Imatinib causes epigenetic alterations of PTEN gene via upregulation of DNA methyltransferases and polycomb group proteins

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We have recently reported the possible imatinib-resistant mechanism; long-term exposure of leukemia cells to imatinib downregulated levels of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) via hypermethylation of its promoter region (Leukemia 2010; 24: 1631). The present study explored the molecular mechanisms by which imatinib caused methylation on the promoter region of this tumor suppressor gene in leukemia cells. Real-time reverse transcription PCR found that long-term exposure of chronic eosinophilic leukemia EOL-1 cells expressing FIP1L1/platelet-derived growth factor receptor-α to imatinib induced expression of DNA methyltransferase 3A (DNMT3A) and histone-methyltransferase enhancer of zeste homolog 2 (EZH2), a family of polycomb group, thereby increasing methylation of the gene. Immunoprecipitation assay found the increased complex formation of DNMT3A and EZH2 proteins in these cells. Moreover, chromatin immunoprecipitation assay showed that amounts of both DNMT3A and EZH2 proteins bound around the promoter region of PTEN gene were increased in EOL-1 cells after exposure to imatinib. Furthermore, we found that levels of DNMT3A and EZH2 were strikingly increased in leukemia cells isolated from individuals with chronic myelogenous leukemia (n = 1) and Philadelphia chromosome-positive acute lymphoblastic leukemia (n = 2), who relapsed after treatment with imatinib compared with those isolated at their initial presentation. Taken together, imatinib could cause drug-resistance via recruitment of polycomb gene complex to the promoter region of the PTEN and downregulation of this gene’s transcripts in leukemia patients.

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Introduction

Epigenetic states are determined by inheritable patterns of changes in chromatin and gene expression without underlying alterations in DNA sequence.1 The polycomb group (PcG) proteins and the DNA methylation systems are intimately involved in heritable repression of gene activity.1,2 PcG protein enhancer of zeste homolog 2 (EZH2), a family of histone methyltransferase, catalyzes trimethylation at lysine 27 of histone H3 (H3K27me3), which serves as an anchorage point for the recruitment of additional PcG proteins and contributes to formation of a repressive chromatin state.3,4 EZH2 works together with other components such as embryonic ectoderm development and suppressor of zeste 12 to form the polycomb repressive complex 2, which interacted with target gene promoters, thereby acting as a transcriptional repressor via chromatin modification.5,6 Recent studies showed that knockdown of EZH2 by RNA interference sensitized drug-resistant ovarian cancer A2780/DDP cells to cisplatin in association with inhibition of H3K27me3. Other studies found that aberrant expression of EZH2 was associated with chemotherapy resistance in cancer cells in vitro and in vivo.7 In addition, H3K27me3 was specifically associated with de novo methylated CpG islands in cancer.8 Thus, aberrant expression of PcG proteins may be involved in both carcinogenesis and drug resistance.

DNA methyltransferases (DNMTs), such as DNMT1, DNMT3A and DNMT3B, are also key epigenetic regulators involved in transcriptional repression.9–12 DNMT3A and DNMT3B are supposed to act as de novo methyltransferase, whereas DNMT1 acts to maintain methyltransferase activity.13 Notably, EZH2 interacted physically with the DNMTs and facilitated their binding to the EZH2-target gene myelin transcription factor 1 (MYT1) promoter, resulting in MYT1 silencing. In addition, both embryonic ectoderm development and suppressor of zeste 12 were shown to interact with DNMTs and to be associated with DNMT enzymatic activity.14

Imatinib (Gleevec) dramatically improved survival of patients with chronic myelogenous leukemia (CML) and Philadelphia-positive acute lymphoblastic leukemia (Ph + ALL) by blocking Abl activity and its downstream signaling.15–18 In addition, imatinib has been shown to be effective in a number of other malignancies that are caused by activated receptor tyrosine kinases, including chronic myelomonocytic leukemia,19 associated with rearrangements of platelet-derived growth factor receptor-β and chronic eosinophilic leukemia, a rare clonal myeloproliferative disorder caused by FIP1L1/platelet-derived growth factor receptor-α.20–21 Development of resistance to imatinib has recently emerged as an important clinical problem in patients with leukemia, most often due to acquired mutations in the target kinase.22 However, the secondary mutation in the target kinase cannot explain all cases of drug resistance.23 Recently, we have identified hypermethylation on the promoter region of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene in association with downregulation of this gene transcripts and activation of pro-survival signaling mediated by Akt in imatinib-resistant leukemia cells isolated from individuals with chronic eosinophilic leukemia, CML and Ph + ALL.24 This study explored molecular mechanisms by which imatinib caused hypermethylation of PTEN in leukemia cells.
Materials and methods

Cells
Chronic eosinophilic leukemia EOL-1 cells were obtained from RIKEN BRC Cell Bank (Tsukuba, Japan). To establish imatinib-resistant EOL-1 cells, imatinib (1 nM) was added every 3 days for up to 4 months to culture media. Leukemic peripheral blood or bone marrow cells were isolated from individual with CML (n = 1) and Ph+ ALL (n = 2) after obtaining informed consent.24

Chemicals
Imatinib and nilotinib were provided by Novartis (Basel, Switzerland). Dasatinib was provided by Bristol-Myers Squibb (New York, NY, USA). DNMT inhibitor 5-aza-2’-deoxycytidine (decitabine, 5-AzadC) was purchased from Sigma (St Louis, MO, USA). These reagents were dissolved in 100% dimethyl sulfoxide to a stock concentration 10 mM and stored at -80°C.

MTT assay
Cells (3 x 10^5 per ml) were cultured with various concentrations of the indicated agents. After 2 days, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.25 All experiments were performed in triplicate and repeated at least three times.

RNA isolation and reverse transcription-PCR
RNA isolation and cDNA preparation were performed as described previously.26 We measured expression of all genes were as follows: 95°C initial activation for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and fluorescence determination at the melting temperature of the product for 20 s on an ABI PRISM 7000 (Applied Biosystems).

Immunoprecipitations and western blot analysis
Whole cell lysates from EOL-1 cells were prepared utilizing RIPA buffer and were immunoprecipitated with an anti-EZH2 (Cell Signaling Technology, Beverly, MA, USA) antibody and protein G Sepharose (Pierce, Rockford, IL, USA). The precipitated samples were subjected to western blot analysis.

Table 1 PCR primers

| Gene     | Direction | Primer                                      |
|----------|-----------|---------------------------------------------|
| DNMT1    | Forward   | 5'-ACCACGCTTCTACTCTCTCAGAGCCCTA-3'         |
|          | Reverse   | 5'-GTTGCAAGCTCTCTGTGAACACTGTGG-3'          |
| DNMT3A   | Forward   | 5'-CAACACAGGAAGCATATCCAGAGTGT-3'           |
|          | Reverse   | 5'-AGTTGACTGGAAGAAAAATATACCC-3'           |
| DNMT3B   | Forward   | 5'-AATGGAATGCCACAGCCAGGAAGGCG-3'           |
|          | Reverse   | 5'-ACTGGAATACCTGACAGGACCCTG-3'            |
| EZH2     | Forward   | 5'-TTCTAGCCAACACCAACACT-3'                 |
|          | Reverse   | 5'-GGGCTCTGCTCTGTATGTTG-3'                |
| EED      | Forward   | 5'-GAGAGGGAAGATGGCGACTG-3'                 |
|          | Reverse   | 5'-CCCCAATCTTTTCCAGAGT-3'                 |
| 18S      | Forward   | 5'-AAACGGGCTACACATTCCAAG-3'               |
|          | Reverse   | 5'-CCTCCAAATGGACTCTGTTA-3'                |

Abbreviations: DNMT, DNA methyltransferase; EED, embryonic ectoderm development; EZH2, enhancer of zeste homolog 2.

The membrane was sequentially probed with anti-DNMT3a (Abcam, Cambridge, UK), -EZH2 (Cell Signaling Technology) and -trimethyl histone H3 (Lys27; Cell Signaling Technology) antibodies.

Methylation analysis by methylation-specific PCR
DNA of 1 μg isolated from EOL-1 cells was used for bisulfite treatment done by the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA) according to the supplier’s protocol.

The primer sets used to amplify the promoter region of the Pten gene were described elsewhere.24 Primers for other PCR products are shown in Table 1. Amplification was carried out in a Mycycler thermal cycler (Bio-Rad, Tokyo, Japan) at 95°C for 3 min, cycled at 95°C for 30 s, 57°C for 30 s and 72°C for 1 min (40 cycles), followed by a 7 min extension at 72°C.

Chromatin immunoprecipitation assay
The cells (5 x 10^5 per ml) were incubated with imatinib (1 nM). After 4 months, formaldehyde was added to cells to a final concentration of 1%, and the cells were incubated at 37°C for 20 min. The cells were collected and subjected to chromatin immunoprecipitation kit (Millipore, Temecula, CA, USA) according to the manufacturer's protocol.

Statistical analysis
When comparing two groups, Student’s t-test was used. All statistical analyses were carried out using SPSS software (Version 11.03; SPSS, Tokyo, Japan), and the results were considered to be significant when the P-value was < 0.05, and highly significant when the P-value was < 0.01.

Results

The effect of imatinib on DNMTs and PcG proteins
We investigated whether short-term exposure (24-120 h) of EOL-1 cells to imatinib induced DNMTs, such as DNMT1, DNMT3A and DNMT3B, by real-time reverse transcription PCR (Figure 1a). Previous studies found that IC50 of imatinib against EOL-1 cells was 0.3 nM.24 High dose of imatinib (1 nM) dramatically induced apoptosis in EOL-1 cells (data not shown) and did not induce expression of these genes after short-term incubation (Figure 1a). On the other hand, low dose of imatinib (0.1 nM) increased levels of DNMTs and PcG proteins such as EZH2 and embryonic ectoderm development in EOL-1 cells by 96 h (Figure 1a). This dose of imatinib (0.1 nM, 96 h) did not significantly inhibit the proliferation of EOL-1 cells, as assessed by MTT assay (Figure 1b). However, low dose of imatinib decreased their proliferation by approximately 60% after 120 h (Figure 1b) in parallel with disappearance of induction of DNMTs and PcG proteins (Figure 1a). Similarly, relatively low
concentration (less than IC50 dose) of dasatinib (1 nM) and nilotinib (10 nM) increased the levels of DNMTs and PcG proteins in these cells (Figures 1c and d). We next attempted to establish imatinib-resistant EOL-1 cells by culturing these cells in the presence of imatinib (1 nM). Interestingly, exposure of EOL-1 cells to imatinib (1 nM) for 4 months significantly increased the levels of DNMTs and PcG proteins (Figure 1e). As expression of these genes increased, EOL-1 cells became resistant to imatinib-mediated growth inhibition (Figure 1f).

EZH2 interacts with DNMT3A to repress PTEN

We examined whether levels of H3K27me3 were increased in EOL-1 cells after exposure to imatinib (Figure 2a). As expected, the levels of H3K27me3 increased after treatment with imatinib for 3 months in EOL-1 cells in parallel with upregulation of EZH2 (Figure 2a). Additional 1-month treatment of EOL-1 cells with imatinib further increased levels of H3K27me3 in conjunction with upregulation of EZH2 and DNMT3A (Figure 2a). We next probed the interaction between imatinib-induced DNMT3A and EZH2 by utilizing Immunoprecipitation

Figure 1 The effect of imatinib on DNMTs and PcG proteins. Real-time reverse transcription (RT)-PCR. (a, c, d) EOL-1 cells were cultured with low dose of imatinib (0.1 nM), dasatinib (1 or 10 nM) and nilotinib (10 or 100 nM) for the indicated time period. After incubation, EOL-1 cells were harvested and RNA was extracted from EOL-1 cells after exposure to imatinib for the indicated time period. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of DNMT1, DNMT3A, DNMT3B, EZH2 and embryonic ectoderm development (EED). Results represent the mean ± s.d. of three experiments performed in triplicate. The statistical significance was assessed by a paired t-test. *P<0.01; **P<0.05. (b) EOL-1 cells (5 x 10^3 per ml) were seeded in 96-well plates and cultured with imatinib (0.1 nM) for 24–120 h. At the indicated time point, their proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results represent the mean ± s.d. of three experiments performed in triplicate. (e) EOL-1 cells were cultured with high dose of imatinib (1 nM) for up to 4 months. After incubation, EOL-1 cells were harvested and RNA was extracted from EOL-1 cells after exposure to imatinib for the indicated time period. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of DNMT1, DNMT3A, DNMT3B, EZH2 and EED. Results represent the mean ± s.d. of experiment performed in triplicate. The statistical significance was assessed by a paired t-test. *P<0.01; **P<0.05. MTT assay. (f) EOL-1 cells were cultured in the presence of imatinib (1 nM) for up to 4 months. At the indicated time point, either control or imatinib-treated EOL-1 cells (5 x 10^5 per ml) were seeded in 96-well plates and cultured with imatinib (1 nM). After 48 h, proliferation was measured by MTT assay. Results represent the mean ± s.d. of experiment performed in triplicate.
Imatinib induces DNA methyltransferases and polycomb group proteins

Figure 1 (Continued).
assay (Figure 2b). Imatinib-induced EZH2 interacted with DNMT3A in imatinib-resistant EOL-1 cells (Figure 2b). We further examined whether DNMT3A and EZH2 bound to the PTEN promoter in EOL-1 cells after treatment with imatinib for 4 months, using chromatin immunoprecipitation assays (Figure 2c). Both DNMT3A and EZH2 were enriched in the PTEN promoter in these cells (Figure 2c). We next examined the promoter region of PTEN gene to confirm if epigenetic modification such as DNA hypermethylation occurred in EOL-1 cells by utilizing methylation-specific PCR (Figure 2d). As shown in Figure 2d, CpG island on the promoter region of the PTEN gene was partially methylated after 3 months exposure to imatinib. However, DNA methylation on the PTEN promoter region was facilitated after 4 months exposure to imatinib (Figure 2d), when the levels of PTEN were downregulated (Figure 2e). In conjunction with downregulation of this gene transcript, EOL-1 cells became resistant to imatinib-mediated growth inhibition (Figure 1e). Furthermore, we compared the levels of DNMT3A and EZH2 mRNA in leukemia cells isolated from individuals with CML (n = 1) and Ph + ALL (n = 2; Figure 2f) at the time of relapse after imatinib treatment with those obtained at initial diagnosis. Notably, the levels of both EZH2 and DNMT3A were increased after treatment with imatinib in these cases (Figure 2f). We previously showed hypermethylation on the promoter region of the PTEN gene in association with downregulation of this gene transcript in these cases.24

**DNMT inhibitor 5-AzadC downregulated the levels of DNMTs in imatinib-resistant EOL-1 cells**

We previously showed that an anti-epigenetic agent DNMT inhibitor 5-AzadC restored PTEN expression, resulting in sensitization of imatinib-resistant EOL-1 cells to imatinib.27 The present study examined whether 5-AzadC downregulated the levels of DNMTs in imatinib-resistant EOL-1 cells by real-time reverse transcription-PCR. As expected, exposure of these cells to 5-AzadC (1 μM) potently decreased the levels of DNMT3A, but not DNMT3B (Figure 3a). Immunoprecipitation assay found that exposure of EOL-1 cells to 5-AzadC (1 μM) prevented interaction of EZH2 and DNMT3A proteins (Figure 3b). At the same time, the methylation on the promoter region of the PTEN was inhibited and the levels of PTEN mRNA were increased by approximately two-fold (Figures 3c and d).

**Discussion**

The present study showed that long-term exposure of EOL-1 cells to imatinib increased the levels of both PcG proteins and
DNMTs (Figure 1e) in parallel with the upregulation of H3K27me3 (Figure 2a). Imatinib-induced DNMT3A formed a complex with EZH2 (Figure 2b), which facilitated their binding to the PTEN promoter and induced DNA hypermethylation of this region (Figures 2c and d), leading to downregulation of PTEN (Figure 2e). Notably, the levels of both DNMT3A and EZH2 were increased in imatinib resistant-leukemia cells isolated from individuals with CML and Ph⁺ ALL (Figure 2f). We previously showed that levels of PTEN were downregulated in these cells.²⁴ We therefore hypothesize that imatinib treatment induced upregulation of DNMT3A and EZH2 in leukemia cells, which caused downregulation of PTEN via hypermethylation of the promoter region of this gene. PTEN is a negative regulator of AKT, an important pro-survival signal mediator. Downregulation of PTEN probably might cause AKT, which causes drug-resistance to imatinib (Figure 4).²⁴

Importantly, exposure of EOL-1 cells to low dose of second-generation tyrosine kinase inhibitors dasatinib and nilotinib also

**Figure 2**  EZH2 interacts with DNMT3A in imatinib-resistant EOL-1 cells. Western blot analysis. (a) EOL-1 cells were cultured with imatinib (1 nM) for 3 or 4 months. Cells were harvested and subjected to western blot analysis to monitor the levels of tri-methyl-histone H3 (Lys27), histone H3, EZH2 and DNMT3A. Each lane was loaded with 20 µg of nuclear protein lysates. Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured as a loading control. Band intensities were quantified with ImageJ software (Wayne Rasband, NIH). Immunoprecipitation. (b) EOL-1 cells were cultured with imatinib (1 nM) for 3 or 4 months. Cells were harvested and proteins were extracted. The EZH2 proteins were immunoprecipitated and subjected to western blot analysis. The membrane was probed sequentially with anti-DNMT3A (top) and anti-EZH2 antibodies (bottom). Binding of EZH2 and DNMT3A in PTEN promoter region. Chromatin immunoprecipitation assay. (c) Binding of DNMT3A and EZH2 in the PTEN promoter was analyzed by ChIP assay. To quantify acetylated DNA precisely, we employed real-time PCR. The amplified sequences were normalized to those from input (the cross-linked DNA/protein complexes, which were not immunoprecipitated with anti-DNMT3A and -EZH2 antibodies). Results represent the mean ± s.d. of two experiments performed in duplicate. The statistical significance was assessed by a paired t-test. *P<0.01; **P<0.05. ChIP, chromatin immunoprecipitation. Methylation-specific PCR. (d) DNA was extracted from EOL-1 cells. DNA with methylated CpG was processed using the EZ DNA Methylation Kit. The recovered DNA was amplified by PCR on methylation of the PTEN promoter. The 162-bp product indicates an unmethylation, whereas the 206-bp product indicates a methylation. Real-time reverse transcription-PCR. (e) RNA was extracted from EOL-1 cells. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of PTEN. Results represent mean ± s.d. of duplicate cultures. *P<0.05; **P<0.01. Real-time RT-PCR. (f) RNA was extracted from peripheral blood mononuclear cells or bone marrow of CML (n=1) and Ph⁺ ALL (n=2) patients before and after treatment. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of PTEN. Results represent mean ± s.d. of duplicate cultures. *P<0.05; **P<0.01.
Figure 2 (Continued).

Figure 3  5-AzadC inhibits the interaction of between DNMT3A and EZH2 in imatinib-resistant EOL-1 cells. Real-time reverse transcription-PCR. (a) EOL-1 cells were cultured with 5-AzadC (1 μM). After 72 h, cells were harvested and RNA was extracted. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of DNMTs. Results represent the mean ± s.d. of three experiments performed in triplicate. The statistical significance was assessed by a paired t-test. *P<0.01; **P<0.05. Aza, 5-AzadC. Immunoprecipitation. (b) EOL-1 cells were cultured with 5-AzadC (1 μM). After 72 h, cells were harvested and proteins were extracted. The EZH2 protein was immunoprecipitated and subjected to western blot analysis. The membrane was probed sequentially with an anti-DNMT3A antibody (top) and an anti-EZH2 (bottom). Aza, 5-AzadC. Methylation-specific PCR. (c) DNA was extracted from EOL-1 cells. DNA with methylated CpG was processed using the E.Z. DNA Methylation Kit. The recovered DNA was amplified by PCR on methylation of the PTEN promoter. The 162-bp product indicates an unmethylation, whereas the 206-bp product indicates a methylation. Real-time RT-PCR. (d) RNA was extracted from EOL-1 cells. cDNAs were synthesized and subjected to real-time RT-PCR to measure the levels of PTEN. Results represent mean ± s.d. of duplicate cultures. *P<0.05; **P<0.01. Aza, 5-AzadC.
Anti-epigenetic agents could be useful to overcome drug resistance.

Other studies have recently shown that the drug resistance in lung cancer cells induced by the tyrosine kinase inhibitors erlotinib was dependent on epigenetic mechanisms. Erlotinib increased levels of histone demethylase such as Junonji AT-rich interactive domain-1A, and caused global changes in the chromatin state in the cells. The drug resistance against tyrosine kinase inhibitors could be overcome by treatment with an anti-epigenetic agent histone deacetylase inhibitor, highlighting a potential therapeutic strategy. In fact, we previously showed that histone deacetylase inhibitor successfully overcame imatinib resistance in EOL-1R cells in association with restoration of PTEN expression. In addition, the present study demonstrated that an anti-epigenetic agent 5-AzaC down-regulated the levels of DNMT3A (Figure 3a) and inhibited its binding to EZH2 (Figure 3b) in imatinib-resistant EOL-1 cells, which probably mediated restoration of PTEN expression in these cells.

EZH2 is essential for DNMT3 to bind to the EZH2-target promoters such as MYT1, KCNA1 and cannabinoid receptor 1 (CNR1), and silences expression of these genes. Imatinib increased levels of both EZH2 and DNMT3A in leukemia cells (Figure 2a), which probably caused global epigenetic aberrations and downregulated the expression of important genes involved in regulation of cell growth, apoptosis and drug metabolism, which could relate to acquisition of drug resistance.

Taken together, long-term exposure of leukemia cells to imatinib induced expression of PcG protein EZH2 and DNMT3A, which probably silenced the PTEN gene expression. Anti-epigenetic agents could be useful to overcome drug resistance in individuals who receive tyrosine kinase inhibitors.

Conflict of interest

The authors declare no conflict of interest.

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Author Contributions

TI contributed to the concept and design, interpreted and analyzed the data and wrote the article. CN performed all experiments and wrote the article. JY provided the technical support. AY provided critical revision and intellectual content. KU provided important intellectual content and gave final approval.

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