

## REVIEW

## Multiple cell cycle regulator alterations in Richter's transformation of chronic lymphocytic leukemia

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To investigate the role of the cell cycle regulators p21<sup>Waf1</sup>, p27<sup>Kip1</sup>, retinoblastoma (Rb), and cyclin D1 in Richter's transformation of chronic lymphocytic leukemia (CLL), we analyzed 19 CLL and eight Richter's syndrome (RS) tumors, previously characterized for p53 and ARF/INK4a abnormalities. p21<sup>Waf1</sup> immunohistochemical expression was negative in 12 of 15 CLL (80%), whereas it was moderate or strong in three of seven RS (43%). p21<sup>Waf1</sup> gene was in germline configuration in all the tumors analyzed. Four immunohistochemical patterns of p53 and p21<sup>Waf1</sup> expression were observed: (1) p53<sup>-</sup>/p21<sup>-</sup> in 10 of 15 CLL (67%), but only in two of six RS (33%); (2) p53<sup>+</sup>/p21<sup>+</sup> in three CLL (20%) and two RS (33%); (3) p53<sup>-</sup>/p21<sup>+</sup> in one RS; and (4) p53<sup>+</sup>/p21<sup>-</sup> in two CLL and one RS. Two p53<sup>+</sup>/p21<sup>+</sup> CLL evolved into RS. p53 mutations clustered around the p53<sup>+</sup>/p21<sup>-</sup> (two CLL and one RS) and p53<sup>-</sup>/p21<sup>-</sup> (one CLL and one RS) tumors. While the majority of CLL displayed strong p27 immunoreactivity, RS tumors were constantly p27-negative. p27<sup>Kip1</sup> gene was in germline configuration in all the tumors analyzed. Most CLL cases were negative for Rb expression. In contrast, all RS exhibited strong Rb expression. Cyclin D1 overexpression was only detected in one CLL evolving into RS and one RS. In conclusion, a p53<sup>+</sup>/p21<sup>-</sup> immunohistochemical pattern is shown exclusively by p53-mutated CLL/RS. Additionally, our results suggest a possible implication of moderate/strong p21<sup>Waf1</sup> expression, loss of p27 expression, and cyclin D1 overexpression in the Richter's transformation of CLL.

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## Introduction

B cell chronic lymphocytic leukemia (CLL) evolves into a B cell diffuse large cell lymphoma in about 13% of patients.<sup>1</sup> This process of histologic transformation, also known as Richter's syndrome (RS), has a profound impact on the natural history of patients with CLL. Thus, RS is usually associated with a rapidly progressive clinical course and dismal outcome.<sup>1</sup> The molecular events that underlie the appearance of RS in CLL are poorly known.

Alterations in apoptosis and cell cycle regulatory genes constitute a widespread mechanism of oncogenesis. Particularly, the tumor-suppressor genes p53 and ARF/INK4a are frequently impaired in human tumors.<sup>2–4</sup> In non-Hodgkin's lymphomas (NHL), the inactivation of these two genes has been mainly associated with poor prognosis, and primary aggressive and histologically transformed tumors.<sup>5–10</sup> Regarding CLL, p53

gene abnormalities have been related to advanced stage, resistance to treatment and poor survival.<sup>11–16</sup> In the specific setting of Richter's transformation, p53 mutations and/or ARF/INK4a homozygous deletions occur in about 60% of cases.<sup>8,15–19</sup>

In addition to p53 and ARF/INK4a, other genes involved in cell cycle regulation have been associated with tumoral aggressiveness, histologic transformation and prognosis in NHL. p21<sup>Waf1</sup> gene is induced by wild-type p53 but not mutant p53, and is implicated in the mechanisms of cell cycle arrest that allow the cell for DNA repair.<sup>20</sup> Although p21<sup>Waf1</sup> alterations are rare in human neoplasms,<sup>21</sup> a possible role for this cyclin-dependent kinase inhibitor (CDKI) in tumorigenesis has been postulated on the basis of its transcriptional control by p53. With respect to p27<sup>Kip1</sup>, it is involved in G1 arrest in response to different extracellular signals.<sup>22</sup> p27<sup>Kip1</sup> gene aberrations are also rare in human neoplasms,<sup>23</sup> but the reduced expression of its protein has been associated with tumor progression and poor prognosis.<sup>24,25</sup> As far as Rb is concerned, it negatively regulates cell cycle progression by sequestering several transcriptional factors, which, in turn, control the expression of proliferation and DNA replication genes.<sup>26</sup> Phosphorylation of Rb by cyclin/CDK complexes leads to the inactivation of its growth-suppressive effect.<sup>27</sup> The inactivation of the Rb gene has been related to the development of aggressive NHL.<sup>28,29</sup> The potential role of CDKI p21<sup>Waf1</sup> and p27<sup>Kip1</sup>, and Rb in transformation of CLL has not been largely investigated. On the other hand, cyclin D1 overexpression has also been detected in a subset of atypical and clinically aggressive CLL.<sup>30,31</sup> Whether or not these cases are more prone to evolve into RS than typical CLL is not clear.

To determine their possible role in the pathogenesis of RS, the genetic and/or expression alterations of the CDKI p21<sup>Waf1</sup> and p27<sup>Kip1</sup>, Rb and cyclin D1, have been analyzed in a group of RS and CLL, previously characterized for the presence of p53 and ARF/INK4a gene aberrations.<sup>8,19</sup>

## Patients, materials and methods

## Case selection

Tumor specimens from 19 CLL and eight RS were selected from the files of the Laboratory of Pathology of the Hospital Clínic, University of Barcelona, Spain, based on the availability of paraffin-embedded and frozen tissue for immunohistochemical and/or molecular analyses, respectively. Patient's characteristics at CLL diagnosis and at the moment of the biopsy are detailed in Table 1. The majority of these tumors

**Table 1** Clinical characteristics of the patients at diagnosis and at biopsy

Patient No.	Biopsy No.	CLL diagnosis		Time from diagnosis (months)	Biopsy		Survival (months)
		Age (years)/Sex	Binet (Rai)		Binet (Rai)	Diagnosis	
1 <sup>a</sup>	7520/2228	28/M	B(I)	0/30	B(I)/C(IV)	CLL/RS	38
2 <sup>a</sup>	577/8324	65/F	B(II)	23/29	C(III)/C(III)	CLL/RS	33
3	12311	39/M	C(IV)	48	C(IV)	CLL	+132
4 <sup>b</sup>	921	49/M	A(O)	48	C(IV)	CLL/RS	48
5	6458	47/M	A(I)	0	A(I)	CLL	+55
6	7003	69/F	A(I)	0	A(O)	CLL	19
7	8406	57/M	A(O)	48	C(IV)	CLL	49
8	1535	49/M	A(I)	112	C(IV)	CLL	119
9	4987	NA	NA	NA	NA	CLL	NA
10	642	44/M	B(I)	0	B(I)	CLL	47
11	3893	51/M	A(I)	0	A(I)	CLL	+57
12	1764	62/F	C(III)	0	C(III)	CLL	12
13	2101	45/M	B(I)	NA	NA	CLL	NA
14	1925	48/F	A(I)	0	A(I)	CLL	+46
15	11668	65/F	B(II)	0	B(II)	CLL	+60
16	11336	81/F	C(IV)	0	C(IV)	CLL	+62
17	14795	70/M	A(O)	26	A(I)	CLL	59
18	1603	51/M	C(IV)	0	C(IV)	CLL	45
19	7794	43/M	A(I)	0	A(I)	CLL	+20
20	507	71/F	B(II)	34	C(IV)	RS	36
21	6016	NA	NA	NA	NA	RS	NA
22	321	60/M	A(O)	36	C(IV)	RS	37
23	294	71/M	B(I)	62	C(IV)	RS	63
24	12310	47/F	A(I)	5	A(I)	RS	NA
25	4563	56/F	A(II)	70	C(III)	RS	75

<sup>a</sup>Patients 1 and 2 had tissue specimens from both the CLL and the RS phase of the disease.

<sup>b</sup>Patient 4 evolved to a leukemic RS, showing the t(11;14). Unfortunately, cyclin D1 expression could not be examined in this sample. M, male; F, female; +, alive; NA, not available.

had been previously analyzed for INK4a/ARF and p53 gene alterations.<sup>8,19</sup>

#### Southern blot analysis of the p21<sup>Waf1</sup> and p27<sup>Kip2</sup> genes

High molecular weight DNA was extracted from frozen material using proteinase K/RNase treatment and phenol-chloroform extraction. Fifteen µg of DNA from each case were digested with EcoRI, HindIII, and/or BamHI and analyzed as previously described.<sup>8</sup> A human p21<sup>Waf1</sup> cDNA probe was obtained by reverse transcriptase-PCR from total RNA and cloned using standard techniques.<sup>32</sup> The p27<sup>Kip1</sup> probe used was a 1.5 kb EcoRI fragment of the p27<sup>Kip1</sup> cDNA clone.<sup>22</sup> The β-actin probe was used as a loading control. The probes were radiolabeled using a random primer DNA labelling kit (Amersham Life Science, Buckingham, UK) with [α-<sup>32</sup>P]dCTP. The membranes were prehybridized, hybridized with the p21<sup>Waf1</sup> and the p27<sup>Kip1</sup> probes, and washed as previously described.<sup>32</sup> The intensity of the autoradiographic signals was quantified using a UVP-5000 video densitometer (UVP, San Gabriel, CA, USA).

#### Immunohistochemical analysis

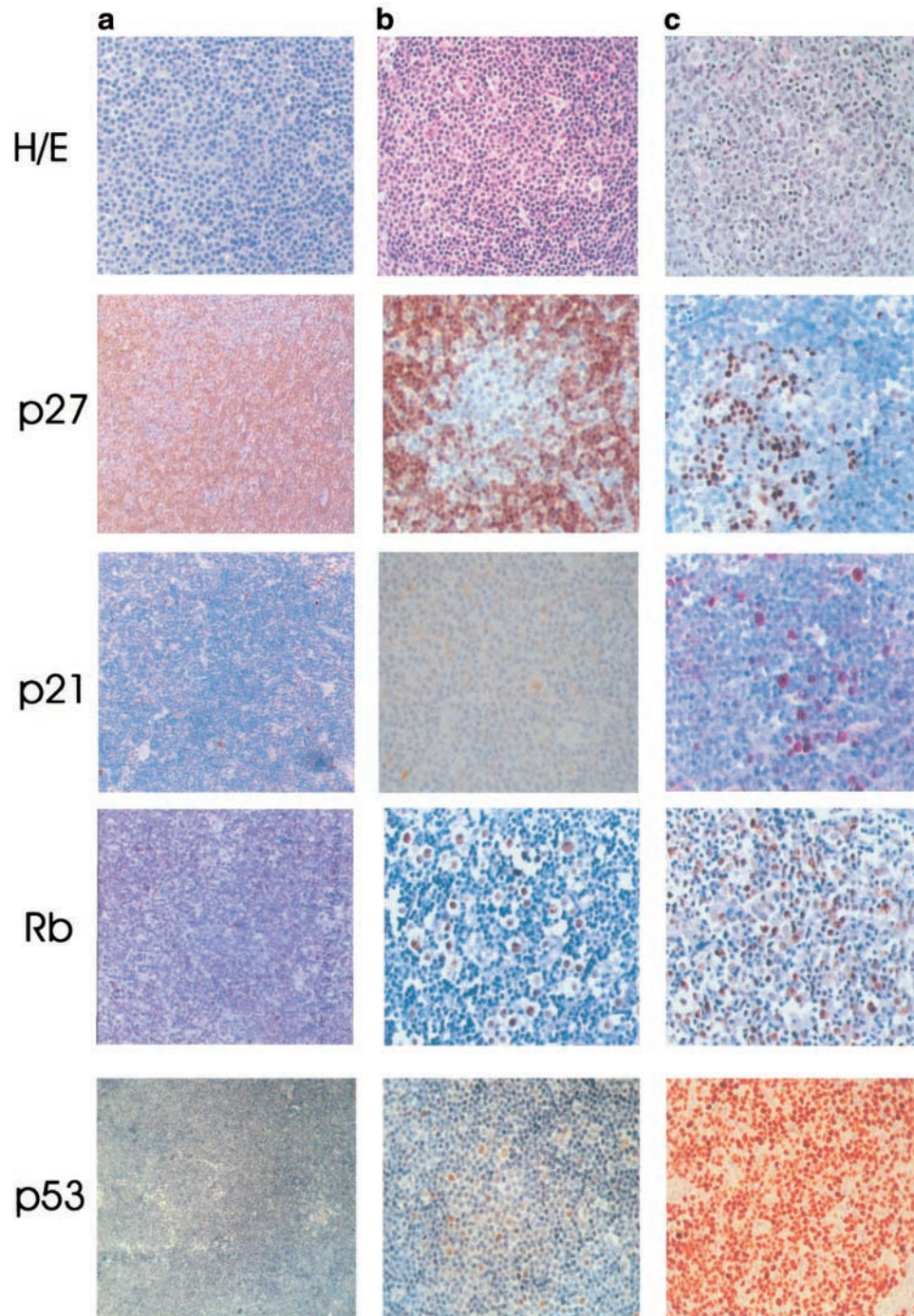
p53, p21<sup>Waf1</sup>, p27<sup>KIP1</sup>, Rb and cyclin D1 protein expression were immunohistochemically assessed on paraffin embedded tissues by using anti-p53 (clone BP53-12; Novocastra, Newcastle on Tyne, UK), anti-p21<sup>Waf1</sup> (clone EA10; Oncogene

Science, Darmstadt, Germany), anti-p27<sup>KIP1</sup> (clone IB-4; Novocastra), anti-Rb (clone Rb I; Novocastra), and anti-cyclin D1 (clone P2D11F11; Novocastra) monoclonal antibodies, respectively. Before the application of the primary antibody, an antigen retrieval technique was performed. The deparaffinized and rehydrated slides were placed in 10 mM citrate buffer, pH 6, and heated in the microwave oven for 15 min at 700 W. The slides were incubated with the monoclonal antibodies for 1 h at room temperature (anti-p53, anti-p21<sup>Waf1</sup>, anti-p27) or overnight at 4°C (anti-Rb, anti-cyclin D1). The immunoreaction was detected by either the streptavidin-biotin-alkaline phosphatase (Biogenex, San Ramon, CA, USA) or the streptavidin-biotin-peroxidase (Dako, Copenhagen, Denmark) techniques. The slides were counterstained with hematoxylin. Quantification of positive cells was evaluated in five or more fields of each tumor until a minimum of 1000 total cells had been examined. The fields in each slide were selected from the most representative areas. The cases were scored as negative (-), moderate (+) or strong (++) when less than 5%, 5–30% or more than 30% of the cells showed nuclear staining. Categorical data were compared using the Exact Fischer's Test (two-sided *P* value).

#### Results

##### p21<sup>Waf1</sup> expression analysis and relationship with p53 gene status

p21<sup>Waf1</sup> expression was immunohistochemically assessed in 22 tumor samples, including 15 CLL and seven RS (Table 2a and b). A general trend to an absence of p21<sup>Waf1</sup> detection was observed in CLL (12 cases; Figure 1), with only three samples



**Figure 1** (a) CLL: lymph node diffusely infiltrated by CLL cells (HE,  $\times 20$ ). Most CLL cells show strong nuclear p27 immunostaining (peroxidase,  $\times 20$ ), but only scattered CLL cells are p21-positive (peroxidase,  $\times 20$ ). Most CLL cells are negative for Rb (alkaline phosphatase,  $\times 20$ ) and p53 (peroxidase,  $\times 10$ ) immunostaining. (b) CLL proliferative growth centers: proliferative growth centers appear as a pale pseudofollicular area in the lymph node section due to an increased number of large cells (HE,  $\times 40$ ). Large cells in the proliferative growth centers are mostly negative for p27 immunostaining (peroxidase,  $\times 40$ ). Some p21-positive large cells are found (peroxidase,  $\times 40$ ). A moderate number of large cells shows nuclear staining for Rb (alkaline phosphatase,  $\times 40$ ) and p53 (peroxidase,  $\times 20$ ). (c) Richter's syndrome: increased number of immunoblastic large cells and of mitotic activity in a case of RS (HE,  $\times 40$ ). Transformed large cells are p27-negative; intermingled p27-positive residual CLL cells also observed (peroxidase,  $\times 40$ ). A moderate number of large transformed cells shows intense p21 nuclear staining (alkaline phosphatase,  $\times 40$ ). A high number of tumor cells are positive for Rb (peroxidase,  $\times 40$ ) and p53 (peroxidase,  $\times 20$ ) immunostaining.

(cases 7520, 577 and 11336) showing moderate p21<sup>Waf1</sup> immunoreactivity (20% of CLL cases; Figure 1). In the latter, p21<sup>Waf1</sup>-positive cells were mainly grouped in proliferative growth centers (Figure 1). p21<sup>Waf1</sup>-positive cells were also observed occasionally in proliferative growth centers of other cases.

However, the number of stained cells was relatively small (<5%). In contrast with CLL, p21<sup>Waf1</sup> expression was consistently moderate (biopsies 2228 and 8324) or strong (biopsy 12310) in three of seven RS (43%). In these cases, the positive cells were diffusely distributed throughout the tumor (Figure 1).

**Table 2** Cell cycle regulators in CLL and Richter's syndrome

Biopsy		p16		p53		p21	p27	Rb	CyD1
No.	Site	SB	MA	MA	IHC	IHC	IHC	IHC	IHC
<i>(a) Cell cycle regulators in CLL</i>									
7520	LN	ND	wt	wt	+	+	++	-	-
577	S	GL	wt	wt	+	+	++	-	-
12311	LN	GL	wt	wt	ND	ND	++	-	-
921	LN	GL	wt	wt	-	-	++	-	++
6458	LN	GL	wt	wt	-	-	++	+	-
7003*	LN	GL	wt	M	-	-	++	+	-
8406*	LN	GL	wt	M	++	-	++	+	-
1535	LN	GL	wt	wt	-	-	++	-	-
4987	LN	ND	wt	wt	-	-	++	-	-
642	LN	ND	wt	wt	ND	ND	++	-	-
3893	T	GL	wt	wt	-	-	++	-	-
1764*	LN	ND	wt	M	++	-	++	-	-
2101	S	ND	M	wt	-	-	++	-	-
1925	LN	GL	wt	wt	-	-	++	-	-
11668	LN	GL	wt	wt	-	-	++	-	ND
11336	LN	GL	wt	wt	+	+	++	-	-
14795	LN	ND	wt	wt	-	-	++	-	-
1603	LN	ND	ND	ND	ND	ND	++	-	-
7794	GI	ND	ND	ND	-	ND	++	-	ND
<i>(b) Cell cycle regulators in Richter's syndrome</i>									
507*	LN	GL	wt	M	-	-	-	++	-
6016*	LN	GL	wt	M	ND	-	ND	ND	ND
321*	LN	DEL	ND	M	++	-	-	++	-
294	LN	GL	wt	wt	-	-	-	++	-
12310*	LN	GL	wt	M	++	++	-	++	-
2228	LN	GL	ND	wt	+	+	-	++	-
8324	LN	DEL	ND	wt	-	+	-	++	-
4563	LN	ND	ND	ND	+	ND	-	++	++

LN, lymph node; S, spleen; T, tonsil; GI, gastrointestinal tract; SB, Southern blot; MA, mutational analysis; IHC, immunohistochemistry; CyD1, cyclin D1; M, mutated; wt, wild type; GL, germline; ND, not done; DEL, deletion.

IHC was scored as negative (-), moderate (+) or strong (++) when less than 5%, 5–30% or more than 30% of the cells, respectively, showed nuclear staining.

\*p53-mutated CLL, case 7003: Δ151–154 (121 stop); case 8406: R175G cgc-ggc; case 1764: R174S agg-agt.

\*p53-mutated RS, case 507: R306ter cga-tga; case 6016: H178D cac-gac; case 321: Δ902–906 (303 stop); case 12310: E171G gag-ggg.

Regarding the relationship between p53 (Figure 1) and p21<sup>Waf1</sup> expression, four main immunohistochemical patterns<sup>33</sup> were observed (Table 3): (1) Negativity for both p53 and p21<sup>Waf1</sup> (p53–/p21–); (2) Relatively similar number of p53 and p21<sup>Waf1</sup> positive cells (p53+/p21+); (3) p53 negativity and moderate p21<sup>Waf1</sup> staining (p53–/p21+); and (4) Strong p53 positivity with negative p21<sup>Waf1</sup> expression (p53+/p21–). The p53–/p21– pattern was observed in 10 of 15 CLL tumors (67%), but only in two of six RS (33%). A wild-type p53 gene had been shown in all these cases, except in one CLL (case 7003) and one RS (case 507) in which a microdeletion of four nucleotides at exon 4, and a missense mutations at exon 8,

both leading to a stop codon, had been demonstrated, respectively.<sup>19</sup> The second pattern, with a relatively similar positivity for both p53 and p21<sup>Waf1</sup>, was observed in three CLL (20%) and two RS (33%). In CLL cases, the number of p53 and p21<sup>Waf1</sup>-positive cells was lower than in RS tumors and, as mentioned above, they were mainly localized in proliferative growth centers, whereas in RS they were dispersed throughout the tumor. In all these cases, a wild-type p53 gene had been disclosed by mutational analysis, except in one RS (case 12310) showing p53 and p21<sup>Waf1</sup> overexpression, in which a missense mutation at exon 5 had been found.<sup>19</sup> Two of the p53+/p21+ CLL cases (biopsies 7520 and 577) evolved into RS (biopsies 2228 and 8324, respectively). The p53–/p21+ pattern was observed in one RS (case 8324), in which the mutational analysis had revealed a wild-type p53.<sup>19</sup> This latter tumor had been previously shown to harbor an INK4a/ARF gene homozygous deletion.<sup>19</sup> The p53+/p21– pattern was observed in three tumor specimens, two CLL and one RS. In these cases, p53 nuclear staining was present in a high number of cells (30–95%), whereas p21<sup>Waf1</sup> expression was negative. Two missense mutations at exon 5 (CLL cases 8406 and 1764) and one microdeletion of five nucleotides at exon 8 (RS case 321), had been detected in these three cases.<sup>19</sup> Negative p21<sup>Waf1</sup> expression was observed in an additional RS (case 6016) with a p53 gene missense mutation at exon 5,<sup>19</sup> in which the p53 immunostaining was not evaluable. Based on

**Table 3** p53/p21 immunohistochemical pattern and relationship with p53 gene status

p53 gene status	CLL		RS	
	wt	M	wt	M
p53–/p21–	9	1	1	1
p53+/p21+	3	0	1	1
p53–/p21+	0	0	1	0
p53+/p21–	0	2	0	1

Wt, wild-type; M, mutated.

our data, a p53+/p21- phenotype associates with the presence of p53 mutations in CLL and RS ( $P = 0.013$ ).

p21<sup>Waf1</sup> gene was analyzed by Southern blot in nine tumor specimens, three CLL (cases 577, 921 and 1535) and six RS (cases 507, 6016, 321, 294, 12310 and 8324). A germline configuration of the gene was demonstrated in all of them.

#### *p27<sup>Kip1</sup> expression analysis*

p27 expression was immunohistochemically assessed in 26 tumor specimens, including 19 CLL and seven RS. In all CLL cases, the majority of neoplastic cells expressed strong p27 nuclear immunoreactivity (Figure 1). Only the larger cells in the proliferative growth centers tended to be negative (Figure 1). On the contrary, large cells of RS cases were constantly negative for the nuclear expression of this CDK inhibitor. In those RS samples in which admixed CLL cells were present, the latter were also positive for p27 expression (Figure 1).

P27<sup>Kip1</sup> gene was analyzed by Southern blot in six tumor specimens, two CLL (cases 921 and 1535) and four RS (cases 507, 6016, 321 and 294). A germline configuration of the gene was demonstrated in all of them.

#### *Rb expression analysis*

Rb protein expression was immunohistochemically assessed in 26 tumors, including 19 CLL and seven RS. Most CLL cases (84%) were considered as negative (Figure 1), with only scattered positive cells being present in the tumor. However, three CLL (cases 6458, 7003 and 8406) showed a moderate number of positive large cells, particularly in the proliferative growth centers (Figure 1). These three cases had a relatively high mitotic index and an increase in the number of large cells, although not diagnostic of RS. Of note, p53 mutations were detected in two of these specimens (cases 7003 and 8406). In contrast to the low number of Rb positive cells in CLL, all cases of RS showed Rb protein expression in a relatively high number of tumor cells (Figure 1).

#### *Cyclin D1 expression analysis*

Cyclin D1 protein overexpression was detected in two tumors, one CLL (case 921) and one RS (cases 4563). Cyclin D1 mRNA overexpression and bcl-1 rearrangement with the p94PS probe had been demonstrated in the first case in a previous study.<sup>34</sup> The second was negative for bcl-1 rearrangement at the MTC locus by PCR, using DNA from formalin-fixed paraffin embedded material.<sup>35</sup>

Both cases matched the clinicobiologic picture previously reported for CLL with t(11;14)/cyclin D1 overexpression, namely hyperleukocytosis, massively enlarged spleen, atypical morphology with a CD5+/CD23-/FMC7+ immunophenotype, and unfavorable outcome.<sup>30,31</sup> Both patients evolved to a RS 48 and 70 months after the initial CLL diagnosis, respectively (Table 1).

#### **Discussion**

The molecular mechanisms underlying RS are poorly understood. We and others have previously shown that disruption of the ARF/INK4a and p53 tumor-suppressor gene pathways

might be pivotal in approximately two-thirds of Richter's cases,<sup>8,17-19</sup> with this suggesting that other cell cycle regulator gene abnormalities may take part in CLL histologic transformation. Thus, we have now analyzed CDK1 p21<sup>Waf1</sup> and p27<sup>Kip2</sup>, Rb and cyclin D1, in a series of CLL and RS tumors well characterized for ARF/INK4a and p53 alterations.<sup>8,19</sup>

Although p53 gene abnormalities have been extensively investigated in CLL,<sup>11-16</sup> the immunohistochemical pattern of p53/p21<sup>Waf1</sup> expression, a well-established marker of the p53 gene status in NHL,<sup>33,36,37</sup> has not been explored in CLL and RS.<sup>33,38</sup> In the present series, a p53+/p21- immunohistochemical pattern was exclusively shown by p53-mutated tumors. Thus, the combined immunohistochemistry of p53 and p21<sup>Waf1</sup> seems a reliable strategy of p53 gene status assessment also in CLL and RS, although it may underscore stop codon mutations, which are characterized by a p53-/p21- pattern of expression, and rare missense mutations leading to both p53 and p21<sup>Waf1</sup> overexpression.

A p53+/p21+ immunohistochemical pattern and a high p21<sup>Waf1</sup> immunoreactivity were seen in RS more commonly than in CLL. Interestingly, this pattern of p53 and p21<sup>Waf1</sup> expression has also been detected in a remarkable proportion of aggressive NHL.<sup>33,36-38</sup> The presence of a wild-type p53 gene in these p53+/p21+ NHL has led to the idea that p53-induced, p21<sup>Waf1</sup>-mediated growth arrest is overcome by a mechanism of inactivation of the p53 G1 checkpoint pathway acting downstream of p21<sup>Waf1</sup>.<sup>38</sup> A similar inactivating mechanism could take place in some CLL cases and, by conferring a growth advantage to CLL cells, hypothetically cooperate in Richter's transformation. In fact, two out of three p53+/p21+ CLL of the present series evolved into RS. Moreover, the possible association of a p53+/p21+ phenotype with histologic transformation is supported by the observation of a higher frequency of p53+/p21+ pattern in high- than in low-grade MALT lymphomas.<sup>39</sup> On the other hand, our finding of a p53-mutated, p53/p21-overexpressing RS, what it has been also reported in some aggressive lymphomas,<sup>38</sup> suggests the existence of p53-independent mechanisms of p21<sup>Waf1</sup> induction,<sup>40</sup> such as the STAT signaling pathway.<sup>41</sup>

An inverse correlation between p27 protein expression and proliferative index is present in the great majority of lymphoid neoplasms.<sup>42-44</sup> Thus, CLL exhibits the highest levels of p27 protein among lymphoproliferative disorders.<sup>45</sup> This is thought to arrest CLL cells in G0 and to be a major mechanism of apoptosis prevention in this 'accumulative' disease.<sup>45</sup> Concordantly, a high level of p27 protein in small lymphoid cells, with negativity of the large and paraimmunoblastic cells within proliferative growth centers, was broadly found in our CLL series, as described by other authors.<sup>42-44,46</sup>

To the best of our knowledge, this is the first instance in which p27<sup>Kip1</sup> protein expression has been analyzed in RS. The absence of p27 expression in nearly all RS analyzed is of note. In this regard, RS parallels the immunohistochemical pattern of p27 immunoreactivity exhibited by most histologically aggressive lymphomas.<sup>42,43,46,47</sup> Due to the lack of p27<sup>Kip1</sup> gene gross aberrations as determined by Southern blot analysis, the p27 protein loss in RS could be explained by a post-translational mechanism, like an enhanced ubiquitin proteasome-mediated degradation, as has been described in MCL.<sup>46</sup> Whether this finding is an epiphenomenon of the high proliferative rate of RS cells or represents a relevant event in histologic transformation is not clear. Support for the role of p27<sup>Kip1</sup> gene in tumor progression comes from recent studies showing that, in Rb-/-/p27+/- mice, the loss of p27 allows the growth and escape of cells that have acquired additional gen-

etic defects (biallelic loss of Rb), resulting in the generation of more aggressive neoplasms.<sup>48</sup> Hence, it is tempting to speculate that the loss of p27-mediated cell cycle restriction may commit CLL cells to proliferate and, by facilitating the acquisition of new genetic abnormalities, finally to evolve into RS cells.

A possible role of Rb in RS evolution has been suggested by the finding of undetectable Rb mRNA levels in some aggressive CLL<sup>49</sup> and the inactivation of Rb gene in a subset of aggressive NHL.<sup>28,29</sup> However, our observation of a low and high Rb expression in CLL and RS, respectively, seems to preclude a hypothetical participation of this tumor-suppressor gene in CLL transformation. Rather, our data support a direct correlation between the amount of Rb product and the proliferative index of these tumors.<sup>28,50</sup> This is further emphasized by the presence of Rb-positive cells in the proliferative growth centers of some CLL cases.

Cyclin D1 was found to be overexpressed in one RS and one CLL, this latter biopsy having been performed at the time of leukemic Richter's transformation. In this latter case, t(11;14) was also present in the peripheral blood immunoblastic cells of the RS phase, which indicates a clonal relationship with CLL cells. To our knowledge, RS development in the context of CLL with t(11;14) has been documented in two occasions,<sup>30,51</sup> one of them resembling the 'Richter's leukemia' developed by one of our patients.<sup>51</sup> In the present series, the clustering of cyclin D1 overexpression in two CLL evolving into RS might suggest a possible relationship between t(11;14)/cyclin D1 overexpression and RS development, either solely or in association with other abnormalities such as p53 mutations, as observed by Cuneo *et al*.<sup>51</sup> The possible relationship of these leukemic lymphoproliferative disorders with morphological and phenotypic characteristics of CLL bearing the t(11;14) translocation and leukemic mantle cell lymphoma is not known.<sup>52</sup>

In summary, a p53+/p21- pattern of immunohistochemical expression is exclusively found among p53-mutated CLL and RS tumors. On the other hand, a trend towards moderate/strong p21<sup>Waf1</sup> expression, loss of p27 expression, and cyclin D1 overexpression seem to be associated with Richter's transformation of CLL. Taken together, our results suggest that multiple cell cycle regulator disruptions might confer an additional growth advantage to CLL cells and thus facilitate the evolution into RS.

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### Editor's note

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