Detection of CXCL12 Gene Polymorphism and CXCR4 Expression in Egyptian Acute Myeloid Leukemia Patients

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Abstract: CXC ligand 12 (CXCL12), a chemokine abundantly produced by the bone marrow (BM) microenvironment, and its receptor CXC chemokine receptor 4 (CXCR4) have crucial roles in malignant cell trafficking. In the present study CXCR4 expression was investigated by flowcytometry and CXCL12 G801A gene polymorphism was detected by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay in 42 patients with de novo acute myeloid leukemia (AML) as well as 35 normal subjects as a control group. The CXCR4 positive expression was found exclusively in AML patients (in 55% of patients). The frequency of the CXCL12 genotypes among AML patients were 55% had a GG alleles genotype while 45% had an A allele genotype while among the control group 83% had a GG alleles genotype and 17% had heterozygous A/G genotype. There was a highly statistical significant relationship between the CXCL12 A allele and extramedullary tissue infiltration (*p* value= 0.01). Also there was a highly statistical significant relationship between each of CXCL12 genotypes and CXCR4 expression and treatment outcome (*p* value= 0.002 & 0.006 respectively). In conclusion CXCR4 expression predicts poor prognosis in AML and CXCL12 G801A polymorphism is a genetic determinant involved in the clinical presentation of leukemia. [Journal of American Science 2010;6(9):318-330]. (ISSN: 1545-1003).

Keywords: PCR-RFLP, CXCR4, Flowcytometry, AML

1. Introduction

Leukemias are complex diseases with a wide range of clinical, morphologic, biologic, cytogenetic, and molecular and immunophenotypic features (1). The world health organization (WHO) classification subdivides acute myeloid leukemia (AML) predominantly according to cytogenetic analysis since recurrent chromosomal abnormalities identify distinct leukemia entities and have a major impact on prognosis (2).

About 40-50% of AML patients show a normal karyotype by conventional cytogenetics & lack a reliable biological marker, thus making the investigations of the etiology and monitoring of minimal residual disease difficult (3).

Patients with AML although initially responsive to current therapy, generally have a poor prognosis and eventually will relapse from minimal residual disease (MRD). The marrow is considered the primary site for MRD where adhesion to stromal elements may protect AML cells from cytotoxic drugs. A better understanding of leukemic cells is needed to identify new prognostic markers and to choose adapted therapeutic strategies (4).

In recent years, attention has been focused on new molecular targets for therapy and biological markers of prognosis. One of these is the CXC chemokine receptor 4 (CXCR4) which is one of a number of chemokine receptors defined by their ability to induce cell migration towards a chemotactic cytokine gradient (chemotaxis). CXCR4 has received much attention because it is the receptor for stromal derived factor (SDF-1a), also known as CXC ligand 12 (CXCL12), and the CXCR4-CXCL12 axis is essential for the migration of normal cells to the bone marrow microenvironment. CXCR4-CXCL12 also appears to play a role in metastatic spread of neoplasms, of both hematopoietic and solid tumors (5).

Myeloid and lymphoid leukemia cells express CXCR4 that induces leukemia cell chemotaxis and migration beneath marrow stromal cells. CXCR4 expression by leukemia cells allows for homing and their retention within the marrow. As such, leukaemia cells appear to utilize CXCR4 to access niches that are normally restricted to progenitor cells, and thereby reside in a microenvironment that favors their growth and survival. Contact between leukemia cells and stromal cells protects leukemia cells from spontaneous and chemotherapy-induced cell death and therefore may represent a mechanism to explain

MRD and subsequent relapses commonly seen in the treatment of these diseases (6).

In AML the immature malignant cells frequently leave the bone marrow, populate the blood, and lodge in extramedullary sites such as the spleen and liver. A potent mechanism in the trafficking of leukemic cells is the interaction of the chemokine receptor CXCR4, which is expressed on AML cells, and its ligand (SDF-1/CXCL12), produced by stromal cells in bone marrow and extramedullary organs such as lymph nodes, liver and spleen. These data provide further evidence that the CXCR4-CXCL12 axis plays a significant role in trafficking and tissue dissemination of AML which subsequently worsen the prognosis (5).

Aim of Work

The aim of this work was to study the expression of CXCR4 receptor by flowcytometry and to study the CXCL12 coding gene polymorphism at codon G801A by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay in Egyptian de novo AML patients and correlate them with the clinical presentation, laboratory data and clinical outcome as CXCL12 gene polymorphism and CXCR4 receptor expression may influence the response to therapy as well as tissue infiltration by malignant cells in these patients.

2. Subject and Methods

Subjects

The present study was conducted on 42 patients with de novo acute myeloid leukemia, their ages ranged between 18 and 70 years with mean value 38.9± 14.4. They were 23 males (55%) and 19 females (45%). Patients were studied prior to chemotherapy and followed up for the disease outcome after induction chemotherapy. Patients were diagnosed and selected among cases referred to Kasr EL-Aini teaching Hospital, Cairo University. Thirty-five age and sex matched individuals with normal peripheral blood picture were also included as a control group.

The diagnosis of leukemia was based on complete history taking, clinical examination and laboratory investigations for diagnosis of AML including complete blood count (CBC), bone marrow aspirate (BMA), cytochemistry and immunophenotyping of leukemic blast cells and special laboratory investigations (for patients and controls):

1- Detection of CXCL12 G801A gene polymorphism by PCR-RFLP assay according to the method described by Dommange et al. (10)
2- Detection of CXCR4 receptor expression by flowcytometry according to the method described by Spoo et al. (12).

Methods

1) Sample Collection:

Four milliliters of venous blood were collected from each patient and each individual of the control group by sterile venipuncture and divided as follows: 1 ml of venous blood for CBC analysis, 2 ml for the study of CXCL12 G801A gene polymorphism by PCR-RFLP assay and 1 ml for the study of CXCR4 receptor expression by flowcytometry.

2) Detection of CXCL12 gene polymorphisms by PCR-RFLP:

I. DNA extraction:

Genomic DNA was extracted from cells by using QIAamp blood DNA isolation kits (Qiagen, Crawley, United Kingdom) according to the manufacturer's protocol.

II- PCR reaction for amplification of CXCL12 gene:

A mixture 25-µL reaction consisted of 2.5 µL genomic DNA, 12.5 µL of PCR master mix, 1 µl of each primer and 8 µL DW (Qiagen).

For CXCL12 G801A polymorphism the following primers (Qiagen) *were used:*
- Forward primer: 5′ CAG TCA ACC TGG GCA AAG CC 3′
- Reverse primer: 5′ AGC TTT GGT CCT GAG AGT CC 3′

Primers were prepared to obtain a primer concentration of 10 pmol/ amplification.

The following cycles were used:
An initial heat denaturation at 94 °C for 3 minutes and 2 loops of amplification: Loop 1 included 7 cycles with the following program: denaturation at 94 °C for 20 sec., annealing at 67 °C for 45 sec., extention at 72 °C for 80 sec. Loop 2 included 28 cycles with the following program: denaturation at 94 °C for 20 sec., anneling at 60 °C for 30 sec., extention at 72 °C for 1 min. In the last cycle extension was prolonged to 5 minutes at 72°C.

The samples were then run in parallel on 2% agarose gel using gel electrophoresis (electro-4, Thermal Hybaid, from Promega) and visualized on a UV transilluminator (wave length 312) to detect the presence or absence of DNA bands. For the CXCL12 polymorphism, a 302- bp fragment was amplified.

III- Digestion of PCR product by specific restriction enzyme for detection of CXCL12 gene polymorphism:

After amplification, the PCR products (302 bp) were digested at 37°C overnight with 0.6 µl *MspI* in the manufacturer's buffer (Helena Biosciences, Sunderland, United Kingdom), generating 2 fragments of 202-bp and 100-bp after digestion in the presence of the **G** allele and it is designated **GG**, wild type (i.e. homozygous for the presence of restriction site), if the A allele exists at position 801, no digestion occurred and only one 302 bp band will emerge and it is designated **AA** (I.e. homozygous for the absence of the restriction site), if the 3 bands 302,202and 100 bp are present it is designated **AG** (i.e. heterozygous) (Figures 1,2).

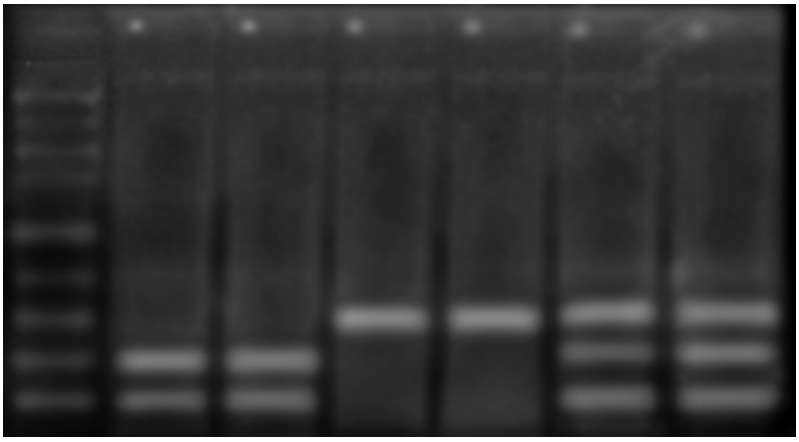

Figure 1. RFLP analysis of CXCL 12 gene polymorphism in AML patients
Lane 1: DNA molecular weight marker (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps).
Lanes 2 & 3: show homozygous GG genotype (100 and 202 bps).
Lanes 4 & 5: show homozygous AA genotype (302 bps).
Lanes 6 & 7: show heterozygous AG genotype (100, 202 and 302 bps).

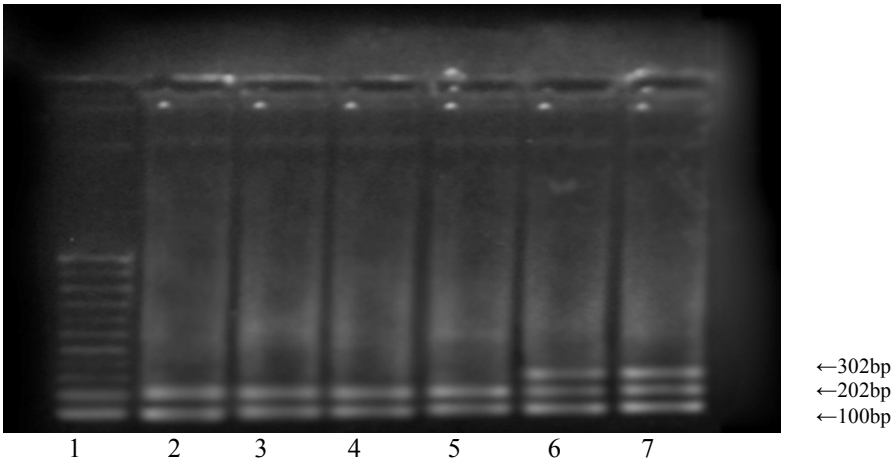

Figure 2. RFLP analysis of CXCL 12 gene polymorphism in the control group
Lane 1: DNA molecular weight marker (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps).
Lanes 2, 3, 4 & 5: show homozygous GG genotype (100 and 202 bps).
Lanes 6 & 7: shows heterozygous AG genotype (100, 202 and 302 bps).

IV- Detection of PCR products:

Bands of CXCL12 gene were identified by using 4% agarose gel. Ethidium bromide staining was used to reveal the fragments under ultra-violet light transillumination.

DNA molecular weight marker (Fermentas AM Egypt), (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bps) was used.

3) Detection of CXCR4 receptor expression by Flowcytometry:

Principle:

Washed cells were incubated with the fluoresein labeled monoclonal antibody, which binds to the cells expressing the CXCR4 receptor. Unbound flurrescein-conjugated antibodies were then washed from the cells. Cells expressing the CXCR4 receptor are fluorescently stained, with the intensity of staining directly proportional to the density of the CXCR4. Cell surface expression of the CXCR4 was determined by flowcytometric analysis using 488 nm wavelength laser excitation.

Flowcytometric analysis:

Ten µl of phycoerythrin (PE) conjugated anti CXCR4 monoclonal antibody and 10µl of fluorescein isothiocyanate (FITS) conjugated anti CD34 monoclonal antibody was added to 100 µl of packed cells (BD Biosciences, San Diego, CA). Tubes were incubated in the dark for 30 min. at room temperature. Unbound anti-CXCR4 and anti CD34 were removed by washing the cells twice in 4 ml PBS buffer supplemented with 0.5% BSA. A non-reactive mAb of the same isotype, and conjugated with the same fluorochrome was used as a negative control. Flowcytometric analysis was performed using (EPICS XL, Coulter Corporation, Hialeah, FL) and analyzed by EXPO analysis software (Beckman Coulter). A cut off value at 20% was set to categorize samples as positive.

The results were declared as the percentage of blasts co-expressing CXCR4 and CD34 within the gated population of blasts:

Positive cases: Those co-expressing CXCR4/CD34 with a value more than 20%.

Negative cases: Those co-expressing CXCR4/CD34 with a value less than 20% or those expressing either CXCR4 receptor or CD34 alone (Figures 3,4).

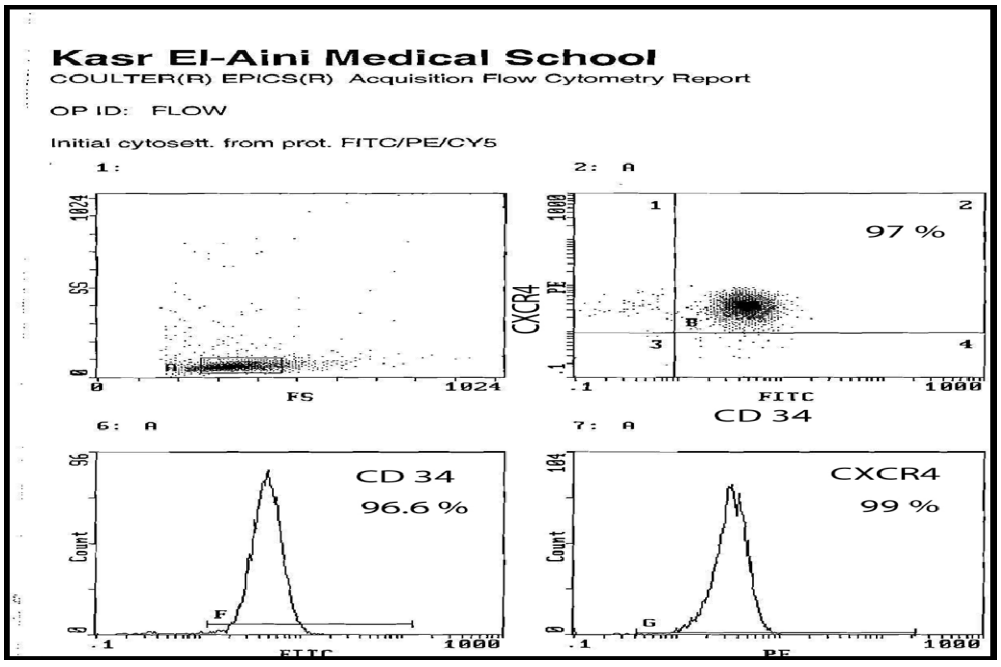

Figure 3. Flowcytometry showing positive CXCR4 / CD34 dual expression

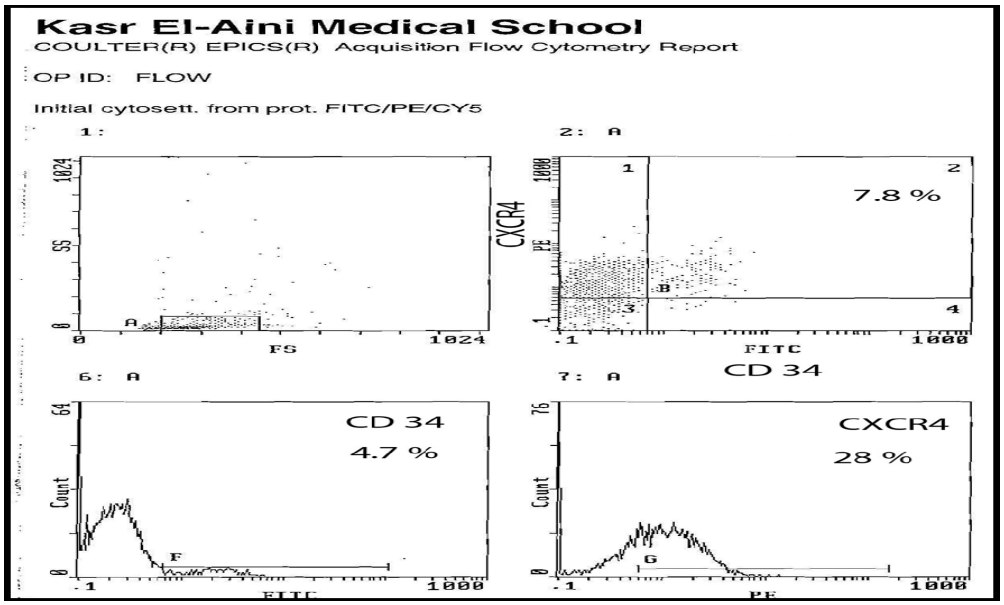

Figure 4. Flowcytometry showing negative CXCR4 / CD34 dual expression

Statistical Analysis

Data was analyzed using SPSS with statistical package version 15. Numerical data were expressed as mean, standard deviation (SD) or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's Exact test was used to examine the relation between qualitative variables. Odds ratio (OR) and 95% confidence interval (CI) were calculated for risk estimation. 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 test). Relation between numerical variables was tested using Pearson product-moment correlation coefficient. P-value less than 0.05 was considered significant and P-value less than 0.01 was considered highly significant.

Declaration of Ethics

A written informed consent was obtained from all patients according to Helsinki guidelines of research ethics.

3. Results

The patients' and the control group characteristics are displayed in table (1) and table (2) respectively.

CXCR4 receptor expression in AML patients and the control group:

Twenty-three AML patients (55%) showed CXCR4/CD34 dual expression on blast cells, while 19 patients (45%) showed no dual expression on their blast cells. Accordingly AML patients were classified into CXCR4 positive group and CXCR4 negative group respectively. The entire control group (100%) showed no CXCR4/CD34 dual expression and considered negative for CXCR4 expression.

CXCL12 G801A gene polymorphism in AML patients and the control group:

Twenty-three AML patients (55%) had (G/G) alleles genotype and 19 patients (45%) had an (A) allele genotype; 16 patients of them (38%) were heterozygous (A/G) & 3 patients (7%) were homozygous (A/A). Accordingly AML patients were classified into CXCL12 (GG) genotype group and CXCL12 (A) allele carrier (A/G & A/A) genotype group respectively. In the control group, 29 individuals (83%) had (G/G) alleles genotype and 6 individuals (17%) had (A) allele genotype; all of them had heterozygous (A/G) type, while none (0%) had homozygous (A/A) type.

Statistical comparison between AML patients and the control group as regard CXCR4 receptor expression and CXCL12 genotypes: Table (3)

Statistical comparison between AML patients and the control group subjects regarding CXCR4/CD34 dual expression and CXCL12 genotypes frequency revealed highly statistical significant difference between the two groups with p-value <0.001 & 0.009 respectively.

Table 1. Clinical and laboratory data of AML patients at diagnosis

Items	AML Patients (No. 42)
Age (years)	
• Range	18 - 70
• Mean ± SD	38.9 ± 14.4
Sex (No.;%)	
• Male	23 ; 55 %
• Female	19 ; 45 %
Clinical data	
Clinical Symptoms (No.; %)	
• Anemic manifestations	42 ; 100 %
• Bleeding tendency	17 ; 40.5 %
• Signs of infection (fever)	24 ; 57 %
Hepatomegaly (No.;%)	
• Present	5 ; 12 %
• Absent	37 ; 88 %
Splenomegaly (No.;%)	
• Present	21 ; 50 %
• Absent	21 ; 50 %
Lymphadenopathy (No.;%)	
• Present	3 ; 7 %
• Absent	39 ; 93 %
Laboratory data	
Hb (gm/dL)	
• Range	3.3 - 11.5
• Mean ± SD	7.1 ± 1.7
TLC (X 10^3 / mm^3)	
• Range	9.0 - 183
• Mean ± SD	53.3 ± 49.1
Platelets (X 10^3 / mm^3)	
• Range	10 - 127.0
• Mean ± SD	47.3 ± 29.4
PB blasts %	
• Range	27 - 98
• Mean ± SD	76.4 ± 18.3
BM blasts %	
• Range	42 - 100
• Mean ± SD	82.5 ± 17.5
LDH (U/L)	
• Range	197 - 900
• Mean ± SD	427.9 ± 173.4
FAB Classification (No.;%)	
• M1	11 ; 26 %
• M2	11 ; 26 %
• M3	6 ; 14 %
• M4	7 ; 17 %
• M5	7 ; 17 %
Immunophenotyping (No.;%)	
• CD 13-33 +ve	42 ; 100 %
• CD 14 +ve	14 ; 33 %
• HLA-DR +ve	36 ; 86 %

Table 2. Clinical and laboratory Data of the control group

Items	Control Group (No. 35)
Age (years)	
• Range	18 - 59
• Mean ± SD	37.3 ± 13
Sex (No.; %)	
• Males	20 ; 57 %
• Females	15 ; 43 %
Laboratory Data	
Hb (gm/dL)	
• Range	10.3 - 16.1
• Mean ± SD	13.2 ± 1.4
TLC (X 10^3 / mm^3)	
• Range	4.1 - 10.9
• Mean ± SD	7.3 ± 1.8
Platelets (X 10^3 / mm^3)	
• Range	154 - 350
• Mean ± SD	261.7 ± 62.3

Table 3. Statistical comparison between AML patients and the control group as regard CXCR4 receptor expression and CXCL12 genotypes

Item	AML patients (No; %)	Control subjects (No; %)	P- value
CXCR4/CD34 Dual expression			
Positive expression	23 ; 55 %	0 ; 0 %	< **0.001**
Negative expression	19 ; 45 %	35 ; 100 %	**HS**
Polymorphism CXCL12 G801A			
(G/G) allele genotype	23 ; 55 %	29 ; 83 %	**0.009**
(A)allele(A/G& A/A)	19 ; 45 %	6 ; 17 %	**HS**

Statistical comparison between CXCL12 (G/G) and (A) allele carrier (A/G & A/A) genotypes in AML patients as regard their clinical and laboratory data:

Comparison revealed no statistical significant difference between CXCL12 (G/G) genotype and (A) allele carrier (A/G & A/A) genotype AML patients regarding demographic data: age, sex, clinical data including; anemic manifestations, fever, bleeding tendency, hepatomegaly & lymphadenopathy; and laboratory data including; hemoglobin level, total leucocyte count (TLC), platelets count, peripheral blood (PB) & bone marrow (BM) blasts %, lactate dehydrogenase (LDH) level and FAB subtypes (p-value > 0.05). However a highly statistical significant difference was found between the CXCL12 (A) allele carrier (A/G & A/A) genotype group and the CXCL12 GG genotype group regarding the presence of splenomegaly; considered as an extramedullary tumor site according to Dommange *et al.* (10) with a p-value 0.005.

Statistical comparison between CXCL12 (G/G) alleles and (A) allele carrier (A/G & A/A) genotypes in AML patients as regard treatment outcome: Table (4)

Comparison revealed highly statistical significant difference between the 2 groups regarding treatment outcome with p-value 0.002.

Statistical comparison between AML patients and control subjects as regards risk of extramedullary tissue infiltration in AML: Table (5).

CXCL12 (A) allele carrier genotypes (Heterozygous A/G and Homozygous A/A) versus CXCL12 (G/G) alleles genotype were associated with increased risk of extramedullary tumor site and tissue infiltration with odds ratio 3.993 and 95% Confidence interval 1.371-11.624.

Comparison between CXCR4 positive and negative AML patients as regard their clinical and laboratory data:

Comparison revealed no statistical significant difference between CXCR4 positive and CXCR4 negative AML patients regarding demographic data:

age, sex, clinical data including; anemic manifestations, fever, bleeding tendency, hepatomegaly & lymphadenopathy and laboratory data including; hemoglobin level, TLC, platelets count, PB & BM blasts % and LDH level (p-value > 0.05). However a statistical significant difference was observed between the CXCR4 positive and negative groups regarding the presence of splenomegaly with p-value 0.03.

To statistically evaluate different FAB subtypes among CXCR4 positive and negative AML patients, they were grouped into 3 groups; (M1, M2) group, (M3) group and (M4, M5) group and the comparison revealed a statistical significant difference between CXCR4 positive and negative AML patients within these FAB subtype groups with p-value 0.003 where the (M4, M5) group showed the higher expression of CXCR4 on blast cells, while the (M1, M2) showed a lower incidence of CXCR4 expression on blast cells.

Statistical comparison between CXCR4 positive and negative AML patients as regard treatment outcome: Table (6)

Comparison revealed highly statistical significant difference between the 2 groups regarding treatment outcome with p-value 0.006, where the CXCR4 positive AML patients had a higher incidence of unfavorable outcome, while on the other hand the CXCR4 negative AML patients had a higher incidence of favorable outcome.

Table 4. Statistical comparison between CXCL12 (G/G) alleles and (A) allele carrier (A/G & A/A) genotypes in AML patients as regard treatment outcome

Treatment outcome No ; %	CXCL12 (G/G) (No. 23)	CXCL12 (A/G&A/A) (No. 19)	P value
Favorable outcome			
Remission	13 ; 87 %	2 ; 13 %	**0.002 HS**
Unfavorable outcome			
Resistant	3 ; 25 %	9 ; 75 %	**0.002 HS**
Death during induction	7 ; 47 %	8 ; 53 %	

Table 5. Risk ratio of CXCL12 genotypes

CXCL12 G801A Polymorphism	AML patients (No;%)	Control subjects (No;%)	Odds ratio	95% Confidence interval	P value
GG alleles genotype	23 ; 55 %	29 ; 83 %	3.993	1.371 – 11.624	**0.009 HS**
A allele (AG &AA) genotype	19 ; 45 %	6 ; 17 %			

Table 6. Statistical comparison between CXCR4 positive and negative AML patients as regard treatment outcome

Treatment outcome (No.; %)	CXCR4 positive AML No. 23	CXCR4 negative AML No. 19	P-value
Favorable outcome			
Remission	4 ; 27 %	11 ; 73 %	**0.006 HS**
Unfavorable outcome			
Resistant	7 ; 58 %	5 ; 42 %	**0.006 HS**
Death during induction	12 ; 80 %	3 ; 20 %	

Association of CXCR4 expression and CXCL12 genotypes in AML patients: Table (7)

The Study reveled that CXCR4 positive expression is significantly associated with CXCL12 (A) allele genotype while CXCR4 negative expression is significantly associated with CXCL12 (G/G) genotype.

Table 7. Association of CXCR4 expression and CXCL12 genotypes in AML patients

CXCL12 genotype	CXCR4 expression		P-value
	Positive (No. 23)	Negative (No. 19)	
A allele carrier (A/G or A/A) (No. 19)	15 ; 65 %	4 ; 21 %	**0.004 HS**
(G/G) Genotype (No. 23)	8 ; 35 %	15 ; 79 %	

Characterization of the AML patients according to CXCR4 expression and CXCL12 genotype status:

According to the association of CXCR4 expression and CXCL12 genotypes as shown in table (11), patients were further grouped into:

- CXCR4 positive / CXCL12 A allele genotype (No. 15).
- CXCR4 positive / CXCL12 GG alleles genotype (No. 8).
- CXCR4 negative / CXCL12 A allele genotype (No. 4).
- CXCR4 negative / CXCL12 GG alleles genotype (No. 15).

Statistical comparison between CXCR4 expression and CXCL12 genotypes in AML patients as regard blast dissemination and extramedullary tissue infiltration: Table (8)

A highly statistical significant difference was detected among these groups as regard the presence of an extramedullar tumor site where CXCR4 positive / CXCL12 A allele genotype group showed the highest incidence of splenomegaly with p-value 0.01. However the difference did not reach a statistical significant level between theses groups regarding PB blasts count.

Table 8. Comparison between CXCR4 expression and CXCL12 genotype in AML patients as regard blast dissemination and tissue infiltration

Item	CXCR4 positive (No. 23)		CXCR4 negative (No. 19)		P-value
	CXCL12 A/G&A/A (No. 15)	CXCL12 GG (No. 8)	CXCL12 A/G&A/A (No. 4)	CXCL12 GG (No. 15)	
Splenomegaly (No.; %)	11 ; 52 %	4 ; 20 %	3 ; 14 %	3 ; 14 %	**0.01 HS**
Hepatomegaly (No.; %)	4 ; 80 %	0 : 0 %	0 : 0 %	1 ; 20 %	0.11 S
Lymphadenopathy (No.; %)	2 ; 67 %	1 ; 33 %	0 : 0 %	0 : 0 %	0.79 NS
PB Blasts Mean ± SD	73.4 ± 17.4	72.5 ± 18.1	85.5 ± 7.4	79.4 ± 18.9	0.37 NS

Statistical comparison between CXCR4 expression and CXCL12 genotypes in AML patients as regard treatment outcome: Table (9)

A highly statistical significant difference was found among these groups regarding treatment outcome, where CXCR4 positive/ CXCL12 A allele genotype group showed the highest incidence of unfavorable prognosis (failed induction and death) while the CXCR4 negative / CXCL12 GG alleles genotype group showed the highest incidence of favorable prognosis with p-value 0.003.

Table 9. Comparison between CXCR4 expression and CXCL12 genotype in AML patients as regard treatment outcome

Treatment outcome (No.; %)	CXCR4 positive (No. 23)		CXCR4 negative (No. 19)		P-value
	CXCL12 A/G&A/A (No. 15)	CXCL12 GG (No. 8)	CXCL12 A/G&A/A (No. 4)	CXCL12 GG (No. 15)	
Resistant to treatment	1 ; 6.5 %	3 ; 20.0 %	1 ; 6.5 %	10 ; 67 %	**0.003 HS**
Failure of induction	7 ; 58 %	0 ; 0 %	2 ;17 %	3 ; 25 %	
Death during induction	7 ; 47 %	5 ; 33 %	1 ; 7 %	2 ; 13 %	

4. Discussion

AML is a genetically heterogeneous disorder characterized by accumulation of acquired genetic alterations in hematopoietic progenitor cells that alter the mechanism of self renewal, proliferation, differentiation and inhibition of apoptosis (7). Despite improvements in outcome of therapy, long-term disease-free survival in AML remains low. A

majority of patients achieve an initial complete remission, but the majority of these patients (60%) eventually will relapse from MRD. The marrow is considered the primary site for MRD where adhesion to stromal elements may protect AML cells from cytotoxic drugs. A better understanding of leukemic cells is needed to identify new prognostic markers and to choose adapted therapeutic strategies (4).

SDF-1; also known as CXCL12 is a CXC chemokine expressed by various cell types playing physiologic roles in the development and function of the immune, cardiovascular, and central nervous systems. In addition, it is a powerful chemoattractant for human progenitor cells mediating their homing to the bone marrow as well as retention, survival, proliferation, and egress to the circulation. SDF-1 signals through its receptor CXCR4 which is functionally expressed on a multitude of tissues and cell types, including the majority of hematopoietic cells (8).

Marrow derived stromal cells constitutively secrete the chemokine SDF-1/CXCL12. CXCL12 acts through its cognate receptor CXCR4 to attract CXCR4-positive tumor cells to marrow niches, where stromal cells secrete high level of CXCL12, and thereby reside in a microenvironment that favors their growth and survival (9). Contact between leukemia cells and stromal cells protects leukemia cells from spontaneous and chemotherapy-induced cell death and therefore may represent a mechanism to explain MRD and subsequent relapses commonly seen in the treatment of these diseases (2). Also, CXCR4-CXCL12 axis plays a significant role in trafficking and tissue dissemination of AML, as the immature malignant cells frequently leave the bone marrow, populate the blood, and lodge in the extramedullary sites such as lymph nodes, liver and spleen, so subsequently worsen the prognosis (5).

The aim of this work is to study the CXCL12 coding gene polymorphism at codon G801A and evaluate its influence on malignant cell dissemination and tissue infiltration in AML and to study the expression of CXCR4 receptor and its prognostic impact on AML patients.

In the present study, as regards CXCL12 genotype 55% of AML patients had a (GG) alleles genotype while 45% had an (A) allele genotype (38% were heterozygous A/G & 7% were homozygous A/A), while among the control group 83% had a (GG) alleles genotype and 17% had an (A) allele genotype (All were heterozygous A/G), the difference between AML patients and the control subjects was highly statistical significant. The results of the present study were nearly similar to the results of Dommange et al. (10) who studied the CXCL12-G801A polymorphism in 86 adult Caucasian AML patients and reported that GG, AG and AA genotypes were 57%, 34% and 9%, respectively for their patients. Also Ponziani et al. (11) studied the CXCL12-G801A polymorphism in 214 adult AML patients and reported that frequency of GG, AG and AA genotypes were 57%, 36% and 7% respectively for their patients.

As regards CXCR4 receptor expression in AML patients and the control group subjects in the present study, the CXCR4 positive expression was found exclusively in AML patients. The incidence of positive CXCR4 receptor expression was 55% of patients, while 45% showed negative CXCR4 expression on their blast cells. This result is in accordance with that recorded by Konoplev et al. (5) who studied CXCR4 expression in 122 de novo AML patients in which the incidence of CXCR4 positive expression was in 57% of their patients and also by Spoo et al. (12) who studied CXCR4 expression in 90 de novo AML patients and reported CXCR4 positive expression in 65% of their patients.

In the present study comparison between CXCL12 (G/G) alleles genotype and (A) allele (A/G & A/A) genotype AML patients regarding age, sex prevalence, clinical presentation and hematological laboratory data revealed no statistical significant difference, this agree with Dommange et al. (10) and Ponziani et al. (11) who could not elicit as well a significant correlation. Also CXCR4 positive AML patients did not show statistically significant difference when compared to CXCR4 negative patients regarding their age, sex prevalence and hematological laboratory data. Similarly, Konoplev et al. (5) and Spoo et al. (12) reported no correlation of CXCR4 expression and age, sex prevalence and laboratory characteristics in AML.

Also, comparison between CXCL12 (G/G) genotype and A allele (A/G & A/A) genotypes AML patients as regards FAB subtypes revealed no statistical significant difference. This is in agreement with Dommange et al. (10) and Ponziani et al. (11) who found that the frequency of CXCL12 genotypes were similar within FAB groups. However there is a highly statistical significant difference between CXCR4 positive and negative AML patients as regards FAB subtypes, where the (M4, M5) group showed higher expression of CXCR4 on blast cells while the (M1, M2) group showed a lower expression of CXCR4 expression on blast cells. These results are in agreement with Mohle et al. (13) who reported differential expression of CXCR4 with lower expression in

cases of undifferentiated (M0), Myeloid (M1/2) & erythroid (M6) AML, and higher expression in myelomonocytic (M4/5) and promyelocytic (M3) AML patients. On the contrary Dommange et al. (10) found that CXCR4 expression showed no statistical difference between FAB groups.

As regards association of CXCR4 expression and CXCL12 genotypes in AML patients, in the present study 65% of patients were CXCR4 positive and have CXCL12 (A) allele genotype (A/G or A/A), and 35% of patients were CXCR4 positive and have CXCL12 (G/G) genotype, on the other hand 21% of patients were CXCR4 negative and have CXCL12 (A) allele genotype (A/G or A/A) CXCL12 genotype and 79% of patients were CXCR4 negative and have CXCL12 (G/G) genotype. Comparison revealed a highly statistical significant difference where CXCR4 positive expression is significantly associated with CXCL12 (A) allele genotype, while CXCL12 (G/G) genotype is significantly associated with negative expression of CXCR4.

This result is in agreement with Dommange et al. (10) who demonstrated correlation between CXCR4 expression and CXCL12 genotype and they attributed that CXCL12 G801A polymorphism might have an influence on the production or transcript half-life of CXCL12 chemokine as it could be associated with lower secretion of CXCL12, an hypothesis supported by the lower CXCL12 level described in the plasma of normal homozygous AA subjects in a study by Soriano et al. (14). This decreased production of CXCL12 might explain the increased capability of malignant cells to egress from the bone marrow microenvironment and infiltrate extramedullary sites and the correlation between CXCR4 positive expression and CXCL12 801A allele (A/G & A/A) genotype patients might result from weaker CXCL12 chemokine level in A allele genotype AML patients (A/G or A/A) with subsequent weaker CXCL12 induced down-regulation of CXCR4 receptor.

As regards percentage of blasts in peripheral blood or in bone marrow, in the present study no statistical significant difference was found between CXCL12 A allele (A/G & A/A) and GG genotypes groups. This comes in approval with Ponziani et al. (11) who found no meaningful difference in percentage of either PB or BM blasts between different CXCL12 genotypes. On the contrary Dommange et al. (10) found that the CXCL12 A allele (A/G & A/A) genotypes were associated with higher blast cell counts in PB, but not in BM and reported that CXCL12 801A carrier status were associated with higher count of circulating AML blasts and higher frequency of extramedullary disease.

To evaluate the role of CXCR4/CXCL12 axis in trafficking of leukemic AML cells in the present study AML patients were classified according to the association of CXCR4 expression and CXCL12 genotypes into four groups: CXCR4 positive / CXCL12 A allele genotype, CXCR4 positive / CXCL12 GG genotype, CXCR4 negative / CXCL12 A allele genotype and CXCR4 negative / CXCL12 GG genotype groups respectively and they were compared as regards PBB percentage however the difference did not reach a statistically significant level between theses groups. On the contrary Dommange et al. (10) reported that CXCR4 positive expression in CXCL12 A allele (A/G & A/A) genotype was correlated with PBB count whereas such a correlation could not be evidenced in CXCL12 GG genotype patients. Their explanation based on the hypothesis of the effect of CXCL12 polymorphism on the intramedullary production of CXCL12 chemokine, the lack of correlation between CXCR4 expression & PBB count in CXCL12 GG genotype patients might be explained by the presence of a critical intermedullary threshold of concentration of CXCL12 below which blasts leave the marrow and that CXCL12 GG genotype patients have a CXCL12 concentration above that threshold, while CXCL12 801A carrier genotype patients might have a concentration below this threshold and therefore present higher PPB count correlated with expression of CXCR4.

As regards extramedullary tissue infiltration, in the present study a highly statistical significant difference was observed between the CXCL12 A allele (A/G & A/A) genotype and the CXCL12 GG genotype groups as regards the presence of splenomegaly (considered as an extramedullary tumor site) which was found in 67% of CXCL12 A allele (A/G & A/A) genotype AML patients, on the other hand it was found only in 33% of CXCL12 (G/G) genotype AML patients. In agreement with Dommange et al. (10), they reported high association of CXCL12 A allele (A/G & A/A) genotype with extramedullary tumor sites which were found in 73.5% of their CXCL12 A allele (A/G & A/A) genotype AML patients and in only 26.5% of CXCL12 (G/G) genotype patients, demonstrating that CXCL12 A allele carrier (A/G & A/A) genotype is a risk factor for tissue infiltration by malignant cells in AML. On the contrary, Ponziani et al. (11) reported evidence for extramedullary dissemination of AML blast cells in only 41% of

their CXCL12 A allele genotype AML patients, while it was found in 59% of CXCL12 GG genotype patients and the difference between their CXCL12 GG and A allele groups was not statistically significant.

In this study, it was found that the CXCL12 801A polymorphism was significantly associated with increased risk of extramedullary tumor sites in AML. This agrees with the results of Dommange et al. (10). On the other hand, Ponziani et al. (11) concluded that their data do not stand in favor of a role for CXCL12–G801A polymorphism in development of extramedullary disease in AML. This may be due to their large sample size compared with smaller sample size in our study and in the study of Dommange et al. (10).

Dommange et al. (10) recommended that it would be interesting to determine in further studies whether CXCL12 genotype has an effect on disease outcome. In the present study we followed this recommendation and compared between CXCL12 GG alleles and A allele carrier (A/G & A/A) genotypes as regards treatment outcome. In CXCL12 (G/G) genotype AML patients; 87% of patients had favorable prognosis (achieved complete remission) and 37% had unfavorable prognosis (either resistance to treatment or death) while in CXCL12 A allele carrier (A/G & A/A) genotypes AML patients; 13% of patients had favorable prognosis and 87% had unfavorable prognosis and the differences were statistically significant between the two groups which clarify a relationship between CXCL12 A allele carrier genotype and poor treatment outcome. On the other hand Ponziani et al. (11) also evaluated whether CXCL12-G801A polymorphism would associate with disease outcome and found the relationship between CXCL12 genotype and disease outcome was statistically non significant. The reason of discrepancy between the present study and that conducted by Ponziani et al. (11) might be attributed to the difference in the treatment protocol and longer period of follow-up for patients.

As regards the effect of CXCR4 expression on disease outcome, the present study revealed a highly statistical significant difference between CXCR4 positive and negative AML patients. In CXCR4 positive group 27% of patients had favorable prognosis and 73% had unfavorable prognosis, while in the CXCR4 negative group 73% of patients had favorable prognosis and 27% of patients had unfavorable prognosis. This approves with Rombouts et al. (15) who demonstrated a correlation between CXCR4 expression on AML cells and poor outcome. Also Konoplev et al. (5) and Spoo et al. (12) confirmed the bad prognostic significance of CXCR4 expression as they reported a strong association between the surface expressions of CXCR4 in AML cells and decreased overall and relapse-free survival. Spoo et al. (12) reported CXCR4 expression by the AML cells favors the enrichment of a more primitive, noncycling subpopulation of AML cells within the stromal layer. These cells may be less susceptible to cytotoxic treatments, and they may represent a reservoir for MRD and subsequent relapses commonly seen in the treatment of AML patients.

As CXCR4 expression predicts poor prognosis in AML patients, CXCR4 represent a novel target for the development of effective treatment of AML and Since the CXCR4/CXCL12 axis plays a central role in homing and maintenance of leukaemia cells in the marrow microenvironment. Therefore, compounds as AMD3100 that target this receptor or its ligand could disrupt the interactions between leukaemia cells and their protective stromal counterparts, antagonize paracrine growth and survival effects of CXCL12, and make leukaemia cells more accessible to conventional therapy (6).

Finally, CXCR4-CXCL12 axis has a role in leukemogenesis and in prognosis or response to therapy, in establishing risk–adapted strategies and help in identifying AML patients at risk of metastasis and could be interesting in the future for new targeted therapies.

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