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Expression of Phosphorylated STAT5 in Chronic Myeloid Leukemia: Relation to Disease Stages

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ABSTRACT

Background: *BCR-ABL* expression is the whole mark of chronic myeloid leukemia (CML). It results in constitutive activation of signal transducer and activator of transcription (STAT) and essentially bypasses cytokine or growth factor-dependent activation of STAT5.

Methods: We investigated the state of STAT5 phosphorylation (pSTAT5) in relation to CML disease stages as a possible indicator of BCR/ABL tyrosine kinase activity. The study was conducted on 39 CML patients including 17 males and 22 females; 22 patients were in chronic phase (Group I) and 17 were in accelerated phase or blastic crisis (Group II). Patients were divided into 3 risk groups according to Hasford score: Low, Intermediate and High. pSTAT5 was measured using Flow Cytometry; its expression was evaluated in relation to various hematological and clinical parameters.

Results: pSTAT5 was expressed in all cases tested. The level was statistically significantly higher in advanced phases than in the chronic phase (p=0.006). CD34+ve cells % was 1.74±1.61% and 21.3±20.4% in Group I and II respectively (p=<0.001). All CD34 positive cells were pSTAT5 positive. CD34-ve cells were pSTAT5-ve (<10%) in 8/22 and 5/17 patients in Group I and II respectively. pSTAT5% expression was significantly higher in Group II as compared to Group I (56.4±27.6% vs. 33.9±21% respectively; p=.006). pSTAT5% expression showed significant +ve correlation with both peripheral blood and bone marrow blast percentage (r=0.39 and 0.37; p=0.017 and 0.02 respectively). No correlation was encountered between pSTAT5 expression on one side and age, Hasford score or duration of chronic phase on the other side.

Conclusions: The level of expression of pSTAT5 is higher in advanced phases of CML reflecting a higher tyrosine kinase activity of the BCR/ABL chimeric protein. This might help making therapeutic decisions.

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Key Words: *Chronic myeloid leukemia (CML) – Signal transducer and activator of transcription 5 (STAT5) – pSTAT5 – CD34.*

INTRODUCTION

Chronic myeloid leukemia (CML) was probably the first form of leukemia to be recognized as a distinct entity. The natural history of CML includes three distinct phases, the chronic phase, the accelerated phase and the blastic phase; CML typically presents in the chronic phase **[1]**.

It is generally believed that CML develops when a single, pluripotent, hematopoietic stem cell acquires a Ph chromosome carrying the *BCR-ABL* fusion gene, which confers on its progeny a proliferative advantage over normal hematopoietic elements and thus allows the Ph-positive clone gradually to displace residual normal hematopoiesis **[2,3]**.

It soon became clear that the BCR-ABL oncoprotein itself is the best molecular target presented by CML cells because it is not expressed by normal cells. Furthermore, the dissection of the signal transduction pathways affected by the deregulated kinase activity of BCR-ABL provided information on additional or alternative signaling steps that could be interrupted in an attempt to eliminate the oncogenic effect of BCR-ABL **[4]**. *BCR/ABL* is not only diagnostic but also pathogenic in CML **[5]**, although the translocation itself may not be sufficient to cause leukemia **[6,7]**.

The JAK/STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors **[8]**. STAT5 plays an important role in hematopoiesis **[9]**. It has been implicated to play an important role in proliferation, differentiation and protection against

apoptosis during hematopoiesis. In addition, it is thought that STAT5 plays an important role in early myeloid differentiation **[9,10]** and to regulate survival in mature myeloid cells. STAT5 has also been implicated in myeloid differentiation induced by IL-3, G-CSF, and GM-CSF **[11-13]**.

In this study, we investigated pSTAT5 expression in relation to CML disease stages as a possible indicator of BCR/ABL tyrosine kinase activity.

PATIENTS AND METHODS

Patients:

The study was conducted on 39 CML patients including 17 males and 22 females with an age range of 19-79, a mean of 39.88±15 and a median of 41.5 years. Twenty two patients were newly diagnosed cases in chronic phase (Group I) and 17 were in accelerated phase (4 cases) or blastic crisis (13 cases) (Group II). All patients presented to the Medical oncology Department of the NCI, Cairo University in the period 1999-2009. The study was conducted between February 2008 and December 2009 at the Bone Marrow Transplantation Lab Unit, Clinical Pathology Department, NCI, Cairo University.

The Study was conducted according to Helsinki declaration; it was approved by the NCI ethical committee and written informed consent was obtained from all patients.

Methods:

Patients were diagnosed as CML and stages defined according to the WHO classification of myeloid neoplasms **[1]**. Patient's files were revised to obtain data at diagnosis and newly diagnosed patients were followed-up for a period of 5-18 months. Patients were divided into 3 risk groups according to Hasford score: Low risk group: Score ≤780, Intermediate risk group: Score 781-1480 and High risk group: Score >1480. The score value for individual patients was calculated by accessing the website www.pharmacoepi.de **[14]**. Hasford score was available for 37 patients including 21 in Group I and 16 in Group II.

Cases were subjected to full history and clinical examination including organomegaly and lymphadenopathy. Routine investigations included abdominal ultrasound, complete blood picture, bone marrow (BM) aspiration, Leukocyte Alkaline Phosphatase (LAP) score, conventional karyotyping, and detection of *BCR/ABL* by RT-PCR. Immunophenotyping using monoclonal antibodies and analyzed on Coulter XL Flow Cytometer was performed for cases in blastic crisis.

Evaluation of STAT5 phosphorylation (pSTAT5):

Analysis of pSTAT5 was performed on EDTA BM samples using double labeling with CD34 PE for direct surface staining and purified antimouse pSTAT5 for indirect intracytoplasmic staining **[15]**.

The intracellular pSTAT5 was performed after fixation and permeabilization using purified monoclonal antibodies followed by FITC-conjugated secondary antibody. The antibodies used are purified mouse anti-human phosphorylated STAT5 (clone 47) obtained from BD Biosciences (cat.No.611964) and PE-conjugated mouse anti-human CD34 monoclonal antibody (clone 563) obtained from BD Biosciences (cat.No.550619). FITC-conjugated polyclonal goat anti-mouse IgG obtained from BD Biosciences (cat.No.555988) served as a secondary antibody for mouse anti-human phosphorylated STAT5. Isotype control included mouse IgG2b/PE for CD34 staining and, as a negative control for indirect staining, FITC-conjugated secondary antibody was only added with no primary antibody.

Analysis of the samples was done using Coulter XL (Hialeah) flow Cytometer.

Results were expressed as percentage positivisty of pSTAT5 in the CD34+ve and CD34 -ve population as well as florescent ratio (FR) by dividing the channel number of the test by the channel number of the isotype (Figs. 1-3).

Statistical methods:

SPSS (Statistical package for social sciences version 17.0) was used for data analysis. Mean and standard deviation were estimates of quantitative data and median with range for non-normally distributed sets. Non-parametric *t*-test (Mann Whitney test) was used for comparison of means of two independent groups (Group I and II). Chi-square or Fischer exact tests checked the hypothesis of proportion independence. Non-parametric correlation analysis "Spearman rho" was used to test association. *p*-value is significant when ≤0.05.

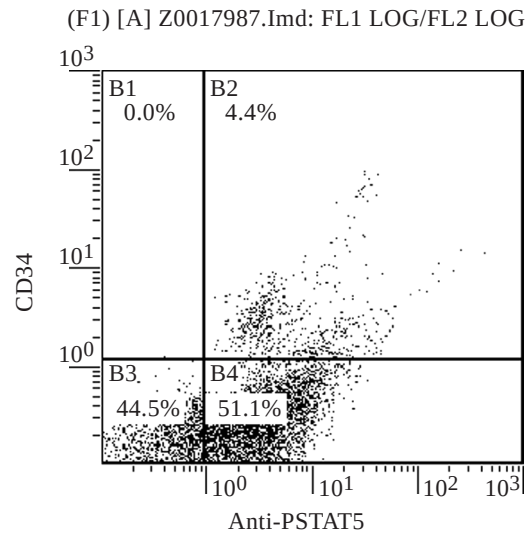

Fig. (1): Co-expression of CD34/STAT5p in a CML case in chronic phase.

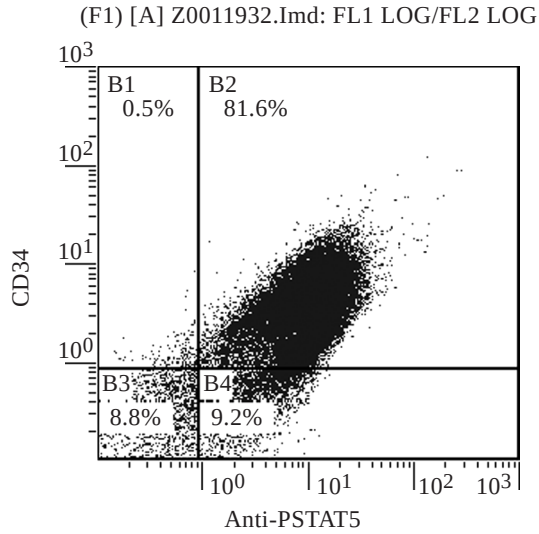

Fig. (2): Co-expression of CD34/STAT5p in a CML case in blastic crisis.

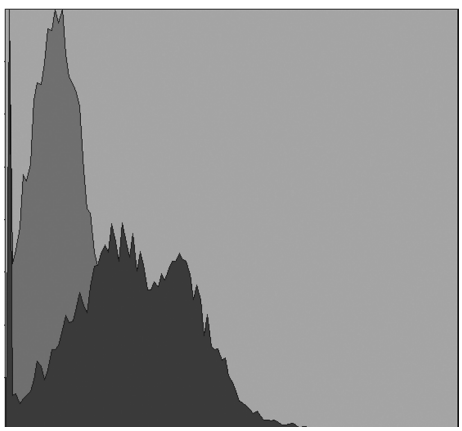

Fig. (3): Histogram showing the overlay of PSTAT5 on the isotypic control.

RESULTS

The patient cohort included 22 CML cases in chronic phase (Group I) and 17 in blastic crisis or accelerated phase (Group II). The latter group included 4 patients in blastic crisis at presentation. The duration of the chronic phase in the remaining 13 patients ranged between 3-96 with a mean of 46.5±26.8 and a median of 12 months. There was no statistically significant difference in the pSTAT5 findings between patients in accelerated phase (4 cases) and those in blastic crises (13 cases); hence they were dealt with as one group in further statistical analysis.

Comparison between Group I and Group II regarding hematological parameters is presented in Table (1). The blasts % was significantly higher in Group II both in PB and BM. Eosinophils % was significantly higher in Group II in the BM, while Hb % and PB granulocytes % were significantly higher in Group I.

Hasford risk stratification for the two patient's groups revealed 8/21 (38.1%) low, 8/21 (38.1%) intermediate and 5/21 (23.8%) high risk in Group I as compared to 4/16 (25%), 8/16 (50%) and 4/16 (25%) in Group II; the difference is statistically insignificant.

CD34 expression and the phosphorylation status of STAT5 are presented in Table (2). CD34 expression was higher in Group II than in Group I. All CD34+ve cells expressed pSTAT5. The pSTAT5% was significantly higher in patients in the accelerated phase or blastic crisis (Group II) than those in the chronic phase (Group I); however the florescent ratio was comparable in both groups. CD34-ve/pSTAT5 +ve% was higher in Group II than in Group I but the difference did not achieve statistical significance.

pSTAT5 % and pSTAT5 FR were comparable among patients in the three Hasford risk groups (Table 3).

Correlations between pSTAT5 expression and different variables:

A statistically significant positive correlation was encountered between pSTAT5 % and both BM and PB blasts % (r=0.37, p=0.02 & r=0.39, p=0.017 respectively) while a statistically significant negative correlation was encountered

between pSTAT5 % and granulocytes % in BM and PB (r=–0.438, p=0.005 and r=–0.435, p=0.007 respectively). A near significant positive correlation between pSTAT5% and Hasford score was encountered in the 37 patients (r=0.29, p=0.08). A fair positive correlation between pSTAT5% and Hasford score was encountered in Group II patients (r=0.28) although the p-value was statistically insignificant (0.28).

No correlation was encountered between CD34–ve/pSTAT+ve % on one side and any of the hematological or clinical parameters on the other side.

Table (1): Hematological parameters of 39 CML patients in relation to disease stage.

Parameter	Group I No=22	Group II No=17	*p* value
Peripheral blood:			
TLC x 10^9/L	157.5±81.9 (5.2-322)*	118.98±128.11 (2-550)	0.26
Hb g/dl	9.4±1.8 (6.3-12.5)	7.7±1.46 (4.1-10.4)	0.003
Platelets x 10^9/L	415.1±241.1 (125-988)	305.18±297.92 (12-949)	0.21
Blasts %	2.91±2.79 (0-10)	20.06±21.27 (1-95)	0.001
Basophils %	4.31±3.30 (0-14)	5.256±5.66 (3-23)	0.2
Eosinophils %	2.95±1.86 (0-6)	2.52±1.62 (0-6)	0.46
Granulocytes %	77.04±9.53 (60-91)	46.52±22.04 (3-87)	0.001
Bone marrow:			
Blasts %	2.5±2.29 (0-9)	27.78±20.66 (0-9)	0.001
Basophils %	2.52±2.29 (0-9)	7.72±7.77 (0-33)	0.06
Eosinophils %	3.78±3.84 (0-18)	4.33±3.82 (0-17)	0.001
Granulocytes %	76.83±12.59 (46-90)	38.77±23.36 (2-78)	0.65

Mean±SD, (range).

Table (2): CD34 and pSTAT5 expression in 39 CML patients.

Parameter	All patients No. 39	Group I† No. 22	Group II‡ No. 17	*p* value
CD34%	10.27±16.55* (0-86)	1.74±1.61 0-7	21.32±20.42 0-86	<0.001
pSTAT5%	43.66±26.32 (2-97)	33.85±21.05 2-75	56.35±27.58 14-97	0.006
pSTAT5 FR§	(1.48-46.03) 13.82±10.64	13.32±10.89 1.48-46.03	14.47±10.61 2.67-39.74	0.74
CD34-ve/pSTAT5+ve %	28.64±26.87 (0-97)	23.35±22.46 0-75	35.18±30.94 2-97	0.19

*Mean±SD, (range). †Group I : Chronic phase.
§FR: Fluorescent ratio. ‡Group II: Accelerated phase and blastic crisis.

Table (3): pSTAT5 expression in 37 CML patients in relation to risk groups according to Hasford score.

Risk group	No.	pSTAT5 %	pSTAT5 FR†
Low	12	39.6±31.7 (2-90)*	15.7±12.5 (1.5-46)
Intermediate	17	45.1±23.2 (6.8-88)	12±8.5 (2.5-27.8)
High	8	45.2±30.1 (20-97)	13±8.6 (4.7-27.2)
Total	37	43.4±27 (2-97)	13.4±9.8 (1.5-46)
p-value		0.79	0.76

*Mean±SD, (range).
†FR: Fluorescent ratio.

DISCUSSION

Leukemogenesis in CML is a complex and incompletely understood process wherein BCR-ABL plays a central role as it influences a large number of signal transduction routes in parallel. Most signal transduction pathways that are involved in the pathogenesis of CML converge at the level of transcription factors, like STAT proteins and C-MYC, and BCL-2 family. These proteins all act synergistically to induce proliferation, while promoting cell survival **[16]**.

STAT proteins are known to be regulated by cytokine receptors and are critical for driving transcription necessary for growth, survival, and differentiation of hematopoietic cells. Experimental evidence indicates that BCR-ABL activates predominately STAT5 and to a lesser extent STAT3 and STAT1. BCR-ABL may activate STAT5 by direct phosphorylation or via phosphorylation by JAK2 **[18]**.

We have investigated the pSTAT5 in 39 patients. pSTAT5 was detected by flow cytometry in all BM samples from CML patients either in chronic or advanced cases in all CD34+ve cells indicating constitutive activation of STAT5 in CML progenitor cells. Gutierrez-Castellanos et al., **[19]** determined pSTAT5 in 27 CML patients including 11 in chronic, 6 in accelerated and 10 in blastic crisis phase. In their study 19 patients (70.3%) were positive for pSTAT5. Frequency of pSTAT5 was higher in patients in blastic crisis (100%) than in patients in accelerated phase and chronic phase (66.6 and 45.3%, respectively, p=0.022). However they tested pSTAT5 by immuneprecipitation and western blotting; the CD34+ve cells in the chronic phase which are pSTAT5+ve are too few to reach the detection limit of this technique. In their work they tested for the pSTAT5 in CD34+ve cells by confocal microscopy but only in the cases that were positive by western blot. In spite of the difference in the technique, our findings are matching theirs with regards to the significantly higher expression of pSTAT5 in advanced phases (blastic crisis and accelerated phase) than in chronic phase (p=0.006). In agreement with our results; Horita et al., **[20]** showed that all CML patients included in their study had pSTAT5 independent of disease stage.

An increase in BCR-ABL level is seen in advanced-phases of CML; blast phase is accompanied by an increase in both BCR-ABL mRNA and protein level and this increase is accompanied by increased tyrosine kinase activity of the BCR-ABL protein **[16]**. In view of our findings related to lower levels of pSTAT5 in chronic phase than in blast crisis, it is suggested that pSTAT5 may be taken as an indicator of BCR-ABL kinase activity.

In our study, pSTAT5 was expressed in 100% of CD34+ cells. In agreement with these results, Gutierrez-Castellanos et al. **[19]** demonstrated pSTAT5 in CD34+ cells in CML patients at different disease stages; however they tested only those cases that were positive by western blot.

Also other studies showed evidence of constitutive STAT5 and/or STAT1 activity in *BCR/ABL*- positive cell lines, peripheral-blood samples from CML patients, and hematopoietic cell lines transfected in vitro with *BCR/ABL*, leading to malignant transformation **[20,21]**.

Although the important role of STAT5 in the pathogenesis of CML was demonstrated in many studies; its role in transformation of the disease from chronic phase into advanced phases was not that much addressed. Up to our best knowledge, only the study conducted by Gutierrez-Castellanos et al., **[19]** investigated the role of STAT5 in the transformation of CML. In a recent study Warsch et al., **[22]** have shown that STAT5 over-expression leads clearly to a TKI-resistant phenotype whereas STAT3 and STAT1 have no effect. High levels of STAT5 protected leukemic cells from TKI toxicity in the absence of JAK2 expression, suggesting that BCR-ABL (or v-ABL) induced JAK2 phosphorylation is not required for STAT5 activation in leukemic cells. In BM samples from patients in advanced CML, high levels of STAT5 mRNA levels have also been shown to correlate with TKI resistance and accordingly, STAT5A and STATB expression (2 highly homologous STAT5 gene products) were found to be increased in CML patients with advanced stage with or without ABL-kinase mutations. These findings together with the report of Nelson et al., **[23]** suggest that STAT5 phosphorylation is a marker of CML progression and it could be an attractive target to circumvent TKI resistance in CML.

In our study, there was a statistically significant positive correlation between pSTAT5%

and blasts % in both BM and PB (r=0.37, p=0.02 and r=0.39, p=0.017 respectively) indicating the relation between the transformation and the STAT5 phosphorylation. This result is in agreement with Gutierrez-Castellanos et al. **[19]** who stated that the percentage of immature cells in chronic phase and advanced phases can influence phosphorylation of STAT5. Our reciprocal finding of a statistically significant negative correlation between the pSTAT5% and the granulocytes % both in the BM and PB ($\boldsymbol{r}$=-0.438 and p=0.005, –0.435 and p=0.007) indicates again that the phosphorylation of STAT5 is dependent on the immature cells.

A near significant positive correlation between pSTAT5% and Hasford score was found in our study (r=0.29, p=0.08). Also pSTAT5% was higher in high risk group than other groups though it was not statistically significant. These findings, though lacking statistical significance, are online with the assumption that pSTAT5 reflects the level of tyrosine kinase activity and the state of disease progression.

Because neoplastic cells are dependent on constitutive STAT activation; targeting STATs causes preferential cancer cell killing with minimal effects on normal cells **[24]**. Although imatinib is very active and well-tolerated in the majority of patients with CML, cure cannot be attained with this or second generation TKI's, necessitating lifelong treatment. Moreover, a substantial number of patients demonstrate insufficient response to these drugs. Quiescence and multiple other mechanisms render CML stem cells drug resistant **[16]**. It is likely that optimal strategy for eliminating leukemic stem cells will involve targeting multiple pathways asking for combination of several agents. Targeting STAT5 could be an appealing approach being downstream of multiple signaling pathways. Being expressed in all CD34+ve cells, the fraction including the leukemia stem cells would further support this approach. Although not tried in CML yet; those new approaches may be of benefit to shut down the signal transduction pathways involved in the progression into advanced phases.

In conclusion, we have reported activation of STAT5 as evidenced by expression of pSTAT5 in all CD34+ve cells in CML patients regardless the disease stage. Its expression is higher in advanced stages known to be associated with higher levels of *BCR/ABL* expression. pSTAT5 may serve as an indicator of the level of *BCR/ABL* expression and STAT5 may serve as a potential therapeutic target in CML.

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