Vascularization predicts overall survival and risk of transformation in follicular lymphoma

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ABSTRACT

Follicular lymphoma patients display heterogeneous overall survival and variable risk of transformation. Recent studies have highlighted the role of the microenvironment. The contribution of microvessel density to follicular lymphoma survival remains controversial. We used a quantitative tumor mapping approach to determine whether the degree of vascularization correlated with outcome in a uniformly treated cohort. Whole-tissue sections of diagnostic biopsies from 84 cases were stained for CD34 and tumor-to-vessel-distance that encompassed 90% of the tumor (TVD_{90}) was determined using image analysis. Twenty-one cases with lower TVD_{90} showed inferior overall survival (P=0.0001) and high risk of transformation (P=0.01). These cases significantly correlated with increased Lymphoma-Associated Macrophages (χ^2=0.025). In multivariate analysis macrophages content, IPI and TVD_{90} were independent predictors of overall survival (P=0.05, P=0.001 and P=0.01, respectively) and IPI and TVD_{90} predicted risk of transformation (P=0.008 and P=0.08, respectively). Increased angiogenesis is an independent marker of inferior survival and may promote transformation.

Key words: follicular lymphoma, vascularization, prognostication.

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Introduction

The clinical course of follicular lymphoma (FL) is unpredictable, with median survivals in the range of ten years. Histological transformation, usually into diffuse large B-cell lymphoma (DLBCL), heralds aggressive clinical behavior and is typically associated with inferior survival. A recent population based analysis from the British Columbia Cancer Agency (BCCA) including 600 newly diagnosed FL patients showed an annual risk of transformation (RT) of 3% continuing beyond 15 years of follow up.[1] The treatment of FL patients is markedly heterogeneous, as no optimal treatment approach has yet been established. The number of prior treatments, tumor burden, advanced-stage and high-risk FLIPI or IPI are the only established clinical features associated with RT.[2] Importantly, none of the clinical variables consistently predict RT. Thus, biological predictors of transformation are needed to individualize therapy and better assess risk. Angiogenesis plays a crucial role in oncogenesis, promoting growth and progression of both solid and hematologic tumors.[3,4] The acquisition of an angiogenic phenotype, referred to as the "angiogenic switch", allows the formation of neovessels that are vital for tumor growth.[5] This "angiogenic switch" results not only from interactions between vessels and cancer cells, but also involves non-neoplastic cells in the microenvironment, including macrophages. Microvessel density (MVD) has, in some tumor types, shown a correlation with survival. Yet, in FL the impact of MVD on prognosis is controversial.[6,7] In most series the number of cases studied is small and/or the therapies markedly heterogeneous, both of which preclude definitive conclusions. In this study, we report the clinical significance of MVD in uniformly treated FL patients using a quantitative tumor mapping approach.

Design and Methods

Patients

Between July 1987 and May 1993, patients with FL were enrolled in a single institution phase II trial using BP-VACOP chemotherapy (bleomycin, cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine and prednisone) followed by involved field irradiation to sites of original nodal involvement. Patients aged 16 to 61 years, with newly diagnosed, treatment-naïve and advanced-stage disease, defined as Ann Arbor stage III or IV, or stage II with B symptoms, non-

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radioencompassable disease, or bulk of 10 cm or more in maximum diameter at any individual tumor site were included. Approval to this study was given by the University of British Columbia BCCA Research Ethics Board. All biopsies were reviewed and classified according to the 2008 WHO classification.[8] Transformation was defined as biopsy proven or clinically diagnosed aggressive lymphoma as described previously.[1]

Histology and immunohistochemistry

Of the 126 cases of FL, 84 had formalin-fixed paraffin blocks with adequate material remaining in the block to be used for whole sections. Immunohistochemistry was performed routinely for CD20 (L26, dilution 1:800, Dako®, Carpentaria, California, USA) and CD34 (QBEend10, dilution 1:30, Dako®, Carpentaria, California, USA) using a Dako® autostainer and the EnVision polymer detection system. $CD68^+$ cell content biomarker, Lymphoma-Associated Macrophages (LAM) data were based on a previous study.[9]

Microvessel density and follicle size

Images of whole sections were stained for CD34 and captured using a cooled CCD camera, a motorized stage and customized NIH software.[10] Thresholding was applied to identify CD34 positive objects. The motorized stage allowed for tiling of adjacent microscopic fields, thereby allowing reassembly of the entire tumour section at high resolution (Figure 1). High-resolution images of tumor vasculature over the entire whole-sections of each case defined "distance maps". The distance from each tumor cell in the tissue to the nearest $CD34^+$ pixel was measured automatically and the mean of distances of all cells to the nearest vessel in the whole section was used to calculate the tumour-to-vessel distance that encompassed 90% of the tumor (TVD_{90}). Average follicle size per case was visually ranked according to the following groups: 0 - loss of follicular pattern; 1 - small follicles (dense staining), and 2 - large follicles (sparse staining).

Statistics and survival analysis

Overall survival (OS) was defined as the interval from date of diagnosis until death from any cause. Survival estimates were calculated using the Kaplan-Meier method[11] and multivariate analysis using the proportional-hazards regression model.[12]

Results and Discussion

There were 84 suitable cases for analysis. The median follow up of the living patients was 14.3 years and 20 patients underwent transformation. The IPI was significant for both OS and risk of transformation (RT) in univariate analysis (P=0.0001 and P=0.01, respectively). Estimated 10-year OS and RT for all patients were 65% and 20%, respectively. TVD_{90} ranged from 50.3 to 144 microns (median 78.4) and these data were divided into quartiles. In univariate analysis the quartile (21 cases) with low TVD_{90} (i.e. high MVD) showed inferior OS (P=0.0001) and an increased RT (P=0.01) (Figure 2). These 21 cases are defined as Low TVD_{90} while the three other quartiles (63 cases) are defined as High TVD_{90}. Clinical characteristics are summarized in Table 1. As most cases showed a distinct perifollicular pattern, we correlated TVD_{90} with follicle size in each case. There was a significant association between low TVD_{90} and small follicle size (χ^2, P=0.01). Low TVD_{90} also correlated with younger age (χ^2, P=0.034), increased IPI (χ^2, P=0.01) and increased $CD68^+$ macrophages (χ^2, P=0.025). In univariate analysis, TVD_{90} and IPI are significant for both OS and RT, but not age or follicle size. In this cohort, $CD68^+$ macrophages predicted OS (P=0.0004), but not RT (P=0.38). In a Cox's model including TVD_{90}, $CD68^+$ and IPI, all three markers were independent variables for OS, TVD_{90} (RR=2.5, 95% CI=1.1-5.0, P=0.01), $CD68^+$ (RR=2.2, 95% CI=1.0-5.0, P=0.05) and IPI (RR=2.9, 95% CI=1.5-5.5, P=0.001). Finally, in a Cox's model for RT, only IPI significantly pre-

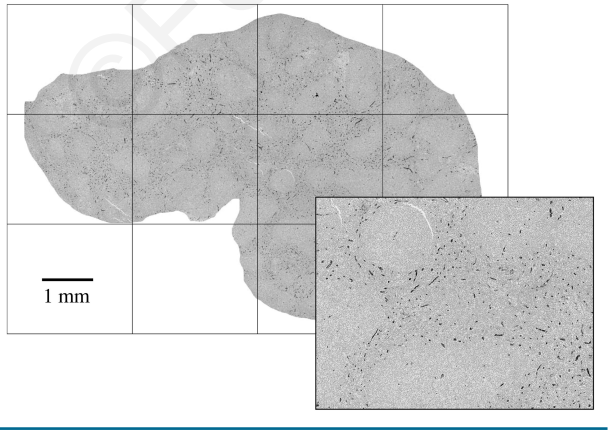

Figure 1. High-resolution image of a CD34 immunostained section allowing the identification of tumor vasculature across the entire section.

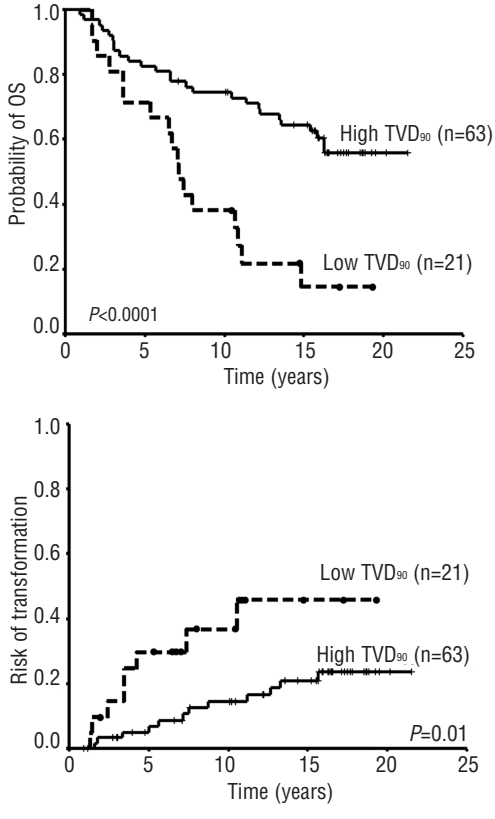

Figure 2. Tumor-to-vessel distance including 90% of malignant cells showing Overall Survival (OS) and Risk of Transformation (RT).

dicted RT (P=0.008), while TVD_{90} was of borderline significance (P=0.08). Angiogenesis plays a crucial role in the growth and progression of human solid tumors.[13] In most tumor types, increased MVD correlates with increased disease progression and decreased survival. Similar results have been reported in hematopoietic tumors, including multiple myeloma and lymphoma.[3,4,14,15] In murine lymphoma models and in lymphoma patients, circulating endothelial cells and serum vascular endothelial growth factor (VEGF) levels appear to correlate with lymphoma volume and increased angiogenesis.[16,17] In contrast, previous studies of MVD in FL have resulted in conflicting results. Koster *et al.* showed increased MVD to be associated with a more favorable OS in a series of 36 uniformly treated patients given CVP and interferon (IFN) α2b followed by IFN maintenance.[6] In a later report, Jorgensen *et al.* analyzed 107 FL cases with heterogeneous treatments and found increased interfollicular MVD predicted inferior OS and increased transformation to DLBCL.[7] In both studies, MVD was calculated using quantification of focal "vessel hot spots" within the tumor. In FL, however, the vessels show a heterogeneous distribution. Therefore, in order to avoid a scoring bias, we studied whole sections of FL biopsies and quantified automatically the average distance from CD34 stained vessel that included 90% of tumor cells: TVD_{90}. We believe this is a more accurate measurement of vessel density. Because of vessel predominance within the interfollicular and perifollicular areas, TVD_{90} was correlated with the average size of the follicles in each case. Interestingly, cases with low TVD_{90} correlated with small sized follicles although follicle size itself did not affect survival or risk of transformation.

In the current study, all 84 patients were treated uniformly with multi-agent chemotherapy and radiation, while in the series by Koster *et al.* all 36 patients were treated with CVP chemotherapy and IFN followed by IFN maintenance. In addition to the difference in cohort size, different therapeutic regimens may explain the contradictory results. IFN has both immunomodulatory and anti-angiogenic effects and thus may have been more effective in tumors with increased MVD.[18] Radiation has been shown to induce tumor cells to secrete cytokines capable of inhibiting apoptosis in endothelial cells, thereby diminishing treatment response.[19,20] It remains possible that this treatment modality influenced survival and transformation risk in our study.

It is well known that angiogenesis in cancer is critically influenced by the local tumor microenvironment.[21] Using the same uniformly treated cohort, we previously showed that increased numbers of Lymphoma-Associated Macrophages (LAM) is associated with adverse outcome.[9] Similar to solid tumor-associated macrophages, these LAM, possibly originating from bone marrow derived myeloid cells, may be attracted by hypoxia and tumor-derived chemotactic factors and show a distinct phenotype that promotes angiogenesis.[22-24] Consistent with this hypothesis, we show a significant association between TVD_{90} and LAM.

In summary, this study confirms the clinical relevance of increased angiogenesis affecting both FL survival and transformation risk in a series of advanced-stage FL patients uniformly treated with chemotherapy and radiotherapy. These findings suggest MVD as a useful biomarker in initial therapeutic decisions of patients with FL, and thus may provide a rationale for trials of anti-angiogenic therapy in FL patients with increased MVD.

Table 1. Distribution of clinical and pathology variables between high TVD90 and low TVD90 cases.

Feature	# Patients with Low TVD 90 (%)	# Patients with High TVD 90 (%)	Total (%)	*P* value (χ^2)
Number	21 (25)	63 (75)	84 (100)	-
Clinical Features				
Median age (y)	39	46	-	0.034
Female (%)	52	48	-	0.63
Median follow up (y)	13.9	14.3	-	0.83
IPI Group 1 (0/1)	8 (38)	45 (71)	62 (61)	0.01*
Group 2 (2/3)	13 (62)	17 (27)	39 (38)	
Group 3 (4/5)	0 (0)	1 (2)	1 (1)	
BM Involvement (%)	14 (66)	28 (44)	42 (50)	0.13
Treatment Response				
CR	8 (38)	39 (62)	47 (56)	0.08**
PR	13 (62)	23 (36)	36 (43)	
NR	0 (0)	1 (2)	1 (1)	
Pathology Features				
FL Grade				
Grade 1	17 (80)	49 (78)	66 (78)	0.76***
Grade 2	4 (20)	10 (16)	14 (17)	
Grade 3	0 (0)	4 (6)	4 (5)	
Large follicles (%)	1 (5)	20 (32)	21 (25)	0.01
Increased CD68+ cells (%)	5 (24)	4 (7)	9	0.025
Transformation (%)	8 (38)	12 (19)	20(24)	0.076

*IPI: International Prognostic Index score, BM: bone marrow, CR: complete remission, PR: partial remission, NR: no response; *IPI group 1 vs. 2/3 **CR vs. PR/NR ***Grade 1 vs. 2/3A.*

Authorship and Disclosures

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