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Imatinib induces demethylation of miR-203 gene: An epigenetic mechanism of anti-tumor effect of imatinib

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ABSTRACT

MicroRNA (miRNA) is an important regulator of cellular proliferation, differentiation and death. Leukemia-specific signature of miRNAs suggests that epigenetic dysregulation of miRNAs is important for leukemogenesis. We focused on the role of DNA methylation of miR-203 which targets *BCR-ABL1* mRNA. The microarray analysis showed that 48 miRNAs of CpG-rich 212 miRNAs were upregulated over 2-fold after imatinib treatment. Imatinib induced the demethylation of the miR-203 promoter region, resulting in low expression of targeted *BCR-ABL1* gene, and loss of proliferation of leukemic cells. In conclusion, demethylation of miR-203 is one of the molecular mechanisms of imatinib-induced inhibition of BCR-ABL1-positive leukemic cells.

1. Introduction

Leukemia has been considered as the consequence of genetic alterations of oncogenes, antioncogenes, proliferation- or cellular death-related genes, and genes encoding transcription factor. These alterations are induced by mutations, translocations or deletions, and lead to the defects of gene functions, such as proliferation, differentiation and apoptosis. Chronic myelogenous leukemia (CML) is characterized by the chimeric gene, *BCR-ABL1*, which codes for a protein with constitutively increased tyrosine kinase activity. The signal transduction downstream of BCR-ABL1 protein promotes the cell proliferation. A tyrosine kinase inhibitor, imatinib mesylate (imatinib), suppresses the proliferation of CML cells by inhibiting specifically the activity of BCR-ABL1 proteins [1].

In the past several years, there are many reports demonstrating that epigenetic alterations correlate to the characteristics of tumor cells, including leukemia [2]. Epigenetic alteration is defined as the changes in the patterns of gene expression that occur without a change in the primary DNA sequence, including DNA methylation and histone modifications [3]. Most reports are about gene silencing caused by promoter methylation, and these epigenetic changes affect tumor progression [4–6]. DNA methylation is a covalent modification of adding a methyl group to the number 5 carbon of the cytosine in a CpG dinucleotide and inhibits the transcription of genes, and this reaction is catalyzed by DNA methyltransferase (DNMT) [7].

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nucleotides in length that regulate a variety of biological processes by post-transcriptionally silencing target mRNA [8,9]. MiRNAs are first transcribed from the genome as long primary transcripts (pri-miRNAs). Pri-miRNAs are cleaved into hairpin-structured precursors (pre-miRNAs) by the complex composed of Drosha, DGCR8 and cofactors. Pre-miRNAs are then transported into the cytoplasm and processed by Dicer to become mature miRNAs. MiRNAs regulate several cellular functions including cell proliferation, differentiation, and apoptosis [10]. Thus, aberrant miRNAs expression, including upregulation of oncogenic miRNAs (oncomir), downregulation of tumor suppressor miRNAs (anti-oncomir), and miRNA deletion, contributes to tumorigenesis [11–14]. MiRNAs are also regulated by methylation of CpG islands within the promoter region of miRNAs [15]. The epigenetic disturbance causes the dysregulation of miRNAs and contributes to malignant transformation during leukemogenesis [16,17].

There are several reports about correlation between CML and DNA methylation. The silencing or downregulation of miRNAs such as miR-15/16 [18], miR-31/155/564 [19], are involved in pathogenesis of CML [20,21]. MiR-203 is epigenetically silenced in human *BCR-ABL1*-positive leukemic cell lines and primary CML cells by the

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methylation of promoter region [16]. Since miR-203 negatively regulates the expression of the ABL1 gene, which has a binding site to miR-203 in a 3′-untranslated region (3′UTR). The silencing of miR-203 by the methylation is a putative mechanism associated with the pathogenesis of *BCR-ABL1*-positive leukemias. Imatinib is highly effective to suppress *BCR-ABL1*-positive leukemic cells growth by down-regulating the expression of *BCR-ABL1* mRNA and protein [22,23], however little is known about the effect of imatinib on miRNA expression profile.

In this study, our data show that imatinib upregulated expression of miRNAs which have CpG islands in its promoter and induced expression of epigenetically silenced miR-203. This is the first report showing that imatinib induces demethylation of miRNA involved in leukemogenesis of CML.

2. Materials and methods

2.1. Cells culture and cell viability

The K562 and HL-60 cell lines were obtained from the American Type Culture Collection (MD, USA), and the KU812 cell line was purchased from RIKEN Cell Bank (Ibaraki, Japan). Cells were plated at 2×10^5 cells/ml and maintained in RPMI 1640 (GIBCO, NY, USA) medium supplemented with 10% fetal bovine serum (GIBCO), and stored at 37 °C in a humidified atmosphere containing 5% CO_2. These cells were treated with 1 μM imatinib mesylate (provided by Novartis Pharmaceutical Co., Ltd. (Basel, Switzerland)). 5-Azacytidine was purchased from Sigma and used as the control for DNA demethylation at the 1 μM concentration. At 72 h, the viability was evaluated by cell counting following trypan blue exclusion method.

2.2. Analysis of gene expression

For RNA, 5×10^5 cells were suspended in RNAlater (Applied Biosystems (ABI), CA, USA) and stocked at −20 °C. Total RNAs were extracted from these cell pellets using the mirVana miRNA Isolation Kit (ABI) according to the manufacturer's instructions. For mRNA and primary miRNA, cDNAs were synthesized using the RNA PCR Kit (Takara, Shiga, Japan). For a quantitative analysis of each gene expression, the amplification of cDNAs by the qRT-PCR method was done using SYBR Premix Ex Taq (Takara) and each primer (*BCR-ABL1*: forward primer, 5′-CAGACTGTCCACAGCATTC-3′, reverse primer, 5′-CTGAGGCTCAAAGTCAGAT-3′, glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′-TCACCACCATGGAGAAGGC-3′, reverse primer, 5′-GCTAAGCAGTTGGTGGTGCA-3′, pri-miR-203: forward primer, 5′-GCTGGGTCCAGTGGTTCTTA-3′, reverse primer, 5′-GACTGTGACTCTGACTCCA-3′) with a Thermal Cycler Dice (Takara). The amounts of *BCR-ABL1* expression were normalized with the level of GAPDH expression.

MiRNAs were transcribed to cDNAs using a High-Capacity cDNA Reverse Transcription Kit (ABI). Looped RT-primers specific for each miRNA were purchased and used according to the manufacturer's instructions (TaqMan MicroRNA Assays Kit, ABI). For detecting miRNAs, the amplification was done using Universal PCR Master Mix (ABI), and Thermal Cycler Dice (Takara). The amounts of each miRNA were normalized with the level of U6B. All experiments were run in triplicate and the average CTs were used for quantification. The relative quantification was analyzed using a comparative CT method.

2.3. Analysis of miR-203 promoter hypermethylation

DNA from cells (1×10^6 cells) were extracted using TRIzol (Invitrogen, CA, USA). Conversion of unmethylated cytosine to uracil was performed with EpiTect Bisulfite Kit (Qiagen, CA, USA). The methylation specific PCR (MSP) for the promoter of pri-miR-203 was done using specific primers; methylated-MSP: forward, 5′-TTTAGACGAGACGGTTCGGGC-3′, reverse, 5′-AAAATAACCCTAACTCAACGACCG-3′, unmethylated-MSP: forward, 5′-TTTAGATGAGATGGTTTGGGT-3′, reverse,

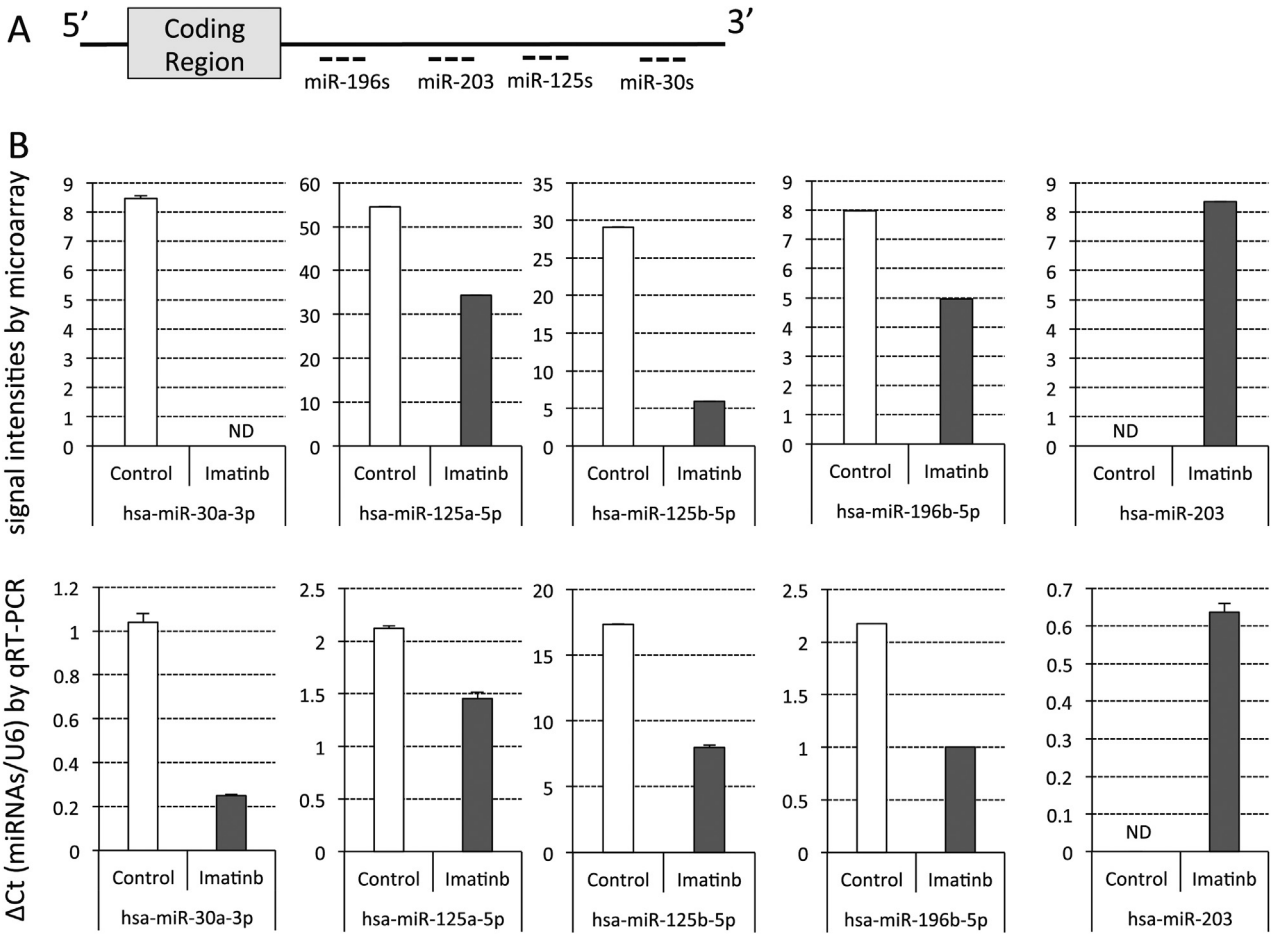

Fig. 1. The expression change of miRNAs targeting *BCR-ABL1* mRNA after imatinib treatment in K562 cells. (A) The map of putative miRNA-binding sites in BCR-ABL1 3′UTR. TargetScan was used to predict miRNA-binding sites. (B) Comparison between microarray and qRT-PCR data in K562 cells after 72 h imatinib treatment (black column) and control (white column). Signal intensities by microarray and $2^{-\Delta Ct}$ (ΔCt = Ct target gene − Ct U6B) by qRT-PCR are shown (ND; not detected).