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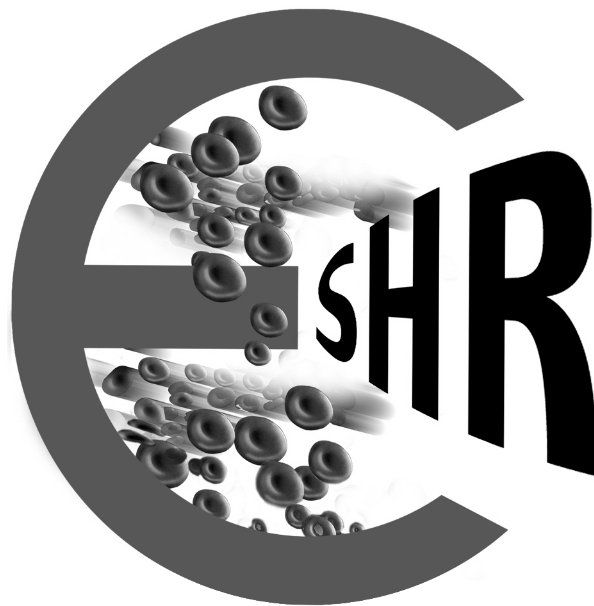

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Role of GSTP1Ile105Val SNP in the Risk of Developing AML and its Clinical Relevance

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ABSTRACT

Background: GSTP1, like other GST (s) plays an important role in the detoxification of several previously activated pro-carcinogens; GST (s) are phase 2 drug metabolizing enzymes responsible for detoxification of many environmental carcinogens. So it is anticipated that polymorphisms of GSTP1Ile105Val resulting in decreased or absent activity might be associated with increased risk of carcinogenesis.

Objectives: To determine the relation of GSTP1 SNPs (single nucleotide polymorphism) with the risk susceptibility to AML and evaluate its clinical relevance regarding response to induction chemotherapy and survival of AML patients.

Patients and Methodos: PCR-RFLP for GSTP1Ile-105Val was done for 60 AML cases and 100 age and sex matched healthy unrelated Egyptian control subjects.

Results: Our study showed that the GSTP1Ile105Val polymorphism distribution in AML cases (n=60; wild 46.7%, heterozygous 40% and homozygous 13.3%) was not significantly different from the control group (n=100; wild 53%, heterozygous 36% and homozygous 11%) (*p*-=0.730). GPST1 150 polymorphism was found to have no effect on response to induction chemotherapy. Although the median survival for patients with mutant GSTP1 was higher than that for patients with wild genotype (4 month versus 2.1 month respectively) but this was not statistically significant (*p*=0.136).

Conclusion: The presence of SNP in GSTP1 has no impact either on the risk of developing de novo AML or on the clinical outcome of patients with AML.

Key Words: *AML – SNP – GSTP1 – PCR-RFLP.*

INTRODUCTION

Acute Myeloid Leukemia (AML) is a clonal disorder characterized by the acquisition of somatic mutations in hematopoietic progenitors leading to disruption of differentiation. Exposition to DNA damaging agents may play an important role in the pathogenesis of AML. Detoxification and Deoxyribonucleic Acid (DNA) repair enzymes protect DNA from damage, due to both endogenous and exogenous sources. When detoxification or repair are ineffective, the DNA damage can cause chromosomal instability leading to severe failure of cell functions, and either apoptosis or oncogenesis. Genetic differences defined by polymorphisms altering the enzymatic activities in detoxification and DNA repair pathways are prime candidates for studies to explain variation in individual susceptibility to develop AML. Individuals with certain polymorphisms in genes metabolizing carcinogens have an increased risk of developing AML [1].

The first line of defense to genotoxic agents is detoxification. This should occur before the agents are able to damage cellular molecules. Metabolism of endogenous and exogenous agents occurs by the same pathways and is divided into two phases. Phase I involves activation of substrates into electrophilic intermediates; these reactions are predominantly catalyzed by the cytochrome P450 (CYP) protein family, many of which harbor polymorphisms which affect their function. The products of the phase I reactions are highly reactive and liable to cause severe cellular damage and the phase II enzymes (conjugation) are required to inactivate the phase I products. Enzymes that participate in phase II include the glutathione S-transferases (GST) and NAD (P) H: Quinine oxidoreductase-1 (NQO1). These enzymes not only detoxify reactive phase I products but also

act on genotoxic agents that do not require phase I activation. The balance of phase I and II activity is critical and a consequence of high levels of phase I activity with low levels of phase II activity is the production of deleterious metabolites which will damage cell components, especially DNA. It follows that polymorphisms affecting the function of either phase I or II proteins, or indeed proteins from both phases, may upset the balance of detoxification activity and predisposes individuals to high levels of damaging agents [2].

Differences in the activities of some GSTs are determined by genetic polymorphisms. Polymorphisms in GSTP1 were first reported by [3]. An A-G polymorphism at nucleotide 313 in exon 5 of GSTP1 gene leads to an amino acid substitution of Isoleucine (IE) by Valine (Val) at 105 amino acid position (Ile105Val). This substitution results in three GSTP1 genotypes: They are isoleucine/isoleucine (Ile/Ile) homozygous wild type, isoleucine/valine (Ile/Val) heterozygote and valine/valine (Val/Val) homozygous variant [4].

GSTs serve two distinct roles in the development of drug resistance via direct detoxification as well as acting as an inhibitor of the MAP kinase pathway. Hence, it is not surprising that high levels of GSTs have been reported in a large number of tumor types. A survey of the NCI cancer drug screening panel of cell lines showed a correlation between GST expression and sensitivity toward alkylating agents [5]. Some of these agents are substrates of GSTs and can be directly inactivated through catalytic conjugation to GSH through thioether bond formation. Many cancer drugs that decompose to produce electrophilic species can be detoxified via glutathione metabolism [6].

Previous studies showed that GSTP1105Val genotype has been associated with favorable prognosis following chemotherapy with drugs known to be GSTP1 substrates in a variety of malignancies such as pediatric acute lymphoblastic leukemia, breast and colon cancers [7-9].

However, the results of GSTs concerning risk and prognosis of acute myeloid leukemia are conflicting in studies conducted in different ethnicities [4,10,11].

PATIENTS AND METHODS

Patients: The study was performed on 60 de Novo AML patients presented to the Medical Oncology Department, NCI, Cairo University in the period from June 2012 to January 2014. Twenty eight patients were (46.7%) males and 32 (53.3%) were females with an age range of 18 to 78 with a median of 32.years. One hundred age and sex matched apparently healthy unrelated individuals selected from blood donors served as a control group; they included 58 (58%) males and 42 (42%) females with an age range of 17 to 59 and a median of 32.5 years. The study was approved by the IRB of the NCI, Cairo University and an informed consent was obtained from each subject before enrollment.

Methods: All patients were subjected to complete history taking and clinical examination as well as baseline chest X-ray and abdominal US and other radiological investigations as indicated. The diagnosis of AML was done according to standard methods (WHO, 2008) and classification was made using the French-American-British (FAB) criteria [12]. The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University. All patients signed informed consent before treatment.

Laboratory investigations included:

- Complete and differential blood picture.
- Bone marrow aspiration and examination of Romanowsky stained smears, supplemented with cytochemical stains such as Peroxidase (MPO) or Sudan Black Stain (S.B.B), Estrases, Acid Phosphatase and PAS when indicated.
- Immunophenotyping using monoclonal antibodies and flow cytometric analysis.
- Conventional karyotyping was performed for all cases.
- FISH as a complementary tool to conventional cytogenetics when indicated.

GSTP1Ile105Val genotyping:

Blood or bone marrow samples were obtained into EDTA tubes. DNA was extracted from WBCs by salting out method (REF) followed by Polymerase Chain Reaction (PCR) as described by Hohaus et al., [13]. Amplification of the extracted DNA was performed in 25µl reaction mix containing 200ng DNA, 200ng

each primer, 1.5mmol/L $MgCl_2$, and 1 unit Taq DNA polymerase in a total volume of 25µL. Following initial denaturation at 95ºC for 7 minutes, 40 PCR cycles were done. Amplification conditions included initial denaturation at 95ºC for 5 minutes followed by 30 cycles of 94ºC for 1 minute, 61ºC for 1minute, and 72ºC for 1 minute with a final elongation step at 72ºC for 7 minutes. The primer sequences were: P105F (5´-ACC CCA GGG CTC TAT GGG AA-3´) and P105R (5´-TGA GGG CAC AAG AAG CCC CT-3´). Enzymatic digestion of the PCR products was performed using one unit Bsm A1 restriction enzyme. Digestion was performed at 37º for 30 minutes in 20µl reaction mix containing 10ul fast digest restriction enzyme mixture (7ul H2O + 2ul buffer + 1u enzyme) + 10ul PCR product. The PCR and the digestion products were visualized with ethidium bromide after electrophoresis on 2% agarose gel at 100 volts for 30min.

Wild type GSTP1Ile/Ile retained the 176bp product of the amplification step, the homozygous GSTP1Val/Val showed 91 and 85bp fragments of complete digestion and the heterozygous GSTP1Ile/Val showed the three bands Fig. (1).

Treatment plan:

Patients received standard induction chemotherapy using cytosine arabinoside and anthracycline as 7 and 3 protocol. Patients who achieved CR were consolidated by the same regimen then HLA typing was done for those below 40 years of age with good general condition, –ve inv (16) & *t* (8,21). Those with identical donor were referred for allogeneic BMT. Those with no HLA identical donor, those with favorable risk (+ve inv 16 or *t* (8,21)) and those 40 years were given 4 cycles of HAM consolidation. Intra-thecal prophylaxis was given only for cases with AML M5 (high risk of CNS disease) after achieving CR by induction chemotherapy. Triple intrathecal prophylaxis was given every 8 weeks for a total of 6 injection using methotrexate 15mg, Ara-C 40mg and dexamethasone 4mg.

Patients with AML M3 were treated with all trans-retinoic acid (ATRA) 45mg/m^2 oral daily in 2 divided doses until CR or for maximum of 90 days in combination with adriamycin 30 mg/m^2 for 3 days every month for 3 months as induction treatment. Patients who achieve CR received maintenance treatment with ATRA (45 mg/m^2 oral daily for 2 weeks every 3 month), 6 mercaptopurine (60mg/m^2 daily) and methotrexate (20mg/m^2 IV once weekly) for 2 years.

Response to induction chemotherapy:

Complete remission was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements, no circulating blast cells, no evidence of extramedullary leukemia and recovery of granulocytes to 1500/µl and platelets to 100,000/µl. Unfavorable outcome included refractory cases (didn't achieve CR) and early death (death within 30 days of diagnosis and before evaluation of the response).

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either Student *t*-test or Mann-Whitney test (non-parametric *t*-test) as apprporiate. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds Ratio (OR) with it 95% Confidence Interval (CI) were used for risk estimation. All tests were two-tailed. A p-value <0.05 was considered significant.

RESULTS

This study included 60 de novo AML patients, as well as 100 age and sex matched healthy controls.

Patient's characteristics:

A total of 60 de novo AML patients included in this study, 28 males (46.7%) and 32 females (53.3%) with an age range of 18-78 years with a median age of 32 years.

The presenting total leucocytic count in the study cases ranged from 2.2 to 183X10^9/L with mean of 60.7±56.5 and a median of 43.5X10^9/L. The platelet count ranged from 6 to 297X10^9/L with a mean of 59.4±54.7 and a median of 40

$X10^9$/L. Hemoglobin ranged from 4.2 to 12 gm/dl, with a mean of 7.69±1.8 and a median of 7.3gm/dl. Blasts in peripheral blood ranged from 3 to 90% with a mean and SD of 40.4% ±28 and a median of 32%. The mean percentage of blasts in marrow was 70.1%±21.4, the median was 77% and the range was 22-97%. The FAB classification of the studied cohort is presented in (Table 1). The most frequent was M2 followed by M4 while the least was M5 and M7; no M6 cases were encountered in our cohort. Cytogentics and molecular genetics findings are presented in (Table 2); the majority of our patients (70%) showed normal karyotype.

GSTP1 105 genotypes in AML vs. control:

The wild type GSTP1105 Ile/Ile (1578AA) was encountered in 28 (46.7%) AML patients compared to 53 (53%) controls, the heterozygous genotype GSTP1 105 Ile/Val (1578AG) in 24 (40%) AML patients compared to 36 (36%) controls and the homozygous genotype GSTP1 105Val/Val (1578GG) in 8 (13.3%) AML patients compared to 11 (11%) controls. The difference was found to be statistically insignificant (*p*.value=0.730). The frequency of heterozygous and homozygous genotypes (mutant types) showed insignificant difference between AML and control groups (*p*.value=0.438).

Association of GSTP1 105 genotypes with various clinical and hematological parameters:

There was no association between GSTP1 genotypes on one side and age (p=0.629), Gender (p=0.744), Hb (p=0.358), TLC (p=0.901), platelets (p=0.493), PB blasts (p=0.883), BM blasts (p=0.625) or cytogenetics (p=0.136) on the other side. As regards the FAB subtypes, we have only 2 cases of M5 and M7 so association could not be statistically analyzed.

Impact of GSTP1 105 genotypes on response to induction chemotherapy:

Complete remission was achieved in 24/60 patients (40%). Twenty two out of 60 patients achieved CR after one course of induction chemotherapy, while 2 patients achieved CR after a second induction. Out of 32 patients with mutant GSTP1, 14 patients (43.7%) achieved CR compared to 10/28 (35.7%) with wild disease (p= 0.526). The CR rate was 50% (4/8) for patients with homozygous GSTP1 105 Val/Val genotype and 41.7% (10/28) for heterozygous lle/Val polymorphism.

Impact of GSTP1 105 genotypes on overall survival:

The median follow-up period for the entire group of 60 AML patients was 2 month (range 0.233-24 month), the median OS for the whole group was 3 month (95% CI 1.463-4.537) and cumulative survival of 47.1% at 3 month Fig. (2). There was no statistically significant difference in the OS of the patients with regards to the GSTP1 105 polymorphism. The cumulative survival at 3 months for patients with GSTP 105 Ile/Ile genotype was 41.2% with a median of 2.1 month (95% CI of0.00-8.355) while the cumulative survival at 3 months for patients with the mutant variant was 85% with a median survival of 4 month (95%CI 0.599-3.734) (p= 0.136) Fig. (3).

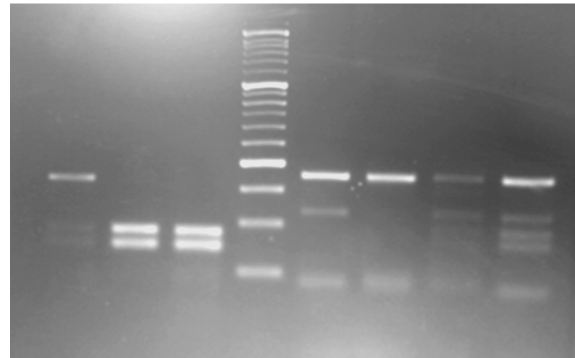

Fig. (1): PCR products for GSTP1Ile105Val after digestion with Bsm A1 restriction enzyme.

Lanes 1, 7, 8 : Heterozygous (Ile/Val), 85, 91, 176bp.
Lanes 2, 3 : Homozygous (Val/Val), 85, 91bp.
Lane 4 : 50bp ladder
Lanes 5, 6 : Wild type (Ile/Ile), 176bp.

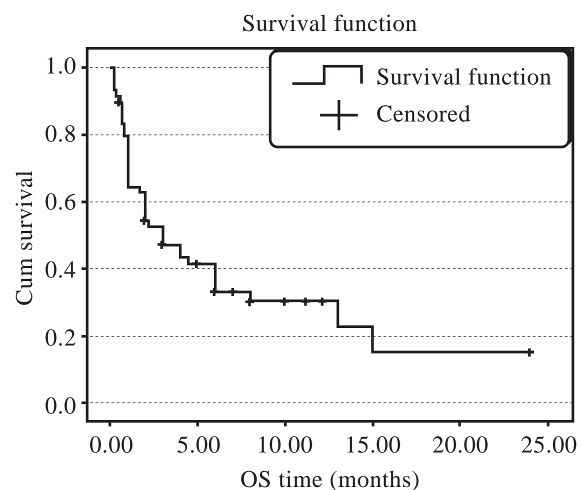

Fig. (2): Cumulative overall survival of 60 adult acute myeloid leukemia patients.

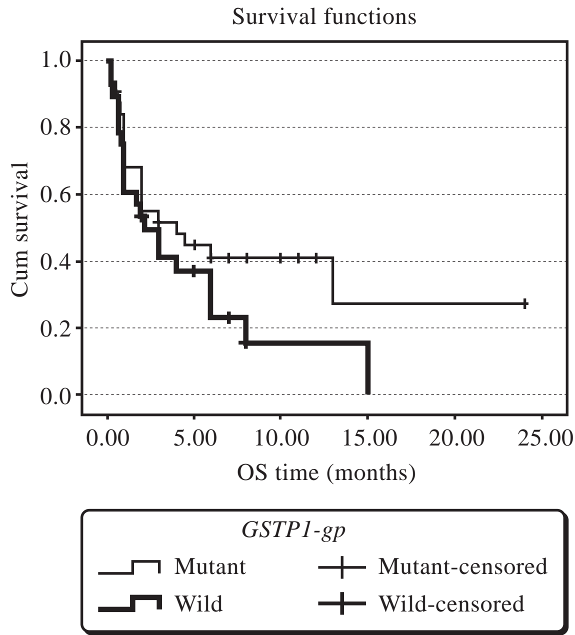

Fig. (3): Effect of GSTP1105 polymorphism on overall survival of 60 adult acute myeloid leukemia patients.

Table (1): French-American-British classification (FAB) of 60 adult acute myeloid leukemia patients.

FAB subtype	No	%
M1	12	20
M2	25	41.7
M3	6	10
M4	13	21.7
M5	2	3.3
M7	2	3.3

Table (2): Karyotyping of 60 adult acute myeloid leukemia patients.

Karyotype	No	%
Normal karyotype	42	70
Abnormal:	18	30
t (8;21)	6	10
t (15;17)	6	10
inv (16)	4	6.7
8 trisomy	1	1.7
Hyperdiploidy	1	1.7

Table (3): Comparison of the frequencies of GSTP1 polymorphisms among healthy Egyptians and other Ethnic groups.

Country (Ethnic)	Exon 5 (codon Ile105Val)				Reference	*p*-value
	No	A/A%	A/G%	G/G%		
Egypt	100	53	36	11	Current study	
Australia	292	39	46*	15**	20	0.123* 0.312**
Finland	293	53	38	9	15	0.881
Germany	64	50	34	16	7	0.586
Italy, Caucasian	70	56	34	10	14	0.911
Japan	50	52	44	4*	16	0.04*
Poland	170	49	44	7	18	0.397
Spain, Caucasian	200	50	44	6	17	0.308
UK, Caucasian	946	49	37	14	21	0.767
USA, PA, NY, Caucasian	163	53	39	8		0.743
African-American	83	58	29	13	19	0.564

p-value: Vs. Egyptians.

DISCUSSION

GSTP1, like other GST (s) plays an important role in the detoxification of several previously activated pro-carcinogens; GST (s) are phase 2 drug metabolizing enzymes responsible for detoxification of many environmental carcinogens. So it is anticipated that polymorphisms of GSTP1 Ile105Val resulting in decreased or absent activity might be associated with increased risk of carcinogenesis on one side and might affect response to therapy, by substrate drugs, on the other side.

In the present study, we tried to elucidate the role of GSTP1 Ile105Val single nucleotide polymorphisms in AML. Their significance was investigated in relation to risk susceptibility, various clinical, laboratory and standard prognostic factors, as well as to treatment response and clinical outcome of patients.

The population frequency of GSTP1 polymorphism among different ethnic groups varies. However, the frequencies of GSTP1 Ile105Val in our study are in concordance with Italian Caucasian reported by Viezzer et al., [14] and

also similar to Finland population [15]. The Japanese population shows slight decrease in GSTP1 Ile105Val homozygous genotype [16] which is statistically different from our population (p-value=0.04). Also Spanish and Polish populations show mild increase in GSTP1 Ile105Val heterozygous polymorphism [17,18]. However, the USA Caucasian population [19], Australians [20] show higher heterozygous type while UK Caucasians show slightly higher homozygous type [14] as shown in (Table 3).

The GSTP1 105Val allele is present in 30% of caucasians and is associated with a decreased activity of the enzyme, when compared to the Ile allele, as measured by the conjugation of the substrate 1-chloro-2, 4-dinitrobenzene and thiotepa [6].

A meta-analysis of case control studies published between 1998 and 2009 was performed to investigate the association of GSTP1 polymorphism with AML risk. Pooled Odds Ratio (ORs) were assessed and heterogeneity between studies was calculated. Overall, OR for GSTP1 Val105 allele was 1.03 with 95% CI (0.80-1.33) and P-value=0.80. Significant heterogeneity was found between studies relating to GSTP1 (p=0.162). From the limited studies on the association of GSTP1 with risk of AML, the role of the gene cannot be fully ascertained [22].

Also, our results are in concordance with Zhou et al., [11] who found that GSTP1 Val/Val carriers had a non-significant risk of AML, with OR of 1.64 and 95 CI of 1.03-2.63 (p>0.05).

However in contrary to the results of the current study, some studies found that GSTP1 Val/Val genotype is associated with the risk of development of acute leukemia [4,23].

Concerning standard prognostic factors, Dunna et al., [4] reported that the proband GSTP1 Val/Val genotype frequency was increased in female AML patients as compared to male patients, whereas sex association was not observed in our patients. Val/Val genotype was also associated with early onset of AML (<20 years) but in our study, all patients are adults (>18 years old). The mean white blood cells count (WBCs) in Dunna study was substantially higher in AML with Val/Val genotype (97.35X10^9/L) compared to 46.06X10^9/L in association with Ile/Ile genotype (p-value <0.05). Also Voso et al., [10] reported lower WBC counts (median 10.8X10^9/L) in AML patients with GSTP1 105Val allele than in patients with GSTP1 105Ile allele (median 18.6X 10^9/L) with p-value of 0.02. However, no association was encountered in our patients.

GSTP1105Val genotype has been associated with favorable prognosis following chemotherapy with drugs known to be GSTP1 substrates in a variety of malignancies such as pediatric acute lymphoblastic leukemia [7].

There is a better survival for patients with multiple myeloma and the GSTP1105Val allele treated with conventional chemotherapy but not in those treated with high-dose therapy [24].

Another study performed on 166 Chinese patients with metastatic colorectal carcinoma treated with first-line FOLFOX-4 showed that patients with Val105 allele variants had a higher response (56.1% vs 37.6%, p=0.04), and a longer progression-free (p<0.01) as well as overall (p<0.01) survival [25].

Voso et al., [10] reported that at a median follow-up of 46 months (range 0-221 months), the GSTP1 105 genotype was significantly associated with Relapse Free Survival (RFS) (p=0.03), whereas the OS was not significantly influenced (p=0.15). Using a competing risk analysis to distinguish between failures due to relapse and failures due to toxicity, they found that the cumulative incidence of relapse was significantly lower for carriers of the variant GSTP1105Val allele (p=0.05), whereas the cumulative incidence of toxic death did not differ according to the GSTP1 105Val allele (p=0.86).

In contrast [4] reported no association between GSTP1 polymorphism and the rate of complete remission failure.

The results of GSTs and risk of acute leukemia are conflicting in studies conducted in different ethnicities. The reason might be inducted by ethnic difference, case selection and variation of clinical characteristics. Further studies are needed to verify the association between GSTP1 polymorphism on AML risk and validate its impact on survival.

In conclusion our data suggested that GSTP1 105 polymorphism has no effect on the risk of development of de novo AML. Also in the

current study, GSTP1 polymorphism failed to show a predictive or prognostic value among the AML patients.

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Role of RAD51G135C SNP in the Risk of Developing AML and its Impact on Survival

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ABSTRACT

Background: RAD51 (Rec A homolog of E.coli) is a polymorphic gene and one of the central proteins in homologous recombination-DNA-double-strand breaks (HR-DNA-DSB) repair pathway, which is vital in maintaining genetic stability within a cell.

Objectives: To determine the relation of RAD51 SNPs (single nucleotide polymorphism) with the risk susceptibility to and impact on the survival of adult AML.

Patients and Methodos: The study included 60 de Novo adult AML patients and 60 age and sex matched healthy unrelated Egyptian control subjects. RAD51G135C was tested by PCR-RFLP. Response to induction chemotherapy was evaluated. Patients were followed-up for Overall Survival (OS).

Results: RAD51G135Cgenotypes distribution in AML cases (wild 75%, variant 25%) was not significantly different from the control group (wild 81.7%, variant 18.3%) (p=0.375). However, the median survival for patients with mutant RAD51 gene was significantly lower than that for patients with wild RAD51 (25 days versus 4.5 months respectively, p 0.005).

Conclusion: The presence of SNP in RAD51has no impact on the risk of developing adult de novo AML but has poor prognostic impact with significant poor survival of the AML patients.

Key Words: *AML – SNP – RAD51 – PCR-RFLP.*

INTRODUCTION

The mechanisms for de novo AML genesis are still rarely understood. Evidence suggests that radiation, smoking, obesity and exposure to chemical carcinogens are considered as its possible risk factors. Nevertheless, AML only develops in a small proportion of people exposed to these environmental and lifestyle risk factors, indicating that the host genetic background might play a critical role in its genesis [1].

DNA damage repair and cell-cycle checkpoints are the most important defense mechanisms against mutagenic exposures. The most important DNA-repair pathways in human cells are: Direct repair, Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR) and double strand break repair (DSB repair). DSB is divided into Homologous Recombination (HR) for example RAD51 and non-homologous end joining (NHEJ) and Translesion DNA Synthesis (TLS). Each pathway repairs a different type of lesion [2].

Polymorphisms in DNA repair genes, including those involved in base excision repair, nucleotide excision repair, mismatch repair and double strand break repair have been implicated in carcinogenesis. Common polymorphisms in DNA repair genes may alter protein function and an individual's capacity to repair damaged DNA. Deficits in repair capacity may lead to genetic instability and tumor genesis [3].

RAD51 is a central protein in the HR repair pathway, binding to DNA and promoting ATP-dependent homologous pairing and strand transfer reactions. The most important polymorphism identified for RAD51 is G135C SNP in 5' untranslated region. The RAD51 G135C polymorphism is associated with RAD51 protein overexpression [4]. RAD51 interacts with and is stabilized by XRCC3, during strand invasion and cross-strand resolution. RAD52 may modulate these activities through its RAD51-interacting region. The ability of RAD52 to induce homologous recombination requires its binding to the p34 subunit of Replication Protein A (RPA); which is a DNA-binding protein that

plays an essential role at replication centers as well as stalled replication forks in the HR repair system [5].

FLT3 (Fms-Like Tyrosine Kinase 3) is activated in about 30% of AML cases. Internal Tandem Duplication (ITD) mutations in FLT3 are associated with the risk of relapse in AML [6]. Some patients with AML have dual mutations of ITD and the tyrosine kinase domain. The dual mutations induce resistance to FLT3 inhibitors and chemotherapeutic agents. The mechanism underlying the resistance was shown to be mediated by STAT5 activation, leading to upregulation of Bcl-x (L) and RAD51. Another study has shown that the FLT3 inhibitor PKC412 and the silencing of FLT3 by RNA interference repress RAD51 in cells with FLT3-ITD mutations but not in cells with intact FLT3. These data suggest that RAD51-mediated HR activity contributes to resistance to therapy in AML with FLT3-ITD mutations [7].

Accumulating evidence suggests that concurrent radiotherapy with Epidermal Growth Factor Receptor (EGFR) inhibitors provides a survival benefit in a variety of cancers, such as those of the lung, head, and neck. The EGFR inhibitor Erlotinib was shown to inhibit radiation-dependent activation of RAD51, indicating that repressed RAD51 contributes to the effect of the concurrent therapy [8].

Thus, some tyrosine kinase inhibitors may not only inhibit growth-promoting signals but also overcome resistance to chemotherapy and radiotherapy by down regulating the HR pathways mediated by RAD51 and its associated proteins [6].

PATIENTS AND METHODS

Patients: The study was performed on 60 de Novo AML patients presented to the Medical Oncology Department, NCI, Cairo University in the period from June 2012 to January 2014. Sixty age and sex matched apparently healthy unrelated individuals selected from blood donors served as a control group; they included 58 (58%) males and 42 (42%) females with an age range of 17 to 59 and a median of 32.5 years. The study was approved by the IRB of the NCI, Cairo University and an informed consent was obtained from each subject before enrollment.

Methods: All patients were subjected to complete history taking and clinical examination as well as radiological investigations as indicated. The diagnosis of AML was done according to standard methods (WHO, 2008) and classification was made using the French-American-British (FAB) criteria [9].

Laboratory investigations included:

- Complete and differential blood count.
- Serum chemistry including liver function and kidney function tests.
- Bone marrow aspiration and examination of Romanowsky stained smears, supplemented with cytochemical stains such as Peroxidase (MPO) or Sudan Black Stain (S.B.B), Estrases, Acid Phosphatase and PAS when indicated.
- Immunophenotyping using monoclonal antibodies and flow cytometric analysis (REF).
- Conventional karyotyping was performed for all cases (REF).
- FISH as a complementary tool to conventional cytogenetics when indicated (REF).

RAD51 G135C genotyping:

Blood or bone marrow samples were obtained into EDTA tubes. DNA was extracted from WBCs by salting out method (REF) followed by Polymerase Chain Reaction (PCR) as described by Voso et al., [10]. Amplification of the extracted DNA was performed in 25µl reaction mix containing 200ng DNA, 200ng each primer, 1.5mmol/L $MgCl_2$, and 1 unit Taq DNA polymerase in a total volume of 25µL. Following initial denaturation at 95ºC for 7 minutes, 40 PCR cycles were done. Amplification conditions included initial denaturation at 95ºC for 5 minutes followed by 30 cycles of 94ºC for 1 minute, 62ºC for 1minute, and 72ºC for 1 minute with a final elongation step at 72ºC for 7 minutes. The primer sequences were:

- RAD51F (5′-TGGGAACTGCAACTCATCTGG-3′) and
- RAD51R (5′-GCGCTCCTCTCTCCAGGCAG-3′).

Enzymatic digestion of the PCR products was performed using one unit MvaI restriction enzyme. Digestion was performed at 37º for 30 minutes in 20µl reaction mix containing 10ul fast digest restriction enzyme mixture (7ul H_2O + 2ul buffer + 1u enzyme) + 10ul PCR product.

The PCR and the digestion products were visualized with ethidium bromide after electrophoresis on 2% agarose gel at 100 volts for 30min.

Wild type RAD51 (GG) showed 86 and 71bp fragments of complete digestion, the homozygous RAD51 (CC) retained the 157bp product of the amplification step and the heterozygous RAD51 (GC) showed the three bands Fig. (1).

Patients were followed-up for a period of 20 days to 72 months with a median of 5 months; Overall survival (OS) and disease free survival were analyzed in context of RAD51 G135C genotypes.

Treatment plan:

Patients received standard induction chemotherapy using cytosine arabinoside and anthracycline as 7 and 3 protocol. Patients who achieved CR were consolidated by the same regimen then HLA typing was done for those below 40 years of age with good general condition ,–ve inv (16) and –ve *t* (8,21). Those with identical donor were referred for allergenic BMT. Those with no HLA identical donor, those with favorable risk (+ve inv 16 or *t* (8,21)) and those 40 years were given 4 cycles of HAM consolidation. Intrathecal prophylaxis was given only for cases with AML M5 (high risk of CNS disease) after achieving CR by induction chemotherapy. Triple intrathecal prophylaxis was given every 8 weeks for a total of 6 injection using methotrexate 15mg, Ara-C 40mg and dexamethasone 4mg.

For patients with AML M3 induction treatment with All Trans-Retinoic Acid (ATRA) (45m/m^2 orally daily in 2 divided doses until CR or for a maximum of 3 months) and adriamycin (30mg/m^2 for 3 days for 3 courses). patients who achieved complete remission received maintenance treatment of ATRA (45mg/m^2 orally daily for 2 weeks every 3 month), methotrexate (20mg/m^2 IV once weekly) and 6 mercaptopurine (60mg/m^2 daily) for 2 years.

Response to induction chemotherapy:

Complete remission was defined as a normocellular BM containing less than 5% blasts and showing evidence of normal maturation of other marrow elements, no circulating blast cells, no evidence of extramedullary leukemia and recovery of granulocytes to 1500/µl and platelets to 100,000/µl. Unfavorable outcome included refractory cases (didn't achieve CR) and early death (death within 30 days of diagnosis and before evaluation of the response).

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either Student *t*-test or Mann-Whitney test (non-parametric *t*-test) as apprporiate. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Odds Ratio (OR) with it 95% Confidence Interval (CI) were used for risk estimation. All tests were two-tailed. A *p*-value <0.05 was considered significant.

Overall survival was measured from the date of diagnosis to death or last follows-up.

RESULTS

This study included 60 de novo AML patients, 28 males (46.7%) and 32 females (53.3%) with an age range of 18-78 years with a median age of 32 years.

The presenting total leucocytic count in the study cases ranged from 2.2 to 183X10^9/L with mean of 60.7±56.5 and a median of 43.5X10^9/L. The platelet count ranged from 6 to 297X10^9/L with a mean of 59.4±54.7 and a median of 40 X10/L. Hemoglobin ranged from 4.2 to 12gm/dl, with a mean of 7.69±1.8 and a median of 7.3 gm/dl. Blasts in peripheral blood ranged from 3 to 90% with a mean and SD of 40.4%±28 and a median of 32%. The mean percentage of blasts in marrow was 70.1%±21.4, the median was 77% and the range was 22-97%. All the studied cases were classified according to FAB classification. The most frequent was M2 followed by M4 while the least was M5 and M7; no M6 cases were encountered in our cohort. Cytogentics and molecular genetics findings showed that the majority of our patients (70%) had normal karyotype. The details of patient's characteristics are shown in (Tables 1,2).

RAD51 G135C genotypes in AML vs. control:

The wild type RAD51 (135 GG) was encountered in 45 (75%) AML patients compared to 49 (81.7%) controls, the heterozygous genotype RAD51 (135 GC) in 14 (23.3%) AML patients compared to 11 (18.3%) controls and the homozygous genotype RAD51 (135 CC) in 1 (1.7%) AML patients compared to 0 (0%) controls. The frequency of both polymorphisms (GC + CC) (mutant type) versus the wild type (GG) was found to be 25% versus 75% in the AML group and 18.3% versus 81.7% in the control group with (p.value=0.375).

Association of RAG51 G135C genotypes with various clinical and hematological parameters:

There was no association between RAD51 genotypes on one side and age (p=0.065), gender (p=0.999), Hb (p=0.322), TLC (p=0.785), platelets (p=0.188), PB blasts (p=0.713), BM blasts (p=0.745) or cytogenetics (p=0.329) on the other side. For FAB subtypes, we had only 2 cases for M5 and M7. So no association could be done statistically for all subtypes.

Response rate:

Complete Remission (CR) was achieved in 24/60 patients (40%). Twenty one out of 45 patients with wild RAD51 genotype showed CR (46.7%), while only 3/15 patients with the variant form achieved CR (20%). Although there is a trend towards better response achievement among patients with wild RAD51, this difference was not statistically significant (p=0.068). The single patient with homozygous RAD51 genotype failed to achieve CR.

Overall Survival:

The median follow-up period for survival of the entire group was 2 month (range: 0.233-24). The median survival for the whole group was 3 month (95% CI 1.463-4.537) with cumulative survival at 6 month of 33.2% Fig. (2). The cumulative survival at 6 months for patients with RAD51 wild genotype was 38% with median survival of 4.5 month (95%CI: 1.739-7.261) versus only 20% cumulative survival at 6 month and median of 25 days (95% CI: 0.518-1.149) for those with RAD51 mutant disease (p 0.005, Fig. (3)). Early death occurred in 13 (21.7%).

Table (1): Characteristics of 60 adult acute myeloid leukemia patients.

Parameter	Findings
Age in years: Median (range)	32 (18-78)
Gender: No. (%)	
Male	28 (46.7)
female	32 (53.3)
TLC X 10^9/L: Median (range)	43.5 (2.2-183)
Hb gm/dL: Median (range)	7.3 (4.2-12)
PLT X 10^9/L; median (range)	40 (6-297)
PBB %, median (range)	32 (3-90)
BMB %, median (range)	77 (22-97)

TLC : Total Leukocyte Count.
PLT : Platelet Count.
PBB : Peripheral Blood Blasts.
BMB : Bone Marrow Blasts.
CR : Complete Remission.
OS : Overall Survival.

Table (2): FAB subtypes and karyotypes of 60 adult acute myeloid leukemia patients.

FAB types	No.	(%)	Cytogenetic	No.	(%)
M1	12	(20)	Normal	42	(70)
M2	25	(41.7)	Inv 16	4	(6.7)
M3	6	(10)	*t* (8;21)	6	(10)
M4	13	(21.7)	*t* (15;17)	6	(10)
M5	2	(1.3)	hyperploidy	1	(1.7)
M7	2	(1.3)	trisomy 8	1	(1.7)

Table (3): Summary of ORs for various contrasts of RAD51G135C polymorphisms among acute myeloid leukemia patients (Li et al., 2014) **[14]**.

Genetic contrast models	Ethnicity	OR (95% CI)	*p*-value
GC vs. GG	Caucasian	1.38 (0.61-3.09)	0.441
	Asian	1.04 (0.81-1.34)	0.762
	Other	1.27 (0.91-1.79)	0.161
	Total	1.22 (0.83-1.79)	0.303
CC vs. GG	Caucasian	1.07 (0.39 3)	0.891
	Asian	1.27 (0.61-2.61)	0.523
	Other	1.24 (0.38-4.08)	0.727
	Total	1.2 (0.71-2.05)	0.494
CC vs. GG/GC	Caucasian	1.02 (0.36-2.86)	0.972
	Asian	1.25 (0.61-2.57)	0.622
	Other	1.19 (0.36-3.93)	0.772
	Total	1.17 (0.69-2)	0.558
CC/GC vs. GG	Caucasian	1.35 (0.61-2.96)	0.457
	Asian	1.06 (0.83-1.36)	0.659
	Other	1.27 (0.92-1.77)	0.153
	Total	1.22 (0.84-1.77)	0.299

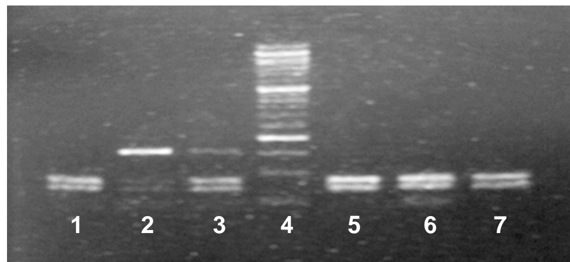

Fig. (1): PCR products after digestion for RD51G135C using MvaI restriction enzyme.

Lanes 1, 5, 6, 7: RD51G135C wild type (86bp, 71bp).
Lane 2: RD51G135C Homozygous (157bp).
Lane 3: RD51G135C heterozygous variant (157bp, 86bp, 71bp).
Lane 4: 50bp ladder.

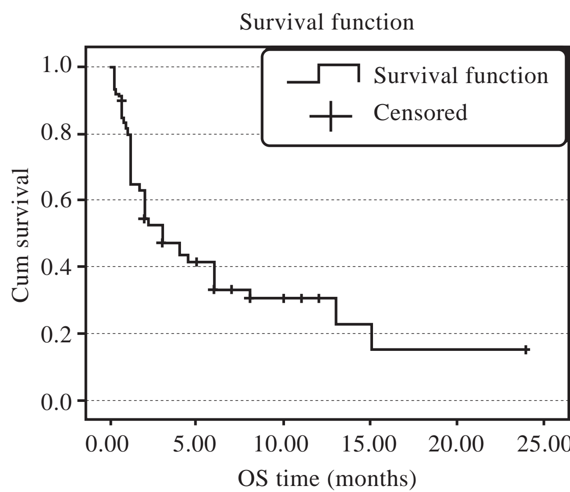

Fig. (2): Overall survival of 60 AML patients.

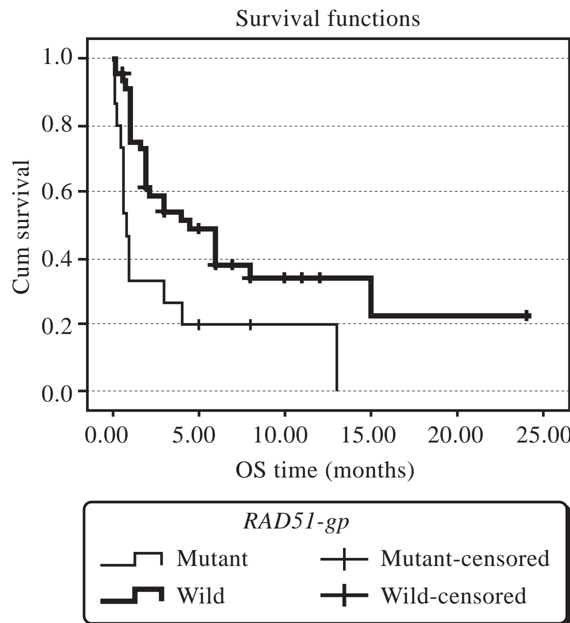

Fig. (3): Overall survival according to RAD51 status in 60 AML cases (*p* 0.005).

DISCUSSION

The polymorphisms of RAD51 which belongs to homologous recombination, part of double strand break repair, was analyzed. Polymorphisms that occur in DNA repair genes affect DNA repair capacity. Common genetic variations in genes involved in DNA repair or response to genotoxic stress may influence both cancer susceptibility and treatment outcome **[11]**.

Some studies supported this hypothesis and showed that RAD51 polymorphism is associated with significant increase in the risk of development of AML **[3,12,13]** in contrast to the results of the current study. However, this association between RAD51 genotypes and development of AML could not be confirmed in 2 Meta analyses. A systematic search of three databases including Pub. Med. and EMBASE for the period up to 20 February 2013 which identified 43 relevant studies was performed by Li et al., **[14]**. Six eligible studies were eventually selected for RAD51 (1764 cases and 3469 controls). Pooled Odds Ratios (ORs) and 95% Confidence Intervals (CIs) for the risk of AML associated with RAD51 were appropriately calculated based on fixed or random effects models. The quality of studies was evaluated using the Newcastle-Ottawa Scale (NOS). Subgroup analyses were performed among Asian, Caucasian and other populations. The pooled results showed that the leukemia risk was not significantly associated with RAD51 and the same results were obtained among any subgroup analysis. This meta-analysis provides evidence that the RAD51 polymorphisms are not associated with an increased risk of AML in the total population as shown in (Table 3).

Also Cheng et al., **[15]** believed that the results of previous studies on the association between RAD51 G135C polymorphism and cancer risk have been inconclusive, partially because of the relatively small sample size of most studies. Therefore, they performed a meta-analysis including 6836 cases and 8507 controls from 22 case-control studies to evaluate the association between RAD51 G135C polymorphism and risk of acute leukemia, Squamous Cell Carcinoma of the Head and Neck (SCCHN), colorectal cancer and ovarian cancer. The overall population analysis showed no significant association between RAD51 G135C polymorphism

and risk of acute leukemia, SCCHN, colorectal cancer or ovarian cancer in any genetic model. However subgroup analysis showed that individuals with GG genotype are more likely to develop SCCHN than other genotypes.

In the present study there was a trend towards better achieving CR among patients with wild RAD51 genotype although not statistically significant (p=0.068). However patients with wild RAD51 genotype had significant better OS versus patients with mutant genotype (p 0.005). In a study by Liu et al., [16] RAD51 (G135C) genotypes were analyzed in 372 Chinese patients with AML by PCR-RFLP or PCR. The Complete Remission (CR) rate, adverse effects, Overall Survival (OS), and Relapse-Free Survival (RFS) were compared among the groups with different genotypes. They concluded that RAD51 gene polymorphism was significantly related to response to therapy, adverse effects, and prognosis of AML with better outcome in patients with wild RAD51 genotype.

Also, Bănescu et al., [17] reported that the RAD51 gene polymorphism showed significant unfavorable outcome among AML patients.

This was explained by Miyagawa [6] who concluded that RAD51-mediated Homologous Recombination (HR) activity contributes to resistance to therapy in AML patients.

On the contrary, Bhatla et al., [18] concluded that RAD51 gene polymorphism did not influence the outcome of AML therapy in the study of de novo AML patients.

Statistical comparison between RAD51 genotypes as regards treatment outcome revealed no statistically significant difference between different genotypes in an Egyptian study of 50 de novo AML patients. Thus, RAD51 gene polymorphism was found to have a non-significant impact on the risk of treatment failure which might be explained by the small sample size. Yet, the percentage of patients with unfavorable outcome (relapse and death) among patients expressing polymorphic RAD51 G135C allele (78% and 100% for heterozygous [G/C] and homozygous [C/C] types. respectively) was higher than that of the patients with favorable outcome (remission) [13].

Also another study of 103 de novo AML patients with positive inv (16)/*t* (16;16) (CBF-beta-MYH1) that were followed-up and retrospectively analyzed found that RAD51 G135C polymorphism has no significant impact on the prognosis among this group of patients [19].

In conclusion, the presence of SNP in RAD51 has no impact on the risk of developing adult de novo AML. However our results showed that RAD51 polymorphism has poor prognostic impact with significant poor survival among patients with mutant variant. Also there was trend towards better response to induction chemotherapy for patients with GG genotypes versus those with CC and GC genotypes.

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Insulin-Like Growth Factor-1 and Interleukin-6 as Key Cytokines in Pathogenesis of Multiple Myeloma

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ABSTRACT

Rationale: Increased angiogenesis has recently been recognized in active Multiple Myeloma (MM) and is associated with poor prognosis. The underlying mechanism for increased angiogenesis in MM remains unclear, with various factors implicated such as Interleukin-6 (IL6) and insulin Growth Factor-1 (IGF1) which promote the proliferation and survival of myeloma cells.

Purpose: The present work is intended to study the level of IL-6 and IGF-1, their role in the pathogenesis of MM and to define the effect of thalidomide on BM angiogenesis and angiogenic cytokines when used as induction therapy

Material and Method: This study includes 40 newly diagnosed MM patients referred to Kasr AL-Ainy Centre of Clinical Oncology and Radiation-Cairo University during the period 2011-2012. ELISA technique was used to measure IGF-1and IL-6 in subjects' sera.

Results: This study showed that IGF-1 and IL-6 post treatment were lower than pretreatment levels but IGF-1 level was more significantly responding to treatment (102.8±61ng/ml before vs. 65.7±51.4ng/ml after, *p*-value 0.028).

Conclusion: Our study delineates the importance of anti-angiogenic drugs such as thalidomide against MM and further suggests the clinical utility of novel treatment paradigms targeting not only the tumor cell directly, but also cellular interactions and cytokine secretion in the BM milieu. We suggest that IL6 and IGF-I should be further studied in future clinical trials as useful monitoring biomarkers and therapeutic targets for MM.

Key Words: *IL6 – IGF1 – MM – Thalidomide.*

INTRODUCTION

Multiple Myeloma (MM) is a malignant disorder characterized by the proliferation of a single clone of plasma cells derived from B cells in the bone marrow. Frequently, there is invasion of the adjacent bone, which destroys skeletal structures and results in bone pain and fractures. Occasionally, plasma cells infiltrate multiple organs and produce a variety of symptoms. The plasma cell clone produces a Monoclonal (M) protein that can lead to renal failure caused by light chains especially λ chain or hyperviscosity from excessive amounts of M protein in the blood. The diagnosis depends on the identification of abnormal monoclonal plasma cells in the bone marrow, M protein in the serum or urine, evidence of end-organ damage and a clinical picture consistent with MM **[1]**.

Recent evidences indicate that angiogenic processes are increased and are fundamental not only in solid tumours but also in hematologic diseases, including MM, as well **[2,3]**. The underlying mechanism for increased angiogenesis in MM remains unclear, with various cytokines, such as Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), interleukin (IL)-6, IL-8 and tumour necrosis factor alpha (TNFa) being implicated **[4]**.

IGF-I is a mitogen and anti-apoptotic cytokine/growth factor/hormone produced by several

types of cells (fibroblasts, hepatocytes, chondroblasts…etc.) [5]. Its potential role as a growth factor for myeloma cells has been deeply analyzed and data of Ge et al suggest that IGF-I significantly contributes to the expansion of MM cells [6].

In spite of the progresses recently registered in the therapy of Multiple Myeloma (MM), the prognosis for patients affected by this disease remains poor [7]. Among innovative treatments, antiangiogenic therapy seems to represent a promising approach, which is based on tumor growth inhibition by starving cancer cells of vital nutrients [8].

The present work is intended to study the level of IGF-1 and IL-6 and their role in the pathogenesis of MM and to define the effect of thalidomide on BM angiogenesis and angiogenic cytokines when used as initial therapy. We measured the levels of these prognostic biomarkers in Egyptian MM patients to help in defining additional therapeutic lines for the disease.

PATIENTS AND METHODS

Study population:

The current study was carried out on 40 Egyptian patients with MM. Patients were chosen during the period of 2010-2012 among cases referred to the Clinical Oncology Department, Cairo University after taking their informed consent. The research was approved by the IRB of the Clinical Oncology Department, Cairo University. Patients were 29 males and 11 females. Their ages ranged between 35 and 69 years with a median value of 49 years.

All patients were subjected to: (1) Routine laboratory investigations including: Complete blood count with differential leukocytic count, metabolic panel (calcium, albumin, and creatinine……) and coagulation tests. (2) Myeloma-specific investigations including: Serum protein electrophoresis, monoclonal protein analysis by immune-electrophoresis, urine protein electrophoresis, serum β2-microglobulin, CRP, LDH and BM aspirate, (3) Skeletal bone survey including: Plain X-ray of spine, pelvis, skull, humeri, and femurs, (4) Specific investigations including: Quantitative assessment of IL6 using AviBion Human IL-6 ELISA Kit that was provided by (Orgenium Laboratories, Helsinki FinlandI) and IGF-1using DRG IGF-I 600 ELISA kit (DRG Instruments GmbH, Germany).

Treatment protocol:

Oral Thalidomide (100mg) given for 28 continuous days (one cycle). After 2 cycles, the patients were referred for Lab. Assessment.

Statistical data analysis:

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. 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). Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. A p-value <0.05 was considered significant.

RESULTS

This study included 40 newly diagnosed Multiple Myeloma (MM) patients and 20 healthy volunteers. Patients were treated with thalidomide (100mg) and dexamethasone (Thal-dex.). They were 29 males (72.5%) and 11 females (27.5.5%). Their ages ranged between 35 and 69 years with median value of 49 years.

MM patients were compared before and after therapy regarding WBCs count, ESR and plasma cells count in BM; all were significantly higher in MM patients before treatment. Other laboratory findings, creatinine, calcium, uric acid, LDH and B2M levels were also significantly higher in MM patients before treatment. As regards Ig subtypes, twenty six patients (65%) had IgG monoclonal band, 14 patients (35%) had IgA. Twenty eight (70%) were Kappa chain positive and 12 patients (30%) were lambda positive. No statistically significant difference was noticed between the two patients groups as regards IL6 levels (p=0.296). However, IGF-1 levels were significantly higher in MM patients before treatment (p=0.028).

Table (1): Comparison between laboratory findings pre and post treatment in 40 MM patients.

Variable	Pre treatment	Post treatment	*p*-value
WBCs X 10^9/L (N: 4-11 X 10^9/L)	5.123±2.7504	4.420±5.001	0.033*
Hb g/dl (Male: 13-17g/dL) (Female: 12-15 g/dL)	10.4±1.8	10.6±1.5	0.444
Platelets X 10^9/L (N: 150-450 X 10^9/L)	32.700±37.035	316.600±99.757	<0.00*1
ESR (Female: 1-25mm/hr) (Male: 0-17mm/hr)	73±21.3	43.4±12.5	<0.001*
Plasma cells count in BM (N: 0-1%)	49.8±15.2	11.3±5.6	<0.001*
Uric acid mg/dl (Female: 2.5-7.5mg/dl) (Male: 3.5-7.5mg/dl)	5.7±2.1	4.4±1.1	<0.001*
Creatinine mg/dl (Female: 0.6-1.2mg/dl) (Male: 0.5-1.1mg/dl)	1.8±1.5	1.1±0.3	0.001*
Calcium mg/dl (N: 8.2-10.2mg/dl)	8.2±0.5	7.8±0.6	0.016*
LDH U/l (N: 0-250U/L)	330.5±81.7	264.8±58.3	0.006*
Albumin g/dl (N: 3.2-5g/dl)	2.8±0.6	3.3±0.5	0.001*
Total protein g/dl (N: 6.0-8.5g/dl)	9.1±2.2	8.4±1.5	0.139
B2M µg/mL (N: 0-3µg/mL)	4.2±3.4	1.9±0.9	<0.001*
IL-6 pg/ml (N: 0-500pg/ml)	38.9±64.7	22.5±36.1	0.296
IGF-1 ng/ml (N: 0-350ng/mL)	102.8±61	65.7±51.4	0.028*

Values are mean ± SD.

DISCUSSION

MM evolution has been shown to be strongly conditioned by angiogenic mechanisms in terms of growth and therapy sensitivity. Several authors tried to explain how angiogenic cytokines **[9,10]** may work influencing the MM cells; consequently, in the recent years, the presence and quantity of several angiogenic factors, their inducers and their signaling mediators have been documented in an effort to explore the possibility to use them as diagnostic, monitoring or prognostic markers of disease evolution and therapy sensitivity.

IL-6 has been demonstrated to be involved in the proliferation of plasmablastic cells in bone marrow and in the differentiation of these cells into mature plasma cells. Apart from its involvement in the development of normal plasma cells, it is clear that IL-6 is a potent myeloma cell growth factor **[11]**.

In this study, 40 MM patient were included and we demonstrated that IL-6 levels posttreatment are lower than pretreatment levels but with no significant statistical difference between them (*p*-value=0.296). This is in agreement with what was previously reported by Klein et al.,

1991 that IL-6 serum levels are increased in patients with multiple myeloma and reflects disease severity; the inhibition of myeloma cell proliferation with antitumoral effects was observed in patients who had been given anti-IL-6 murine monoclonal antibodies **[12]**.

Another molecule involved in MM biology is IGF-I, a mediator cytokine, known to be a growth promoter for several tumors, including MM, acting through its anti-apoptotic/proliferative **[13-15]** effects and interaction with angiogenic factors, such as the anti-proliferative TGF-beta1 **[16]**.

Data regarding serum level of IGF-I in MM are scarce and partially contrasting **[17]**. The present study clearly shows that the serum IGF-I concentrations significantly decreased after treatment with thalidomide (p-value=0.028). Some hypotheses suggest that thalidomide or its breakdown product (s) inhibits the stimulatory effects of insulin like growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2) on angiogenesis **[18,19]**. Also, because there is increasing evidence that IGF-1 is also an important growth and survival factor in MM **[20]** inhibition of IGF-1 signaling by thalidomide may contribute to its anti-MM activity. Specifically, IGF-1 triggers phosphatidylinositol-3-kinase (PI-3K) signaling, with downstream Mitogen Activated Protein Kinase (MAPK) activation and proliferation, as well as activation of Akt with downstream phosphorylation and inactivation of the proapoptotic Bcl-2 family member Bad, thereby inhibiting caspase activity. Finally, IGF-1 enhances the growth of the MM cell line OPM-6 in severe combined immunodeficiency mice, further supporting its role in MM pathophysiology and raising the possibility that inhibition of IGF-1 may account, at least in part, for the anti-MM effects of thalidomide **[21]**.

Our study delineates the importance of anti-angiogenic drugs such as thalidomide against MM and further suggests the clinical utility of novel treatment paradigms targeting not only the tumor cell directly, but also cellular interactions and cytokine secretion in the BM millieu. We suggest that IL6 and IGF-I should be further studied in future clinical trials as possible monitoring markers and possible therapeutic targets for MM.

Conclusion:

The results of this study may be considered as important hypothesis-generating observations and need further validation with larger numbers of patients. Further research in understanding the BM micro-enviroment role of angiogenesis and other angiogenic cytokines in various stages of MM is needed.

Conflicts of interest:

None

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Role of GM-CSF in Myeloid Neoplasms

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ABSTRACT

Background: Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) cytokine stimulates growth, differentiation and function of myeloid progenitors.

Aim: The present work aimed to study the role of GM-CSF gene expression, its protein and antibodies in acute myeloid leukemia/myelodysplastic syndromes (AML /MDS) patients and their correlation to disease behavior and treatment outcome. The study included 50 AML/MDS Egyptian patients in addition to 20 healthy volunteers as controls.

Patients and Methods: Assessment of GM-CSF gene expression was performed by quantitative real-time PCR. GM-CSF proteins and antibodies were assessed by ELISA.

Results: There was significant decrease in GM-CSF gene expression (p-value=0.008), increase in serum level of GM-CSF protein (p-value=0.0001) and anti GM-CSF antibodies (p-value=0.001) in AML/MDS patients in comparison to healthy controls. Also, there was significant negative correlation between serum level of GM-CSF and initial PB blasts percentage as well as response to therapy.

Conclusion: Any alteration in GM-CSF gene expression could have implication in leukemogenesis, also GM-CSF serum level could be used to predict outcome of therapy. GM-CSF antibodies may as well play a role in the pathogenesis of AML/MDS. The use of these GM-CSF parameters for disease monitoring and as markers of disease activity needs further research.

Key Words: *GM-CSF – Gene expression – Protein concentration – Antibodies – AML/MDS.*

INTRODUCTION

Acute Myeloid Leukemia (AML) is a heterogenous group of leukemias that results from a genetic event or series of events occurring in an early hematopoietic precursor that both blocks differentiation and allows uncontrolled proliferation. The abnormally proliferating leukemic cells accumulate in the marrow space, eventually replacing normal marrow progenitors, with consequent diminished production of red cells, white cells and platelets. This, in turn, leads to the common clinical manifestations of AML [1].

The Myelodysplastic Syndromes (MDS) include a large spectrum of clonal hemotopoietic stem cell disorders that are characterized by peripheral cytopenia (s), morphologic dysplasia, ineffective hematopoiesis and a variable propensity to transform to AML [2].

GM-CSF stimulates multipotent progenitor cells depending on its concentration, the proliferation of macrophage progenitors, followed by granulocytes, erythroid, eosinophil, megakaryocyte and multipotent progenitors. It also stimulates the differentiation of myeloid leukemic cells and controls eosinophil function in some instances [3].

Human GM-CSF gene is approximately 2.5 kbp long. The gene is located on the long arm of chromosome 5 (5q21-q32). It has 4 exons that are separated by 3 introns [4]. Besides the GM-CSF gene, genes encoding various cytokines like IL4, IL5, IL9, IL12, M-CSF and EGR1 gene are located at the 5q31.1 locus of chromosome 5 [5]. The (5q-) syndrome has elucidated the role of these cytokines in development of clonal hematopoietic stem cells [6]. The current study aimed to investigate and understand the role of GM-CSF in pathogenesis, progression and response to therapy in Egyptian AML/MDS patients.

SUBJECTS AND METHODS

Study population:

The present study included 50 patients with AML/MDS. Patients were recruited from the outpatient clinic and the inpatient wards of Kasr

Al-Ainy Clinical Hematology Unit, Internal Medicine Department and Clinical Oncology Department, Faculty of Medicine, Cairo University.

Patients in complete remission or those with history of recombinant human GM-CSF intake were excluded. Twenty age and sex matched healthy volunteers were included in the study as a control group. The study was approved by the Research Ethical Committee of Internal Medicine Department, Faculty of Medicine, Cairo University and informed consents were obtained from all participants prior to enrollment in the study.

For both patients and controls, 4ml EDTA blood and serum samples were collected under complete aseptic conditions for routine workup, molecular studies and ELISA techniques respectively.

Quantitative assessment of GM-CSF gene expression:

Extraction of total RNA was performed by QIAamp RNA Blood Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Total RNA was reverse transcribed using random primers with a high capacity cDNA archive kit (Applied Biosystems, Foster city, CA, USA).

GM-CSF gene expression was detected by real-time PCR based on Taqman technology using ABI Prism 7700 (Applied Biosystems, USA).

The primers and probes for GM-CSF as well as the house keeping gene (GAPDH) were provided by Qiagen® (USA). GM-CSF gene: F: 5'-CTGCTGAGATGAATGAAACAG-3'and R:5'-TCCAAGATGACCA TCCTGAG-3', FAM probe:5'-ACTCCCACCATGGCTGTGG-3' (TaqMan GM-CSF, access No: M11220, Applied Biosystem, USA). The thermocycler program conducted was initial denaturation at 50ºC for 2min. followed by 40 cycles of denaturation at 95ºC for 10min., annealing at 95ºC for 0.15min. and extension at 60ºC for 1min. The Relative Quantification (RQ) of gene expression was assessed by $2^{-\Delta\Delta Ct}$ method (ΔΔCt= {[Ct (GM-CSF sample)-Ct (GAPDH sample)]-[Ct (GM-CSF calibrator)-Ct (GAPDH calibrator)]}. The calibrator was the average ΔCt value of 20 controls [7].

Assessment of GM-CSF protein and anti GM-CSF antibodies:

The concentration of GM-CSF was measured in appropriately diluted sera from all AML/MDS patients as well as healthy controls by using a specific ELISA assay (Quantikine human GM-CSF kit, catalog # SGM00, R & D Systems, Inc., Minneapolis, USA). The minimum detectable dose of GM-CSF by this reagent is typically less than 3pg/ml.

The concentration of GM-CSF Abs was measured in appropriately diluted sera from 42AML/MDS patients as well as healthy controls by using a specific ELISA assay (anti GM-CSF Ab kit, catalog # MBS162797, My Biosource, USA). The minimum detectable dose of GM-CSF Abs by this reagent is typically less than 0.52ng/ml.

Treatment regimen and response to therapy:

ALL patients were treated according to the adopted protocol of the Internal Medicine Department, Faculty of Medicine, Cairo University. AML patients (except M3 cases) were subjected to 7-3 protocol for induction of remission. M3 patients were subjected to All-Trans-Retinoic Acid (ATRA). Treatment of AML depends on the fitness of the patient. Fit patients (<60 years) received intensive therapy. Treatment includes induction and postremission therapy (consolidation). Less fit patients (70-75 years and older, or younger patients with significant co-morbidities) receive low-intensity therapy. For induction therapy, combination of cytarabine and anthracycline or anthracenedione is recommended (Cytarabine 100-200mg/m^2 continuous IV infusion for 7 days plus Idarubicin 12 mg/m^2/day for 3 days or Daunorubicin 60-90 mg/m^2/day for 3 days). Follow-up by bone marrow examination to assess remission is typically done 7-14 d after completion of induction chemotherapy. For postremission therapy, all patients should be assessed for risk of relapse. Specific drug regimens are recommended based on a patient's risk of relapse (e.g: High-dose Cytarabine 3g/m^2 IV over 3h every 12h on days 1, 3, and 5 for 4 cycles).

Complete Remission (CR) status was defined by normalization of the neutrophil count (≥1.5/μL) and platelet count (>100X10^3/mm^3), and marrow examination that demonstrates at least 20% cellularity, less than 5% blasts and no Auer

rods, as well as absence of extramedullary infiltration. Resistance to treatment (RD) is defined as more than 25% blasts in the BM, lack of regeneration of normal hematopoiesis, persistence of peripheral blood blasts and/or extramedullary leukemia after induction. Relapse was defined as re-infiltration of the bone marrow by 5% or more leukemic blasts or proven leukemic blasts at any site. Death during induction is defined as death during or after the first course of therapy with aplastic or hypocellular marrow [8].

The only known curative modality for patients with MDS is Stem Cell Transplantation (SCT). Therefore, all appropriate candidates should be considered for SCT. They include patients younger than 70 years, with a reasonable performance status and no significant comorbidity Fig. (1).

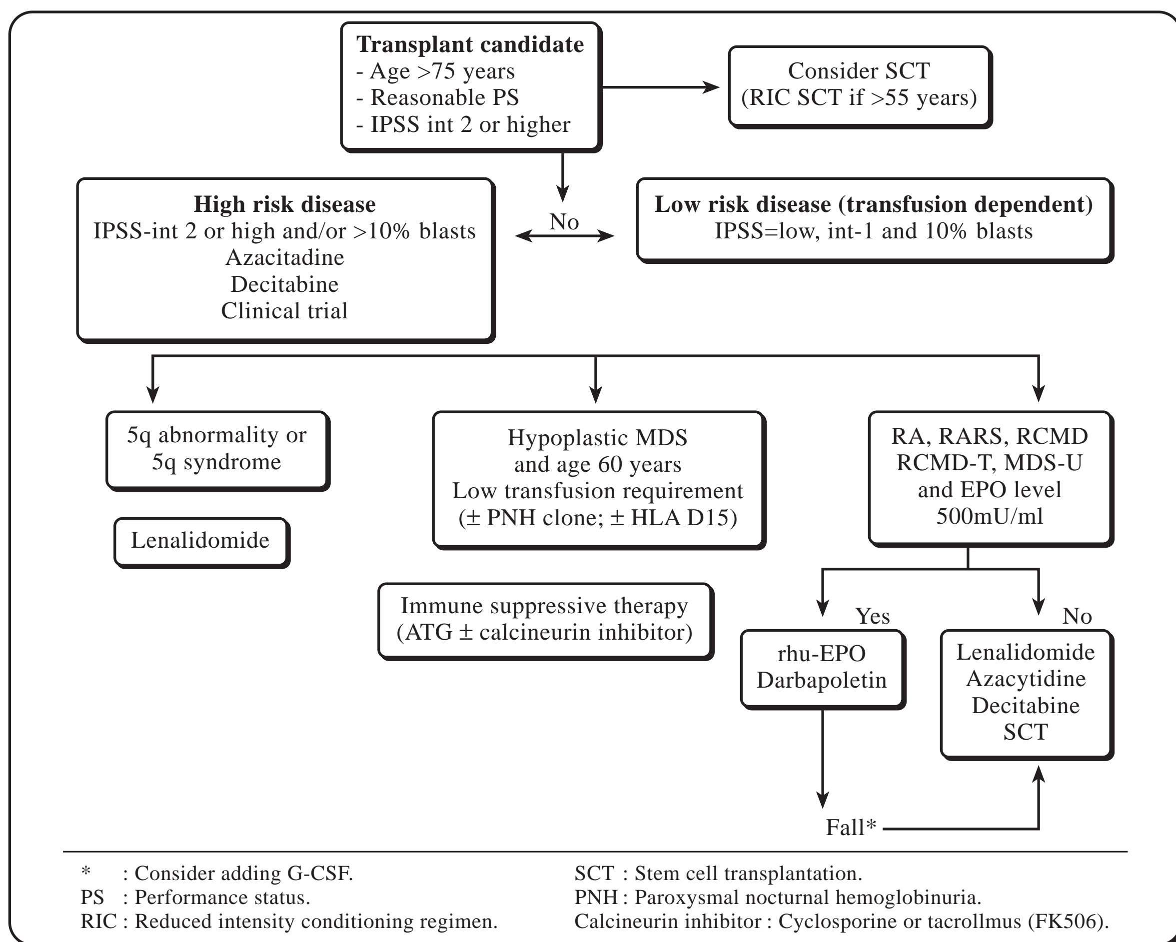

Fig. (1): Algorithm for management of a patient with MDS.

Data analysis:

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as number and percentage. Statistical differences between groups were tested using Chi Square test for qualitative variables. For non parametric quantitative data, comparison between groups was done using Mann-Whitney test and Wilcoxon Signed Ranks 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. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. A p-value <0.05 was considered significant.

RESULTS

The patients were 27/50 (54%) males and 23/50 (46%) females. Their age ranged between 20 and 91 years with a median of 45.5 years. According to disease subtypes, 44 patients (88%) were AML and 6 patients (12%) were MDS. According to FAB classification of AML, 14 patients (28%) were FAB-M1, 9 (18%) were M2, 3 (6%) were M3, 9 (18%) were M4, 4 (8%) were M5, while 5 patients (10%) were M7.

Patients who did not receive chemotherapy due to bad general condition, poor performance or just refusal were 17. Among patients who received chemotherapy, CR rate was 15/33 (45.4%), failure of induction rate was 6/33 (18.1%) and death rate was 12/33 (36.36%). Out of 15 patients who achieved CR, relapse was reported in 5/15 patients (33.3%) at a follow-up period of 24 months. Overall Survival (OS) ranged between 1 and 24 months with a mean value of 4±4.04 months, while the OS for patients achieving CR ranged between 2 and 12 months with a mean value of 7.6±3.8 months.

GM-CSF gene expression:

GM-CSF gene expression values in the 20 healthy controls ranged between 0.659 and 1.515 with a mean value of 0.62±0.09, while in the 50 AML/MDS patients it ranged between 0.0126 and 3.249 with a mean value of 0.629± 0.09. GM-CSF gene expression levels were significantly lower in AML/MDS patients compared to controls (*p*-value=0.008). We didn't find significant correlation between GM-CSF gene expression and age, gender, clinical data, TLC, initial PB blasts percentage, treatment outcome, period of CR and OS [(Table 1) & Fig. (2)].

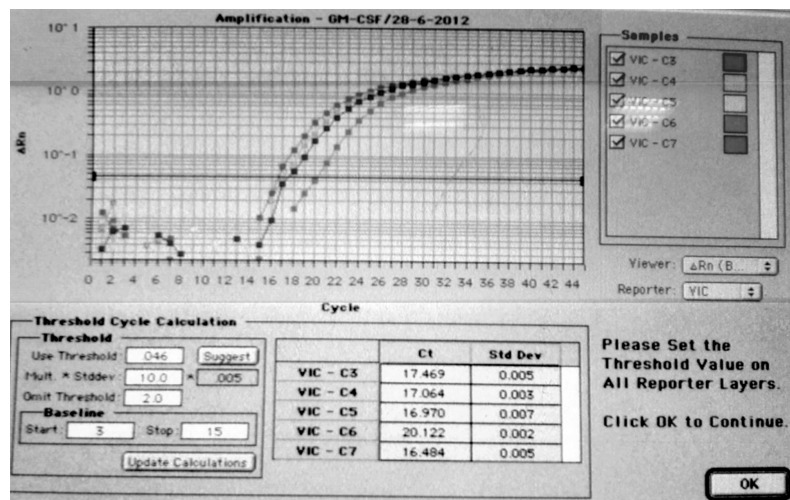

Fig. (2): GM-CSF amplification plot.

GM-CSF protein and anti GM-CSF antibodies:

Serum GM-CSF protein concentration in the healthy controls ranged between 7 and 8.6 pg/mL with a median of 7.8pg/mL, while in the 50 AML/MDS patients it ranged between 12 and 80pg/mL with a mean value of 41.1±18.4 pg/mL. We found higher GM-CSF protein concentration in AML/MDS patients compared to controls (*p*-value=0.0001). We found significant negative correlation between GM-CSF protein concentration and initial PB blasts percentage (*r*=–0.357, *p*-value=0.011), treatment outcome (*r*=–0.339, *p*-value=0.016). Otherwise, we didn't find significant correlation between GM-CSF protein concentration and age, gender, clinical data, other laboratory data, period of CR and OS (Table 1).

Serum anti GM-CSF antibodies in the healthy controls ranged between 10 and 18 ng/mL with a median of 14ng/mL, while in 42/50 (84%) AML/MDS patients it ranged between 37 and 240ng/mL with a mean value of 64.4±42.33ng/mL. We found higher serum anti GM-CSF antibodies in AML/MDS patients compared to controls (*p*-value=0.001). We didn't find significant correlation between serum anti GM-CSF antibodies and age, gender, clinical data, laboratory data, treatment outcome, period of CR and overall survival OS (Table 1).

Table (1): Correlation between GM-CSF and different clinical and laboratory parameters of 44 AML/6 MDS patients.

Parameter	GM-CSF gene expression		GM-CSF protein		Anti GM-CSF antibodies	
	r	*p*	*r*	*p*	*r*	*p*
Age	–0.045	0.755	0.229	0.110	–0.129	0.414
Gender	–0.032	0.825	0.086	0.551	0.117	0.461
TLC	–0.141	0.328	–0.180	0.212	–0.540	0.735
PB blasts	–0.026	0.858	–0.357	0.011*	–0.255	0.102
Outcome	0.163	0.258	–0.339	0.016*	–0.021	0.616
Survival	0.02	0.891	0.072	0.621	0.035	0.828
Period of CR	–0.193	0.491	0.003	0.991	0.102	0.521
GM-CSF protein	–0.197	0.171	–	–	0.052	0.746
Anti GM-CSF antibodies	–0.038	0.813	0.052	0.746	–	–
GM-CSF gene expression	–	–	–0.197	0.171	–0.038	0.813

*Significant correlation.

Response to therapy given to AML/MDS patients:

- After 24 months observation period, the mean survival time was 4±4.04 ranging from 1-24 months, while the mean complete remission period was 7.6±3.8 ranging from 2-12 months.
- In patients who did not receive chemotherapy, the mean survival time was 4.2±2.4 months while in patients who received chemotherapy it was 8.2±4.5 months. There was highly significant association between mean survival time and receiving chemotherapy (p=0.006).

DISCUSSION

Growth and progression of leukemic cells are mediated by alterations in the microenvironment often caused by an aberrant expression of growth factors and receptors [9]. GM-CSF is an autocrine/paracrine cytokine which stimulates growth, differentiation and function of normal and leukemic myeloid progenitors. Antibodies to GM-CSF are also implicated in the process of leukemogenesis.

The aim of the current study was to investigate the role of GM-CSF gene expression, serum GM-CSF protein concentration and anti GM-CSF antibodies in AML/MDS patients in relation to treatment outcome and overall survival to help in understanding their impact in the pathogenesis of the disease, and hence predict prognosis as well as response to treatment.

The present study revealed that GM-CSF gene expression levels were significantly lower in AML/MDS patients compared to controls. This is in accordance with previous studies [10, 11] who reported that GM-CSF gene expression by RT-PCR was lower in leukemia patients than in healthy volunteers. Also, it has been demonstrated that in vivo autocrine production of GM-CSF is not common in unperturbed AML, but allow the possibility that either autocrine or paracrine GM-CSF activity could be induced in leukemic cells under stress conditions [12]. Furthermore, abnormalities in GM-CSF gene expression levels may contribute to the pathogenesis and abnormal proliferation of leukemia [13,14].

In the current study, serum GM-CSF protein concentration was significantly higher in AML/MDS patients compared to controls. This is in accordance with previous reports [15,16]. Highly significant increase in serum levels of GM-CSF was previously reported in 14 Egyptian AML patients compared to the reference control group [17]. This was denied by another study that reported comparable concentrations of serum GM-CSF in AML and healthy controls [11].

The current finding of negative correlation between serum levels of GM-CSF and initial PB blasts percentage could be correlated to defect in the biological functions of GM-CSF which may be attributed to a functional alteration of GM-CSF receptor or disturbances of signal transduction pathways which needs larger future studies. Furthermore, lower serum level of GM-CSF was associated with better response to therapy which suggests that GM-CSF protein concentration could be used as a biomarker to predict the outcome of therapy in AML/MDS cases.

In the present study, higher GM-CSF antibodies were found in AML/MDS patients compared to controls. Our results are in agreement with a previous study that reported a high prevalence of anti GM-CSF antibodies in patients with myeloid leukemia and MDS and higher titer of GM-CSF antibodies in patients with active disease compared to patients in complete remission [11].

In conclusion, our study results suggest that GM-CSF expression level might have an implication in leukemogenesis. The use of GM-CSF protein level and GM-CSF antibodies as prognostic markers of disease activity needs further investigations.

Conflicts of interest:

The authors declare that they have no conflicts of interest.

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Expression Pattern of Chemokine Receptors CXCR3, CXCR4 and CCR7 in B-Cell Lymphoproliferative Disorders

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ABSTRACT

Background: Chemokine receptors are expressed by lymphoid cells and function to mediate cell trafficking under both physiological and pathological conditions. In lymphoproliferative disorders they may play a role in the dissemination of malignant cells.

Aim: To identify the expression of chemokine receptors CXCR3, CXCR4 and CCR7 in malignant B lymphoproliferative disorder and to evaluate their prognostic impact.

Patients and Methods: A total of 60 newly diagnosed cases of B-Lymphoproliferative disorders (30 CLL/SLL, 17 B-ALL/LBL, and 13 B-NHL) and 12 controls were enrolled in our study. The expression of CXCR3, CXCR4 and CCR7 was detected by Flow Cytometry.

Results: Chemokine receptor expression in CLL patients was 90% for both CXCR3 and CCR7, and 73% for CXCR4 that showed a significant correlation with Bone Marrow (BM) lymphocytes, and between CCR7% and Absolute Lymphocyte Count (ALC). In Non-Hodgkin's Lymphoma (NHL), chemokine receptor expression was 53.8%, 69.2% and 61.5% for CXCR3, CXCR4% and CCR7%, respectively; with significant decrease of CXCR4 Mean Florescence Intensity (MFI) in patients with splenomegaly and hepatomegaly and CCR7% and MFI in patients with splenomegaly. A significant positive correlation was found between CXCR3 expression and Total Leukocytic Count (TLC) and ALC. A significant higher overall survival was detected in cases with higher CXCR3 MFI and CCR7. Chemokine receptor expression in B-ALL/LBL group was 64.7%, 82.4% and 23.5% for CXCR3, CXCR4 and CCR7, respectively. There was a significant positive correlation between CXCR3 expression and TLC, and between CXCR4 expression and Hemoglobin (Hb) level.

Conclusions: The expression of some chemokine receptors is heterogenous in lymphoproliferative disorders with a significant higher expression in CLL/SLL.

Key Words: *Chemokine receptors – CXCR3 – CXCR4 – CCR7 – Lymphproliferative disorders.*

INTRODUCTION

Chemokines are a large family of structurally homologus cytokines that stimulate leukocyte movements and regulate the migration of leukocytes from blood to tissues. They were first characterized as chemo-attractants that mediate cell trafficking and localization, and have subsequently been shown to have many functions in homeostasis and pathophysiology [1]. Studies have identified 50 human chemokines and 20 chemokine receptors [2].

Chemokines regulate their activity through interaction with their specific G-proteins coupled receptors superfamily. They were shown to play a critical physiological role to establish the complex architecture of secondary lymphoid organs [2], as well as pathological conditions including tumor metastasis, growth, survival and angiogenesis [3]. In addition, they were found to regulate antitumor immunity [4].

A given cell can express multiple chemokine receptors. Many of chemokine receptors can bind more than one ligand allowing extensive overlap and redundancy of chemokine functions. They are expressed by many cells, including lymphoid cells. Normal B cells have been reported to express CXCR3, CXCR4, CXCR5, CCR6, and CCR7 [5].

B Cell Lymphoproliferative Disorders (BCLPDs) often represent a defined stage in the normal lymphoid differentiation pathway at which the neoplastic transformation takes place. Neoblastic cell infiltration of extramedullary organs is a character of many BCLPDs

and variation in their chemokine receptor expression could partly explain this behavior [5]. Migration and homing to Bone Marrow (BM) and lymphoid tissue may be critical for growth support and rescue from apoptosis that the microenvironment provides for these malignant B cells [6].

The identification of the critical role of chemokine receptors in tumor development and metastasis could lead to the development of new and potent anticancer drugs that target those receptors [4].

In this work we studied the expression pattern of chemokine receptors (CXCR3, CXCR4 and CCR7) in B-CLPDs and their clinical relevance. Also, the changes in the expression of chemokine receptors during early stages of B cells development (ALL) and the more mature ones (CLL) were evaluated.

PATIENTS AND METHODS

Patients:

Sixty newly diagnosed cases of B-CLPD were enrolled from the out patient clinic at the National Cancer Institute (NCI), Cairo University. Twelve healthy, hematological free, age and sex matched subjects were included as normal controls.

Patients were divided into three groups: Group I: Thirty patients with Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL). Diagnosis of CLL was based on morphological and phenotypic scoring system; all cases showed score ≥3 with dim expression of surface κ (17 cases) or λ (13 cases). Group II: Thirteen patients diagnosed by lymph node and BM biopsy and immunohistochemistry as stage IV B-Non Hodgkin Lymphoma (B-NHL). Group III: Seventeen patients with B-Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma (B-ALL/LBL); they were diagnosed by BM morphology, cytochemistry and immunophenotyping by Flowc cytometry, 11/17 were C-ALL (64.7%) and 6 Pre B ALL (35.3%).

The existence of hepatomegaly, splenomegaly, lymphadenopathy, infiltration to other organs, and the overall survival were checked to determine extent of disease and relation to the studied markers. This study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University.

Methods:

Direct surface staining for chemokine receptors:

Fresh bone marrow or peripheral blood EDTA samples were used. The leucocytic count was adjusted to 5-10 X 10^3 cell/µl. Cells were washed by PBS three times before adding the monoclonal. Monoclonal antibodies (Mo Abs) labeled with Phycoerythrien (PE) against human CXCR3 (FAB160p), CXCR4 (FAB170p) and CCR7 (FAB197p) and appropriate isotype control supplied by R and D systems Inc (Minneapolis, Minnesota, USA) were used. Fifty µl of the washed sample were dispensed and 10µl of each monoclonal antibody were added to appropriately labeled tubes, properly mixed and incubated at 2-8ºC for 30 minutes in the dark. The RBCs were lysed by adding 2ml of erythrocyte lysing solution (BD. 349202), mixed gently and incubated for 5 minutes at room temperature, centrifuged at 1500g. Cells were washed twice with 3ml PBS and re-suspended in 200-400ml of PBS buffer for immediate analysis.

Flow cytometric analysis:

Immunophenotypic expression was measured by (FAC Vantage): Becton Dickinson, San Diego, USA) using the Cell Quest software program (Becton Dickinson). Analytical gates were set on the clone of malignant lymphoid cells based on forward and side scatters combined with exclusion of normal cells using a CD45 gating protocol. In short, we first gated on the CD45 dim population (G1) then reflected those cells (G1) on the FSC/SCC dot plot to get gate 2 (G2) that is fixed in all tubes and used for analysis of all markers.

Marker expression was determined as percent positivity of stained cells within the lymphoid population as well as Mean Florescent Intensity (MFI) obtained by dividing the channel number of the specific marker by that of the isotype control [3].

Statistical analysis:

Data was analyzed using SPSS statistical package version 16. Numerical data were expressed as mean ± standard deviation, median, and range. Qualitative data were expressed as frequency and percentage. Chi-square 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) because the variables are not normally distributed. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA test) followed by post-Hoc comparison test "Schefe test" on the rank of variables was used to compare pairs of groups.

Survival analysis was done using Kaplan-Meier method. Comparison between two survival curves was done using Log-rank test. Relation between different numerical variables was tested using Spearman's rho correlation. Correlation coefficient (*r*) of 0.5 was considered fair correlation, if more than 0.5 to 0.75, it was considered good correlation, and if more than 0.75, it was considered as very good correlation.

Receiver Operating Characteristic (ROC) curves were plotted for identification of the cut off values for CXCR4, CXCR3, CCR7 both % and MFI.

Probability (*p*-value) <0.05 was considered significant and less than 0.001 considered as highly significant.

RESULTS

Clinical parameters of the studied patient's groups are shown in (Table 1).

Chemokine receptor expression in CLL patients:

Table (2) shows the expression of Chemokine receptors in CLL patients.

Splenomegaly and/or hepatomegaly had no impact on the expression of CXCR3, CXCR4 or CCR7 (*p*=>0.05). Neither did the performance state (*p*=0.09, 0.85, 0.29, respectively) or the modified Rai stage (*p*=0.18, 0.87, 0.65, respectively).

Also, expression of κ (17 patients, 57%) or λ (13 patients, 43%) had no significant effect on CXCR3, CXCR4 or CCR7 expression (*p*= 0.6, 0.14, 0.13, respectively) neither CD38 positive expression (5 patients, 17%) (*p*=0.19, 0.9, 0.27, respectively).

CXCR3 expression was comparable to control group while both CXCR4 and CCR7 were significantly higher in CLL compared to control group. No significant differences from other groups were encountered except for CCR7 which was higher than ALL and NHL significantly in both % expression and MFI for ALL (*p*=<0.001) and in MFI for NHL (*p*=0.05) (Table 3).

Correlation between chemokine receptors expression and hematological data is presented in (Table 4). Significant positive correlation was encountered between CXCR4 and BM Lymphocytes (*p*=0.02) as well as between CCR7% and absolute lymphocyte count (*p*=0.04).

The follow-up period of CLL/SLL patients ranged between 1 and 91 with a median of 19 months. The Cumulative Overall Survival (CS) was 72.7%. Chemokine receptors expression had no impact on and overall survival, Fig. (1).

As regards Disease Free Survival (DFS), 14/30 patients (47%) achieved Complete Remission (CR) after initial chemotherapy. Time to Disease Progression (TDP) ranged from 1 to 32 with a median of 16 months with no impact of chemokine receptors expression (*p*>0.05), Fig. (2).

Chemokine receptor expression in NHL patients:

Chemokine receptor expression in NHL patients is shown in (Table 2).

Patients with splenomegaly (11/13, 85%) showed significantly lower values of CXCR4 MFI (6.6±6.7), CCR7% (27.8±30.2) and CCR7 MFI (4.2±4.9) when compared to patients without splenomegaly (CXCR4 MFI, 23.3±13.4, CCR7% 87.2±4.5 and CCR7 MFI 30.2±13.1); *p*-values were 0.04, 0.04 and *p*=0.03, respectively.

The mean ± SD of CXCR4 MFI expression was 4.4±4.6 in NHL patients with hepatomegaly (8/13, 62%) which was significantly lower than patients without hepatomegaly (mean ± SD 16.7±11.1) (*p*=0.02).

There was no significant association of the chemokine receptor expression with other parameters (*p*>0.05).

CXCR4% and CXCR4 MFI were significantly higher in patients with performance state 0, I (4/13, 31%) compared to (9/13, 69%) in patients with performance state (II, III). The CXCR4% and MFI were 61.9±19.7 and 14.6± 10.2, respectively in patients with performance

state 0, I, compared to 21±25 and 2.8±2.4 in patients with performance state (II, III) (p=0.02 and 0.006, respectively). No association of CXCR3 or CCR7 expression and performance status was encountered (p=0.75 and 0.1, respectively).

Significant positive correlation was encountered between CXCR3 expression and TLC (r=0.6), ALC (r=0.65) and peripheral blood lymphocytes % (r=0.54) (Table 4).

The follow-up period of B-NHL patients ranged between 1 and 64 with a median of 14 months. The cumulative overall survival (CS) was 39% with a mean ± SE (13.7±5.1).

Significant higher overall survival was associated with higher CXCR3 MFI (p=0.005) and with higher CCR7 positive cases (p=0.011). There was no statistically significant association of CXCR4 expression and overall survival in NHL patients (Table 5), Figs. (3,4).

In B-NHL patients, only 3/13 cases responded to the initial chemotherapy and entered in Complete Remission (CR) but relapsed after that. The follow-up period of DFS for those three B-NHL patients ranged (16-26), with a median follow-up of 16.2 months and the mean ± SD was 19.4±5.7. The CS at 16 months was 67.7% with a median ± SE 16.1±0.0. No significant relation with chemokine receptors expression could be estimated.

Chemokine receptor expression in B-Lymphoblastic leukemia/lymphoblastic lymphoma (B-ALL/LBL) group:

Table (2) shows the expression of Chemokine receptors in ALL patients.

In ALL, there was no impact of splenomegaly or hepatomegaly on the expression of CXCR3, CXCR4 or CCR7 (p=0.4, 0.2, 0.2 for splenomegaly and 0.18, 0.87, 0.65 for hepatomegaly respectively).

Significant positive correlation was encountered between CXCR3% and TLC (r=0.5, p= 0.04) and significant negative correlation between CXCR4% and Hb (r=–0.61, p=0.009) (Table 4).

Follow-up period of B-ALL/LBL ranged from 6 to 40 with a median of 37.6 months. The CS at 37.5m was 70.6% with no impact of chemokine receptor expression (Table 5).

Follow-up period for Disease Free Survival (DFS) ranged from 3-39 with a median of 35 months; 15/17 (88%) patients responded to the initial chemotherapy and achieved Complete Remission (CR) with no impact of chemokine receptor expression on DFS.

Table (1): Clinical and laboratory data of the studied groups at diagnosis.

Characteristic	CLL (n=30)	NHL (n=13)	ALL (n=17)
Sex: Male/female	19/11	7/6	13/4
Age*	60.5 (37-75)	58 (36-73)	6 (1.2-17)
TLC X 10^9/L*	80.5 (12-278)	40.1 (5.2-15)	17.8 (2-60)
Hb g/d*	10 (3.1-13.5)	11 (6.9-13.8)	7.4 (3-12.6)
Platelets X 10^9/L*	168 (79-474)	144 (83-379)	40 (6-70)
B cell population in PB%*	88.5 (39-99)	72 (30-89)	60 (20-95)
B cell population in BM%*	78.5 (21-97)	6.5 (0.2-84)	90 (20-97)
Presence of B Symptoms**	2 (7%)	4 (30.8)	8 (41.7)
Lymphadenopathy**	27 (90%)	12 (92.3)	11 (64.7)
Splenomegaly**	25 (83%)	11 (84.6)	9 (52.9)
Hepatomegaly**	17 (57%)	8 (61.5)	9 (52.9)
Number achieved CR**	14 (47%)	3 (23.1%)	15 (88.2%)
*Survival status**:*			
- Alive	28 (93.3%)	8 (61.5)	16 (94.1)
- Dead	2 (6.7%)	5 (38.5)	1 (5.9)

CLL : Chronic Lymphocytic Leukemia.
NHL : Non-Hodgkin's Lymphoma.
CR : Complete Remission.
* : Median (range).
** : No (%).

Table (2): Chemokine receptors expression among the studied groups.

Parameter	CLL 30 cases	NHL 13 cases	ALL 17 cases
CXCR3%*	27 (90%) 16.3 (1-83)	7 (4%) 6.5 (0.2-84)	11 (5%) 4.4 (1-35)
CXCR3 MFI**	7 (23%) 23 (77%)	5 (9%) 8 (2%)	7 (41.2%) 10 (9%)
CXCR4%*	22 (73%) 46.6 (1.2-97)	9 (69.2%) 43 (1.3-80)	14 (82.4%) 167 (2.4-25)
CXCR4 MFI**	8 (27%) 22 (73%)	5 (9%) 8 (2%)	6 (35.3%) 11 (65%)
CCR7%*	27 (90%) 68.8 (1-96)	8 (2%) 27 (1.2-90.4)	4 (4%) 8.1 (3.1-17)
CCR7 MFI**	5 (17%) 25 (83%)	6 (46.2%) 7 (4%)	10 (9%) 7 (41.2%)

CLL : Chronic lymphocytic leukemia. *% expression:* * Frequency (%) *MFI**:* Low: Frequency (%)
NHL: Non-Hodgkin's lymphoma. Median (range) High: Frequency (%)
ALL : Acute lymphoblastic leukemia.

Table (3): Comparison between the expression of chemokine receptors in the different studied groups and control.

Groups \ Chemokine	CXCR3 (%)	CXCR3 (MFI)	CXCR4 (%)	CXCR4 (MFI)	CCR7 (%)	CCR7 (MFI)
CLL vs. control	0.14	0.28	0.02	0.01	<0.001	0.009
NHL vs. control	0.73	0.59	0.14	0.24	0.24	0.9
ALL vs. control	0.51	0.95	0.12	0.79	0.9	0.5
CLL vs. NHL	0.75	0.98	0.98	0.78	0.11	0.05
CLL vs. ALL	0.8	0.51	0.96	0.09	<0.001	<0.001
ALL vs. NHL	0.9	0.83	1.0	0.69	0.5	0.18

CLL : Chronic lymphocytic leukemia. NHL: Non-Hodgkin's lymphoma. ALL : Acute lymphoblastic leukemia.

Table (4): Correlations between the expression of chemokine receptors and some prognostic hematological parameters in the different studied groups.

Parameter	CXCR3%		CXCR4%		CCR7%	
	r	*p*	*r*	*p*	*r*	*p*
Chronic lymphocytic leukemia:						
TLC ($X10^9$/L)	0.3	0.11	0.13	0.49	0.27	0.15
HB (g/dL)	–0.19	0.33	0.001	0.99	0.13	0.49
PLT ($X10^9$/L)	–0.14	0.45	–0.16	0.39	–0.35	0.057
ALC	0.28	0.13	0.17	0.35	0.36	0.04*
BM lymphocyte (%)	0.19	0.9	0.5	0.02*	0.28	0.13
Non-Hodgkin's Lymphoma:						
TLC ($X10^9$/L)	0.6	0.04*	0.04	0.89	0.43	0.14
HB (g/dL)	–0.25	0.41	0.52	0.86	–0.09	0.77
PLT ($X10^9$/L)	–0.2	0.5	–0.02	0.96	–0.27	0.37
ALC ($X10^9$/L)	0.65	0.02*	0.15	0.62	0.5	0.1
PB lymphocyte %	0.54	0.048*	0.49	0.08	0.21	0.49
BM lymphocyte %	0.23	0.45	–0.36	0.22	0.19	0.52
Acute lymphoblastic leukemia:						
TLC ($X10^9$/L)	0.5	0.04*	0.17	0.5	0.35	0.17
HB (g/dL)	–0.44	0.08	–0.61	0.009*	–0.45	0.07
PLT ($X10^9$/L)	–0.28	0.27	–0.08	0.77	–0.26	0.32
PB Blasts %	0.37	0.15	0.18	0.49	0.23	0.38
BM Blasts %	0.12	0.65	0.36	0.15	0.02	0.94

*significant (p=<0.05).

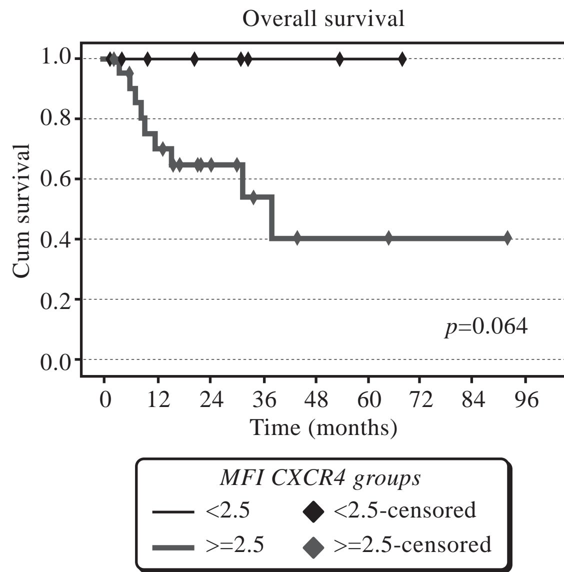

Fig. (1): Impact of CXCR4 MFI on overall survival in CLL/SLL patients

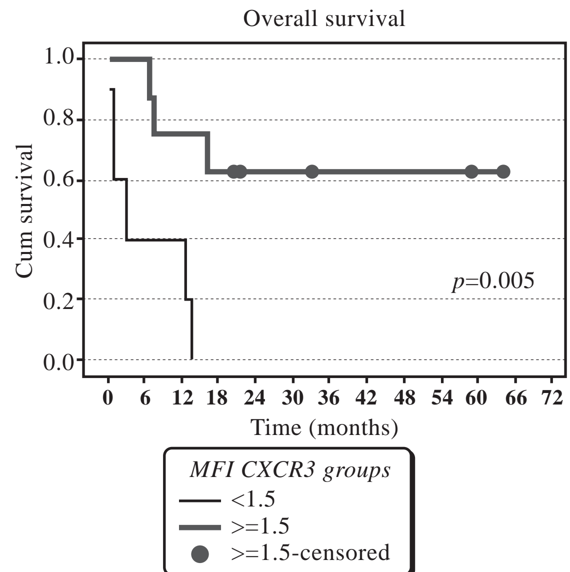

Fig. (3): Impact of CXCR3 MFI on overall survival in B-NHL patients.

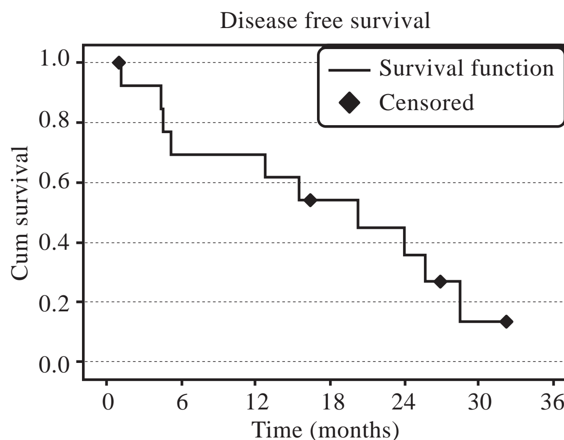

Fig. (2): Disease free survival of CLL/SLL patients.

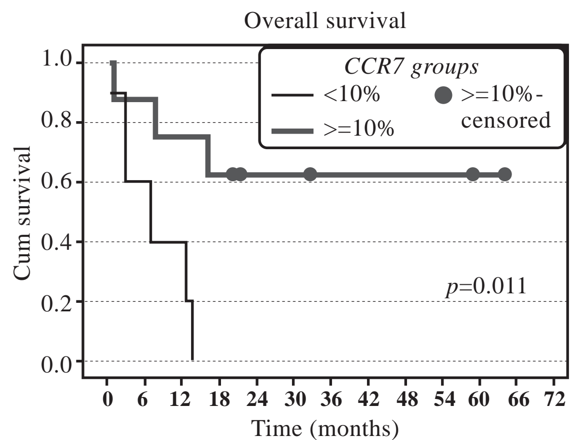

Fig. (4): Impact of CCR7% of expression on overall survival of B-NHL patients.

Table (5): Relation between chemokine receptors expression and survival in B-NHL and B-ALL/LBL patients.

Parameter		NHL			B-ALL/LBL		
		No	Overall survival %	*p*-value	No	Cumulative survival %	*p*-value
Overall group		13	38.5%		17	70.6%	
CXCR3 %:	Negative	6	16.7%	0.098	6	66.7%	0.854
	Positive	7	57%		11	72.7%	
CXCR3 MFI:	Low	5	0	0.005**	7	71.4%	0.899
	High	8	62.5%		10	70%	
CXCR4 %:	Negative	4	0%	*	3	100%	*
	Positive	9	55.6%		14	64.3%	
CXCR4 MFI:	Low	5	20%	0.169	6	66.7%	0.854
	High	8	50%		11	72.7%	
CCR7 %:	Negative	5	0%	0.011**	13	69.2%	*
	Positive	8	62.5%		4	75%	
CCR7 MFI:	Low	6	17%	0.114	10	70%	1.00
	High	7	57.1%		7	71.4%	

**: Statistically significant: ($p<0.05$). *: No *p*-value because of small no of cases within group.

DISCUSSION

Chemokines are a family of peptide hormones that were originally identified as chemoattractant cytokines and later as growth factors for hematopoietic cells and other cell types. Chemokines comprise of a large family of molecules that have been implicated in numerous different functions including the regulation of cellular adhesion, migration, proliferation and survival through their specific G protein coupled receptors [7].

In the present study 90% of CLL/SLL patients were expressing CXCR3, 73% were expressing CXCR4 and 90% were expressing CCR7. These results are comparable to results obtained by Ghobrial et al., [3] who stated that CXCR3, CXCR4 and CCR7 are constitutively expressed by B-CLL/SLL, suggesting that these receptors might represent a hall mark of this disease.

In agreement with Wong and Fulcher [5], there was no significant difference between CXCR3 expression percent or intensity when compared to the control ($p>0.05$). However, others reported a significant increase in CXCR3 expression in B-CLL/SLL patients when compared with the normal B-lymphocytes of control samples. This difference could be attributed to technical issues or due to the small sample size [3].

We detected a statistical significant increase of both CXCR4% and CXCR4 MFI in CLL/SLL cases when compared with the control group. This result is in agreement with other publications [8-10]. Our results and other's suggest that the pathogenesis of B CLL may be linked to an over expression of CXCR4 and the importance of CXCR4-SDF-1 system for tissue localization and increased survival of B-CLL cells.

Also, an over expression of CCR7% and MFI in the CLL/SLL group was detected in our study when compared to the control group. This is in agreement with previous reports [11-13].

As regards B-NHL group, 53.8% of cases expressed CXCR3, 69.2% expressed CXCR4% and 61.5% expressed CCR7%. Comparable results were previously reported [14-16], which may indicate the involvement of chemockine receptors in lymphoma cell trafficking and homing.

In the current study, the expression of CXCR3% and MFI in NHL was comparable to control. Similar results were previously reported [5,14], while significant higher expression of CXCR3 in NHL was reported by another study [15].

Also CXCR4 expression % and MFI were not significantly different from control. This is in concordance with some [8] but not others [15,17].

The same for CCR7% and MFI, we did not find any significant difference compared to the control group. This is similar to another report [18].

In the current study, 5% of B-ALL/LBL patients expressed CXCR3, 82.4% expressed CXCR4 and 4% expressed CCR7 with no significant difference from control. Similar data was previously reported [5,14,19]. In contrast, other authors reported a significant increase in CXCR4 expression in B-ALL/LBL patients [5,8].

Regarding the relation between CXCR3, CXCR4, and CCR7 expression and presence of hepatomegaly or splenomegay as a sign of extramedullary spread of the disease in our studied patients, no significant relation could be detected in CLL/SLL patients or B-ALL/LBL. This result is in accordance with Lopez-Giral et al., [18]. Their data and ours could indicate that CXCR3, CXCR4 and CCR7 chemokine receptors expression on lymphocytes from CLL and blasts of B-ALL/LBL have no role in extramedullary spread of the disease. On the contrary, Crazzolara and Bernhard [19] reported higher expression of CXCR4 in B-ALL/LBL patients with extramedullary infiltration.

On the other hand, B-NHL showed significant decrease of CXCR4 MFI in cases with splenomegaly and hepatomegaly and significant decrease of both CCR7% and CCR7 MFI in patients with splenomegaly. These results may indicate that down regulation of CXCR4 and CCR7 could play a role in tumor cell migration outside the LN and metastatic destination in B-NHL.

In the current study, there was no association between CLL/SLL Rai stage and CXCR3, CXCR4 or CCR7 expression. This is in agreement with previous reports [8,9,20] regarding CXCR4 expression. On the other hand, Ghobrial et al., [3] reported a significant higher expression of CCR7 in patients with advanced Rai stage (III, IV).

In the studied B-NHL cases, there was a significant increase of both CXCR4% and MFI in patients with PS (0, I) compared to patients with PS (II, III). This may suggest that down regulation of CXCR4 can play a role in advanced clinical stage and disease progression.

Within the CLL group, no significant relationship could be detected between CD38 expression and chemokine receptors. In agreement with our work, many authors could not find a relation between CXCR4 and CD38 expression [8,9,20] or between CCR7 and CD38 expression [16,18]. For CXCR3 expression, however, some authors reported similar results to ours [3,20] while one author reported association of higher expression of CXCR3 with low CD38 expression [2]. In the current study, CLL patients showed no association of CXCR3 expression with the hematological prognostic factors. This is in agreement with previous reports [2,20]. While in B-NHL a significant positive correlation was found between CXCR3 (% and MFI) and TLC (r=0.6), ALC (r=0.65) and peripheral blood lymphocytes percentage (r=0.54). Similar results were previously reported [14,15]. Also, a significant positive correlation between CXCR3% and TLC (r=0.5) was detected in B-ALL/LBL patients. These results might indicate a role of the expression of CXCR3 on neoplastic cells of B-ALL/LBL and B-NHL in their trafficking to the peripheral blood.

In our studied CLL patients, a significant positive correlation between CXCR4 and BM Lymphocytes (p=0.02, r=0.5) was detected. This result is in agreement with many publications [3,6,9], which reflect the important role of CXCR4/SDF-1 system in the physical interaction between leukemic cells and the microenvironment regulating apoptosis. Also it was found that; it has both chemotactic effect and pro-survival effect on CLL cells, being a crucial mechanism through which stromal cells support CLL cells in vitro [25]. Also, a significant positive correlation was detected between CCR7% and peripheral blood lymphocytes (r=0.56) as previously reported [3]. This can be explained by the fact that CCR7 plays an important role in migration of lymphocytes from the BM to LN [26].

In the current study, chemokine receptor expression had no impact on survival in CLL/SLL patients. In contrast, shorter survival was reported in patients with positive as compared to those with negative CXCR3 [2].

Survival analysis of B-NHL cases revealed a significant higher overall survival in cases expressing CXCR3 with a higher MFI and in cases expressing CCR7. These results could provide an evidence for the role of these chemokine receptors and may be considered as a good prognostic marker in B-NHL.

In the current study, chemokine receptor expression had no impact on disease free survival in ALL/LBL patients. However, it was previously reported that over expression of CXCR4 in B-ALL/LBL patients is associated with shorter disease free survival [19]. Based on this finding, therapeutic use of CXCR4 inhibitors might improve patient's survival. Previous studies have demonstrated that CXCR4 mediates bone marrow microenvironment signalling. Using a xenograft model of ALL with MLL rearrangement, Sisson et al., [22] found that targeting leukaemia-stroma interactions with CXCR4 inhibitors may prove useful in this high-risk subtype of pediatric ALL. Along the same line Welschinger et al., [23] demonstrated that mobilizing agents, like CXCR4 antagonist Plerixafor (AMD3100), can increase the therapeutic effect of cell cycle dependent chemotherapeutic agents. Similar to the CXCR3/ CXCL10 circuit, another chemokine/receptor couple CXCL12/CXCR4 has been shown to mediate crosstalk between BM stroma and tumor cells in a number of hematologic malignancies [24].

In conclusion, expression of the chemokine receptors; CXCR3, CXCR4 and CCR7 is heterogenous in B-lymphoproliferative disorders with a significant higher expression of CXCR4 and CCR7 in CLL when compared to the control group. SDF-1/CXCR4 pathway is an attractive target for the development of novel therapeutic approaches that can be specifically blocked by

monoclonal antibodies against CXCR4 (CXCR4 antagonists) leading to elimination of residual CLL and ALL cells hiding in stromal niches within the marrow and the lymphatic tissue. Positive CXCR3 and CCR7 expression were associated with higher OS of B-NHL patients. CXCR3 and CCR7 expression may be a good prognostic marker of B-NHL patients.

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