Improved demonstration of immunohistochemical prognostic markers for survival in follicular lymphoma cells

Francisca I Camacho¹, Carmen Bellas², Cesáreo Corbacho², Alexia Caleo³, Reyes Arranz-Sáez⁴, Jimena Cannata⁴, Javier Menárguez⁵, Lydia Sánchez-Verde⁶, Leocricia González-Camacho⁷, M^a Elena Pérez-Martín⁸, Miguel A Martínez-González⁸, Tomás Álvaro⁹, Manuela Mollejo¹⁰, Carmen Ruíz-Marcellán¹¹, Carlos Montalbán⁷ and Miguel A Piris⁶

¹Department of Pathology, Hospital Universitario de Getafe, Madrid, Spain; ²Department of Pathology, Hospital Puerta de Hierro-Majadahonda, Madrid, Spain; ³Biomorphological and Functional Sciences Department, 'Federico II' University, Naples, Italy; ⁴Department of Haematology, Hospital de La Princesa, Madrid, Spain; ⁵Department of Pathology, Hospital General Universitario Gregorio Marañón, Madrid, Spain; ⁶Lymphoma Group, Molecular Pathology Program, Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid, Spain; ⁷Department of Pathology, Hospital Ramón y Cajal, Madrid, Spain; ⁸Department of Pathology, Hospital Universitario 12 de Octubre, Madrid, Spain; ⁹Department of Pathology, Hospital Verge de la Cinta, Tortosa, Tarragona, Spain; ¹⁰Department of Pathology, Hospital Virgen de la Salud, Toledo, Spain and ¹¹Department of Pathology, Hospital Universitario Vall d'Hebron, Barcelona, Spain

Follicular lymphoma (FL) is one of the most common forms of the low-grade non-Hodgkin's lymphoma in adults, with a characteristic translocation, t(14;18)(q32;q21) that deregulates the expression of the *BCL2* gene. The clinical course of FL patients is variable, whereby a subset of patients survive for long periods even without relapses, whereas the majority have frequent relapses with shorter survival. We have analyzed a series of 186 FLs, studying the correlation between clinical outcome and the tumor cell expression of a set of immunohistochemical markers, using an automated procedure for tissue microarrays to reduce the subjectivity of scoring. The results identified several markers associated with differences in overall survival (OS) in univariate analyses, such as Cyclin E, Mdm2, CD10, p21, IgD, Bcl-xL, CD30, and E2F6. Cases with a higher level of expression of Cyclin E, Mdm2, p21, IgD, Bcl-xL, CD30, and E2F6 were associated with a significantly shorter OS. On the other hand, strong CD10 expression was linked to a significantly better outcome. A Cox model was then constructed, integrating the Follicular Lymphoma International Prognostic Index (FLIPI) score and a restricted selection of three immunohistochemical markers: Cyclin E, Mdm2, and CD10 expression. A potentially useful finding is that the integrated FLIPI plus immunohistochemical model can be used to identify a subset of 26 patients (almost 20% of the total series), with a survival probability of 100% at 5 years. This not only confirms that a group of FL cases may have a very good clinical course, but also indicates that this group can be identified using this integrated clinical and immunohistochemical approach.

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Correspondence: Dr FI Camacho, MD, PhD, Department of Pathology, Hospital Universitario de Getafe, Carretera de Toledo, km 12.5, E-28905: Getafe, Madrid, Spain.
E-mail: ficamacho.hugf@salud.madrid.org
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Follicular lymphoma (FL) is one of the most common forms of the low-grade non-Hodgkin's lymphoma in adults. It is characterized by the presence of the translocation t(14;18)(q32;q21), which deregulates the expression of the *BCL2* gene. Clinical course in FL patients is variable, whereby

some patients survive for long periods even without relapses, although the majority has frequent relapses with shorter survival.

Currently, the prognosis of FL patients is essentially based on the clinical parameters of the International Prognostic Index (IPI) or the Follicular Lymphoma International Prognostic Index (FLIPI),¹ the latter having been designed specifically for this type of B-cell lymphoma. Many immunohistochemical and molecular markers have been considered to be potential prognostic markers in FL, carrying information from the tumoral cells or the microenvironment, but none of them are used in routine clinical diagnosis. Significant and potentially useful data have recently emerged regarding the role of the microenvironment in the prognosis of FL.²⁻⁷ Less attention has been paid to the role of tumoral cell markers in the prognosis of FL patients, partially because of the difficulty of obtaining reproducible measurements of protein expression from paraffin-embedded samples.

Previous studies have demonstrated that predictors based on a number of biomarkers that can be obtained at diagnosis in tissue microarrays (TMAs) have the potential to improve the prediction of overall survival (OS) in FL.⁸ Here, we have analyzed a large series of FL samples to identify potentially useful markers that could be merged in a biological prognostic model. The series has an exceptionally long follow-up, with a mean of 173 months, thus making it possible to use OS as the end point. Markers were selected on the basis of previous studies of the pathogenesis or prognosis of FL. To increase the reliability of the data generated, the immunohistochemical markers were analyzed with a quantitative image analysis system that improves the reproducibility of the results.⁹

Materials and methods

This series included 186 cases of nodal FL collected from seven institutions, diagnosed between 1980 and 2002, with clinical follow-up and available clinical information. All cases were reviewed by two pathologists (FIC and CB) and reclassified according to the WHO classification.¹⁰ The grade of every case was evaluated simultaneously by both the pathologists, and disagreements were resolved through a joint review using a multiheaded microscope. The only criteria used for patient selection were the diagnosis confirmation, the availability of a paraffin block for TMA analysis, and of the appropriate clinical information. The study was conducted under the supervision of the ethical committee of the Hospital La Paz, Madrid, Spain. This same series has also been used to analyze the impact of the reactive microenvironment in the prognosis of FL.^{5,6}

We used a tissue arrayer device (Beecher Instruments, Silver Springs, MD, USA) to construct eight TMA blocks, according to conventional protocols.⁹

All cases were subjected to routine H&E staining and were histologically reviewed according to the criteria of the WHO classifications.¹⁰ Two representative 1.5-mm diameter cylinders, from two pre-selected areas in the paraffin block, were included in each case, along with 16 separate controls to ensure the quality, reproducibility, and homogenous staining of the slides. Selected controls included reactive lymph nodes and tonsils, and paraffin-embedded FL cell lines.

Immunohistochemistry was performed using standard procedures⁹ to demonstrate the expression of 28 selected proteins (Table 1) related to apoptosis control (extrinsic and intrinsic pathways), cell cycle, B-cell differentiation, and signaling. The association of these molecules with survival probability was evaluated.

Fluorescence *in situ* hybridization was performed on TMA slides according to standard procedures.¹¹ LSI IGH/BCL2 dual color, dual fusion translocation probe (Vysis, Downers Grove, IL, USA) was used to detect the *BCL2* translocation. Samples were considered positive when a distinctive pattern of one red, one green, and two red/green (yellow) fusion signals was present in more than 10% of nuclei.

All hybridized TMAs were scanned using the Bacus Laboratories Incorporated Slide Scanner system (Bacus Laboratories, Lombard, IL, USA), which uses three CCD RGB sensor optically coupled to a microscope. TMAs were quantitatively scored

Table 1 Range and mean $\pm$ s.d. of protein expression of 28 markers analyzed on TMA

Marker	N	Minimum (%)	Maximum (%)	Mean (%)	s.d. (%)
p53	176	0.01	79.98	5.36	11.883
Bax	170	0.00	93.83	7.56	21.324
BCL2	170	0.01	89.93	42.29	24.687
MIB1	181	3.18	50.39	20.82	8.515
pRB	176	0.00	46.10	3.24	6.088
Survivin	163	0.01	91.74	30.87	27.032
BCL6	179	0.01	65.24	13.66	16.211
Cyclin A	169	0.02	89.41	7.20	11.037
Cyclin B1	170	0.00	77.02	1.36	7.229
MUM1	178	0.07	58.60	6.01	9.475
CD38	177	0.02	79.06	16.35	16.441
Cyclin D3	159	0.00	26.23	0.56	2.251
CDK6	169	0.01	65.94	6.46	11.144
CD27	171	0.00	75.48	16.58	18.766
PU 1	179	0.00	72.91	9.50	14.164
Cyclin E	179	0.00	4.09	0.07	0.348
E2F6	182	0.11	48.65	8.90	8.525
CDK2	177	0.00	76.68	3.28	8.526
Caspase 3a	181	0.01	16.29	0.96	1.883
p27	182	0.08	96.27	55.65	24.723
Mdm2	178	0.00	12.44	0.80	1.744
CD30	179	0.00	24.88	0.47	2.313
Bcl-xL	182	0.72	63.80	15.95	10.984
CD10	182	0.05	90.56	49.78	27.368
Skp2	174	0.00	50.27	2.40	5.426
p21	185	0.00	49.24	3.05	5.998
IgD	170	0.08	87.20	17.95	19.310
p18	165	0.05	45.34	10.38	8.435

with TMA score v.1.0 image analysis software, which uses WebSlide v.1.0 virtual slides (all Bacus Laboratories). The system is designed to quantify immunohistochemical staining using images obtained by scanning, and specific parameters fixed for each marker. Protein expression was measured as the percentage of the total core areas that was positively stained. The scanning system measured the total area occupied by cells, avoiding fibrosis or other negligible areas. The data were then exported to spreadsheets or database management systems for further analysis (Figure 1). The higher of the two values of positive expression was selected from each case.

To relate the expression of the different markers to the clinical data, the series was divided into quintiles with respect to the expression of each protein (Tables 1 and 2).

Overall survival was the end point of all statistical analyses. Only deaths attributable to the disease or to toxicity of treatment were considered. Survival curves were performed using the Kaplan–Meier estimator and compared using the log–rank test. Cox univariate analysis was also performed independently for each variable to estimate relative risk (Exp (B)). Values of $P < 0.05$ were considered significant. Survival probability was assessed by a Cox multivariate analysis that included all the variables with a value of $P < 0.1$. An individual risk for each patient was assigned using the value of X- β , which was obtained from the integrated markers (protein-based survival predictor) in the Cox multivariate analysis. All patients were ranked with respect to X- β value and divided into equal groups before applying the Kaplan–Meier estimator and log–rank test. All statistical analyses were carried out using SPSS v.15.0 (Chicago, IL, USA).

Results

Patients

The series was comprised of 186 cases of nodal FL collected from seven institutions, diagnosed between 1980 and 2002, with clinical follow-up and available clinical information (Table 1). The median age of patients was 56 years, with a range from 17 to 85 years, of whom 82 were men and 104 were women. The FLIPI score was obtained for 150 cases and the distribution with respect to this index was as follows: low-risk, 46 cases; intermediate-risk, 59 cases; and high-risk, 45 cases. The IPI score was calculated for 170 cases with the following distribution: 80 low-risk cases, 82 intermediate-risk cases, and 8 high-risk cases. The series included 35 patients with low clinical Ann-Arbor stages (I–II) and 147 patients with high clinical stages (III–IV).

With respect to histological grade, 80 cases were considered as grade 1, 57 cases as grade 2, 36 cases as grade 3a, and 13 cases as grade 3b. No significant

differences in OS were found between these four groups.

OS ranged from 1 to 250 months and the mean follow-up was 173 months. A total of 46 patients died because of the disease. Transformation to diffuse large B-cell lymphoma (DLBCL) was observed in six cases, with four of them dying of the disease.

Approximately, 36% of informative cases (41 out of 114) were negative for the (14;18)(q32;q21) translocation. A trend towards better prognosis was observed in negative t(14;18)(q32;q21) cases, with six out of 41 patients dying of the disease, in contrast with 21/73 cases positive for t(14;18)(q32;q21). However, these differences were not statistically significant. These data have been previously published.¹²

Treatment protocols varied considerably within the series, over time, and among institutions. The protocols included ‘watch and wait’ approach, different regimens of chemotherapy, radiotherapy, and bone marrow transplantation as first-line therapy. A total of 74 patients were treated with cyclophosphamide, vincristine, and prednisone (CVP), including 61 patients treated with CVP plus immunomodulator (58 with α -interferon and three with anti-CD20 monoclonal antibody). Other non-aggressive combinations without alkylating agent were used as first-line therapy in five cases. Aggressive combination chemotherapy was the therapy option in 86 cases, out of whom 65 received cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), including six CHOP plus α -interferon and five CHOP plus anti-CD20 monoclonal antibody. The other 21 patients were treated by chemotherapy with alkylating agents (C-MOOP, etc). Monotherapy with cyclophosphamide or clorambucil plus prednisone or leukeran plus prednisone was given to eight patients, and localized radiation therapy was used to treat four patients. A ‘watch and wait’ approach was taken in nine patients. In this series, eight patients were initially treated with anti-CD20 monoclonal antibody, whereas eight patients were treated with autologous stem cell transplantation or non-myeloablative allogeneic stem cell transplantation. The different therapeutic approaches made it essential to collect samples from different clinical institutions and over a relatively long period.

Survival Analysis

Univariate analysis identified several clinical parameters with the capacity to predict OS (Table 3). The group of patients aged 60 years or more, at high Ann-Arbor stage, bone marrow involvement, and high performance status had significantly shorter OS, measured by the clinical FLIPI and IPI scores.

However, several markers were associated with differences in OS in univariate analyses, such as

Figure 1 Scheme of quantification system using TMAcore v.1.0 image analysis software, which uses WebSlide v.1.0 virtual slides (all from Bacus Laboratories). The image shows the quantitative expression of a nuclear marker (Mdm2) in a selected core. (a) Low-power view of the immunostaining. (b) High-power view, with an insert showing a selected field. (c) Measuring the area occupied by cells. (d) Positive immunostaining in the selected field. (e) Example of quantitative results.

Cyclin E, Mdm2, CD10, p21, IgD, Bcl-xL, CD30, and E2F6. Those cases with a higher level of expression of cyclin E, Mdm2, p21, IgD, Bcl-xL, CD30, and E2F6 were associated with a significantly shorter OS. On

the other hand, strong CD10 expression was linked to a significantly better outcome. We did not find any differences between the five groups defined by Ki67 expression (Table 4).

Table 2 Mean $\pm$ s.d. of each quintile of the expression of the eight markers with differences in OS probability

Marker	Groups	1	2	3	4	5	Total
Cyclin E	Mean $\pm$ s.d. N	— —	0.00 $\pm$ 0.000 70	0.01 $\pm$ 0.00 41	0.03 $\pm$ 0.009 30	0.3 $\pm$ 0.7 38	0.08 $\pm$ 0.3 179
Mdm2	Mean $\pm$ s.d. N	0.01 $\pm$ 0.01 32	0.06 $\pm$ 0.02 38	0.2 $\pm$ 0.09 37	0.6 $\pm$ 0.1 36	3.1 $\pm$ 2.9 35	0.8 $\pm$ 1.7 178
CD10	Mean $\pm$ s.d. N	5.5 $\pm$ 6.1 36	35.5 $\pm$ 8.7 37	57.1 $\pm$ 5.7 36	69.9 $\pm$ 2.9 37	80.8 $\pm$ 3.5 36	49.8 $\pm$ 27.4 182
p21	Mean $\pm$ s.d. N	0.07 $\pm$ 0.05 39	0.4 $\pm$ 0.1 35	1 $\pm$ 0.3 37	2.4 $\pm$ 0.7 37	11.4 $\pm$ 9.5 37	3 $\pm$ 6 185
IgD	Mean $\pm$ s.d. N	1.2 $\pm$ 0.7 34	4.3 $\pm$ 1.1 34	10.2 $\pm$ 2.1 34	23.5 $\pm$ 5 34	50.5 $\pm$ 14.6 34	17.9 $\pm$ 19.3 170
Bcl-xL	Mean $\pm$ s.d. N	4.7 $\pm$ 1.7 36	9.4 $\pm$ 1.2 37	13.5 $\pm$ 1.4 36	19.1 $\pm$ 1.8 37	33.2 $\pm$ 10.7 36	15.9 $\pm$ 11 182
CD30	Mean $\pm$ s.d. N	0 $\pm$ 0 30	0.01 $\pm$ 0.005 44	0.04 $\pm$ 0.01 35	0.1 $\pm$ 0.03 35	2.2 $\pm$ 4.9 35	0.5 $\pm$ 2.3 179
E2F6	Mean $\pm$ s.d. N	1.6 $\pm$ 0.7 37	3.6 $\pm$ 0.5 36	6.2 $\pm$ 0.9 36	10 $\pm$ 1.6 37	23.2 $\pm$ 8.2 36	8.9 $\pm$ 8.5 182

Table 3 Outcome and survival analysis according to group as defined by different clinical parameters and histological grading

Parameter	N total	N	5-year OS (%)	10-year OS (%)	Log-rank test, P
<i>Gender</i>	186				NS
Female		104	88	71	
Male		82	82	57	
<i>Age</i>	185				0.005
<60 years		109	91	74	
$\geq$ 60 years		76	78	48	
<i>Cell type</i>	186				NS
Grade 1		80	86	60	
Grade 2		57	83	68	
Grade 3a		36	84	69	
Grade 3b		13	100	67	
<i>Ann-Arbor stages</i>	182				0.001
I-II		35	97	97	
III-IV		147	83	56	
<i>Number of nodal sites</i>	186				NS
0-4		119	88	72	
>4		67	82	53	
<i>Bone marrow involvement</i>	172				0.01
Absence		80	88	77	
Presence		92	82	51	
<i>Serum LDH</i>	171				NS
$\leq$ ULN		144	85	66	
>ULN		27	88	58	
<i>Hemoglobin level</i>	157				NS
$\geq$ 120 g/l		126	89	64	
<120 g/l		31	89	75	
<i>FLIPI</i>	150				0.002
0-1		46	97	89	
$\geq$ 2		104	86	55	
<i>IPI</i>	170				0.0001
0-1		80	93	80	
$\geq$ 2		90	77	47	
<i>Follow-up (months)</i>	186				
Mean ^a		173			
Total range		1-251	86	65	
DOD		46			

Abbreviations: DOD, death of disease; FLIPI, Follicular Lymphoma International Prognostic Index; IPI, International Prognostic Index; LDH, lactate dehydrogenase; NS, not statistically significant; OS, overall survival; ULN, upper limit normal.

^aMedian not reached.

Significant *P*-values are in bold.

Cyclin E showed a range of protein expression (%) from 0.00 to 4.09, with a mean of 0.07; Mdm2 expression ranged from 0.00 to 12.44, with a mean of 0.80; CD10 expression ranged from 0.05 to 90.56, with a mean of 49.80; p21 showed a range of expression from 0.00 to 49.24, with a mean of 3.05; the expression of IgD protein ranged from 0.08 to 87.20, with a mean of 17.95; Bcl-xL expression ranged from 0.72 to 63.80 with a mean of 15.90; CD30 protein expression ranged from 0.00 to 24.90 with a mean expression of 0.50; and E2F6 protein expression ranged from 0.11 to 48.65, with a mean of 8.90 (Table 2).

The Cox multivariate analysis showed that Cyclin E, Mdm2, and CD10 expression (Figure 2) were markers with a predictive capacity that was independent of FLIPI (Table 4).

To represent the survival curves defined in this way, the individual risk for each patient was assigned using the value of X- β , which was obtained from the integrated markers (IHC survival predictor or protein model) identified in the Cox multivariate analysis. All patients were ranked with respect to X- β value, and divided into equal groups before applying the Kaplan–Meier estimator and carrying out a log-rank test (Figure 3). A 5-year survival for patients in the low-risk group was 95% and for high-risk group was 77%, the difference being statistically significant (log-rank test; $P < 0.0001$).

This protein expression model was then used for both the low and intermediate-high FLIPI groups using the Kaplan–Meier method, and proved to be a significant prognostic marker for both. The expression of the three markers divided the low-risk FLIPI

Table 4 Cox univariate and multivariate analyses

Marker	Univariate analysis					Multivariate analysis			
	95% CI for Exp (B)				P	95% CI for Exp (B)			P
	N	Exp (B)	Lower	Upper		Exp (B)	Lower	Upper	
Cyclin E	68 vs 111	2.8322	1.5342	5.2284	0.0009	2.0302	1.079	3.8198	0.0281
Mdm2	146 vs 32	12.1814	1.6744	88.6216	0.0135	10.3266	1.4109	75.5812	0.0215
CD10	73 vs 109	1.964	1.0776	3.5793	0.0275	2.0678	1.1038	3.8737	0.0233
p21	74 vs 111	3.0518	1.6645	5.5953	0.0003	—	—	—	—
IgD	34 vs 136	2.6001	1.3959	4.843	0.0026	—	—	—	—
Bcl-xL	36 vs 146	1.9517	1.0343	3.6826	0.0390	—	—	—	—
CD30	70 vs 109	2.3942	1.3125	4.3672	0.0044	—	—	—	—
E2F6	36 vs 146	2.3176	1.2414	4.3269	0.0083	—	—	—	—

Significant *P*-values are in bold.

Figure 2 Immunohistochemical expression of markers with differences in overall survival and included in the Cox multivariate analysis ($\times 40$).

group into two groups with 100 vs 93% survival probabilities at 5 years and 100 vs 68% at 10 years (log-rank test; $P < 0.05$). This was also the case for the intermediate-high risk FLIPI groups in which two groups were identified with 97 vs 81% and 76 vs 39% survival probabilities at 5 and 10 years, respectively, were found (log-rank test; $P < 0.01$) (Figure 4).

No significant differences in OS were observed between FL histological grades 1–3 (Table 3). However, the protein expression model was able to discriminate different survival probabilities in low-grade (grades 1 and 2) and high-grade (grade 3) histological groups. Cases with low-grade histology and a low protein score had 95 and 82% OS probabilities at 5 and 10 years, respectively, in

Figure 3 Survival curves of FL patients divided into two equal groups by integration of the three markers identified in the multivariate analysis (X-β assigned on the basis of the outcome of the Cox analysis).

contrast with 72 and 40% in cases with low-grade histology and high protein score. High histological grade cases and low protein score showed 95 and 89% survival probability at 5 and 10 years, respectively, whereas cases with the same histological grade but high protein score had 81 and 48% survival probability at 5 and 10 years, respectively (log-rank; $P < 0.0001$) (Figure 5).

Protein score was also independent of the therapy received. To analyze the interaction between the protein score and the therapy, the series were divided into non-aggressive and aggressive therapy groups. The distribution of protein expression scores revealed two non-aggressive therapy subgroups with 94 vs 71% survival probabilities at 5 years and 94 vs 49% at 10 years. Cases treated with aggressive therapy were divided using the protein score into a group with 95 vs 87% at 5 years and 77 vs 29% at 10 years (log-rank test; $P < 0.0001$) (Figure 6).

Discussion

In this study, we identified a set of markers expressed by neoplastic cells that were associated with low OS in FL patients. These included Cyclin E, Mdm2, CD10, p21, IgD, Bcl-xL, CD30, and E2F6. Their expression was estimated using an automated procedure that reduces the subjectivity of scoring. Some of these markers, or others related with them, have already been shown to be prognostic markers in FL patients.^{13,14} A Cox model was then built integrating FLIPI and a restricted selection of three markers: Cyclin E, Mdm2, and CD10 expression.

A potentially useful finding is that the integrated FLIPI plus IHC model was able to identify a subset of 26 FL patients (almost 20% of the entire series) with a survival probability of 100% at 5 years. This not only confirms that a group of FL cases may have a very good clinical course, but also indicates that

Figure 4 Survival curves of two groups defined by X-β (protein model) in low-risk FLIPI (a) and intermediate-high-risk FLIPI series of patients (b). Log-rank test; $P = 0.001$.

Figure 5 Survival curves of two groups defined by X-β (protein model) in low-histological grade (a) and high-grade series of FL cases (b). Log-rank test; $P < 0.0001$.

Figure 6 Survival curves of two groups defined by X-β (protein model) in non-aggressive chemotherapy (a) and aggressive chemotherapy (b) groups of patients. Log-rank test; $P = 0.001$.

this group can be identified using this integrated clinical and IHC approach.

Cyclin E is a cyclin involved in the initiation of DNA replication, genomic stability, and the centrosome cycle. The cyclin E/CDK2 complex is able to activate the cascade of molecules needed in S-phase and that are critical in G1-to-S transitions during the cell cycle. In fact, on the basis of results of studies of human cancers such as breast cancer, leukemia, and lymphoma, it is considered to be an oncoprotein.¹⁵ Our findings reveal higher expression of cyclin E and its kinase partner, CDK2, in a group of patients with poorer outcome, with cyclin E being an independent factor identified in the multivariate analysis.

CD10 is considered to be a marker of follicular center B-cell differentiation. This prognostic marker has previously been described.¹³ PU-1 is a transcription factor that is also involved in B-cell differentiation, and a significant positive association has been

observed between a high level of GC antigen expression (PU-1, CD27, CD75, CD20, Bcl-6, CD10, etc.) and longer OS.¹⁶ In our study, a significantly better clinical outcome was related with strong expression of CD10, independent of FLIPI, suggesting that the strong GC differentiation signature in FL could also be defined a group of lymphomas with better prognosis, similarly to the situation described in DLBCL.¹⁷

The Mdm2 oncoprotein is a cellular inhibitor of p53 that is frequently deregulated in human cancer. Univariate analysis of our series revealed a highly statistically significant shorter OS for higher levels of Mdm2 protein expression, the better prognostic group having almost negative Mdm2 expression ($P = 0.01$). Moreover, this protein was included in the final multivariate model. Mdm2 protein overexpression has previously been reported in B-cell lymphoma and in transformed FL cases, independent of TP53 status.^{18–24}

A potentially related finding is the strongly raised level of p53 expression observed in 5% of the series, which is associated with shorter OS, and confirms the results about the prognostic value of the p53 mutation.²⁵ We have not included p53 in the final model, as the proportion of positive cases was substantially below the required threshold (20% cases).

This study has focused on the features of the tumoral cells, rather than other cell components of the FL microenvironment that have been already demonstrated to have a prognostic significance. FL genesis, progression, and treatment escape represent a scenario of complex relationship between molecules and signal from tumoral and non-tumoral cells.² Further analyses integrating both sources of data are still to be performed, but seem likely to enrich the results shown here.

Histological grading in the diagnosis of FL is still routinely included in the pathological report, following the WHO recommendations.¹⁰ In this series, no significant difference in OS was found according to the histological grading. Moreover, the integration of the protein model and grade demonstrated that the model was independent of the histological grading, with strong OS predictive capacity for both low and high-grade groups.

This series is not based on a standardized treatment, and includes patients treated with different protocols, reflecting the absence of any standard approach to the treatment of FL. However, the model here identified was independent of the treatment, when patients were divided into two groups considering the different therapeutic options. Prospective prognostic studies in FL are hampered by the relatively indolent clinical course that requires long follow-up. In contrast, this retrospective study offers the opportunity of including patients with a long follow-up, thereby increasing the capacity of the study to identify prognostic markers. In this study we have followed the model of patient selection previously used in the development of the FLIPI.¹

The study does not consider whether changes in treatment protocols can modify these prognostic markers, as has been shown for some microenvironment markers.²⁶ The specific effect of Rituximab should be analyzed after a longer follow-up than that currently available.

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Disclosure/conflict of interest

Authors declare no conflict of interest.

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