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CXCL12 G801A Gene Polymorphism and the Risk of Tissue Infiltration in Acute Leukaemia

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ABSTRACT

Background: In acute leukemia blasts invade the blood stream and may localize in extramedullary sites. Stromal cell-derived factor-1 (SDF-1), coded by CXCL12 gene, is a chemokine that plays an important role in stem cell homing and malignant cell trafficking. CXCL12 G801A polymorphism could influence blast dissemination and tissue infiltration in acute leukemia.

Aim of Work: To characterize the expression pattern of CXCL12 G801A gene polymorphism in patients with acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) in relation to clinical features and laboratory findings at diagnosis, and its correlation with disease progression and outcome in an Egyptian patient's cohort.

Patients and Methods: CXCL12 G801A was analyzed in 65 AML and 35 ALL patients together with 30 normal controls using a polymerase chain reaction-restriction fragment length polymorphism assay [PCR-RFLP].

Results: CXCL12 801A/A, and 801A/G were associated with a higher PB blasts, BM blasts, compared to 801G/G homozygous patients (p=0.001 and 0.03 respectively) in AML and (p=0.005, 0.008 respectively) in ALL. The number of extramedullary tumor sites was higher in the A allele carriers (p=0.001) in AML, but in ALL the difference was near statistical significance (p=0.006). Patients with 801A allele relapsed and died more frequently (62.5%, 76.9%) than those with 801G/G (37.5%, 23.1%) in AML and ALL respectively; yet the difference was not statistically significant (p=0.08 in both AML and ALL).

Conclusion: CXCL12-G801A polymorphism is associated with higher mobilization of BM blasts, its infiltration to extramedullary tumor sites; it is also associated with treatment resistance, early relapse and mortality. CXCL12-G801A is an adverse prognostic factor and may define an important risk group in acute leukemia patients.

Key Words: *SDF-1 – CXCL12 G801A – ALL – AML – Extramedullary infiltration.*

INTRODUCTION

Acute myelogenous leukemia (AML) is characterized by uncontrolled proliferation within the bone marrow of myeloid progenitors arrested in their maturation process **[1]**. In contrast to normal hematopoiesis, it is usually associated with egress of immature cells from the bone marrow into the circulation and in some AML subtypes, these cells may anchor in extramedullary locations, such as in the liver and spleen **[2,3]**. Peripheral blood blast (PBB) count and the number of extramedullary tumor sites are extremely variable from one patient to another and depend, in part, on AML subtype **[3,4]**.

Stromal cell-derived factor-1 (SDF-1), which now is designated as CXCL12, is a homeostatic chemokine that signals through its receptor CXCR4, which in turn plays an important role in hematopoiesis, development, and organization of the immune system **[3]**. They also contribute to stem cell homing and may play a role in the trafficking of leukemic cells **[1]**.

SDF-1 is constitutively produced in the bone marrow by immature osteoblasts lining the endosteum region and by stromal and endothelial cells. SDF-1 is also produced by different hematopoietic cells as well as by AML blast cells, which express varying amounts of functionally active CXCR4 **[4,5,6]**. It is also secreted by stromal and endothelial cells of other organs such as heart **[7]**, skeletal muscle **[8]**, liver **[9]**, brain **[10]**, and kidney **[11]**. Moreover, its secretion increases during tissue damage such as heart infarct **[12]**, limb ischemia **[13]**, toxic liver

damage **[9]**, excessive bleeding **[14]**, total body irradiation, and after-tissue damage related to chemotherapy **[15,16]**.

CXCR4 and its ligand SDF-1 were also shown to have an important role in breast **[17]**, prostate **[18]**, and sympathetic nervous system cancer metastasis **[19]**, as well as in the migration assays of malignant cells from pancreatic cancers **[20]**, non-Hodgkin B cell lymphomas **[21]**, chronic lymphocytic leukemia **[22]**, chronic myeloid leukemia **[23]**, and acute leukemia **[4,5,6]**. However, the role of SDF-1/CXCR4 interactions in the control of human AML cell trafficking and disease progression is poorly understood **[2]**.

Previous studies have reported an association between the mobilizing capacity of haematopoietic progenitor cells (HPCs) and a single nucleotide polymorphism (SNP) in CXCL12 **[24]**, the SDF-1-encoding gene. This polymorphism is located at nucleotide position 801 (G to A transition, counting from the ATG start codon) in the 3' untranslated region (3'UTR) of the SDF-1 transcript. The ability of blasts to exit from the bone marrow microenvironment, circulate in the peripheral blood, and anchor in extramedullary locations might thus depend on the CXCL12 genotype **[4]**.

In this study, we analyzed the expression of the CXCL12 (G801A) gene polymorphism in 100 patients with acute leukemia, 65 with de novo AML and 35 with acute lymphoid leukemia (ALL), aiming to determine whether CXCL12. G801A polymorphism is important for the dissemination of malignant cells in acute leukemia and also to determine its role in the progression of the disease.

PATIENTS AND METHODS

Patients: One hundred Egyptian patients with newly diagnosed acute Leukemia referred to the National Cancer Institute (NCI)) and Beni Suef University hospital were included in this study. They were 65 AML and 35 ALL patients.

Diagnosis of Acute leukemia was based on (1) morphologic findings from Giemsa stained smears of bone marrow (BM) aspirates, (2) cytochemical stains criteria such as negativity for myeloperoxidase (MPO) and sudan black B (SBB) in cases of Acute Lymphoblastic Leukemia [ALL] or their positivity in cases of Acute Myeloid Leukemia [AML] and positivity for acid phosphatase in T-cell Acute Lymphoblastic Leukemia [T-ALL] and (3) immunophenotyping criteria as CD10 +/-,CD19+,CD20+,CD22+ for B-ALL,CD2+/-,CD3+,CD5+/-,CD7+ for T-ALL, and positivity of CD13 and CD33 for AML cases. Follow-up of patients was carried out for one year to study any possible association between CXCL 12 G801A polymorphism and the response of patients to therapy.

Concerning the 65 AML patients, they were 37 males and 28 females with a mean age of 43.2±16.6 (26-75) and median 46 years. Patients were classified according to the French-American-British (FAB) classification into: 11 M0, 15 M1, 18 M2, 12 M4, 4 M5, 4 M6 and only 1 case of M7.

As for the 35 ALL patients, they were 21 males and 14 females with a mean age of 23.5±11 (21-54) and median 24 years. They were classified according to immunophenotyping into 22 B-lineage (B-ALL) and 13 T-lineage (T-ALL) ALL.

Thirty age and sex matched individuals were included as a control group.

DNA isolation and CXCL12 genotype analysis:

Mononuclear cells (MNCs) were isolated from 2ml BM aspirate or peripheral blood at diagnosis by Ficoll density gradient centrifugation. Genomic DNA was extracted using QIAamp DNA Mini Blood kit (cat. no. 51304) (Qiagen, Germany) according to the manufacturer's instructions.

CXCL12 G801A polymorphism was determined with a polymerase chain reaction-restriction fragment length polymorphism assay [PCR-RFLP]. The PCR primers were:

- 5'-CAGTCAACCTGGGCAAAGCC-3'(F)
- and 5'-AGCTTTGTGCCTGAGAGTCC-3'(R) **[4]**.

PCR assay was performed for each sample in a final reaction volume of 25µL, using 5µL genomic DNA, 12.5µL universal master mix, 1µL CXCL12 G801A forward primer, 1µL CXCL12 G801A reversed primer, together with 5.5µL distilled water (DW).

The PCR conditions were as follows: 35 cycles of amplification consisting of, denaturation at 95°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 30 seconds then a final extension at 72°C for 10 minutes was performed **[4]**. All reactions were done using the thermal cycler Applied Biosystems (Berkin Elmer 9600).

The PCR product was digested with the restriction endonuclease MspI **[4]** (Fermentas, Fast Digest ® MspI # FD 0544) and put at 37°C for 30 minutes. The products were then resolved on 2% agarose gel electrophoresis containing ethidium bromide, then visualized using UV transilluminator. DNA molecular weight marker (QIAGEN GelPilot 50 bp Ladder (100) (cat no. 239025) was used to assess the size of the PCR-RFLP products.

Because of the elimination of the MspI restriction site, MspI digestion of the PCR product, results in two fragments of 100 and 202bp for the 801G allele and in one fragment of 302bp for the 801A variant **[4]**. So the homozygous G/G results in two fragments of 100 and 202bp, the homozygous variant A/A results in one fragment of 302bp, while the heterozygous variant A/G results in three fragments of 100, 202bp and 302bp (Fig. 1).

Remission status was assessed after completion of induction chemotherapy. Complete remission (CR) was defined as follows: Granulocyte count of $\geq 1.5 \times 10^9$/L, platelet count of $\geq 100 \times 10^9$/L, no PB blasts, BM cellularity of ≥20% with maturation of all cell lines and <5% blasts, and no extramedullary leukemia. Relapse was defined as reappearance of PB blasts, >5% blasts in BM, or appearance of extramedullary manifestations after CR was achieved.

Statistical methods:

Data were analyzed using SPSS version 17. The data were summarized using descriptive statistics: Mean, standard deviation, median, range (minimal and maximum values) for quantitative variables and number and percentage for qualitative values. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric *t*-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" on rank of variables was used for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. A *p*-value ≤0.05 was considered significant **[25]**.

RESULTS

The present study included 100 patients with acute leukemia, 65 AML and 35 ALL together with 30 healthy controls.

Table (1) represents CXCL12 G801A among all studied groups. The frequency of 801 A allele does not differ significantly between AML, ALL patients and controls.

Tables (2,3) represent comparison between AML and ALL patients. There was no statistically significant difference between AML and ALL patients as regards gender, WBCs, Hb, platelets count, hepatomegaly, splenomegaly (HSM) or any extramedullary sites. After follow-up of patients for one year, there was no statistically significant difference between AML and ALL patients as regards CR (50.8% in AML versus 62.9% in ALL patients). There was a statistically significant difference between AML and ALL patients as regards age, (median 46 vs. 24 years), PB blasts and BM blasts were higher in ALL than AML patients. ALL patients presented with higher frequency of lymph nodes (LN) enlargement than AML patients (42.9% vs. 20%).

Tables (4,5) compare between characters of AML patients as regards CXCL12 G801A polymorphism. There was no statistical significant difference between wild type G/G and mutant type (A/A or A/G) as regards gender, age, WBCs, Hb, or platelets count. There was a statistical significant difference between the wild and the mutant type as regards HSM, LN enlargement, and extramedullary tumor sites being higher in patients harboring the A allele. Also PB blasts and BM blasts were significantly associated with the A allele. Although AML patients with bad outcome (relapse or mortality) showed higher frequency of the mutant type (A/A or A/G) than in the wild type (G/G) (62.5% vs. 37.5%), yet the difference did not reach statistical significance (p=0.08).

Tables (6,7) compare between characters of ALL patients as regards CXCL 12 G801A polymorphism. There was no statistically significant

difference between wild type G/G and mutant type (A/A or A/G) as regards gender, age, WBCs, Hb, or platelets count. Although A allele was more frequent in patients with HSM (70% vs. 30%) and with LN enlargement (73.3% vs. 26.7%); yet the difference was not statistically significant (p=0.09 and 0.1 respectively). The same applied to extramedullary tumor sites (68% vs. 32%); yet the difference was near statistical significance (p=0.06). As for the immunophenotyping, there was no statistically significant difference between B lineage (40.9% in G vs. 59.1% in A allele) and T lineage ALL (46.2% in G vs. 53.8% in A allele). The only statistically significant difference between the wild and the mutant type in ALL patients was in PB blasts and BM blasts which both were significantly higher in A allele than G allele (p=0.005 and 0.008 respectively). As for response to treatment, ALL patients with mutant type had bad response (relapse or mortality) more frequent than those with wild type (76.9% vs. 23.1%), but the difference did not reach statistical significance (p=0.08).

Table (1): Comparison between acute leukemia patients and control as regards CXCL 12 G801A polymorphism.

Group	No.	Wild Type Homozygous G/G	Mutant type		*p*-value
			Heterozygous A/G	Homozygous A/A	
		No. (%)	No. (%)	No. (%)	
AML	65	32 (49.2%)	30 (46.2%)	3 (4.6%)	
ALL	35	15 (42.9%)	18 (51.4%)	2 (5.7%)	0.75
Control	30	18 (60%)	11 (36.7%)	1 (3.3%)	

Table (2): Clinical and haematological parameters of AML and ALL patients.

Parameter	AML (65 patients)	ALL (35 patients)	*p*
Gender:			
Male: No. (%)	37 (56.9%)	21 (60%)	
Female: No. (%)	28 (43.1%)	14 (40%)	0.9
Age at diagnosis (years)	43.2±16.6 (26-75)*	23.5±11 (21-54)	
	46**	24	<0.001
Total leucocytic count x10^9/L	62.8±87.8 (2.5-340)	79.4±84.3 (1.4-274.7)	
	32	41.3	0.3
Hemoglobin gm/dl	7.8±1.8 (2.7-11.6)	7.3±2.5 (2.3-11.6)	
	8.1	7.9	0.8
Platelets x10^9/L	58.2±49 (7-200)	74.5±71.7 (5-296)	
	45	54	0.4
Peripheral Blood blasts	26±21.2 (0-78)	47.9±24.7 (12-95)	
	21	45	<0.001
Bone Marrow blasts	55.3±31.2 (21-99)	86.5±14.4 (29-99)	
	57	89	<0.001
Hepatomegaly: No. (%)	35 (53.8%)	20 (57.1%)	0.8
Splenomegaly: No. (%)	36 (55.4%)	20 (57.1%)	0.8
LN enlargement: No. (%)	13 (20%)	15 (42.9%)	0.02
Extramedullary sites: No. (%)	40 (61.5%)	25 (71.4%)	0.3

*Mean ± SD (range), **Median.

Table (3): Comparison between AML and ALL patients as regards clinical outcome.

Parameter	AML (65 patients)	ALL (35 patients)	*p*-value
Complete Remission (CR)	33 (50.8%)*	22 (62.9%)	0.2
No CR (relapsed or death)	32 (49.2%)	13 (37.1%)	
Death	17 (26.2%)	6 (17.1%)	0.3

* No. (%).

Table (4): Clinical and Hematological findings in 65 AML patients in relation to CXCL 12 G801A polymorphism.

Parameter	Wild Type G/G (32 cases)	Mutant Type A/G + A/A (33 cases)	*p*
Gender:			
Male: No. (%)	17 (45.9%)	20 (54.1%)	0.6
Female: No. (%)	15 (53.6%)	13 (46.4%)	
Age at diagnosis: years	39.1±17.9 (26-75) *	35.3±15.3 (27-62)	
	36.5**	35	0.4
Total leucocytic count: $x10^9$/L	71.8±97.9 (2.7-40)	54.2±77.3 (2.5-327)	
	35.7	32	0.6
Hemoglobin: gm/dl	7.9±1.7 (3.5-11.6)	7.7±2 (2.7-11.4)	
	8	8.1	0.7
Platelets: $x10^9$/L	59.3±52.1 (7-198)	57.1±46.5 (8-200)	
	40	50	0.8
Peripheral Blood blasts: %	14.2±12.2 (0-50)	37.5±21.9 (0-78)	
	12	38	<0.001
Bone Marrow blasts: %	47.3±29.9 (23-98)	63.1±30.9 (21-99)	
	43.5	70	0.03
Hepatomegaly: No. (%)	13 (37.1%)	22 (62.9%)	0.04
Splenomegaly: No. (%)	13 (36.1%)	23 (63.9%)	0.02
LN enlargement: No. (%)	0 (0%)	13 (100%)	<0.001
Extramedullary sites: No. (%)	13 (32.5%)	27 (67.5%)	0.001
FAB subtype: No (%)			
M0	2 (18.2%)	9 (81.8%)	
M1	5 (33.3%)	10 (66.7%)	
M2	11 (61.1%)	7 (38.9%)	
M4	6 (50%)	6 (50%)	
M5	4 (100%)	0 (0%)	
M6	3 (75%)	1 (25%)	
M7	1 (100%)	0 (0%)	

*Mean ± SD (range).
FAB: French American British classification.

Table (5): Impact of CXCL 12 G801A polymorphism on clinical outcome of AML patients.

Parameter	Wild Type G/G (32 cases)	Mutant Type A/G + A/A (33 cases)	*p*-value
Complete Remission (CR)	20 (60.6%)*	13 (39.4%)	0.08
Not in CR (relapse or death)	12 (37.5%)	20 (62.5%)	
Death	7 (41.2%)	10 (58.8%)	0.5

* No. (%).

Table (6): Clinical and Hematological findings in 35 ALL patients in relation to CXCL 12 G801A polymorphism.

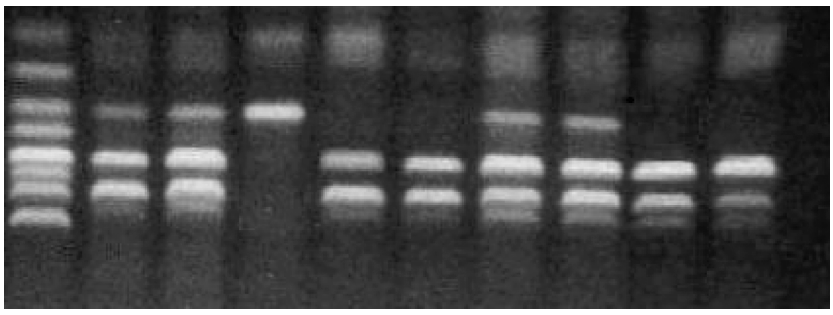
Parameter	Wild Type G/G (15 cases)	Mutant Type A/G + A/A (20 cases)	*p*
Gender:			
Male: No. (%)	11 (52.4%)	10 (47.6%)	
Female: No. (%)	4 (28.6%)	10 (71.4%)	0.2
Age at diagnosis: years	28.9±10.1 (24-38) *	29.9±11.8 (21-54)	
	26**	28.5	0.9
Total leucocytic count: $x10^9$/L	69.4±82.6 (1.4-270)	86.9±86.9 (2.1-274.7)	
	39.8	42.5	0.5
Hemoglobin: gm/dl	7.1±2.8 (2.3-10.5)	7.5±2.2 (2.5-11.6)	
	7.3	8.1	0.7
Platelets: $x10^9$/L	88.6±87.5 (5-296)	63.9±57.3 (6-257)	0.5
	54	54	
Peripheral Blood blasts: %	34.8±19.6 (12-92)	57.8±23.8 (13-95)	0.005
	34	62.5	
Bone Marrow blasts: %	79.5±18.3 (29-98)	91.9±7.4 (70-99)	0.008
	85	94	
Hepatomegaly: No. (%)	6 (30%)	14 (70%)	0.09
Splenomegaly: No. (%)	6 (30%)	14 (70%)	0.09
LN enlargement: No. (%)	4 (26.7%)	11 (73.3%)	0.1
Extramedullary sites: No. (%)	8 (32%)	17 (68%)	0.06
Immunophenotype:			
B lineage: 22 cases	9 (40.9%)	13 (59.1%)	0.7
T lineage: 13 cases	6 (46.2%)	7 (53.8%)	

*Mean ± SD (range). **Median.

Table (7): Impact of CXCL 12 G801A polymorphism on clinical outcome of ALL patients.

Characteristic	Wild Type G/G (15 cases)	Mutant Type A/G + A/A (20 cases)	*p*-value
Complete Remission (CR)	12 (54.5%)*	10 (45.5%)	0.08
No CR (relapse or death)	3 (23.1%)	10 (76.9%)	
Death	2 (33.3%)	4 (66.7%)	0.6

*No. (%)



M 1 2 3 4 5 6 7 8 9

Fig. (1): PCR-RFLP analysis of CXCL 12 G801A polymorphism using MspI restriction enzyme:
M: DNA molecular weight marker: 500 bp.
Lane 1, 2, 6, 7: Heterozygous (A/G): 3 bands 100, 202 & 302bp.
Lane 3: Homozygous (A/A): One band at 302bp.
Lane 4, 5, 8, 9: Wild type (G/G): 2 bands at 100 and 202 bp.

DISCUSSION

In contrary to solid tumors that invade into the BM usually in the late stage of the disease, acute leukemias originate in the BM **[2]**. In the marrow microenvironment, acute leukemia cells are in close contact with marrow stromal cells that provide growth and survival signals through surface-bound or secreted factors **[3]**.

Some studies emphasized the idea that tumor cell migration and organ-specific metastasis are critically regulated by chemokines and their receptors **[2]**. SDF-1 is constitutively produced in the BM by immature osteoblasts lining the endosteum region, stromal and endothelial cells **[26]**.

SDF-1 was claimed to have numerous biological roles. In addition to controlling cell motility, SDF-1 can regulate cell proliferation, induce cell cycle progression, and act as a survival factor for both human and murine stem cells **[27,28]**.

Previous studies have indicated that the chemokine SDF-1a encoded by the CXCL12 gene and its receptor CXCR4 play an important role in metastatic cancers **[4,29]**. CXCL12 secretion by stromal cells attracts cancer cells, acting through its cognate receptor, CXCR4, which is expressed by both hematopoietic and non-hematopoietic tumor cells **[3]**. SDF-1/CXCR4 signaling is active in many cancer cells, including those of solid tumors and hematological malignancies **[4]**.

Previous studies have reported an association between the mobilizing capacity of normal hematopoietic progenitor cells and polymorphism at position 801 (G to A transition) in CXCL12, the SDF-1-encoding gene **[4,24,30]**. The ability of leukemic cells to exit from the bone marrow microenvironment, circulate in the peripheral blood and anchor in extramedullary locations might thus depend on the CXCL12 genotype **[30]**.

In this study, CXCL12 (G801A) gene polymorphism was determined in 65 AML and 35 ALL patients, together with 30 controls.

We found that the mutant type (A/A or A/G) was associated with higher PB and BM blasts in both AML and ALL patients compared with the wild type G/G. This finding of PB blasts in AML patients is in accordance with previous studies **[4,30]**. Up to our best knowledge, there are no reported studies for this polymorphism in ALL patients till know; this is the first study.

Also, we found that the homozygous and heterozygous 801A carriers (A/A or A/G) had higher frequency of extramedullary tumor sites compared with the 801G/G wild type patients.

The functional significance of this polymorphism has not been characterized **[31]**. It was hypothesized in 1998 that this mutation could be associated with increased marrow stromal cell secretion of SDF-1 **[32]**, without confirmation to date. On the other hand, it could be associated with lower secretion of SDF-1, a hypothesis supported by the lower SDF-1 level observed in the plasma of normal homozygous 801A subjects **[33]**. This decreased production of SDF-1 might explain the increased capability of malignant cells to egress from the bone marrow microenvironment **[4]**.

As for the response to treatment, we found that the 801A carriers (A/A or A/G) were associated with poor response to treatment as they relapsed and died more frequently than the 801G/G AML or ALL patients; yet the difference was not statistically significant. This might be explained by the finding that SDF-1 enhanced in vitro survival of normal human CD34+ cells and murine stem cells in the absence of growth factors **[27,28]**. This effect of SDF-1 in stress deprivation conditions may give an advantage in the growth and dissemination of blast cells, especially in view of the fact that BM SDF-1 levels increase after irradiation and chemotherapy **[15]**. Because VLA-4 activation was shown to be involved in the pathogenesis of AML minimal residual disease **[34]** and SDF-1 can activate the major integrins VLA-4 and VLA-5 **[35]**, therefore SDF-1 may be important for the persistence of BM minimal residual disease that causes AML relapse after chemotherapy **[2]**.

AML cells express SDF-1 and in vitro treatment of AML cells with neutralizing anti-CXCR4 Abs, anti-SDF-1 Abs, decrease cell survival, implying autocrine regulation of AML cell survival by endogenous SDF-1 **[2]**. However, the fact that blocking CXCR4 or SDF-1 reduced but did not completely inhibit the proliferation and survival of AML cells suggest that factors

and pathways other than CXCR4/SDF-1 interactions are also involved in the regulation of these processes **[2].**

Different studies found that the CXCL12-3'A allele was associated with higher yield of CD34+ cells **[36]**, faster recovery of both granulocytes and platelets after BM transplantation **[37]** and good mobilization capacity **[38]**.

In conclusion we found that the CXCL12 G801A gene polymorphism is a genetic determinant involved in the clinical presentation of leukemia. It is associated with increased release of blasts from the BM to the blood and higher frequency of distal dissemination. It is also associated with resistance to treatment, more frequent relapse and early mortality. So it could be a risk factor for extramedullary dissemination. Accordingly assessment of CXCL12 G801A polymorphism might help in identifying patients at risk of early relapse and mortality.

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