No Influence of bcl-2, p53, and p21^{waf1} Protein Expression on the Outcome of Pediatric Hodgkin Lymphomas

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Summary: In Argentina, lymphomas account for 13.6% of all pediatric tumors and 47% of them are Hodgkin lymphoma. Previous studies of lymphoma series have reported the expression of apoptotic and cell cycle proteins. Our aim was to study these markers in our pediatric patients and correlate them with their outcome. Immunohistochemical staining with monoclonal antibodies anti-p53, bcl-2, p21^{waf1}, and mdm2 were performed on formalin-fixed paraffin-embedded Hodgkin lymphoma lymph node biopsies from 54 pediatric patients. The analyzed oncogenes p53, bcl-2, p21^{waf1}, and mdm2 exhibited 81%, 44%, 76%, and 90% positive staining, respectively. The most prevalent p53/p21^{waf1} expression pattern was p53+/p21^{waf1}+, in 57% of cases, whereas concerning p53/mdm2 expression pattern p53+/mdm2+ was observed in 61% of cases. We failed to find any statistically significant correlation between oncogene expression and patient's survival. It seems that p53 plays an important role in lymphomagenesis in our studied population, because it is overexpressed in 81% of Hodgkin lymphoma cases and in more than 50% of cases, it might be able to activate its cellular effectors. Bcl-2 staining observed in 44% of our cases could represent a failure in bcl-2 down-regulation that leads to a rescue event in defective germinal center B-cells, that allows them to develop into Reed-Sternberg and Hodgkin cells.

Key Words: Hodgkin lymphoma, childhood, p53, bcl-2, p21^{waf1}, mdm2

(*J Pediatr Hematol Oncol* 2006;28:552–558)

Lymphoma accounts for 10% of all childhood cancers in developed countries, which ranks third in relative frequency after acute leukemia and brain tumors. Worldwide incidence of Hodgkin lymphoma (HL) displays different patterns. Epidemiologic studies demonstrated 3 distinct forms of HL: a childhood form (in patients 14 y old or younger), a young adult form (in patients 15 to 34 y old), and an older adult form (in patients 55 to 74 y old).[1] The epidemiologic features of these 3 forms suggest that HL may have several different causes. A definite bimodal age peak present in the incidence of HL is not seen for most other lymphomas. In industrialized countries, the early peak occurs in the middle to late 20s and the second peak after the age of 50 years, whereas in developing countries, the early peak is shifted before adolescence.[1] In Argentina, all cases of pediatric HL diagnosed in public hospitals are registered in the Registro Oncopediátrico Hospitalario Argentino Fundación Kaleidos.[2] They reported 3342 pediatric tumors during the period 2000 to 2002. Of these 13.6% were lymphomas including 47% diagnosed as HL and the remaining 53% as non Hodgkins lymphoma (NHL).[2] The Reed-Sternberg (RS) and Hodgkin (H) cells are the malignant cell population of Hodgkin lymphoma. These cells harbor clonally rearranged and somatically mutated immunoglobulin genes, indicating that, in most cases, they are derived from germinal center (GC) B cells.[3]

The analysis of cell cycle regulation in different types of lymphoid neoplasms reveals that increased clinical aggressiveness is associated with the accumulation of genetic and epigenetic alterations, which lead to the alteration and/or inactivation of several cell cycle regulating pathways.[3] Several studies have shown certain relationship between the expression of activation or differentiation markers in the neoplastic H and RS cells and both cell cycle and apoptosis deregulation.[4]

Among the huge diversity of genetic pathways involved in cancer progression, p53 seems to represent a focal point of deregulation of cell proliferation irrespective of the tissue and cellular origin of the tumor. Normal p53 acts as a tumor suppressor gene. Mutations in this pathway are observed in the majority of invasive cancers.[5] Loss of functional p53 is thought to permit the propagation of genetic defects that would otherwise be eliminated during p53-mediated apoptosis or repair process.[6]

Mdm2 is a key negative regulator of the tumor suppressor p53. Activated p53 induces the transcription of mdm2, which binds to p53 and inactivates it, and which functions as an ubiquitin E3 ligase to target p53 to the proteosome for destruction.[7]

Received for publication January 03, 2006; accepted May 23, 2006.
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This study was supported in part by a grant from National Agency for Science and Technology Promotion (PICT no 01–10977, Resol. no 008/04). P.A.Ch. was supported by a postdoctoral fellowship from the CONICET.
M.V.P. is a member of the CONICET Research Career Programme.
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The control of cell cycle also involves a family of proteins that bind to and inhibit cyclin-dependent kinases (Cdks). One of the best known of these inhibitors is $p21^{waf1}$, which is a potent downstream effector of p53 function. $P21^{waf1}$ affects the function of most known cyclin/Cdk complexes, blocking DNA replication and cell cycle progression into S phase.[8]

Moreover, bcl-2 is particular among oncogenes because it exerts its oncogenic effects via inhibition of apoptosis instead of enhancing cell cycle progression.[9] Deregulated expression of bcl-2 by translocation and other mechanisms is involved in the pathogenesis of hemopoietic malignancies including HL.[10]

Previous studies have shown a relationship between the expression of both apoptotic and cell cycle proteins and patients outcome.[3,4,10–20] Particularly, it is crucial to evaluate these mechanisms in those cases which chemotherapic agents and radiotherapy induce apoptosis of tumor cell, to predict treatment response. Therefore, an accurate prediction of the results of treatment might eventually allow the identification of patients who are likely to benefit from reduced therapy, or those who have a low probability of having a sustained response to standard treatment.[4,20] The aim of this study was to look for the expression of the main proteins involved in cell cycle and apoptosis pathways, namely bcl-2, p53 and its functional effectors ($p21^{waf1}$ and mdm2) in a pediatric HL series, as well as to correlate it with patient's survival to establish their usefulness as molecular markers to predict treatment response.

MATERIALS AND METHODS

Patients and Tissue Preparation

Formalin-fixed paraffin-embedded HL tissue samples from 54 pediatric patients were collected retrospectively from files at Ricardo Gutiérrez Children's Hospital in Argentina, from 1990 to 2002. Institutional guidelines regarding human experimental investigation were followed.

Diagnosis was made from biopsies taken from the primary tumor. The histologic classification was performed according to schemes for HL.[21] Classic HL types: 32 mixed cellularity (MC), 15 nodular sclerosis (NS), 1 lymphocyte depletion, and 4 lymphocyte-rich classic HL; and 2 nodular lymphocyte-predominant type.

Patient's median age was 8 years (range 2 to 16 y), with a male: female ratio of 7:2. Follow-up period ranged from 5 to 201 months (median: 76 mo).

Detection of p53, $p21^{waf1}$, mdm2, and bcl-2 by Immunohistochemistry (IHC)

Immunostains were applied to localize and quantitate p53, bcl-2, mdm2 and $p21^{waf1}$ expression in tumor cells using monoclonal antibodies DO7 (Dako, Carpintería, dilution 1:100), bcl-2/100/D5 (Novocastra, Newcastle, UK, dilution 1:100), SMP 14 (Dako, Carpintería, dilution 1:50), and SX118 (Dako, Carpintería, dilution 1:40), respectively. Microwave antigen retrieval with 0.01 M citrate buffer pH 6 was performed. Immunohistochemical detection of monoclonal antibodies was performed using a streptavidin-biotin complex-peroxidase detection system (Vectastain Elite ABC, Vector, Burlingame) according to the manufacturer's instructions. As positive control, for p53, mdm2, and $p21^{waf1}$ we used a biopsy of a p53, mdm2, and a $p21^{waf1}$ positive breast cancer and for bcl-2, a normal tonsil biopsy. A quantitative evaluation of antigen expression was performed assigning to each case a different score obtained by evaluating the percentage of positive cells: −, 0% to 10%; +, > 10% to 25%; ++, > 25% to 50%; +++, > 50% to 100%.

Statistical Analysis

Statistical analysis was carried out with GraphPad Prism 4 software. Event-free survival (EFS) was defined as the time from initiation of treatment to the event. An event in EFS was failure to achieve complete remission, relapse after a prior complete remission, or death from any cause. Overall survival was defined as the time from initiation of treatment to death from any cause.

Survival distributions were estimated according to the Kaplan-Meier method.[22] Differences in survival distributions were tested with the log-rank test.[23]

For the univariate analysis, Fisher exact test was used to assess the association between categorical variables.

All tests were 2-sided, and a P value of less than 0.05 was considered to indicate statistical significance.

TABLE 1. Correlation of HL Subtype and Clinical Stage

		Clinical Stage				
Hodgkin Disease Subtype		**I N (%)**	**II N (%)**	**III N (%)**	**IV N (%)**	**Total N (%)**
Classical HL	MC	1 (2)	21 (39)	7 (13)	3 (6)	32 (59)
	NS	1 (2)	7 (13)	4 (7)	3 (6)	15 (28)
	LRCHL	3 (5)	0 (0)	1 (2)	0 (0)	4 (7)
	LD	0 (0)	0 (0)	0 (0)	1 (2)	1 (2)
Nodular lymphocyte predominance		0 (0)	2 (4)	0 (0)	0 (0)	2 (4)
Total		5 (9)	30 (56)	12 (22)	7 (13)	54 (100)

LD indicates lymphocyte depletion; LRCHL, lymphocyte-rich classic HL; MC, mixed cellularity; NS, nodular sclerosis.

TABLE 2. Correlation of HL Subtype and Oncogene Expression

Hodgkin Disease Subtype		p53 N/tot	p53 (%)	bcl-2 N/tot	bcl-2 (%)	p21^{waf1} N/tot	p21^{waf1} (%)
Classic HL	MC	27/32	84	18/32	56	24/32	75
	NS	14/15	93	3/15	20	10/15	67
	LRCHL	2/4	50	2/4	50	4/4	100
	LD	0/1	0	0/1	0	1/1	100
Nodular lymphocyte predominance		1/2	50	1/2	50	2/2	100
Total		44/54	81	24/54	44	41/54	76

LD indicates lymphocyte depletion; LRCHL, lymphocyte-rich classic HL.

RESULTS

Histologic distribution according to patients clinical stage are summarized in Table 1. Advanced stages III and IV, were present in 35% of patients, and the most frequent histologic subtype was MC, observed in 59% of cases.

P53, bcl-2, and p21^{waf1} immunohistochemical expression are summarized in Table 2.

P53 positive staining restricted to nuclei of RS cells were detected in 44 out of 54 (81%) cases, (Fig. 1) (Table 2), with positive cells ranging from 15% to 80%, and positive cells percentage account for 25% to 50% (++) in 54% cases (Table 3). Most p53 positive cases were included in MC (84%) and NS (93%) subtypes.

Twenty-four out of 54 (44%) cases displayed bcl-2 granular cytoplasmic positive staining in malignant RS cells (Fig. 2) (Table 2). Bcl-2 positive cases where distributed among all HL subtypes, with the exception of lymphocyte depletion. bcl-2 positive cells varied from 10% to 75%, with most cases displaying < 25% (+) of positive cells (Table 3).

The p53 functional effector, p21^{waf1}, exhibited, like p53, a high percentage of positive cases, 41 out of 54 (76%), confined to nuclei of RS neoplastic cells (Fig. 3) (Table 2), and was uniformly distributed among all histologic subtypes. The proportion of p21^{waf1} positive cells also fluctuated from 25% to 90% (++ to +++) (Table 3). When analyzing oncogene coexpression, only bcl-2 and p21^{waf1} were statistically significant associated ($P = 0.0126$, Fisher exact test).

To illustrate more accurately the relationship between p53 and its functional effector, p21^{waf1}, we defined 5 staining patterns, modified from those described by Chilosi et al[24]: type I, characterized by the complete lack of p53 and p21^{waf1} expression (p53−/p21^{waf1}−); type II, with neoplastic cells expressing both p53 and p21^{waf1} (p53+/p21^{waf1}+); type III, with neoplastic cells expressing only p21^{waf1} but not p53 (p53−/p21^{waf1}+); type IV, (p53+/p21^{waf1}−) with low proportion (< 25%) of p53 positive cells and without p21^{waf1} expression; and type V (p53++/p21^{waf1}−), with intermediate and high proportions (> 25%) of p53 positive cells and completely lacking p21^{waf1} expression. The most prevalent pattern observed in our series was type II (p53+/p21^{waf1}+), accounting for 57% (31/54) of cases (Table 4). This expression pattern was mainly observed in MC and NS subtypes. Types IV (p53+/p21^{waf1}−) and V (p53++/p21^{waf1}−) patterns, correspond to the overexpression of nonfunctional p53, that represent 24% (13/54) of our series and is present in MC subtype.

With the aim to describe an alternative p53 activation pathway, we analyzed mdm2 expression (Fig. 4), another p53 functional effector in a subgroup of 31 patients, whose formalin-fixed paraffin-embedded tissue samples were available. mdm2, which is induced by p53, regulates p53 protein concentrations through the ubiquitin-proteasome pathway. We defined 4 expression patterns of p53/mdm2 relationship: type A, characterized

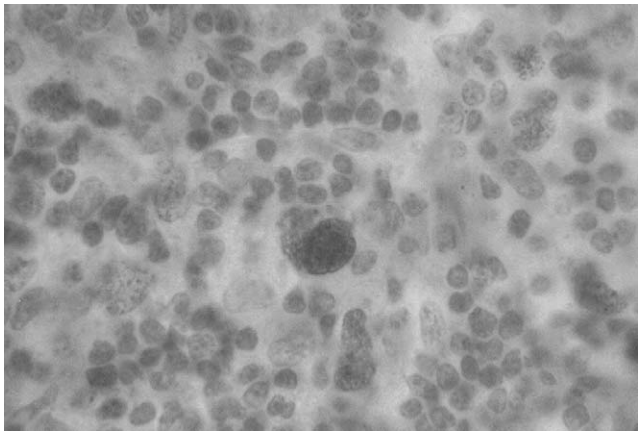

FIGURE 1. MC HL. p53 abundant nuclei positive staining with monoclonal antibodies anti-p53. ×100.

TABLE 3. Positive Cells Percentage of Oncogene Expression in Pediatric HD

Positive Cells (%)	p53 No. (%)	bcl-2 No. (%)	p21^{waf1} No. (%)
0–10 (−)	10 (18)	30 (55)	13 (24)
> 10–25 (+)	4 (7)	15 (28)	3 (5)
> 25–50 (++)	29 (54)	5 (9)	20 (37)
> 50–100 (+++)	11 (20)	4 (7)	18 (33)
Total	54 (100)	54 (100)	54 (100)

MC indicates mixed cellularity; NS, nodular sclerosis.

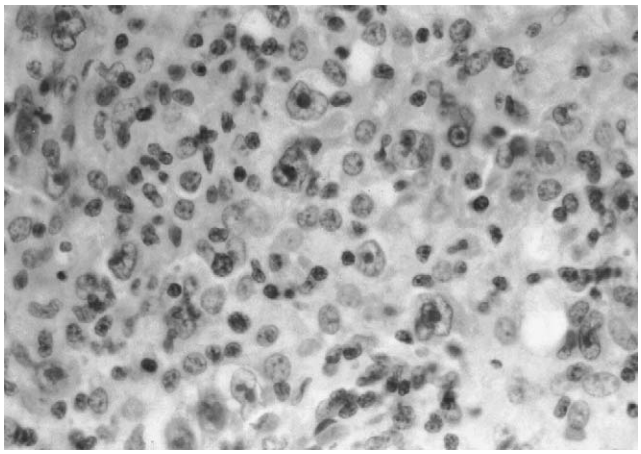

FIGURE 2. MC HL. Bcl-2 cytoplasms reactive with monoclonal antibodies anti-bcl-2 are evident. × 40.

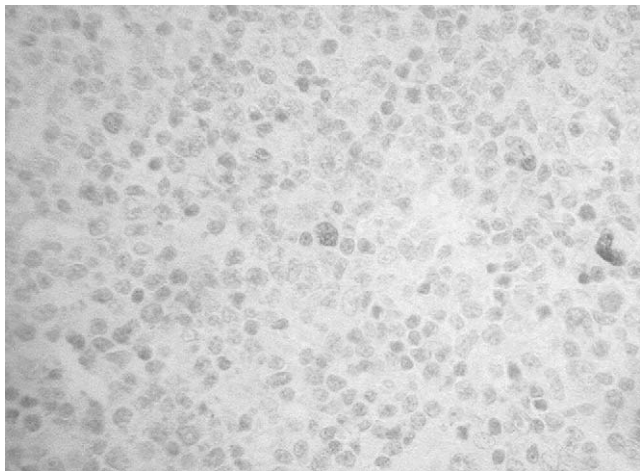

FIGURE 4. MC HL. Mdm2 reactive nuclei with monoclonal antibodies anti-mdm2 in tumor cells. × 40.

by the lack of p53 and mdm2 expression (p53 − / mdm2 −); type B, with RS cells expressing both p53 and mdm2 (p53 + /mdm2 +); type C, with tumor cells expressing only mdm2 (p53 − /mdm2 +); type D, with neoplastic cells expressing p53 and lacking mdm2 expression (p53 + /mdm2 −).

Sixty-one percent (19/31) (Table 5) of our series exhibited a type B expression pattern (p53 + /mdm2 +). Furthermore, type C (p53 − /mdm2 +) expression pattern, which indicates that mdm2 is capable to down-regulate p53 expression, was observed in the 29% (9/31) of cases. However, type D (p53 + /mdm2 −) expression pattern was seen in just a few cases (2/31, 6%).

No statistically significant association was found between each oncogene expression and either clinical stage or histologic subtype. Concerning patient's outcome, the estimated 5 years EFS was 69%, and the estimated 5 years overall survival was 88%. Survival analysis indicates that p53, bcl-2, and p21^{waf1} expression did not show statistically significant associations with patients EFS ($P > 0.05$, log-rank test) (Fig. 5).

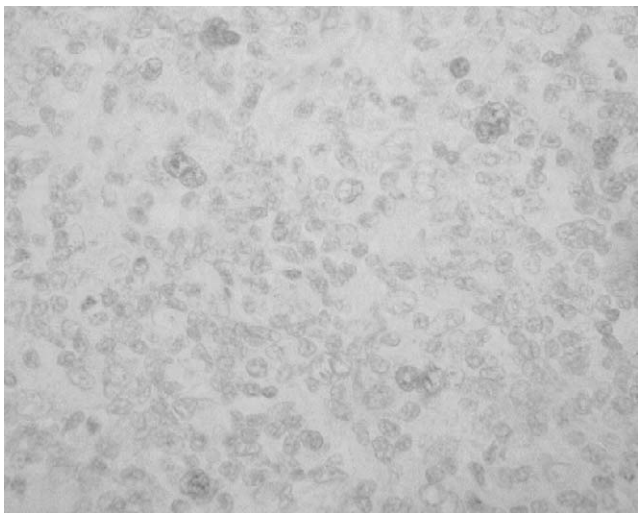

FIGURE 3. MC HL. P21^{waf1} nuclear positive staining with monoclonal antibodies anti-p21^{waf1} in Reed-Sternberg cells. × 40.

DISCUSSION

HL has a favorable outcome in most patients; however, there are a proportion of patients who do not respond to standard treatments. It is assumed that this is a consequence of the disruption of specific molecular mechanisms.[4] Different research groups have explored the potential use of biologic markers as determinants of clinical outcome in adult HL,[4] but this scenario remains still poorly studied in the pediatric counterpart.

It was demonstrated that GC B cells are the cellular origin of H and RS cells. Classical H/RS cells carrying mutated immunoglobulin gene rearrangements that are often ''crippled'' and lack intraclonal diversity, are likely derived from preapoptotic GC B cells.[25] Bcl-2 protein displays a remarkably restricted topographic distribution within mature lymphoid tissues and is essentially absent in GCs.[26] Because GC B cells with unfavorable or crippling mutations are usually quickly eliminated by apoptosis, and only a fraction of unfavorable mutations that cause apoptosis of GC B cells are easily identifiable as crippling mutations, HRS cells as a rule may represent preapoptotic GC B cells rescued by a transforming event.[25] This rescue event in our 44% of cases, similar to that reported in the literature,[10,14–18] could be due to a failure in bcl-2 down-regulation in GC B cells, that could trigger malignant transformation in these cells. Furthermore, it could also contribute with another transformation factor, such as p53.

p53 was overexpressed in 81% of HL cases studied, indicating that p53 inactivation is an important cofactor in neoplastic transformation. Indeed, similar figures were observed in our previous study of p53 on pediatric

TABLE 4. Immunohistochemical Patterns of p53/p21^{waf1} Expression

		p53/p21^{waf1} Immuhistochemical Patterns				
Hodgkin Disease Subtype		**I (%) (p53 − /p21 −)**	**II (%) (p53 + /p21 +)**	**III (%) (p53 − /p21 +)**	**IV (%) (p53 + /p21 −)**	**V (%) (p53 + + /p21 −)**
Classic HL	MC	0 (0)	19 (35)	5 (9)	1 (2)	7 (13)
	NS	0 (0)	9 (17)	1 (2)	1 (2)	4 (7)
	LRCHL	0 (0)	2 (4)	2 (4)	0 (0)	0 (0)
	LD	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)
Nodular lymphocyte predominance		0 (0)	1 (2)	1 (2)	0 (0)	0 (0)
Total		0 (0)	31 (57)	10 (19)	2 (4)	11 (20)

LD indicates lymphocyte depletion; LRCHL, lymphocyte-rich classic HL; MC, mixed cellularity; NS, nodular sclerosis.

NHL,[27] but they were higher than both reported for HL adult series,[12,15,28] and for our previous smaller HL pediatric series.[16]

Detection of large amounts of p53 prompted us to verify the biologic significance of such overexpression. Furthermore, it has been reported that p53 overexpression was not often correlated with p53 gene mutations in HL and in other malignant pathologies,[7,29] therefore in our series it could also be a consequence of other wild type p53 inactivation pathways. To describe more precisely the p53 ability to act as a transcriptional activator, we decided to analyze 2 p53 downstream pathways, p21^{waf1} and mdm2. In a previous study in a smaller series, we have observed a high percentage of p21^{waf1} and mdm2 expression but without performing the interrelated analysis.[16]

The synthesis of p53 at the G1-S boundary induces the synthesis of Cdk inhibitor p21^{waf1}, which promotes cell growth arrest. Overexpression of nonfunctional p53 could lead to the absence of p21^{waf1} expression. On the other hand, p21^{waf1} accumulation correlates with the overexpression of functional wt-p53 protein.

We defined five p53/p21^{waf1} expression patterns.[24] Fifty-seven percent of cases displayed type II (p53 + / p21^{waf1} +) expression pattern, prompting us to hypothesize that p53 successfully induces p21^{waf1} synthesis, as it has been previously suggested for NHLs by other groups.[24,30,31] In our cases, the neoplastic process might involve either a functional failure of p21^{waf1} and/or of other genetic targets downstream p21^{waf1}, or a deregulation of other p53 cell cycle control pathways that do not involve p21^{waf1}. Conversely, patterns IV (p53 + / p21^{waf1} −) and V (p53 + + /p21^{waf1} −) could be correlated with overexpression of nonfunctional p53, and in our series, accounted just for 24%, demonstrating that only these cases could express, either wild type or mutated, nonfunctional p53.

Mdm2 is a wild-p53 inducible protein, which may form a complex with p53, abrogating its function. We defined four p53/mdm2 expression patterns. In a high percentage of cases, 61% exhibited a p53 + /mdm2 + expression pattern. Because p53 transactivates mdm2 and mdm2 down-regulates p53 through the ubiquitin-proteasome pathway, this pattern could represent 2 plausible situations: (a) active p53 is able to induced mdm2 transcription, observed by immunohistochemical staining, and further complex formation, but it reflects a failure at mdm2 level or downstream in the ubiquitin-proteasome pathway, (b) inactive p53 cannot induce mdm2 transcription, then mdm2 overexpression may be due to p53-independent activation pathways.[32] On the contrary, 29% of cases showed type C pattern (p53 − / mdm2 +), where mdm2 is able to target p53 for degradation, but it remains to be elucidated if in these patients, mdm2 overexpression could attenuate p53 activity and therefore increase tumor susceptibility. Only 6% of cases showed p53 + /mdm2 − expression, leading to a transcriptionally inactive p53 protein that accumulates within a cell to high levels, because it could be unable to transactivate mdm2, and, therefore, cannot be degraded.

Several previous reports of adult HL have observed a statistically significant correlation between bcl-2 and/or p53 expression and clinical outcome,[3,10,17,28,33] suggesting

TABLE 5. Immunohistochemical Patterns of p53/mdm2 Expression

		p53/mdm2 Immunohistochemical Patterns			
Hodgkin Disease Subtype		**A (%) (p53 − /mdm2 −)**	**B (%) (p53 + /mdm2 +)**	**C (%) (p53 − /mdm2 +)**	**D (%) (p53 + /mdm2 −)**
Classic HL	MC	0 (0)	12 (39)	5 (16)	2 (6)
	NS	0 (0)	4 (13)	1 (3)	0 (0)
	LRCHL	0 (0)	2 (6)	2 (6)	0 (0)
	LD	1 (3)	0 (0)	0 (0)	0 (0)
Nodular lymphocyte predominance		0 (0)	1 (3)	1 (3)	0 (0)
Total		1 (3)	19 (61)	9 (29)	2 (6)

LD indicates lymphocyte depletion; LRCHL, lymphocyte-rich classic HL; MC, mixed cellularity; NS, nodular sclerosis.

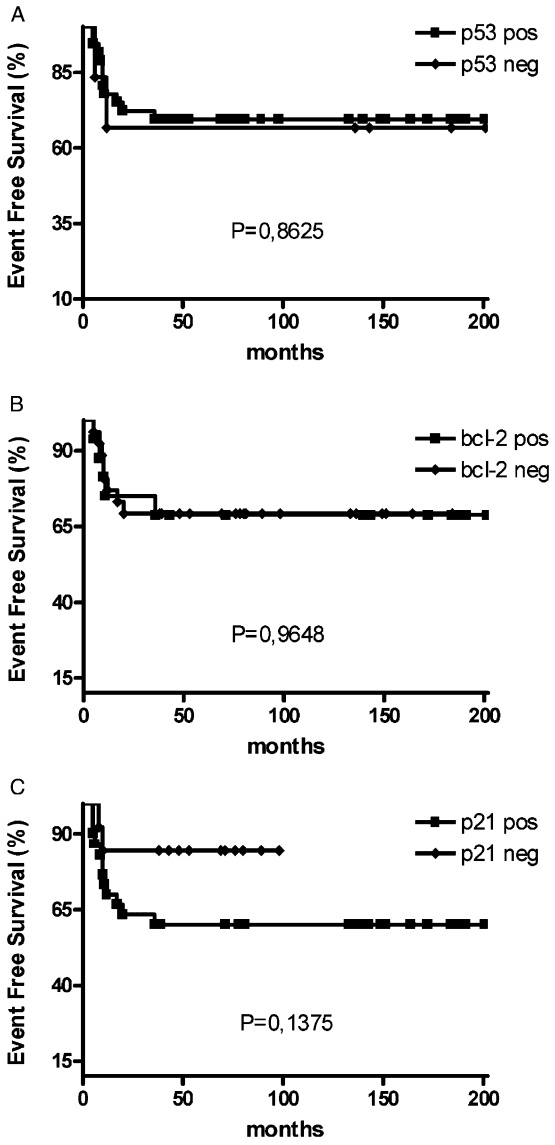

FIGURE 5. Kaplan-Meier analysis. Patients EFS curves correlated with oncogene expression: A, p53; B, bcl-2; C, $p21^{waf1}$.

that they could be used as prognostic factors. On the other hand, Spector et al[34] have recently reported no correlation between p53 and bcl-2 expression and clinical outcome in adult HL in Brazil. In this current large pediatric HL series, we have performed Kaplan Meier survival analysis to improve the understanding of the clinical significance of these oncoprotein expressions. The differences in survival distributions were not statistically significant for any analyzed oncogene. Moreover, no statistically significant association was found between each oncogene expression and either clinical stage or histologic subtype. So, in our hands they could be not applied as molecular prognosis markers. Our study confirms the findings of Spector et al[34] and also ruled out $p21^{waf1}$ as prognostic factor. Nevertheless further studies of pediatric HL series in developed and developing countries will be necessary to confirm these results.

In conclusion, lymphoid carcinogenesis is a complex process, as a result of the deregulation of diverse interacting oncogene pathways. Loss of normal function of any of these oncogenes could be the first step leading to neoplastic transformation, and this might induce the failure of the other pathways. Bcl-2, p53 and its functional effectors, mdm2, and p21waf1 proved to be involved in the pediatric HL series studied. However, they did not provide evidence of being useful as predictive biomarkers of disease progression or therapeutic response.

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