Journal of the Egyptian Society of Haematology & Research, Vol. 13, No. 1, March: 19-24, 2017

B-Cell Specific Moloney Murine Leukemia Virus Integration Site-1 (BMI-1) Gene Expression in Chronic Myeloid Leukemia

HALA M. FARAWELA, M.D.*; HAMDY M. ZAWAM, M.D.**; HANAN A. AL-WAKEEL, M.D.*; MONA H. EL-NAGDY, M.D.*; FATMA A. EL-REFAEY, M.D.*** and HALA A. ABD EL-RAHMAN, M.D.*

The Departments of Clinical & Chemical Pathology and Radiation Oncology & Nuclear Medicine (NEMROC)**, Faculty of Medicine and The Department of Clinical Pathology, National Cancer Institute***, Cairo University, Egypt*

ABSTRACT

Background: B-cell specific moloney murine leukemia virus integration site-1 (BMI-1) gene is a stem cell gene that modulates stem cell pluripotency and is also implicated in the regulation and accumulation of Leukemic Stem Cells (LSCs).

Objectives: The current study aimed at characterizing BMI-1 gene expression in Chronic Myeloid Leukemia (CML) patients in chronic phase (CML-CP), in those who have achieved deep Molecular Response (MR^4) and in patients in Accelerated and Blastic Phase (AP/BP) to be able to pinpoint its role in leukemogenesis and CML disease progression.

Patients and Methods: Real-Time Polymerase Chain Reaction (RT-PCR) was used to assess BMI-1 gene expression in 52 CML patients: 31 CML-CP, 11 in MR^4 and in 10 in AP/BP as well as in 21 non malignant bone marrow samples.

Results: No statistically significant difference was detected between the three patients' group of CML as regards BMI-1 gene expression. No significant relationship between BMI-1 expression in CML-CP patients and different patient characteristics was detected as well as their Progression-Free Survival (PFS).

Conclusion: The current study could not demonstrate a role for BMI-1 gene in the pathogenesis of CML or in the disease progression. On account of the small numbers, further studies on larger patient groups are needed to further elucidate the role and functional consequences of BMI-1 in myeloid leukomogenesis.

Key Words: *BMI-1 – Chronic myeloid leukemia – Leukemic stem cells.*

INTRODUCTION

CML originates in a Hameatopoietic Stem Cell (HSC) with the reciprocal translocation t(9;22). The resulting Philadephia (Ph) chromosome produces BCR/Abl (Breakpoint cluster region/Abelson), a constitutively active tyrosine kinase that drives expansion of leukemic progeny. Most patients harbor residual leukemic cells, and disease recurrence usually occurs when therapy is discontinued. Although various mechanisms to explain leukemia cell persistence have been proposed, the critical question from a therapeutic standpoint whether disease persistence is BCR/Abl dependent or independent has not been answered **[1]**.

In CML, it appears that even after years of treatment with tyrosine kinase inhibitors and the presence of cytogenetic and molecular remission, residual disease is retained in many patients. Often, these patients relapse after tyrosine kinase inhibitor therapy is stopped. This led to contemplating that at least some leukemic stem cells are able to enter a quiescent state which renders them resistant to the therapeutic effect **[2]**.

BMI-1, one of the polycomb group proteins, is reported to play an important role in self-renewal of stem cells. It also plays a vital role in differentiation of progenitor and leukemic cells **[3]**; and is also associated with a number of human malignancies. Studies suggest that BMI-1 is involved in the initiation of cancer and targeting BMI-1 by gene therapy abolishes chemoresistance in tumor cells **[4,5]**. BMI-1 has also been reported to prevent senescence and immortalize cells through the activation of telomerase **[6]**.

The aim of the current study is to characterize the BMI-1 gene expression in CML patients in chronic phase, in deep Molecular Response (MR^4) and in CML disease progression (AP/BP).

PATIENTS AND METHODS

This study comprised 52 CML patients. Cases were selected from the Medical Oncology Department, Kasr Al-Ainy School of Medicine, Cairo University. The research protocol was approved by the Research Ethics committee of the Clinical Pathology Department, Faculty of Medicine, Cairo University, and informed consent was obtained from all participants.

Patients were: A) CP patients (n=31) before the start of therapy (diagnosed according to the WHO 2008 criteria); B) Patients who achieved MR^4 (n=11): Defined as ≥4-log reduction of BCR-ABL1 transcripts when they are expressed on an International Scale (IS) as a percentage, with 100% BCR-ABLIS corresponding to the International Randomized Study of Interferon and STI571 (IRIS) study standardized baseline. MR4 is either (I) Detectable disease 0.01% BCR-ABLIS or (II) Undetectable disease in cDNA with 10_000-31_999ABL1 transcripts or 24_000-76_999 GUSB transcripts [7]; and C) Patients in AP/BP (n=10), diagnosed according to the 2008 WHO criteria [8].

Twenty-one age and gender matched control subjects were also selected in this study. They were patients undergoing bone marrow aspiration for reasons other than malignancy, such as bone marrow transplant donors or prior to splenectomy in patients with hypersplenism.

Patient medical records were reviewed for history and clinical data. Reviewed data included complete blood counts, BCR/ABL1 fusion gene detection by cytogenetic and molecular techniques.

Demographic and clinical data of patient and control groups are presented in (Table 1).

Treatment:

Management and regular monitoring were done according to the 2013 guidelines of European Leukemia Network (ELN) [9].

Methods:

Detection of expression levels of BMI-1 gene by SYBR Green Real-Time PCR:

Mononuclear Cells (MNCs) were separated from EDTA anticoagulated venous blood sample or bone marrow by density gradient using 1.077g/ml Ficoll Hypaque (Invitrogen, USA) and stored at –20ºC for later use. RNA was isolated using QIAamp RNA Blood Mini Kit, (Qiagen, Germany). Total RNA was reversely transcribed in a total volume of 20µl reaction using high capacity cDNA reverse transcription kit (Qiagen, Germany). This was followed by amplification of cDNA by RT-PCR using Quantitect SYBR Green Master Mix (Qiagen, Germany). The real time cycler (Applied Biosystems 7500, USA) was programmed as follows: Initial denaturation at 95ºC for 2 minutes, followed by 60ºC for 40s and extension at 72ºC for 15s. This was repeated for 40 cycles. The signal from the RT-PCR product was normalized to the signal from the internal control (β2-microglobulin) which was amplified by another set of primers. The primer sequences used were: For BMI-1 gene: F-5'- TTCATTGATGCCACAACCAT-3'. R-5'CAGCATCAGCAGAAGGATGA-3' [10]; for β2-microglobulin: F 5'-TACACTGAATTCACCCCCAC-3'; R 5'-CATCCCAATCCAAATGCGGCA-3' [11].

The relative expression of BMI-1 was determined using the delta C_T method. A Comparative Threshold cycle (C_T) was used to determine the gene expression relative to a normal control (calibrator) and used for comparison between patients at different stages of the disease. Briefly, each sample of either patient or control was normalized for the expression of β2 microglobulin (housekeeping gene) using the formula $\Delta C_T = C_T$ of the gene – C_T of β2 microglobulin. The mean expression of the control samples was then chosen as a normal calibrator, and relative BMI-1 expression for every patient were calculated using $2^{-\Delta\Delta CT}$ formula, where $\Delta\Delta C_T = \Delta C_T$ sample $-\Delta C_T$ calibrator. BMI-1 expression levels were expressed as an n-fold difference relative to the calibrator. Therefore; a $2^{-\Delta\Delta CT}$ value of >1 was considered as a high expression of the gene as compared to the control and a value of <1 was considered low expression of the gene [12].

Statistical methods:

Data was analyzed using IBM SPSS advanced statistics Version 22 (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 or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric *t*-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" was used for pair-wise comparison based on Kruskal-Wallis distribution. 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. All tests were two-tailed. A *p*-value <0.05 was considered significant.

RESULTS

BMI-1 mRNA transcripts were detected in 29% (9/31) of CML-CP patients, 36.4% (4/11) of CML-MR^4 patients and was detected in 70% (7/10) of CML-AP/BP patients (Table 2). No statistically significant difference was seen between the controls and patient groups (p= 0.093). Regarding expression of BMI-1 gene in different CML phases, BMI-1 gene expression was higher in CML-AP/BP (median=0.84, range 0.000-13.890) than in CML-CP (median 0.000, range 0.000-18.150) and CML-MR^4 (median 0.000, range 0-6.37), without reaching a statistical significant difference (p=0.184). No statistical significance was detected between CML-CP, CML-MR^4, and CML AP/BP patients as regards BMI-1 low and high expression percentage (p=0.593).

No correlation was found between BMI-1 gene expression and the patients' age (r=–0.305, p=0.095). A trend towards a statistically significant difference (p=0.058) was seen when comparing patients with low/intermediate Sokal scores and those with high Sokal scores regarding BMI-1 gene expression. The latter group had a higher median fold change in expression (median=1.843) as compared to low/ intermediate groups (median=0.000). No statistically significant difference in BMI-1 expression was observed in CML-CP patients as regards gender (p=0.226) and presence of organomegaly (p= 0.827).

No correlation was found between BMI-1expression and hemoglobin levels (r=0.212, p=0.269), total leucocytic count (r=0.045, p= 0.815), platelets (r=–0.063, p=0.745), peripheral blood basophils (r=–0.133, p=0.526), or peripheral blood blasts (r=–0.053, p=0.806).

No statistically significant difference was found in BMI-1 expression levels in CML-CP patients as regards the need for second-line TKIs (p=0.251).

On comparing CML-CP patients showing low and high BMI-1 expression with different parameters, no statistically significant difference was observed between the 2 groups as regards age (p=0.187), hemoglobin concentration (p= 0.088), total leucocytic count (p=0.924), platelets (p–=0.672), or PB blasts (p=0.901), or PB basophils (p=0.745). No statistically significant difference was found between the 2 groups as regards hematological or clinical parameters (Tables 3, 4).

CML patients with BMI-1 median fold change <1 had a median PFS of 15.670 and ranged between 3.170 and 35.130 months. CML patients with BMI-1 median fold change >1 (n=4) had an undetermined median PFS time as none of the patients lost their MMR. Their PFS ranged between 7.670 and 30.470 months. No statistically significant difference was found between the 2 groups (p=0.200) Fig. (1).

Table (1): Clinical and demographic data of 52 chronic myeloid leukemia patients.

Parameter	CML-CP No.=31	CML-MR^4 No.=11	CML-AP/BP No.=10
Age: Median (range)	39 (21-75)	43 (19-65)	40.5 (19-75)
Gender (M:F)	18: 13	5: 6	6: 4
Organomegaly: No (%)	25 (80.6%)	9 (81.8%)	10 (100%)
Sokal score: No (%):			
Low/intermediate	25 (83.3)	5 (83.3)	4 (40)
High	5 (16.7)	1 (16.7)	6 (60)

CP : Chronic Phase.
MR^4 : Deep Molecular Response.
AP/BP : Accelerated Phase/Balstic Phase.

Table (2): Comparison of BMI-1 gene expression between 52 Chronic Myeloid Leukemia (CML) patients and normal controls.

Group	No.	BMI-1		p-value
		Detected	Not detected	
Controls	21	11 (52.4)*	10 (47.6%)	0.093
CML-CP	31	9 (29)	22 (71)	
CML-MR4	11	4 (36.4)	7 (63.6)	
CML-AP/BP	10	7 (70)	3 (30)	

CP : Chronic Phase.
MR4 : Deep Molecular Response.
AP/BP : Accelerated Phase/Balstic Phase.
* : No (%).

Table (3): Comparison between chronic myeloid leukemia patients in chronic phase with low and high BMI-1 expression as regards age and peripheral blood parameters.

Parameter	BMI-1 expression				p-value
	Low: No.=23		High: No.=8		
	Median	Range	Median	Range	
Age: Years	38	21-75	44	36-70	0.187
Hb: g/dl	9.8	8.0-13.3	10.5	10.3-14.0	0.088
TLC: X 10^3/uL	147.0	21.6-636	192.0	56.0-300	0.924
Plts: X 10^3/uL	397.5	154-662	383.0	134-600	0.672
PB blasts: %	2.0	0-8	3	0-5	0.901
PB basophils: %	3.0	0-8	3.0	0-6	0.745

Hb : Haemoglobin.
TLC : Total Leucocytic Count.
Plts : Platelets.
PB : Peripheral Blood.

Table (4): Comparison between chronic myeloid leukemia patients in chronic phase with low and high bmi-1 expression as regards clinical parameters.

Parameter	BMI-1 expression		p
	Low: No=23	High: No=8	
Gender:			
Male	11 (47.8)*	7 (87.5)	0.095
Female	12 (52.2)	1 (12.5)	
Organomegaly:			
Present	19 (82.6)	6 (75)	0.634
Absent	4 (17.4)	2 (25)	
*Sokal score**:*			
Low/intermediate	21 (91.3)	4 (57.1)	0.068
High	2 (8.7)	3 (42.6)	
*Second-line TKIs***:*			
Yes	6 (26.1)	0 (0)	ND
No	13 (56.5)	5 (62.5)	

* : No (%) ND: Not determined due to small no. of patients.
** : Low expression n=23/high expression n=7.
*** : Low expression n=19/ high expression n=5.

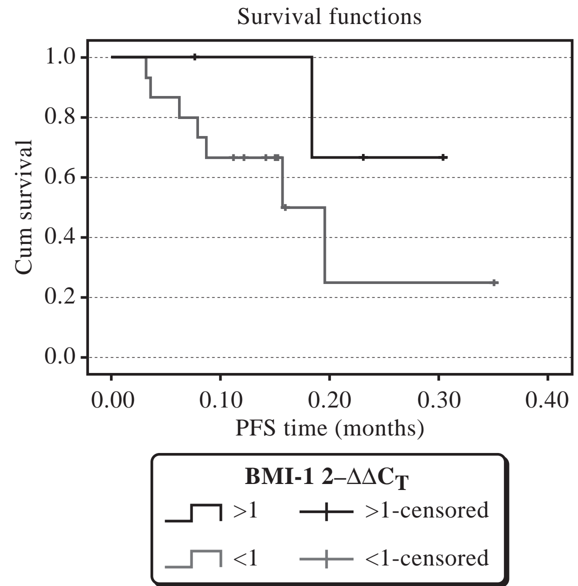

Fig. (1): Kaplan-Meier survival curve for PFS data according to BMI-1 gene expression.

DISCUSSION

BMI-1 gene expression was higher in CML-AP/BP (median=0.84, range 0.000-13.890) than in CML-CP (median 0.000, range 0.000-18.150) and CML-MR4 (median 0.000, range 0-6.37), without reaching a statistical significant difference (*p*=0.184). No statistical significant difference was detected in the percent of low and high expression cases in different CML phases (*p*=0.593).

Most studies agree that BMI-1 is significantly overexpressed in CML-CP patients in relation to healthy controls **[11,13-15]**. However, when BMI-1 mRNA transcripts were analysed in CD34+ cells, a higher level was found as compared to unfractionated whole blood **[13,15]**. Therefore standarisation of samples in control subjects as well as across studies has to be taken into account.

Research suggests high BMI-1 expression in CML patients which increased more with disease progression **[11,15,16]**. However, discrepancy between BMI-1 protein level and mRNA expression levels were seen in many studies. Bhattacharya et al. **[13]** found that, despite a significant high level of BMI-1 protein among CD34+ cells in CML-CP in comparison to control group, this level further increased during disease progression, mRNA levels were almost consistent during disease progression. This was attributed to the fact the proteosomal inhibitors

can enhance protein but not mRNA levels. Moreover, levels of BMI-1 were enhanced by BCR-ABL1 transfection. Ultimately, further investigation is required to evaluate expression of BMI-1 in peripheral blood and bone marrow at both the protein and mRNA levels. In addition, a study by Merkerova et al., [14] proved that BMI-1 inhibition affected neither the proliferation rate nor the cell cycle of CML cell lines suggesting that there are some parallel signaling pathways which are fundamental for CML disease progression independent of BMI-1 overexpression.

In CML-CP patients, no significant relation was found between BMI-1gene expression and patients' age, sex, presence of organomegaly, peripheral blood findings and their need for second-line Tyrosine Kinase Inhibitors (TKIs) (p>0.05). No significant difference was seen between CML-CP patients with low and high SALL4 expression as regards these parameters (p-value>0.05). This was also demonstrated by Ghannam et al., (2012) & Saudy et al., (2014). However, Saudy et al., (2014); found increased basophil count with BMI-1 high expression groups. Our study also found a higher SALL4 gene expression in CML-CP patients with high Sokal scores as compared with low/intermediate Sokal scores. The difference was not statistically significant yet there was a trend (p=0.068). No studies were found to validate this.

Kaplan Meier analysis was done to assess effect of BMI-1 gene expression on CML-CP patients PFS. No statistically significant was observed between low and high gene expression patient groups as regards their PFS (p=0.200) Fig. (1). Other studies correlated low BMI-1 expression with longer OS times [15,17]. However no studies were done to correlate its effect in progression or event-free survival of CML patients.

In conclusion, no role has been found in our study as regards BMI-1 gene in CML-CP. However, a larger study is needed to delineate the role of BMI-1 in CML disease progression.

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