



Assessment of Expression of Phosphorylated CXCR4 in AML Blast cells and Its Relation to the Disease Prognosis

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Abstract: Background: The G protein-coupled receptor CXCR4 is activated by CXCL12 (stromal cell-derived factor 1) and is involved in the control of migration and homing of cells notably for engraftment of normal and neoplastic hematopoietic cells (including AML) in the bone marrow (BM) including AML. CXCR4 phosphorylation has been shown to affect its internalization. CXCR4 endocytosis has been reported to modify its signaling and other downstream signaling pathways including ERK cascade activation. **The aim:** evaluating (immunohistochemically) the CXCR4 (phosphorylated and unphosphorylated) expression in bone marrow biopsy blast cells and the correlation of these markers with AML prognosis. **Patients & methods:** The study was performed on 20 adult patients with newly diagnosed AML, their age ranged from 20 to 64 years. All patients were subjected to the following: detailed history, clinical examination, routine investigations for AML diagnosis and immunohistochemistry of bone marrow biopsy samples to detect CXCR4 and phosphorylated CXCR4-S339 expression. **Result:** By analysis of survival and using kaplanmeier survival analysis, it has been found that expression of ph-CXCR4 is more significant than CXCR4 expression. Ph-CXCR4 correlated well with survival data (OS & DFS) unlike CXCR4 which did not play a significant role. **Conclusion:** Homing of these blasts actively dividing to the bone marrow niche is suggested to be regulated by G protein-coupled receptor CXCR4 specifically its active phosphorylated form, which has been proved to be associated with bad prognosis of AML.

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Keywords: Assessment; Expression; Phosphorylated; CXCR4; AML; Blast cell; Relation; Disease; Prognosis

1. Introduction

Acute myeloid leukemia (AML) is one kind of aggressive clonal hematopoietic disorders, with the differentiation of hematopoietic progenitor cells blocked and the normal regulation of proliferation disturbed (Lee et al., 2014).

Acute myeloid leukemias (AMLs) are neoplastic proliferations arising in hematopoietic precursor cells. They result in overgrowth of myeloblasts and other immature cells of myeloid lineage. The malignant cells replace the bone marrow cells, circulate in the blood and may accumulate in other tissues, including the lymph nodes, liver and spleen. Eighty to ninety percent of leukemia in adults are AML (Jabbour et al., 2006).

The G protein-coupled receptor CXCR4 is activated by CXCL12 (stromal cell-derived factor 1) and is involved in the control of migration and homing of cells notably for engraftment of normal and neoplastic hematopoietic cells in the bone marrow (BM) (Tavor et al., 2004).

CXCR4 function depends on its cell surface expression which is regulated at the transcriptional and

posttranscriptional level by endocytosis, intracellular trafficking and recycling (Alkhatib, 2009).

CXCR4 phosphorylation has been shown to affect its internalization. CXCR4 endocytosis has been reported to modify its signaling and other downstream signaling pathways including ERK cascade activation (Brault et al., 2014).

Some studies proved that CXCR4 expression on AML blast is associated with poor prognosis (Rombouts et al., 2004).

2. Patients and Methods

1 Patient

This prospective study was carried out in the clinical pathology department of the National Cancer Institute (NCI), Cairo University during the period between 2017 and 2019.

Inclusion criteria:

Adult patients of age more than 18 years, Patients were newly diagnosed as AML, no sex difference, males and females was included and they had not received treatment at time of sample withdrawal.

Exclusion criteria:

Any patients with malignancy other than AML and treated AML patients were also excluded.

All patients were subjected to the following:

Detailed history: with special emphasis on age, sex, presence of leukemia-associated symptoms (fever, easy fatigability, bleeding tendency and bone aches), as well as duration of the disease.

Clinical examination:

Laying stress on the presence and extent of leukemia involvement including: pallor, purpuric eruptions, size of liver and spleen, lymphadenopathy and CNS involvement.

Investigations for diagnosis of AML:

Morphological examination of peripheral blood and / or bone marrow aspirate smears, **Cytochemical** analysis of air-dried peripheral blood and/ or bone marrow smears. The cytochemical stains included Myeloperoxidase (MPO) and Nonspecific esterase and **Immunophenotyping** of blast cells in BM aspirate samples using Becton Dickinson (BD) FACSCalibur to confirm diagnosis stem cell markers (CD34, HLA-DR) for blasts; (cyt MPO, CD13, CD33, CD117) as a primary panel for myeloid lineage; (CD14, CD36, CD11b, CD11c, CD64) for M4 and M5; (glycophorin A) for M6; (CD41, CD42) for M7; and (CD19, CD10, CD79a, CD3, cyt CD3) for lymphoid lineage.

2. Method

Immunohistochemical staining for BMP, BMA and peripheral blood samples was done by using Primary Antibody for CXCR4ph-CXCR4. CXCR4 diluted primary Ab (1:200) is a synthetic peptide (KLH conjugated) corresponding to the region of the human CXCR4, comp (Biospes), Code (YPA1454). Sections were incubated overnight at 4 C. phosphorylated CXCR4-S339 (ph-CXCR4) diluted primary Ab (1:200) is a synthetic phosphopeptide

(KLH conjugated) corresponding to the region of the human CXCR4 around the phosphorylated site of Ser339, comp (Biospes), Code (YPA1461). Sections were incubated overnight at 4 C.

Data interpretation:

The slides were microscopically examined using light microscopy 10x – 40x – 100x (Olympus BX 41-Japan). At least 200 blast cells were assessed, and the percentage of positive blast cells is calculated. Cases were considered as CXCR4 positive when the percentage of stained blasts per case was above 20% and Cases were considered as phosphorylated CXCR4 positive when the percentage of stained blasts per case was above 15% (**Brault et al., 2014**).

Follow up was done by frequent examination of peripheral blood and BM smears after induction therapy. Follow up of hematological remission at day 28. A patient had **CR** when the BM blast >5% with normalization of neutrophils and platelets count (neutrophils \geq 1000/cmm and platelets \geq 100000/cmm). Blast in BM >5%, recovery of neutrophils and platelets and absence of extramedullary disease considered as the cornerstone of haematological CR in patient with AML (**Breems et al., 2005**). A patient was **relapsed** when the BM blast \geq 5%, reappearance of blast cells in peripheral blood or development of extramedullary disease (**Breems et al., 2005**). The patients were followed up for one year then the time at which the patient achieved remission, relapsed, died or last seen alive was detected for calculation of overall survival (**OS**) and the disease free survival (**DFS**).

3. Result

Age and sex did not differ significantly between positive versus negative CXCR4 cases or between positive versus negative Ph-CXCR4 cases ($p > 0.05$ for each) (table 1).

Table (1): Relationship between CXCR4 expression and Ph-CXCR4 and age and sex of the studied AML cases

Sex		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Male	N	9	3	0.125	7	5	0.444
	%	52.9%	100.0%		53.8%	71.4%	
Female	N	8	0		6	2	
	%	47.1%	.0%		46.2%	28.6%	
Age		CXCR4 BM biopsy			Ph CXCR4 BM biopsy		
		+ve	-ve	p. value	+ve	-ve	p. value
		43.24 ± 13.76	49.00 ± 25.12	0.559	44.08 ± 14.23	44.14 ± 18.01	0.993

Expression of both CXCR4 and ph-CXCR4 did not differ significantly in patients presented with different karyotyping and FLT3 mutation (table 2).

Table (2): Relationship between CXCR4 expression and Ph-CXCR4 and (molecular study and karyotyping) in the studied AML cases

Karyotyping		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Intermediate risk	N	11	1	0.119	9	3	0.169
	%	78.6%	33.3%		81.8%	50.0%	
Better risk	N	3	2		2	3	
	%	21.4%	66.7%		18.2%	50.0%	

Molecular FLT3		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Wild	N	14	3	0.517	10	7	0.253
	%	87.5%	100.0%		83.3%	100.0%	
Muted	N	2	0		2	0	
	%	12.5%	.0%		16.7%	.0%	

No significant association was observed between CXCR4 and ph-CXCR4 expression and many laboratory data of the patients including hemoglobin, platelet and WBCs count. However Serum level of LDH was found to be significantly associated with CXCR4 and Ph-CXCR4 expression, with higher significant association with ph-CXCR4 (table 3).

Patients presented with fever and hepatosplenomegaly showed significant expression of both CXCR4 and Ph-CXCR4 with higher significance with Ph-CXCR4. Patients presented with lymph node enlargement and bleeding tendency showed significant relation only with Ph-CXCR4 expression (table 4).

Expression of CXCR4 and Ph-CXCR4 is higher in patient failed to reach complete remission and relapsed patients compared to patient reached complete remission with the highest significant relation with ph-CXCR4 expression (table 5).

By analysis of survival and using kaplanmeier curve, it has been found that expression of ph-CXCR4 is more significant than CXCR4 expression. Ph-CXCR4 correlated significantly with survival data (OS & DFS) unlike CXCR4 which did not play a significant role. Thus the study highlights the importance of investigating the expression of ph-CXCR4 as an evaluating marker for patient prognosis (figure 1).

Table (3): Relationship between CXCR4 expression and Ph-CXCR4 and (laboratory data) in the studied AML cases

	CXCR4 BM biopsy			Ph CXCR4 BM biopsy		
	+ve	-ve	p. value	+ve	-ve	p. value
LDH	1092.12 ± 518.38	435.00 ± 40.63	0.046*	1204.38 ± 511.74	602.00 ± 318.95	0.011*
HB	8.12 ± 1.62	8.57 ± 0.38	0.650	8.06 ± 1.85	8.43 ± 0.45	0.616
TLC	66.47 ± 44.12	119.00 ± 68.17	0.094	61.38 ± 39.18	98.43 ± 61.67	0.116
Plt	67.59 ± 42.79	87.00 ± 13.89	0.455	60.85 ± 44.89	88.43 ± 22.36	0.147

Table (4): Relationship between CXCR4 expression and Ph-CXCR4 and (clinical data) in the studied AML cases

Fever		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Yes	N	12	0	0.021*	11	1	0.002*
	%	70.6%	.0%		84.6%	14.3%	
No	N	5	3		2	6	
	%	29.4%	100.0%		15.4%	85.7%	

LN		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Yes	N	7	0	0.168	7	0	0.016*
	%	41.2%	.0%		53.8%	.0%	
No	N	10	3		6	7	
	%	58.8%	100.0%		46.2%	100.0%	

HSM		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Yes	N	11	0	0.038*	10	1	0.007*
	%	64.7%	.0%		76.9%	14.3%	
No	N	6	3		3	6	
	%	35.3%	100.0%		23.1%	85.7%	

Bleeding tendency		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
Yes	N	8	0	0.125	8	0	0.007*
	%	47.1%	.0%		61.5%	.0%	
No	N	9	3		5	7	
	%	52.9%	100.0%		38.5%	100.0%	

Table (5): Relationship between CXCR4 expression and Ph-CXCR4 and (clinical outcome) in the studied AML cases

Clinical outcome		CXCR4 BM biopsy		P-value	Ph CXCR4 BM biopsy		P-value
		+ve	-ve		+ve	-ve	
CR	N	4	3	0.038*	1	6	0.002*
	%	23.5%	100.0%		7.7%	85.7%	
Failure of CR	N	6	0		6	0	
	%	35.3%	.0%		46.2%	.0%	
Relapsed	N	7	0	6	1		
	%	41.2%	.0%	46.2%	14.3%		

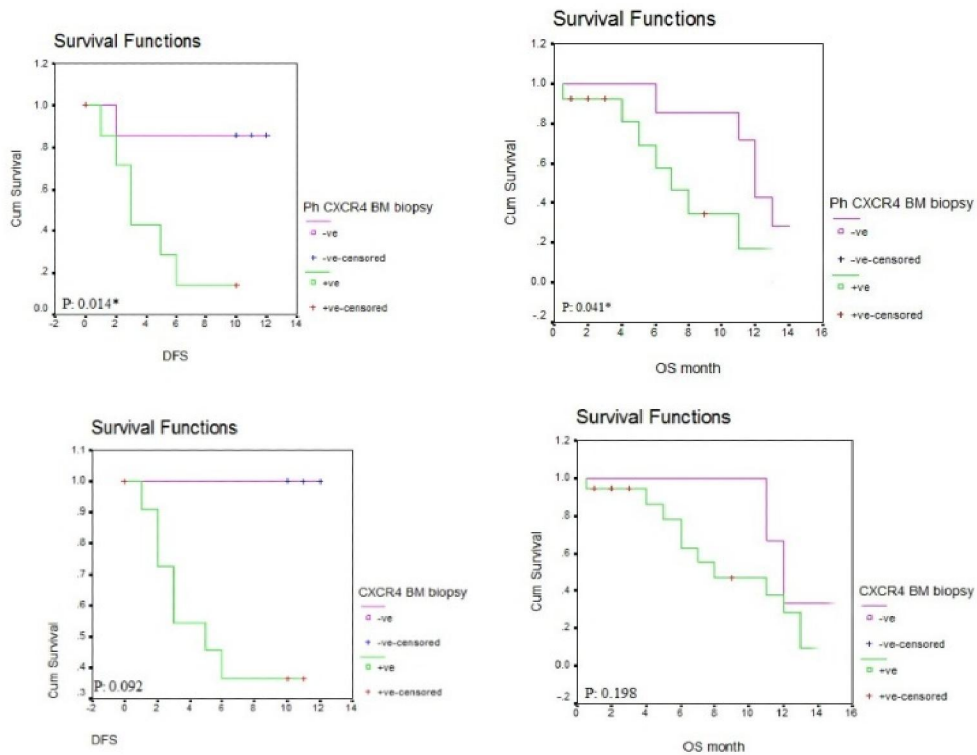


Figure (1) DFS & OS for both CXCR4 and ph-CXCR4 express

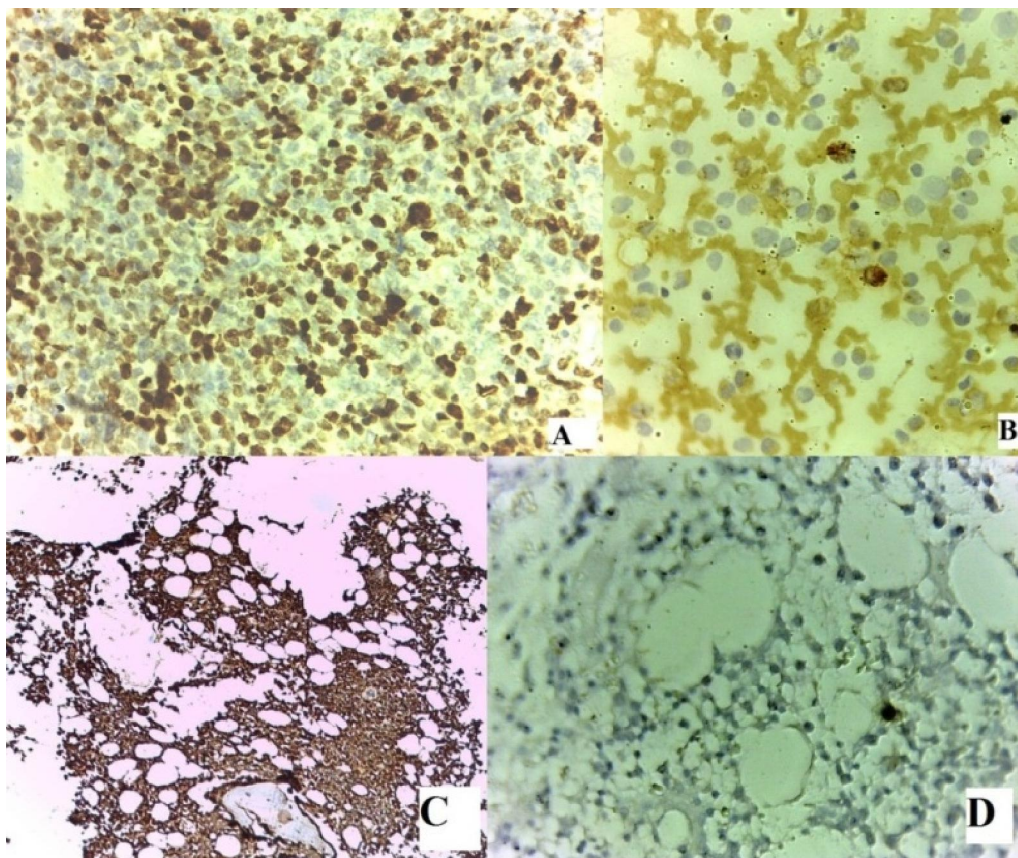


Figure (2) These photomicrographs from immunohistochemical studies reveal (A) Positive (ph- CXCR4 expression) x40 (B) Negative (ph- CXCR4 expression) x 100 (C) positive (CXCR4 expression) x10 (D) Negative (CXCR4 expression) x40

4. Discussion

Acute myeloid leukemia (AML) is a heterogeneous group of diseases in which the hematopoietic stem cells and progenitors go through uncontrolled proliferation with a decreased ability to differentiate into mature cells (Peled and Tavor, 2013).

Unsatisfactory outcomes persist for the majority of patients with AML. As a result, new molecular targets for therapy and biological markers of leukemia pathogenesis and prognosis are needed. There is also continued need for adequately powered prospective clinical trials to evaluate new treatments and strategies in different subsets of AML (Short et al., 2018).

CXCR4 is a 352 amino acid rhodopsin-like G protein coupled receptor with no intrinsic kinase activity that selectively binds CXC chemokine stromal cell-derived factor 1 alpha (SDF-1 α), also known as CXCL12. The CXCR4/ SDF-1 α interaction is essential for hematopoiesis as well as for general development, organogenesis, and vascularization (Nam et al., 2017).

CXCR4 expression is tightly controlled at several levels. At the transcriptional level, a number of signaling molecules can either increase or attenuate

CXCR4 expression at the post-translational level, phosphorylation is a key mechanism of CXCR4 regulation. SDF-1 α -promoted tyrosine phosphorylation activates the JAK/ STAT pathway. Whereas cytokine-induced tyrosine phosphorylation promotes ligand-independent internalization of CXCR4. CXCR4 is phosphorylated in response to ligand binding in a G-protein-coupled receptor kinase 2-dependent fashion (Zhao et al., 2015).

Receptor phosphorylation stimulates the interaction of β -arrestin with the carboxy terminus. This interaction terminates CXCR4-mediated activation of G α i but promotes dynamin-dependent, clathrin-mediated receptor endocytosis and enhances CXCR4-RAF-dependent signaling. Phosphorylation of CXCR4 also can occur in response to the activation of other receptors and involves additional kinases, such as protein kinase C20-22 or tyrosine kinases. CXCR4 expression has been detected in 23 cancers of various origins and is the most common chemokine receptor expressed by cancer cells (Balkwill, 2004).

Regulation of phosphorylation and internalization has significant effects on CXCR4-mediated cellular responses, and for this reason some

authors believe that the descriptions of total CXCR4 expression alone are inadequate (Konoplev et al., 2013).

It was suggested that interference at CXCR4-S339 phosphorylation with small-molecule inhibitors targeting the involved kinases like PIM1, GRK6 or PKC might offer a strategy to increase the mobilization of potentially relapse-initiating leukemic stem cells out of bone marrow niche (Brault et al., 2014).

It was suggested that circulating blasts will be less susceptible to treatment with cell-cycle phase specific agents such as cytosine arabinoside, the mainstay of current AML therapy. Nevertheless, by virtue of not having entered quiescence, these cells may be capable of rapid proliferation if they recirculate back to the BM (Braess et al., 2001).

This is particularly relevant to the potential use of CXCR4 inhibitors to mobilize blast cells from the bone marrow niche into the circulation prior to the administration of chemotherapy (Nervi et al., 2009).

The present study was performed on 20 adult patients with newly diagnosed AML, their age ranged from 20 to 64 years. The aim was to evaluate (immunohistochemically) CXCR4 (phosphorylated and unphosphorylated) expression in bone marrow biopsy blast cells and Correlate these markers with AML prognosis.

CXCR4 and Ph-CXCR4 expression was evaluated by cut off value of 20% of blast cells for CXCR4 and 15% for Ph-CXCR4. CXCR4 was found positive in 85% of patients and negative in 15% of them. The phosphorylated CXCR4 was found positive in 65% of cases and negative in 35% them.

A study conducted by Konoplev et al, CXCR4 expression was detected in 64% of patients and pCXCR4 expression was detected in 26% of patients (Konoplev et al., 2013).

In a study done by Brault and his colleague to determine the role of CXCR4-S339 phosphorylation in AML, immunohistochemical staining of pCXCR4-S339 and CXCR4 in BM core biopsies of 75 untreated AML patients. Eighty eight (88)% were CXCR4 positive, but only 57% were positive for pCXCR4-S339 (Brault et al., 2014).

The patients were diagnosed on the basis of clinical presentation, cytomorphology, cytochemistry, as well as immunophenotyping. AML cases were classified according to FAB classification into four cases M1 20%, four M2 20.0%, one M3 5%, nine M4 45.0%, and two M7 10.0%. CXCR4 and phosphorylated CXCR4 expression did not differ significantly in the different AML/FAB subclasses.

Bone marrow cellularity plays a role in treatment outcome. hypercellularity at baseline was associated with a lower likelihood of achieving full count

recovery after treatment. Patients that were hypercellular after cycle 1 of treatment were more likely to have been hypercellular at baseline. Neither baseline nor post treatment hypocellularity had an association with hematological recovery (Dugan et al., 2017).

Bone marrow aspirate was hypercellular in 85% and normocellular 15% of patients. BM trephine biopsy was hypercellular in 90% and normocellular in 10% of studied cases.

No significant difference was found between CXCR4 and ph-CXCR4 expression and BM aspiration cellularity, Peripheral blood cellularity or BM biopsy cellularity.

Patients with AML commonly present with leukocytosis, anemia and thrombocytopenia. Uncontrolled proliferation of blast cells in BM and its release in circulation leading to leucocytosis (Appelbaum, 2009). The infiltration of the BM by the leukemic blasts suppresses the normal hematopoietic cells as late erythroid progenitor (LEP) and erythroid colony-forming unit (CFU-E) and quantitative defect in this subset associated with anemia (Iskander et al., 2015). Leukemic blast infiltration has been documented to leads to inadequate production of platelet and decreased platelet survival (Döhner et al., 2015).

Leukocytosis is one of the prognostic factors of AML. Leukocytosis more than 100,000/cc is considered bad prognostic factor of the disease. hyper leukocytosis correlates with shorter overall survival due to both increased induction deaths and an association with flt3 mutations (Wintrobe, 2008).

Serum LDH is an important prognostic factor, predicting for clinical outcome in both haematological and non-haematological malignancy. By utilizing this simple laboratory test, alone or in combination with other prognostic factors (Dalley et al., 2001).

No significant association was observed between CXCR4 and ph-CXCR4 expression and laboratory data of the patients including hemoglobin, platelet and WBCs count.

Serum level of LDH was found to be significantly associated with CXCR4 and Ph-CXCR4 expression, with higher significant association with ph-CXCR4.

Spoo et al noticed higher WBC counts and higher LDH with higher CXCR4 expression (Spoo et al., 2007).

Fever was the commonest presentation among studied patients. Sixty (60.0) % of studied cases presented with fever, 55.0% presented with hepatosplenomegaly, 40.0% presented with bleeding tendency and 35% presented with LN enlargement.

Patients presented with fever and hepatosplenomegaly showed significant expression of

both CXCR4 positivity and Ph-CXCR4 with higher significance with Ph-CXCR4. Patients presented with lymph node enlargement and bleeding tendency showed significant relation only with Ph-CXCR4 expression.

The identification of recurrent cytogenetic abnormalities associated with distinct clinical presentation in acute myeloid leukemia paved the way for the incorporation of genetic markers into clinical decision-making. The observation that most cytogenetic abnormalities are non-overlapping and their distinct associations with clinical presentation, therapeutic response, relapse rates, and overall survival formed the basis for the development of molecular classification and risk stratification schemas by the World Health Organization (WHO) (Moarii and Papaemmanuil, 2017).

The combination of baseline biologic parameters (karyotyping, molecular biology) enables a better definition of discrete prognostic categories. This approach may potentially allow improved tailoring of postconsolidation therapy aimed at avoiding undertreatment or overtreatment (Buccisano et al., 2010).

Identification of FLT3 mutation as a negative prognostic marker, serves to highlight the importance of FLT3 testing at diagnosis and again at relapse. Earlier identification of FLT3 mutations will help provide a better understanding of the patient's disease and enable targeted treatment that may help patients achieve longer and more durable remissions (Daver et al., 2019).

Expression of both CXCR4 and ph-CXCR4 did not differ significantly in patients presented with different karyotyping and FLT3 mutation. Brault et al documented no significant differences in Karyotyping and FLT3 mutation regarding to Ph-CXCR4 positivity (Brault et al., 2014).

Patients received induction therapy for AML according to (7+3) regimen which includes cytarabine for 7 days through an intravenous line (IV) and anthracycline in a single IV dose for the first 3 days of treatment. Follow up was done by frequent examination of peripheral blood and BM smears after induction therapy. Follow up was monitored by hematological complete remission (CR) by day 28.

Thirty five (35)% of patients reached complete remission, 30% did not reach complete remission after 28 days and 35% reached complete remission by the day 28 and then relapsed.

Expression of CXCR4 and Ph-CXCR4 is higher in patient failed to reach complete remission and relapsed patients compared to patient reached complete remission with the highest significant relation with ph-CXCR4 expression.

Patients had been followed up for one year from the start of the study then the time at which the patient achieved remission, relapsed, died or last seen alive was recorded for calculation of overall survival (OS) and the disease free survival (DFS).

By analysis of survival and using kaplanmeier curve, it has been found that expression of ph-CXCR4 is more significant than CXCR4 expression. Ph-CXCR4 correlated significantly with survival data (OS & DFS) unlike CXCR4 which did not play a significant role. Thus the study highlights the importance of investigating the expression of ph-CXCR4 as an evaluating marker for patient prognosis.

Spoo et al study showed higher relapses, and deaths in patients with higher CXCR4 expression (Spoo et al., 2007).

Brault et al demonstrated that patients with positive staining for CXCR4 did not show significant trend toward longer overall survival. However, the presence of pCXCR4-S339 in these patients was significantly associated with shorter overall survival (Brault et al., 2014).

A study by Konoplev et al, proved that shorter OS was significantly associated with CXCR4 expression, but failed to prove a significant association with ph-CXCR4 expression. The study also noted that shorter DFS was associated with both CXCR4 and ph-CXCR4 expression (Konoplev et al., 2013).

Taken together, our observations indicate the important value of bone marrow biopsy in AML at diagnosis and follow up. It also indicate the role of ph-CXCR4 in homing and migration of AML blasts to the bone marrow niche, so investigating the phosphorylated form of CXCR4 expression in BM biopsy is could to be an important tool for AML patients follow up and prognosis. Homing of leukemic blasts actively dividing to the bone marrow niche is suggested to be regulated by G protein-coupled receptor CXCR4 specifically its active phosphorylated form, which has been proved to be associated with bad prognosis of AML.

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