Bcl-xL Expression and *STAT5* Phosphorylation in Chronic Myeloid Leukemia at Different Disease Stages

Thesis

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By

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Abstract

Introduction: CML is characterized by the presence of the Ph chromosome in hematopoietic stem cells. Clinically, it is manifested in three distinct phases: chronic, accelerated, and blastic. Most patients present in the chronic phase, a stage that is typically indolent in nature. The natural history of the disease includes progression into an accelerated phase followed by blastic crisis, which is inevitably fatal (Melo and Barnes, 2007).

Signal transducer and activator of transcription (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 (Ilaria and Van Etten, 1996). BCR-ABL expression results in constitutive activation of STAT5 and essentially bypasses cytokine or growth factor-dependent activation of STAT5. Activation of STAT5 contributes to increased expression of the anti-apoptotic Bcl-2 family members Mcl-1 and Bcl-xL (Huang et al., 2002).

Aim of work: The aim of our work is to investigate the role of phosphorylated STAT5 and Bcl-xL expression in the pathogenesis of CML and progression into advanced phases.

Subjects and methods: Forty patients with chronic myeloid leukemia were included in this study. Twenty two patients were females and eighteen patients were males. Their age ranged between 7-79 years with a mean of 39.88±15 and a median of 41.5. Analysis of PSTAT5 was done using flow cytometry for 39 patients among whom 22 patients were in chronic phase and 17 were in blastic crisis or accelerated phase. Bcl-xL expression was assessed by RT-PCR in 32 patients among whom 18 patients were in chronic phase and 14 were in blastic crisis or accelerated phase.

Results: Our study revealed a statistically significant higher level of PSTAT5 expression in advanced phases than in the chronic phase ($p=0.006$). Bcl-xL

expression did not differ according to the disease stages but showed a statistically significant higher expression in patients in high risk group according to Hasford score than patients in intermediate or low risk groups (p=0.01). With follow up some of our patients (18 patients); a statistically significant better outcome was observed in patients with Bcl-xL negative than in patients with Bcl-xL positive (p=0.05).

Conclusion: The level of expression of PSTAT5 is higher in advanced phases of CML which may lead to activation of pathways that render the cells more resistant to apoptosis and to treatment. Bcl-xL can serve as a prognostic factor in CML indicating whose patient will respond to the conventional therapy and who will need Gliveec from the start. But still the pathogenesis beyond the progression into blastic crisis remains to be declared. Also, the value of the Bcl-xL as a prognostic factor and its role in the progression into more advanced phases should be more investigated.

Key words:

- Chronic Myeloid Leukemia (CML)
- Signal Transducer and activator of transcription 5 (STAT5)
- Bcl-xL- Bcl-2 family
- CD34

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List of Abbreviations

- ABL: Abelson proto-oncogene
- AIF: Apoptosis Induced Factor
- ALPS: Autoimmune Lymphoproliferative Syndrome
- AML: Acute Myeloid Leukemia
- Arg: ABL-related gene
- AS: Anti-sense
- ATP: Adenosine Tri-phosphate
- ATR: Ataxia Telangectasia Related
- Bak: Bcl2-antagonist/killer
- Bax: Bcl-2-associated X protein
- BCL: B- Cell Lymphoma gene
- BCR: Breakpoint Cluster Region
- BH: Bcl-2 Homology
- CML: Chronic Myeloid Leukemia
- DDR-1: Descoidin Domain Receptor-1
- EBMT: European group of Bone Marrow Transplantation
- ER: Endoplasmic reticulum
- EBV: Epstein-Barr Virus
- EndoG: Endonuclease G
- FISH: Fluorescent in situ Hybridization
- FTI: Farnesyl Transferase Inhibitor
- G-CSF: Granulocytes- Colony Stimulating Factor
- GM-CSF: Granulocyte-Monocyte Colony Stimulating Factor
- GPCRs: G Protein Coupled Receptors
- Grb: Growth factor receptor- bound protein
- HSP: Heat Shock Protein
- IBMTR: International Bone Marrow Transplantation Registery
- ICSBP: Interferon Consensus Sequence Binding Protein
- IFN: Interferon
- IL: Interleukin
- InsP3R: Inositol 1,4,5-triphosphate Receptor

- IRS: Insulin Receptor Substrate
- ITAMs: Inducible Tyrosine-based Activation Motifs
- ITD: Internal Tandem Duplication
- JAK: Janus Kinase
- JNK: JUN Kinase
- LMP: Lysosomal Membrane Potential
- M bcr: Major breakpoint cluster region
- m bcr: Minor breakpoint cluster region
- µ bcr: Micron breakpoint cluster region
- MAPK: Mitogen Activated Protein Kinase
- MMR: Mitochondrial Membrane Permeabilization
- MPD: Myeloproliferative disease
- NLS: Nuclear Localization Signal
- PBS: Phosphate buffer saline
- PCR: Polymerase Chain Reaction
- PDGFR: Platelet Derived Growth Factor Receptor
- Ph: Philadelphia chromosome
- PI3K: Phophoinositol 3 Kinase
- PIAS: Protein that Inhibit Activated STAT
- PTPs: Protein Tyrosine Phosphatases
- RARα: Retinoic Acid Receptor Alpha
- ROS: Reactive Oxygen Species
- RTK: Receptor Tyrosine Kinase
- SH: Src Homology
- SOCs: Suppressors of Cytokine Signaling
- SoS: Son of Sevenless protein
- STAMs: Signal Transducing Adaptor Molecules
- STAT: Signal Transducer and Activator of Transcription
- StIP: Stat-Interacting Protein
- Th : T-helper lymphocytes
- TKI: Tyrosine Kinase Inhibitor
- TRAIL: Tumor necrosis factor Related Apoptosis Inducing Ligand
- WHO: World Health Organization

CONTENTS

CHRONIC MYELOID LEUKEMIA

Introduction

Chronic myeloid leukemia (CML) was probably the first form of leukemia to be recognized as a distinct entity. In 1845, two patients were described as having massive splenomegaly associated with leukocytosis, which seemed to be a novel entity not explained by the other causes of splenomegaly, such as tuberculosis, that were already widely accepted in the 1840s (Geary,2000). The first important clue to its pathogenesis came only very much later, when in 1960 even before development of basic G-banding techniques newly developed techniques for studying human cells in mitosis allowed Nowell and Hungerford to detect a consistent chromosomal abnormality, later termed the Philadelphia (Ph [1], or just Ph) chromosome and identified as 22q-,(Nowell and Hungerford, 1960). Banding technology later showed the marker chromosome to be a translocation between the breakpoint cluster region (BCR) on chromosome 22q11.2 and the Abelson proto-oncogene (ABL) on chromosome 9q34. Further advances in cytogenetics have also contributed to the understanding, diagnosis, and treatment of CML. Fluorescent in situ hybridization (FISH) has revealed cryptic translocations in most cases of Ph negative CML (Haigh and Cuthbert, 2004; Zagaria et al., 2004). Additional rare chromosomal variant translocations have been discovered as well (Harris et al., 1999; Vardiman et al., 2002). As the treatment of CML evolved, the cytogenetic monitoring of the disease has become increasingly important, with the discovery that a major cytogenetic response to interferon-alpha therapy leads to improved survival (Kantarjian et al., 1995). Cytogenetic monitoring has also been useful in following the clinical course of seemingly cured patients after hematopoietic cell transplantation (Mark et al., 2006). Finally, understanding the cytogenetic pathophysiology of CML has led to the use of imatinib mesylate (Gleevec; Novartis, Basel, Switzerland) as treatment for this disease with spectacular success (Druker et al., 2001; Kantarjian et al., 2002a;

Kantarjian et al., 2002b; Kantarjian et al., 2002c; Talpaz et al., 2002; Kantarjian et al., 2003; Marin et al., 2003; Deininger et al., 2005). This is one of the first examples of exploiting the known pathophysiology of a malignant disease for the development of treatment targeted specifically for the molecular derangement. Over the 50 years since being identified as the first cytogenetic disease, CML has become the greatest success in translating the basic science of oncology into the treatment of patients with cancer (Kantarjian et al., 2003).

Prior to the introduction of imatinib, CML was a life-threatening disease with a median life expectancy of around six to seven years, the only exception being the minority of patients who could receive a stem cell transplant. In most cases, it is now a chronic condition with good quality of life and it is expected that at least three out of four patients will be alive at ten years and overall survival may be even higher than this (Mark et al., 2006). Prior to imatinib; CML used to progress slowly during the chronic phase of the disease. This phase lasted four to six years on average and then underwent a transformation to a more rapidly progressing State. In about two-thirds of patients this change was to an accelerated phase, which lasted about three to nine months. This then proceeded to a final or blast phase. In some patients the disease proceeded directly from chronic phase to blast phase. The term “advanced phase” is sometimes used to describe both the accelerated phase and blast phase (Leukemia Reseach, 2008).

Staging of chronic myeloid leukemia

The natural history of CML includes three distinct phases. CML typically presents in the chronic phase, which may not be detected for several years if patients have infrequent medical contact. In this phase, cell counts can easily be controlled with hydroxyurea prior to or concurrent with the institution of specific anti-CML therapy. In the imatinib era, these patients have had the best chance of responding to therapy, the best quality of response, and the longest duration of

response (Mark et al., 2006). However, it is an open question at this time whether these patients will ever need treatment of their disease with measures more aggressive than imatinib. The accelerated phase is poorly defined but may include increasing blast counts and/or various combinations of blood or marrow cell count abnormalities, an increasingly difficult to control white blood cell count, and additional cytogenetic lesions beyond a single Philadelphia chromosome. Characteristics of the accelerated phase under the definitions of the World Health Organization (Vardiman et al., 2002) and the Center for International Blood and Marrow Transplant Research (formerly the International Bone Marrow Transplant Registry) (Speck et al., 1984) are presented in Table 1. In the blast phase, also known as blast crisis, the disease clinically resembles acute leukemia and can be of either myeloid or lymphoid phenotype. Blastic-phase CML is characterized by 20% or more blasts in the peripheral blood or bone marrow. When 20% or more blasts are present in the face of fever, malaise, and progressive splenomegaly, the patient has entered blast crisis (Cortes et al, 2006).

Chemotherapy regimens typically used for acute myeloid leukemia with or without the addition of imatinib are required to gain control of the malignant clone at this phase, and only hematopoietic cell transplantation can lead to sustained remission of blastic CML (Radich et al., 2003).

At diagnosis, patients with CML may be entirely asymptomatic, with the disease being discovered on a complete blood count obtained for other reasons. If symptoms are present, they may be referable to hepatosplenomegaly. In addition, patients can present with the classic symptoms of fever, night sweats, and weight loss and more rarely, hyperviscosity syndromes such as retinal hemorrhage, stroke, or priapism (Mark et al., 2006). Physical examination at presentation may be completely normal or may be significant for organomegaly or for signs of anemia or thrombocytopenia. If splenomegaly is detected, spleen size should be noted, because it has prognostic significance (Sokal et al., 1988). Once the diagnosis of CML is suspected, the absence of splenomegaly on

physical exam is important as well and should also be specifically noted (Mark et al., 2006).

Table 1. Criteria for accelerated phase of CML

Reference	**Vardiman et al., 2002**	**Speck et al., 1987**
Organization	WHO[a]	IBMTR[a]
Leukocytosis	Increasing WBC count and splenomegaly unresponsive to therapy	WBC count difficult to control with busulfan or hydroxyurea or WBC doubling in 5 days or less or increasing splenomegaly
Early myeloid cells	Blasts 10% to 19% of peripheral blood white cells or bone marrow cells	Peripheral blood blasts ≥10% or bone marrow blasts ≥10% or blood or marrow blasts + promyelocytes ≥20%
Basophilia or eosinophilia	Peripheral blood basophils at least 20%	Blood eosinophils + basophils ≥20%
Other cytopenias	Persistent thrombocytopenia ($<100 \times 10^9$/l) unrelated to therapy	Anemia or thrombocytopenia unresponsive to busulfan or hydroxyurea
Thrombocytosis	Persistent thrombocytosis ($>1000 \times 10^9$/l) unresponsive to therapy	Persistent thrombocytosis
Cytogenetics	Cytogenetic evidence of clonal evolution	New cytogenetic abnormalities
Pathologic findings	N/A	Chloromas or myelofibrosis
Disease	N/A	Second chronic phase after blast crisis is considered the accelerated phase

Criteria of blast crisis of CML (Vardiman et al., 2002)

Diagnosed if one or more of following is present:

Blasts 20% or more of peripheral blood white cells or bone marrow cells

Extramedullary blast proliferation

Large foci or clusters of blasts in bone marrow biopsy

Biology of chronic myeloid leukemia

It is generally believed that CML develops when a single, pluripotential, 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 (Eaves et al.,1997; Goldman and Melo,2003).

Until the 1980s, there was uncertainty as to whether substantial numbers of normal stem cells were still present in the bone marrow (or elsewhere) in patients with a new diagnosis of CML. However, several findings — the demonstration of the presence of Ph-negative progenitors in myeloid cell cultures,(Coulombel et al.,1983) the observation that Ph-negative progenitor cells can be identified in the blood after high-dose chemotherapy, (Carella et al.,1993) and the ability of interferon alfa to induce Ph-negativity in the marrow — all constitute persuasive circumstantial evidence that the Ph-positive clone displaces normal hematopoiesis but does not destroy residual normal stem cells. The final proof comes from the observation that imatinib mesylate, can induce complete or nearly complete cytogenetic remissions in up to 80 percent of patients (Druker et al., 2001; O'Brien et al., 2003).

Through the contribution of various researchers, the past 25 years have brought considerable knowledge on the molecular and cell biology of CML, creating the essential platform for targeted therapy to be engineered. 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. More recently, attention has also been focused on immunological means of recognizing and destroying the leukemic clone, and these approaches look promising, particularly in the context of eliminating residual disease after various sorts of debulking therapy (Melo et al., 2003).

Signaling in Myeloid progenitors

To understand the defect ongoing in the signal transduction in CML cells, it was important to study the signal transduction in myeloid progenitors.

As shown in figure 1, Interferon-α (IFN-α) induces ICSBP (interferon consensus sequence binding protein) transcription through signal transducer and activator of transcription 1 (STAT1). Increased ICSBP mediates an antileukemic effect through an unknown mechanism. The BCR–ABL kinase represses ICSBP transcription through an unknown mechanism, but also activates multiple signaling pathways, including RAS-MAPK (leading to induction of Bcl-2 gene transcription), STAT5 (leading to Bcl-X gene transcription), PI3K (through a (growth factor receptor-bound protein) 2Grb2–Gab2 interaction) leading to Akt activation, and Src family kinases (Lyn and Hck). The net effect of BCR–ABL activity is to promote Bcl-2 and Bcl-X expression and to inhibit ICSBP transcription. In contrast, 12/15-lipoxygenase may either activate PTEN or inhibit PDK1, both regulators of Akt, leading to increased phosphorylation and cytoplasmic localization of ICSBP, an effect mediated in part through an unknown tyrosine kinase. This may increase survival in myeloid progenitors through relief of ICSBP-mediated inhibition of Bcl-2 and Bcl-X (Van Etten,2007).

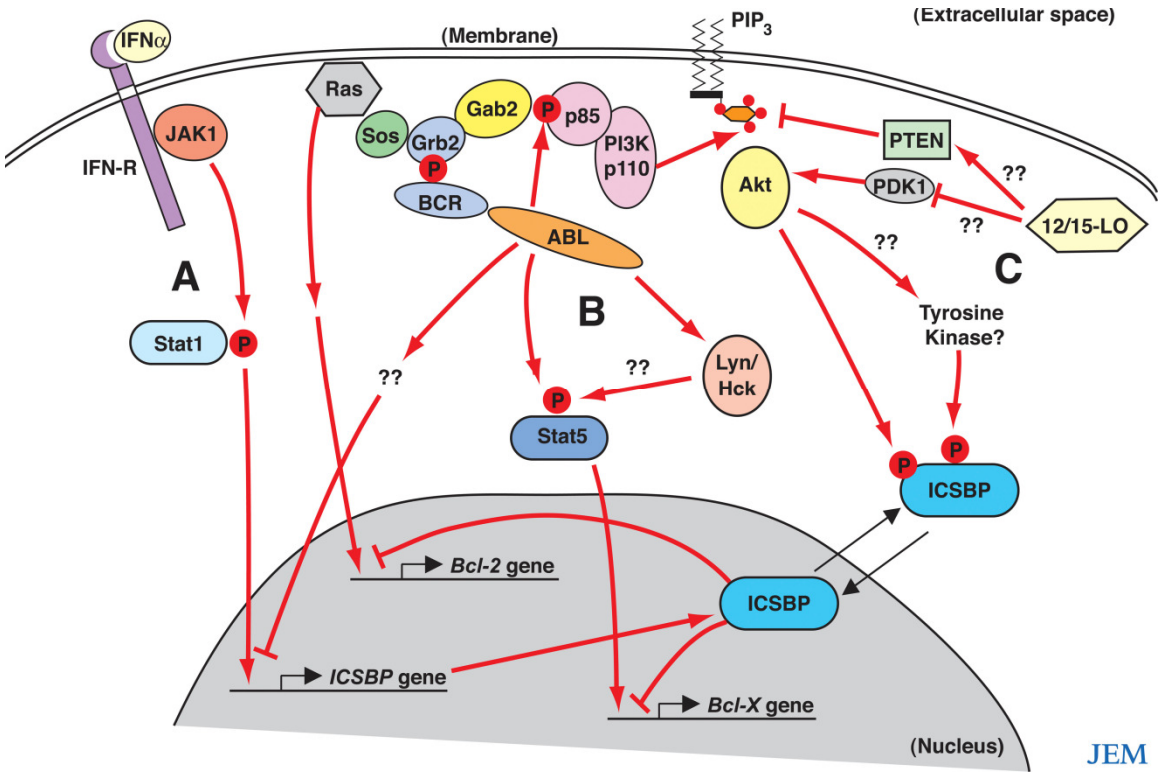

Figure 1: Schematic presentation of signal transduction in myeloid progenitors

(A) IFNα stimulates ICSBP transcription via STAT1 activation

(B) ABL enhances phosphorylation of STAT5 and hence Bcl-X gene expression

(C) 12/15-LO activates PTEN or inhibits PDK1; both regulators of Akt leading to increase phosphorylation of ICSBP. (Van Etten,2007)

BCR-ABL oncoprotien

BCR/ABL is not only diagnostic but also pathogenic in CML (Sawyers, 1999), although the translocation itself may not be sufficient to cause leukemia (Biernaux et al., 1995**;** Bose et al., 1998). Evidence that the inciting event occurs at the level of the stem cell includes the observation that cells of all lymphohematopoietic lineages express the BCR/ABL fusion gene (Fialkow et al., 1977; Bernheim et al.,1981; Fialkow et al., 1981). The ABL proto-oncogene codes for a tyrosine kinase that leads to downstream signal transduction events resulting in cell division and resistance to apoptosis (Melo et al., 2005). BCR is

also considered to be a signaling protein (Mark et al., 2006), and the chimeric BCR/ABL protein is known to be constitutively active (Sawyers, 1999).

In CML, the mRNA molecules transcribed from the hybrid gene usually contain 1 of 2 BCR-ABL junctions, designated e13a2 (formerly b2a2) and e14a2 (b3a2) (Fig.2).Both mRNAs are translated into an oncoprotein of 210 kDa molecular weight. The leukemogenic potential of the $p210^{BCR-ABL}$ resides in the fact that the normally regulated tyrosine kinase activity of the ABL protein is constitutively activated by the juxtaposition of “alien” BCR sequences. BCR acts by promoting dimerization of the oncoprotein such that the 2 adjacent BCR-ABL molecules phosphorylate their respective partners on tyrosine residues in their kinase activation loops. The uncontrolled kinase activity of BCR-ABL then usurps the physiological functions of the normal ABL enzyme by interacting with a variety of effector proteins, the net result of which is deregulated cellular proliferation, decreased adherence of leukemia cells to the bone marrow stroma and reduced apoptotic response to mutagenic stimuli. The enzymatic (tyrosine kinase) activity of the normal ABL protein ($p145^{ABL}$), is kept under tight control, probably by the intramolecular binding of an N-terminal cap region encompassed by the first exon (1b or 1a) and the first part of exon a2 (Fig 3) (Goldman and Melo, 2003). In the BCR-ABL fusion protein ($p210^{BCR-ABL}$), lack of the ABL cap region and a dimerization domain encoded by the first exon of BCR are responsible for constitutive activation of the ABL SH1 domain, resulting in uncontrolled signal transduction and an abnormal cellular phenotype (Deininger et al.,2000).Other variant breakpoints and fusion can give rise to full-length, functionally oncogenic BCR-ABL protiens, notably $p190^{BCR-ABL}$ (associated with e1a2 mRNA junction) and $p230^{BCR-ABL}$ (associated with an e19a2 mRNA junction), but they are rather rare in classic CML (Pane et al.,1997).

The BCR-ABL protein structure and the biochemical pathways in which it is involved have been extensively investigated. Knowledge of the function of several structural domains ‘inherited’ from the parental BCR and ABL proteins allows one to test for certain properties of the fusion product. Thus, the tyrosine