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ORIGINAL ARTICLE

miR-17 in imatinib resistance and response to tyrosine kinase inhibitors in chronic myeloid leukemia cells

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Summary

Purpose: *In this study we examined the expression levels of miR-17 which possesses oncogenic activities through downregulation of CDKN1A, p21 and E2F1 tumor suppressor genes, in imatinib sensitive and resistant chronic myeloid leukemia (CML) cells. On the other hand, we also determined the expression levels of miR-17 in response to tyrosine kinase inhibitors imatinib, nilotinib and dasatinib used for the treatment of CML.*

Methods: *The expression profiles of miR-17 were analysed by Stem-Loop reverse transcription (RT) polymerase chain reaction (PCR).*

Results: *The results revealed significant increase in the expression levels of miR-17 in imatinib sensitive and resistant cells compared to peripheral blood mononuclear cells (PBMCs). On the other hand, significant decrease was observed in miR-17 levels in response to imatinib, nilotinib and dasatinib.*

Conclusion: *These results may imply that miR-17 can be used for diagnosis and treatment of CML.*

Key words: *chronic myeloid leukemia, dasatinib, drug resistance, imatinib, miR-17, nilotinib*

Introduction

miRNAs are about 20-22 nucleotides long, single-stranded, non-protein-coding RNA molecules [1]. This abundant class of small RNA molecules is found in most of the animal and plant cells [2]. They are supposed to play critical roles in gene regulation and they downregulate the target genes either by degrading the target mRNAs with their perfect match with the cognate mRNAs or by inhibiting the translation of the target mRNAs due to their imperfect complementarity with cognate mRNAs [3,4].

Transcription of miRNA genes that are encoded within the genome of eukaryotic organisms begins in the nucleus, when the transcription factors bind to the promoter of miRNA genes. In this way, primary-miRNA (pri-miRNA) is formed and then it is converted into precursor–miRNA (pre-miRNA) by Drosha. The newly formed pre-miRNAs are then transferred from the nucleus to cytoplasm by RanGTP-dependent dsRNA-binding protein (Exportin 5). After the transfer of the pre-miRNAs to the cytoplasm through the exportin transfer system, the pre-miRNAs are cleaved to form miRNA:miRNA duplexes which are between 19-24 base pairs, by the ribonuclease (RNase) Dicer. Dicer is known as an endoribonuclease belonging to RNase III protein family that cleaves dsRNAs and pre-miRNAs into short, dsRNA fragments and it has the potential to form RNA-inducing silencing complex (RISC) which recognizes the mature miRNA, while the complementary region of the mature miRNA is degraded. The incorporated miRNA into the RISC complex is directed to the target mRNA for degradation or the inhibition of translation of it [1,5,6].

Inhibition of the translation of target mRNAs or even degradation of them by miRNAs make these specific RNA fragments crucial and

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potential targets for anticancer therapy [7]. It has been shown that each miRNA has its signature for a specific malignancy and normal tissue [8]. It has been also revealed that the abnormal gene expression of some miRNAs playing critical roles as oncomiRs in some tissues can result in cancer development and progression [8]. Thus, the detection of these types of miRNAs seems to be an important step for the detection and classification of various cancer types or even prediction of their severity [9].

miR-17 family which is within the miR-17-92 cluster, has crucial oncogenic activities in different cancer cell types, including CML [10,11]. Overexpression of miR-17 in various types of cancer emphasizes its roles in clinical diagnosis and treatment of cancers [10,11].

CML is characterized by the increased number of myeloid cells in the peripheral blood, resulting from malignant disorder of hematopoietic stem cells [12-14]. CML is characterized with the presence of Philadelphia (Ph) chromosome, originating from the reciprocal translocation between *ABL1* gene on chromosome 9q34 and BCR gene on chromosome 22q11.2 [12-14]. The resulting BCR/ABL oncogene induces leukemogenesis with its constitutive tyrosine kinase activity [15].

Imatinib, nilotinib and dasatinib are anticancer agents used for the treatment of CML. They specifically recognize, bind and inactivate tyrosine-kinase activity of the BCR/ABL oncoprotein and thus inhibit leukemogenesis [12,13].

Recent miRNA-dependent cancer studies are based on the comparison of the expression levels of specific miRNAs in different cancer cell types with normal cells in the same tissue [4]. Thus, regarding the specific miRNA expression profiles in specific tumors, abnormal upregulation (oncomiRs) or downregulation (tumor-suppressor miRNAs) of particular miRNAs in various cancer types are addressed for classifying human cancer types [4]. Although for the detection of miRNA profiles the techniques of cloning, northern blotting and microarray are commonly used, it has been argued that their efficiency in detecting quite small amounts of miRNAs is low [16]. That's why Stem-Loop RT-PCR which is quick, efficient and exact for the detection of miRNA gene profiling is proposed [16]. Since the specific primers are used in this new technique, it is quite sensitive and efficient to detect even one nucleotide difference within the specific miRNAs, distinguish homologous miRNAs, and overcome low throughput of convential techniques besides not being affected from DNA contamination [16,17].

In the present study, we aimed to examine the expression levels of miR-17 in the PBMCs and in the imatinib sensitive and resistant CML cells by Stem-Loop RT-PCR. We also determined the expression levels of miR-17 in CML cells in response to tyrosine kinase inhibitors imatinib, nilotinib and dasatinib.

Methods

Ethical permission of this study was obtained from Ege University, Faculty of Medicine, Clinical Research Ethical Comission.

Target and primers

miR-17 was specifically selected from the microRNA database miRBase (http://www.mirbase.org). miR-17 and U6 snRNA, an internal positive control, stem-loop reverse transcriptases, forward and reverse primers were designed manually according to Chen et al. [16].

hsa-miR–17 miRNA sequence 5'-CAAAGUGCUUA CAGUGCAGGUAG-3';
Stem-Loop RT Primer
3'-TCCATCCAACCGAGACCACGCTTATGGAGCCTGGGA CGTGGTCTCGGTTG-5';
Forward Primer
5'-CGGCAAAGTGCTTACAGTGC-3'
Universal Reverse Primer
5'-GTGCAGGGTCCGAGGT-3';
UPL Probe
5'-TGGCTCTG-3'.
U6 snRNA Stem-Loop Primer
5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACA GAGCCAACACGATT-3';
Forward Primer
5'-CCTGCGCAAGGATGAC-3';
Reverse Primer
5'-GTGCAGGGTCCGAGGT-3';
UPL Probe
5'-TGGCTCTG-3'.

Peripheral blood mononuclear cell isolation

PBMCs were isolated from the blood of a healthy donor from our lab. Eight ml of blood were mixed with 32 ml phosphate buffered saline (PBS). Then, the diluted blood was transferred carefully over 12 ml of Ficoll-Paque in 50 ml falcon tube and centrifuged at 2500 rpm for 25 min at 20 ^{0}C. After centrifugation, the mononuclear cell layer was carefully transferred into a new falcon tube and PBMCs were washed. After centrifugation at 1600 rpm for 10 min at 4 ^{0}C, the supernatant was removed carefully and this step was repeated

for three times. Finally, the PBMC pellet was diluted with 10 ml of PBS and cells were counted using Thoma Counting Chamber.

Cell lines and conditions of cell culture

Human K562 CML cells were obtained from the German Collection of Microorganisms and Cell Cultures (Germany). Subpopulations of K562 cells that could grow in the presence of 3 μM imatinib (referred to as K562/IMA-3) were developed in our research laboratory [18]. K562 and K562/IMA-3 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in CO_2 incubator at 37 ^{0}C. The medium was refreshed three times in a week.

Total RNA isolation

RNA isolation process was performed using Trizol Reagent for obtaining miRNAs efficiently, since it is more sensitive to small RNA fragments and miRNAs. 2x10^6 PBMC, K562 and K562/IMA-3 cells were collected and centrifuged at 1000 rpm for 10 min. After removing the supernatant from the pellets, 1 ml Trizol Reagent was added to each cell tube. The pellets were lysed by repetitive pipetting and incubated at 20 ^{0}C for 5 min. Then, 0.2 ml chloroform was added to the homogenized mixture and incubated at 20 ^{0}C for 3 min. Afterwards, samples were centrifuged at 12000 g for 10 min at 4 ^{0}C. The RNA containing upper phase was transferred into a new eppendorf tube and 0.5 ml isopropyl alcohol was added to the tube. Samples were incubated at 20 ^{0}C for 5 min and centrifuged at 12000 g for 10 min at 4 ^{0}C. After centrifugation, RNAs observed were washed with 1 ml of 75% ethanol, vortexed and centrifuged at 7500 g for 5 min at 4 ^{0}C. Finally, RNAs were resuspended with 40 μl of RNase-free distilled H_2O and the quality and amount of each sample was measured by Nanodrop ND-2000 spectrophotometer (Thermo Scientific, DE, USA).

Reverse transcriptase reactions and real-time PCR

Stem-loop RT-primers specifically designed for miR-17 and U6 snRNA were used instead of random primers. After the mixtures for each sample were prepared, they were incubated at 25 ^{0}C for 10 min, at 42 ^{0}C for 1 h, at 70 ^{0}C for 10 min, and then stored at 4 ^{0}C. The mixture for reverse transcriptase reactions was 1 μl Stem-Loop RT-Primer (1μM, for miR-17 or U6 snRNA), total RNA (50 ng), 1 μl dNTP mixture (10 mM), 1 μl RNase inhibitor (50 U/μl), 4 μl Reverse Transcriptase Buffer (10X), 1 μl Reverse Transcriptase (200 U/μl) and RNase-free dH_2O up to 20 μl.

For RT-PCR reactions, specifically designed forward and reverse primers of miR-17 and U6 snRNA were used. The reactions were run at 50 ^{0}C for 2 min, at 95 ^{0}C for 10 min, then at 95 ^{0}C for 15 sec, at 59 ^{0}C for 30 sec, at 72 ^{0}C for 30 sec x 40 cycles and finally at 72 ^{0}C for 10 min in LightCycler Real-Time PCR Machine. The mixture of the reactions was prepared as follows: 12.5 μl SYBR Green Mix (2X), 1 μl primer mix (forward and reverse primers for miR-17 and U6 snRNA, 5 pmol), 1 μl reverse transcript, 10.5 μl distilled H_2O. All reactions were performed in triplicate.

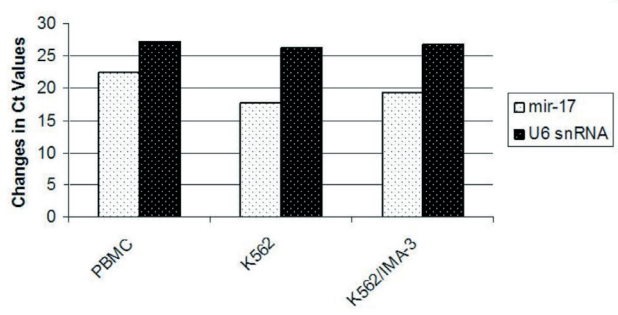

Figure 1. Changes in Ct values in PBMC, K562 and K562/IMA-3 cells.

Statistics

The change of miR-17 gene expression profiling was analysed by using the Livak method (ΔΔCT method). For realizing the Livak method to the samples, there are 3 steps to follow. These are normalization of the Ct value of the target gene to the Ct value of the reference gene, normalization of the ΔCt value of the test sample to the ΔCt value of calibrator, and calculation of the expression difference which is fold change.

Results

Significant increase was observed in K562 and K562/IMA-3 cells

We generated K562/IMA-3 cells that showed 52-fold more resistant to imatinib as compared to parental sensitive K562 cells previously [18]. In this study, we aimed to examine the changes in expression levels of miR-17 in healthy, imatinib sensitive and resistant cells.

The results showed that Ct values were down in CML cells, revealing that the expression levels of miR-17 were going up in CML cells as compared to healthy PBMCs while there were no significant changes in the expression levels of U6 snRNA (Figure 1).

1. ΔCt (PBMC) = Ct (miR-17, PBMC) – Ct (U6 snRNA, PBMC)ΔCt (PBMC) = 22.5 – 27.3 = -4.8
   ΔCt (K562) = Ct (miR-17, K562) - Ct (U6 snRNA, K562)
   ΔCt (K562) = 17.7 – 26.2 = -8.5
2. ΔΔCt = ΔCt (K562) - ΔCt (PBMC)
   = -8.5 – (- 4.8) = -3.7
3. $2^{-\Delta\Delta Ct}$ = Normalized fold change
   $2^{-(-3.7)}$ = 12.99

The calculated fold change indicated that K562 cells expressed miR-17 12.99-fold higher than PBMCs (Figure 2).

ΔCt (PBMC) = Ct (miR-17. PBMC) – Ct (U6 snRNA, PBMC)
ΔCt (PBMC) = 22.5 – 27.3 = -4.8
ΔCt (K562/IMA-3) = Ct (miR-17, K562/IMA-3) - Ct (U6 snRNA, K562/IMA-3)
ΔCt (K562/IMA-3) = 19.2– 26.8 = -7.6
ΔΔCt = ΔCt (K562/IMA-3) - ΔCt (PBMC)
= -7.6 – (- 4.8) = -2.8
$2^{-\Delta\Delta Ct}$ = Normalized fold change
$2^{-(-2.8)}$ = 6.96

The calculated expression difference indicated that K562/IMA-3 cells expressed miR-17 at a 6.96-fold level higher than PBMCs (Figure 2).

ΔCt (K562/IMA-3) = -7.6
ΔCt (K562) = -8.5
ΔΔCt = ΔCt (K562-sensitive) - ΔCt (K562 imatinib-resistant) = -8.5 – (-7.6) = -0.9
$2^{-\Delta\Delta Ct}$ = $2^{-(-0.9)}$ = 1.87

The expression levels of miR-17 were 1.87-fold higher in K562 cells as compared to K562/IMA-3 cells (Figure 2).

Tyrosine kinase inhibitors decreased the expression levels of miR-17 in a dose-dependent manner in K562/IMA-3 cells

The effects of nilotinib and dasatinib on the expression level of miR-17 in K562/IMA-3 cells were also examined. The average Ct values were calculated for each sample (Figure 3). The results showed that Ct values were going up in response to imatinib, dasatinib and nilotinib treatment, indicating that the expression levels of miR-17 were going down after drug treatment, while there were no significant changes in the expression levels of U6 snRNA (Figure 3).

The fold changes in the expression of miR-17 in each sample were calculated by using the Livak method by normalizing each sample to K562/IMA-3 cells. As shown in Figure 4, there was 77% decrease in the expression levels of miR-17 in response to 5 μM imatinib as compared to K562/IMA-3 cells and normalized to U6 snRNA. On the other hand, 87 and 90% decreases in the expression levels of miR-17 were detected in K562/IMA-3 cells treated with 2 and 5 μM nilotinib, respectively (Figure 4), while 0.2 and 0.5 μM dasatinib application resulted in 93 and 96% decreases in the expression levels of miR-17, respectively.

These results showed that imatinib, nilotinib and dasatinib reduce the expression of miR-17 in imatinib-resistant CML cells. On the other hand, extreme fold changes in the expression levels of miR-17 in CML cells in the presence of different tyrosine kinase inhibitors treatment proved that miR-17 may be a crucial target for the treatment of CML.

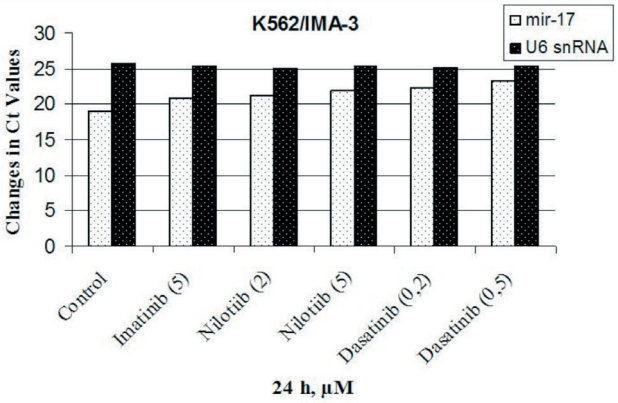

Figure 3. Changes in Ct values in K562/IMA-3 cells treated with imatinib, dasatinib and nilotinib.

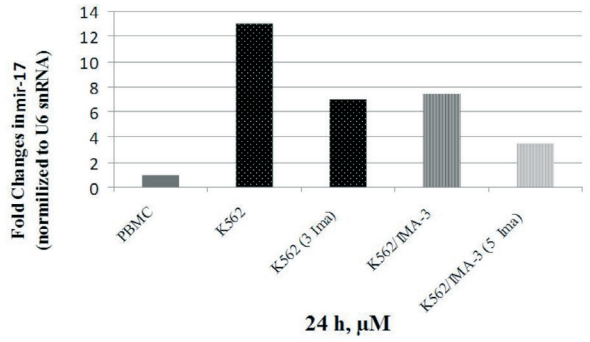

Figure 2. Fold changes in expression levels of miR-17 in PBMC, K562, 3 μM imatinib treated K562, K562/IMA-3, and 5μM imatinib treated K562/IMA-3 cells.

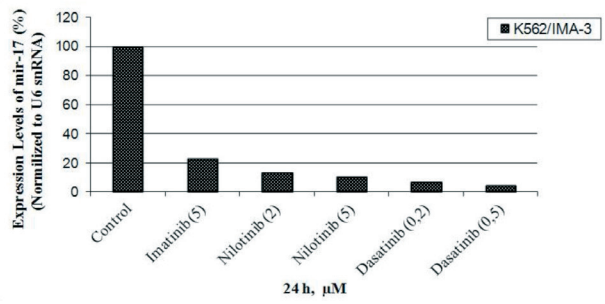

Figure 4. Percent changes in expression levels of miR-17 in K562/IMA-3 cells treated with imatinib, dasatinib and nilotinib.

Discussion

It was shown by Zhang et al. that miR-17 plays critical roles in the proliferation and metastasis of colon cancer [19]. It was also demonstrated that miR-17 can be used as a molecular marker for the detection of gastric cancer [20]. Additionally, miR-17 has oncogenic role in colorectal carcinoma cells as compared to the normal tissues and hence it could be accepted as a therapeutic target for the treatment of colorectal carcinoma [21].

Taking together, the results of our group and of some other groups show that miR-17 has significant roles in the initiation of cancer. Thus, this study is important in showing the involvement of miR-17 in the initation of CML, and the development of drug resistance. Treatment of CML cells with different concentrations of imatinib, nilotinib and dasatinib reduced the expression levels of miR-17 in drug-resistant cells. Since the upregulation of miR-17 was detected in CML cells, miR-17 can be used as a diagnostic and/or therapeutic target for detection and/or treatment of CML.

Aknowledgement

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