Effect of High VEGF-C mRNA Expression on Achievement of Complete Remission in Adult Acute Myeloid Leukemia

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Abstract

Although vascular endothelial growth factor-C (*VEGF-C*) is known to be expressed in acute myeloid leukemia (AML) blasts, the relevance of *VEGF-C* in the clinical setting remains to be fully explored. We examined the effect of *VEGF-C* on achievement of complete remission (CR) in adult *de novo* AML and immune cell population profiles according to *VEGF-C* mRNA expression. In comparison of *VEGF-C* expression between the no-CR and CR groups, the CR group showed a trend toward higher levels of plasma VEGF-C ($P = .088$), whereas mRNA expression of *VEGF-C* was downregulated ($P = .008$). Next, patients with continuous data for *VEGF-C* were divided into two groups (low vs. high) by a ROC curve analysis. The low- versus high-level groups for plasma VEGF-C (RR of 0.20, $P = .030$), mRNA expression of *VEGF-C* (RR of 18.75, $P = .003$), and the ratio of plasma level to mRNA expression (RR of 0.05, $P = .007$) were potential predictors of CR on univariate analysis. After adjusting for potential clinical factors including genetic group, multivariate analyses revealed that high *VEGF-C* mRNA expression was an independent risk factor for failure of induction chemotherapy. Furthermore, patients with high *VEGF-C* mRNA expression had a lower frequency of NKT and CD8^{+} cells and showed a trend for a lower frequency of NK cells. These results suggest that interruption of VEGF-C signaling might be a potential therapeutic target for antileukemic treatment in AML patients.

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Introduction

Vascular endothelial growth factor-C (*VEGF-C*) is a lymphangiogenic growth factor that generally transmits intracellular signals resulting in cell proliferation and survival [1,2]. Previous studies have reported that *VEGF-C* is important for cancer progression based on the autocrine VEGF-C loop promoting the invasion and metastasis of cancer cells, as well as the spread of cancer cells by active recruitment of new lymphatics by tumor-derived VEGF-C.

Six *VEGFs (A-F)* are known [3,4], of which *VEGF-A* and *VEGF-C* were reported to be expressed by acute myeloid leukemia (AML) cells [5,6]. VEGF-C signals through kinase insert domain receptor (KDR, i.e., VEGF receptor-2) and fms-related tyrosine kinase 4 receptor (FLT4; i.e., VEGF receptor-3) [7,8]. A few studies demonstrated that high *VEGF-C* mRNA expression of AML blasts was related to increased *in vitro* and *in vivo* drug resistance and could predict adverse long-term outcomes. Dias et al. showed that exogenously added VEGF-C promotes *in vitro* cell proliferation, survival, and resistance to chemotherapy by signaling

through FLT4 [9]. De Jonge et al. reported that endogenous *VEGF-C* mRNA expression levels of primary AML cells were related to increased *in vitro* resistance to typical AML drugs. In addition, they showed a relationship between high *VEGF-C* mRNA expression and slow AML blast disappearance during induction treatment *in vivo* [10].

In addition to the effect of the autocrine VEGF-C loop, we previously reported that natural killer (NK) cells expressing high levels of FLT4 in AML patients have a low killing potential and low levels of

interferon (IFN)-γ [11,12] and, furthermore, showed that FLT4 inhibition restored NK cell killing function with increased IFN-γ levels in a leukemic mouse model [13]. Currently, the importance of immune reinforcement in cancer therapy has been emphasized, and restoration of the immune system is known to be important in AML treatment [14,15]. Based on these findings, in this prospective study, we explored the effect of VEGF-C on achievement of complete remission (CR) in adult de novo AML with consideration of other established prognostic markers, such as cytogenetics and gene mutation. In addition, we investigated immune cell population profiles according to VEGF-C mRNA expression.

Materials and Methods

Patients and Treatment Procedures

First, the expression of VEGF-C was compared among BM samples taken from NDAML (n = 58), complete remission status (n = 26), and refractory/relapsed AML (n = 15). Second, to evaluate the predictive role of VEGF-C for achievement of CR, 58 consecutive adult patients with NDAML between June 2015 and March 2016 were analyzed in this study (Figure 1). Patients who had acute promyelocytic leukemia, therapy-related AML, and secondary AML were excluded. Among the enrolled patients, 48 were included in response assessment after induction chemotherapy; 10 patients who were treated with induction chemotherapy with reduced intensity due to age and severe comorbidity (n = 4), experienced early mortality (n = 5), or were transferred (n = 1) were not assessed. The induction chemotherapy regimen consisted of '3+7' idarubicin (12 mg/m^2) plus ARA-C (100 mg/m^2 continuously infused for 24 hours). To include plasma levels and mRNA expression of VEGF-C in addition to baseline biological characteristics as variables affecting the achievement of CR, BM samples were collected at the time of diagnosis. All clinical data including response assessment were prospectively collected. Cytogenetic and molecular genetic data were available for all patients, and genetic group (favorable, intermediate-I, intermediate-II, and adverse) was defined according to the European LeukemiaNet [16]. Written informed consent was obtained from each patient before participation in the study. This study was approved by the Institutional Review Board of the Catholic University of Korea and was conducted in accordance with the Declaration of Helsinki.

Isolation of Bone Marrow Mononuclear Cells

Mononuclear cells were separated from BM samples by density gradient centrifugation using Ficoll Paque Plus (17-1440-03; GE Healthcare Life Sciences, Piscataway, NJ). Plasma samples were stored at –80°C, and mononuclear cells were stored in a liquid nitrogen tank.

VEGF-C ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed using commercially available kits from Abcam (Cambridge, MA) according to the manufacturer's instructions. The results represent the mean values of duplicate determinations.

Quantitative Reverse Transcription (qRT)-PCR Analysis

Total RNA was extracted from mononuclear cells of BM samples. RNA (1 µg) was reverse transcribed into cDNA at 42°C for 60 minutes in a 20-µl reaction mixture using a Transcriptor First Strand cDNA Synthesis Kit (04 897 030 001; Roche, Mannheim, Germany). Primer sequences are given in Supplemental Table 1. qRT-PCR was performed with the fluorescent dye SYBR Green I Master (04 707 516 001; Roche, Mannheim, Germany) using LightCycler 480 (Roche). All data were normalized to the amount of GAPDH expression, with samples run in duplicate.

Flow Cytometric Analysis

Mononuclear cells were suspended in 100 µl of phosphate-buffered saline and incubated with antibodies. After washing, cells were analyzed using a FACSCalibur flow cytometer equipped with Cell Quest software (BD Biosciences, San Diego, CA) and FlowJo software. The antibodies used to detect cells included APC-conjugated anti-human CD1d tetramer (Proimmune, D001-4A), FITC-conjugated anti-human CD3 (BD, 555339), APC-conjugated anti-human CD56 (BD, 555518), FITC and PE-conjugated anti-human CD3/CD16+CD56 (340042, BD Simultest), PE-Cy5-conjugated anti-human CD4 (555348, BD Pharmingen), PerCP-conjugated anti-human CD8 (347314, BD Pharmingen), and PE-conjugated anti-human CD19 (340364, BD Pharmingen). Flow cytometric data were analyzed using appropriate controls of proper isotype-matched IgG and unstained samples.

Definitions and Statistical Analysis

The study objectives were to identify the association of VEGF-C expression with achievement of CR after induction chemotherapy
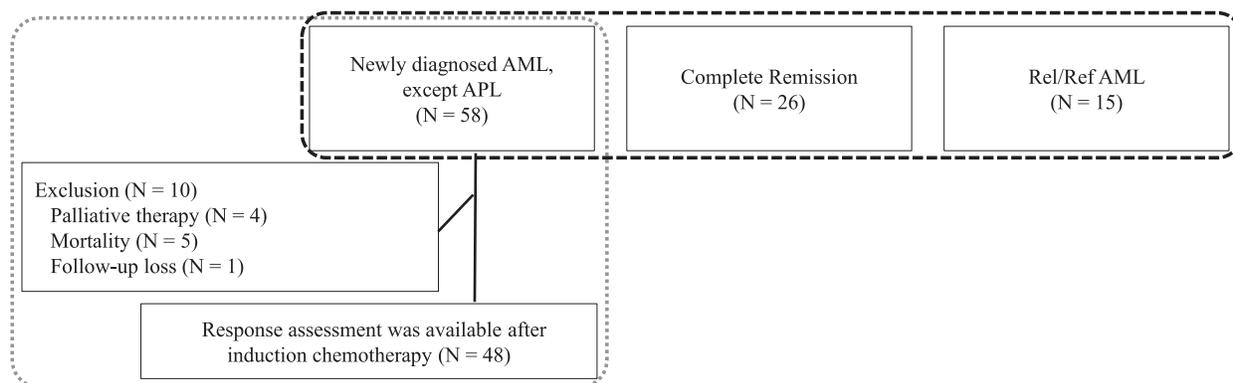

Figure 1. Diagram of analyzed patients. Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; Rel/Ref; relapsed/refractory.
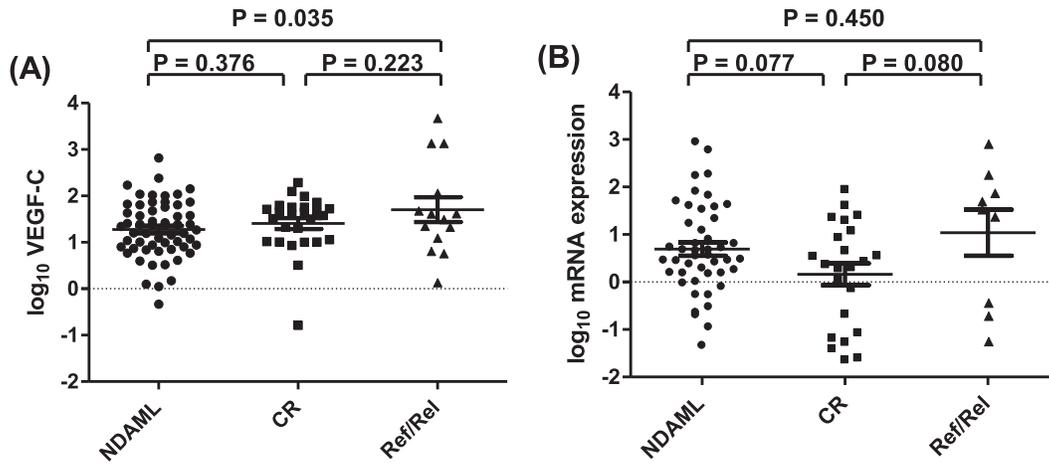
Figure 2. Comparison of *VEGF-C* expression between newly diagnosed AML (NDAML), complete remission (CR), and relapsed/refractory (Ref/Rel) AML. Bone marrow samples were taken from patients with NDAML ($n = 58$), CR ($n = 26$), and Ref/Rel AML ($n = 15$). The plasma levels of VEGF-C (A) and mRNA expression of *VEGF-C* (B) were compared between NDAML, CR, and Ref/Rel AML using the values after log transformation. Data are presented as mean ± SEM, and *t* test was used to compare the continuous variables.

and to evaluate the difference in immune cell population profiles according to *VEGF-C* mRNA expression. For the response assessment, BM aspirates were independently evaluated under a microscope by two examiners blinded to clinical outcomes. In all cases of disagreement between examiners, a common reading was organized to achieve a consensus on the count. Differential counts were recorded after a 500-cell count, and definition of CR was based on BM blasts <5%, absence of blasts with Auer rods, absence of extramedullary disease, and hematologic recovery. The Chi-square test and Fisher's exact test were used to test the correlation of categorical variables. The 2-tailed Student's *t* test was used to analyze

Table 1. Patient Characteristics

Parameter	All Patients ($n = 48$) (%)
Age (years), median (range)	46 (20-72)
Sex	
Female	23 (47.9)
Male	25 (52.1)
WBC (×10⁹/l), mean ± SE	45105.4 ± 74878.2
PB blast (%), mean ± SE	50.9 ± 31.1
NPM1[a]	
Negative	37 (84.1)
Positive	7 (15.9)
FLT3-ITD[a]	
Negative	37 (84.1)
Positive	7 (15.9)
CEBPA[a]	
Negative	31 (70.5)
Positive	13 (29.5)
BALLC RQ-PCR,[b] mean ± SE	2.3 ± 3.5
WT1 RQ-PCR,[b] mean ± SE	0.5 ± 0.4
Genetic group (ELN)	
Favorable	10 (20.8)
Intermediate-I	18 (37.5)
Intermediate-II	10 (20.8)
Adverse	10 (20.8)

ELN, European Leukemia Net; *NA*, not available; *RQ-PCR*, quantitative real-time polymerase chain reaction; *PB*, peripheral blood; *WBC*, white blood cell.

[a] Not available ($n = 4$).

[b] RQ-PCR levels represent the ratio of molecular expression compared to expression of normal *ABL1* (copies/10⁴ *ABL1*).

continuous variables. The predictive value of plasma levels and mRNA expression of *VEGF-C* for achievement of CR was assessed using logistic regression in a cohort including patients who received standard induction chemotherapy regimen. Covariates with a *P* value less than .1 in univariate analyses were added to the multivariate analysis model.

Results

VEGF-C Expression of AML Patients

Expression of *VEGF-C* in the bone marrow (BM) of newly diagnosed *de novo* AML (NDAML) patients prior to induction chemotherapy ($n = 58$) was compared with that of patients with CR status ($n = 26$) and RRAML ($n = 15$), (Figure 2). Plasma levels of VEGF-C for NDAML, CR status, and RRAML were 47.87 ± 12.4 pg/ml, 44.57 ± 8.4 pg/ml, and 519.2 ± 319.9 pg/ml, respectively. When these values were compared after log transformation, the plasma level of VEGF-C in NDAML was lower than that in RRAML ($P = .035$) but not significantly different from that in CR status ($P = .376$) (Figure 2A). In contrast, mRNA expression of *VEGF-C* in CR status showed a trend toward a lower level than that in NDAML ($P = .077$) and RRAML ($P = .080$) (Figure 2B).

Relationship Between VEGF-C and CR Achievement

Next, we evaluated the association of *VEGF-C* with achievement of CR after induction chemotherapy. Forty-eight evaluable patients who received standard induction chemotherapy were divided into two groups (13 in the no-CR group versus 35 in the CR group). The distribution of favorable, intermediate-I, intermediate-II, and adverse genetic groups based on the European LeukemiaNet was 21%, 38%, 21%, and 21%, respectively. Table 1 lists patient demographic information. We measured the plasma levels and mRNA expression of *VEGF-C* in BM samples taken at the time of diagnosis and compared them between the no-CR and CR groups. The CR group showed a trend toward a higher level of plasma VEGF-C ($P = .088$) (Figure 3A), whereas mRNA expression of *VEGF-C* was downregulated ($P = .008$) (Figure 3B).
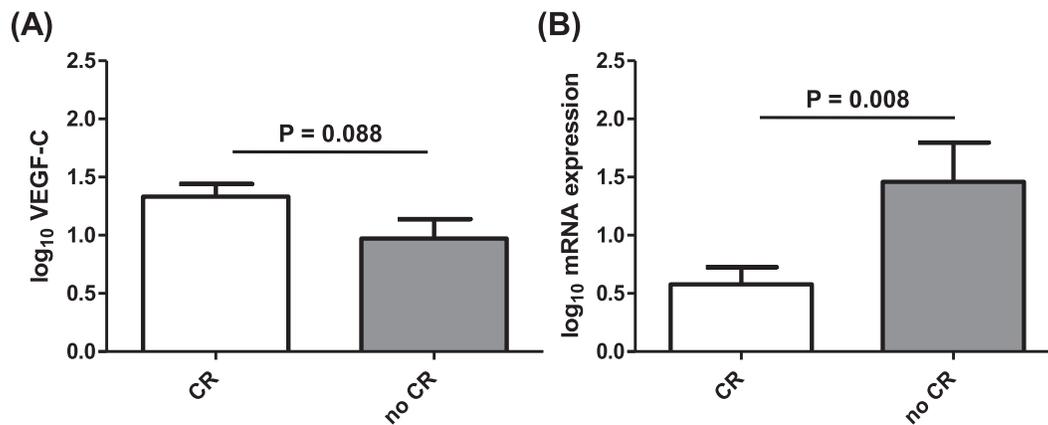
Figure 3. Predictive role of *VEGF-C* for achievement of complete remission; analyses of consecutive samples. The plasma levels of VEGF-C (A) and mRNA expression of *VEGF-C* (B) in BM samples taken at the time of diagnosis are shown after log transformation. The CR group showed a trend toward higher level of plasma VEGF-C, whereas mRNA expression of *VEGF-C* was downregulated. Data are presented as mean ± SEM, and *t* test was used to compare the continuous variables.

Predictive Factors for Achievement of CR

Next, patients with continuous values of *VEGF-C* were divided into two groups (low vs. high levels) by an ROC curve analysis. The low- versus high-level groups for plasma VEGF-C (RR of 0.20, *P* = .030), mRNA expression of *VEGF-C* (RR of 18.75, *P* = .003), and the ratio of plasma level to mRNA expression (RR of 0.05, *P* = .007) were potential predictors of CR on univariate analysis. Age, sex, *FLI3-ITD*, and genetic group were also associated with achievement of CR (Table 2). Because the genetic group based on the European LeukemiaNet was evaluated based on cytogenetic and molecular genetic data, factors with multicollinearity were not simultaneously entered into the multivariate models, and *VEGF-C* parameters were entered into separate multivariate models (Model I-III). After adjusting for factors identified on univariate analysis, low mRNA expression of *VEGF-C* (*P* = .017) and high ratio of plasma level to mRNA expression (*P* = .029) were independently associated with achievement of CR (Table 2).

Immune Cell Population According to VEGF-C mRNA Expression

To identify the characteristics of immune cell populations in BM according to *VEGF-C* mRNA expression, the patients with available continuous data (*n* = 44) were divided into two groups (lower Q1-Q3 vs. higher Q4) by quartiles of mRNA expression of *VEGF-C*. As shown in Figure 4, *A-E*, the frequency of NKT cells (CD3$^+$CD1d tetramer$^+$), NK cells (CD3$^-$CD16$^+$CD56$^+$), CD4$^+$ cells, CD8$^+$ cells, and CD19$^+$ cells in BM of the patients in Q4 for *VEGF-C* expression (high group) was compared with that of the lower-expression group. Patients with a high mRNA expression of *VEGF-C* had a lower frequency of NKT cells (0.04% vs. 0.09%, *P* = .002) (Figure 4*A*) and CD8$^+$ cells (4.40% vs. 7.42%, *P* = .036) (Figure 4*D*) and showed a trend of lower frequency of NK cells (1.26% vs. 2.20%, *P* = .057) (Figure 4*B*).

Discussion

In this study, we demonstrated that AML patients with high *VEGF-C* mRNA expression have inferior drug responsiveness based on CR achievement after induction chemotherapy. We also measured VEGF-C level in the patients' plasma using ELISA and found that VEGF-C plasma level was higher in the CR group than the no-CR group, suggesting the possibility of the internalization of VEGFR-3 after VEGF-C exposure [13,17,18]. To provide further insight into the possible mechanisms responsible for the VEGF-C–related poor response rate in AML, we analyzed immune cell population profiles of

Table 2. Univariate and Multivariate Analyses of Factors Affecting Achievement of CR

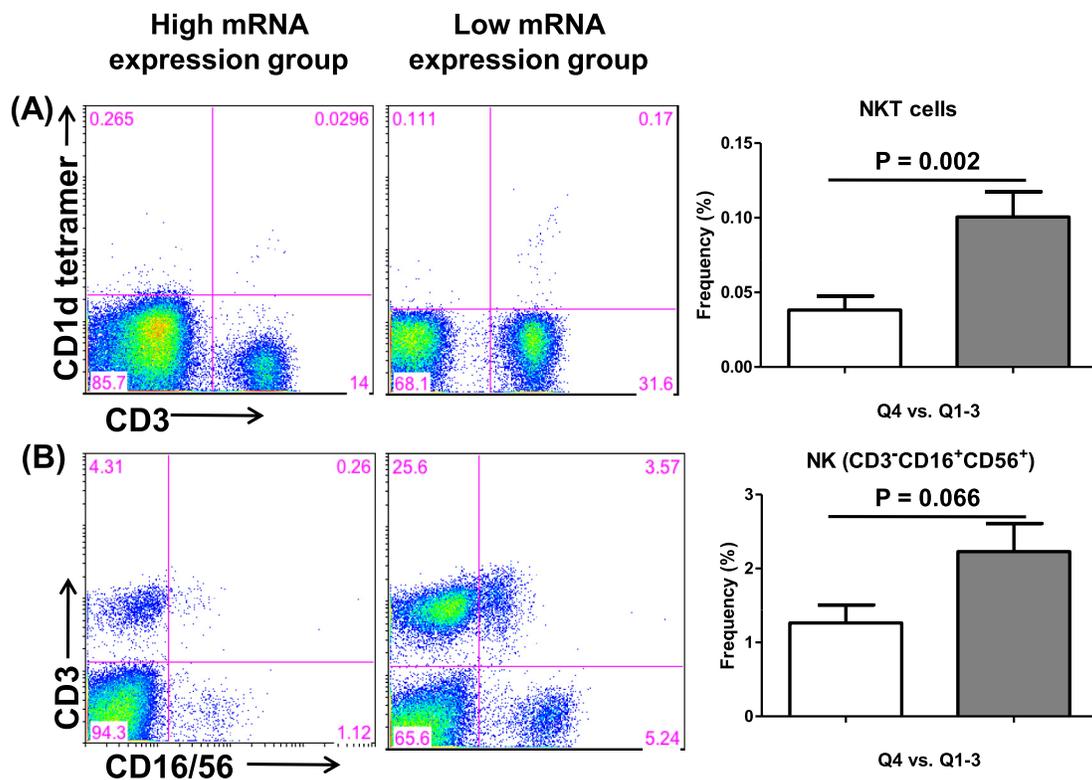| Variables	Univariate Analysis		Multivariate Analysis (Model I)		Multivariate Analysis (Model II)		Multivariate Analysis (Model III)	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (years), continuous	0.96 (0.92-1.00)	0.060	0.93 (0.87-0.99)	0.027	0.89 (0.81-0.98)	0.023	0.86 (0.76-0.98)	0.027
Sex (male vs. female)	0.23 (0.05-0.96)	0.044	0.23 (0.04-1.33)	0.101	0.19 (0.01-2.54)	0.207	0.06 (0.01-1.65)	0.097
WBC (×10^9/l), continuous	1.00 (1.00-1.00)	0.257	-	-	-	-	-	-
NPM1 (positive vs. negative)	1.06 (0.18-6.30)	0.951	-	-	-	-	-	-
FLT3-ITD (positive vs. negative) a	0.24 (0.05-1.29)	0.096	-	-	-	-	-	-
CEBPA (positive vs. negative)	1.59 (0.36-7.07)	0.544	-	-	-	-	-	-
BALLC RQ-PCR, continuous	1.06 (0.86-1.31)	0.563	-	-	-	-	-	-
WT1 RQ-PCR, continuous	0.86 (0.19-3.88)	0.844	-	-	-	-	-	-
Genetic group (ELN) (favorable/INT-I vs. INT-II/Adverse)	4.91 (1.24-19.46)	0.024	7.66 (1.23-47.56)	0.029	4.47 (0.44-45.18)	0.205	10.60 (0.75-149.97)	0.081
Log$_{10}$VEGF-C plasma level (low vs. high)	0.20 (0.05-0.86)	0.030	0.20 (0.04-0.79)	0.193	-	-	-	-
Log$_{10}$ *VEGF-C* mRNA expression (low vs. high)	18.75 (2.76-127.51)	0.003	-	-	26.81 (1.80-400.47)	0.017	-	-
Plasma level to mRNA expression ratio (low vs. high)	0.05 (0.01-0.43)	0.007	-	-	-	-	0.01 (0.01-0.63)	0.029

CI, confidence interval; *HR*, hazard ratio.

a The genetic group (ELN) is evaluated based on cytogenetic and molecular genetic data. Considering these close relationships, factors with multicollinearity were not simultaneously entered into the multivariate models.

44 AML patients in the same cohort. This analysis showed that AML patients in the high–*VEGF-C* mRNA expression group had a lower frequency of NKT cells and CD8^{+} cells, and a trend of lower frequency of NK cells, compared with patients in the low–*VEGF-C* mRNA expression group. Our results are in line with a few previous publications showing that high *VEGF-C* expression level was associated with reduced complete remission rate, overall survival, and event-free survival in AML [19,20]. Although the patient numbers were relatively small, all of the patients in this study were fully characterized with regard to *FLT3-ITD*, *NPM1*, *CEBPA*, cytogenetics, and clinical prognostic markers. After adjusting for potential factors, multivariate analyses revealed that high *VEGF-C* mRNA expression was an independent risk indicator for failure of induction chemotherapy. However, further and larger studies are needed to generate more conclusive results regarding *VEGF-C* mRNA expression and treatment response in specific cytogenetic subgroups.

VEGF-C is known to promote lymphangiogenesis by inducing proliferation, migration, and sprout formation of existing lymphatic endothelial cells [21–24], and active recruitment of new lymphatics by tumor-derived VEGF-C contributes to the spread of cancer cells from a primary tumor [22,24,25]. Several studies have reported another function of VEGF-C in cancer progression based on an autocrine VEGF-C loop promoting the invasion and metastasis of cancer cells independent of lymphangiogenesis [21,26–32]. Fiedler et al. detected *VEGF-C* and *KDR/FLT4* expression in AML blasts at protein and/or mRNA levels [6]. In addition, *VEGF-C*/FLT4 expression was found to be significantly higher in patients with

AML compared with healthy controls by immunohistochemical staining [33]. *In vitro* studies of Dias et al. [9] identified a role for exogenously added VEGF-C in survival and proliferation of leukemic cells and in a protection mechanism against chemotherapy-induced apoptosis by BCL-2 induction through FLT4 signaling. Furthermore, AML blasts are also themselves able to express VEGFRs, leading to autocrine signaling in AML [34,35]. Interestingly, in this study, we found that high *VEGF-C* mRNA level of AML blasts, but not high plasma VEGF-C concentration, was correlated with a lower CR achievement after induction chemotherapy. This result may indicate that the role of VEGF-C in the pathogenesis of AML depends on autocrine VEGF-C signaling.

AML blasts are known to produce VEGF-C and its receptor FLT4, unlike normal hematopoietic cells [9]. In a previous report, we showed that NK cells expressing high levels of FLT4 in AML patients have a low killing potential with low levels of IFN-γ [11], and our next report demonstrated restoration of both the killing potential and IFN-γ expression through FLT4 inhibition in defective NK cells from leukemic mice [13]. Therefore, our previous observations suggested that AML blasts, which release VEGF-C, might impair the function of NK cells via FLT4, and NK function could be rescued by inhibition of FLT4. These findings are concordant with results from Gunji et al. that showed that FLT4 inhibition could restore NK cytotoxicity against YAC-1 target cells by restoring high IFN-γ expression [36]. Consistent with these findings, Kalkunte et al. demonstrated that VEGF-C mediated TAP-1 expression on target cells and was directly involved in noncytotoxic behavior in uterine NK cells with increasing MHC-I expression [37], illustrating the
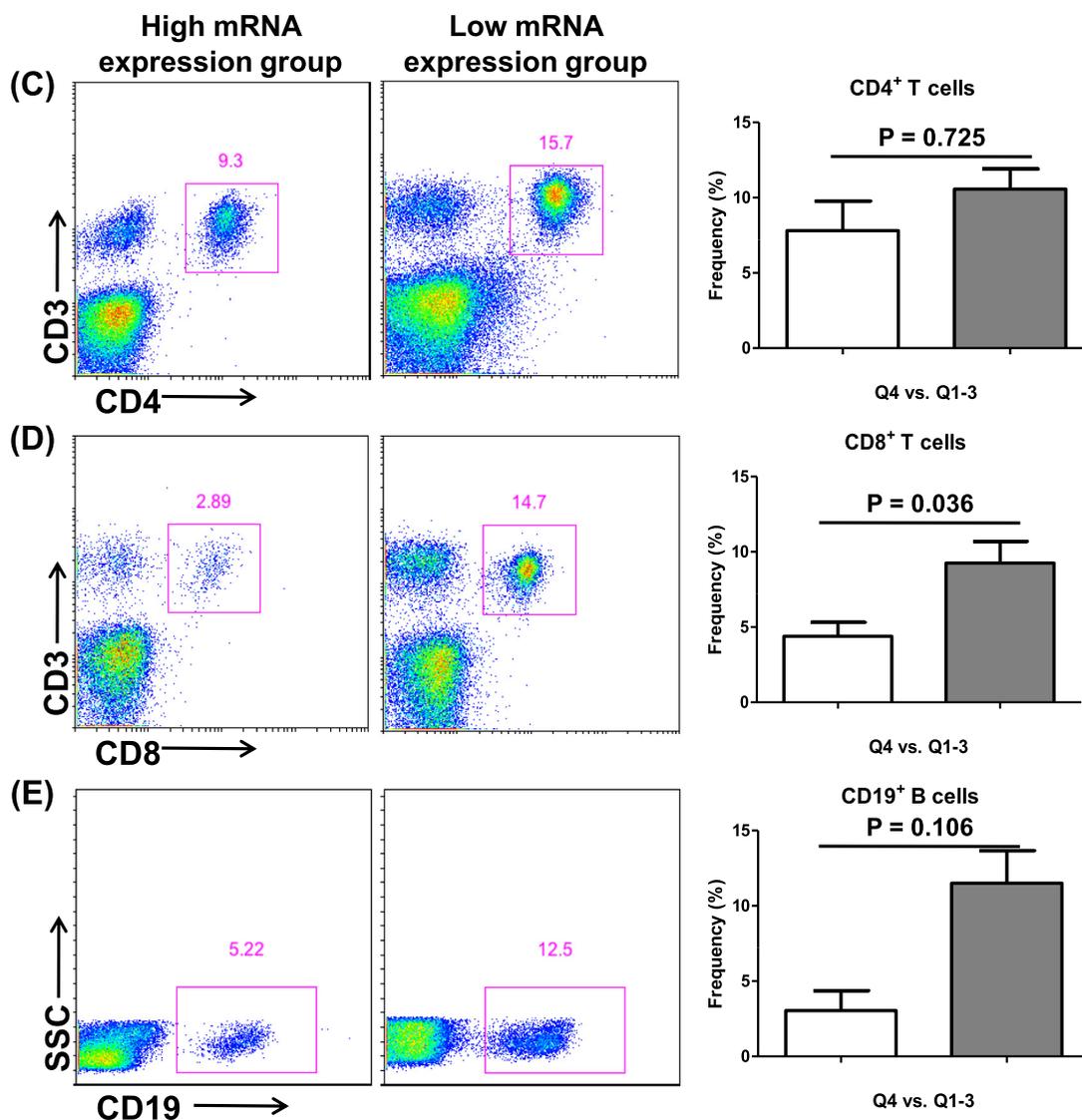

Figure 4. Comparison of immune cell populations in bone marrow between high– and low–*VEGF-C* mRNA expression groups. The patients were divided into two groups (Q4 vs. Q1-3) by quartiles of Log$_{10}$ *VEGF-C* mRNA expression. The frequency of NKT cells (CD3^{+}CD1d tetramer^{+}) (A), NK cells (CD3^{-}CD16^{+}CD56^{+}) (B), CD4^{+} cells (C), CD8^{+} cells (D), and CD19^{+} cells (E) were compared. Data are presented as mean ± SEM, and *t* test was used to compare the continuous variables.

Figure 4. (*continued*.)

importance of the VEGF-C/FLT4 axis in the killing activity of NK cells. In this study, to address the relationship between immune cell populations and *VEGFR-3* mRNA expression in a leukemic BM microenvironment, we compared the frequency of various immune cells according to high versus low *VEGF-C* mRNA expression and showed decreased frequencies of NKT cells and CD8$^+$ cells and a trend for decreased frequency of NK cells in the high–*VEGF-C* mRNA expression group compared with the low–*VEGF-C* mRNA expression group. This result has clinical implications in terms of a possible role for *VEGF-C* as a biomarker and in therapeutic approaches through correction of the microenvironment in AML patients.

Therapeutic targeting of the pathway involving VEGF-C and FLT4 in AML patients is supported by several possible mechanisms. De Jonge et al. showed that high mRNA levels of endogenously expressed *VEGF-C* in primary AML cells are related to increased *in vitro* resistance to typical AML drugs such as gemcitabine, fludarabine, cladribine, 6-thioguanine, daunorubicin, and etoposide, but not to acute lymphoblastic leukemia drugs such as vincristine and L-asparaginase [10]. Similar to these findings, Dias et al. reported that exogenously added VEGF-C protected AML cell lines from *in vitro*

chemotherapy-induced apoptosis for daunorubicin, etoposide, and cytarabine, and this protective effect of VEGF-C *in vitro* after FLT4 binding was associated with an increased Bcl2/Bax ratio [9]. Another mechanism could be VEGF-C binding to FLT4, which might attenuate the function of immune cells that play a pivotal role such as the function of NK and T cells in cancer cell clearance, especially against leukemia. Recent studies have shown the therapeutic efficacy of blocking lymphangiogenesis to attenuate allogeneic immune responses. Prolonged allograft survival in experimental models of islet, tracheal, cardiac, and corneal transplantation has been reported [38–43], and in addition, anti-FLT4 antibody ameliorated aGVHD and improved survival in murine models [44]. Our data demonstrated that *VEGF-C* expression was related to CR achievement after induction chemotherapy and changes in immune cell frequency according to *VEGF-C* mRNA expression in humans. Because our results considered only the frequency of immune cells and not their functional characterization, the potential mechanisms associated with the VEGF-C/FLT4 axis in immune cells remain to be further explored using antibodies against FLT4. In addition, some limitations of our study need to be discussed. Because the included patients had incomplete long-term follow-up, the effect of *VEGF-C* expression on

long-term outcomes was not assessed in this study. Further research on the pathway involving *VEGF-C* and FLT4 in leukemic stem cells is also warranted for a better understanding of the mechanisms of *VEGF-C* in the pathogenesis of AML.

Conclusions

We showed that *VEGF-C* expression was associated with CR achievement in AML patients. Especially, in multivariate analysis, high *VEGF-C* mRNA expression emerged as an independent factor for achievement of CR. Furthermore, the frequencies of NK, NKT, and T cells were different between high– and low–*VEGF-C* mRNA expression groups. These results suggest that interruption of VEGF-C signaling might be a potential therapeutic target for antileukemic treatment in AML patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.02.018.

Acknowledgements

Authorship Contributions

H.-J.K. had primary responsibility for study design, collection and assembly of data, data analysis and interpretation, and manuscript writing; S.-E.L. interpreted and analyzed data, performed statistical analysis, and wrote the manuscript. A.-R.H. and H.-S.H. performed laboratory work, and J.-Y.L. and W.-S.M. contributed to interpretation.

Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

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