Orijinal Araştırma / Original Investigation
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Global DNA Methylation Analysis of Imatinib Resistant and Sensitive K562 Cells

İmatinibe Dirençli ve Duyarlı K562 Hücrelerinin Global DNA Metilasyon Analizi

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ÖZET

AMAÇ: Kronik miyeloid lösemi (KML), Philadelphia kromozomunun (Ph+) varlığı ile bilinen hematolojik bir hastalıktır. BCR-ABL proteini Ph+ kromozomu tarafından ifade edilir ve sürekli olarak artmış tirozin kinaz aktivitesi göstermektedir. İmatinib (IMA), KML'de ilk basamak tedavi olarak kullanılan bir tirozin kinaz inhibitörüdür (TKI). Tedavinin bir noktasında IMA direncinin ortaya çıkması tedavi başarısızlığına yol açar. DNA metilasyonu en stabil epigenetik değişiklik olarak kabul edilir ve epigenetik değişikliklerin ilaç direncinde rol oynayabileceği çeşitli çalışmalarda gösterilmiştir. Bu çalışmada, IMA'ya karşı gelişen dirençte ve bu direnç nedeniyle fenotipte görülen değişimde epigenetik yeniden programlamanın rol alıp almadığını belirlemek amacıyla, IMA'ya duyarlı K562S, IMA'ya dirençli K562R ve IMA'ya dirençli ve yapışan K562R (K562R-adh) hücrelerinin global metilasyon profilini araştırdık.

GEREÇ VE YÖNTEM: Bu çalışmada, global DNA metilasyon profilini analiz etmek için morfolojik olarak farklılık gösteren, IMA'ya karşı duyarlı K562S ve 5μM IMA'ya karşı dirençli K562R ve K562R-adh in-vitro KML hücre modelleri kullanılmıştır. Hücrelerden DNA izole edildikten sonra, eşit miktardaki DNA'lar kullanılarak ELISA yöntemi ile global 5mC DNA metilasyon profilleri araştırılmıştır.

BULGULAR: K562R'nin global metilasyonu K562S'ye göre kıyaslandığında, DNA metilasyon profilinde artış saptanırken, metilasyondaki bu artış istatistiksel olarak anlamlı bulunmamıştır. Buna karşılık, K562R-adh ile K562S ve K562R-adh ile K562R kıyaslandığında, K562R-adh hücrelerinin global DNA metilasyon profilinde K562S ve K562R hücrelerinine göre istatistiksel olarak anlamlı derecede artış saptanmıştır.

SONUÇ: K562S, K562R ve K562R-adh hücre hatlarında CpG adalarındaki 5metil-Sitozin (5mC)'de gözlemlediğimiz farklılıklar, dirençli hücrelerdeki DNA metilasyon değişikliğinin fenotip değişimine kısmen katkıda bulunabileceğini düşündürmektedir.

Anahtar Kelimeler: kronik miyeloid lösemi, imatinib direnci, global metilasyon, EMT

ABSTRACT

OBJECTIVE: Chronic myeloid leukemia (CML) is a hematological disease which is known for the presence of Philadelphia chromosome (Ph+). BCR-ABL protein is expressed by Ph+ chromosome, represents constant increased tyrosine kinase activity. Imatinib (IMA) is a tyrosine kinase inhibitor (TKI) which is utilized as a first line treatment in CML. Emergence of IMA resistance at some point of therapy leads to treatment failure. DNA methylation is considered to be the most stable epigenetic change and several studies have shown that epigenetic changes may play a role in drug resistance. In this Study, we investigated the global methylation profile of IMA-sensitive K562S, IMA-resistant K562R and IMA-resistant and adherent K562R (K562R-adh) cells to determine whether epigenetic reprogramming is involved in the resistance to IMA and the change in phenotype due to this resistance.

MATERIALS AND METHODS: In this study, morphologically distinct, IMA-sensitive K562S and 5μM IMA-resistant K562R and K562R-adh in-vitro CML cell models were used to analyze the global DNA methylation profile. After DNA was isolated from the cells, global 5mC DNA methylation profiles were investigated by ELISA using equal amounts of DNA.

RESULTS: Compared to K562S, the global methylation of K562R showed an increase in DNA methylation profile, but this increase in methylation was not statistically significant. Whereas, a slight hypermethylation was observed in the DNA of the K562R-adh vs K562S and K562R-adh vs K562R which is statistically significant. We observed slight hypermethylation in IMA-resistant cells lines versus to the IMA-sensitive cell line.

CONCLUSION: Our observed differences in 5methyl-Cytosine on CpG islands (5mC) in K562S versus K562R and K562R-adh cell lines suggest that the DNA methylation alteration in resistant cells may partly contributed in phenotype switching.

Keywords: chronic myeloid leukemia, Imatinib resistance, Global methylation, EMT

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INTRODUCTION

Chronic myeloid leukemia (CML) is a type of hematological malignancy which is known to impact hematopoietic stem cells. The Philadelphia chromosome's (Ph+) presence is the hallmark of CML (1, 2). The reciprocal translocation of chromosomes 9 and 22 results in the generation of the BCR-ABL fusion protein production. The protein is expressed by Ph+ chromosome, represents constant increased tyrosine kinase activity (2). Imatinib (IMA) is a tyrosine kinase inhibitor (TKI) which is used as a first line therapy to overcome the constitutive tyrosine kinase activity of BCR-ABL protein in CML (3). Although IMA treatment is successful in clinical routine, patients develop IMA resistance at some point of therapy (4). In most cases IMA resistance caused by the mutations of the kinase domain and /or the amplification of BCR-ABL gene (5). Different BCR-ABL independent mechanisms, including overexpression of efflux transporters, inhibition of apoptosis pathway, aberrant epigenetic modification and dysregulation of microRNA expression are involved in IMA resistance (6, 7). Phenotype switching is the mechanism which is contributed to drug resistance in different cancer types (8). One of the processes causing the epithelial-to-mesenchymal transition (EMT) is probably epigenetic regulation (9).

Epigenetic processes are categorized in three classes: DNA methylation, histone modifications and noncoding RNAs (10). In CML, aberrant DNA methylation frequently occurs, with gene hypermethylation predominately over hypomethylation (11). DNA methylation is considered as the most stable epigenetic alteration (11). This process related to the attachment of a methyl group (CH_3) at the 5'carbon position of cytosine residues in CpG islands (12). There are studies reported that, DNA methylation in hematological diseases including CML can happen regardless of genetic background (13). Considering DNA methylation's significant role in leukemogenesis, it can be a valuable biomarker for leukemia prognosis and treatment response prediction (14).

In our previous study, we showed that constant exposure of IMA-resistant K562R cells to 5 µM IMA led adherent phenotype development (15).

In this study, to determine epigenetic reprograming of each cell type on cell morphology, we analyzed the global methylation pattern of IMA-sensitive K562S, IMA-resistant K562R and K562R-adh cells.

MATERIAL & METHODS

Cell Culture

The CML cell line K562S (IMA-sensitive K562) cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) media at 37°C under 5% CO_2 supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The same media supplemented with 5 µM IMA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to sustain the K562R (IMA- resistant K562) cells (6). The stock solution of IMA was prepared at a final concentration of 10 mM since IMA is soluble in water. Every month, it was prepared fresh and kept at -20°C. Every three days, the cell line was passaged and the IMA-containing medium was added.

A subpopulation of adherent cells (K562R-adh) is generated when 5 µM IMA is present in the medium of K562R cells. Adherent cells grew in the presence of IMA for 18 months after discarding the K562R suspended cells (15).

DNA extraction

Using the Quick-DNA TM Miniprep Plus Kit, genomic DNA was isolated from $5x10^6$ cells of the K562S, K562R and K562R-adh using (Zymo research/Irvine, CA, USA) according to the manufactures' instructions. Cells were centrifuged at 500g for 2 min. Then, supernatant was discarded and the pellets were suspended in 1 ml PBS. Later, cells were centrifuged at 500 g for 5 min and resuspended in 200 µl of BioFluid & Cell buffer. In the next step, cells were treated with 20 µl of Proteinase K for 10 min at 55°C. Genomic binding buffer (1 volume) was added to the sample and mixed for 10-15 sec. Later, the mixture was transferred to the Zymo-SpinTM IIC-XLR Column in a Collection Tube. The cells were centrifuged at 12000 g for 1 min. Spin column transferred to the new collection tube and incubated with DNA Pre-Wash Buffer (400 µl). Then, the cells were centrifuged at 12000 g for 1 min. Cells were washed with g-DNA Wash Buffer (200 µl) and centrifuged at 12000 g for 1 min, this step repeated twice. The flow-through collection tube was disposed of. Spin columns were transferred to the lean microcentrifuge tube and 50 µl DNA Elution Buffer was added to each sample. A spectrophotometer was used for quantity and quality assessment of the isolated DNA (BioTek Instruments, Inc./Winooski, VT, USA).

Global DNA Methylation Analysis

To analyze global methylation level in K562S, K562R and K562R-adh cell, we utilized the 5-mC DNA ELISA Kit (Zymo Research/ Irvine, CA, USA). The global DNA methylation level analysis according to the manufacturer's protocol. For analysis, 100 ng of each DNA sample was used.

The absorbance values of seven standards that were created by combining the positive and negative controls included with the kit were used to create the standard curve.

The absorbance of each sample was measured at 405 nm using an ELISA plate reader (BioTek Instruments, Inc. / Winooski, VT, USA).

Statistical analysis

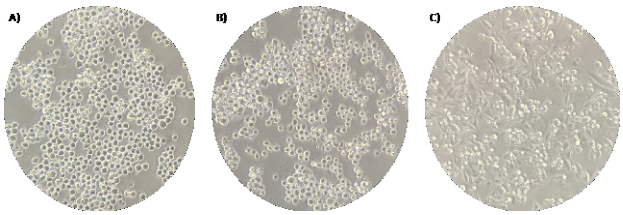
Statistical software Graph Pad Prism was utilized for the analysis of the differences between the K562S, K562R and K562R-adh cells. The sample means were compared using a one-way ANOVA, and the groups that were significantly different were compared pairwise using the Tukey Multiple Comparison test. The results were represented as mean ±SD. $P < 0.05$ was considered as statistically significant.

RESULTS

Global DNA Methylation in CML Cell lines

The mean global level of DNA methylation in K562S, K562R and K562R-adh cells (Figure 1) were 1.2% ± 0.014, 1.305% ± 0.035, and 1.63% ± 0.084 respectively. The mean methylation levels of K562R-adh cells is slightly higher than K562S, K562R cells, which are statistically significant $p < 0.05$ (Figure 2).

Figure1. *Phase-contrast microscopic images of CML cells before DNA isolation (magnification 400). A) K562S cells; B) K562R cells; C) K562R-adh cells.*

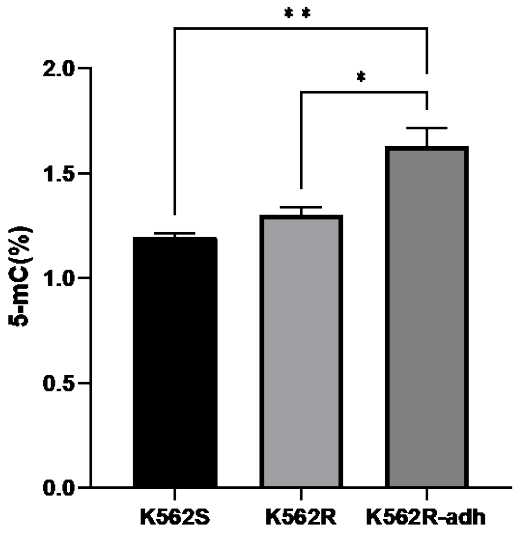


DISCUSSION

CML is a hematopoietic disorder, which is distinguished by the presence of BCR-ABL fusion protein (16). In addition to the BCR-ABL dependent mechanisms, there are BCR-BL independent mechanisms, which are involved in IMA resistance in CML (6). Phenotype switching is one of the alternative mechanism which was reported by Kemper and colleagues in 2014 (8).

DNA methylation profile changes based on the differentiation stage and type of the cell. For instance, compared to lymphoid cells, myeloid progenitors exhibit lower levels of global DNA methylation, which inhibits the myeloid differentiation pathway (17). DNA methylation suppresses gene transcription and cytosine residues at promoter regions in CpG islands (10). There are some studies have reported that epigenetic modifications accompany the phenotypic changes underlying EMT(18, 19). DNA methylation is one of the well- characterized mechanisms of epigenetic gene regulation and it may also play a role in EMT regulation (18). Moreover, gene hypermethylation and loss of function are frequently associated to drug resistance (5).

Figure 2. *5-mC (%) Global methylation level in K562S, K562R and K562R-adh. * P < 0.05 and **P < 0.001*



In this study, by using IMA sensitive and resistant CML cell line models, which are different in morphology, we aimed to evaluate the role of DNA methylation in IMA resistance and phenotype switching of the IMA resistant cells.

Several studies, which reported the increased methylation, profile in early phase of CML (17). In cancer, abnormalities in DNA methylation can be correlated with aberrant gene expression. Ko and colleagues performed a methylation analysis and RNA-seq analysis on CD^{34+} isolated from peripheral blood and bone marrow samples of healthy, CP (Chronic phase) and BP (Blast phase) of CML patients (20).

They reported that DNA hypermethylation events (>80%), frequently at promoters, are the main characteristic of BC transformation (20). Another study reported the aberrant promoter methylation of DAPK1, RASSF1A, p14ARFARF, RIZ1 and P16INK4A genes which have a strong association with poor IMA response, poor overall survival rates, and CML progression to advanced clinical stages (21).

In the study by Kaehler and colleagues, genome wide methylation analysis represented a slight increase in total methylation in IMA resistance K562 cells, which are resistant to 0.5 and 2 µM IMA. According to their study, PDE4DIP (phosphodiesterase 4D interacting protein), BCL2 (B-cell lymphoma 2), NMU (neuromedin U), IFI30 (gamma-interferon-inducible lysosomal thiol reductase) and DNASE2 (deoxyribonuclease-2-alpha) were identified as potential genes that were downregulated in IMA resistance-potentially by DNA methylation in their promoter region (7). Based on our results, there is not significant changes in the DNA methylation profile of K562R vs K562S. Whereas, a slight hypermethylation was observed in the DNA of the K562R-adh vs K562S, K562R-adh vs K562R. This slight increase which is observed between these groups is statistically significant. In our previous study, we performed bioinformatics analysis on the GSE120932 dataset. Based on our analysis and data reported by Baykal-Kose and colleagues, there were not dramatic differences in the number of the upregulated and down regulated genes between K562R vs K562S, K562R-adh vs K562S and K562R-adh vs K562R (15, 22). We did not observe dramatic changes in DNA methylation comparing resistant cells lines versus control CML cell line. Nevertheless, our observed differences in 5mC in IMA sensitive K5652S versus IMA resistant cell lines K562R and K562R-adh cell lines suggest that the DNA methylation alteration in resistant cells may partly contribute in phenotype switching.

Our results suggested that, in addition to the drug concentration, which is crucial in methylation patterns of the cells, switching the shape of the cells which can be caused by the induction of EMT or DNA hypermethylation in these cells.

CONCLUSION

It is not clear whether the observed epigenetic modifications are causally underlying the phenotype switching, or whether phenotype switching is merely a consequence of epigenetic modifications. Different mechanisms could involve in the methylation changes. In the future studies, the methylation profile of DNA, both on promoter sequences of EMT-related genes can be analyzed.

Etik: Bu çalışmanın etik kurulu alınmıştır.

Ethics committee approval had been taken.

Yazar katkı durumu; Çalışmanın konsepti; YH, dizaynı; YH, Literatür taraması; YH, verilerin toplanması ve işlenmesi; YH, istatistik; YH, yazım aşaması; YH,

Author contribution status; The concept of the study; YH, design; YH, literature review; YH, collecting and processing data; YH, statistics; YH, writing phase; YH,

Yazarlar arasında çıkar çatışması yoktur.

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