



Serum cytokines in follicular lymphoma. Correlation of TGF- β and VEGF with survival

Sana Intidhar Labidi, Christine Ménétrier-Caux, Sylvie Chabaud, Catherine Chassagne, Catherine Sebban, Thérèse Gargi, Pierre Biron, Jean-Yves Blay, Hervé Ghesquières

► To cite this version:

Sana Intidhar Labidi, Christine Ménétrier-Caux, Sylvie Chabaud, Catherine Chassagne, Catherine Sebban, et al.. Serum cytokines in follicular lymphoma. Correlation of TGF- β and VEGF with survival. *Annals of Hematology*, 2009, 89 (1), pp.25-33. 10.1007/s00277-009-0777-8 . hal-00535069

HAL Id: hal-00535069

<https://hal.science/hal-00535069>

Submitted on 11 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Serum cytokines in follicular lymphoma. Correlation of TGF- β and VEGF with survival

Sana Intidhar Labidi · Christine Ménétrier-Caux · Sylvie Chabaud · Catherine Chassagne · Catherine Sebban · Thérèse Gargi · Pierre Biron · Jean-Yves Blay · Hervé Ghesquières

Received: 6 March 2009 / Accepted: 16 June 2009 / Published online: 7 July 2009
© Springer-Verlag 2009

Abstract The prognosis of follicular lymphoma could vary with the tumor immune microenvironment. We evaluated the prognostic value of serum levels of ten cytokines. Our study cohort included 60 follicular lymphoma patients and 20 controls. Serum was available at diagnosis in 31 patients, at first relapse in 18, and complete remission in 11. Bioplex technology was used for determination of nine cytokines [interleukin (IL)-1Ra, IL-6, IL-7, IL-10, IL-13, tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (b-FGF)]. Transforming growth factor beta (TGF- β) was measured by sandwich enzyme-linked immunosorbent assay. IL-1Ra, IL-6, IL-7, IL-10, IL-13, TNF- α , VEGF, and PDGF levels were found increased in follicular lymphoma patients compared to controls. Multivariate analysis identified early

stage and high TGF- β levels as independent predictors of overall survival associated with improved outcome. High lactate dehydrogenase and VEGF levels were independently associated with poorer progression-free survival. These results show the prognostic value of TGF- β and VEGF in follicular lymphoma and suggest their contribution to tumor microenvironment alterations.

Keywords Cytokine · Follicular lymphoma · TGF- β · VEGF · Prognosis

Introduction

Follicular lymphoma (FL) is the second most frequent type of adult B-cell non-Hodgkin lymphoma (NHL) [1]. Considerable clinical heterogeneity and wide variation in survival among patients are found: In some patients, the disease is indolent and slowly progressive over a period of many years, whereas in others, it progresses rapidly, often to aggressive lymphoma, and patients die of their disease. The median survival is 8–10 years [2], but recent reports suggest that prognosis has been improved [3, 4]. A number of clinical prognostic variables have been described. The Follicular Lymphoma International Prognostic Index (FLIPI) [5], which measures five variables including age, Ann Arbor stage, hemoglobin level, number of nodal sites, and lactate dehydrogenase (LDH) level, identifies three prognostic groups with distinct survival probabilities. The FLIPI score is also helpful to compare different patient series, but its relevance for evaluating new treatments at diagnosis remains controversial [6].

Recent studies have shown that the cellular microenvironment influences the prognosis of patients with FL [7]. Whole-genome microarray analysis of diagnostic biopsies

This work was presented in part at the 49th American Society of Hematology (ASH) Annual Meeting, Atlanta, 8–11 December 2007 (Poster Session).

S. I. Labidi (✉) · C. Sebban · P. Biron · J.-Y. Blay · H. Ghesquières
Department of Medical Oncology, Centre Léon Bérard,
28 Rue Laennec,
69008 Lyon, France
e-mail: labidi@lyon.fnclcc.fr

C. Ménétrier-Caux · J.-Y. Blay
Inserm U590, Equipe Cytokines et Cancers, Centre Leon Berard,
Lyon, France

S. Chabaud · T. Gargi
Department of Biostatistics, Centre Leon Berard,
Lyon, France

C. Chassagne
Department of Pathology, Centre Leon Berard,
Lyon, France

has revealed that FL patients with poor and good outcomes are distinguishable by immune microenvironment-associated gene signatures [8]. The gene signature associated with favorable outcome is rich in expression of T-cell genes, whereas the signature associated with poor outcome is characteristic of macrophages. These results have been confirmed by immunohistochemical analyses of diagnostic FL biopsies [9, 10], which have demonstrated a prognostic correlation with the number of forkhead box protein P3 (FOXP3) T-regulatory (Treg) cells present within the tumor. In contrast, high numbers of CD68⁺ macrophages are an independent prognostic factor of worse outcome [11], but this prognostic value is lost in patients treated with rituximab and chemotherapy [12].

Cytokines are produced by both the tumor cells and the surrounding immune cells and play a crucial role in the regulation of key pathways of immunity. They are implicated in the pathogenesis of many lymphomas. Increased concentrations of various cytokines and angiogenic factors have been reported as biomarkers for use in early diagnosis and prognosis in Hodgkin [13] and non-NHLs [14–16]. Most published studies have investigated heterogeneous series of NHL with relatively few patients within each subtype [14–17]. Our literature search revealed few specific studies of cytokines in FLs [18].

Here, we tested the hypothesis that serum levels of ten cytokines largely involved in lymphoid development, pro-inflammatory or anti-inflammatory pathways, angiogenesis, and Th1/Th2 balance are associated with FL outcome.

Materials and methods

Study population

This study was done using the extensive bank of blood samples prospectively collected at the Centre Leon Berard in the 1990s. Serum cytokine levels [interleukin (IL)-1Ra, IL-6, IL-7, IL-10, IL-13, tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor, and transforming growth factor beta (TGF- β)] were measured in 60 consecutive FL patients for whom samples were available and consent had been obtained according to the legislation in place between 1991 and 2002. Samples taken at diagnosis ($n=31$), at first relapse ($n=18$), and in complete remission (CR) ($n=11$) were available. Samples from 20 healthy volunteers obtained from the National Blood Service (*Etablissement Français du Sang*, Lyon, France) after informed consent served as controls. Clinical staging was performed according to the Ann Arbor classification system [19]. Staging included evaluation of patient clinical status,

chest radiography, computerized tomography (CT) of the chest and abdomen, and bone marrow biopsy.

Measurements of cytokine levels

We measured ten cytokines known to be involved in Th1/Th2 balance (IL-6, IL-10, and IL-13), angiogenesis [VEGF, PDGF, and basic fibroblast growth factor (b-FGF)], anti-inflammatory pathways (TGF- β and IL-1Ra), and lymphoma pathogenesis (TNF- α and IL-7). Peripheral blood samples were collected using sterile tubes. Sera were isolated by centrifugation at 2,000 rpm and stored at -80°C until assayed. The Bioplex technology (BioRad, Marne la Coquette, France) combines the principle of a sandwich immunoassay with fluorescent bead-based technology, allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well [20]. The Bioplex assay for nine cytokines (IL-1Ra, IL-6, IL-7, IL-10, IL-13, TNF- α , VEGF, PDGF, and b-FGF) was carried out in 96-well microplates according to the manufacturer's instructions. Samples were analyzed in duplicate. Analysis of experimental data was done using five-parametric curve fitting. Inter-assay variability for individual cytokines was in the range of 1.0–9.8% and intra-assay variability in the range of 3.6–12.6% (information provided by BioRad). TGF- β human sandwich enzyme-linked immunosorbent assay was performed on HCl/NaOH-treated serum samples as recommended by the manufacturer (R&D Systems, Lille, France).

Statistical analysis

All assessable patients were entered into the analysis and evaluated using SAS 8.2 software (SAS institute, Cary). Quantitative variables were described by the median and number of missed values. Comparisons between different cytokine levels were performed using the Wilcoxon non-parametric test or t test. Correlations between cytokine levels and clinicopathological characteristics were estimated using the Pearson test.

Survival estimates were calculated using the Kaplan–Meier method [21]. Differences in survival were assessed by the log-rank test. The p values were given as an exploratory indication; a level of 0.05 was considered significant. In multivariate analysis, we used Cox proportional hazards modeling and the likelihood ratio test to evaluate survival differences between groups. Cytokines levels were introduced in the models as continuous parameters, and results were expressed as the relative risk ratio for one unit change. Candidate variables for the selection procedure were those statistically significant at 0.1 level in the univariate analysis. Although not statistically significant in the univariate analysis, clinical status was also

included in the model. The adequacy of the Cox model was tested, and the proportional hazards assumption was found to be reasonable [22].

Results

Clinical characteristics of the patients

Patient characteristics, diagnosis, and staging are reported in Table 1. The study included 26 women and 34 men. Median age at diagnosis was 53.4 years (44–87). Ten patients had stage I disease, eight had stage II, ten had stage III, and 31 had stage IV. Different treatment modalities were used. Patients with localized disease received involved-field radiotherapy alone or were followed without lymphoma-specific treatment. One patient received single-agent rituximab. Patients with disseminated disease were treated with either cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP)-like regimens or chlorombucil and/or prednisone. The median follow-up for surviving patients was 9 years (95% CI, 7.3–12.4). Overall survival (OS) rates at 5 and 10 years were 80.9% (95% CI, 70.8–91.1) and 57.5% (95% CI, 47.2–72.4), respectively (Fig. 1).

Serum cytokines concentration in FL patients and healthy controls

The serum concentrations of cytokines were compared between the samples from patients at diagnosis ($n=31$), first relapse ($n=18$), or complete remission ($n=11$) and from the 20 healthy controls. Median levels of cytokines (IL-1Ra, IL-6, IL-7, IL-10, IL-13, TNF- α , VEGF, PDGF, b-FGF, and TGF- β) are summarized in Table 2.

Overall, serum levels of IL-1Ra, IL-6, IL-7, IL-10, IL-13, TNF- α , VEGF, and PDGF were higher in FL patients than in controls. In contrast, TGF- β levels were significantly lower in FL patients compared to healthy controls ($p=0.00326$; Table 2). Only few patients had detectable serum concentrations of b-FGF (12 of 60).

IL-6 levels correlated positively with IL-7 ($R=0.45$, $p<0.001$), IL-10 ($R=0.58$, $p<0.001$), IL-13 ($R=0.57$, $p<0.001$), and IL-1Ra ($R=0.91$, $p<0.001$). Similarly, IL-10 correlated with IL-6 ($R=0.58$, $p<0.001$), IL-13 ($R=0.82$, $p<0.001$), and IL-1Ra ($R=0.5$, $p<0.001$). VEGF correlated with PDGF ($R=0.31$; $p=0.0027$). TGF- β correlated with IL-7 ($R=0.4$, $p<0.001$) and PDGF ($R=0.43$, $p<0.001$).

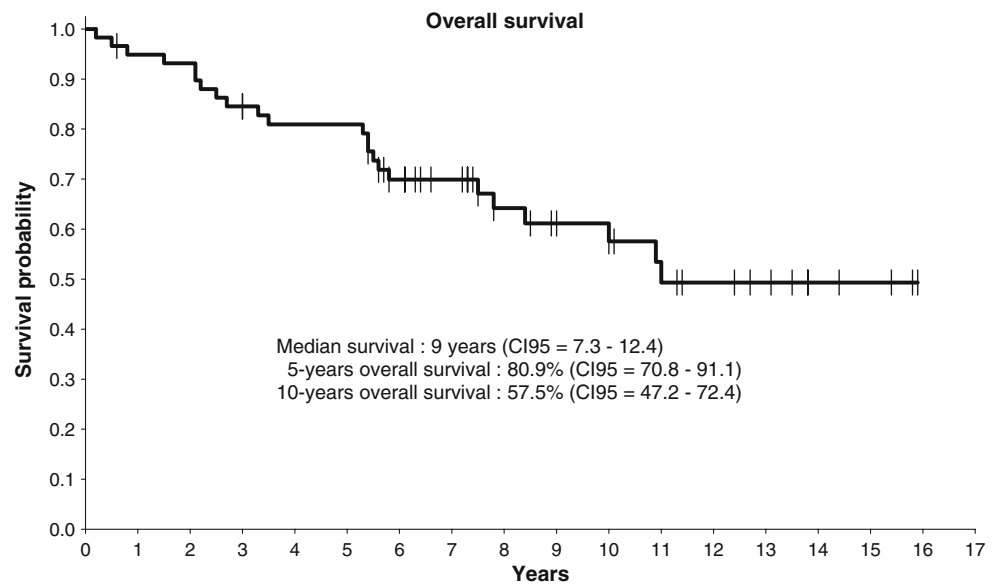
Correlations between cytokine levels and clinicopathologic characteristics of FL patients

We analyzed the correlations between the serum levels of each cytokine and the clinicopathologic characteristics of

Table 1 Clinico-pathologic characteristics of the 60 patients with follicular lymphoma

Variables	Number of patients (%)
Age	
<60 years	44 (73.3%)
≥ 60 years	16 (26.7%)
Gender	
Male	34 (57%)
Female	26 (43%)
Stage	
I	10 (17%)
II	8 (14%)
III	10 (15%)
IV	31 (54%)
Unknown	1
B symptoms	
Yes	8 (15%)
No	45 (85%)
Unknown	7
Performance status	
0–1	52 (96%)
≥ 2	2 (4%)
Unknown	6
Bulky tumor	
Yes	29 (53%)
No	26 (47%)
Unknown	5
LDH	
Normal	32 (64%)
> normal	18 (36%)
Unknown	10
FLIPI score	
0–1	14 (30%)
2–3	31 (68%)
4–5	1 (2%)
Unknown	14
WHO grade	
1	11 (30%)
2	24 (67%)
3	1 (2%)
Unknown	26
t (14;18)	
Yes	18 (90%)
No	2 (10%)
Unknown	40

the 31 FL patients tested at diagnosis. IL-6 levels correlated only with LDH ($p=0.003$). High IL-10 levels correlated with advanced stage ($p=0.05$), bone marrow involvement ($p=0.05$), and high FLIPI score (trend, $p=0.07$). IL-13 levels correlated with β -2 microglobulin ($p=0.05$). A high

Fig. 1 Overall survival of the 60 patients with follicular lymphoma

platelet count was strongly associated with high VEGF ($p=0.0057$), PDGF ($p=0.0147$), and TGF- β ($p=0.0112$). No correlation was observed between leukocyte count and cytokines levels.

Univariate and multivariate analyses of prognostic factors

We analyzed prognostic factors for OS and progression-free survival (PFS) in the entire cohort of FL patients. The issue most likely to confound the relationship between cytokine levels and survival was patient clinical status (diagnosis, relapse, or complete remission) at time of cytokine measurement. We attempted to account for this confounding factor by including clinical status in the univariate analysis and found differences in favor of CR patients. These differences were not significant for either OS ($p=0.14$) or PFS ($p=0.29$).

Factors associated with shorter PFS in the univariate analysis were intermediate or high FLIPI score ($p=0.0034$), high LDH levels ($p=0.0083$), advanced stage ($p=0.0206$), and high VEGF levels ($p=0.0625$; Table 3). Patients with advanced stage (III–IV; $p=0.0324$), intermediate or high FLIPI score (2–5; $p=0.0786$), high serum levels of IL-10 f ($p=0.0267$), and β -2 microglobulin ($p=0.0707$) had shorter OS. In contrast, high serum levels of TGF- β were associated with long survival ($p=0.0149$; Table 3).

In multivariate analysis, the prognostic value of VEGF level (HR=1.003; $p=0.0393$) for PFS was independent of LDH level (HR=2.675; $p=0.005$) and clinical status (Table 4; Fig. 2). Independent prognostic factors associated with improved OS were early stage (I–II) (HR=4.967; $p=0.00178$) and high serum levels of TGF- β (HR=0.932; $p=0.0328$; Table 4, Fig. 3): A 1 ng/ml increase in TGF- β levels was coupled with a 7% decrease of the risk of death.

Table 2 Cytokine levels in the sera of 60 FL patients with different clinical status (diagnosis, complete remission or first relapse) and 20 healthy controls

	Diagnosis	Complete remission	Relapse	Controls	<i>p</i> value
IL-1Ra (pg/ml)	136.19 (23.97–12,197.46)	65.28 (0–216.85)	114.24 (0–900.02)	78.70 (0–363.21)	0.007
IL-6 (pg/ml)	13.32 (0–1,000.98)	10.96 (2.53–45.21)	15.53 (0–421.69)	8.91 (2.98–70.38)	0.064
IL-7 (pg/ml)	16.75 (0–106.4)	14.88 (0–39.14)	10.99 (0–32.85)	12.38 (6.1–70.09)	0.022
IL-10 (pg/ml)	5.46 (1.62–1,202.92)	4.67 (1.71–7.42)	8.13 (1.77–899.64)	2.28 (0–22.17)	<0.001
IL-13 (pg/ml)	3.91 (0–124)	2.19 (0–6.79)	0.66 (0–31.08)	0.94 (0.46–4.23)	0.033
TNF- α (pg/ml)	10.88 (0–11,916.27)	0.00 (0–81.81)	0.00 (0–97.62)	0.00 (0–35.63)	0.036
PDGF (ng/ml)	89.76 (3,090.26–23,683.91)	10.14 (1,439.45–19,522.18)	4.53 (795.29–20,185.02)	5.74 (738.24–13,512.12)	0.001
VEGF (pg/ml)	174.14 (44.9–666.87)	138.48 (33.87–302.88)	129.71 (30.48–439.58)	105.43 (20.41–438.68)	0.046
TGF- β (ng/ml)	20.05 (1.98–67.5)	19.83 (7.44–53.75)	12.70 (3.92–27.8)	25.85 (11.7–32.95)	0.003
b-FGF (pg/ml)	0.00 (0–156.79)	0.00 (0–0)	0.00 (0–740.02)	0.00 (0–14.35)	

Results are expressed as median and (range)

Table 3 Univariate analysis of progression-free survival (PFS) and overall survival (OS) in patients with follicular lymphoma according to clinical characteristics and serum cytokine levels

Variables	Code	Univariate analysis of PFS				Univariate analysis of OS			
		PFS at 6 years (%)	HR	95% CI	<i>p</i> value	OS at 6 years (%)	HR	95% CI	<i>p</i> value
Age	<60 years (R)	27			0.6398	72			0.4540
	≥60 years	20	1.165	0.614–2.212		63	1.405	0.577–3.419	
β-2 microglobulin	Normal (R)	33			0.8292	79			0.0707
	> Normal	24	1.096	0.478–2.514		56	2.849	0.915–8.868	
Bulky disease	Yes(R)	25			0.1655	71			0.4630
	No	30	0.648	0.350–1.197		67	0.719	0.298–1.736	
FLIPI score	0–1 (R)	57			0.0034	93			0.0786
	≥2	23	3.799	1.557–9.274		64	3.763	0.859–16.475	
Hemoglobin	<12 g/dl		1.101	0.806–1.503	0.5455		0.984	0.633–1.530	0.9443
	LDH								
LDH	Normal (R)	41			0.0083	71			0.6237
	>Normal	11	2.418	1.255–4.658		71	0.784	0.297–2.071	
Gender	M (R)	26			0.9899	65			0.3224
	F	24	1.004	0.562–1.792		76	0.651	0.279–1.523	
Stage of disease	I/II (R)	38			0.0206	89			0.0324
	III/IV	20	2.239	1.132–4.429		60	3.766	1.118–12.690	
Clinical status	Diagnosis (R)	33			0.2917	76			0.1429
	Complete remission	20	1.353	0.597–3.068		79	0.445	0.095–2.090	
	Relapse	13	1.683	0.874–3.238		56	1.771	0.722–4.343	
IL1-Ra			1.000	1.000–1.000	0.1413		1.000	0.999–1.001	0.8526
IL-6			1.001	0.999–1.004	0.2554		1.000	0.996–1.004	0.8228
IL-7			0.982	0.950–1.014	0.2636		0.997	0.956–1.040	0.8971
IL-10			1.003	0.998–1.007	0.2329		1.005	1.001–1.010	0.0267
IL-13			0.954	0.886–1.028	0.2138		1.032	0.944–1.127	0.4919
PDGF			1.000	1.000–1.000	0.9305		1.000	1.000–1.000	0.9505
TGF-β			0.993	0.964–1.022	0.6240		0.929	0.875–0.986	0.0149
VEGF			1.002	1.000–1.004	0.0625		1.002	0.999–1.005	0.2863
TNF-α			1.000	0.997–1.002	0.7118		0.998	0.988–1.007	0.6276

Normal range for LDH is 105–313 IU/l; normal level for serum β-2 microglobulin ≤3 mg/ml. *p*=0.05 for log-rank test

HR hazard ratio, R reference variable, 95% CI confidence interval, M male, F female

Discussion

Gene expression profiling and immunohistochemistry studies suggest that the tumor microenvironment influences the biological behavior and clinical outcome of FL [8–11]. Cytokines are key regulators of immune functions. In the tumor microenvironment, they influence the survival and proliferation of both tumor and stromal cells. A recent study of single nucleotide polymorphisms (SNPs) in various cytokines has highlighted their importance in FL survival [23].

The present work focused on the cytokines possibly implicated in this tumor-microenvironment cross-talk. Many of these cytokines have been measured in the sera of lymphoma patients [14–17], but most of the mentioned studies involved heterogeneous groups of NHL and rarely

evaluated the prognostic value of cytokines in each different subtype [18]. We specifically screened patients with FL using a novel multiplex technology [20].

The median follow-up for our FL patients was 9 years, which is consistent with the median OS in this disease. Survival rates in our study were in accordance with those reported in the literature [5].

Overall, serum levels of IL-1Ra, IL-6, IL-7, IL-10, IL-13, TNF-α, VEGF, and PDGF were higher in FL patients than in healthy controls. We found a correlation between the different Th2 cytokines (IL-6, IL-10, and IL-13), and between two angiogenic cytokines (VEGF and PDGF). Few patients (*n*=12) had detectable b-FGF, and this cytokine did not influence outcome; neither did PDGF.

Univariate analysis of prognostic factors indicated that disease stage, FLIPI score, β-2 microglobulin, LDH and

Table 4 Multivariate analysis of progression-free survival (PFS) and overall survival (OS) in patients with FL

Variables	Code	Multivariate analysis of PFS				Multivariate analysis of OS			
		PFS at 6 years (%)	HR	95% CI	<i>p</i> value	OS at 6 years (%)	HR	95% CI	<i>p</i> value
FLIPI score	0–1 (R)	57				93			NS (1)
	≥2	23				64			
Stage of disease	I/II (R)	38			NS(1)	89			
	III/IV	20				60	4.967	1.140–21.632	0.0178
TGF-β							0.932	0.879–0.988	0.0328
IL-10									NS (2)
Clinical status	Diagnosis (R)	33			0.0184	86			NS(3)
	Complete remission	20	1.583	0.600–4.177		90			
	Relapse	13	3.087	1.413–6.746		81			
VEGF			1.003	1.000–1.005	0.0393				
LDH	Normal (R)	41			0.0050	71			
	>Normal	11	2.675	1.345–5.319					

NS not significant, HR hazard ratio, R reference item, 95% CI confidence interval

also IL-10, VEGF, and TGF-β serum levels influenced patient outcome. Confirmation of the prognostic value of disease stage and LDH levels by multivariate analysis supports the validity of our series. Besides, two additional prognostic factors for FL were identified: VEGF and TGF-β. We did not include treatment in our analysis because we consider that this variable was highly dependent on patient clinical characteristics: Patients with localized disease received radiotherapy or were followed up without treatment, whereas patients with disseminated disease received chemotherapy, and our multivariate model already included clinical status at time of cytokine measurement.

IL-10 is a crucial mediator of inflammation, apoptosis, Th1/Th2 balance, and functions as autocrine growth factor in lymphoid tumors [24, 25]. Our finding of an adverse

prognostic impact of increased IL-10 serum levels is in accordance with previous reports in high and intermediate NHL [14, 26]. Correlation between tumor mass indicators (advanced stage, medullar involvement, and high FLIPI score) and IL-10 serum levels suggests that tumor B cells might be the source of this anti-inflammatory cytokine [14, 27].

TGF-β is a pleiotropic cytokine that affects the proliferation, survival, and differentiation of normal and malignant cells, including lymphocytes [28, 29]. We were intrigued by the finding that TGF-β serum levels were lower in FL patients than in healthy controls. Indeed, high serum levels of TGF-β were predictive of improved survival in FL patients. These preliminary findings suggest an implication of TGF-β in the physiopathology of FL. Interestingly, several recent studies have explored the involvement of the

Fig. 2 Progression-free survival of patients with follicular lymphoma according to serum VEGF levels. Median cytokines levels were used as cut-off values

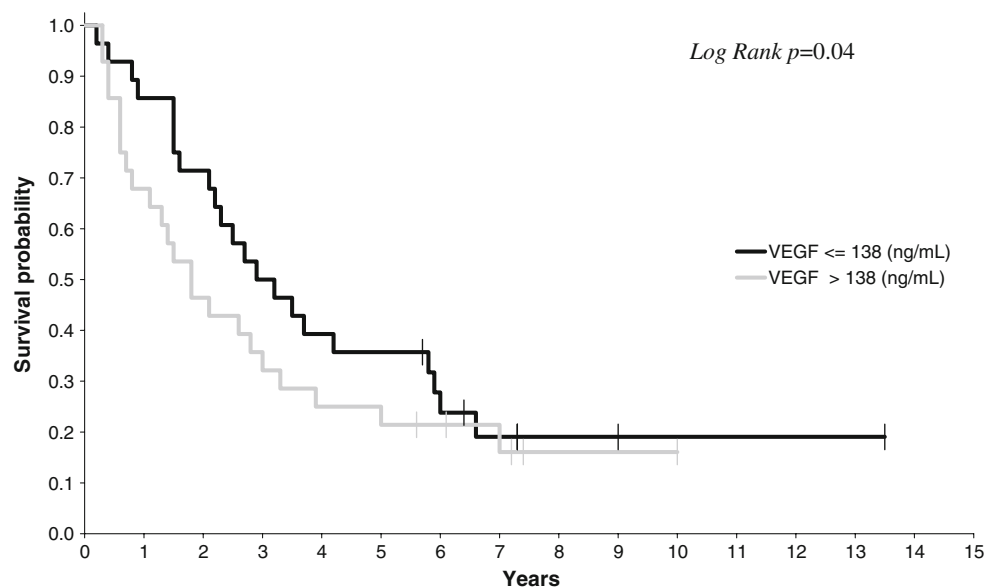
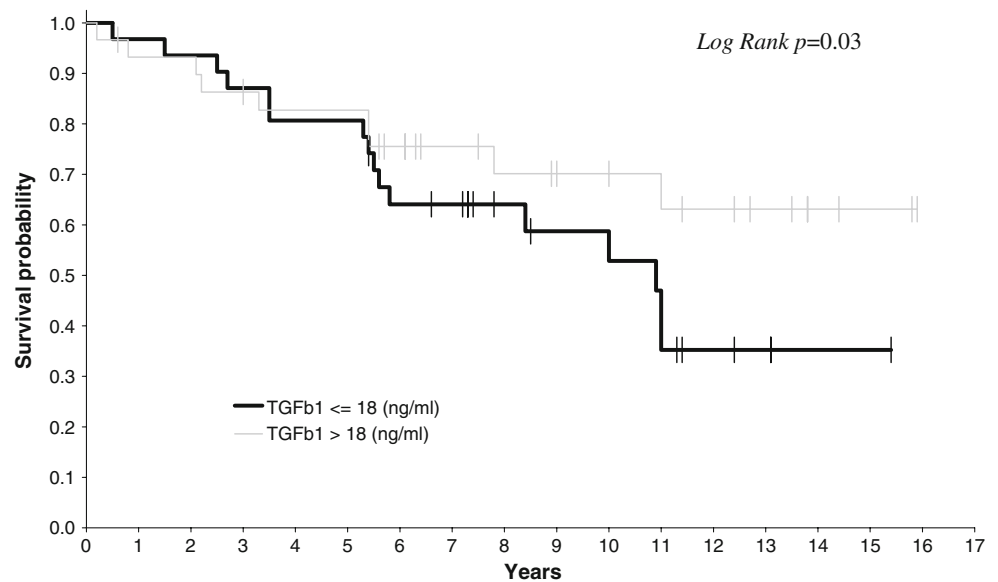


Fig. 3 Overall survival of patients with follicular lymphoma according to serum TGF- β levels. Median cytokine levels were used as cut-off values



TGF- β pathway in FL. Tvdirk et al. have studied the effects of TGF- β in vitro, specifically in the FL cell line DoHH2, and have shown that it blocks cell-cycle progression [30, 31]. Husson et al. [32], using gene expression profiling, have identified Smad1 as the most differentially overexpressed gene in FL as compared with normal germinal center B cells. They have identified a new pathway involving TGF- β and Smad1, which controls cell proliferation and is present in vivo in FL cells. More recently, Huse K et al. [33] have reported that TGF- β inhibits specifically the proliferation of FL cell lines but not of other B-cell lymphoma cell lines including diffuse large B cell lymphoma. Taken together, these in vivo and in vitro results demonstrate the negative impact of TGF- β on FL cell proliferation, which could explain, at least in part, the benefit of high serum levels of TGF- β in FL patients. Identifying the cellular source of this cytokine should provide further insight into the pathogenesis of the disease. The observation that serum TGF- β level did not correlate to tumor burden (LDH, β -2 microglobulin, and clinical stage) could suggest a non-tumoral origin. The role of immune cells, including T lymphocytes and macrophages, as a TGF- β source remains to be explored.

VEGF is one of the most potent angiogenic stimulants with several biologic effects, including the maintenance and the survival of newly formed blood vessels [34]. Its expression can be evaluated with different methods. We found that high serum concentrations of VEGF were associated with poorer PFS. This observation has also been reported in heterogeneous NHL series including different subtypes [15, 16]. One study has evaluated VEGF expression by immunochemical staining specifically in FL and has shown that patients whose biopsies display a diffuse VEGF staining have poorer OS than those with focal staining [35]. The same study has shown that the high

microvessel density found in interfollicular areas of FL is predictive of progressive disease, poorer OS, and transformation to DLBCL [35]. These observations and ours suggest a potential role of angiogenesis in the physiopathology of FL. Several antiangiogenesis agents, including bevacizumab [36] and sorafenib, are currently under investigation in lymphoma clinical trials [37]. Testing these agents specifically in FL patients would provide insight into the pathogenesis of this disease.

In conclusion, our results on serum cytokine levels in patients with FL identified two cytokines of probable importance in the outcome of this disease. However, our study has several limitations. It was based on a retrospective analysis of a limited and heterogeneous series of FL patients receiving different treatments before the era of monoclonal antibodies. Rituximab, which is currently a standard treatment of FL, alters the cytokine milieu. Since only one patient of our series received rituximab, our results should be interpreted with some caution, and prospective validation of our findings is necessary.

Another limitation of our study is that cytokine measurements were performed in patients with different clinical status (diagnosis, relapse, or complete remission). Clearly, clinical status had an impact on cytokine serum levels, with better PFS for patients in CR. However, the fact that serum VEGF levels remained independently predictive of prognosis in multivariate analysis of PFS confirmed the importance of this cytokine in the physiopathology of FL.

Our data suggest that VEGF may play a role in FL progression. In contrast, we found that high serum levels of TGF- β were associated with improved survival. To our knowledge, these results have not been previously reported in the literature. Elucidating TGF- β and VEGF pathways in FL would be helpful to identify novel targets for tumor immunotherapy.

Acknowledgments The authors thank Damien Jeantet, Ecole Normale Supérieure, Lyon, for technical assistance and Marie Dominique Reynaud, Centre Leon Berard, for editorial assistance.

Declaration of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Financial support This study was supported in part by research funding from Roche France laboratories.

Authorship Ghesquière, Blay, and Labidi made the design of the study. Ghesquière obtained funding. Labidi, Gargi, Sebban, and Biron obtained biological and clinical data. Chassagne carried out pathology revision. Labidi and Caux performed serum cytokine dosage. Chabaud made the statistical analysis. Labidi and Ghesquière drafted the manuscript. All authors revised the manuscript and have reviewed and approved the final manuscript.

References

- Armitage JO, Weisenburger DD (1998) New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol* 16:2780–2795
- Horning SJ (1993) Natural history of and therapy for the indolent non-Hodgkin's lymphomas. *Semin Oncol* 20:75–88
- Liu Q, Fayad L, Cabanillas F, Hagemeister FB, Ayers GD, Hess M et al (2006) Improvement of overall and failure-free survival in stage IV follicular lymphoma: 25 years of treatment experience at The University of Texas M.D. Anderson Cancer Center. *J Clin Oncol* 24:1582–1589
- Coiffier B, Brice P, Delarue R, Haioun C, Souleau B, Mounier N et al (2007) Effect of rituximab and/or high-dose therapy with autotransplant at time of relapse in patients with follicular lymphoma. *Blood* (ASH annual meeting abstracts) 110, Abstract 386
- Solal-Celigny P, Roy P, Colombat P, White J, Armitage JO, Arranz-Saez R et al (2004) Follicular lymphoma international prognostic index. *Blood* 104:1258–1265
- Salles GA (2007) Clinical features, prognosis and treatment of follicular lymphoma. *Hematology* 2007:216–225
- De Jong D (2005) Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. *J Clin Oncol* 23:6358–6363
- Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC et al (2004) Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N Engl J Med* 351:2159–2169
- Carreras J, Lopez-Guillermo A, Fox BC, Colomo L, Martinez A, Roncador G et al (2006) High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* 108:2957–2964
- Lee AM, Clear AJ, Calaminici M, Davies AJ, Jordan S, MacDougall F et al (2006) Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. *J Clin Oncol* 24:5052–5059
- Farinha P, Masoudi H, Skinnider BF, Shumansky K, Spinelli JJ, Gill K et al (2005) Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). *Blood* 106:2169–2174
- Canioni D, Salles G, Mounier N, Brousse N, Keuppens M, Morschhauser F et al (2008) High numbers of tumor-associated macrophages have an adverse prognostic value that can be circumvented by rituximab in patients with follicular lymphoma enrolled onto the GELA-GOELAMS FL-2000 trial. *J Clin Oncol* 26:440–446
- Casanovas RO, Mounier N, Brice P, Divine M, Morschhauser F, Gabarre J et al (2007) Plasma cytokine and soluble receptor signature predicts outcome of patients with classical Hodgkin's lymphoma: a study from the Groupe d'Etude des Lymphomes de l'Adulte. *J Clin Oncol* 25:1732–1740
- Blay JY, Burdin N, Rousset F, Lenoir G, Biron P, Philip T et al (1993) Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. *Blood* 82:2169–2174
- Pedersen LM, Klausen TW, Davidsen UH, Johnsen HE (2005) Early changes in serum IL-6 and VEGF levels predict clinical outcome following first-line therapy in aggressive non-Hodgkin's lymphoma. *Ann Hematol* 84:510–516
- Salven P, Orpana A, Teerenhovi L, Joensuu H (2000) Simultaneous elevation in the serum concentrations of the angiogenic growth factors VEGF and bFGF is an independent predictor of poor prognosis in non-Hodgkin lymphoma: a single-institution study of 200 patients. *Blood* 96:3712–3718
- Fayad L, Cabanillas F, Talpaz M, McLaughlin P, Kuzrock R (1998) High serum interleukin-6 levels correlate with a shorter failure-free survival in indolent lymphoma. *Leuk Lymphoma* 30:563–571
- Ansell SM, Novak AJ, Ziesmer S, LaPlant B, Cerhan JR, Yang ZZ et al (1997) Serum cytokine levels predict time to progression after rituximab as initial therapy in patients with follicular grade 1 non-Hodgkin lymphoma. *Blood* (ASH Annual Meeting Abstracts) 110, abstract 3410
- Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M (1971) Report of the committee on Hodgkin's disease staging classification. *Cancer Res* 31:1860–1861
- Vignali DA (2000) Multiplexed particle-based flow cytometric assays. *J Immunol Methods* 243:243–255
- Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457–481
- Therneau T, Grambsch P (2000) Modeling survival data: extending the Cox model. Springer, New York
- Cerhan JR, Wang S, Maurer MJ, Ansell SM, Geyer SM, Cozen W et al (2007) Prognostic significance of host immune gene polymorphisms in follicular lymphoma survival. *Blood* 109:5439–5446
- Khatri VP, Caligiuri MA (1998) A review of the association between interleukin-10 and human B-cell malignancies. *Cancer Immunol Immunother* 46:239–244
- Levy Y, Brouet JC (1994) Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* 93:424–428
- Lech-Maranda E, Baseggio L, Bienvenu J, Charlot C, Berger F, Rigal D et al (2004) Interleukin-10 gene promoter polymorphisms influence the clinical outcome of diffuse large B-cell lymphoma. *Blood* 103:3529–3534
- Masood R, Zhang Y, Bond MW, Scadden DT, Moudgil T, Law RE et al (1995) Interleukin-10 is an autocrine growth factor for acquired immunodeficiency syndrome-related B-cell lymphoma. *Blood* 85:3423–3430
- Lebman DA, Edmiston JS (1999) The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. *Microbes Infect* 1:1297–1304
- Golstein P, Wyllie AH (2001) T cell death and transforming growth factor beta1. *J Exp Med* 194:F19–F22
- Tvrđik D, Dundr P, Povysil G, Pytlík R, Planková M (2006) Up-regulation of p21WAF1 expression is mediated by Sp1/Sp3

- transcription factors in TGFbeta1-arrested malignant B cells. *Med Sci Monit* 12:BR227–BR234
31. Tvrdik D (2004) The effect of TGFbeta1 on the expression and phosphorylation of key cell-cycle regulators in malignant B cells. *Med Sci Monit* 10:BR447–BR454
 32. Husson H, Carideo EG, Neuberg D, Schultze J, Munoz A, Marks PW et al (2002) Gene expression profiling of follicular lymphoma and normal germinal center B cells using cDNA arrays. *Blood* 99:282–289
 33. Huse K, Bakkebo M, Oksvold M, Smeland EB, Myklebust JH (2007) B cell lymphomas escape bone morphogenetic protein (BMP) induced growth inhibition: downregulation of BMP receptors and upregulation of inhibitory Smads. *Blood* (American Society of Hematology) 110, Abstract 2605
 34. Ganjoo K (2007) Antiangiogenesis: a new approach to the treatment of lymphoma. *Leuk Lymphoma* 48:454–455
 35. Jorgensen JM, Sorensen FB, Bendix K, Nielsen JL, Olsen ML, Funder AM et al (2007) Angiogenesis in Non-Hodgkin Lymphoma: clinico-pathological correlations and prognostic significance in specific subtypes. *Leuk Lymphoma* 48:584–595
 36. Stopeck AT, Bellamy W, Unger J, Rimsza L, Lannone M, Fisher RI et al (2005) Phase II trial of single agent bevacizumab (Avastin) in patients with relapsed, aggressive non-Hodgkin's lymphoma (NHL): Southwest Oncology Group Study S0108. *J Clin Oncol* (ASCO Annual Meeting), Abstract 6592
 37. Sorafenib in treating patients with recurrent diffuse large B-cell non-Hodgkin's lymphoma (2008) <http://clinicaltrials.gov/ct2/show/record/NCT00131937>