Correlation between High Vascular Endothelial Growth Factor-A Serum Levels and Treatment Outcome in Patients with Standard-Risk Acute Lymphoblastic Leukemia: A Report from Children’s Oncology Group Study CCG-1962

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Abstract  Purpose: Many molecular pathways, including cell cycle control, angiogenesis, and drug resistance, mediate tumor growth and survival. Vascular endothelial growth factor-A (VEGF-A) serum levels (<40 and >100 pg/mL) have been associated with good and poor prognoses, respectively. Experimental Design: The hypothesis was that serum VEGF-A levels in standard-risk acute lymphoblastic leukemia pediatric patients at induction are predictive of event-free survival (EFS). One hundred seventeen patients were entered in CCG-1962 study and randomized into the native and polyethylene glycolated asparaginase arms. VEGF-A levels were quantified by an ELISA assay. Results: All patients had a decrease in VEGF-A levels by day 14 of induction, but they later dichotomized; EFS group levels remained low and event group levels increased. A correlation exists between high VEGF-A levels at entry to induction and time to event. Moreover, 6-year EFS patients have lower end of induction VEGF-A levels (28 ± 6 pg/mL) than event patients (>100 pg/mL; P < 0.01). Kaplan-Meier curves using various VEGF-A values were produced; with ≤30 at entry into induction (day 0) and ≤60 pg/mL at the end of induction (day 28), patients with low VEGF-A levels had superior EFS (P < 1e−4). Furthermore, patients who had an increase in VEGF-A during induction (∆VEGF-positive, days 0-28) were more likely to have an event (P < 1e−4). Bifurcation by asparaginase treatment arm did not alter these results. Conclusions: These observations strongly support that high VEGF-A levels in induction are an asparaginase treatment-independent predictive marker for EFS. Hence, an anti-VEGF-A therapy should be tested in acute lymphoblastic leukemia.

Acute leukemias are aggressive disorders characterized by accumulation of immature malignant cells in the bone marrow. The risk for relapse varies considerably between patients and depends on many genetic abnormalities and influences of growth factors. An inverse relationship has been seen between blast proliferation and the magnitude of response to growth factors. An inverse relationship has been seen between blast proliferation and the magnitude of response to growth factors. Drug resistance to anthracyclines. In leukemia, the clonal population is characterized by a hierarchical organization similar to the normal hematopoiesis and a subset of the stem cells retain their undifferentiated “stem cell” morphology. The cytokine effect on acute lymphoblastic leukemia (ALL) stem cells, especially childhood T-cell ALL cells, seems to be similar to normal hematopoietic bone marrow cells. Pleiotropic and lymphopoietic cytokines, such as stem cell factor, lactoferrin, interleukin-3, interleukin-6, interleukin-2, and other growth factors involved in the physiologic regulation of hematopoiesis, are known to provide growth signals, prevent apoptosis, and induce a shift from G0 to G1 phase of the cell cycle in both B-cell and T-cell lineage ALL blasts (3, 4). Moreover, in progenitor cells, signaling through vascular endothelial growth factor (VEGF) receptor-2 specifically mediates the chemotactic effect of VEGF-A (5).

One of the most potent growth factors, VEGF, exists in several isoforms as a result of alternative splicing from a single gene, which differ in their binding to extracellular compounds (6, 7). The freely diffusible VEGF protein in the extracellular space available to endothelial cells is the bioactive form of VEGF-A (7). Little is known about the regulation of VEGF in normal human tissues, but some evidence indicates that sex steroids (estradiol and possibly progesterone) affect VEGF production in normal breast tissue (8). Similarly unknown are the influences of normal growth hormones in normal and malignant hematopoiesis in childhood, and this uncertainty may play an important role in the development and progression of leukemic blast cells (9). In support of this hypothesis, the recently identified autocrine loop by which VEGF may regulate and control hematopoietic stem cell replication and survival may be different from its paracrine
effects regulating angiogenesis (9). Furthermore, stimulatory autocrine and paracrine loops have been shown to modulate the effects of individual cytokines on bone marrow microenvironment, both normal and malignant blast cells (10–12). Moreover, T-cell leukemia and B-cell lymphoma cells express N-cadherin, which may facilitate the binding of the blast cells to bone marrow stromal cells, thus finding their stem cell “niche” and facilitating their pathologic features (5, 13).

Recent clinical studies suggest that local bone marrow angiogenesis with increased blood vessel density is important both for disease development and chemosensitivity in acute myeloid leukemia (14–16). Moreover, it is well established that angiogenesis plays a critical role in tumor growth and development in solid tumors and hematologic malignancies (17, 18). Many studies have shown that patients with leukemia and lymphoma have increased microvascularization as well as increased levels of proangiogenic vascular growth factors, including VEGF-A (19–22). More recently, accumulating evidence has linked angiogenesis in the pathophysiology of leukemias. An association between the enhanced marrow vasularity after chemotherapy and duration of response has been established in acute and chronic myeloid leukemias, lymphoproliferative diseases, non-Hodgkin’s lymphoma, and hairy cell leukemia (21–27). In a recent article (28), higher levels of interleukin-1 receptor α, interleukin-8, VEGF receptor 1, and VEGF receptor 2 were predictive of poor survival in adult ALL patients, but the ligand was not found to be predictive. Therefore, angiogenesis-inducing growth factors and their receptors play an important role in the survival, growth, and pathogenesis of hematologic malignancies in both pediatric and adult patients.

Recently, the mitotic inhibitor drugs vinblastine, vincristine, and Taxotere were shown to inhibit the secretion of angiogenesis-inducing VEGF-A by wild-type and drug-resistant human leukemia T-cell lines, whereas the protein inhibitor of ALL blasts, native E. coli asparaginase, did not (29, 30). Tyrosine kinase–containing receptors specific to endothelial cells are also expressed on certain subsets of leukemias, like VEGF receptors 1, 2, and 3 (7, 31, 32). Clinical studies have also provided strong evidence that VEGF-A intracellular levels in B-cell chronic lymphocytic leukemia patients correlated with disease characteristics and prognosis (23, 33, 34). In adult acute myeloid leukemia studies, higher VEGF-A protein levels in the leukemia cells corresponded with poorer patient survival (15, 35). This evidence provided a direct relationship between soluble VEGF levels and leukemic blast survival as a function of this growth factor, which may be distinct from its role in angiogenesis. Additional studies on the mechanism of survival of leukemia cell lines showed that VEGF-A and its mutant form that lacks the KDR-binding motif induced receptor phosphorylation and increased Bcl-2 expression, leading to protection from apoptosis and leukemia proliferation (31, 36).

The main goal of this study was to determine the effect of VEGF-A serum concentrations may have on the outcome of standard-risk ALL (CCG-1962 study) pediatric patients (37).

### Materials and Methods

**Patient characteristics, treatment, and serum samples.** Between May 1997 and November 1998, 117 children with standard-risk ALL were enrolled in CCG-1962, a two-arm study that compared native versus polyethylene glycolated (PEGylated) asparaginase in induction and two delayed-intensification phases (37). Multiple specimens from each patient were assayed for VEGF-A concentrations for a total of 598 serum specimens in 86 patients in the event-free survival (EFS) group and 242 serum specimens in patients in the 26 event group (total of 840 specimens).

**VEGF-A ELISA Assays.** The ELISA for VEGF-A used in numerous studies was used for our studies. The ELISA method has extensively been used in our laboratory (30, 32). The limit of detection was 0.01 pg/mL (theoretical assay zero). Serum aliquots (100 μL) from all collected patient sera were used to determine VEGF-A concentration in induction, delayed intensification-1, and delayed intensification-2, with Institutional Review Board approval.

**Prognostic significance of VEGF-A serum concentrations.** The prognostic significance of serum VEGF-A protein in standard-risk ALL pediatric patients was evaluated using nonparametric analyses by computing Life Table analyses and analyzing survival (failure time) data. Results were analyzed in three outcome groups: no relapse event, central nervous system (CNS) relapse, and bone marrow or bone marrow plus CNS relapse. In each group, the VEGF-A levels were averaged per treatment phase. The means and SDs were also calculated for each outcome group. Nonpaired t tests were used to find statistical significance. Average levels of VEGF-A were calculated according to certain intervals during induction to follow changes of VEGF-A levels in the three outcome groups. Associations or correlations were sought between mean VEGF-A serum levels during induction or end of induction and time of relapse. Because there were no statistical differences in the CNS or CNS plus bone marrow event patient values, the results for the final analyses in all the event patients were combined as one group and compared with EFS group. In addition to parametric t tests for differences in VEGF-A levels (which are based on statistical methods for normally distributed data), nonparametric methods were used as well. This approach used the Wilcoxon rank test and compared VEGF-A levels to seek correlations between event (relapse) and EFS groups. The “time to event” or EFS survival was calculated from the date of ending of treatment to the date of first event (or death). For events, data for patients who died from causes other than leukemia were considered at the time of death. EFS curves were computed by using various cutoff values of VEGF-A by the Kaplan-Meier method. Univariate analyses of the probability for EFS according to VEGF-A serum levels at entry and at the end of induction were done in 112 patients with complete VEGF-A and outcome parameter profiles and with the use of a two-sided log-rank test.

A series of VEGF-A serum concentrations were analyzed by separating the matrix of VEGF concentrations in high, intermediate, and low risk for an event by using various cut points of VEGF-A starting from 10, 20, 30, 40, 50, 60, 80, 100, 120, and 300 pg/mL and the most significant were selected [i.e., at entry into induction (≥30 pg/mL) or at the end of induction (≥60 pg/mL); refs 38, 39]. The goal was to identify cutoff point of VEGF-A concentrations as an optimal pharmacodynamic predictor parameter that had both “high sensitivity” and “high selectivity” at the same time. The cutoff points of 30 pg/mL at entry into the study (induction) and 60 pg/mL at the end of induction were the most optimal in bifurcating these patients per EFS versus event groups. The log-rank test was used to compare Kaplan-Meier EFS survival curves.

Multivariate analyses of EFS and other factors [age, WBC at time of diagnosis (standard-risk ALL patients) <50,000/μm^3] and clinical status were done with the use of the Cox proportional hazards model with both forward and backward stepwise inclusion of factors, with a criterion of P < 0.05 and the opposite (exclusion criterion of P > 0.05). Further, the change in VEGF-A (ΔVEGF-A) in induction was evaluated and compared with VEGF-A levels in delayed intensification-1 and delayed intensification-2. The STATA-9 program (College Station, TX) was used to produce the Kaplan-Meier curves and related outcome analyses.
Results

Patient characteristics. Twenty-six of 112 fully evaluable patients enrolled in CCG-1962 study had relapses by the summer of 2005. There were 14 isolated CNS relapses, 9 isolated bone marrow bone marrow relapses, and 3 combined bone marrow plus CNS relapses. Fifteen relapses were in the native asparaginase arm and 11 in the PEGylated asparaginase arm. Among the 86 event-free patients, 44 had been randomized to native asparaginase and 42 to the PEGylated asparaginase. The detailed patient characteristics, treatment scheme, and outcomes have been reported (37).

Treatment efficacy and toxicities. All patients achieved complete remissions by day 28 of induction. Two patients in the bone marrow relapse group had T-cell ALL (randomized to native asparaginase arm). A considerable number of toxicities were observed in both of these patient groups, the majority of which were in the event group. Detailed characteristics of all CCG-1962 patients have been reported in the CCG-1962 study population (37).

Serum VEGF concentrations. The average VEGF-A serum levels on days 0 to 4 of induction were 28.3 ± 55.5 pg/mL (mean ± SD; median, 0.01; range, 0.01-285.3 pg/mL; n = 86 patients) and 149.6 ± 156.7 pg/mL (median, 125.0; range, 0.01-503.2 pg/mL) for the EFS and event groups, respectively. The average VEGF-A levels in serum were higher in the bone marrow relapse group than in the CNS relapse group, but not statistically different (P = 0.4). There was no statistical difference between CNS or bone marrow relapse patients and bone marrow plus CNS relapse patients. Thus, these event groups were combined (n = 26).

All the serum VEGF-A concentrations during induction for the EFS and event groups are shown in Table 1. During induction, VEGF-A levels in EFS patients remained low. In contrast, VEGF-A levels in 26 patients with events remained the same or declined until day 14 and then increased to a maximum VEGF-A concentration of 289.9 ± 44.6 pg/mL by day 28 of induction. During days 7 to 14 of induction, VEGF-A concentrations remained the same or declined, averaging 5.4 ± 12.2 and 45.9 ± 53.3 pg/mL (mean ± SD) in the EFS and event groups of patients, respectively (P = 0.001). The mean VEGF-A values by end of induction (day 28; range, 24–35 days of sample) averaged 42.5 ± 53.2 and 213 ± 150.5 pg/mL for the EFS and event groups, respectively (P < 1e–5). When the assay zero VEGF-A values were removed from the calculations, similar trends were seen (Table 1). During delayed intensification-1, the mean VEGF values decreased to 98 ± 81 pg/mL in the CNS event patients, whereas they increased to 144 ± 106 pg/mL in the bone marrow event patients. During delayed intensification-2, VEGF levels in the bone marrow event patients increased to 200 ± 198 pg/mL from 100 ± 95 pg/mL induction values (P = 0.03). In contrast, average VEGF-A levels in the EFS group were similar to those at the end of induction and declined further during delayed intensification-2.

Figure 1 depicts the average VEGF levels in induction treatment phase bifurcated as the event and EFS groups of patients. The average VEGF-A levels in the event patients clearly show a sharp decline by day 14 after initiation of treatment, but subsequently increased to a higher value than before induction treatment began. In contrast, VEGF-A levels of EFS patients started low or remained lower than the event group, mostly unaffected by induction treatment, with a moderate trend for an increase by day 28. The EFS patients (n = 86) had lower average VEGF-A serum concentrations, which were statistically significant, at entry into induction treatment (P = 6e–4), on day 14 of induction (P = 0.001), and at the end of induction (P = 5.2e–6) than the 26 event patients. The differences of VEGF-A serum concentrations at the end of induction in favor of the EFS patients were also seen at delayed intensification-1 (P < 0.005) and delayed intensification-2 (P < 0.05) treatment phases (data not shown).

Prognostic significance of VEGF-A serum concentrations. The hypothesis was that low serum VEGF-A levels in standard-risk ALL pediatric patients at entry of induction or at the end of induction are predictive of EFS. The average VEGF-A concentrations at all time points of induction treatment in the two groups of event patients (n = 26) and EFS patients (n = 86) were examined and compared with time to event. Figure 2 shows the average VEGF-A levels in serum of patients at entry to induction (days 0-4). These values are clustered by the nearest “time to event” method of event timing. The patients with the earliest events had the highest VEGF-A levels at entry into the study. The highest VEGF-A values averaged >400 pg/mL for

<table>
<thead>
<tr>
<th>Patients</th>
<th>Serum VEGF-A levels per CCG-1962 treatment phase (mean ± SD ± SE, pg/mL)</th>
<th>Total VEGF-A (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IND day 0&lt;sup&gt;0&lt;/sup&gt;</td>
<td>P (day 0: days 7–14)</td>
</tr>
<tr>
<td>Event-free VEGF-A conc.</td>
<td>86</td>
<td>28.3 ± 55.5 ± 5.9</td>
</tr>
<tr>
<td>Maximum VEGF-A conc.</td>
<td></td>
<td>74.97 ± 136.4 ± 2.55</td>
</tr>
<tr>
<td>[minus assay 0 (0.01)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Event (BM and BM + CNS + isolated CNS)</td>
<td>26</td>
<td>149.6 ± 156.7 ± 30.7</td>
</tr>
<tr>
<td>Maximum VEGF-A conc.</td>
<td></td>
<td>212.7 ± 150.5 ± 29.5</td>
</tr>
<tr>
<td>[minus assay 0 (0.01)]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: IND, induction; BM, bone marrow.
<sup>0</sup>Day 0 values are from specimens obtained on days 0 to 4.
<sup>†</sup>P values from nonpaired t test evaluations between mean VEGF-A serum levels in the treatment phases per group of patients.
<sup>‡</sup>P values from nonpaired t test evaluations of mean VEGF-A serum levels between event-free and event groups of patients.
Serum VEGF-A and Outcome in ALL

Univariate and multivariate analyses. The CCG-1962 study was designed for a pharmacologic evaluation of two forms of asparaginase, and it was not powered for an outcome difference between these two treatment arms. However, the long-term EFS was defined for a pharmacologic evaluation of two forms of asparaginase was administered per randomization. There was no statistical difference in the VEGF-A values obtained on days 0 to 4 of induction (Fig. 2; P = 0.05) treatment phases. The EFS or continuous complete remission patients (n = 86 patients) had lower average VEGF serum concentrations at entry into induction treatment (P = 6e-4), on day 14 of induction (P = 0.001), and at the end of induction (P = 5.2e-6) than the n = 26 event patients. The significant differences of high or low VEGF-A serum concentrations at the end of induction in favor of the EFS patients were also detected at delayed intensification-1 (P = 0.005) and delayed intensification-2 (P = 0.05) treatment phases. Points, mean of n = 26 to 75 from patients with events and 86 to 270 per data points (n = 86 EFS patients). The results of the multivariate analysis of host factors predictive of EFS are presented in Table 3. Forward and backward stepwise procedures led to the same final model. Separate Kaplan-Meier analyses were made by bifurcation of the databases per form of asparaginase (native versus PEGylated asparaginase) randomization arm of the CCG-1962 study. This analysis did not reveal an independent association of therapy with clinical outcome in addition to the existing significant differences of high VEGF-A (≥60 pg/mL; data not shown). Hence, the high VEGF-A surrogate marker for event is treatment independent.

Among the other disease variables usually associated with time to an event, neither WBC (<50,000/mm$^3$ blast count) nor day 7 bone marrow rapid early response (RER) were statistically significant. The failure to find a relationship with WBC is not surprising because patients in this study were standard-risk ALL and all required to have a WBC <50,000/mm$^3$. Age was the first two subsets of patients with the earliest time to events (<500 days). A second-order curve-linear relationship exists between the time of event and average VEGF-A serum concentrations over time in the 26 event patients. The values declined gradually as the time to event lengthened, reaching an average of 100 pg/mL at ~1,500 days of time to events (Fig. 2).

Conversely, the overall average value of the EFS patients is 28.3 pg/mL (P < 0.01 for all subset value t tests; Table 1). All patients had a serum specimen before induction treatment begun. In addition to day 0, serum specimens had been obtained in these patients on days 1 to 4 post vincristine-prednisone treatments. On day 3 or 4, native or PEGylated asparaginase was administered per randomization. There was no statistical difference in the VEGF-A values obtained on days 0, 1, 2, 3, 4, and 7. There were not many specimens between days 4 and 7. Thus, if we apply statistical exclusion criteria, we could not separate the day 0 values from the average of days 0 to 4. Therefore, serum VEGF-A levels (day 0 or days 0-4 of induction; Fig. 2) were predictive of time of event. When the maximum single value per patient at any time in induction was examined versus time to event, a similar correlation was found. These analyses suggested that high serum VEGF-A levels at entry into induction were predictive of a short duration of remission. Conversely, patients with VEGF-A levels <40 to 50 pg/mL had no events after 6 or more years of follow-up.

Univariate and multivariate analyses. The CCG-1962 study was designed for a pharmacologic evaluation of two forms of asparaginase, and it was not powered for an outcome difference between these two treatment arms. However, the long-term EFS for standard-risk ALL patients was inversely correlated with the level of VEGF-A at entry or at the end of induction. More importantly, univariate analyses showed that the ≥6-year EFS was correlated with VEGF-A levels ≥30 pg/mL at entry or with ≥60 pg/mL at the end of induction (Table 2). The 6-year EFS were significantly better among patients with low levels of VEGF-A in serum at entry and at the end of induction (P < 1e-4). Thus, high VEGF-A serum levels had a predictive value for leukemia event. Kaplan-Meier survival curves with the patient groups bifurcated at various VEGF-A concentrations are shown in Fig. 3. The low-risk VEGF-A concentration was defined as <30 pg/mL at entry into induction or ≤60 pg/mL at end of induction (Fig. 3; P < 1e-4, two-sided log-rank test). The summary of these analyses is shown in Tables 2 and 3.

When the VEGF-A concentrations were examined in the subsequent treatment phases, delayed intensification-1 and delayed intensification-2, no apparent additional relationship was found between VEGF-A concentrations and time to relapse. However, this is to be expected because there were no significant changes in VEGF-A levels from the end of induction to delayed intensification-1 or delayed intensification-2 treatment phases (data not shown).

Fig. 1. Average VEGF-A concentrations in serum (means ± SE) during the 28 days of induction in standard-risk ALL pediatric patients treated on CCG-1962 protocol. The EFS or continuous complete remission patients (n = 86 patients) had lower average VEGF serum concentrations at entry into induction treatment (P = 6e-4), on day 14 of induction (P = 0.001), and at the end of induction (P = 5.2e-6) than the n = 26 event patients. The significant differences of high or low VEGF-A serum concentrations at the end of induction in favor of the EFS patients were also detected at delayed intensification-1 (P = 0.005) and delayed intensification-2 (P = 0.05) treatment phases. Points, mean of n = 26 to 75 from patients with events and 86 to 270 per data points (n = 86 EFS patients).

Fig. 2. Averages (mean ± SD) of maximum serum VEGF concentrations (average, days 0-4) at induction from the 26 relapsed patients (events: combined CNS and bone marrow + CNS) grouped according to nearest time of relapse. Points, mean of the serum concentrations at days 0 to 4 of induction for three or four patients who had relapses close in time (time axis, median). The best-fit line is a second-order fit that showed an inverse log-linear correlation at r² = 0.98 between the higher VEGF-A concentration in serum and time to event. The last data point is the average VEGF-A serum concentrations during induction in 86 EFS patients. All individual means from the event patients were statistically higher than the EFS mean value (P < 0.01).
Table 2. Statistical analyses in 112 standard-risk ALL pediatric patients: log-rank test for equality of survivor functions

<table>
<thead>
<tr>
<th>Testing function</th>
<th>Treatment (pg/mL)</th>
<th>Events observed</th>
<th>Events expected</th>
<th>Log rank P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomization, N1-N2*</td>
<td>N1</td>
<td>11</td>
<td>12.90</td>
<td>0.456</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>15</td>
<td>13.10</td>
<td>0.456</td>
</tr>
<tr>
<td>Pre-Tx VEGF-A</td>
<td>&lt;30</td>
<td>7</td>
<td>18.66</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>≥30</td>
<td>19</td>
<td>7.34</td>
<td>0.044</td>
</tr>
<tr>
<td>Post IND-Tx VEGF-A</td>
<td>&lt;60</td>
<td>3</td>
<td>16.87</td>
<td>&lt;1e-4</td>
</tr>
<tr>
<td></td>
<td>≥60</td>
<td>23</td>
<td>9.13</td>
<td>&lt;1e-4</td>
</tr>
<tr>
<td>Tx = N1 plus VEGF-A</td>
<td>&lt;60</td>
<td>2</td>
<td>13.41</td>
<td>0.456</td>
</tr>
<tr>
<td></td>
<td>≥60</td>
<td>1</td>
<td>3.46</td>
<td>0.456</td>
</tr>
<tr>
<td>Tx = N1 plus VEGF-A</td>
<td>≥60</td>
<td>5</td>
<td>5.26</td>
<td>0.456</td>
</tr>
<tr>
<td>Tx = N2 plus VEGF-A</td>
<td>&lt;60</td>
<td>18</td>
<td>3.87</td>
<td>&lt;1e-4</td>
</tr>
<tr>
<td></td>
<td>≥60</td>
<td>18</td>
<td>3.87</td>
<td>&lt;1e-4</td>
</tr>
<tr>
<td>Tx-N1 plus VEGF-A, Pre-Tx + end of IND</td>
<td>Pre-Tx &lt;30 + end of IND &lt;60</td>
<td>1</td>
<td>5.08</td>
<td>0.456</td>
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<tr>
<td></td>
<td>Pre-Tx ≥30 + end of IND ≤60</td>
<td>1</td>
<td>1.58</td>
<td>0.456</td>
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<tr>
<td></td>
<td>Pre-Tx &lt;30 + end of IND ≥60</td>
<td>1</td>
<td>3.09</td>
<td>0.456</td>
</tr>
<tr>
<td></td>
<td>Pre-Tx ≥30 + end of IND ≥60</td>
<td>8</td>
<td>1.26</td>
<td>&lt;1e-4</td>
</tr>
</tbody>
</table>

N1, PEGylated asparaginase arm; N2, native asparaginase arm.

*When the asparaginase treatment was considered, there was no change in the significance of VEGF-A 30 or 60 pg/mL as cutoff values.

\( b \) The first two groups N1 or N2 were not significantly different.

\( c \) The N2 randomized patients had a superimposable pattern as the N1 patients.

\( d \) In the treatment N1 (PEGylated asparaginase arm), when the serum VEGF-A concentrations were >30 pg/mL at entry to induction treatment plus >60 pg/mL and the end of induction treatment, these parameters were highly significantly predictive factors for event (log-rank, \( P < 1e-4 \)).

significantly related to the risk of an event, with patients of ages >8 years having 2.87 times the risk of an event than the patients of ages ≤8 years (\( P = 0.011; \) 95% confidence interval, 1.27-6.44). A multivariate Cox proportional hazards model indicated that both age and VEGF-A risk grouping were significantly related to time to events (Tables 2 and 3).

Lastly, we examined the significance of serum VEGF-A ≥30 at entry plus ≥60 pg/mL at the end of induction in all 112 patients (Table 3). The findings showed that high VEGF-A levels at entry to induction plus higher levels at the end of induction was the most powerful significant treatment-independent factor for event (\( P < 1e-4 \); log-rank test; Table 2). The combined effect of low VEGF-A, <30 pg/mL at entry to induction or <60 pg/mL at the end of induction treatment, showed that there were four subsets of patients. Set 1 had 2 events in 51 patients (3.92% events or long-term 96% EFS) who had VEGF-A levels <30 pg/mL at entry and <60 pg/mL at the end of induction treatment; set 2 had 1 event in 13 patients (7.7% events or 92% EFS) who had ≥30 pg/mL at entry to induction and ≤60 pg/mL at the end of induction. In contrast to these patients, set 3 had 5 events in 22 patients (20.8% events or 79% EFS) who had <30 pg/mL at entry to induction and ≥60 pg/mL at the end of induction (positive ΔVEGF-A). Lastly, set 4 had 18 events in 24 patients (75% events or 25% EFS) who had VEGF-A concentrations ≥30 pg/mL at entry to induction and ≥60 pg/mL at the end of induction treatment (high positive ΔVEGF-A).

**Discussion**

We have shown that high or increasing VEGF-A serum concentrations during induction are correlated with events and poor survival of standard-risk ALL pediatric patients. The VEGF-A levels changed during therapy. As induction treatment proceeds, at least two processes are in effect: leukemic cell die and hematopoietic stem cell growth is inhibited. However, towards the end of induction, the chemotherapy-induced myelosupression promotes many “host” responses, one of which is the stromal and epithelial cell response of secreting many growth factors, including VEGF-A (9, 12, 14). Some hematopoietic growth factors stimulate marrow regeneration and prevention of infections associated with chemotherapy-induced neutropenia (19). Aguayo et al. (23) have shown that VEGF-A and related growth factors enhance the bone marrow microvasculature in bone marrow biopsies in relapsed leukemia patients. Quiescent residual leukemia stem cells may also be stimulated into cell replication by VEGF-A and other growth factors (2, 4, 5, 9, 12, 15, 23, 24, 31, 39, 40).

Neoangiogenesis is the process by which the vasculature is expanded and remodeled to form the networks of the growing microvasculature in bone marrow biopsies in relapsed leukemia patients. Quiescent residual leukemia stem cells may also be stimulated into cell replication by VEGF-A and other growth factors (2, 4, 5, 9, 12, 15, 23, 24, 31, 39, 40).

We have shown that high or increasing VEGF-A serum concentrations during induction are correlated with events and poor survival of standard-risk ALL pediatric patients. The VEGF-A levels changed during therapy. As induction treatment proceeds, at least two processes are in effect: leukemic cell die and hematopoietic stem cell growth is inhibited. However, towards the end of induction, the chemotherapy-induced myelosupression promotes many “host” responses, one of which is the stromal and epithelial cell response of secreting many growth factors, including VEGF-A (9, 12, 14). Some hematopoietic growth factors stimulate marrow regeneration and prevention of infections associated with chemotherapy-induced neutropenia (19). Aguayo et al. (23) have shown that VEGF-A and related growth factors enhance the bone marrow microvasculature in bone marrow biopsies in relapsed leukemia patients. Quiescent residual leukemia stem cells may also be stimulated into cell replication by VEGF-A and other growth factors (2, 4, 5, 9, 12, 15, 23, 24, 31, 39, 40).

Fig. 3. Correlation between high VEGF-A levels and EFS of standard-risk ALL pediatric patients. Kaplan-Meier survival plots of EFS dichotomized by the VEGF-A concentrations in serum, >60 pg/mL at the end of induction treatment in the event (n = 26) and EFS (n = 86) of standard-risk ALL pediatric patients. There was a significant difference in EFS (log-rank, P < 1e-4; Table 2) between patients with high and low VEGF-A concentrations in serum.
cells in vitro has been shown to reduce VEGF-A secretion from leukemia three vincristine doses during induction in CCG-1962, which hematopoiesis. remission may be related to the renewal of normal basic fibroblast growth factor in pediatric ALL patients in concluded that the increment in both serum VEGF-A and control values obtained at diagnosis (26, 48). These studies trend of serum VEGF levels during remission, reaching patients who had complete remission had an increasing patients at diagnosis was statistically significantly lower than solid tumors, experience in childhood ALL has been limited overexpression of VEGF-receptor-2 (KDR) and VEGF-A secre- tion have been reported in acute myeloid leukemia patients, with the latter being an independent inverse prognostic factor for survival without relapse (21, 35, 40, 47). Although VEGF-A and basic fibroblast growth factor have been suggested as reliable prognostic indicators and important tools for treatment approach in malignant hematopoietic and solid tumors, evidence in childhood ALL has been limited to only one study on angiogenesis and basic fibroblast growth factor (26). The median level of serum VEGF in ALL patients at diagnosis was statistically significantly lower than in the control group and at remission. In that study, 26 of 31 patients who had complete remission had an increasing trend of serum VEGF levels during remission, reaching control values obtained at diagnosis (26, 48). These studies concluded that the increment in both serum VEGF-A and basic fibroblast growth factor in pediatric ALL patients in remission may be related to the renewal of normal hematopoiesis.

In our study, standard-risk ALL patients were treated with three vincristine doses during induction in CCG-1962, which has been shown to reduce VEGF-A secretion from leukemia cells in vitro (30). In these studies, VEGF-A levels in the EFS patients averaged 28 pg/mL at the end of induction and <44 pg/mL in the other phases of treatment. Similar values for VEGF-A were reported recently in other studies in patients with solid tumors, acute myeloid leukemia patients, and in healthy blood donors (48–51). Relapsed patients who had significantly elevated levels of VEGF-A in serum at the beginning of induction responded to vincristine treatment by a log-linear decrease of these concentrations up to day 14 (Fig. 2). However, the serum levels increased soon afterwards and increased further at delayed intensification-1 and delayed intensification-2 to levels that exceeded 120 pg/mL. Conversely, most patients in long-term EFS had either low VEGF-A levels in serum at entry or the levels became undetectable during induction treatment. These low VEGF-A levels from event-free patients have recently been reported in sera of healthy blood donors or in patients with adult leukemias (43, 50). In our study, the average VEGF-A levels at entry to induction were correlated with time of relapse (Fig. 3). We hypothesize that the high VEGF-A in patients who eventually relapsed may have allowed more leukemic blasts to survive in sanctuary sites.

The findings from the Kaplan-Meier analyses suggested that, because VEGF-A serum concentrations during induction ther- apy are independently predictive of treatment outcome with a combination chemotherapy regimen, the existence of distinct leukemic subgroup blast populations (leukemic stem cell) exhibiting either intrinsic or acquired chemotherapy resistance, facilitated by the ligand-VEGF-receptor signal transduction, leads to further activation of antiapoptotic proteins and survival. Furthermore, these data suggested that an anti-VEGF-A modality treatment should be considered to be used during the last 2 weeks of induction. This evidence is in accordance with the other adult oncology studies in acute and chronic myeloid leukemias and lymphomas (44–46, 48–51). Lastly, similar correlative studies are in progress to verify these findings in the high-risk ALL study (CCG-1961) and in a prospective new study in leukemias.

In summary, the results of this study contributed to a better understanding of the prognostic significance of serum VEGF-A levels and proposed that high levels may facilitate the survival and growth of leukemic blasts and disease progression. Evidence was provided that VEGF-A at entry or at the end of induction treatment is of paramount importance and is correlated with factors predictive for an unfavorable prognosis for pediatric standard-risk ALL patients with high serum levels. Overall, a better understanding of the importance of this growth factor in circulation exists and it may open new avenues for therapeutic interventions aimed to decrease the serum VEGF-A levels and minimize or prevent relapses in ALL patients. Finally, the results have clearly demonstrated that VEGF-A concentrations in induction are a clinically relevant factor for screening high-risk ALL patients and in monitoring their treatment outcome.

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### Table 3. Multivariate results of Cox proportional hazards model for VEGF-A risk groups and age

<table>
<thead>
<tr>
<th>Time to event</th>
<th>Hazard rate</th>
<th>SE</th>
<th>P &gt;</th>
<th>95% Confidence interval</th>
</tr>
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<tr>
<td>Reference IND &lt;60, age ≤8 y</td>
<td>1.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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<td>Pr &lt;30, IND &gt;60</td>
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<td>4.74</td>
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<td>20.47</td>
<td>0.000</td>
<td>9.38-111.79</td>
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<tr>
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<td>1.26</td>
<td>0.009</td>
<td>1.32-6.87</td>
</tr>
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</table>

Abbreviation: Pr, pretreatment.
References


Correlation between High Vascular Endothelial Growth Factor-A Serum Levels and Treatment Outcome in Patients with Standard-Risk Acute Lymphoblastic Leukemia: A Report from Children's Oncology Group Study CCG-1962

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