Journal of the Egyptian Society of Haematology & Research, Vol. 5, No. 1, March: 35-39, 2009

Study of Vascular Endothelial Growth Factor Gene Polymorphism in Acute Myeloid Leukemia Patients

DALIA NAFEA[1]; DALIA EL-NILLY[2] and RANIA SOWELAM[2]

The Departments of Internal Medicine[1] and Clinical Pathology[2], Alex University

ABSTRACT

Introduction: Acute myeloblastic leukemia (AML) represents a group of clonal hematopoietic stem cells. Angiogenesis is a prerequisite for the growth and progression of malignancies. Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide. Several polymorphisms have been described in the VEGF gene, some of these variants are in the promoter region (Locus-2578c>A), 5' untranslated region (Loci-1154 G>A,–634G>A) and 3' untranslated region (+936 c>T) were found to be associated with variations in VEGF protein production.

Aim of the Work: To evaluate the ability of VEGF polymorphisms to predict prognosis in AML patients.

Patients and Methods: The study was performed on 45 newly diagnosed AML patients. Genotypes of VEGF were determined using a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. ELISA was used for quantitative assay of VEGF in serum. Patients were re-evaluated post induction.

Results: Patients with 936CC genotype were associated with significant worse response to induction than those with CT or TT genotypes (p=0.021). Patients with 634 CG genotype had significant bad response (p=0.035). Patients with-2578 AA/CC genotypes were associated with significant better CR in comparison to patients with –2578 CA genotype (p=0.0001). Regarding –1154 AA, GA, GG different polymorphisms, no significant correlation with CR. Hardy-Weinberg equilibrium was observed for all polymorphisms. The association of genotype AA, GG, CT for loci –2578/–634/936 in the same patient, as well as CC/CC/TT were associated significantly with CR (p=0.036 & 0.02). Combination polymorphisms which were associated with significantly wase CR were CA/CC/CC for loci –2578/–634/936 (p=0.021), CA/CG/CC (p=0.012), CA/CG/CT (p=0.036).

Conclusion: We found that certain combination polymorphisms are associated significantly with remission while others with worse response to induction chemotherapy.

Key Words: *AML – VEGFA – Genotype – Polymorphism.*

INTRODUCTION

Acute myeloblastic leukemia (AML) represents a group of clonal hematopoietic stem cells disorder that results from genetic alterations in normal hematopoietic stem cells **[1]**. A number of clinical and biologic features are used to predict clinical outcome **[2]**.

Constitutive genetic characteristics of the patient may play an important role in the prognosis, yet this has scarily been investigated in AML **[3]**.

In this regard, it is well recognized that most drugs exhibit wide interpatient variability in their efficacy and toxicity **[3,4]**.

Moreover, recent studies have shown that genetic polymorphisms can be used to predict the clinical outcome of malignancies **[5,6]**.

Angiogenesis, the process of new blood vessel formation from endothelial precursors, is a prerequisite for the growth and progression of malignancies. Vascular endothelial growth factor (VEGF) a soluble, 34-46 KDa, heparin binding glycoprotein is a potent angiogenic peptide with diverse biological activities including angiogenesis **[7,8]**. VEGF is located on chromosome 6p21.3 and composed of eight exons and seven introns **[8]**. Dysregulation of VEGF production was suggested to have a major impact on leukaemic growth and constitutes an important step in the progression of AML **[9]**.

Several polymorphisms have been described in the VEGF gene, some of these variants are in the promoter region (Locus-2578c>A), 5' untranslated region (Loci-1154 G> A,–634G>A) and 3' untranslated region (+936 C>T) were

found to be associated with variations in VEGF protein production **[10,11]**.

As the prognosis of AML patients may correlate with the degree of angiogenesis and VEGF, the current study evaluated the ability of VEGF polymorphisms to predict prognosis in AML patients.

PATIENTS AND SAMPLES

The study was performed on 45 newly diagnosed AML patients from Haematology division Faculty of Medicine, Alexandria University, with age range of 16-60 years, median 24 years (mean ± SD 25.63±10.45). Patients were treated with standard cytarabine and daunorubicin protocol. (Daunorubicin 45mg/m^2/day for 3 consecutive days and cytarabine 100mg/m^2/day continuous infusion for 7 consecutive days). For patients over 50 years the anthracycline dose was reduced by one third. Patients diagnosed as AML M3, received all trans-retinoic acid (ATRA) 45mg/m^2/day and idarubicin 12 mg/m^2/day on day 1 through 4.

Genotyping of the VEGF gene polymorphisms:

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood by a standard extraction method using blood DNA extraction kit (OMEGA BIO TEK, USA). The nucleotide sequence of four VEGF gene polymorphisms which were in the promoter region at –2578 and –1154, in the 5' untranslated region (UTR) at –634 and in the 3'-UTR at 936 was amplified by polymerase chain reaction (PCR).

Genotypes were determined using a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method as previously described **[12-14]**.

Briefly, the PCR primers for –2578C/A, –1154G/A, –634C/G and 936C/T were 5'-GGCCTTAGGACACCATACC-3' (forward) and 5'-CACAGCT TCTCCCCTATCC-3' (reverse); 5'-TCCTGCTCCCTCCTC GCCAATG-3' (forward) and 5'-GGCGGGGA CAGGCGAGCCTC-3' (reverse); 5'-CGACGGCTTGGGGAGATTGC-3' (forward) and 5'-GGGCGGTGTC TGTCTGTCTG-3' (reverse); and 5'-AGGGTTTCGGG AACCAGATC-3' (forward) and 5'-CTCGGTGATTT AGCAGCAAG-3' (reverse), respectively. PCR was performed in a final volume of 25µl, using 2 x Dream Tag Green PCR Master Mix (Fermentas, EU) containing, 20pmol/ul of each primer, and 50-100ng of genomic DNA. After the initial denaturation step at 95°C for 10min, 35 cycles consisted of denaturation at 95°C for 45s, annealing at 62°C for 45s, extension at 72°C for 30s, followed by final extension lasting 10min at 72°C. Genotypes were determined by restriction fragment length polymorphism (RFLP). The restriction enzymes which detect –2578C/A, –1154G/A, –634C/G and 936C/T are BstYI, MnlI, BsmFI and NlaIII respectively. Amplified DNA was digested with 1-3U of endonucleases for overnight at 37°C as indicated by the manufacturer (Fermentas, EU), and then electrophoresed on 3% agarose gel. The restriced DNA size of each polymorphism type was as follow (Fig. A,B):

VEGF –2578: CC (438bp), CA (438, 231, 207bp), AA (231, 207bp).

VEGF –634: CC (274bp), CG (274, 156, 118bp), GG (156, 118bp).

VEGF 936: CC (326bp), CT (326, 271, 55bp), TT (271, 55bp).

VEGF-1154; GG (206bp), GA (206,184, 22bp), AA (184,22bp).

ELISA assay:

Serum samples were collected and centrifuged at 1000g for 10min within 30min from collection. Serum was aliquoted and stored at –20°C until VEGF evaluation. VEGF-A (Platinum ELISA eBioscience) concentrations were determined in serum according to the manufacturer's instructions. Concentrations are reported as picograms per milliliter. Normal range value of VEGF is up to 42.6pg/ml for serum.

Statistical analysis:

The Data was collected and entered into the personal computer. Statistical analysis was done using Statistical Package for Social Sciences (SPSS/version 17) software. Arithmetic mean and standard deviation were used for categorized parameters, Chai square test was used while for numerical data, *t*-test was used to compare two groups while for more than two groups ANOVA test was used. The level of significance was considered 0.05.

Hardy-Weinberg: Hardy-Weinberg describes genetic balance within a studied group. The law is used to determine whether the number of harmful mutations in patients is increased.

RESULTS

We evaluated VEGF polymorphism at different loci among 45 AML patients (6 were FAB M0 11.1%, 10 M2 22.2%, 5 M3 11.1%, 11M4 24.4%, 11 M5 24.4%, 3 M7 6.7%).

Among 45 patients 20 (44.4%) achieved complete remission (CR was defined as the presence of no more than 5% blast cells in the bone marrow aspirate). Table (1) presents a CR status in relation to different polymorphisms of VEGF.

Patients with 936 CC genotype were associated with significant worse response to induction chemotherapy than those with CT or TT genotypes (p=0.021); (among 28 patients with CC genotype, 20 did not achieve CR).

Patients with –634 CG genotype had significant worse response (p=0.035); (among 25 patients who had this polymorphism 17 [68%] did not achieve CR) while all patients with -634 GG genotype achieved CR. Patients with –2578 AA/CC genotypes were associated with significant better CR in comparison to patients with –2578 CA genotype (p=0.0001). Regarding –1154 AA, GA, GG different polymorphisms, we did not find significant correlation with remission.

VEGFA polymorphism and disease characteristics:

Disease characteristics including clinical (splenomegaly, hepatomegaly, lymphadenopathy, gum hypertrophy), peripheral WBCs mean ± SD 68.457±80.538, sex and age at diagnosis did not show any significant correlation with the four genotypes of VEGFA polymorphisms. While level of VEGFA mean ± SD 626.933± 577.980 was significantly higher in 936CC genotype in comparison with CT, TT genotypes.

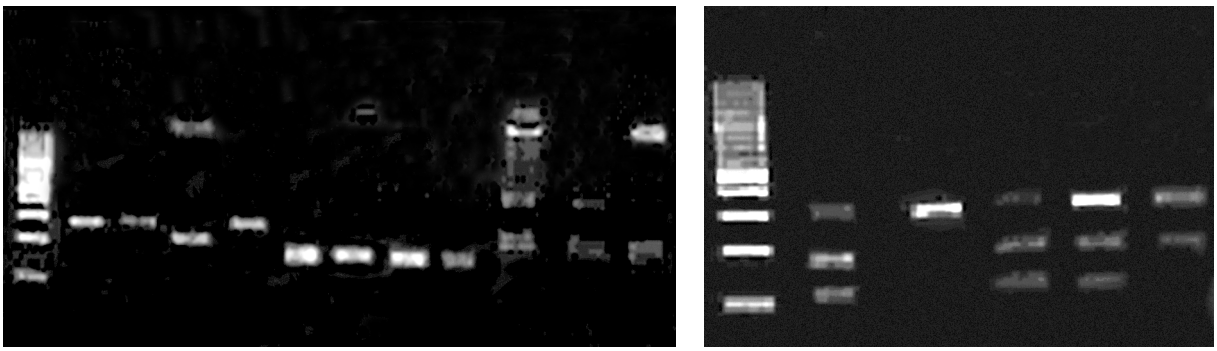
Hardy-Weinberg equilibrium was observed for all polymorphisms. Accordingly, we analysed VEGFA polymorphism based on three genotypes at loci 936, –634, –2578 and we correlated them with achievement of CR (Table 2). The combination of genotype AA, GG, CT for loci –2578/ –634/936 in the same patient, and CC/CC/TT were associated with significant CR (p=0.036). Combination polymorphisms which were associated with significant wase CR were CA/CC/ CC for loci –2578/–634/936 (p=0.021), CA/CG/ CC (p= 0.012), CA/CG/CT (p=0.036).

Table (1): VEGFA polymorphism and achievement of CR after first cycle of induction chemotherapy.

	Response to induction chemotherapy				
	Remission		No Remission		*p*
	No.	%	No.	%	
936 C>T					
C/C	8	40.0	20	80.0	
C/T	6	30.0	3	12.0	
T/T	6	30.0	2	8.0	.021
–634 C>G					
C/C	9	45.0	8	32.0	
C/G	8	40.0	17	68.0	
G/G	3	15.0	0	0.0	.035
–1154 G>A					
A/A	5	25.0	8	32.0	
G/A	0	0.0	3	12.0	
G/G	15	75.0	14	56.0	.201
–2578 C>A					
A/A	3	15.0	0	0.0	
C/A	2	10.0	22	88.0	
C/C	15	75.0	3	12.0	.0001

Table (2): Associations of different VEGFA polymorphisms in relation to CR.

–2578 C>A	–634 C>G	936 C>T	Response to induction chemotherapy				Total	*p*
			Remission		No Remission			
			No.	%	No.	%		
A/A	G/G	C/T	3	100	0	0.0	3	0.036
C/A	C/C	C/C	0	0.0	5	100	5	0.021
C/A	C/G	C/C	2	14.3	12	85.7	14	0.012
C/A	C/G	C/T	0	0.0	3	100.0	3	0.036
C/A	C/G	T/T	0	0.0	2	100.0	2	–
C/C	C/C	C/C	3	50.0	3	50.0	6	0.85
C/C	C/C	T/T	6	100.0	0	0.0	6	0.020
C/C	C/G	C/C	3	100.0	0	0.0	3	0.85
C/C	C/G	C/T	3	100.0	0	0.0	3	0.85



(A) **(B)**

Fig. (1): Polymorphisms of the VEGF. (A) Lane 1: 100-bp DNA ladder. Lanes 2, 3 & 5: VEGF-936 CC genotype (326-bp bands), lane 4: VEGF 936 TT genotype (271-bp band), lanes 6-9: VEGF-1154G/G genotype (206-bp bands), lanes 10 & 11: VEGF 2578C/A genotype (438, 231 & 207-bp bands), and lane 12: A/A genotype of the same SNP. (B) Lane 1: a 100-bp DNA ladder. Lanes 2, 5 & 6: VEGF-634 C/G genotype (274, 156, and 118-bp bands), lane 3: C/C genotype (274-bp band) of the same SNP, and lane 4: G/G genotype (118 & 156-bp bands).

DISCUSSION

Data suggest that VEGF is an important pathogenetic factor in myeloid leukemia. VEGFA has been described as a mediator of leukemia-dependent angiogenesis and as an autocrine growth regulator of AML cells **[15]**.

Our study demonstrated the importance of VEGFA polymorphism for predicting the prognosis in patients with AML. The VEGFA polymorphisms, 936 CT/TT were associated with better CR than 936 CC which was associated with significant worse response to induction chemotherapy. This finding coincide with previous data which found that 936 CT or TT alleles had favourable prognosis in AML, while 936 CC had unfavourable outcome **[16]**.

In the present work, –2578 AA or CC genotypes have been shown to be associated with significant good response to treatment. Others found that the wild and polymorphic types of VEGFA at position –2578 were associated with the development of certain diseases while carriers of wild type-polymorphic type were not **[13]**. Previous data found that –2578CC genotype was associated with shortened OS in patients with ovarian cancer **[17]**.

The current study assessed polymorphism of –1154 locus and did not find correlation with prognosis in AML in contrast to other researches who found that GG allele was associated with risk of RA **[13]**.

Our data showed that CG polymorphism was associated with significant worse response to induction therapy. In contrast to Soo-Han Hun et al who found that –634 CC or CG polymorphisms were associated with better OS and PFS in patients with colorectal cancer compared with GG genotype **[18]**.

It has been demonstrated that the pathogenesis of acute leukemia involves complex inter-

actions between host susceptibility, chromosomal damage and possibly the incorporation of genetic informations into susceptible progenitor cells **[19,20].**

We reported that the level of VEGF was significantly higher in 936 CC genotype in comparison with CT and TT genotypes; this is comparable to previous data which found that higher levels of VEGF are associated with poor outcome in patients with Hodgkin and Hodgkin diseases **[21-23].**

We correlated the association of certain polymorphisms at different loci for VEGFA with prognosis; we found that certain association polymorphisms were associated with significant favorable response while others with worse outcome. Young Park et al. studied the haplotypes of certain loci on VEGFA and found that CTG haplotype on loci 25781/405/460 was associated with worse prognosis **[16].**

In conclusion, further studies integrating VEGF polymorphism with other prognostic parameters are needed to verify if it would stand a multivariate analysis as independent prognostic parameter to refine therapeutic decision.

REFERENCES

1- Löwenberg G. Strategies in the treatment of acute myeloid leukemia. Haematologica. 2004, 89: 1029-32.

2- Stone RM. AML: Current landscape and future directions. Hematology (Am Soc Hematol Educ program). Hematology 2004, 98-117.

3- Monzo M, Brunet S. Genomic polymorphisms provide prognostic information in intermediate risk acute myeloblastic leukemia. Blood 2006, 107: 4871-9.

4- Evans WE, Johnson JA. Pharmacogenomics: The inherited basis for interindividual differences in drug response. Ann Rev genomics Hum Genet. 2001, 2: 9-29.

5- Ruzzo A, Gaziano F, Kawakami K, et al. Pharmacogenetic profiling and clinical outcome of patients with advanced gastric cancer treated with palliative chemotherapy J Clin Oncol 2006, 24: 1883-91.

6- Li D, Frazier M, Evans DB, et al. Single nucleotide polymorphisms of Rec Q1, RAD54 L, and ATM genes are associated with reduced survival of pancreatic cancer. J Clin Oncol 2006, 24: 1720-8.

7- Thomas KA. Vascular endothelial growth factor a potent and selective angiogenic agent. Journal of biological chemistry. 1996, 271: 603-6.

8- Vincenti V, Cassano C, Rocchi M. Assignment of the vascular endothelial growth factor gene to human chromosome 6 p21.3. Circulation, 93: 1493-5.

9- Thomas DA, Giles FJ, Cortes J. Albitar. Antiangiogenic therapy in leukemia. Acta Haematologica. 2001, 106: 190-207.

10- Prior SJ, Hagberg JM, Paton CM, Brown. DNA sequence variation in the promoter region of the VEGF gene impacts VEGF gene expression and maximal oxygen consumption. American Journal of Physiology. Heart and circulatory physiology 2006, 290: 1848-55.

11- Stevens A, Soden J, Brenchley PE. Haplotype analysis of the polymorphic human vascular endothelial growth factor gene promoter. Cancer Res. 2003, 63: 812-6.

12- Perrey C, Turner SJ, Pravica V, Howell WM, Hutchinson IV. ARMS PCR methodologies to determine IL-10, TNF-alpha, TNF-beta and TGF-beta 1 gene polymorphisms Transpl Immunol. 1999, 7: 127-8.

13- Han SW, Kimi GW, Seo JS, Kang YM. VEGF gene polymorphisms and susceptibility to rheumatoid arthritis. Rheumatology. 2004, 43: 1173-7.

14- Sunyoung L, shahlaM J, Granka V. Processing of VEGFA by matrix metalloproteinases regulates bioavailability and vascular patterning intumors. JCB 2005, 169 (4): 681-91.

15- Padro T, Bieker R, Ruiz S, et al. Overpression of vascular endothelial growth factor (VEGF) and its cellular receptor KDR (VEGVR-2) in the bone marrow of patients with acute myeloid leukemia. Leukemia 2002, 16: 1302-10.

16- Dong Hwan, Nan Young Lee, Jae Yong Park. Vascular endothelial growth factor (VEGF) gene (VEGFA) polymorphism can predict the prognosis in acute myeloid leukaemia patients. British Journal of Hematology. 2007, 140: 71-9.

17- Hefler LA, Mustea A, Lonsgen D, et al. Vascular endothelial growth factor gene polymorphisms are associated with prognosis in ovarian cancer. Clin cancer Res. 2007, 13: 898-901.

18- Kim JG, Chae YS, Sohn SK, Jun SH. Vascular endothelial factor gene polymorphisms associated with prognosis for patients with colorectal cancer. Clin Cancer Res. 2008, 14 (1): 62-6.

19- Ayton PM, Cleary ML. Transformation of myeloid progenitors by MLL an oncoproteins is dependent on Hoxa 7 and Hoxa 9. Genes Dev. 2003, 17: 2298-307.

20- Garber JE, Offit K. Hereditary cancer predisposition syndromes. J Clin Oncol. 2005, 23: 276-92.

21- Koomagi R, Zintl F, Volum M. Vascular endothelial growth factor in newly diagnosed and recurrent childhood acute lymphoblastic leukemia as measured by real-time quantitative polymerase chain reaction. Clinical cancer Research. 2001, 7: 3381-4.

22- Aguayo A. The role of angiogenesis in the biology and therapy of myelodysplastic syndromes. Current Hematology Reports. 2004, 3: 184-91.

23- Hazar B, Paydas S, Sahin B, Tuncer I. Prognostic significance of microvessel density and vascular endothelial growth factor (VEGF) in non-Hodgkin's lymphoma. leuk lymphoma. 2003, 44 (12): 2089-93.