

# Does Chemotherapy Change Expression of VEGF A&C and MVD in Acute Myeloid Leukemia?

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## ABSTRACT

**Introduction:** Acute Myeloid Leukemia is a malignant transformation of hematopoietic tissue, bone marrow infiltration of undifferentiated cells known as blasts that interfere with the production of normal cells. Vascular endothelial growth factor (VEGF) is persistently secreted from myeloid cells and high levels can be detected in patients' serum.

**Methods:** Twenty-one AML patients, who were chemotherapy candidates were evaluated in a clinical trial. Serum VEGF was measured by ELISA. VEGFA, VEGFC mRNA and bone marrow MVD were measured in all patients before and after chemotherapy and then all results were analyzed.

**Results:** There were 10 (48%) female and 11 (52%) male patients ranged in age from 20 to 60 years, with an average age of  $39.5 \pm 14.1$  years. The mean amount of MVD was reduced from  $10.8 \pm 3.6$  before chemotherapy to  $7.6 \pm 3.3$  after chemotherapy ( $P=0.008$ ). VEGF was also reduced from  $0.59 \pm 0.16$  before chemotherapy to  $0.24 \pm 0.03$  after chemotherapy ( $P=0.005$ ). Gene expression differences for VEGFA mRNA was  $4.6 \pm 1.4$ , while it was  $120.7 \pm 93.2$  for VEGFC mRNA, showing the significance only for VEGFA mRNA ( $P=0.02$ ).

**Conclusion:** Regarding reduced angiogenesis, we can conclude that anti-angiogenic preparations can be effective in treatment course of AML in combination with chemotherapy regimen.

**KEYWORDS:** Angiogenesis, VEGF, AML, MVD

## INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by accumulation of immature malignant myeloid cells in the bone marrow and blood due to their clonal proliferation without substantial maturation.<sup>1</sup> The crucial role of angiogenesis in the growth, persistence, and metastases of solid tumors has been indicated in many studies.<sup>2, 3</sup> Moreover, the importance of angiogenesis in the pathogenesis of hematologic malignancies has been recognized recently. Previous studies have shown that

microvessel density (MVD) increases in the bone marrow of AML patients compared to normal groups.<sup>4, 5, 6</sup> Angiogenesis is controlled by a balance between proangiogenic and antiangiogenic growth factors and cytokines.<sup>7, 8</sup> One of the most key regulators of angiogenesis is the vascular endothelial growth factor (VEGF), increasing permeability and promoting proliferation, migration, and differentiation of endothelial cells. The most responsible factor for angiogenesis is hypoxia which induces expression of VEGF.<sup>8</sup> The VEGF family includes 5 glycoproteins: VEGF-A,

VEGF-B, VEGF-C, VEGF-D, and PGF.<sup>9</sup> Among the VEGF family, it is known that VEGF-A and VEGF-C are expressed by AML cells.<sup>10,11</sup>

It has been shown that VEGF stimulates a mitogenic response in hematologic malignancies and promotes self-renewal of leukemia progenitors.<sup>12, 13</sup> The role of VEGF-A as a proangiogenic factor in AML has been well documented.<sup>14</sup> Furthermore, recent studies have revealed the contribution of VEGF-C in the progression of hematologic malignancies.<sup>15, 16, 17</sup> However, in spite of the evidence of the angiogenic role of VEGF in AML, there are investigations reporting lower VEGF-C<sup>23, 24</sup> and VEGF-A<sup>18</sup> expression in the AML patient's bone marrow. Studies have shown that VEGF in blast cells of patients with acute myeloid leukemia is continuously produced and secrete increased levels in serum of patients.<sup>19</sup> According to numerous studies with varying results, we designed this study to evaluate gene expression of VEGF-A and VEGF-C and MVD in AML patients before and after chemotherapy. On the other hand, if angiogenesis decreases after chemotherapy, anti-angiogenic medication can be used as an adjunct in the treatment of acute myeloid leukemia.

## MATERIALS AND METHODS

### Study design

Twenty-one patients with AML were enrolled in a clinical trial conducted between 2009 and 2010 in the Hematology-Oncology Research Center, Iran, Tabriz.

### Patients and Samples

The initial diagnosis of AML and its subtypes were determined according to the French-American-British classification. AML smears were routinely investigated at the same hospital and subtyping was confirmed by Flow Cytometry. Bone marrow aspiration (BMA), bone marrow biopsy (BMB) and serum level of VEGF were measured, before chemotherapy (pre-treatment) and 30 days after chemotherapy (post-treatment). The level of VEGF was determined by a specific enzyme-linked immunosorbent assay (IBL, Hamburg, Germany) with a detection limit of 20 pg/mL of VEGF. All patients were treated with standard chemotherapy

with Cytarabine/ Daunorubicin (7 + 3) protocols. Remission status was evaluated after the completion of cancer therapy according to conventional criteria. This study was approved by the local ethics committee.

### Analysis of Microvascular Density (MVD)

Immunohistochemical staining was performed on formalin fixed, paraffin-embedded specimens. Hematoxylin and Eosin stained sections of patients before and after chemotherapy were examined and appropriate tissues for immunohistochemistry were further processed. Before staining, tissue sections (4 mm thick) were dewaxed, micro waved and rehydrated; endogenous peroxidase activity and non-specific binding were blocked by incubation with 3% hydrogen peroxide and non-immune serum, respectively. Vessels were highlighted by staining endothelial cells for VWF (DAKO Corporation, working dilution 1/500) antigens according to standard avidin- biotin method. Three hot spot points (representing areas of most intense vascularity) were determined by scanning the slide with low power lens (100 X magnifications) after agreement between 2 observers. The image was captured by 400X power and was analyzed for vessel number. For analysis of vascular number, the number of micro-vessels in the representative high power field was counted and the results were calculated for the number of vessels being present in 1-2 mm of the bone marrow. Only micro-vessels were considered and megakaryocytic (which strongly stain with anti-VWF antibody) areas of vascularization adjacent to the bone or dense connective tissue were omitted from analysis. Clearly discrete single-sprout endothelial cells as well as lumen containing vessels were counted (no vessel was counted in the absence of VWF). Mean vessel number of the 3 selected 400X fields were expressed as micro vascular density (MVD) of the bone marrow for each patient at any given time point.

### Bone marrow sampling and Leukemic cell isolation:

EDTA-added bone marrow aspirate was collected from AML patients. Mononuclear Cells were isolated by Ficoll- Hypaque density gradient

centrifugation. The remaining pellet was T-cell-depleted by sheep RBCs and separated over Ficoll density gradient. The remaining blast cell population contained >95% AML cells and is referred to hereafter as AML cells.

#### RNA Extraction and First-strand cDNA synthesis:

Total RNA was extracted from AML cells using Trizol Reagent (Invitrogen, USA) according to the manufacturer's description and treated with RNase-free DNase to remove any residual genomic DNA. Single stranded cDNAs were synthesized by incubating total RNA (1µg) with Revert Aid H Minus M-MuL V Reverse transcriptase (200 U), oligo-(dT) primer (5 µM), Random Hexamer Primer (5 µM), dNTPs (1 mM) and RiboLock RNase inhibitor (20 U) for 5 min at 37°C, followed by 5 min at 25°C and 60

min at 42°C in a final volume of 20 µl, respectively. Reaction was terminated by heating at 70°C for 5 min.

#### Quantitative Real-Time RT-PCR:

Quantitative Real Time PCR was done using the Corbett Life Science (Rotor-Gene 6000) system using 2 µL of a 2-fold diluted cDNA, 150 nM of primers and 1 × FastStart SYBR Green Master (Roche) in a final volume of 20 µL. β-actin (ACTB) mRNA expression levels were used to calculate the relative expression levels. The specificity of the PCR reactions was verified by melting curve analysis and electrophoresis in a 1.5% agarose gel. The relative quantification was performed by  $2^{-\Delta\Delta Ct}$  formula. (Table-1)

**Table-1:** Sequences of primers

Gene	Forward Primer	Reverse Primer
VEGFA	GAGTGTGTGCCACTGAGGAGTCCAAC	CTCCTGCCCGGCTCACCGCCTCGGCTT
VEGFC	GATCTGGAGGAGCAGTTAGG	GAGTTGAGGTTGGCCTGTTC
B-actin	GCTGTGCTACGTCCGCTG	GGAGGAGCTGGAAGCAGCC

#### Statistical analysis

Expression of VEGF-A and VEGF-C was obtained through the Corbett Rotor-Gene 6000. Normal distribution of data was evaluated using Stata software with q-norm program version 11. Data was analyzed by statistical SPSS software, version 16. Variables that had normal distribution were reported as means and standard deviations. Medians were reported for the variables whose distribution deviated from the normal distribution. Comparisons between independent groups were performed with the Mann–Whitney test. Differences between diagnostic groups were evaluated using the Kruskal–Wallis test and comparisons between gene expression levels obtained before and after chemotherapy were evaluated with the Wilcoxon signed-rank test. Correlation between continuous variables was studied with the Spearman's rank correlation (rs). All tests were two-tailed and a 5% significance level was applied.

#### RESULTS

Twenty-one patients were enrolled in this study. There were 10 (48%) female and 11(52%) male patients ranged in age from 20 to 60 years, with an average age of 39.5 ±14.1 years. All of selected patients had WBC > 10 × 10<sup>9</sup>/L. AML subtypes (according to the French-American-British) classification were 3(14.3%) M1, 8(38.1%) M2, 2(9.5%) M3, 4(19%) M4 and 4(19%) M5.

#### Bone Marrow Microvascular Density (MVD)

There was a significant reduction in bone marrow hot-spot vascular density of patients, as calculated by mean vessel number in 3 hot-spot fields. Bone marrow vascular density was reduced as identified by anti-VWF immunohistochemistry staining (mean before treatment = 10.8 mm<sup>-2</sup> ± 3.6(SEM), mean after treatment = 7.6±3.3 (SEM) (p=0.008). (Table-2)

**Table 2:** A comparison of changes in MVD and VEGF in AML

Variable	Pre Chemotherapy (Mean±SD)	Post Chemotherapy (Mean±SD)	P value
MVD	10.8±3.6 (4.6-18)	7.6±3.3 (3.3-15.3)	P=0.008
VEGF(pg/ml)	0.59±0.16 (0.1-2.9)	0.24±0.03 (0.1-0.75)	P=0.005

### Expression and Secretion of VEGF

The variable levels of VEGF were found before chemotherapy ( $0.59 \pm 0.74$ ) and after chemotherapy ( $0.24 \pm 0.15$ ) ( $P=0.05$ ) (Table-2)

There was no significant relationship between VEGF and AML subtypes ( $P=0.41$ ) (Table -3).

**Table 3:** A comparison of the mean level of VEGF and AML subtype

FAB classification	Mean pre Chemotherapy VEGF( <sup>pg/ml</sup> )	Mean post Chemotherapy VEGF( <sup>pg/ml</sup> )
M1, n=3	0.2	0.1
M2, n=7	0.586	0.257
M3, n=2	1.65	0.475
M4, n=4	0.4	0.225
M5, n=4	0.55	0.25

### Association between VEGF-A and VEGF-C expression in leukemic cells and MVD

There were no significant correlations between VEGF-A and VEGF-C transcripts in leukemic cells before treatment and after completion of treatment with MVD. ( $r_s = -0.090$ ,  $P=0.751$  or  $r_s = -0.043$ ,  $P=0.875$  and  $r_s = 0.423$ ,  $P=0.150$  or  $r_s = -0.216$ ,  $P=0.458$ ).

### Gene Expression

#### VEGF-A and VEGF-C mRNA expression and response to chemotherapy:

Data analysis showed significant differences in median expression of VEGF-C and VEGF-A mRNA in leukemic cells before and after chemotherapy ( $4.6 \pm 1.4$ ,  $120.7 \pm 93.2$ ). ( $P=0.02$ , and  $P=0.21$ )

#### Association between VEGF-A or VEGF-C expression in leukemic cells and clinical features:

There is no significant correlation between gene expression levels of VEGF-A or VEGF-C and FAB subtypes, peripheral white blood cells (WBC) count, blast count, hemoglobin value, platelet count, age and sex.

### DISCUSSION

Myeloblasts are continuously produced and secreted by VEGF in serum of patients with acute myeloid leukemia.<sup>19</sup> VEGF binds to cell surface, causes phosphorylation and transmission of messages into the cell and accelerates the cell proliferation. Also, the effect of VEGF on the endothelial cells of blood vessel causes release of

growth factors, leading to the proliferation of malignant cells.<sup>20</sup>

In a study by Jeha et al, the expression levels of VEGF in adult and pediatric patients with AML were studied.<sup>21</sup> The results showed that VEGF levels were significantly lower in children than in adults, whereas no significant difference in serum levels of VEGF was found between adults and children with AML.<sup>21</sup> In our study, conducted only on adults with AML, the median VEGF before and after chemotherapy was  $0.59 \pm 0.16$  pg/ml and  $0.24 \pm 0.03$  ( $P=0.005$ ), respectively.

In a study by Teng et al, MVD in patients with AML was 7.29.<sup>18</sup> In our study, MVD was reported  $10.8 \pm 3.6$  and  $7.6 \pm 3.3$  before and after chemotherapy, respectively ( $P=0.008$ ).

Fielder et al. reported the VEGF-C expression in the leukemic cells of most AML patients.<sup>22</sup> Furthermore, Dias et al. demonstrated that VEGF-C, released from the endothelium, induced proliferation and promoted survival of AML cells.<sup>16</sup>

Liersch et al evaluated the relationship between VEGF-C and the extent of angiogenesis in leukemic patients. The results suggest that serum VEGF-C levels were higher in patients than in normal subjects, but was not much helpful in predicting patient survival.<sup>20</sup> In our study, we showed significant differences between median expression of VEGF-C and VEGF-A mRNA in leukemic cells before and after chemotherapy ( $4.6 \pm 1.4$ ,  $120.7 \pm 93.2$ ). ( $P=0.02$ , and  $P=0.21$ )

Imai et al., studied the effect of monoclonal antibodies against VEGF, inhibiting cell growth in AML patients.<sup>23</sup> The results showed that the

inhibition of VEGF or angiogenesis in AML patients in remission was associated with greater success. On the other hand, they mentioned the use of thalidomide as an anti-angiogenic drug.<sup>23</sup>

The results of our study demonstrated a reduction in angiogenesis after chemotherapy, and anti-angiogenic drugs such as thalidomide as well as chemotherapy regimen used in this study were considered effective in the treatment of acute myelogenous leukemia.

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#### CONFLICT OF INTEREST

All authors declare no conflict of interest.

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