/ARF pathway is located) had p53 mutation. Another striking finding is the absence in the group of BLs of double p53/p16 inactivation, only observed in the LBCL group.

The majority of the cases (11 of 14 LBCLs and 2 of 5 BLs) with a high level of expression of p27 have simultaneous p53 or p16 alterations. When the relationship between p27 overexpression and alterations in p53 and/or p16 were investigated in LBCLs, a significant association was found (chi-square, 4.202;  $P = 0.040$ ; Yates' correction test), which was absent in the BL group. Nevertheless, not all of the p53/p16 alterations were associated with p27 overexpression. Thus, there were 13 LBCLs and

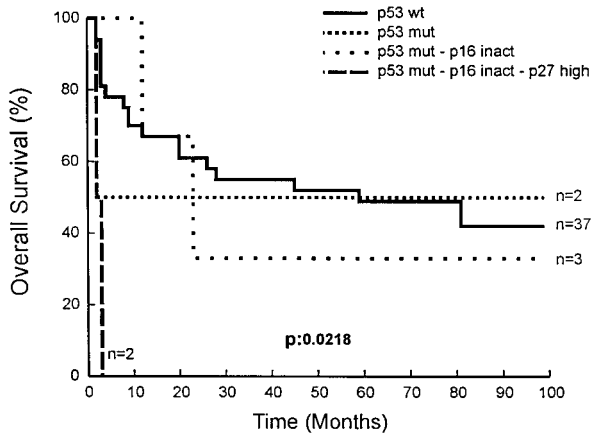


**Figure 4.** Different patterns of p27 and Ki67 protein expression in aggressive B-cell lymphomas. **A:** p27 expression of case LB17, considered to be negative. P27 expression could only be seen in small lymphocytes. **B:** Ki67 expression in the same case (LB17). **C:** p27-positive (+) case (LB47) with the corresponding Ki67 immunostaining (**D**). **E:** A highly p27-positive (++) case (LB169), also displaying high Ki67 protein expression (**F**).

**Table 3.** Summary of p53 and p16 Molecular Alterations, p27 and Ki67 Expression and Clinical Outcome Data in Aggressive B-Cell Lymphomas (46 LBCL and 16 BL)

Case	p53 status	p16 status	p27 IHQ	Ki67 IHQ	Clinical outcome	
					OS	Status
LBCL						
LB1			+	50	59	Death
LB4		MET	-	60	125	Death
LB8		HD	-	50	77	Life
LB14	MUT	MET	-	80	2	Death
LB17			-	70	9	Death
LB20		MUT	++	95	2	Death
LB21	MUT	MET	+	95	3	Death
LB24		MET	-	95	69	Life
LB47	MUT	MET/MUT	+	90	N.A.	N.A.
LB48	MUT		-	90	23	Death
LB51			-	90	94	Life
LB52			-	85	9	Death
LB53			-	60	19	Life
LB54	MUT	MET	-	75	50	Life
LB55			-	50	39	Death
LB61			-	65	36	Life
LB62		LOH	+	75	78	Life
LB65			-	45	4	Death
LB66			+	80	3	Death
LB68		MET	+	60	81	Death
LB69			-	90	3	Death
LB81			-	75	41	Life
LB139		MET	+	95	N.A.	N.A.
LB146	MUT		-	90	12	Death
LB168			-	85	180	Life
LB169		MET	++	80	22	Death
LB173		MET	+	95	28	Death
LB174		MET	-	50	9	Life
LB183		MET	-	100	75	Life
LB184			-	80	7	Life
LB188			-	50	10	Life
LB193			-	60	3	Death
LB194	MUT?	MET	+	70	2	Death
LB196			+	60	48	Life
LB198			-	60	115	Death
LB211		MET	+	95	28	Life
LB271	MUT		+	100	122	Life
LB311			-	60	42	Life
LB322			-	50	22	Life
LB340			-	60	8	Death
LB342			-	70	26	Death
LB415		MET	-	90	7	Life
LB510		MET/LOH	-	60	3	Death
LB530		LOH	-	70	3	Death
LB540		MET	-	25	52	Life
LB568			-	75	2	Death
BL						
BL1			++	100	3	Death
BL7		MET	++	95	4	Death
BL10			-	90	6	Death
BL13	MUT		++	90	96	Life
BL15		MET	-	95	55	Life
BL16		HD	N.A.	N.A.	N.A.	N.A.
BL18			++	100	28	Death
BL82			++	100	10	Life
BL88		LOH	-	90	111	Life
BL172	MUT		-	90	2	Death
BL181			-	100	25	Death
BL414			-	100	42	Life
BL323		HD	-	90	45	Life
BL324	MUT		-	85	110	Life
BL343		MET	-	95	8	Death
BL3035			-	90	71	Life

N.A., data not available; MUT, mutated; MET, methylated; HD, homozygous deletion; LOH, loss of heterozygosity.



**Figure 5.** Overall survival of LBCL patients according to accumulation of alterations in p53, p16, and p27. Inact, inactivated; Mut, mutated. —, p53 wt; ·····, p53 mut-p16 inact; ·····, p53 mut; - - -, p53 mut-p16 inact-p27 high.

6 BLs with p53 and/or p16 alteration, not showing p27 overexpression.

### The Effect of the Simultaneous Inactivation of Different CKIs on Overall Survival

Survival analysis was performed to investigate the impact on the survival probability of the alterations in the p53, p16, and p27 gene/protein here analyzed. Additionally to p21 and p16 silencing, p27 overexpression was included here as an unfavorable marker, because it has been previously shown that, in aggressive lymphomas, it is the result of the formation of complexes with cyclin D3, where p27 is sequestered.<sup>21</sup> In the group of BLs none of the alterations was significantly related with the survival probability, thus this group was not considered for this objective.

In the LBCL group, four different subgroups were compared: group A with wt p53; group B, composed of those cases with p53 mutations; group C with simultaneous p53 and p16 inactivation; and group D that included cases with accumulation of p53, p16, and p27 alterations. Patients with alteration in the three gene/proteins had a significantly worse prognosis than those with p53 mutations or without alterations ( $P = 0.0218$ ) (Figure 5).

**Table 4.** Correlation between p27 Protein Expression and Status of p16 and p53 Genes in LBCL

	p27+	p27-
WT		
p53 WT-p16 WT	3	19
ALT		
p53 MUT-p16 WT	1	2
p16 ALT-p53 WT	7	9
p53 MUT-p16 ALT	3	2
Total ALT	11	13

$P = 0.040$ ;  $n = 46$ .  
 WT, wild type; MUT, mutated; ALT, altered (p16 altered by mutation, deletion, or promoter hypermethylation).

### Discussion

This study examined the status of p53 and p16 tumor suppressor genes in a series of aggressive B-cell lymphomas, together with p27 protein expression, in an attempt to elucidate if the accumulation of alterations cooperate in the pathogenesis of aggressive lymphomas.

p53 mutations and p16 inactivation have been described as the most frequent alterations in human cancer, having been found in approximately half of tumors studied.<sup>4,5</sup> In this study, p53 mutations were detected in 17.7% (11 of 62) of the aggressive B-cell lymphomas analyzed [3 of 16 BL cases (18.8%) and 8 of 46 (17.4%) LBCL cases], this being approximately consistent with previous reports that have shown a percentage of p53 mutations in non-Hodgkin's lymphoma ranging from 19 to 35% of cases, most of them in aggressive tumors.<sup>10,11</sup> Alterations of p16 were observed in 43.5% of cases [6 of 16 BLs (37.5%) and 21 of 46 LBCLs (45.6%)]. In 20 of 62 (32.2%) we found promoter hypermethylation; 9p21 homozygous or hemizygous deletion were found in 7 of 44 cases (16%) and point mutations in 2 of 62 cases (3.2%). These results are consistent with previous reports on p16 alterations.<sup>12-15,45-49</sup> p27 anomalous overexpression was detected in 19 of 61 cases (30.6%), 14 of 46 LBCLs (30.4%), and 5 of 15 BLs (31.3%), a percentage comparable to those described previously.<sup>16,21,50</sup>

A total of 33 cases (53.2%) showed inactivation of p16 and/or p53 (Table 3). Five of these (8.1%, all LBCLs) showed concurrent p53 mutation and p16 inactivation, and all of these cases had p16 inactivated by methylation (one case also shows a mutation in p16 together with promoter hypermethylation), thereby confirming the existence of a subset of cases with simultaneous inactivation of the p53 and Rb pathways, as was recently shown by Pinyol and colleagues.<sup>38</sup> Strikingly, none of the analyzed BLs showed concurrent p53 and p16 alterations, and the frequency of loss of 9p21 was higher in this group, this showing that the inactivation of these growth suppressor pathways (ARF/p53 and p16/Rb) is dependent of the histological type and the underlying specific molecular alterations, such as *c-myc* overexpression and others.

The INK4a/ARF locus in the 9p21 chromosomal region codifies for p14 and p16 proteins; this locus is the nexus between the two main pathways that control cell cycle progression, the Rb/p16 and p53/ARF pathways.<sup>51</sup> None of the nine cases with LOH at 9p21 included in this series showed p53 mutation, which confirms previous observations that these two alterations may be mutually exclusive.<sup>52</sup>

Anomalous p27 protein overexpression, detected in 19 of 61 cases of aggressive B-cell lymphomas analyzed, tends to occur in cases with p16 and/or p53 alteration in aggressive lymphomas (11 of 14 LBCLs). This association is statistically significant ( $P = 0.040$ ). The cause of the anomalous overexpression of p27 in lymphomas with a high growth fraction has previously been demonstrated to be the existence of cyclin D3/p27 complexes that lead to increased stabilization of the protein.<sup>21,22</sup> In experimental assays it has also been shown that p15 induces p27 displacement from the cyclin D/CDK4 complex to

cyclin E/CDK2 in response to transforming growth factor- $\beta$ .<sup>23</sup> The presence of p16 has also been shown to modulate the reassortment of p27 from cyclin D/CDK4 to cyclin E/CDK2 complexes.<sup>26</sup> The results of this series seem to support the hypothesis that in LBCLs p27 accumulation is secondary to loss of the p16 and p21 CKIs, and therefore extends the findings of *in vitro* observations to a specific subset of human tumors. The absence of p16 or p21 precludes the redistribution of p27 from complexes containing CDK4/cyclin D, where p27 is stabilized and probably inactive, to others containing CDK2/cyclin E, where p27 is active in arresting cell proliferation. However, there are 17 cases with p53 or p16 inactivation that do not show simultaneous p27 overexpression, thus indicating the existence of additional factors (*c-myc* or other possible targets) to explain p27 accumulation.<sup>53,54</sup>

Several *in vitro* studies have provided evidence of the synergy between p53 and p16, and concurrent alterations of p16 and p53 have been shown to confer a higher aggressiveness on different tumor types.<sup>35,38</sup> This series analyzing LBCLs shows that the concurrent inactivation of these CKIs [p21 (provided by p53 mutations), p16, and p27], operating in different but co-operative pathways has an accumulative effect on overall survival of LBCL patients ( $P = 0.0218$ ). Whether this phenomenon is valid only in LBCLs or could be extrapolated to other tumors is a debatable point. Thus, in this same series of data, BL patients seem to behave in a different way than LBCLs, p53 and p16 alteration being mutually exclusive and the association with p27 anomalous expression is not clinically significant. Nevertheless, more comprehensive studies in BLs and other tumoral types could bring new data concerning the existence and characteristics of the simultaneous inactivation of different CKIs, and clarify the role that *c-myc* or cyclin D could play in this complex interaction.

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