Alterations of the p53, Rb and p27 Tumor Suppressor Pathways in Diffuse Large B-cell Lymphomas

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Abstract. *Diffuse large B-cell lymphomas (DLBCL) display defects in cell cycle and apoptosis regulation. Therefore, the immunohistochemical expression patterns of the proteins p14, p21, Hdm2 and cyclin D2 were analyzed in relation to the previously reported expression of other major cell cycle proteins (p53, Rb, p16, p27, Ki-67 and cyclins A, B1, D2, D3 and E), apoptosis-associated proteins (bcl2, bcl-xl, bax, bak, bad and bid) and the B-cell differentiation immunophenotypes. Expression of the proteins p14, p21, Hdm2 and cyclin D2 was observed in 62/71 (87%), 22/76 (29%), 35/74 (47%) and 11/77 (14%) cases, respectively. Immunohistochemical alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) pathways were found in 56/77 (73%), 53/79 (67%) and 54/79 (68%) cases, respectively. Concomitant alterations of the p53-Rb, p53-p27 and Rb-p27 pathways were found in 40/77 (52%), 38/77 (50%) and 36/79 (46%) cases, respectively. Three concomitant alterations of the p53-Rb-p27 pathways were found in 28/79 (35%) cases. The main findings of the present study were the following: alterations of the p27 pathway were associated with higher expression of Ki-67 ($p=0.023$); concomitant alterations of the p53-Rb pathways and the p53-p27 pathways were associated with higher expression of cyclin A ($p=0.015$ and $p=0.021$, respectively) and concomitant alterations of the p53, Rb and p27 pathways were associated with higher expression of cyclin A ($p=0.013$). Since cyclin A supports DNA replication, centrosome duplication and mitosis, these findings indicate that concomitant alterations of the p53, Rb and p27 pathways in DLBCL may have cooperative effects resulting in increased neoplastic cell proliferation. This might explain, at least partially, the association between concurrent aberrations of the p53, Rb and p27 pathways and aggressive clinical behavior in DLBCL.*

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Diffuse large B-cell lymphomas (DLBCL) account for approximately 30-40% of all non-Hodgkin's lymphomas of the Western world and are characterized by heterogeneous clinical, histological, immunophenotypic and genetic features (1-9). Several lines of evidence have suggested that diverse mechanisms disrupting the molecular pathways that regulate the cell cycle are involved in the pathogenesis of DLBCL (6-8, 10-35).

Cell cycle progression is achieved through a family of serine/threonine kinase holoenzyme complexes consisting of regulatory cyclin subunits that bind to and activate catalytic cyclin-dependent kinases (CDK) (6, 7, 36-39). The cyclins D1, D2, D3 and E are important for the G1/S transition. Cyclin A supports DNA synthesis, S-phase completion, centrosome duplication and preparation for mitosis. Cyclins B1 and B2 control the onset, sequence of events and completion of mitosis. The kinase activity of the complexes composed of cyclins and CDKs is negatively-regulated by cyclin-dependent kinase inhibitors (CDKI) (6, 7, 36-39). There are two known families of CDKIs. The INK4 family includes p16/INK4A, p15/INK4B, p18/INK4C and p19 (p14)/INK4D, which bind to CDK4 and 6. The CIP/KIP family includes p21/CIP1, p27/KIP1 and p57/KIP2, which target CDK2, 4 and 6.

Cell cycle progression is controlled by the p53, Rb and p27 tumor suppressor pathways (6, 7, 36-38). The p53 (p14-Hdm2-p53-p21) pathway regulates cell cycle arrest in the G1- and G2-phases. p53-Dependent G1/S and G2/M arrest can be mediated through p53-mediated induction of p21 and by repression of the promoters of cyclin B1 and CDK1, respectively (7, 38). The activity and the stability of the p53 protein is regulated *via* interactions with proteins such as Hdm2, which allows targeting of p53 to the ubiquitin-mediated proteolytic network (7, 36, 37). The Rb (p16-cyclin D-CDK4-Rb) pathway regulates the restriction point by inhibiting transcription of genes necessary for the transition from G1- to S-phase. Phosphorylation (inactivation) of the Rb protein, which is stimulated by cyclin D-CDK4/6 complexes and inhibited by p16, results in release of the transcription factor E2F1 in order to activate S-phase entry

(7, 36, 37). The p53 and Rb pathways are linked through the 9p21 locus in which reside two CDKI genes, *CDKN2A* and *CDKN2B*. The CDKN2A gene encodes the p16 protein whereas the p14/ARF protein binds to Hdm2 and stabilizes the p53 protein in the nucleus by blocking its cytoplasmic transport and Hdm2-mediated degradation of p53 (7, 36, 37). Central to the p27 (p27-cyclin E-CDK2) pathway is the CDKI p27 which acts as a mediator of G1 arrest and is phosphorylated by cyclin E-CDK2. This modification signals the proteolytic degradation of the p27 protein *via* ubiquitination-proteasomal degradation; in this process, the SKP2 protein mediates degradation of p27 by acting as ubiquitin ligase for the p27 protein (7, 37).

Recently, three molecularly distinct histogenetic groups of DLBCL have been identified on the basis of B-cell differentiation gene expression profiles by using cDNA and oligonucleotide microarrays: the germinal center (GC) B-cell-like DLBCL express genes of the normal GC B-cells (*e.g. bcl6, CD10, CD38*); the activated B-cell-like (ABC) DLBCL express genes that are normally induced during *in vitro* activation of peripheral blood B-cells while the type 3 DLBCL do not express either set of genes at a high level (40-46). The B-cell differentiation profiles of DLBCL have recently been analyzed by immunohistochemistry using markers such as bcl6, CD10, MUM1 and CD138, and a good correlation between immunophenotypic and microarray data was reported (47-51). In this respect, we have shown that increased expression of bcl6 and CD10 was associated with increased apoptosis and proliferation in DLBCL and that DLBCL with a GC B-cell differentiation immunophenotype was associated with a high apoptotic index, high expression of the proteins bax, bak and bid and low expression of the protein bcl-xl (52-54). The aforementioned findings, taken together, indicate links between B-cell differentiation and cell cycle and apoptosis profiles in DLBCL.

Although the expression of various cell cycle regulators has been reported in DLBCL (10-35), the expression patterns of p21, p14, Hdm2 and cyclin D2 proteins in relation to other major cell cycle proteins, the apoptosis profile and the B-cell differentiation immunophenotypes, to the best of our knowledge, have not been extensively analyzed in DLBCL. Therefore, 79 cases of DLBCL were assessed by immunohistochemistry for the expression of p21, p14, Hdm2 and cyclin D2 in relation to the previously reported expression of other major cell cycle proteins (p53, Rb, p16, p27, p16, Ki-67 and cyclins A, B1, D2, D3 and E), apoptosis-associated proteins (bcl2, bcl-xl, bax, bak, bad and bid), the apoptotic index and the B-cell differentiation immunophenotypes (28, 35, 52, 53). Furthermore, since concurrent alterations of the p53, Rb and p27 pathways were reported in DLBCL (7, 26-28, 32), the p53-Hdm2-p21-p14, Rb-p16-cyclin D (D2 or D3) and p27-cyclin E combined expression patterns in relation to the expression levels of Ki-67, cyclin A and cyclin B1 were analyzed in order to examine whether concurrent impairment of the p53, Rb and p27 pathways may affect tumor cell proliferation.

Materials and Methods

Materials. Seventy-nine cases of *de novo* diffuse large B-cell lymphoma (37 nodal and 42 extranodal) classified according to the WHO classification (1, 2) were selected from the files of the Department of Pathology of the University of Ioannina on the basis that sufficient formalin-fixed, paraffin-embedded tissue material was available for performing multiparameter immunohistochemical analysis.

Immunohistochemistry. Immunostainings were performed on formalin-fixed, paraffin-embedded tissue sections by the labelled streptavidin-avidin-biotin method (LSAB kit, Dako SA, Glostrup, Denmark) using the monoclonal antibodies for cyclin D2 (DCS-3.1, Novocastra, dilution 1:50), p21 (EA10, Oncogene, dilution 1:50) and Hdm2 (IF2, Oncogene, dilution 1:10) and the rabbit polyclonal antibody for p14 (Diagnostic BioSystems, catalogue number PR084, dilution 1:500). The counting of immunopositive cells was performed as described previously (52, 53). Briefly, a continuous score system was adopted by using the x40 objective lens and counting at least 10 fields selected on the basis that they contained immunopositive cells. The number of immunopositive cells was divided by the total number of the counted cells and the expression was defined as the percentage of positive cells in the total number of the counted cells. The cut-off point of positivity was 10% for the expression of p21 and cyclin D2, 30% for the expression of Hdm2 and 50% for the expression of p14. These cut-off points were defined after analyzing the distribution of data. Reactive lymph nodes, normal thymuses and lymphomas from our previous studies were used as positive controls (55-57). Negative controls were included and consisted of the same immunohistochemical method with omission of the primary antibody. Immunohistochemical alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) tumor suppressor pathways were defined taking into consideration previously published criteria (18, 21, 23, 24, 26-30, 35). Briefly, increased expression of p53, Hdm2, cyclin D (D2 or D3) and cyclin E and low/null expression of p27, p21, p14, p16 and Rb were considered as altered expression patterns.

Statistical analysis. The Mann-Whitney test, Chi-square test and Spearman's correlation coefficient test were used for statistical analysis. The results were considered as statistically significant when $p<0.05$. The program SPSS for Windows Release 10 was used for statistical analysis.

Results

Expression patterns of the proteins p14, p21, Hdm2 and cyclin D2. Immunohistochemical expression of the proteins p14, p21, Hdm2 and cyclin D2 was observed in 62/71 (87%), 22/76 (29%), 35/74 (47%) and 11/77 (14%) cases, respectively (Figure 1). The expression of the proteins p53, Rb, p27, p16, Ki-67, cyclins A, B1, D2, D3 and E, bcl2, bcl-xl, bax, bak, bad and bid, the apoptotic index and the bcl6/CD10/MUM1/

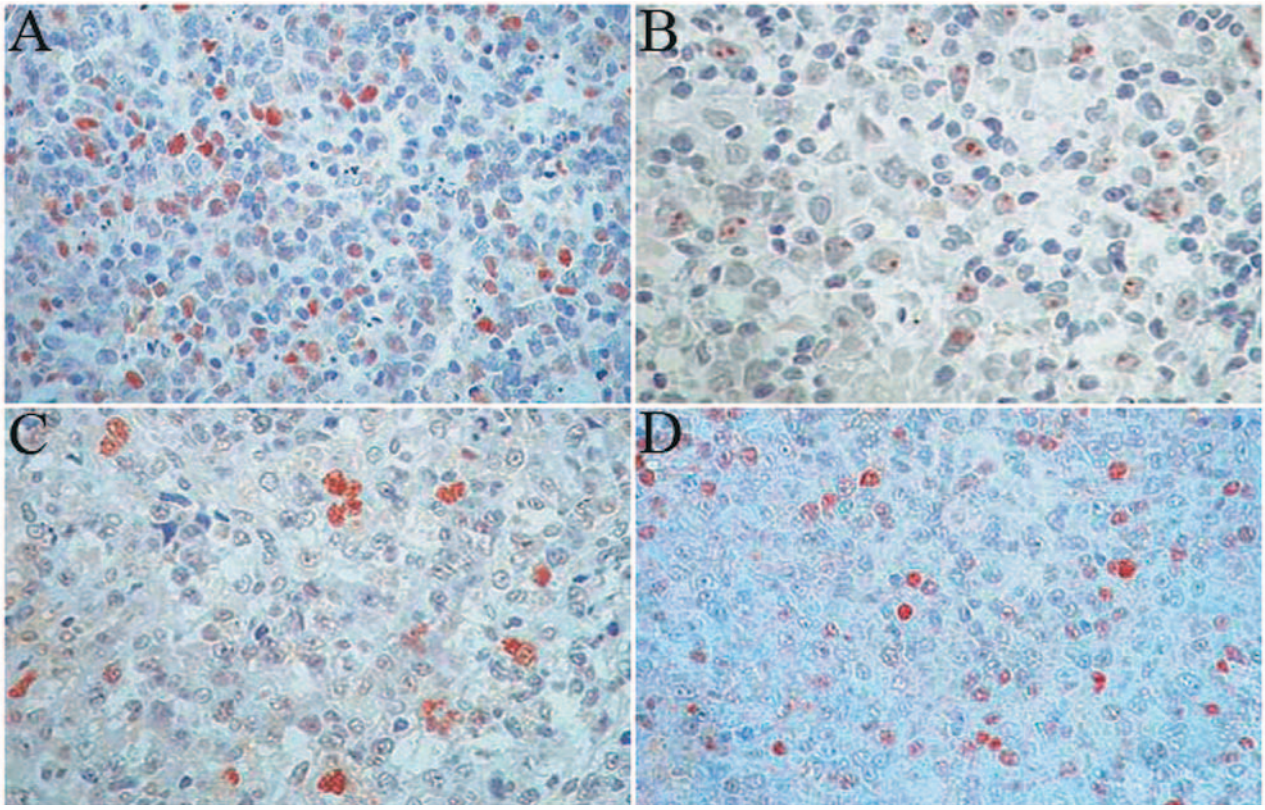

Figure 1. *Immunohistochemical expression of A) p21, B) p14, C) Hdm2 and D) cyclin D2 proteins (original magnification x400).*

CD138 B-cell differentiation immunophenotypes were reported in our previous studies (28, 35, 52, 53). Using Spearman's correlation coefficient test (for assessment of correlation between the expression levels of two proteins taken as continuous variables), no statistically significant correlations were found between the expression of p14, p21, Hdm2 and cyclin D2 proteins and the expression of p53, Rb, p27, p16, Ki-67, cyclins A, B1, D2, D3 and E and bcl2, bcl-xl, bax, bak, bad and bid proteins. The combined expression patterns of the CDK-I p21, p27, p16 and p14 were analyzed by Chi-Square tests in order to investigate whether there was a tendency for concomitant or mutually exclusive expression in DLBCL. The differences were not statistically significant (Table I).

Alterations of the p53, Rb and p27 pathways. Immunohistochemical alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) pathways were found in 56/77 (73%), 53/79 (67%) and 54/79 (68%) cases, respectively. Concomitant alterations of the p53 and Rb, p53 and p27 and Rb and p27 pathways were found in 40/77 (52%), 38/77 (50%) and 36/79 (46%) cases, respectively. Two or three concomitant alterations of the p53, Rb and p27 pathways were found in 58/77 (75%) cases. Three concomitant alterations of the p53, Rb and p27 pathways were found in 28/79 (35%) cases (Table II).

Correlations of the alterations of the p53, Rb and p27 pathways with proliferation-associated proteins, apoptosis-associated proteins and the B-cell differentiation immunophenotypes. The analysis of the alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 [p27-cyclin E] pathways with respect to the expression levels of Ki-67, cyclin A and cyclin B1 was performed using the Mann-Whitney test. This analysis showed that: alterations of the p27 pathway were significantly correlated with higher expression of Ki-67 ($p=0.023$); concomitant alterations of the p53 and Rb pathways and concomitant alterations of the p53 and p27 pathways were significantly correlated with higher expression of cyclin A ($p=0.015$ and $p=0.021$, respectively) and three concomitant alterations of the p53, Rb and p27 pathways were significantly correlated with higher expression of cyclin A ($p=0.013$) (Tables III and IV). No significant correlations were found between the alterations of the p53, Rb and p27 pathways, the apoptosis-associated proteins, the apoptotic index and the

Table I. *Correlations between the immunohistochemical expression patterns (positive vs. negative cases) of the CDK-inhibitors p14, p16, p21 and p27 (Chi-square test).*

		Group p21	Group p21	Total	*p*-values
		1	2		
Group P14	1	18	44	62	
Group P14	2	3	6	9	
Total		21	50	71	$p=1.0$
		Group p16	Group p16	Total	*p*-values
		1	2		
Group P14	1	50	12	62	
Group P14	2	8	1	9	
Total		58	13	71	$p=1.0$
		Group P27	Group P27	Total	*p*-values
		1	2		
Group P14	1	24	38	62	
Group P14	2	2	7	9	
Total		26	45	71	$p=0.470$
		Group p16	Group p16	Total	*p*-values
		1	2		
Group p21	1	16	6	22	
Group p21	2	46	8	54	
Total		62	14	76	$p=0.212$
		Group P27	Group P27	Total	*p*-values
		1	2		
Group p21	1	9	13	22	
Group p21	2	19	35	54	
Total		28	48	76	$p=0.794$
		Group P27	Group P27	Total	*p*-values
		1	2		
Group p16	1	24	41	65	
Group p16	2	4	19	14	
Total		28	51	79	$p=0.760$

Group 1: positive cases for expression of p14, p16, p21 and p27; Group 2: negative cases for expression of p14, p16, p21 and p27.

Table II. *Immunohistochemical alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) tumor suppressor pathways.*

Pathways	Cases with alterations/ Total number of cases
p53 (p53-Hdm2-p21-p14),	56/77 (73%),
Rb (Rb-p16-cyclin D [D2 or D3])	53/79 (67%)
p27 (p27-cyclin E)	54/79 (68%)
Concomitant alterations of p53 and Rb pathways	40/77 (52%),
Concomitant alterations of p53 and p27 pathways	38/77 (50%)
Concomitant alterations of Rb and p27 pathways	36/79 (46%)
Two or three concomitant alterations of p53, Rb and p27 pathways	58/77 (75%)
Three concomitant alterations of p53, Rb and p27 pathways	28/79 (35%)

Table III. *Correlations between alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) pathways and the expression levels of Ki-67, cyclin A and cyclin B1 (Mann-Whitney test).*

	P53 pathway	Number of cases	Mean rank	*p*-values
Ki-67	1	56	37.61	
	2	21	42.71	
	Total	77		$p=0.370$
Cyclin A	1	56	41.43	
	2	21	32.52	
	Total	77		$p=0.119$
Cyclin B	1	56	39.64	
	2	21	37.29	
	Total	77		$p=0.680$
	Rb pathway	Number of cases	Mean rank	*p*-values
Ki-67	1	53	40.80	
	2	26	38.37	
	Total	79		$p=0.656$
Cyclin A	1	53	40.85	
	2	26	38.27	
	Total	79		$p=0.638$
Cyclin B	1	53	39.35	
	2	26	41.33	
	Total	79		$p=0.718$
	P27 pathway	Number of cases	Mean rank	*p*-values
Ki-67	1	54	43.98	
	2	25	31.40	
	Total	79		$p=0.023$*
Cyclin A	1	54	42.56	
	2	25	34.46	
	Total	79		$p=0.143$
Cyclin B	1	54	42.59	
	2	25	34.40	
	Total	79		$p=0.139$

p53, Rb and p27 pathways: Group 1: alterations of the pathway, Group 2: no alterations of the pathway; *significant *p*-values.

Table IV. *Correlations between concomitant alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) pathways and the expression levels of Ki-67, cyclin A and cyclin B1 (Mann-Whitney test).*

	Concomitant p53-Rb alterations	Number of cases	Mean rank	*p*-values
Ki-67	1	40	39.42	
	2	37	38.54	
	Total	77		$p=0.862$
Cyclin A	1	40	44.97	
	2	37	32.54	
	Total	77		$p=0.015$*
Cyclin B	1	40	39.65	
	2	37	38.30	
	Total	77		$p=0.790$
	Concomitant p53-p27 alterations	Number of cases	Mean rank	*p*-values
Ki-67	1	38	43.53	
	2	39	34.59	
	Total	77		$p=0.078$
Cyclin A	1	38	44.95	
	2	39	33.21	
	Total	77		$p=0.021$*
Cyclin B	1	38	43.32	
	2	39	34.79	
	Total	77		$p=0.094$
	Concomitant Rb-p27 alterations	Number of cases	Mean rank	*p*-values
Ki-67	1	36	43.63	
	2	43	36.79	
	Total	79		$p=0.197$
Cyclin A	1	36	43.19	
	2	43	37.33	
	Total	79		$p=0.256$
Cyclin B	1	36	40.86	
	2	43	39.28	
	Total	79		$p=0.760$
	Concomitant P53-Rb-p27 alterations	Number of cases	Mean rank	*p*-values
Ki-67	1	28	44.88	
	2	51	37.32	
	Total	79		$p=0.160$
Cyclin A	1	28	48.63	
	2	51	35.26	
	Total	79		$p=0.013$*
Cyclin B	1	28	42.77	
	2	51	38.48	
	Total	79		$p=0.426$

Concomitant P53-Rb, p53-p27, Rb-p27 and p53-Rb-p27 alterations: Group 1: concomitant alterations of the pathways, Group 2: no concomitant alterations of the pathways; *significant *p*-values.

two major B-cell differentiation immunophenotypic profiles: the GC B-cell-like profile and the non-GC B-cell-like profile (data not shown).

Correlation with clinicopathological parameters. No significant correlations were found between tumor localization (nodal *vs.* extranodal) or tumor stage (I-IV) and alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) pathways alone or in combination (data not shown).

Discussion

In the present study, expression of the proteins p14, p21 and Hdm2 was observed in 87%, 29% and 47% cases of DLBCL, respectively. These results concur with previous findings, although there are variations in the reported percentages of positive tumor cells in DLBCL (12, 15, 20, 25, 27, 30-32). These variations are likely to be due to different antibodies and/or different cut-off points used for assigning immunohistochemical positivity. The present and previous findings (12, 15, 25, 27, 30-32) have indicated that the expression of the CDK-Is p14 and p21 is variable in DLBCL. This could be due to abnormalities in gene structure and/or expression. For example, the p21 negative-p53 positive immunophenotype was frequenty associated with missense p53 gene mutations resulting in the inability of the mutated p53 to transactivate its target gene p21 and the absence of p14 immunoexpression was associated with promoter hypermethylation and loss of heterozygosity (reviewed in 6-8, 12, 23, 26, 30). Furthermore, the combined expression patterns of the CDK-Is p21, p27, p16 and p14 was analyzed in order to assess whether there was a tendency for concomitant or mutually exclusive expression in DLBCL. It has been suggested that p27 protein accumulation may be secondary to loss and subsequent inactivation of p21 and/or p16 CDK-Is (27). In the present study, the expression patterns p27 positive-p21 negative, p27 positive-p16 negative were found in 19/76 (25%) and 4/79 (5%) cases, respectively. It is possible that the absence of p21 or p16 precludes the redistribution of p27 from complexes containing CDK4/cyclin D, where p27 is stabilized and probably inactive, to other complexes containing CDK2/cyclin E, where p27 is active and may arrest cell proliferation (27). On the other hand, the cases with the expression patterns p27 positive-p21 positive (9/76; 12 %) and p27 positive-p16 positive (24/79; 30 %) may indicate p27 protein accumulation without concomitant inactivation of p21 and p16, respectively. In these cases other factors (such as c-myc, altered ubiquitin-mediated proteolytic degradation of p27 protein) might explain the p27 protein accumulation (11, 17, 27).

In agreement with recent studies (10, 47), we observed expression of the protein cyclin D2 in 14% of cases of DLBCL. This may result from constitutive activation of NF-κB, which characterizes the ABC-DLBCL (45, 46), and may be related to the findings that the cyclin D2 promotor contains NF-κB binding sites (58). It could be suggested that induction of cyclin D2 expression may support the proliferation of DLBCL cells in a few of these tumors which are likely to represent ABC-DLBCL. Interestingly, increased cyclin D2 protein expression was also observed in Hodgkin and Reed-Sternberg cells in most classical Hodgkin's lymphomas (54), which are also characterized by constitutive activation of NF-κB (6, 7).

In the present study, alterations of the p53 (p53-Hdm2-p21-p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) tumor suppressor pathways were found in 73%, 67% and 68% cases of DLBCL, respectively. These findings are in keeping with previous data (10-20, 23-30) and indicate that most DLBCL display alterations of at least one of the three major tumor suppressor pathways. In addition, previous studies using immunohistochemical and/or genetic analysis (26-28, 30, 32, 35) showed two or three concurrent aberrations of the p53, Rb and p27 tumor suppressor pathways in a sizable fraction of DLBCL. However, to the best of our knowledge, the combined (p53-Hdm2-p21-p14), (Rb-p16-cyclin D [D2 or D3]) and (p27-cyclin E) immunohistochemical expression patterns have not been analyzed previously in DLBCL. On the basis of these patterns, two or three concomitant alterations of p53, Rb and p27 tumor suppressor pathways were identified in 75% cases of DLBCL. Interestingly, concurrent aberrations of the p53, Rb and p27 tumor suppressor pathways have been associated with aggressive clinical behavior in DLBCL (26, 27, 32). Indeed, concurrent disruption of the p16 and ARF-p53 pathways was an independent negative prognostic factor in DLBCL whereas selective disruption of either the p16 or the ARF-p53 pathway did not significantly influence the clinical outcome in that series (26). In addition, anomalous p27 protein overexpression tends to occur in cases of DLBCL with p16 and/or p53 alterations and DLBCL with concurrent p53, p16 and p27 alterations had the lowest overall survival probability (27). Furthermore, concurrent disruptions of the p16 and Rb and p14 and p53 pathways are associated with a worse prognosis in DLBCL with GC B-cell differentiation immunophenotypes (32). In this respect, we have previously shown that combined alterations in the p27, p53, Rb and p16 immunohistochemical expression status were significantly correlated with increased expression levels of cyclin A and cyclin B1 (28). In the present study, the expression of the proteins p14, p21, Hdm2 and cyclin D2 was included and the p53 (p53, Hdm2, p21 and p14), Rb (Rb-p16-cyclin D [D2 or D3]) and p27 (p27-cyclin E) combined immunohistochemical expression status was subsequently analyzed with respect to the expression levels of Ki-67, cyclin A and cyclin B1. The present results showed that two concomitant alterations of p53 and Rb pathways, two concomitant alterations of p53 and p27 pathways and three concomitant alterations of p53, Rb and p27 pathways were significantly correlated with higher expression levels of cyclin A. Since cyclin A supports DNA replication, centrosome duplication and mitosis (39), the above findings indicate that the combined alterations of the p53, Rb and p27 pathways in DLBCL may have cooperative effects resulting in increased neoplastic cell proliferation.

In conclusion, the main finding of the present study was the association between concomitant alterations of p53, Rb and p27 pathways and higher expression of cyclin A. This finding further indicates that combined alterations of these pathways in DLBCL may have cooperative effects resulting in increased neoplastic cell proliferation. This might explain, at least partially, the association between concurrent aberrations of the p53, Rb and p27 pathways and aggressive clinical behavior in DLBCL (26, 27, 30, 32) since increased neoplastic cell proliferation is a negative prognostic factor in these tumors (15).

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