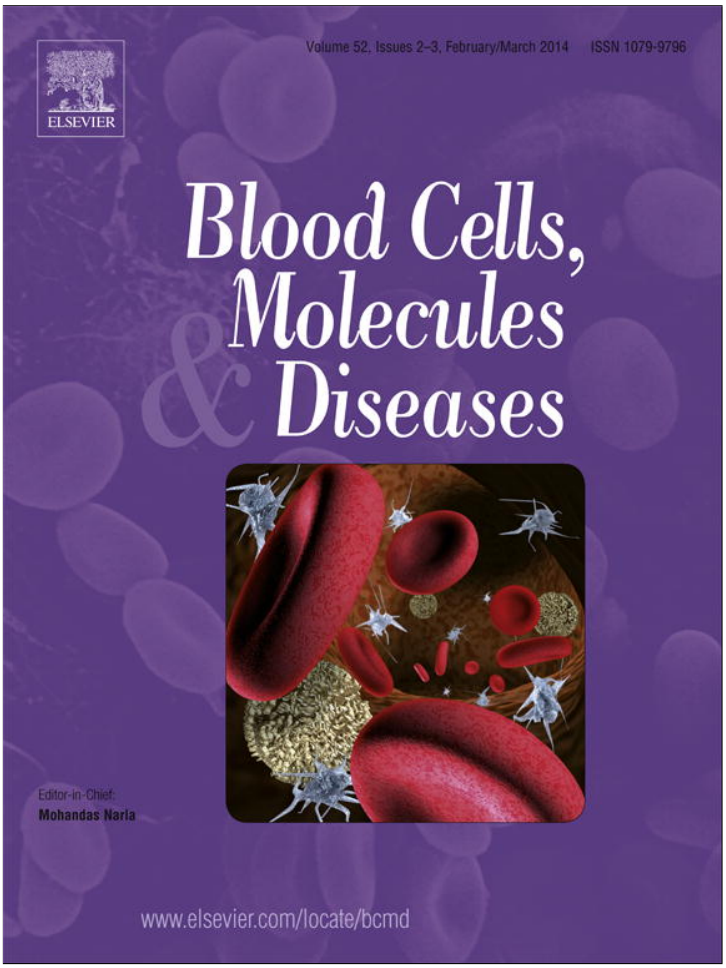

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Expression of *LYN* and *PTEN* genes in chronic myeloid leukemia and their importance in therapeutic strategy

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ABSTRACT

Tyrosine kinase inhibitors (TKIs), imatinib, nilotinib and dasatinib, are the current treatment of chronic myeloid leukemia (CML). *BCR-ABL1* point mutations are the principal cause of resistance to treatment; however other mechanisms could be involved in failure to TKI therapy. LYN is a *src* kinase protein that regulates survival and responsiveness of tumor cells by a *BCR-ABL1* independent mechanism. *PTEN* tumor suppressor gene is downregulated by BCR-ABL1 in CML stem cells and its deletion is associated with acceleration of disease. In this study we evaluated the expression of *LYN*, *PTEN* and the ratio of both genes in 40 healthy donors (HD) and in 139 CML patients; 88 of them resistant to TKI in different phases of disease and 51 in chronic phase classified as optimal responders (OR) to TKI [40 treated with imatinib or nilotinib (OR-IN) and 11 treated with dasatinib (OR-D) therapy]. When we analyzed the gene expression values of *LYN*, an increase was observed only in advanced stages of the disease, however, when we analyzed the ratio between *LYN* and *PTEN* genes, the group of resistant patients in chronic phase in imatinib or nilotinib treatment (CP-IN) also showed a significant increase. Resistant patients treated with dasatinib, a *src* kinase inhibitor, presented a similar ratio to the observed in HD. In addition, the *LYN*/*PTEN* ratio and the *LYN* expression showed a direct significant correlation with *BCR-ABL1* transcript levels in unmutated resistant patients treated with non-*src* kinase inhibitors. We were able to identify 8/35 (23%) of cases in CP-IN and 4/12 (33%) in accelerated phase and blast phase (AP/BC-IN), in which resistance could be associated with an increase in the ratio of the *LYN*/*PTEN*. Our data suggest that the *LYN*/*PTEN* expression ratio may be a sensitive monitor of disease progression in unmutated CML patients under imatinib or nilotinib treatment. This ratio could detect cases when resistance is related to altered *LYN* expression, suggesting that the treatment change to a *src* kinase inhibitor would be most suitable to overcome resistance.

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder, characterized by the presence of the *BCR-ABL1* fusion gene as the essential detectable genetic abnormality. [1] The *BCR-ABL1* fusion encodes a chimeric oncoprotein ($p210^{BCR\text{-}ABL1}$) that displays constitutively elevated tyrosine kinase activity and drives the pathogenesis of the disease [1,2]. Treatment of CML with tyrosine kinase inhibitors (TKIs), imatinib, nilotinib and dasatinib can induce durable responses in the vast majority of patients. However, the emergence of resistant leukemia clones bearing mutations in the *BCR-ABL1* kinase domain (KD) represents a major mechanism of disease recurrence [3]. However, the absence of a *BCR-ABL1* KD mutation does not exclude drug resistance [4], only 30%–50% of resistant cases could be explained by *BCR-ABL1* KD mutation [5,6]. Other mechanisms of resistance can be acquired, including, *BCR-ABL1* gene amplification, transcript overexpression, alterations in drug-efflux kinetics and upregulation of other kinase pathways [7].

BCR-ABL1 expression alters many signaling pathways that increase cell survival and cell cycle progression [8–10], which may promote additional chromosomal alterations and mutations. These changes may lead to acceleration of the disease and play a role in the aggressive nature of late-stage CML. Although many changes have been described in late-stage disease, some evidence suggests that additional tyrosine kinases that function downstream of *BCR-ABL1* such as *LYN* (Lck/Yes-related novel protein tyrosine kinase) and *HCK* (hemopoietic cell kinase) contribute to late-stage disease [11,12].

LYN and HCK are *src* family kinases that are expressed in CML cells and activated by BCR-ABL1 kinase [13,14]. Results of gene knockout

Abbreviations: CML, chronic myeloid leukemia; KD, kinase domain; TKI, tyrosine kinase inhibitors; HD, healthy donors; OR, optimal responders; OR-IN, optimal responders to TKI treated with imatinib or nilotinib; OR-D, optimal responders to TKI treated with dasatinib; CP-IN, chronic phase treated with imatinib or Nilotinib; CP-D, chronic phase treated with dasatinib; AP/BP-IN, accelerated phase/blast phase treated with imatinib or nilotinib; AP/BP-D, accelerated phase/blast phase treated with dasatinib; *C*t, threshold cycle.

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Table 1
Patients' characteristics.

		Resistant patients				
	OR	CP	AP and BP	Mutated	Total resistant	Total patients
Imatinib	35	29	9	2	40	75
Nilotinib	5	6	3	8	17	22
Dasatinib	11	11	10	10	31	42
Total	51	46	22	20	88	139

OR: Optimal responders, CP: chronic phase, AP: accelerated phase, BP: blastic phase.

studies support a role for LYN in BCR-ABL1 kinase mediated transformation and leukemogenesis [11,12].

BCR-ABL1 controls the activation of LYN and HCK kinases in freshly isolated primary cells from CML patients [15]; however, activation of LYN and HCK kinases may also be controlled by other mechanisms. For example, *LYN* kinase is over-expressed in some imatinib-resistant CML cell lines and its activation is independent of BCR-ABL1 kinase [16,17]. It was observed that inhibition of myeloid *src* family kinase activity (Hck, Lyn, and Fyn) induces growth arrest and apoptosis in *BCR-ABL1* transformed cells, suggesting that cell transformation by BCR-ABL1 involves *src* family kinases [18]. Expression of a deregulated LYN kinase may interfere with the TKI inhibition of one or more members of the BCR-ABL1 signaling complex, including CRKL, STAT5 (signal transducer and activator of transcription 5), and MAPK (mitogen-activated protein kinase) [18,19].

In human cancers the tumor progression to more advanced stages is characterized by acquiring additional genetic alterations, and by inactivation of tumor suppressor genes. Phosphatase and tensin homolog (PTEN) is often deleted or inactivated in many tumor types. *PTEN* is downregulated by *BCR-ABL1* in CML stem cells and its deletion causes acceleration of the disease [20–22]. PTEN is a phosphatase that dephosphorylates phosphatidylinositol-3-trisphosphate (PIP3) [23,24], which is a direct product of phosphoinositide 3-kinase (PI3K) activity and plays a critical role in the regulation of cell survival and growth by activating the serine/threonine protein kinase (PDK1) and its downstream target AKT [25,26]. Activated AKT mediates several well-described PI3K responses that include survival, cellular metabolism, angiogenesis, migration and cell growth.

Considering that many mechanisms may be responsible for the lack of response to TKIs, the aim of this study was to evaluate the expression of *LYN*, *PTEN* and the ratio of both genes (*LYN*/*PTEN*) in different phases of the disease in unmutated CML patients.

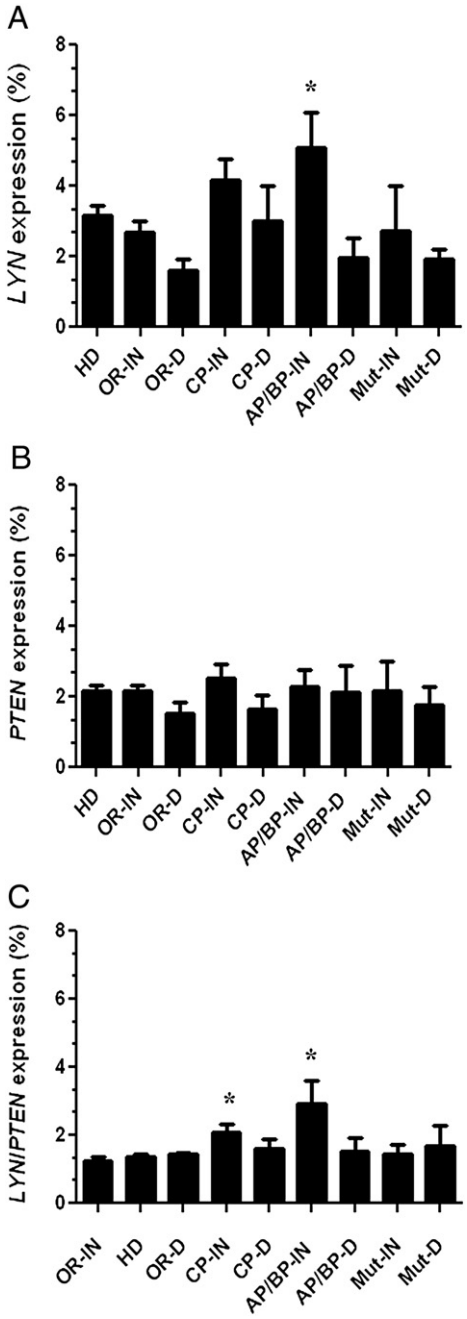

Fig. 1. Percentage of the mean of *LYN*, *PTEN* and *LYN*/*PTEN* expression. A: *LYN* expression was statistically higher in AP/BP-IN and was significantly lower in patients treated with dasatinib. B: The *PTEN* expression was constant in the different groups analyzed independently of treatment. C: *LYN*/*PTEN* ratio showed significant differences in two resistant groups: CP-IN and AP/BP-IN. *: Significant differences (upper) values of expression with respect to HD. #: Significant differences (down) values of expression with respect to HD. HD: Healthy donor, OR-IN: optimal responder to imatinib or nilotinib treatment, OR-D: optimal responder to dasatinib treatment, CP-IN: resistant patients in chronic phase to imatinib or nilotinib treatment, CP-D: resistant patients in chronic phase to dasatinib treatment, AP/BP-IN: resistant patients in accelerated phase/blastic phase to imatinib or nilotinib treatment, AP/BP-D: resistant patients in accelerated phase/blastic phase to dasatinib treatment.

Table 2
The expression of *LYN* gene in CML patients treated with tyrosine kinase inhibitors.

Groups	*LYN* (X ± SE)	*p* value
HD (controls) (n = 40)	3.176 ± 0.2624	
OR-IN (n = 40)	2.693 ± 0.3225	0.2486
Resistant patients in CP-IN (n = 35)	4.174 ± 0.5954	0.1139
Resistant patients in AP/BP-IN (n = 12)	5.075 ± 1.027	0.0119[a]
Resistant mutated patients Mut-IN (n = 10)	2.722 ± 1.300	0.5833
OR-D (n = 11)	1.606 ± 0.3305	0.0048[a]
Resistant patients in CP-D (n = 11)	3.022 ± 1.002	0.8313
Resistant patients in AP/BP-D (n = 10)	1.947 ± 0.5856	0.0460[a]
Resistant mutated patients Mut-IN (n = 10)	1.914 ± 0.3077	0.0260[a]

HD: Healthy donors, OR-IN: optimal responders treated with imatinib or nilotinib, CP-IN: chronic phase treated with imatinib or nilotinib, AP/BP-IN: accelerated phase/blast phase treated with imatinib or nilotinib, Mut-IN: mutated patients treated with imatinib or nilotinib.
OR-D: Optimal responders treated with dasatinib, CP-D: chronic phase treated with dasatinib, AP/BP-D: accelerated phase/blast phase treated with dasatinib, Mut-D: mutated patients treated with dasatinib.
[a] Significant differences with respect to control group.

Methods

Patients' characteristics

The study was carried out according to an institutional review board-approved laboratory protocol. Informed consent was obtained from the subjects prior to their inclusion in the study. Peripheral blood specimens

Table 3
The expression of *PTEN* gene in CML patients treated with tyrosine kinase inhibitors.

Groups	*LYN* (X ± SE)	*p* value
HD (controls) (n = 40)	2.172 ± 0.1683	
OR-IN (n = 40)	2.156 ± 0.1750	0.9452
Resistant patients in CP-IN (n = 35)	2.509 ± 0.4251	0.4600
Resistant patients in AP/BP-IN (n = 12)	2.271 ± 0.4829	0.8069
Resistant mutated patients Mut-IN (n = 10)	2.186 ± 0.8203	0.9792
OR-D (n = 11)	1.545 ± 0.3049	0.0783
Resistant patients in CP-D (n = 11)	1.545 ± 0.3049	0.0783
Resistant patients in AP/BP-D (n = 10)	2.144 ± 0.7613	0.9553
Resistant mutated patients Mut-D (n = 10)	1.781 ± 0.4859	0.3479

HD: Healthy donors, OR-IN: optimal responders treated with imatinib or nilotinib, CP-IN: chronic phase treated with imatinib or nilotinib, AP/BP-IN: accelerated phase/blast phase treated with imatinib or nilotinib, Mut-IN: mutated patients treated with imatinib or nilotinib.
OR-D: optimal responders treated with dasatinib, CP-D: chronic phase treated with dasatinib, AP/BP-D: accelerated phase/blast phase treated with dasatinib, Mut-D: mutated patients treated with dasatinib.
*Significant differences with respect to control group.

were obtained from 139 CML patients with different TKI treatment (20 of them harboring mutations in the tyrosine kinase domain of *BCR-ABL1* and 119 were unmutated patients) and 40 healthy donors (HD) as control group. From 119 unmutated patients, 40 were optimal response (OR) to imatinib or nilotinib (OR-IN) and 11 OR to dasatinib (OR-D) (OR is defined as *BCR-ABL1*/*ABL1* < 0.1%), the remaining 68 cases showed resistance to treatment, 46 in chronic phase (CP) and 22 in accelerated phase/blast phase (AP/BP). The presence of mutations was determined by direct sequencing and a high sensitivity screening and detection method applying high resolution melting and ARMS-qPCR [27]. Table 1 shows patient's characteristics and TKI treatment.

Sample preparation

Total RNA was extracted from total leukocyte cells from peripheral blood by Trizol solubilization (Invitrogen®). The extraction procedure was followed according to the manufacturer's manual; cDNA synthesis was performed using random hexamer primers and M-MLV reverse transcriptase (Promega®). A 201 bp PCR fragment of *LYN* sequences was amplified using primers (Fw: accaaggtggctgtgaaaac, Rv: accttcatcgctcttcagga) and a *PTEN* PCR fragment of 207 bp was amplified using primers (Fw: cagtcagaggcgctatgtgtatta, Rv aacttgtcttcccgtcgtgtg); as a housekeeping gene, a 97 bp β-*actin* fragment was amplified using (Fw: ccagaggcgtacagggatag, Rv: ccaaccgcgagaagatga).

Table 4
LYN/*PTEN* expression ratio in CML patients treated with tyrosine kinase inhibitors.

Groups	*LYN*/*PTEN* ratio (X ± SD)	*p* value
HD (controls) (n = 40)	1.35 ± 0.61	
OR-IN (n = 40)	1.25 ± 0.69	0.4727
Resistant patients in CP-IN (n = 35)	2.01 ± 1.39	0.0031[a]
Resistant patients in AP/BP-IN (n = 12)	2.91 ± 2.34	0.0003[a]
Resistant mutated patients Mut-IN(n = 10)	1.43 ± 0.89	0.2810
OR-D (n = 11)	1.44 ± 0.07	0.6271
Resistant patients in CP-D (n = 11)	1.60 ± 0.97	0.3026
Resistant patients in AP/BP-D (n = 10)	1.53 ± 1.31	0.5512
Resistant mutated patients Mut-D (n = 10)	1.70 ± 1.82	0.8394

HD: Healthy donors, OR-IN: optimal responders treated with imatinib or nilotinib, CP-IN: chronic phase treated with imatinib or nilotinib, AP/BP-IN: accelerated phase/blast phase treated with imatinib or nilotinib, Mut-IN: mutated patients treated with imatinib or nilotinib.
OR-D: Optimal responders treated with dasatinib, CP-D: chronic phase treated with dasatinib, AP/BP-D: accelerated phase/blast phase treated with dasatinib, Mut-D: mutated patients treated with dasatinib.
[a] Significant differences with respect to control group.

LYN and PTEN expression

Real-time PCR of *LYN* and *PTEN* expression was performed using RotorGene (Qiagen), as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles to 95 °C for 15 s and 60 °C for 1 min. The melting curve analysis and gel electrophoresis experiments were used to ensure that only one product of the expected size was amplified (Mezcla real—Biodynamic, Argentine). Non template controls were included in each run. Expression values were obtained from the threshold cycle (*Ct* value) using serial dilutions of K562 cell line as standard curve.

The expression of *LYN* and *PTEN* was normalized by subtracting the *Ct* value of the housekeeping gene (β-*actin*) from the *Ct* value of the target gene (ΔCt). The fold increase, relative to the control, was obtained by using the formula $2^{-\Delta Ct}$, and then expressed as a percentage. All samples were measured in duplicate. The ratio values were obtained as follows: *LYN* expression/*PTEN* expression.

BCR-ABL1 transcript quantification

RT-qPCR assay was performed on total RNA extracted from peripheral blood using the RotorGene (Qiagen). The *BCR-ABL1*/*ABL1* ratio was determined using the *BCR-ABL1* RT-qPCR kit (Molecular MD), according to the manufacturer's instructions. The methodology that we have employed to quantify *BCR-ABL1* expression was cross-validated with the Institute of Medical and Veterinary Science (Adelaide, South Australia) [28].

Statistical analysis

Differences in expression levels were analyzed using the one-way analysis of variance (ANOVA) and an unpaired *t* test to compare differences between groups. The statistical analyses were performed using GraphPad Prism 5.0. In order to determine a value allowing the identification of cases with increased *LYN*/*PTEN* expression ratio with respect to the HD control, a cutoff from the HD group was calculated as follows: mean + 3SD: 1.35 + 3 × 0.61 = 3.18.

Spearman test was used to determine the correlation between *LYN*/*PTEN* expression ratio and *BCR-ABL1* transcripts.

Results

The *LYN* expression levels were compared among the different groups of patients analyzed in order to evaluate its role in disease progression and resistance to treatment. The gene expression values of *LYN* in different groups showed significant increase only in the accelerated phase/blast phase group treated with imatinib or nilotinib (AP/BP-IN) (Table 2, Fig. 1A). There were no significant differences between HD and OR-IN patients; these results suggest that patients with good response to TKI treatment present similar results to the HD group. As expected in patients treated with dasatinib (*src* kinase inhibitor) the *LYN* expression values were lower than the control group in three patient groups (Table 2). The *PTEN* expression was similar in all patient groups ($p = 0.4275$) (Table 3, Fig. 1B). When we analyzed both genes as a ratio (*LYN*/*PTEN*) significant differences were observed in resistant unmutated patients in AP/BP-IN and CP-IN groups, suggesting that the ratio between both genes might be more informative for the CP-IN group than each separately (Table 4, Fig. 1C). Resistant patients with mutations in the KD showed no increased in *LYN*, *PTEN* and *LYN*/*PTEN* ratio with respect to HD group, independently of the treatment received, indicating that in mutated patients the expression of these genes likely would not be involved in a resistance mechanism (Tables 2, 3, 4). In order to estimate the utility of the expression of *LYN* and the *LYN*/*PTEN* ratio in the progression of the disease a correlation with *BCR-ABL1*/*ABL1* transcripts was performed. In this analysis we include only those unmutated cases on imatinib or nilotinib treatment. Cases receiving dasatinib treatment were excluded because this dual inhibitor suppresses *LYN* expression. The *LYN* expression showed a direct significant correlation

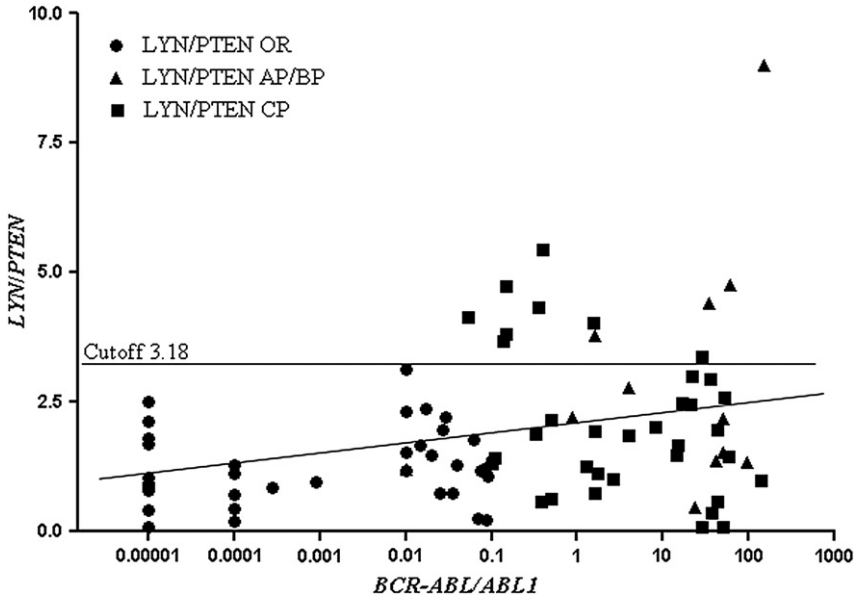

Fig. 2. Correlation between *LYN*/*PTEN* ratio and *BCR-ABL1* transcripts in CML patients treated with non-*src* kinase inhibitors. Spearman correlation test between the *LYN*/*PTEN* ratio and *BCR-ABL1* transcripts ($p < 0.0036$); inclined line shows the positive Spearman correlation ($r = 0.31$). Horizontal line indicates the *LYN*/*PTEN* cutoff obtained from healthy donors. Chronic myeloid leukemia unmutated patients are classified at different stage of the disease. OR: Optimal responder to imatinib or nilotinib treatment, CP: resistant patients in chronic phase to imatinib or nilotinib treatment, AP/BP: resistant patients in accelerated phase/blastic phase to imatinib or nilotinib treatment.

with level of *BCR-ABL1*/*ABL1* transcripts (Spearman $p < 0.0066$) (data not shown). Similarly, the correlation between levels of *BCR-ABL1*/*ABL1* transcripts and *LYN*/*PTEN* ratio was done (Spearman $p < 0.0036$) (Fig. 2). Taking into account the cutoff value in *LYN*/*PTEN* ratio obtained from HD ($X + 3SD \geq 3.18$), we identified 23% of cases in CP (8/35) and 33% in AP/BP (4/12) with high expression *LYN*/*PTEN* ratio among resistant unmutated patients receiving non-*src* kinase inhibitors (Fig. 3). In these cases the resistance to treatment could be associated with the increased *LYN*/*PTEN* ratio.

Discussion

The lack or loss of response to TKI in CML is related to *BCR-ABL1*-dependent or independent mechanisms of drug resistance. Although mutations of BCR-ABL1 tyrosine kinase domain are the predominant cause of resistance, evidence suggests that additional tyrosine kinases that function downstream of *BCR-ABL1* are activated in leukemic blasts contributing to late-stage disease [11,29]. CML patients in disease progression show increased expression of *LYN*, a regulatory element involved in signal transduction in hematopoiesis. Any deregulation may interfere with normal cellular growth and function, resulting in different hematological phenotypes.

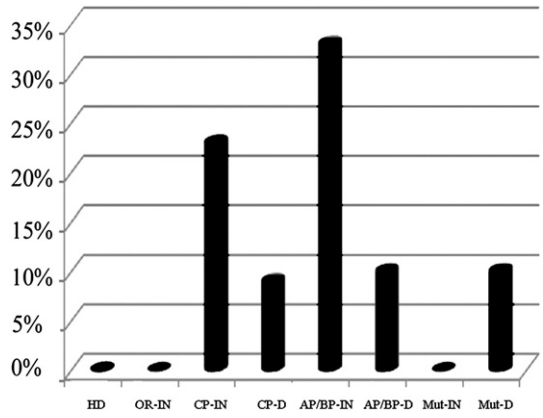

Fig. 3. Frequency of cases with *LYN*/*PTEN* expression ratio ≥ 3.18. Percentage calculated taking into account the cutoff value obtained from HD ($X + 3SD = 3.18$). High expression levels of the *LYN*/*PTEN* ratio among resistant unmutated patients receiving non-*src* kinase inhibitors, was 23% in CP and 33% in AP/BC cases. In the remaining groups, *LYN*/*PTEN* expression ratios were less than 10%. HD: Healthy donors, OR: optimal responders, CP: chronic phase, AP: accelerated phase, BP: blast phase, I: imatinib, N: nilotinib, D: dasatinib, Mut-IN: mutated patients in imatinib or nilotinib treatment, Mut-D: mutated patients in dasatinib treatment.

PTEN is often deleted or inactivated in many human cancers; overexpression causes cell cycle arrest and increased apoptosis of leukemia cells. Moreover, down-regulation of *PTEN* is dependent upon *BCR-ABL1* kinase activity [20]. In this study, we evaluated the expression of the *LYN*, *PTEN* and the ratio of both genes in CML patients in different phases of the disease, with and without response to TKI. It is known that low levels of *PTEN* may cause resistance to treatment in CML patients; however, this gene in the studied patients did not show much change in the expression levels.

Increased expression of *LYN* was observed in AP/BP-IN and CP-IN groups, but was statistically significant only in the first group.

The *LYN*/*PTEN* ratio showed a similar expression level between HD and OR indicating that in these cases with good response to treatment the oncoprotein $p210^{BCR\text{-}ABL1}$ is efficiently blocked. On the other hand, a significant increase in *LYN*/*PTEN* ratio, with respect to HD was observed in CP-IN or AP/BP-IN resistant unmutated cases treated with non-*scr* kinase inhibitors (imatinib or nilotinib). Therefore; the *LYN*/*PTEN* ratio allowed us a broader view and would indicate that measurement of this ratio is more informative than the evaluation of *LYN* and *PTEN* gene expression separately.

These results suggest that *LYN* overexpression or *PTEN* subexpression might be associated with resistance to imatinib or nilotinib treatment. These finding are in agreement with previous reports where persistent *LYN* signaling was observed in imatinib-resitance CML cases with mutation-negative *BCR-ABL1* [30] and downregulation of *PTEN* by *BCR-ABL1* [31].

In this study none of the resistant cases treated with dasatinib in different phases of disease, showed a difference in the *LYN*/*PTEN* ratio versus control group. Since *LYN* is a *src* kinase, treatment with dasatinib may inhibit its expression, which shows that the mechanism of resistance in these patients would not be related to altered expression of *LYN* and *PTEN* genes.

Cases with mutations in the *BCR-ABL1* kinase domain showed low levels of the *LYN*/*PTEN* ratio, similar to the HD group, regardless of treatment indicating that the resistance mechanism is dependent on *BCR-ABL1* mutations and independent of *LYN* and *PTEN*.

Taking into account that resistance cannot always be explained by *BCR-ABL1* dependent mechanisms, it is important to note that we were able to associate the resistance due to increased *LYN*/*PTEN* ratios in 23% of CP-IN and 33% of AP/BP-IN unmutated cases.

The underlying mechanisms of resistance and clonal expansion are not fully understood; only a limited percentage of resistance patients express detectable *BCR-ABL1* point mutations suggesting that other resistance mechanisms could be involved.

The *LYN*/*PTEN* ratio showed a direct significant correlation with the level of *BCR-ABL1* transcripts, indicating the higher *LYN*/*PTEN* ratio was related to disease progression. We considered that the *LYN*/*PTEN* ratio could be an important tool in unmutated cases under non-*src* kinase treatment. This ratio could detect cases where resistance is related to altered *LYN* and *PTEN* expression, suggesting that a change to dual inhibitor treatment would be most suitable to overcome resistance.

Conflict of interest

The authors declare that they have no conflict of interest.

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