RESEARCH ARTICLE

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Re-Expression of Bone Marrow Proteoglycan-2 by 5-Azacytidine is associated with STAT3 Inactivation and Sensitivity Response to Imatinib in Resistant CML Cells

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Abstract

Background: Epigenetic silencing of tumor suppressor genes (TSG) is involved in development and progression of cancers. Re-expression of TSG is inversely proportionate with STAT3 signaling pathways. Demethylation of DNA by 5-Azacytidine (5-Aza) results in re-expression of silenced TSG. Forced expression of PRG2 by 5-Aza induced apoptosis in cancer cells. Imatinib is a tyrosine kinase inhibitor that potently inhibits BCR/ABL tyrosine kinase resulting in hematological remission in CML patients. However, majority of CML patients treated with imatinib would develop resistance under prolonged therapy. **Methods:** CML cells resistant to imatinib were treated with 5-Aza and cytotoxicity of imatinib and apoptosis were determined by MTS and annexin-V, respectively. Gene expression analysis was detected by real time-PCR, STATs activity examined using Western blot and methylation status of PRG2 was determined by pyrosequencing analysis. **Result:** Expression of PRG2 was significantly higher in K562-R+5-Aza cells compared to K562 and K562-R ($p=0.001$). Methylation of PRG2 gene was significantly decreased in K562-R+5-Aza cells compared to other cells ($p=0.021$). STAT3 was inactivated in K562-R+5-Aza cells which showed higher sensitivity to imatinib. **Conclusion:** PRG2 gene is a TSG and its overexpression might induce sensitivity to imatinib. However, further studies are required to evaluate the negative regulations of PRG2 on STAT3 signaling.

Keywords: CML- PRG2- imatinib- 5-Azacytidine- STAT3

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Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy in which there is a reciprocal translocation between chromosomes 9 and 22, t (9;22) (q34;q11) forming Philadelphia chromosome (Goldman and Melo, 2003). The translocation results in formation of a fusion oncogene called BCR/ABL (Deininger et al., 2000; Villuendas et al., 2006). BCR/ABL is presented in more than 99% of CML patients and approximately 30% of ALL patients. The BCR/ABL enhances the development of leukemia through stimulation of several signaling including STAT signaling (Ilaria and Van Etten, 1996; Chai et al., 1997; Warsch et al., 2011). Signal transducers and activators of transcription (STAT) signaling pathway is negatively regulated by tumor suppressor genes (TSG) such as SHP-1, SOCS-1, SOCS-3 and PIAS genes (Yoshikawa et al., 2001; Shuai and Liu, 2003; Roman-Gomez et al., 2004; Qiu et al., 2012; Stec et al., 2013). Furthermore, constitutive activation of STAT3 and STAT5 are common events in myeloid leukaemia and involved in resistance to TKIs (Benekli et al., 2002; Zhou et al., 2009; Bar-Natan et al., 2012). The activation of STAT3 is an important mechanism of resistance to imatinib (Bewry et al., 2008; Al-Jamal et al., 2014).

Imatinib is a tyrosine kinase inhibitor (TKI) that potentially inhibits BCR/ABL tyrosine kinase and described as the standard first-line therapy for CML patients (Druker et al., 2006). However majority of patients treated with imatinib would develop resistance under prolonged therapy (Esposito et al., 2011).

Bone marrow proteoglycan 2 (PRG2) is a protein-coding gene that mainly expressed in eosinophils (Gleich, 2000; Weyer and Glerup, 2011). PRG2 is one of the major constituents of eosinophil granules, functions to defence against parasites (Gleich, 2000; Weyer and Glerup, 2011). Overexpression of PRG2 in myeloid cells blocked G-CSF-dependent proliferation and increased apoptosis (Liu and Dong, 2012). However, epigenetic silencing of PRG2 induced proliferation and lowered apoptosis in pancreatic cancer cell lines (Hagihara et al., 2004b), suggesting tumor suppressor function of PRG2

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gene.

Epigenetic silencing due to hypermethylation of CpG islands is a frequent mechanism of inactivation of TSG in a variety of human cancers including acute myeloid leukemia (AML) (Leone et al., 2003). The 5-Azacytidine (5-Aza) and 5-Aza-2-deoxycytidine (5-Aza2dc) are chemotherapeutic drugs that induce DNA demethylation and approved to be the standard care for patients with myelodysplastic syndromes (MDS) (Bhalla, 2005; Garcia-Manero, 2008). Treatment with 5-Aza and cytotoxic anticancer drugs exhibits synergistic activity in AML and non-small cell lung cancer (NSCLC) cells (Fuller et al., 2015). In addition, re-expression of PRG2 by 5-Aza treatment could enhance sensitivity to PKC-412 in AML cells (Al-jamal et al., 2015).

It was hypothesized that, enhanced expression of JAK/STAT negative regulators such as SOCS-1, SOCS-3 and SHP-1 as well as PRG2 by 5-Aza could be involved in the restoration of sensitivity response to imatinib in resistance CML cells. Therefore, cytotoxicity to imatinib, gene expression and methylation analysis were performed in a BCR/ABL positive CML cells resistant to imatinib before and after treatment with 5-Aza compared to parental cells.

Materials and Methods

Imatinib Mesylate

Imatinib was purchased from LC Laboratories (Woburn, MA, USA) and prepared as described in published data (Al-Jamal et al., 2014).

Development of resistant cells

K562 leukemic cells were obtained from Hematology Department, Universiti Sains Malaysia (USM), originally purchased from American Type Culture Collection (ATCC). Resistant cell lines to imatinib was developed as described in previous published data (Al-Jamal et al., 2014).

5-Azacytidine treatment

5-Azacytidine (5-Aza) (Sigma-Aldrich Corp. MO, USA) was dissolved in RPMI-1640 for stock and working preparation. The treatment of resistant cells (K562-R) was by sub-culture in working preparation as described in published data (Al-jamal et al., 2015).

Growth inhibition assay

Leukemic cells (K562, K562-R and K562-R+5-Aza) were seeded in 96-well culture plates at a density of 1 X 10^4 viable cells/100 µL/well in triplicates, and were treated with serial concentrations of 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).

Apoptosis assay

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

RNA extraction

Total RNA was extracted from leukemic cells using Rneasy® Mini Kit (Qiagen, Valencia, CA, USA), the purity and concentration was measured by NanoDrop ND-1000 spectrophotometer V3.3.0 (NanoDrop Technologies, Berlin, Germany).

Quantitative RT-PCR

Quantitative RT-PCR (RQ-PCR) was performed for gene expression analysis using High Capacity RNA-to-cDNA kit to synthesis cDNA according to the manufacturer's protocol (Applied Biosystem, Foster City, California, USA). TaqMan Gene Expression assays (Applied Biosystems) were performed on Applied Biosystem 7500 Fast Real-Time PCR System according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. ABI 7,500 software v2.0.6 (Applied Biosystem) was used to perform relative quantification of SOCS-3 and PRG2 using the comparative threshold cycle (Ct) method.

DNA extraction

DNA was extracted from K562, K562-R and K562-R+5-Aza cells using extraction kit NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The concentration and purity of DNA were measured by NanoDrop.

Methylation analysis

DNAs from the leukemic cell lines were sent to EpigenDx (EpigenDx, Hopkinton, MA, USA) for pyrosequencing analysis. The assay was designed to target 4 CpG islands in the promoter region of PRG2 gene.

Western Blot analysis

Total protein was extracted from treated K562, K562-R and K562-R+5-Aza cells with 400 nM imatinib for 3 days, using RIPA buffer (Sigma-Aldrich, MO, USA). BioRad protein dye (BioRad, Hercules, California, USA) and spectrophotometer (BioPhotometer Plus, Eppendorf, Germany) were used 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 Kruskal Wallis tests were employed for statistical analyses. All statistical analyses were performed using the SPSS software package (Version 20, SPSS, Armonk, NY, USA) and p value <0.05 was considered as significant. The level of significance was considered at p value <0.017 in Multiple Mann-Whitney test with Bonferruni correction.

Results

Higher sensitivity to imatinib in K562-R+5-Aza

Cell growth inhibition by imatinib was evaluated by the MTS assay and K562 and K562-R+5-Aza cells were inhibited by low-doses, however the resistant K562-R cells were only inhibited by higher dose. The IC50 of imatinib on K562-R+5-Aza was 290 nM compared to K5462-R cells 3953 nM (p=0.040). However there was no significant difference in the IC50 between K562 and K5462-R cells (p=0.201) (Figure 1).

Higher apoptosis in K562 and K562-R+5-Aza in response to imatinib

Upon incubation of cells in the presence of imatinib, there was a significant increase in the apoptotic cells by increasing imatinib concentration (p<0.001) after treatment of resistant K562-R with 5-Aza (Figure 2).

Restoration of PRG2 gene expression in K562-R+5-Aza cells

To speculate the possible correlation of re-expression of PRG2 and induction of sensitivity response to imatinib in K562-R+5-Aza cells, gene expression using RQ-PCR were performed. There was a significant up-regulation of PRG2 in K562-R+5-Aza cells compared to K562-R and K562 cells (p=0.001). However, there was no difference in the expression of SOCS-3 between K562-R and K562-R+5-Aza cells (Figure 3).

Low methylation of PRG2 gene in K562-R+5-Aza cells

To relate the expression of PRG2 with methylation status, pyrosequencing analysis was performed on DNA from all three cell lines. The results revealed a significant lower methylation of four CpG islands in the promoter region of PRG2 gene when K562-R cells were treated with 5-Aza compared to untreated K562-R and K562 cells (p=0.021). However, there was no significant difference (p=0.284) in methylation of CpG islands in the promoter region of PRG2 in K562 cells compared to K562-R cells (Table 1 and Figure 4).

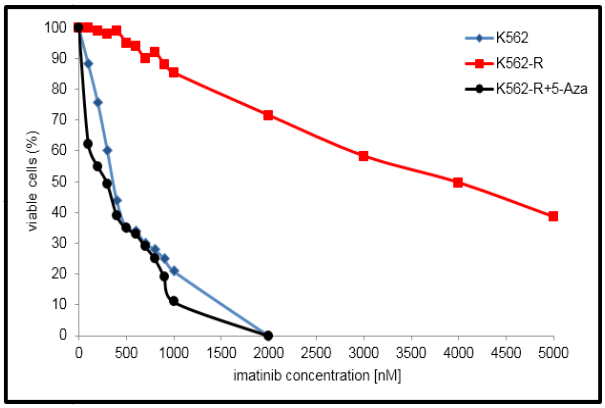

Figure 1. Cell Growth Inhibition by Imatinib in K562, K562-R and K562-R+5-Aza Cells. MTS result shows a significant lower of IC50 of imatinib on K562-R+5-Aza cells (290 nM) compared to K562-R cells (3953 nM). The cells were exposed to serial concentration of imatinib for 72 hours and the IC50 was quantified by cell proliferation assay. Each result is presented as the median percentage of proliferation to unexposed control cultures.

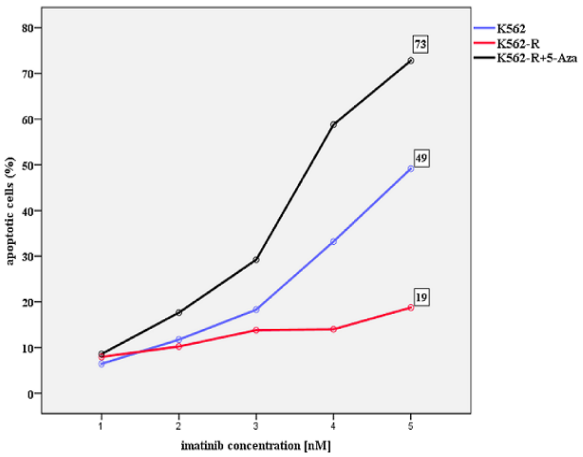

Figure 2. The Profile Plot of Apoptotic Cells for Serial Concentrations of Imatinib. Repeated measure ANOVA between groups based on concentrations was applied. The profile plot shows the adjusted mean (estimated marginal means) of apoptotic cells for serial concentrations imatinib. Although the mean percentage of apoptotic cells before incubations with imatinib were almost equal for K562, K562-R-pkc and K562-R+5-Aza cells, there was a sharp increase in K562 and K562-R+5-Aza apoptotic cells with an increase in imatinib concentrations to reach 49 and 79%, respectively at 400 nM. In contrast, there was no significant increase in the apoptotic cells in K562-R with an increase in concentration to reach only 19% apoptosis at 400 nM.

Inactivated STAT3 after treatment of resistant K562-R cells with 5-Aza

Although, STAT3 was activated in K562-R cells, it showed inactivation in K562-R+Aza cells. STAT1 and STAT5 showed no differences in phosphorylation status in all cells (Figure 5).

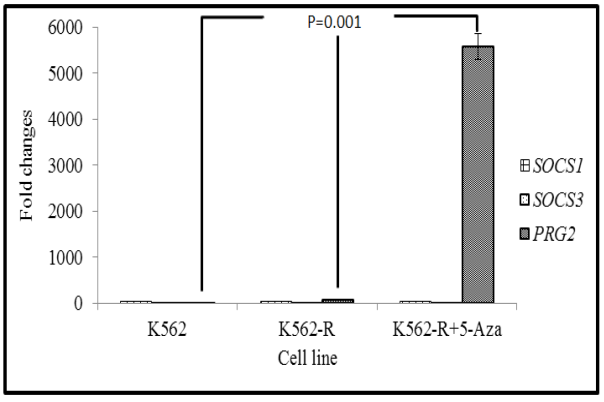

Figure 3. Expression of PRG2 Gene was Restored in K562-R+5-Aza Cells. Real-time quantitative PCR (RQ-PCR) results revealed re-expression of PRG2 with the highest fold changes in K562-R+5-Aza cells compared to in K562-R cells. The results shows more than 5000 times higher of PRG2 expression in K562-R+5-Aza cells compared to K562-R cells but no restoration of expression of SOCS-1 or SOCS-3 in these cells.

Table 1. Percentage of Methylation of CpG Islands in the Promoter Region of PRG2 Gene

Sample ID	CpG -6	CpG -5	CpG -4	CpG -3	mean	min	max
K562	37.6	45.6	44.6	48.5	44.1	37.6	48.5
K562-R	57.8	36.2	48.8	52.3	48.8	36.2	57.8
K562-R+5-Aza	3.4	6.1	11.6	17.1	9.6	3.4	17.1
Low Meth Control	2.3	2.6	1.2	1.3	1.9	1.2	2.6
Med Meth Control	46.8	39.5	25.8	26.7	34.7	25.8	46.8
High Meth Control	96.7	85.3	49.3	50.4	70.4	49.3	96.7

Pyroseqencing analysis showing methylation levels of 4 CpG islands of PRG2 gene. It shows a significant lower methylation in the CpG islands of PRG2 in K562-R+5-Aza cells compared to K562 and K562-R cells (p=0.021)

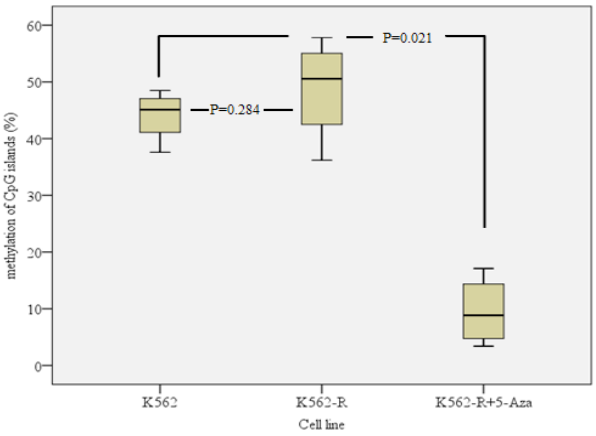

Figure 4. Low Methylation of PRG2 Gene in K562-R+5-Aza Cells. Pyrosequencing results show a significant lower in methylation level of PRG2 genes in K562-R+5-Aza cells compared to K562 and K562-R cells (p=0.021). However, there was no significant difference in methylation of PRG2 genes between K562 and K562-R cells (p=0.284).

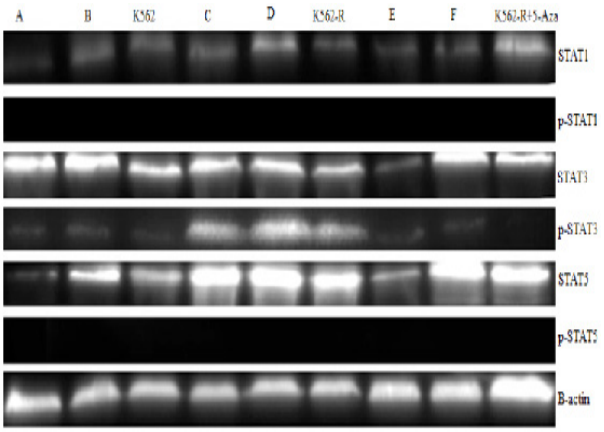

Figure 5. Activation of STAT3 in Resistant Cells. Western blot analysis depicts expression of STAT1, STAT3 and STAT5 proteins in K562, K562-R and K562-R+5-Aza cells. Although, STAT3 was phosphorylated in K562-R cells, it showed de-phosphorylation in K562-R+5-Aza cell. However, STAT1 and STAT5 display no differences in phosphorylation status in all cell lines.

Discussion

The acquired resistance to imatinib remains a challenge in the treatment of CML patients. Transcriptional silencing of SOCS-1, SOCS-3 and PRG2 due to hypermethylation has been documented in leukemic cells (Capello et al., 2008; Zhou et al., 2009; Al-Jamal et al., 2014; Al-jamal et al., 2015). Resistance of K562-R cells to imatinib were acquired and confirmed by cytotoxicity and apoptosis assays as described in published data (Al-Jamal et al., 2014).

Aberrant methylation of TSG such as SOCS-1, SOCS-3 and SHP-1 has been documented in variety of cancers including hematological malignancies (Oka et al., 2001; Leone et al., 2003; Johan et al., 2005; Hatirnaz et al., 2007; Uhm et al., 2009; Al-Jamal et al., 2014; Li et al., 2014). PRG2 gene is poorly described in cancers, but it has been silenced due to hypermethylation of CpG islands in the promoter region in pancreatic cancer cells (Hagihara et al., 2004a) and AML cells (Al-jamal et al., 2015). In consistency, the results of this study revealed a significant decrease in methylation level of CpG island in the promoter region of PRG gene in K562-R+5-Aza cells compared to K562-R and K562 cells ($p<0.021$). The hypomethylation was associated with marked re-expression of PRG2 genes in K562-R+5-Aza cells with more than 5,000 times higher compared to K562-R and K562 cells (p=0.001). These findings were similar to that reported previously by Hagihara et al., (2004b) in which PRG2 was silenced due to methylation in pancreatic cancer cells and re-expressed by hypomethylation using 5-Aza2dc. The findings of this study are also consistent to previous published data in which PRG2 gene re-expressed significantly in AML cells resistant to PKC-412 after treatment with 5-Aza (Al-jamal et al., 2015).

The activation of STAT3 is an important mechanism of resistance to imatinib (Bewry et al., 2008; Al-Jamal et al., 2014). TSG function on signaling pathways to control proliferation and subsequently prevent tumor formation (Sever and Brugge, 2015). On the other hand, re-expression of SHP-1, SOCS-1, SOCS-2 and SOCS-3 by 5-Aza or 5-Aza2dc results in inactivation of STAT3 and consequently confer sensitivity to TKIs (Zhang et al., 2000; Han et al., 2006; Zhou et al., 2009; Esposito et al., 2011; Witzig et al., 2014). Additionally, overexpression of PRG2 was associated with inhibition of myeloid cells proliferation and higher apoptosis (Liu and Dong, 2012; Al-jamal et al., 2015). In accordance, the re-expression of PRG2 in K562-R+5-Aza cells was associated with STAT3 inactivation. These findings suggest that, PRG2 re-expression could be involved in the inhibition of STAT3.

Moreover, K562-R+5-Aza cells showed a significant higher sensitivity to imatinib compared

to the resistant K562-R cells (p=0.010). The results also demonstrated a significant increase of apoptotic cells in K562-R+5-Aza cells by increasing imatinib concentration compared to slightly increased in resistant cells (p<0.001). These finding are supported by previous reports in which, the inactivation of STAT3 enhances apoptosis and reverts sensitivity response towards TKIs (Bewry et al., 2008; Redell et al., 2011). These finding suggest that, the over-expression of PRG2 gene could be involved in the suppression of STAT3 activity resulting in induction of apoptosis and higher sensitivity to imatinib.

In conclusion, this study addressed silencing of PRG2 gene in leukemic cells is due to DNA hypermethylation and re-expression of this gene by 5-Aza is associated with higher sensitivity to imatinib. However, further studies are needed to clarify the correlation between the re-expression and sensitivity to imatinib.

Acknowledgements

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