Leukemia Research 45 (2016) 24–32

Contents lists available at ScienceDirect

Leukemia Research

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

BCR/ABL increases EZH2 levels which regulates XIAP expression via miRNA-219 in chronic myeloid leukemia cells

Chie Nishioka, Takayuki Ikezoe*, Jing Yang, Akihito Yokoyama

Department of Hematology and Respiratory Medicine, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan

ARTICLE INFO

Article history:
Received 29 November 2015
Received in revised form 10 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keyword:
EZH2
BCR/ABL
XIAP
miR-219

ABSTRACT

In this study, we showed that the levels of EZH2 in bone marrow mononuclear cells (BMMNCs) isolated from individuals with chronic myeloid leukemia (CML) (n = 12) were significantly greater than those in BMMNCs isolated from healthy volunteers (n = 6) as well as individuals with Philadelphia chromosome-negative myeloproliferative neoplasms. Lentiviral transduction of the *BCR/ABL* gene in Ba/F3 cells increased EZH2 levels in parallel with phosphorylation of STAT5. Notably, chromatin immunoprecipitation assays showed that STAT5A bound to a promoter region of the *EZH2* gene, resulting in an increase in the transcriptional activity of EZH2 in leukemia cells. Importantly, downregulation of EZH2 by short hairpin RNAs (shRNAs) inhibited the expression of XIAP and increased the miR-219 levels associated with a decrease in hypermethylation of *miR-219-1* CpG islands. Moreover, overexpression of miR-219 decreased the levels of XIAP in CML cells. Since the 3′-untranslated region (3′-UTR) of XIAP contains miR219-5p-complementary binding site, miR-219 might modulate the expression of XIAP through binding of miR-219 on the 3′-UTR of XIAP.

Taken together, BCR/ABL positively regulates the expression of EZH2 via STAT5 signaling. EZH2 modulates epigenetic changes at DNA methylated regions encoding miR-219 and downregulates the level of miR-219, resulting in upregulation of XIAP.

1. Introduction

Polycomb group (PcG) proteins are multifaceted regulators of normal and cancer stem cells and are involved in transcriptional regulation of stem cell self-renewal and fate decisions [1].

PcG protein enhancer of zeste homolog 2 (EZH2), a member of the family of histone methyltransferases, catalyzes trimethylation at lysine 27 of histone H3 (H3K27me3), which serves as an anchor for the recruitment of additional PcG proteins and contributes to formation of a repressive chromatin state [2,3]. EZH2 overexpression is associated with poor prognosis in many aggressive cancers [4–6]. Conversely, downregulation of EZH2 has been shown to reduce both the growth of invasive breast carcinoma and tumor angiogenesis [7,8].

We previously reported that long-term exposure of leukemia cells to imatinib induced the activation of AKT, ERK, and signal transducers and activators of transcription 5 (STAT5) signaling and an increase in levels of EZH2. Furthermore, the aberrant expression of EZH2 was associated with resistance to tyrosine kinase inhibitor (TKI) imatinib in leukemia cells [9]. Expression of EZH2 is regulated by the ERK/AKT signaling pathway in colon cancer cells [10]; however, the regulation of EZH2 in hematological malignant cells remains to be fully elucidated.

Imatinib was Food and Drug Administration (FDA) approved for first-line treatment of chronic myeloid leukemia (CML). However, treatment with imatinib causes resistance due to the presence or acquisition of mutation in the kinase domain that prevented imatinib binding and activity in many cases [11]. Thus, second and third generation TKIs such as dasatinib, nilotinib, and ponatinib are developed to overcome imatinib resistance/intolerance and approved for treatment for CML or Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ALL) [12,13].

The EZH2 inhibitor 3-deazaneplanocin (DZNep)-induces degradation of the PRC2 complex and reduces levels of EZH2 [14,15]. Previous studies have shown that DZNep inhibited the binding of EZH2 to the promoter region of microRNAs (miRNAs) such as miR-1246, mi*R*-302a, and miR-4448 and activated these miRNAs in human gastric cancer cells [16]. MiRNAs are 19–25-nucleotide noncoding RNAs that induce translational inhibition or cleavage of their target mRNAs, resulting in the regulation of gene expression [17]. The mature miRNAs are processed from a larger stem-loop precur-

* Corresponding author. Department of Hematology and Respiratory Medicine, Kochi University, Oko-cho, Nankoku, Kochi 783-8505, Japan.
E-mail address: ikezoet@kochi-u.ac.jp (T. Ikezoe).

http://dx.doi.org/10.1016/j.leukres.2016.03.012
0145-2126/

sor (pri-miRNA). These mature miRNAs bind to the 3′-untranslated region (3′-UTR) of the target mRNA and promote degradation of its target mRNA in association with translational repression of its target gene [18–20].

Epigenetic changes at the methylated regions encoding miRNAs are relatively frequent in sporadic colorectal adenomas [21]. DNA methylation on CpG islands contributes to the transcriptional status of the miRNA. The expression of miRNAs can be suppressed by DNA methylation on GpG islands in normal or tumoral tissues [22]. For example, the expression of BCL2 was repressed by miRNA-15a and miR16-1, resulting in induction of apoptosis in leukemic cells [23]. In addition, miR-7 downregulated XIAP expression, resulting in suppression of cell growth and promotion of apoptosis in cervical cancer cells [24].

BCR/ABL, a critical genetic abnormality in CML, activates STAT5, phosphoinositide 3 kinase (PI3K)/AKT, RAS/MAPK/ERK, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in these cells [25–29]. STAT proteins are latent transcription factors located in the cytosol of resting cells. Following stimulation with cytokines or growth factors, these proteins are rapidly phosphorylated at the tyrosine residues, allowing them to dimerize and translocate to the nucleus, where they bind to specific response elements in promoters of target genes, and transcriptionally activate these genes. The STAT family consists of several transcription factors including STATs 1, 2, 3, 4, 5A, 5B, and 6 [30–32]. STAT5 was shown to play a key role in development of CML in parallel with upregulation of Bcl-xl [25].

Therefore, in this study, we examined the relationship between STAT5 and EZH2 and found that STAT5 directly regulated expression of EZH2 and proliferation of CML cells.

2. Materials and methods

Cells. BCR/ABL-expressing K562 and KU812 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Ph^+ ALL PALL-2 and BCR/ABL-expressing KCL22 cell lines have been described elsewhere [33,34]. Dasatinib-resistant K562 (designated as K562DR) cell lines were established by culturing with increasing concentrations of dasatinib (10 nM) for 12 months [35]. Ba/F3 cells infected with control lentiviral particles were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and IL-3 (1 ng/mL, Pepro Tech, Rocky Hill, NJ). Ba/F3 Bcr/Abl cells were generated by lentiviral infection of Ba/F3 cells with lentiviruses encoding the Bcr/Abl followed by selection with 4 μg/mL blascitidin (Invitrogen) for 2 weeks and maintained in culture without IL-3. Informed written consent was obtained from each subject in accordance with the Declaration of Helsinki. After obtaining written informed consent and Kochi University Institutional Review Board approval, bone marrow mononuclear cells (BMMNCs) or peripheral blood mononuclear cells (PBMCs) were isolated from individuals with Ph^+ ALL (n = 8), Ph^- ALL (n = 9), Ph^- myeloproliferative neoplasms (MPN) including essential thrombocythemia (ET), myelofibrosis (MF), and polycythemia vera (PV) (n = 13), CML (n = 12), acute myelogenous leukemia (AML) (n = 12), and healthy volunteers (n = 6) after obtaining informed consent from the subjects in addition to approval from the Kochi University Institutional Review Board.

2.1. Chemicals

Dasatinib, imatinib, and ponatinib were obtained from Bristol-Myers Squibb (NY, USA), Novartis (Basel, Switzerland), and Med Chem Express (NJ, USA), respectively. The EZH2 inhibitor (DZNep) was purchased from Sigma-Aldrich (St. Louis, MO, USA). These reagents were dissolved in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and stored at −80 °C.

Table 1
PCR primers.

Gene	Direction	Primer
EZH2 v1	Forward	5′-TTCATGCAACACCCAACACT-3′
	Reverse	5′-GGGCCTGCTACTGTTATTGG-3′
STAT5A	Forward	5′-ACATTTGAGGAGCTGCGACT-3′
	Reverse	5′-CCTCCAGAGACACCTGCTTC-3′
18S	Forward	5′-AAACGGCTACCACATCCAAG-3′
	Reverse	5′-CCTCCAATGGATCCTCGTTA-3′

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells (3×10^5/mL) were cultured with various concentrations of the indicated agents. After 24–96 h, cell viability was measured by the MTT assay as previously described in Ref. [9]. All experiments were performed in triplicate and repeated at least three times.

RNA isolation and reverse transcription-polymerase chain reaction. RNA isolation and cDNA preparation were performed as described previously in Ref. [9]. We measured the expression of 18S rRNA for normalization of the sample values as previously described in Ref. [9]. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) as described previously in Ref. [9]. Primers for PCR are shown in Table 1.

2.3. Reporter plasmid, transfection, and reporter gene assay

The human EZH2 promoter-luciferase construct (pEZH2 (-349/+88)-Luc) was prepared as follows. Primers complementary to the published human EZH2 promoter sequence containing NheI and XhoI restriction sites for the forward (5′-GCGGCTAGCGTTCCCGCCACCTATCCT-3′) and reverse (5′-GCGCTCGAGCTCCACTGCCTTCTGAGTCC-3′) primers, respectively, were synthesized. Human genomic DNA (636401, Clontech, Heidelberg, Germany) was mixed with the primers and PCR was performed using PrimeSTAR GXL DNA Polymerase (Takara Bio, Otsu, Japan). The PCR product and pGL4.10 [Luc2] vector (E6651, Promega, Madison, WI, USA) were digested with NheI (1241A, Takara Bio) and XhoI (1094A, Takara Bio) restriction endonucleases. The PCR product was ligated into the pGL4.10 [Luc2] vector using T4 DNA ligase (2011A, Takara Bio). We also generated the pEZH2 (-349/+88)-Luc mutant vector with 4-bp deletions (TTCA) in the binding site of STAT5A using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio). The transfection and reporter gene assay were performed as described previously in Ref. [35]. Briefly, K562 and KCL22 cells were cotransfected by nucleoprotein (Amaxa Biosystems, Gaithersburg, MD, USA) according to the manufacture's protocol (solution V, program T-016) using pEZH2 (-349/+88)-Luc (4 μg), pRL vector (0.5 μg), and either STAT5A (4 μg) or empty vector (4 μg). After 24 h, cell lysate luciferase activity was measured using the Dual Luciferase assay system (Promega). Lysate luciferase activity was normalized to that of Renilla luciferase, which was used as a control.

2.4. STAT5A vector

The STAT5A expression vector was transfected into K562 and KCL22 cells using FuGENE HD (Promega KK, Tokyo, Japan). After 48 h, RPMI medium with 10% heat inactivated fetal bovine serum (FBS) was replaced with that containing blastisine (10 μg/mL) to select for stably transfected cells.