Leukemia Research 36 (2012) 509–513

Contents lists available at SciVerse ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

The study of resistant mechanisms and reversal in an imatinib resistant Ph+ acute lymphoblastic leukemia cell line

Hongyun Xing[a,b], Xi Yang[a], Ting Liu[a], Juan Lin[a], Xiaoyi Chen[a], Yuping Gong[a,*]

[a] Department of Hematology, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital of Sichuan University, Chengdu, China
[b] Department of Hematology, the Affiliated Hospital of Luzhou Medical College, Luzhou, China

ARTICLE INFO

Article history:
Received 15 June 2011
Received in revised form
16 November 2011
Accepted 22 December 2011
Available online 29 January 2012

Keywords:
Imatinib resistance
Ph+ acute lymphoblastic leukemia
Tyrosine kinase inhibitor
mTOR signaling pathway
Proteasome inhibitor

ABSTRACT

In this study, we established an imatinib resistant Ph+ acute lymphoblastic leukemia (ALL) cell line SUP-B15/RI in vitro and studied the mechanism of imatinib resistance. Our results showed that the BCR-ABL1 fusion gene and the mdr1 gene were 6.1 times and 1.7 times, respectively, as high as that of parental SUP-B15 cell line. We found no mutation in the Abl kinase domain of SUP-B15/RI. Furthermore, the detection of cell signaling pathway of PI3K/AKT/mTOR, RAS/RAF, NF-κB, JNK and STAT showed the up-regulation of phosphorylation of AKT, mTOR, P70S6K, and RAF, ERK, and MEK, down-regulation of PTEN and 4EBP-1, and no change in other cell signaling pathways in SUP-B15/RI. However, dasatinib and nilotinib showed partial resistance. Interestingly, bortezomib had no resistance. Imatinib combination with rapamycin had synergistic effect on overcoming the resistance. Altogether, over-expression of BCR-ABL1 and mdr1 gene were involved in the resistance mechanisms, and up-regulation of the cell signaling pathways of PI3K/AKT/mTOR, RAS/RAF in SUP-B15/RI cell line may be correlated with them. The SUP-B15/RI cell line was also resistant to the second generation tyrosine kinase, dasatinib, and nilotinib, not bortezomib. The combination of imatinib with rapamycin can partially overcome the resistance and blockade of the ubiquitin–proteasome can be also a promising pathway to overcome imatinib resistance.

1. Introduction

The translocation t(9;22)(q34;q11), known as the Philadelphia (Ph) chromosome, and formed fusion genetic BCR-ABL1, is the most common cytogenetic abnormality and the most unfavorable prognosis in adult acute lymphoblastic leukemia (ALL) [1]. The most exciting breakthrough in the treatment of Ph+ leukemia has been the development of imatinib, a tyrosine kinase inhibitor [2]. Although imatinib produced high rates of clinical and cytogenetic responses in the chronic phase of CML and achieved complete response in approximately 95% of newly diagnosed Ph+ ALL, the onset of resistance and clinical relapse in the advanced phases of CML and Ph+ ALL is rapid [3–5].

The main mechanisms of resistance to imatinib involve BCR-ABL1 dependent mechanisms, such as amplification or mutations in the ABL1 portion of the BCR-ABL1 fusion gene [6,7], and BCR-ABL1 independent mechanisms of resistance, such as up-regulation of P-glycoprotein (P-gp), down regulation of drug influx transporters [8,9], and alternative signaling pathway activation Ras/Raf/Mek, PI3K/mTOR, JAK/STAT, which may contribute to the acquisition of resistance to imatinib [10–13]. Therefore, a strategy for overcoming imatinib resistance in Ph+ leukemia is an important requirement.

Although the mechanism of imatinib resistance in CML has been intensively investigated, fewer studies have examined imatinib resistance in Ph+ ALL [14]. Herein, we investigated the mechanism and reversal of imatinib induced resistance in Ph+ ALL cell line.

2. Materials and methods

2.1. Cell lines and reagents

We obtained Ph+ SUP-B15 ALL cell line from the American Type Culture Collection (ATCC, Manassas, USA) and maintained in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) (Thermo Scientific Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen/Gibco, USA). Imatinib resistant cell line SUP-B15/RI was generated by culture with gradually increasing imatinib concentrations in our lab. Generally, exposure of imatinib to sensitive SUP-B15 ALL cell line started with 0.2 μM and increased every 7 days by 0.2 μM, but only in case of greater than 70% viability in culture, as assessed with the trypan blue exclusion method. The imatinib concentration remained unchanged if the viability was between 30% and 70% and IM was withdrawn in case of viability of 30% or less, which was referred to as rescue. Rescue periods depended on recovery times. Imatinib was added to 50% of the last achieved imatinib level with 90% viability in the culture. Imatinib resistant cell line SUP-B15/RI was collected and checked when imatinib concentration rose up to 6 μM, as described below.

Imatinib and nilotinib were purchased from Novartis Pharma (Basel, Switzerland) and were prepared in dimethylsulfoxide (DMSO) and stored as a 10 mM solution at −20 °C. Dasatinib was purchased from Bristol-Myers Squibb (New York,

* Corresponding author at: Department of Hematology, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital of Sichuan University, 37# Guo Xue Xiang, Chengdu 610041, China. Tel.: +86 018980601257; fax: +86 2885423921.
E-mail address: xinghy52003@yahoo.com.cn (Y. Gong).

0145-2126/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2011.12.018

Table 1
The primers, reaction condition and PCR products of *bcr-abl*, *mdr1*, *hoct1* gene.

Gene	Primer (5′ → 3′)	Annealing temperature (°C)	PCR products
bcr-abl(P190)	CTGGCCCAACGATGGCGA CACTCAGACCCTGAGGCTCAA	58	92bp
mdr1	CCCATCATTGCAATAGCAGG GTTCAAACTTCTGCTCCTGA	55	157bp
hoct1	GGGCAGCCTGCCTCGTCATG ACACCATCACTCCGAGGTTC	57	165bp
β-Actin	CCAAGGCCAACCGCGAGAAGATGAC AGGGTACATGGTGGTGCCGCCAGAC		587bp

USA) and prepared and stored under the same condition. Rapamycin was purchased from Sigma (Saint Louis, MO, USA). Bortezomib was purchased from Millenium Pharmaceuticals Inc (Cambridge, MA, USA) and was dissolved in phosphate-buffered saline (PBS) as a 2 mM stock solution. The stock solutions were diluted to the required concentrations with serum-free culture medium before use.

2.2. Proliferation assay

Cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid (MTT) colorimetric reduction method, as described by the manufacturer (Sigma Chemical). Measures were taken as quadruplicates after 72 h of culture without the presence as well as in the presence of inhibitor at indicated concentrations. Absorbance at 570 nm was measured in an OptiMax microplate reader (Microplate Spectrophotometer type 1500, Thermo Scientific, USA).

2.3. RT-PCR amplification of BCR-ABL1, mdr1, hoct1 gene

BCR-ABL1, mdr1 and hoct1 mRNA were amplified using reverse transcription polymerase chain reaction (RT-PCR) amplification. The primers of each gene and reaction condition were listed in Table 1.Mutational analysis of ABL kinase domain by direct sequencing

Heminested PCR was performed essentially as described by Pfeifer et al. [15] using the following primers: Step 1, BCR-C (BCR exon 1; ACCGCATGTTCCGGGACAAAAG) plus A7− (ABL1 exon 7, AGACGTCGGACTTGATGGAGAACT); Step 2, AN4+ (Abl exon 4, TGGTTCATCATCATTCAACGGTGG) plus A7−. A 15 μL aliquot of the PCR product (675 bp) encoding the BCR-ABL1 ATP binding pocket and the activation loop was purified (QIAquick PCR Purification kit; Qiagen, Hilden, Germany), and sent to a commercial laboratory (Invitrogen Biotech, USA) for direct sequencing. Sequences were compared with the unmutated sequence using Jellyfish Alignment (Genebank ABL, accession no. X16416.1). For each fragment, sequence analysis was performed on both strands.

2.5. Immunoblotting

Protein lysates were prepared from the cells (1×10^7 cells), resuspended in loading buffer, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8%, 10%, and 15% acrylamide gel, and electrophoretically transferred onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were probed into using standard techniques with the primary antibodies and then with the secondary antibodies. The enhanced chemiluminescence (ECL) detection kit and Hyperfilm ECL (Millipore, Billerica, MA) were used to visualize the presence of proteins. Phospho-mTOR (Ser2448) Antibody, Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb, Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb, phospho-PTEN, phospho-Akt, phospho-ERK1/2, phospho-MEK1/2, phospho-RAF, phospho-NF-κB p65 (E498), IKKα, IKKβ phospho-STAT3,5, phospho-JNK (Cell Signaling Technology, Danvers, MA, USA) and actin (A5316) (Sigma, Saint Louis, MO, USA) were used as the primary antibodies (all from Cell Signaling Technology, Berverly, MA). Goat anti-rabbit IgG HRP conjugated antibody (Santa Cruz Biotechnology, Inc, USA) was used as the secondary antibody.

2.6. Statistical analysis

The cell proliferation assay between two groups was analyzed with two-sided unpaired Student's *t* tests using SPSS 16.0. The results were considered statistically significant at $p < 0.05$. The coefficient of drug interaction (CDI), calculated as CDI = AB/(A × B), was used to analyze effects of drug combinations. According to the absorbance of each group, AB is the ratio of the combination groups to control group; A or B is the ratio of the single agent group to control group. Thus, CDI value <1, =1, or >1 indicates that the drugs are synergistic, additive, or antagonistic, respectively. CDI less than 0.7 indicates that the drugs are significantly synergistic. The results of RT-PCR agarose gel electrophoresis and western blots electrophoresis were analyzed with quantity one software for quantitative analysis.

3. Results

3.1. Establishment of the Ph+ ALL imatinib-resistant cell line SU-B15/RI

In order to obtain the imatinib-resistance, the SUP-B15 cell line was cultured initially with a low dose of imatinib (0.2 μM) and then with gradually increasing imatinib concentrations. After approximately 6 months, the cell line continued to proliferate even in the presence of 6 μM of imatinib. The resistant cell line, which has acquired significant resistance to imatinib, was generated and named as SUP-B15/RI cell line. Cell proliferation was assessed by the MTT assay. IC_{50} and resistance-fold (RF) was calculated. The IC_{50} of SUP-B15/RI to imatinib was 22.37 ± 1.16 μM, which was 20 times higher than that of the parental sensitive cell line, with IC_{50} to imatinib being only 1.09 ± 0.14 μM. The IC_{50} of SUP-B15/RI retained the same level after withdrawing imatinib from culture medium after one month.

3.2. The expressions of BCR-ABL1, mdr1, hoct1 mRNA in the SUP-B15/RI cell line

We investigated the level of BCR-ABL1 and mdr1 gene mRNA expression by RT-PCR. The analysis of grey strip indicated marked, statistically significant 6-fold amplification of the BCR-ABL1 gene mRNA in the SUP-B15/RI cell. The mdr1 gene on the other hand increased approximately 1.7-fold. No up-regulation of the hoct1 mRNA expression was observed in SUP-B15/RI cell line.

3.3. Analysis of point mutation in the ABL1 kinase domain in the SUP-B15/RI cell line

We performed mutation analysis on the ABL1 kinase domain by sequencing the heminested PCR products obtained from the two cell lines. The results confirmed no mutation in the ABL1 kinase domain of the SUP-B15/RI cell line.

3.4. Up-regulation of the PTEN/PI3K/AKT/mTOR and RAF/ERK/MEK signaling pathway in the SUP-B15/RI cell line

The expression of the PTEN/PI3K/AKT/mTOR and RAF/ERK/MEK signaling pathways were investigated using western blot analysis in SUP-B15 and SUP-B15/RI under common culture conditions. The results showed up-regulation of phosphorylation of AKT, mTOR, and P70S6K and down-regulation of phosphorylation of PTEN, 4EBP1 in SUP-B15/RI cell line (see Fig. 1). Likewise, phosphorylation of RAF, ERK, and MEK was also up-regulated in SUP-B15/RI cell line (Fig. 1). Therefore, these changes indicated that the activated two signaling pathways contributed to imatinib resistance. NF-κB, STAT3, STAT5, and JNK signaling pathways were also examined. We confirmed there were no different expression of the phosphorylated STAT3, STST5, JNK, and the molecules downstream of the NF-κB pathway protein between the two cell lines (data was not