Indolent lymphoplasmacytic and marginal zone B-cell lymphomas: absence of both IRF4 and Ki67 expression identifies a better prognosis subgroup

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Background and Objectives. Small B-cell indolent lymphomas postulated to be of a post-germinal center origin include marginal zone lymphomas of the spleen (S-MZL) or lymph nodes (N-MZL) and mucosa-associated lymphoid tissue (MALT) lymphomas and lymphoplasmacytic lymphomas (LPL). The existence of rather aggressive cases stresses the need for new biological prognostic markers.

Design and Methods. We analyzed 90 tumors (20 LPL, 41 MALT lymphomas, 12 N-MZL, 17 S-MZL), investigating the expression of CD5, CD10, CD20, CD23, CD27, CD38, CD79a, CD138, Bcl6, cyclin D1, IRF4 and Ki67 antigens by immunohistochemistry. Results were compared to the histology, the standard clinical and biological parameters, and the global survival.

Results. Tumors were all positive for CD20 and CD79a, occasionally positive for CD5, CD23, CD138 and cyclin D1, and all negative for Bcl-6 and CD10. CD38, CD27 and IRF4 expression was heterogeneous. IRF4 expression was correlated with plasma-cell differentiation (p=0.0017). Ki67 expression was increased mainly in N-MZL (66%) and LPL (45%). In terms of overall survival, Ki67, IRF4 and C-reactive protein levels were found to be the 3 independent parameters associated with a worse outcome. Lack of both Ki67 and IRF4 expression was associated with a longer survival (median overall survival 9.8±1.1 years versus 3.6±1.3 years in the other group) (p=0.0011).

Interpretations and Conclusions. Absence of expression of both Ki67 and IRF4 is likely to define a group of memory B-cell lymphomas with a better prognosis. This may have an important impact in the staging of patients since expression of these markers is easily assessed in routine diagnosis.

Key words: lymphoma, memory B-cells, IRF4, Ki67.

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Among the indolent B-cell lymphomas postulated to be of post-germinal center origin, marginal zone lymphomas (MZL) encompass three recently described entities of indolent B-cell lymphomas, i.e. extranodal MZL or mucosa-associated lymphoid tissue (MALT) lymphomas, splenic MZL (S-MZL) and nodal MZL (N-MZL).[1] MZL display cellular heterogeneity and variations in growth patterns, independently of their anatomical site, but share immunophenotypic, genetic and chromosomal similarities.[2] These lymphomas are associated with morphological features of plasma-cell differentiation; the degree of this differentiation varies among patients. Morphologically, these histological categories overlap with lymphoplasmacytic lymphomas (LPL) including Waldenström's macroglobulinemia (WM), which have an extra-follicular pattern and features of plasmacytic differentiation.[3] A monoclonal paraprotein may be found in the serum of patients with MZL but, as in LPL,[4] the levels of monoclonal IgM secretion are not related to plasma-cell differentiation in the tumor. Among LPL, the diagnosis of WM is based on the detection of monoclonal IgM, but, again, IgM levels do not correlate with the degree of morphological plasma-cell differentiation in the tumor. LPL are usually localized in the spleen and/or lymph nodes although bone marrow infiltration is also frequent. Although now considered relatively rare, extra-nodal localizations of LPL are possible and include the lung and gastro-intestinal tract.[3] N-MZL and S-MZL may arise from both mutated and non-mutated transformed B-lymphocytes.[5,6] In contrast, IgVH somatic mutations are constantly found in LPL.[7,9] Some prognostic factors have been reported in these lymphomas. Nevertheless, in daily clinical practice, it is nearly impossible to predict the outcome of the patients.[10] N-

MZL seem to have a worse prognosis than MALT lymphomas[11] or S-MZL.[12] In WM, the level of serum albumin, cytopenia and age have been reported to be of prognostic value.[13] LPL seem to have a worse prognosis than the three other categories.[14] Chacon *et al.* showed that bone marrow involvement and lack of response to therapy are predictive of poor outcome in S-MZL.[10] These authors confirmed the relative indolence of these lymphomas, but also evidenced the existence of more aggressive cases, pointing out the need for new biological markers allowing a better discrimination of the patients at diagnosis[10] in order to be able to adapt the therapy or, even better, to propose therapeutic abstention, as recommended by some authors.[12] The aim of this work was to characterize the immunophenotypic profile of LPL, focusing on the degree of plasma-cell differentiation and the proliferation index, in order to identify new, simple biological markers of prognosis.

Design and Methods

Patients' selection and follow-up

For this retrospective study, a series of 130 biopsies from patients diagnosed with a small B-cell lymphoma between 1989 and 2003 in the Department of Pathology of Dupuytren Hospital (Limoges, France) was reviewed. All the biopsies selected had been performed at initial diagnosis. Tumors with a partial aspect of transformation and/or more that 50% Ki67 positive cells were excluded.

Diagnosis and classification

All tumors were reviewed by one of us (BP). The selection of retained cases was based on an initial diagnosis of small B-cell lymphoma with an indolent clinical course, excluding chronic lymphocytic leukemia, follicular lymphoma or mantle cell lymphoma. The final selection comprised 90 cases. Histologic and immunohistochemical characteristics of the tumors were used to reclassify them according to the WHO classification.[1] Patients with an extra-nodal location of lymphoma, histologic features of marginal zone lymphoma, and either splenic or nodal involvement were considered as having disseminated MALT lymphomas.[15] Lymph node tumors displaying a parafollicular/perisinusoidal distribution of neoplastic cells or a nodular pattern, and composed of an admixture of centrocyte-like cells, monocytoid cells, scattered blasts and small cells with plasmacytoid differentiation were classified as nodal marginal zone lymphomas. As reported by Andriko *et al.*,[14] LPL comprise tumors with a diffuse pattern of infiltration without any residual germinal center and an intermediate or marked plasma-cell differentiation, or with a partial lymph node involvement corresponding to an inter-follicular pattern and prominent features of plasma-cell differentiation. Tumors with features of progression to large B-cell lymphoma were excluded from the study. Moreover, plasma cell differentiation was analyzed morphologically. Plasmacytoid features of the tumor cells were defined by eccentric nuclei, condensed *clock face* chromatin, and abundant cytoplasm. The plasmacytic differentiation was semi-quantified as follows: negative, less than 5%; intermediate, 5-20%; high, more than 20%.

Immunohistochemical analysis

Immunolabeling was performed with antibodies specific for B-cell lineage antigens (CD20/L26, Dako SA, Glostrup, Denmark, CD79a/JCB 117, Dako, CD23/1B12, Novocastra Labs, UK), T-cell lineage antigens (CD3/PC3/188A, Dako, CD5/4C7, Novocastra), antigens expected to be absent in post-germinal center B-cells (CD10/56C6, Novocastra, Bcl-6/PG-B6p, Dako, cyclin D1/DCS-6, Dako) and the proliferation marker Ki67 (MIB-1, Dako). Plasma-cell immunohistochemical characterization was evaluated with antibodies directed against CD38 (SPC32, Novocastra), CD138 (MI15, Dako SA), CD27 (137B4, Novocastra) and IRF4 (goat polyclonal immune serum from Santa-Cruz, CA USA).

At initial diagnosis, the 90 biopsies were routinely fixed in formol or Bouin's, and paraffin-embedded. For immunohistochemical analysis, they were re-embedded 6 at a time in paraffin blocks, resulting in 15 paraffin blocks of tumors. For each marker tested, immunolabeling was performed on 5 μm sections of these 15 paraffin blocks. Antigen retrieval pre-treatment included heating at 100°C for 40 min in 0.01 mol/L citrate buffer pH 6 (for formol-fixed tumors) or in ethylene diamine tetra acetic acid (EDTA)-NaOH buffer pH 8 (Bouin's fixed tumors). Immunolabeling was performed with a standard indirect immunoperoxidase technique using an avidin-biotin complex method, with diaminobenzidine chromogen as the substrate, on an automated immunostainer (Ventana Medical Systems SA, Illkirch, France). A semi-quantitative evaluation of immunostaining was performed by two of us (BP, JF). Except for Ki67, the expression of each marker was estimated in a semi-quantitative manner by the percentage of tumor cells positive for that marker: negative, 0% to 20%; intermediate, 20 to 50%; high, >50%. Ki67 expression was quantified as follows: negative, <5% positive cells; significant, 5%-20% positive cells; increased, 20%-50% positive cells.

Statistical analysis

Qualitative data were compared by the χ^2 test using Yates' correction when appropriate. Overall survival was defined as the time between diagnosis (first biopsy) and the time of death or the last follow-up. Actuarial survival curves were calculated using the Kaplan-Meier method, and differences between parameters were tested for significance with the log-rank test.[16,17] Variables shown to be of significant impact by univariate analysis were included in a multivariate analysis using a Cox proportional hazard model, to determine independent prognostic factors for survival. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed with Statview software (SAS institute, Cary, NC, USA).

Results

Clinical characteristics

The 90 tumors studied included 20 LPL, 12 N-MZL, 17 S-MZL and 41 MALT lymphomas. The 90 patients were 41 males and 49 females (sex ratio, 0.84), with a median age of 67 years old (range, 30 to 96 years), without differences between histological subtypes (Table 1). Except for those with MALT lymphomas, most patients had stage III or IV disease according to the Ann Arbor staging classification (Table 1). The localization of MALT lymphoma was ocular (17/41), gastro-intestinal (12/41), pulmonary (4/41), salivary (4/41), thyroid (3/41) and breast (1/41). Monoclonal IgM was detected in 14% cases, mostly in the LPL category (Table 1). High C-reactive protein serum levels were observed in 70% of the cases. Bone marrow infiltration was frequent in LPL (79%) and S-MZL (87%). A leukemic phase of the disease was found in 70% S-MZL, 16% of LPL and was rare in the other cases. Patients with LPL were submitted to initial monochemotherapy (42%) or polychemotherapy (53%). Seventy-five percent of the patients with N-MZL were treated with polychemotherapy, and one of them received an autologous bone marrow transplantation. Most patients with S-MZL benefited from surgery alone (59%) or surgery supplemented by monochemotherapy (12%). Patients with MALT lymphomas were treated according to various therapeutic schemes (27% surgery alone, 16% surgery supplemented by monochemotherapy, 16% monochemotherapy alone and 30% polychemotherapy).

Morphological and immunophenotypic analysis of the tumors

Plasma-cell differentiation was evaluated morphologically. Taking all cases together, intermediate or marked features of plasma-cell differentiation were

Table 1. Clinical, morphological, and biological characteristics of the 90 patients included in this analysis.

Diagnosis	No. of patients	Age (year)	Sex ratio (M/F)	Ann Arbor stage III/IV*	Bone Marrow Infiltration*	Leukemic phase*	CRP (>5mg/L)	IgM peak >3g/L
LPL	20	62 (30-83)	1	16 (80%)	15/19 (79%)	3/19 (16%)	10/12 (83%)	6/16 (37%)
N-MZL	12	68 (37-80)	0.33	8 (75%)	4/9 (44%)	2/11 (18%)	5/10 (50%)	1/10 (10%)
S-MZL	17	69 (45-79)	0.89	15 (88%)	14/16 (87%)	12/17 (70%)	9/11 (82%)	1/17 (6%)
MALT	41	66 (30-96)	1	8 (19%)	5/29 (17%)	2/36 (5%)	16/24 (66%)	5/32 (16%)

observed in 57% of them and 100% of LPL as it was a criterion for this diagnosis. Marked plasma-cell differentiation of tumor cells was noted in 75% LPL, 17% N-MZL, 18% S-MZL and 2% MALT lymphomas ($p<0.0001$). As expected,[4] plasma-cell differentiation of tumors cells did not correlate with the presence of monoclonal IgM (*data not shown*). All tumors were positive for CD20 and CD79a and negative for Bcl-6 and CD10. Most cases were CD5 and CD23 negative. In agreement with the reported occasional expression of cyclin D1 in these lymphomas,[18] 5 cases were cyclin D1 positive (2 LPL, 2 MALT lymphomas and 1 S-MZL). These 5 tumors were CD5, CD23 and IgD negative. CD138 was expressed only in 5 cases (3LPL and 2 S-MZL) that showed pronounced features of plasma-cell differentiation and expressed CD38 and IRF4 strongly. Two of the CD138 positive cases (1 S-MZL and 1 LPL) were associated with serum monoclonal IgM. The expression of CD27, CD38, Ki67 and IRF4 was heterogeneous (Table 2 and Figure 1 for IRF4). CD27 expression was observed in 24% of the cases, without any significant correlation with the histologic subtype or plasma-cell differentiation (Table 2). Intermediate or high levels of CD38 expression (>20% positive cells) were observed in 40% of LPL, 18% of N-MZL, 7% of MALT lymphomas and never in MZL. IRF4 was expressed in more than 20% of the tumor cells in 40% of LPL, 25% of N-MZL, 12% of S-MZL and 5% of MALT lymphomas. Both IRF4 and CD38 expression were partly associated with morphological plasma-cell differentiation ($p=0.002$ and $p=0.001$, respectively). Twenty-nine of the 90 patients had tumors with significant (>5%) or increased (>20%) expression of Ki67 (Figure 1). Ki67 expression was noted mainly in cases of N-MZL and LPL (67% and 45%, respectively, $p=0.01$, Table 2) and was not associated with plasma-cell differentiation ($p=0.6$).

**Table 2.** Expression of CD38, IRF4 and Ki67.

Diagnosis	>20% CD38+cells*	> 20% IRF4+cells*	> 5% Ki67+cells*	> 20% Ki67+cells	>20% CD27+cells
LPL	8/20 (40%)	8/20 (40%)	9/20 (45%)	1/20 (5%)	4/13 (30%)
MALT	3/41 (7%)	2/41 (5%)	9/41 (22%)	2/41 (5%)	6/24 (25%)
N-MZL	2/11 (18%)	3/12 (25%)	8/12 (66%)	2/12 (16%)	1/10 (10%)
S-MZL	0/11 (0%)	2/17 (12%)	3/17 (17%)	1/17 (5%)	4/15 (27%)
All cases	17%	18%	33%	7%	24%

*$p(\chi^2) < 0.05$.

Table 3. p value of each factor for overall survival after univariate analysis.

Factor	p value
Monoclonal IgM	0.03
Ann Arbor stage I/II versus III/IV	0.03
High CRP level	0.007
CD38 expression	0.024
Ki67 expression	<0.0001
LPL / Others	0.0016
IRF4 expression	<0.0001

Correlation between immunological profile and survival

Our next aim was to evaluate the prognostic significance of the markers investigated (Table 3). In univariate analysis, LPL was the histological subtype with the worst prognosis ($p=0.0016$), whereas there was no significant difference in overall survival between cases of N-MZL, S-MZL and MALT lymphomas (*data not shown*). Ann Arbor stage III or IV was also associated with a worse prognosis ($p=0.03$). The only prognostically significant biochemical marker was C-reactive protein, CRP, high levels being associated with a worse outcome ($p=0.007$). Three immunohistological markers were prognostically significant when expressed in more than 20% of the tumour cells, CD38 ($p=0.024$), Ki67 ($p<0.0001$) and IRF4 ($p<0.0001$) (Figure 2). Multivariate analysis identified 3 significant independent parameters: Ki67, IRF4 and CRP levels. Kaplan-Meier analysis of the whole series suggested that global survival of patients
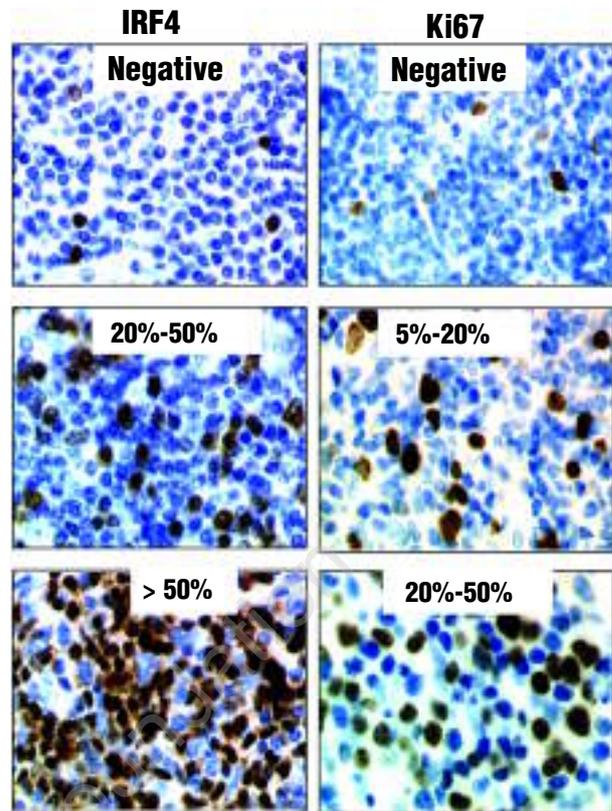
Figure 1. Detection of Ki67 and IRF4 expression by immunohistochemistry.

with >20% of tumor cells positive for Ki67 and/or IRF4 had a markedly reduced overall survival when compared to the others (Figure 2). A distribution histogram of overall survivals clearly suggested the existence of two groups of patients: those with a short survival of less than 5 years and the other group with a longer survival of up to 13 years (*not shown*). Ki67 and IRF4 expression were both associated with the group of patients with a short term survival ($p=0.0003$ and $p0.0001$, respectively). Altogether, this suggests that patients lacking expression of both IRF4 and Ki67 in tumor cells may have a much better prognosis than the other patients. We, therefore, grouped the patients into two categories: those whose tumor cells were both Ki67 and IRF4 negative and all others. The median overall survival of patients with Ki67/IRF4 double negative tumors was 9.8±1.1 years, while it was only 3.6±1.3 years for the other patients ($p=0.0011$). The median overall survival of the whole series was 8.6±1 years.

Discussion

The aim of this study was to evaluate the potential prognostic significance of the proliferation index and

plasma-cell differentiation, in terms of morphological aspect and immunophenotypic profile, in LPL and MZL.

The cases studied were indolent lymphomas that were not chronic lymphocytic leukemias (CLL), follicular lymphomas or mantle cell lymphomas. Reanalysis of the morphology led us to change the initial diagnosis for 25% of the tumors biopsied before 1998. The major difficulty was to differentiate N-MZL from LPL. The diagnosis of N-MZL was made when tumor cells were infiltrating an area surrounding the mantle zone, with both centrifugal extension to the extrafollicular area and centripetal infiltration of the mantle zone, combined with persistent residual germinal centers. A helpful but not always present additional criterion for N-MZL diagnosis was the cytological aspect of tumor cells resembling monocytoid cells with a large pale cytoplasm. In case of partial interfollicular or diffuse infiltration, the differential diagnosis between LPL, lymphoplasmocytoid variant of CLL and MZL with lymphoplasmacytosis may be difficult. The diagnosis of lymphoplasmocytosis variant of CLL was based mainly on expression of the CD5 marker.[19] The diagnosis of LPL was achieved when characteristic lymphoplasmacytic cells were prominent.[14] Overall, 20/90 patients of our series were definitely classified as having LPL. It is noteworthy that, with these criteria, patients with monoclonal IgM were more frequent in the LPL category than in that of MZL. Moreover, we observed that the prognosis of patients with LPL was worse than that of patients with the other categories of indolent lymphomas. These results are in agreement with those of Andriko et al.,[14] and clearly suggest that LPL is a genuine clinico-pathologic entity distinct from MZL, even though immunochemistry was unable to identify a specific marker for either of these categories.

CD27, CD38, IRF4 and Ki67 were the 4 markers with heterogeneous expression, and thus those we investigated further for prognostic significance. No significant association was found between CD27 expression and any other clinical or biological parameter. Theoretically, the main interest of the CD27 marker is that its negativity has been reported to be physiologically typical of naive B-cells.[20] The heterogeneous expression of CD27 in our series of MZL and LPL was not significantly related with the histological subtype, even though it was less expressed in N-MZL, and was not related to plasma-cell differentiation. The frequency of CD27 negative tumors may reflect the loss of this antigen during the transformation process of memory B-cells since a soluble form of CD27 can be generated after activation of memory B cells following CD70 interaction.[21-25] Alternatively, it may reflect the dynamics of phenotypic evolution during recircula-
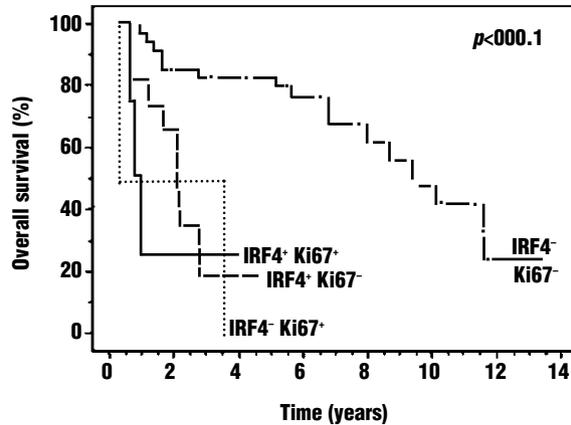

Figure 2. Overall survival according to the expression of IRF4 and Ki67.

tion of tumor cells, as discussed by Franco et al.[26] Finally, it is noteworthy that the marginal zone is a physiologically heterogeneous area containing not only T-cell-dependent mutated memory B cells, but also B cells responding to T-cell-independent antigens and virgin B cells.[27] This could also explain the molecular heterogeneity of MZL, since N-MZL and S-MZL may arise from both mutated and non-mutated transformed B lymphocytes.[5,6] Thus, the significance of CD27 expression in lymphomas postulated to be of post-germinal center origin, both in our series and in the literature series, is not understood and needs further studies such as correlation with the immunoglobulin gene mutational status.

After univariate analysis, the significant parameters for prognosis were LPL histology, CRP level, and overexpression of CD38, IRF4 and Ki67. Ki67 expression is likely to reflect the proliferative activity of tumors. We found that Ki67 was more frequently expressed in N-MZL than in the other categories, without any relationship with plasma cell differentiation. It is noteworthy that, although not specific, the other 4 parameters are all related to plasma-cell differentiation. Indeed this is a morphological criterion for LPL histology, while CRP levels are related to the secretion of interleukin-6 (IL-6), a cytokine that promotes plasma-cell differentiation and survival of myeloma cells. Moreover, both CD38 and IRF4 are expressed at high levels on plasma cells. Regarding the prognostic significance of these markers, our results are in agreement with those of Andriko et al., who recently identified LPL as the histological subtype with the worst prognosis.[14] Moreover, CRP levels are also predictive of outcome in myeloma.[28] Both CRP and IL-6 levels predict poor prognosis in B-cell NHL.[29,30] CD38 is expressed on a variety of lymphomas, including CLL, and is correlated with a poor

prognosis in this group of tumors.[31] IRF4, also called MUM1, has been cloned from a t(6;14)x(p25;q32) translocation in a myeloma cell line.[32] IRF4 is a member of the interferon regulatory factor family and is associated with plasma-cell differentiation.[33] In *IRF4*-/- mice, peripheral B-cell development is blocked at the germinal center stage, and no plasma cells are present.[34] IRF4 is highly expressed on a subset of cells in the light zone of the germinal center that also expresses CD138 and Blimp-1 and appears to be committed to a plasma cell fate.[35,36] IRF4 represses the transcription of class I MHC.[37] In association with PU.1, IRF4 activates both κ and λ light chain enhancers.[38-40] In fact, IRF4 is frequently expressed in a variety of lymphomas including CLL, DLCL[41] and N-MZL.[33,42] Conflicting results have been reported on the prognostic value of IRF4 in CLL.[43,44] Outside this context, the prognostic value of IRF4 has not been evaluated in indolent lymphomas so far, although IRF4 expression has recently been shown to be characteristic of activated diffuse large B-cell lymphomas, a subgroup of high grade lymphomas associated with a poor prognosis.[45,46]

One of the key questions of the management of LPL and marginal zone lymphomas is when to treat patients or to propose therapeutic abstention, since the majority of patients have an indolent course. Some clinicians prefer therapeutic abstention in S-MZL patients.[12] However, it is known that a subset of patients have more aggressive disease and a shorter survival.[10,47] To date, there is no consensus on the therapeutic options, probably because of the lack of prognostic factors allowing stratification. In our study, multivariate analysis showed that Ki67, IRF4 and CRP were three independent prognostic markers. Because the expression of IRF4 and Ki67 can easily be assessed by immunohistochemistry, we combined these 2 markers in an attempt to define new prognostic criteria. We found that patients with tumors negative for both IRF4 and Ki67 had a much better overall survival than the others, with a dramatic difference in terms of median overall survival between Ki67/IRF4 double negative patients (9.8 + 1.1 years) and the others (3.6 + 1.3 years).

In conclusion, the identification of a subset of patients lacking expression of both IRF4 and Ki67 on tumor cells may be important in the staging of indolent memory B-cell lymphomas, since these patients clearly have a better prognosis. It would be interesting to determine in prospective studies whether patients with LPL or MZL negative for both Ki67 and IRF4 would benefit or not from therapeutic abstention.

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