

# High Levels of Vascular Endothelial Growth Factor Receptor-2 Correlate with Shortened Survival in Chronic Lymphocytic Leukemia

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## ABSTRACT

**Vascular endothelial growth factor receptor-2 (VEGFR-2), also termed KDR, is a high-affinity vascular endothelial growth factor (VEGF) receptor. VEGFR-2 plays a role in *de novo* blood vessel formation and hematopoietic cell development. Recently, we found that chronic lymphocytic leukemia (CLL) cells express high levels of VEGF. Therefore, we sought to investigate the role of VEGFR-2 in CLL. Using Western blot analysis, we first determined that VEGFR-2 is present in peripheral blood CLL cells. We then quantified the cellular levels of VEGFR-2 protein using a solid-phase radioimmunoanalysis in peripheral blood cells from 216 patients with CLL. As control, we used peripheral blood mononuclear cells (PBMNCs) from 31 hematologically normal individuals. The median of VEGFR-2 levels detected in the control samples was assigned a value of 1.0, and VEGFR-2 protein levels were normalized to the control median value. The median level of VEGFR-2 in CLL cells was 1.57. Patients with VEGFR-2 levels higher than 1.57 had elevated lymphocyte counts, severe anemia, elevated  $\beta_2$ -microglobulin and advanced-stage disease. Elevated VEGFR-2 levels were also associated with statistically significantly shorter survival (35.4 versus 60.1 months;  $P < 0.01$ ). Our data indicate that cellular VEGFR-2 levels may serve as a prognostic factor in CLL. Further studies should investigate the biological implications of these findings and the effect of the interaction between VEGF and VEGFR-2 on CLL cell proliferation.**

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## INTRODUCTION

VEGFR-2,<sup>2</sup> also termed KDR, is a high-affinity VEGF receptor (1). A member of the tyrosine kinase receptor superfamily (2), VEGFR-2 consists of 1338 amino acid residues and comprises an extracellular domain formed by seven immunoglobulin-like loops, a transmembrane sequence, and a split tyrosine-kinase intracellular domain (3).

Although mainly localized on vascular endothelial cells, VEGFR-2 also plays a crucial role in hematopoiesis (4). Knock-out mice lacking the VEGFR-2 gene fail to develop hematopoietic cells (5). Furthermore, VEGFR-2, the gene of which is located on the long arm of human chromosome 4 at 4q11–13 (6), is markedly elevated in normal human bone marrow (7). Furthermore, Ziegler *et al.* (8) have recently shown that functional pluripotent hematopoietic cells express VEGFR-2. These investigators have found that CD34<sup>+</sup> cells expressing VEGFR-2 engraft and give rise to cells of all hematopoietic lineages and suggested that these cells are precursors of both human hematopoietic and human endothelial lineages. Aguayo *et al.* (9) recently found that CLL cells produce high concentrations of VEGF. Given these findings, we hypothesized that VEGFR-2 may also be elevated in CLL and facilitate cell proliferation. We measured VEGFR-2 protein levels in CLL cells and correlated these levels with various clinical characteristics and patient survival. We found that VEGFR-2 is overexpressed in CLL cells and that patients with high VEGFR-2 levels have marked lymphocytosis, severe anemia, and shortened survival.

## PATIENTS AND METHODS

**Subjects.** Peripheral blood samples were obtained from 216 patients with CLL during routine diagnostic evaluation at the Department of Leukemia at M. D. Anderson Cancer Center between 1994 and 1997. The diagnosis of CLL was established based on morphology, flow cytometric analysis (CD5<sup>+</sup>, CD19<sup>+</sup>, CD23<sup>+</sup>), and molecular analysis (JH and  $\kappa$  or  $\lambda$  rearrangement). All samples were obtained in accordance with the protocols approved by the Human Subjects Committee of the M. D. Anderson Cancer Center, and all patients gave individual informed consent. The characteristics and clinical data of the patients are presented in Table 1. PBMNCs from 31 hematologically normal individuals were used as controls.

**Cell Fractionation.** PBMNCs were separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation, washed twice with PBS, and stored at  $-70^{\circ}\text{C}$  until analyzed. All

<sup>2</sup> The abbreviations used are: VEGFR-2, vascular endothelial growth factor receptor-2; AML, acute myelogenous leukemia;  $\beta_2\text{M}$ ,  $\beta_2$ -microglobulin; CLL, chronic lymphocytic leukemia; PBMNC, peripheral blood mononuclear cells; VEGF, vascular endothelial growth factor.

Table 1 Patient characteristics

The median age was 61 years (range, 22–87 years).

	No. of patients	%
Sex		
Female	67	31
Male	149	69
Rai stage		
0	34	16
I	88	41
II	28	13
III	15	7
IV	44	21
Not available	3	2
Hemoglobin (g/dl)		
<11	41	19
11–13	67	31
>13	107	50
Platelets ( $\times 10^9$ /liter)		
<100	46	21
100–150	50	24
>150	118	55
Lymphocytes ( $\times 10^9$ /liter)		
<50	105	49
51–100	60	28
>100	50	24
Lymphocytes in bone marrow (%)		
<50	29	14
50–70	28	13
>70	87	40
Data not available	72	33
$\beta_2$ M (mg/liter)	2.8 <sup>a</sup> (1.1–15.4) <sup>b</sup>	

<sup>a</sup> Median.<sup>b</sup> Numbers in parentheses, range.

analyzed samples contained >70% CD19- and CD5-coexpressing cells as assessed by flow cytometry. In eight patients, CD19<sup>+</sup> and CD3<sup>+</sup> cells were isolated using magnetic beads and MACS columns (Miltenyi Biotec, Auburn, CA). Flow cytometric analysis of these cell subpopulations using anti-CD20 and anti-CD7 antibodies showed that at least 90% of the cells were pure B cells and 70% pure T cells, respectively. The levels of VEGFR-2 in sorted CD19<sup>+</sup> and CD3<sup>+</sup> cells were compared with the level in the entire sample (8).

**Protein Extraction.** Protein was extracted as previously described (10). Briefly, cell pellets were lysed in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% NP40, and 150 mM NaCl buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml pepstatin for 30 min on ice with frequent vortexing and then left on ice for 1 h. Lysates were clarified by microcentrifugation for 1 h at 14,000 rpm. After protein concentration was determined using the Bradford method, 200  $\mu$ g of cellular protein extract was run on 9.5% SDS-PAGE gel and stained with Coomassie Blue R-250 to determine the protein profile and to verify the amount of protein loaded.

**Western Blot Analysis of VEGFR-2 Protein.** Cellular protein extract (200  $\mu$ g) from patients with CLL and from the hematologically normal individuals were electrophoretically separated on 9.5% SDS-PAGE gels and transferred to nitrocellulose membrane paper. The nitrocellulose membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20

and 0.01% sodium azide for 6–8 h at room temperature. The blots were incubated overnight at 4°C with 1  $\mu$ g/ml mouse anti-VEGFR-2 antibody (Sigma, St. Louis, MO) in PBS containing 2.5% nonfat milk, 2.5% BSA, and 0.1% Tween 20. The membranes were washed with PBS containing 0.1% Tween 20. The blots were then incubated with 1:2000 diluted antimouse IgG linked to horseradish peroxidase (Sigma) in PBS containing 1% nonfat milk and 0.1% Tween 20. Immunoreactive bands were developed using the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). After enhanced chemiluminescence detection, the primary and secondary antibodies were stripped off the membranes under conditions recommended by the manufacturer. The stripped membranes were then blocked and probed with antiactin monoclonal antibodies (Amersham) to ensure that the protein was loaded equally in each lane.

**Solid-Phase RIA.** Quantification of the VEGFR-2 level was determined using solid-phase RIA as previously described (10). Briefly, microtiter plates were coated overnight at 4°C in 50  $\mu$ l of PBS containing 5  $\mu$ g of protein extracted from each of the patients with CLL and from each of the hematologically normal individuals. The RIA plates were washed with PBS and blocked with 100  $\mu$ l of 1% BSA (Amersham) in PBS for 1 h at 37°C. The plates were incubated overnight at 4°C with 50  $\mu$ l of mouse anti-VEGFR-2 antibody (Sigma) diluted 1:1000 in PBS containing 0.1% BSA. The plates were then washed with PBS and amplified with mouse antimouse IgG antiserum (Sigma) diluted 1:1000 in PBS containing 0.1% BSA for 2 h at 37°C. After being washed, the plates were developed with excess <sup>125</sup>I-labeled protein G [200,000 cpm (50 IU)] in PBS containing 0.1% BSA for 2 h at room temperature, washed with PBS, and then separated into individual wells that were counted in a gamma counter (Pharmacia LKB Biotechnology, Uppsala, Sweden). The assays were performed in triplicate. The results were corrected for the nonspecific binding (1–2%) detected in control wells, which were not coated with a test antigen but which were blocked with BSA. A second set of plates was incubated with antiactin antibodies to confirm the use of equal amounts of total cellular protein from each sample.

**Statistical Analysis.** The median values for clinical variables were calculated. Association between patient characteristics (covariates) was determined for pairs of numeric variables using Spearman's correlation coefficient; the differences between two categorical and/or continuous variables were evaluated with the Mann-Whitney *U* test and the Kruskal-Wallis analysis by ranks for more than two variables or groups. The primary end point of this study was the survival time. Survival was evaluated by the Kaplan-Meier method (11), and the differences between groups were assessed using the log rank test. *P* < 0.05 was considered significant.

## RESULTS

**Variation in the Expression of VEGFR-2 in CLL.** CLL samples showed high levels of VEGFR-2 protein by Western blot analysis. As is shown in Fig. 1, there was variation between the samples. VEGFR-2 was not detectable on Western blot in PBMNCs from hematologically normal subjects.

Solid-phase RIA was used to quantify VEGFR-2 intracel-

Fig. 1 Representative Western blot analysis of VEGFR-2 in 13 CLL patients. Lane N, PBMNCs from a hematologically normal individual; Lanes 1–13, the variation in the expression of VEGFR-2 in patients with CLL. Bottom abscissa, corresponding solid-phase RIA values for VEGFR-2.

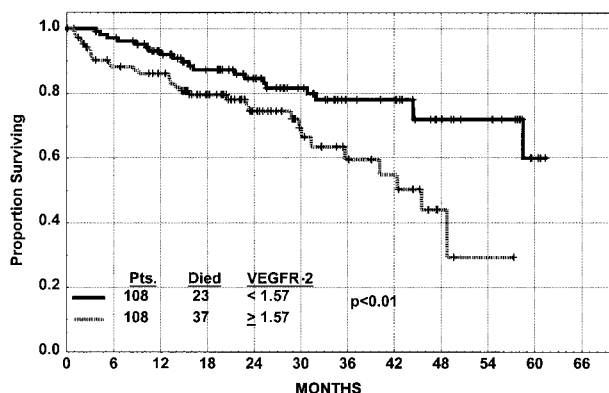
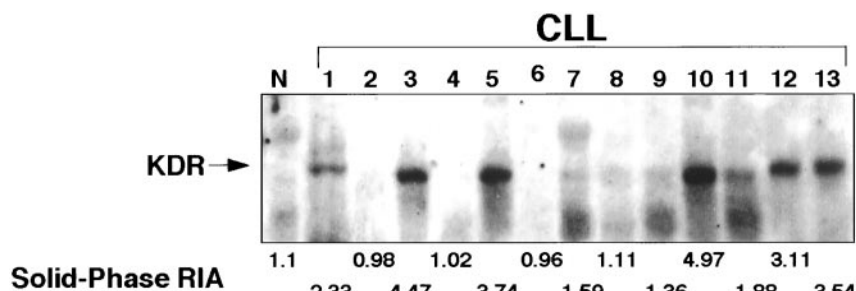


Fig. 2 Kaplan-Meier survival curves for CLL patients. The curves show shorter survival in patients (Pts.) with high levels of VEGFR-2.

lular protein content. The median cpm for the CLL samples was normalized to the median cpm for 31 hematologically normal individuals, which was assigned the arbitrary value of 1.0. The median value for VEGFR-2 in 216 CLL samples was 1.57 times higher (range, 0.49–13.9) than that in the hematologically normal individuals.

Because the analyzed samples contained residual normal cells, albeit <30%, we sought to determine whether this dilution significantly affected the values obtained in an analysis of total cells. Levels of VEGFR-2 in CD19<sup>+</sup> (tumor) cells were compared with those in CD3<sup>+</sup> cells and in unfractionated total PBMNCs from eight patients with CLL. There was no significant difference between the levels of VEGFR-2 protein in CD19<sup>+</sup> cells and unfractionated PBMNCs ( $P = 0.07$ , Kruskal-Wallis test). However, there was a significant difference in the VEGFR-2 levels between CD19<sup>+</sup> and CD3<sup>+</sup> cells ( $P < 0.001$ ). These findings confirm that although the residual normal cells slightly decreased the values obtained in an analysis of total cells, this decrease was not statistically significant.

**Correlation between High Levels of VEGFR-2 and Aggressive Disease.** The clinical characteristics of the 216 patients with CLL are summarized in Table 1. The survival time of patients expressing VEGFR-2 levels higher than the relative median of 1.57 was significantly shorter ( $P = 0.003$ ) than those of patients with levels below this median (Fig. 2). Of the 108 patients with VEGFR-2 level below the relative median of 1.57, 85 (79%) were alive at the time of this analysis, compared with 71 (66%) of the 108 patients with VEGFR-2 level  $\geq 1.57$ . The

Table 2 Correlation between VEGFR-2 and clinical variables

	R	P
Age	0.03	0.62
Rai stage	0.17	0.01
$\beta_2$ M	0.13	0.08
Hemoglobin	-0.24	<0.001
Platelets	-0.08	0.21
% PB <sup>a</sup> lymphocytes	0.25	<0.001
% BM lymphocytes	-0.21	0.01
WBC	0.44	<0.001
VEGF	-0.02	0.77

PB, peripheral blood; BM, bone marrow.

median survival times for the two groups were 60.1 and 35.4 months, respectively. If we use a cutoff point of 4.2, which separates the upper quartile, patients with high levels of VEGFR-2 had shorter survival ( $P = 0.001$ ). VEGFR-2 was also strong predictor of survival when used as a continuous variable in a Cox proportional hazard model ( $P < 0.001$ ). VEGFR-2 levels remained predictive of survival in a multivariate analysis model incorporating  $\beta_2$ M, hemoglobin, Rai staging, and platelet count ( $P = 0.01$ ). When VEGFR-2 levels were correlated with patient age, Rai stage,  $\beta_2$ M levels, and hematological parameters, the following associations were found (Table 2): elevated VEGFR-2 levels correlated with decreased hemoglobin levels (Spearman's correlation coefficient,  $r = -0.24$ ;  $P < 0.001$ ) and elevated lymphocyte count ( $r = 0.25$ ;  $P < 0.001$ ). Elevated VEGFR-2 levels also correlated with advanced Rai stage (III and IV) and elevated  $\beta_2$ M levels ( $r = 0.17$  and  $0.13$ , respectively), but this association was not statistically significant. When patients grouped into three risk groups (low, intermediate, and high) according to Rai staging, there was a trend for increasing VEGFR-2 levels with advanced stages (Fig. 3A), but this was not significant. However, there was significant difference in VEGFR-2 levels between patients with low (<2.5 mg/liter) and patients with high (>2.5 mg/liter)  $\beta_2$ M ( $P = 0.03$ ; Fig. 3B).

## DISCUSSION

Recent studies demonstrated that tumor growth depends on the development of new blood vessels (12) and that the density of tumor microvasculature correlates with invasion, metastatic potential, and prognosis (13). Increased bone marrow microvessel density and elevated levels of urinary basic fibroblast growth factor were also found in children with acute lymphocytic

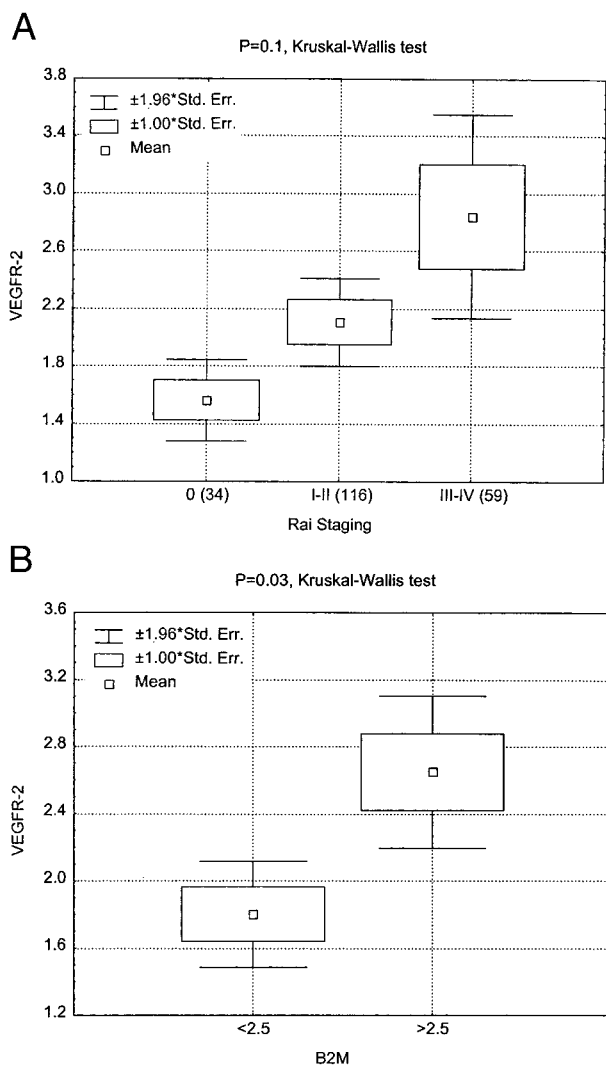


Fig. 3 Levels of VEGFR-2 in various clinical stages of CLL. Box plots showing the levels of VEGFR-2 in various Rai stages (A) and in patients divided into two groups according to their  $\beta_2M$  levels (B).

leukemia (14). Bellamy *et al.* (7) evaluated VEGF mRNA and protein levels in leukemia and lymphoma cell lines. Our data and data of other investigators (15) showed increased intracellular VEGF levels in fresh AML cells. Interestingly, we found that elevated cellular levels of VEGF had a negative prognostic significance in AML (16) and that increased levels of intracellular VEGF correlated with less aggressive disease in CLL (9). Despite elevated intracellular VEGF levels, there was no increase in CLL bone marrow vascularity as compared with that of hematologically normal subjects (17). VEGF was also detected in the peripheral blood of CLL patients, and Molica *et al.* (18) reported elevated serum levels of VEGF in 12 (17.6%) of 68 patients with CLL.

These findings prompted us to investigate the role of VEGFR-2 in CLL. VEGFR-2, a high affinity VEGF receptor, is a marker for early hematolymphopoietic precursors and is abundantly expressed by normal hematopoietic and stromal cells in

bone marrow biopsies of patients with multiple myeloma (7). Fiedler *et al.* (15) detected VEGFR-2 expression in 20% of the AML samples, whereas Bellamy *et al.* (7) did not detect VEGFR-2 expression in 12 leukemia cell lines. However, the presence of VEGFR-2 was not studied in CLL cells thus far.

In the present study, we demonstrated that whereas patients with CLL vary in their cellular VEGFR-2 levels, CLL cells from patients with aggressive disease produce high levels of VEGFR-2 protein. Therefore, overproduction of VEGFR-2 characterizes a subgroup of CLL patients with shorter survival.

The ability to detect VEGFR-2 may depend on the sensitivity of the assay used in the analysis. We used flow cytometry to test some of our CLL samples that had shown high VEGFR-2 expression on Western blot analysis and were unable to detect any expression using the method described by Ziegler *et al.* (8).

In contrast to the paucity of observations in hematological malignancies, there is clear evidence of up-regulation of VEGFR-2 in solid tumors. In an experimental model of angiosarcoma, Arbiser *et al.* (19) showed that the level of VEGFR-2 expression was higher than that observed in benign hemangiomas originating from the same cell line. Similarly, VEGFR-2 has been identified as the major mediator of VEGF-induced tumor development in murine hepatocellular carcinoma cells (20). Carroll *et al.* (21) described the activation of VEGFR-2 in 37 astrocytic neoplasms. Kranz *et al.* (22) found the expression of activated VEGFR-2 in patients with breast cancer to be much higher in the malignant tissue than in the neighboring nonneoplastic regions.

To our knowledge, the present study is the first to describe the overexpression of VEGFR-2 protein in CLL cells. Because the cellular levels correlated with survival, it is possible that VEGFR-2 is a stimulatory factor. The apparent paradox and correlation of higher VEGF expression with better outcome reported by Aguayo *et al.* (9) may indicate that VEGFR-2 is an essential factor stimulating cell growth in CLL and may explain the lack of increased vascularity in CLL. This issue is of potential therapeutic importance, because specific agents targeting VEGFR-2 have generated exciting results in preclinical studies (23). Monoclonal antibodies neutralizing VEGFR-2 have been shown to induce significant tumor regression in xenografts of neuroblastoma (24). Interestingly, the effect was more significant when these monoclonal antibodies were combined with standard chemotherapy. SU5416, a novel tyrosine kinase inhibitor targeting VEGFR-2, has been shown to inhibit the development of liver metastases in a murine colon cancer model (25).

Our data suggest that VEGFR-2 is involved in disease progression and may be a prognostic factor in CLL. Further studies are needed to elucidate the function of VEGF/VEGFR-2 in CLL.

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