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Phosphorylation of spleen tyrosine kinase at tyrosine 348 (pSyk^{348}) may be a marker of advanced phase of Chronic Myeloid Leukemia (CML)

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ABSTRACT

We investigated Syk as a potential marker of CML progression. We observed a significant over-expression of Syk mRNA and constitutive phosphorylation of Syk Y348 in blast cells from six AP or BP-CML, but not in 15 CML in chronic phase. We could follow *in vivo* the recurrence of pSyk^{348} throughout blast cell escape, despite observing storage of dasatinib in blast cells. A combination of dasatinib and R406 did not improve therapeutic efficacy *in vitro*. Our results strongly suggest that Syk activation could be a relevant biomarker of disease progression and dasatinib resistance but is probably not a molecular target.

1. Introduction

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder of hematopoietic stem cells characterized by the *t*(9, 22)(q34, q11) reciprocal translocation which juxtaposes the ABL gene located on chromosome 9 and the BCR gene on chromosome 22 [1]. The resulting BCR–ABL fusion gene encodes a chimeric oncoprotein that displays constitutively elevated tyrosine kinase activity and drives the pathogenesis of the disease [2]. Without treatment, CML evolves in three distinct phases which are increasingly aggressive: the chronic phase (CP), the accelerated phase (AP) and the blast crisis phase (BP), which is the ultimate disease phase with short survival. At this stage, CML cells are characterized by cessation of cell differentiation, increased proliferation and survival, self-renewal, an inflammatory profile and genetic instability.

Even if the mechanisms leading to CML progression remain unclear, the evolution of disease is probably the consequence of several molecular abnormalities, some independent of the BCR–ABL protein. These profound modifications of cell metabolism could explain, to a certain extent, why therapeutic response to TKI in the advanced phase of CML is not as favorable as during the chronic phase. There is no consensus on an optimal therapeutic strategy for these patients, but it has been clearly established that early treatment of the disease is vital to control its aggressiveness. Similarly, detecting the blast phase as early as possible is relevant for effective treatment management. Currently, apart from the number of blast cells and rising BCR–ABL transcript levels, no marker can be used as an early sign of disease progression or treatment failure. Several groups are working on such molecules and recently, Gioia's team showed that resistance to nilotinib was dependent on Syk expression, which regulates phosphorylation and interaction of Lyn and Axl [3]. However, for treating blast crisis, dasatinib (DAS) appears more attractive, owing to its large spectrum of target molecules.

We have been interested in Syk expression and activation in CML because it is involved in different signaling pathways: (i) it potentially interacts with Src kinases, activated by BCR–ABL, and tyrosine kinase receptors [4,5]; (ii) it is involved in the molecular complexes

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activating actin and the cytoskeleton [6,7] and in integrin signaling pathways [8] regulating cell adhesion, a property that is impaired in CML [9]; (iii) it interacts with the PI3K/Akt pathway [10], activated by BCR–ABL [11]. We thought it interesting to assess the benefit of Syk phosphorylation as a biomarker, and as a potential therapeutic target during advanced phases (AP and BP) of CML.

2. Material and methods

2.1. Reagent

Dasatinib was obtained from Sequoia Research Product (Pangbourne, UK) and R406 from Euromedex (Souffelweyersheim, France). The two compounds were dissolved in sterile dimethyl sulfoxide (DMSO) to obtain a 10 mM stock solution and stored in aliquots at −20 °C.

2.2. Samples

K562, a BCR–ABL positive cell line derived from patients with CML in blast crisis, was purchased from ATCC, and TF1 cells were a gift from Dr. V. Maguer-Satta (CRCL U1052 UMR5286, Lyon, France). K562 cells were cultured in Iscove's modified Dulbecco's medium (Lonza, Verviers, Belgium) and TF1 in RPMI 1640 (Lonza). For the two cell lines, culture media was supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), 1% L-glutamine (Lonza), and 1% ciprofloxacin (Merck). All the cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2.

Blood samples were collected from patients newly diagnosed with CML in chronic phase, and from patients with CML in AP or BP phase. All samples were obtained after informed consent. Peripheral blood samples from healthy adults were obtained from blood remaining after routine analysis. These samples could be used for research because patients had been informed and did not verbally express any disagreement, as stipulated by French law.

2.3. Real-time quantitative polymerase chain reaction

Total RNA was extracted using Nucleospin RNA II kit (Macherey Nagel, Hoerdt, France), according to the manufacturer's instructions. After quantification using a spectrophotometer (Eppendorf 6131), 1 μg of RNA was reverse-transcribed into cDNA with the High Capacity cDNA reverse transcription kit (Applied Biosystem, Saint Aubin, France). The amount of Syk transcript was then quantified by real-time quantitative polymerase chain reaction using the TaqMan Gene Expression Assays Hs00895377_m1 for Syk and Hs00984230_m1 for β2-microglobulin (B2M) (Applied Biosystems). The Syk mRNA level was normalized to the endogenous reference gene (B2M) and results were expressed using the $2^{-\Delta\Delta Ct}$ method; K562 cells were used as the calibrator.

2.4. Flow cytometry

For all experiments, cell analysis was performed using a Coulter Epics Elite™ flow cytometer (Beckman Coulter, Roissy Charles de Gaulle, France) equipped with an Innova 90C-4 UV laser (Coherent, Orsay, France).

We evaluated the expression of Syk and pSyk^{348} in K562 cells, in polymorphonuclear cells from three healthy donors (HD-PMN), and in primitive CML cells from 15 patients in chronic phase (CP) at diagnosis, in the blast cells from four patients in accelerated phase (AP) (patient #16, #17, #18 and #19) and from two patients in blast crisis (BC) (patient #20, #21) (Table 1). Briefly, 1×10^6 cells were fixed using BD Fix and Lyse buffer, and then permeabilized with BD phosflow perm buffer III (Becton Dickinson, Le Pont de Claix, France). The cells were washed twice with stain buffer and incubated for 1 h at room temperature, either with Syk or pSyk^{348} antibody or isotype matched control (Becton Dickinson). After staining, cells were successively washed with stain buffer and PBS before analysis. Results were expressed as the ratio between mean fluorescence intensity (MFI) on the labeled sample and the MFI of the isotype control.

Table 1
Patient characteristics.

Characteristics	CP	AP/BP
Number of patients	15	6
Median age at CML diagnosis, y (range)	59 (36–87)	47 (11–70)
M/F	7/8	1/5
Sokal risk group at diagnosis		
Low	5	–
Intermediate	2	2
High	6	4

CP: chronic phase; AP: accelerated phase; BP: blast phase.

The level of intracellular dasatinib was evaluated by an original flow cytometry method quantifying its fluorescence under UV excitation [12]. The process was standardized using UV fluorescent beads (Flow-Check™ Fluorospheres, Beckman Coulter).

2.5. Statistical analysis

All results from *in vitro* experiments were expressed as means ± SEM. Statistical analysis was performed using Student's *t*-test. *P* less than 0.05 was considered statistically significant.

3. Results

3.1. Syk expression was deregulated in primary cells from patients in advanced phase of CML

We first evaluated Syk expression at the RNA and protein level in polymorphonuclear cells from healthy donors (HD-PMN), CP-CML and AP/BP-CML. As shown in Fig. 1A there was no variation of Syk mRNA level in CP-CML compared to HD-PMN ($P = 0.67$). At the protein level we even observed a 2-fold decrease in CP-CML cells (MFI/isotype control ratio: 2.13 ± 0.23 and 0.84 ± 0.10, respectively; $P < 0.001$) (Fig. 1B). We then studied Syk phosphorylation on tyrosine 348 (pSyk^{348}), which enhances Syk signaling both by increasing the activity of Syk and by generating docking sites for binding of other signaling molecules. We did not observe any expression of pSyk^{348} in HD-PMN or granulocytes from CP-CML ($n = 15$) (Fig. 1C).

In contrast, in blast CML cells, while the Syk expression has only an insignificant trend to be increased at mRNA and protein levels (MFI/isotype control ratio: 3.84 ± 1.30; $P = 0.3$) (Fig. 1A and B), Syk was constitutively phosphorylated in Y348 without any overlapping with the chronic phase CML or healthy donor values ($n = 6$; MFI/isotype control ratio: 2.32 ± 0.36 *vs* 0.61 ± 0.07 and 0.55 ± 0.06 respectively; $P < 0.001$) (Fig. 1C).

To ensure that this phenotype was not dependent on cell immaturity, we analyzed CD34$^+$ from CP-CML clones without being able to detect Syk phosphorylation in CD34$^+$ CP-CML cells ($n = 6$) as shown by the demonstrative example (Fig. 1D). These data suggests that the constitutive activation observed in blast cells was related to disease progression rather than to differentiation arrest. In this hypothesis, we then checked that Syk was expressed and also activated in the K562 cell line, derived from the cells of a female patient in the undifferentiated blast phase of the disease (Supplemental Figs. S1A and B). The analysis of both CP and BP cells from one patient (patient #17) reinforced this hypothesis; we found no pSyk^{348} expression in CP cells, whilst the patient was resistant to imatinib, then to nilotinib and dasatinib (Fig. 1E). Syk activation only appears at the time of disease progression (Fig. 1F).