

RESEARCH ARTICLE

Silencing of Suppressor of Cytokine Signaling-3 due to Methylation Results in Phosphorylation of STAT3 in Imatinib Resistant BCR-ABL Positive Chronic Myeloid Leukemia Cells

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Abstract

Background: Silencing due to methylation of suppressor of cytokine signaling-3 (SOCS-3), a negative regulator gene for the JAK/STAT signaling pathway has been reported to play important roles in leukemogenesis. Imatinib mesylate is a tyrosine kinase inhibitor that specifically targets the BCR-ABL protein and induces hematological remission in patients with chronic myeloid leukemia (CML). Unfortunately, the majority of CML patients treated with imatinib develop resistance under prolonged therapy. We here investigated the methylation profile of SOCS-3 gene and its downstream effects in a BCR-ABL positive CML cells resistant to imatinib. **Materials and Methods:** BCR-ABL positive CML cells resistant to imatinib (K562-R) were developed by overexposure of K562 cell lines to the drug. Cytotoxicity was determined by MTS assays and IC₅₀ values calculated. Apoptosis assays were performed using annexin V-FITC binding assays and analyzed by flow cytometry. Methylation profiles were investigated using methylation specific PCR and sequencing analysis of SOCS-1 and SOCS-3 genes. Gene expression was assessed by quantitative real-time PCR, and protein expression and phosphorylation of STAT1, 2 and 3 were examined by Western blotting. **Results:** The IC₅₀ for imatinib on K562 was 362nM compared to 3,952nM for K562-R (p=0.001). Percentage of apoptotic cells in K562 increased upto 50% by increasing the concentration of imatinib, in contrast to only 20% in K562-R (p<0.001). A change from non-methylation of the SOCS-3 gene in K562 to complete methylation in K562-R was observed. Gene expression revealed down-regulation of both SOCS-1 and SOCS-3 genes in resistant cells. STAT3 was phosphorylated in K562-R but not K562. **Conclusions:** Development of cells resistant to imatinib is feasible by overexposure of the drug to the cells. Activation of STAT3 protein leads to uncontrolled cell proliferation in imatinib resistant BCR-ABL due to DNA methylation of the SOCS-3 gene. Thus SOCS-3 provides a suitable candidate for mechanisms underlying the development of imatinib resistant in CML patients.

Keywords: Methylation - SOCS-3 - STAT3 - imatinib - BCR-ABL - K562

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Introduction

The reciprocal translocation t(9;22) in chronic myeloid leukemia (CML) generates a *BCR-ABL* fusion gene that results in the expression of a leukemia-specific oncoprotein, BCR-ABL. BCR-ABL is a constitutive active tyrosine kinase that presents in more than 95% patients (Sawyers, 1999; Shah et al., 2002) and results in the underlying process of pathogenesis of CML (Shah et al., 2002; Binato et al., 2009; Smith et al., 2012). Imatinib, a tyrosine kinase inhibitor is used as the treatment of CML specifically targets BCR-ABL protein and induces hematological remission in these patients (Melo et al., 2007). However, majority of CML patients treated with imatinib would develop resistance under-prolonged therapy (Druker et al., 2006; A et al., 2010; Esposito et al., 2011). Point mutation in the kinase domain of BCR-

ABL is the main mechanisms underlying the development of imatinib resistance in CML patients with progressing disease (Shah et al., 2002; Branford et al., 2003). Moreover, expression profiling and in vitro studies predict the involvement of additional genes in imatinib resistance and disease progression (Radich et al., 2006). However, most of those genes have not been thoroughly investigated or described in clinical specimens from CML patients.

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is one of the most important signaling cascades that regulates various cell biological activities including immune response, cell growth and differentiation (Niwa et al., 2005). Abnormalities in the regulation of JAK/STAT pathway were reported in cancers including hematological malignancies (Ihle, 1995; Croker et al., 2008; Jatiani et al., 2010; Furqan et al., 2013). STAT3 and STAT5 are

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constitutively activated in many myeloid tumors (Bar-Natan et al., 2012). STAT3 involved in the development and progression of colorectal cancer and a candidate gene of interest in the colorectal cancer therapy (Gruber et al., 2012).

Constitutive activation of STAT3 confers resistance to apoptosis in multiple myeloma tumor cells (Catlett-Falcone et al., 1999). The JAK/STAT signaling pathway, is subject to negative regulation by three protein families; the SH2-containing phosphatases (SHP), the suppressors of cytokine signaling (SOCS) and the protein inhibitors of activated STATs (PIAS) (Yoshikawa et al., 2001; Roman-Gomez et al., 2004; Qiu et al., 2012; Furqan et al., 2013). Aberrant DNA methylation of promoter CpG dinucleotides is associated with the silencing of many proteins in human malignancies, including the negative regulators, *SOCS-1* and *SOCS-3*. *SOCS-1* binds to the conserved regulatory tyrosine in the activation loop of the JAK2 kinase domain through its SH2 domain and inhibits JAK kinase activity (Rottapel et al., 2002), while *SOCS-3* bind to phosphorylated tyrosine residues on activated cytokine receptors (Kubo et al., 2003). Reduced *SOCS-1* levels, resulting from aberrant methylation, were initially reported in hepatocellular carcinoma (Yoshikawa et al., 2001). Recent study suggested *SOCS-1* methylation as a predictive marker for hepatocellular carcinoma patients (Saelee et al., 2012). The miR-19a is one microRNA that function as an oncogene and enhances gastric cancer progression by targeting *SOCS-1* tumor suppressor gene (Qin et al., 2013) and the activation of the JAK/STAT signalling due to *SOCS-1* methylation has been documented in chronic myeloid leukaemia (Liu et al., 2003). Frequently methylated *SOCS-3* with transcriptional silencing has been reported in myeloproliferative neoplasia (MPN) and acute myeloid leukemia post-MPNs (Capello et al., 2008).

We hypothesized that DNA methylation of *SOCS-1* and *SOCS-3* would lead to uncontrolled activation of JAK/STAT signaling by phosphorylation of STAT3 causing the resistance to the imatinib. Thus we developed a resistant cell line (designated as K562-R) by long-term coculture of the human leukemia cell line, K562 (CML, BCR/ABL positive), with imatinib mesylate. We investigated the DNA methylation profiles of *SOCS-1* and *SOCS-3* genes, their expression analysis and effects on JAK/STAT pathway in both parental and resistant cells to reveal the relationship between DNA methylation and the mechanism of resistance.

Materials and Methods

Imatinib mesylate

Imatinib mesylate was purchased from LC Laboratories (Woburn, MA, USA) and dissolved in distilled H₂O before use. The stock solutions were stored at 1 mM.

Cell lines and development of resistant clones

K562, a CML cell line, obtained from the Department of Hematology, Universiti Sains Malaysia (USM) originally purchased from American Type Culture Collection (ATCC). K562 carries the Philadelphia

chromosome with a *BCR-ABL* fusion gene. *BCR-ABL* was confirmed by multiplex PCR. K562 cells were cultured with RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY, USA) at a density of 5×10^4 cells/mL in a humid incubator with 5% CO₂ at 37°C. Resistant cell line to imatinib was developed according to the protocol described by Coley (2004). Log phase growing K562 cells were cocultured at a starting dose of 50 nM imatinib followed by a step-wise increasing concentration of 20-30 nM for 9 months or until the cells still survive at the IC₅₀ of imatinib on parental K562 (300 nM). The resistant lines were grown in normal medium without imatinib for at least 48 hours before start of each experiment.

Cell proliferation assay

Leukemic cells were seeded in 96-well culture plates at a density of 2×10^4 viable cells/100 μ L/well in triplicates and were treated with imatinib. Colorimetric CellTiter 96 Aqueous One Solution Cell Proliferation assay (MTS assay; Promega, Madison, WI, USA) was used to determine the cytotoxicity. The IC₅₀ values were calculated using GraphPad Prism 3.02 (San Diego, California, USA). Each experiment was in triplicate.

Apoptosis assay

Annexin V-FITC binding assay (BD Pharmingen, San Diego, CA, USA) was used as recommended by the manufacturer and analyzed by flow cytometry (BD FACSCanto™, San Jose, California, USA). Analysis was performed with Diva software (FACS Diva, 6.1.2, San Jose, California, USA). Each experiment was in triplicate.

Methylation specific polymerase chain reactions (MS-PCR)

DNA was extracted from resistant and parental cell lines using the DNA extraction kit NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The concentration and purity of DNA were measured by NanoDrop ND-1000 spectrophotometer V3.3.0 (NanoDrop Technologies, Berlin, Germany). One microgram DNA was treated with bisulphite using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, NY, USA) according to the manufacturer's instructions. MS-PCR was performed as described (Herman et al., 1996) and modified DNA was subjected to two separate PCRs. MS-PCR primers were designed to amplify the methylated (M) or unmethylated (U) alleles. *SOCS-1* (GenBank: NM_003745) and *SOCS-3* (GenBank: NM_003955) were amplified. The primers were as described by Tischoff et al. (2007). Universal methylated DNA (Zymo Research, Irvine, NY, USA) was used as a positive control. The 50 μ l PCR reaction contained 200 ng of bisulphite-treated DNA, 1X GoTaq® Green Master Mix (Promega Bioscience, San Luis Obispo, CA, USA) and 0.2 μ M of each primer. MS-PCR cycling conditions was: 95°C for 2 min, 40 cycles of 95°C for 30 s, annealing for 2 min (*SOCS-1* U at 60°C, *SOCS-1* M at 52°C and *SOCS-3* at 58°C), 72°C for 1 min and a final extension at 72°C for 5 min. PCR

products were electrophoresed on 2% agarose gels, and visualized by ethidium bromide staining under ultra violet transillumination. Results from triplicate experiments were used to determine methylation status.

DNA sequencing

The extent of CpG methylation was confirmed by automated DNA sequencing. MS-PCR products were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), sequenced bidirectionally and analyzed on an automated DNA sequence analyzer (MWG, Ebersberg, Germany). Sequences were compared with wild-type sequence with each CpG in the region of interest being converted to TpG in the predicted sequence.

Quantitative RT-PCR (RQ-PCR)

Total RNA was extracted using Rneasy® Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was set up for the synthesis of cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystem, Foster City, California, USA) according to the manufacturer's protocol. TaqMan Gene Expression assays (Applied Biosystems) were used and analyzed by RQ-PCR performed on Applied Biosystem 7500 Fast Real-Time PCR System according to the manufacturer's protocol. TaqMan® Fast Advanced Master Mix (Applied Biosystems) was used as recommendation by the manufacturer. Glyceraldehyde-3- phosphate dehydrogenase (GAPDH, Assay ID Hs99999905_m1) was used as internal control. ABI 7500 software v2.0.6 (Applied Biosystem) was used to perform RQ of target genes using the comparative CT method.

Western blot analysis

Proteins from K562 and K562-R cells were extracted by RIPA buffer (Sigma-Aldrich, MO, USA). BioRad protein dye (BioRad, Hercules, California, USA) and spectrophotometer (BioPhotometer Plus, Eppendorf, Germany) were employed for measurement of protein concentrations. Preparation of immunoblotting was performed as described previously (Frohling et al., 2007). Antibodies used were anti-STAT1, anti-p-STAT1, anti-STAT3, anti-p-STAT3, anti-STAT5, anti-p-STAT5 and anti-β-actin (Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Repeated Measure ANOVA and nonparametric Mann-Whitney test (at $p < 0.05$) was employed to determine the differences in the mean of IC_{50} values between the K562R and parental K562 cell lines. All statistical analyses were performed using the SPSS software package (Version 20, SPSS, Armonk, NY, USA).

Results

Response of BCR-ABL CML cell lines to imatinib

In order to verify the resistance of K562-R cells to imatinib, we investigated the rate of cell growth inhibition of imatinib in both K562-R and the parental K562 cell lines. Each cell line was cultured with increasing concentrations of imatinib (50-400 nM). Cell proliferation

was assessed by the MTS assay (Figure 1). K562 cells were inhibited by low-dose imatinib, whereas the resistant K562-R cells were only inhibited by higher dose. The 50% inhibitory concentration (IC_{50}) of imatinib for K562-R was approximately 10-fold higher than that for K562, indicating that K562-R cell line is resistant to imatinib ($Z \text{ stat} = -3.361, p = 0.001$).

Inhibition of apoptosis in resistant cells

The vitality and fraction of apoptotic and necrotic cells of K562 and K562-R after various incubations with imatinib is shown in Figure 2. Upon incubation of K562 cells in the presence of 400 nM of imatinib, a reduction of cell viability of 90% down to 50% was detected. In contrast, the resistant cell line K562-R showed only a reduction of vitality of 10%, with still showing 80% viable cells ($p < 0.001$). Figure 3 depicts the course of apoptotic (Q2 and Q4) and necrotic (Q1) cells over 72h measured

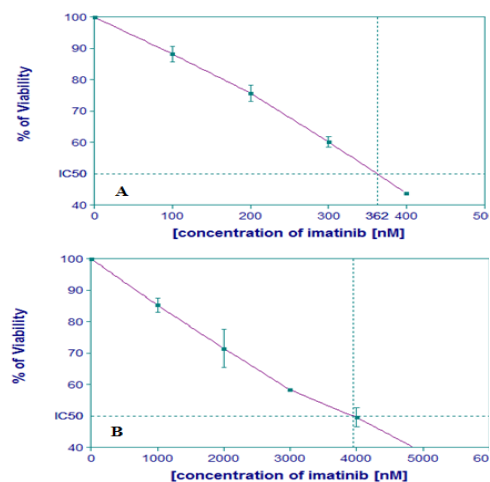


Figure 1. Cell Growth Inhibition by Imatinib in K562 and K562-R. A) K562 cells ($IC_{50} = 362$ nM) and B) K562-R ($IC_{50} = 3952$ nM) exposed to imatinib for 72 hours were quantitated by cell proliferation. Each result is presented as the mean percentage of proliferation of unexposed control cultures

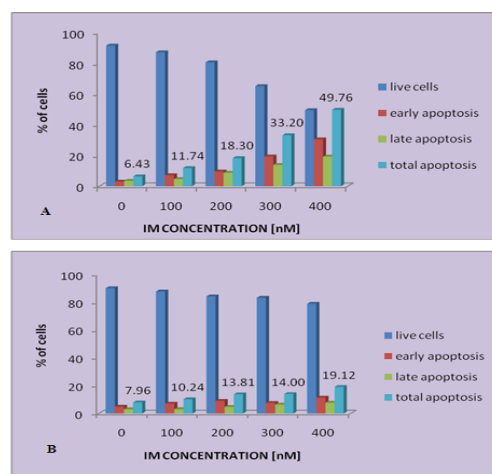


Figure 2. The Vitality and Fraction of Apoptotic and Necrotic Cells in K562 and K562-R Cells. Apoptotic cells increased significantly in A) K562 compared to B) K562-R cells after addition of imatinib at increasing concentrations. There was a reduction of cell viability of 90% down to 50% in K562 in contrast to only 10% reduction in the resistant cell line K562-R ($p < 0.001$)

by Annexin V/FITC-FACS analysis in K562 and K562-R after addition of 100, 200, 300 and 400 nM of imatinib. The resistant cells showed a significant increase in the

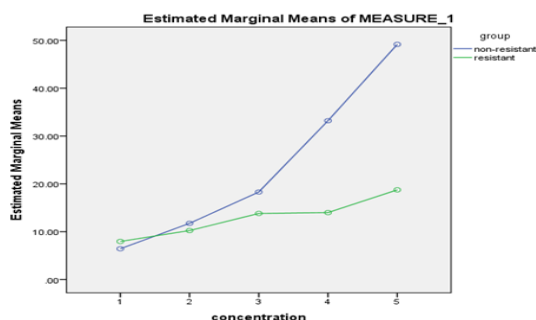


Figure 4. The Profile Plot of Apoptotic Cells for All Concentrations of Imatinib. Repeated Measure-ANOVA between groups based on concentration was applied. The profile plot shows the adjusted mean (estimated marginal means) of apoptotic cells for all concentrations of imatinib (0, 100, 200, 300 and 400 nM). Despite the mean percentage of apoptotic cells before treatment with TKIs were almost equal for resistant and non-resistant cells, there was a sharp increase in the mean percentage of apoptotic cells (50% at 400 nM) in non resistant cells at increasing concentration of imatinib. However, there was no significant increase in resistant cells (< 20% at 400 nM), $p < 0.001$

Imatinib concentrations (nM)

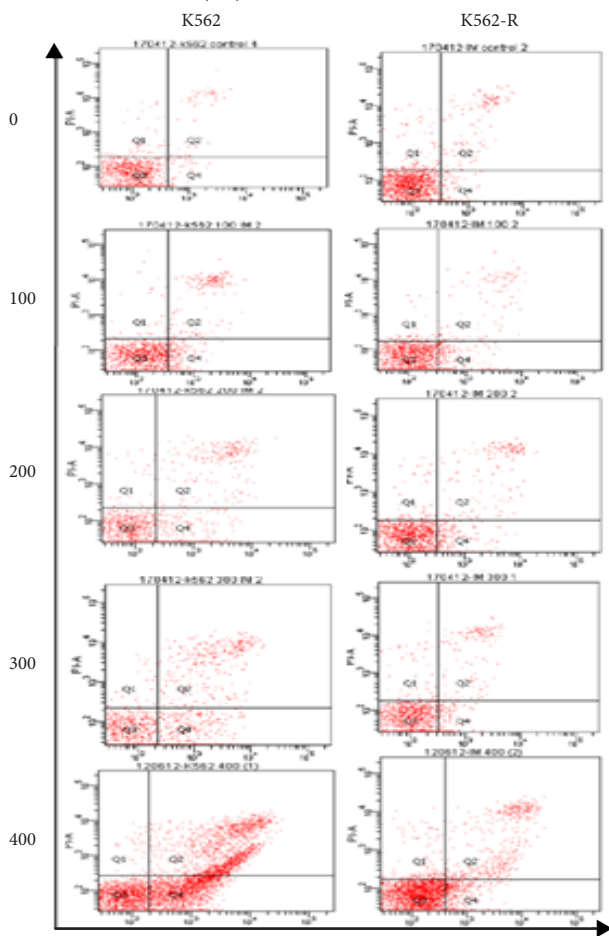


Figure 3. Imatinib-induced Decrease of Vitality in K562 and K562-R. Flow cytometric scatterplots of K562 and K562-R after addition of various concentrations of imatinib cells at 72 hours. Imatinib-induced decrease of vitality is concentration-dependent and decreased in K562-R compared with K562

viability with obvious decreased in apoptotic cells after the incubations compared to the parental cell lines. Figure 4 showed that despite the mean percentage of apoptotic cells before incubations with imatinib were almost equal for both K562 and K562-R cells, there was a sharp increase in K562 with an increase in concentrations (100, 200 and 300 nM) to reach 50% apoptosis at 400 nM. In contrast, there was no significant increase in the apoptotic cells in K562-R with an increase in concentrations to reach only 20% apoptosis at 400 nM ($p < 0.001$).

Methylated SOCS-3 in resistant cells

Methylation specific polymerase chain reaction (MS-PCR) showed a complete methylation of SOCS-3 in K562R but not in K562 (Figure 5a). SOCS-1 was partially methylated and showed no changes between resistant and

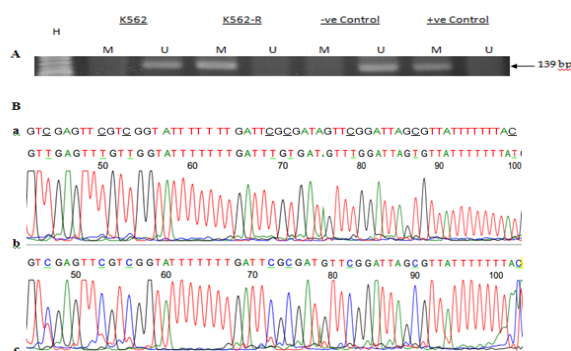


Figure 5. DNA Hypermethylation in K562-R Cells. Analysis of methylation status of SOCS-3 gene. (A) Complete methylation of SOCS-3 in K562-R compared to unmethylated in K562 by MS-PCR. U, unmethylated DNA; M, methylated DNA; H, hyperladder IV marker (Bioline, U.K.). (B) Direct bisulfite sequencing of SOCS-3 from the MS-PCR product using primers for methylated sequence. a. Unmodified or wild-type DNA sequence, b. Bisulfite modified DNA of K562, c. Bisulfite modified DNA of K562-R. All cytosines (C) were altered to thymine (T) on bisulfite-modified DNA of K562 but the 5' cytosine of guanine (CpG) remained as such on bisulfite-modified DNA of K562-R. This denotes DNA methylation at all CpG of K562-R and confirmed the complete methylation

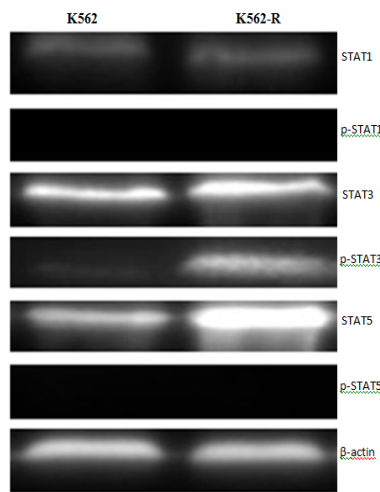


Figure 6. Activation of STAT3 in K562-R Cells. Protein Phosphorylation Status of STAT1, STAT3 and STAT5 in K562 and K562-R Cell Lines Assessed by Western Blotting. STAT3 was phosphorylated in K562-R but not K562

non resistant cells. In addition, we also performed direct bisulfite sequencing in K562 and K562-R. All cytosines (C) were altered to thymine (T) on bisulfite-modified DNA of K562. However, 5' cytosine of guanine (CpG) remained as such on bisulfite-modified DNA of K562-R denotes DNA methylation in all CpGs. Figure 5 shown part of direct bisulfite sequencing of SOCS-3 from the PCR product by MS-PCR method using primers for methylated sequence.

Down-regulation of SOCS-3

Real-time PCR-based approach was used to compare the gene expression amongst the two cells. The SOCS family (SOCS-1, 2 and 3) genes were down-regulated (Table 1). Consistent with the methylation results, SOCS-3 was down-regulated due to methylation, however there was no different in the methylation status of SOCS-1 in either resistant or non-resistant cells. This indicate that hypermethylation of SOCS-3 in resistant cells may have some important roles in the resistance to tyrosine kinase inhibitors.

Activation of STAT3

To determine the role of STATs in the resistance of the K562-R cell lines, we investigated the expression of STAT1, STAT3 and STAT5 by Western blot analysis under common culture conditions. The results showed phosphorylation of STAT3 in K562-R but not in K562 cells. However, STAT1 and STAT5 did not show any phosphorylation in both K562 and K562-R cell lines (Figure 6).

Discussion

The resistance to imatinib tyrosine kinase inhibitor remains the challenges in the treatment of CML patients. Gene expression profiles of patients with CML have been studied to identify the cause of resistance to imatinib

(Villuendas et al., 2006; Binato et al., 2009). Loss of tumor-suppressor function has been associated with the process of resistance to imatinib in BCR-ABL positive CML patients (Villuendas et al., 2006; Binato et al., 2009). In our study, we have developed an imatinib resistant BCR-ABL positive cell lines by overexposure of the drug to the cells. This was confirmed significantly by cytotoxicity and apoptosis analysis on parental and resistant cells. A more than ten folds higher IC₅₀ of imatinib on K562-R compared to that of parental K562 cells (p=0.001) was a similar finding with that reported previously on development of resistant cell lines to three tyrosine kinase inhibitors, gefitinib-(GEF-R), erlotinib-(ERL-R) and vademitanib-(VAN-R) from CALU-3 and HCT116 (Morgillo et al., 2011). Our results also demonstrated a higher increased in the percentage of apoptotic cells in K562 by increasing the imatinib concentrations compared to slightly increased in K562-R (p<0.001). However, there was no significant different when increasing the imatinib concentration by 100 nM in K562-R compared to K562. Therefore, there was a distinct resistant to imatinib in K562-R due to higher vitality rates and less apoptotic cells compared to parental K562 cells.

It is our interest to identify the mechanism of resistant by studying the DNA methylation and corresponding gene expression. Our results suggested that the mechanism of resistant to tyrosine kinase inhibitor in K562-R is due to constitutive activation of JAK/STAT signaling pathway as a result of silencing of SOCS-3 gene due to DNA hypermethylation. Hypermethylation of the negative regulator genes of JAK/STAT pathway leads to their transcriptional silencing in other hematological malignancies cells such as MV4-11-R, an Acute Myeloid Leukemia (AML) resistant to tyrosine kinase (Zhou et al., 2009). These negative regulators include SOCS-1, -2 and -3 were also silenced due to methylation in other cancer cells (Sutherland et al., 2004). However, our finding suggested that, DNA methylation of SOCS-3 but not SOCS-1 might involve in the mechanism of resistance in K562-R.

Overexpressions of STAT pathways were shown in resistant to tyrosine kinase AML cells resulted from, at least in part, decreasing expression of SOCS molecules inhibitors (Benekli et al., 2002; Zhou et al., 2009; Bar-Natan et al., 2012). Constitutive activation of STAT3 and STAT5 are common events in myeloid leukemia and it

Table 1. The Expression of SOCS Family was Down Regulated in K562-R as Compared to K562 Cells

Gene Name	Assay ID	Fold change
SOCS-1	Hs00705164_s1	-1.78
SOCS-2	Hs00919620_m1	-2.51
SOCS-3	Hs02330328_s1	-1.34

Table 2. Concentration Effects of Apoptotic Cells within Non-resistant and Resistant Groups

Drug concentration	Non-resistant			Resistant		
	MD (95% CI)		p value	MD (95% CI)		p value
0-100	-5.311 (-12.916, 2.294)		0.281	-2.275 (-4.850, .300)		0.092
0-200	-11.867 (-22.036, -1.698)		<0.021	-5.850 (-8.571, -3.129)		0.001
0-300	-26.767 (-34.227, -19.306)		<0.001	-6.038 (-10.354, -1.721)		0.008
0-400	-42.722 (-55.633, -29.811)		<0.001	-10.788 (-16.263, -5.312)		0.001
100-200	-6.556 (-12.080, -1.032)		<0.019	-3.575 (-5.385, -1.765)		0.001
100-300	-21.456 (-29.871, -13.040)		<0.001	-3.762 (-6.871, -.654)		0.018
100-400	-37.411 (-29.871, -26.501)		<0.001	-8.513 (-14.868, -2.157)		0.01
200-300	-14.900 (22.998, 6.802)		0.001	-.188 (-2.848, 2.473)		>0.95
200-400	-30.856 (-39.620, -22.091)		<0.001	-4.938 (-10.444, 0.569)		0.86
300-400	-15.956 (-29.380, -2.531)		<0.019	-4.750 (-10.198, 0.698)		0.098

*Repeated Measure ANOVA within group analysis was applied followed by pairwise comparison with 95% confidence interval adjustment by Bonferroni correction; MD=mean difference. There was no significant increase in the apoptotic cells in K562-R with an increase in concentrations by 100 nM compared to significant increases in that in K562

results in resistance to tyrosine kinase inhibitors (Benekli et al., 2002; Zhou et al., 2009; Bar-Natan et al., 2012). SOCS-3 plays critical roles in the suppression of STAT3 phosphorylation and the knockdown of SOCS3 expression results in uncontrolled constitutive activation of STAT3 signaling (Liang et al., 2013). The anti-proliferative effect of trimethoxyl stilbene (TMS) in lung cancer cell line was through the inhibition of STAT3 and STAT5b proteins but not by inhibition of JAK2 (Liu et al., 2011). SOCS-3 specifically targets STAT3 to inhibit its activation (Tamiya et al., 2011) and STAT3 represents the central transcription factor for many signaling pathway (Benekli et al., 2009). Similarly, our results shown that, silencing of SOCS-3 due to methylation lead to constitutive activation of STAT3 signaling and it represents an important mechanism of resistant in K562-R cells.

In conclusions, development of cells resistant to tyrosine kinase inhibitor is feasible by overexposure of the drug to the cells. This was confirmed significantly by cytotoxicity and apoptosis analysis on parental and resistant cells. Hypermethylation of SOCS-3 might be responsible for the development of resistance to tyrosine kinase inhibitor in BCR-ABL positive CML as a result of activation of STAT3 protein that lead to uncontrolled cell proliferation. Thus SOCS-3 provides a suitable candidate for the mechanisms underlying the development of imatinib resistant in CML patients.

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