RESEARCH ARTICLE

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Enhanced Expression of miR-638-5p May Suppress Acute Myeloid Leukemia *in vitro* Cell Proliferation Through *PGK1 and PIM1*

ABSTRACT

Objective: miR-638-5p is a crucial tumor suppressor miRNA in several cancer types including, Acute Myeloid Leukemia (AML). This study aimed to analyze the role of miR-638-5p and its potential target genes in HL-60 and NB4 acute promyelocytic leukemia cell lines using *in vitro* method.

Method: After the miR-638-5p mimic transfection into AML cells, the effect on cell viability was examined by the WST-8 method, and the effect on apoptosis was measured via the Caspase-3 quantification method. *In silico* tools such as miRWalk, miRDB, and miRTarBase were used to select the possible target genes of miR-638-5p. The expression levels of selected genes were investigated by qRT-PCR. The overall survival (OS) rate of AML patients was explored via the BloodSpot database, the Enrichr tool was used for enrichment analysis, and correlation analysis was performed using the Correlation AnalyzeR tool.

Results: Decreased proliferation and increased apoptosis were determined in miR-638-5p mimic transfected cells compared to the controls. *MECP2*, *PIM1*, *MEF2C*, *PGK1*, and *SPAG1* genes were selected as the potential targets of miR-638-5p for *in vitro* study. *PGK1* and *PIM1* expression levels were significantly suppressed in cells transfected with the miR-638-5p mimic. The OS investigation revealed that overexpression of *MECP2*, *MEF2C*, and *PGK1* does not affect the survival of AML patients; however, overexpression of *SPAG1* and *PIM1* has a detrimental effect on AML survival. Also, a positive correlation was detected between *PIM1* and *PGK1* genes via enrichment analysis.

Conclusions: miR-638-5p may contribute to AML pathogenesis by targeting the *PGK1* and *PIM1* genes, and this situation may indicate its potential as a biomolecule for regulating cell proliferation in AML cells.

Keywords: miR-638-5p, Acute Myeloid Leukemia, HL-60, NB4, microRNA Mimic Transfection.

miR-638-5p'nin Ekspresyon Artışı *PGK1* ve *PIM1* Aracılığıyla Akut Miyeloid Lösemide *in vitro* Hücre Proliferasyonunu Baskılayabilir

ÖZET

Amaç: miR-638-5p, Akut Miyeloid Lösemi (AML) dahil olmak üzere çeşitli kanser türlerinde önemli bir tümör baskılayıcı miRNA'dır. Bu çalışma, miR-638-5p'nin ve potansiyel hedef genlerinin HL-60 ve NB4 akut promyelositik lösemi hücre hatları üzerindeki rolünü *in vitro* ortamda incelemeyi amaçlamaktadır.

Yöntem: miR-638-5p mimik transfeksiyonundan sonra AML hücrelerinin canlılığı üzerindeki etki WST-8 yöntemi ile apoptoz üzerindeki etki ise Caspase-3 kantifikasyon yöntemi ile incelendi. miRWalk, miRDB ve miRTarBase gibi *in silico* araçlar miR-638-5p'nin olası hedef genlerini seçmek için kullanıldı. Seçilen genlerin ifade düzeyleri qRT-PCR ile araştırıldı. AML hastalarının genel sağkalım (OS) oranı BloodSpot veritabanı üzerinden araştırıldı, zenginleştirme analizi için Enrichr aracı kullanıldı ve korelasyon analizi Correlation AnalyzeR aracı kullanılarak gerçekleştirildi.

Bulgular: Kontrollerle karşılaştırıldığında miR-638-5p mimik transfekte hücrelerde azalmış proliferasyon ve artmış apoptoz belirlendi. *MECP2*, *PIM1*, *MEF2C*, *PGK1* ve *SPAG1* genleri *in vitro* çalışma için miR-638-5p potansiyel hedefleri olarak seçildi. *PGK1* ve *PIM1* ekspresyon seviyeleri miR-638-5p mimic transfekte her iki hücrede de önemli ölçüde baskılandı. OS araştırması, *MECP2*, *MEF2C ve PGK1*'in aşırı ekspresyonunun AML hastalarının sağkalımını etkilemediğini; ancak *SPAG1* ve *PIM1*'in aşırı ekspresyonunun AML sağkalımı üzerinde zararlı bir etkiye sahip olduğunu ortaya koydu. Ayrıca, zenginleştirme analizi yoluyla *PIM1* ve *PGK1* genleri arasında pozitif bir korelasyon tespit edildi.

Sonuç: miR-638-5p'nin *PGK1* ve *PIM1* genlerini hedef alarak AML patogenezine katkıda bulunabileceği ve bu durumun AML hücrelerinde hücre proliferasyonunu düzenleyen bir biyomolekül olarak potansiyeline işaret edebileceği düşünülmektedir.

Anahtar Kelimeler: miR-638-5p, Akut Miyeloid Lösemi, HL-60, NB4, mikroRNA Mimik Transfeksiyonu.

INTRODUCTION

Acute Myeloid Leukemia (AML) is a type of leukemia that occurs with the accumulation of myeloid cells that cannot mature during the blood cell formation process. It is the most common type of acute leukemia among adults. It is responsible for 80% of adult acute leukemias and is a rare type of childhood leukemia. The disease mostly occurs due to de novo mutations in healthy individuals (1, 2).

MicroRNAs (miRNAs) are small, non-coding RNAs, consisting of 17- 25 nucleotides (3). It is possible to functionally investigate the roles of miRNAs in cellular processes through target genes by providing gain or loss of miRNA function in the cell through miRNA mimic and inhibitor transfection into the cell. (4). Although there are different methods, miRNA mimic transfection is generally applied via the lipofectamine-mediated method (5). miRNAs bind to target mRNA transcripts, causing transcript degradation or negative regulation of gene expression by repressing translation (6, 7). Sequences encoding miRNAs, whose main function is to regulate protein levels by causing mRNA degradation, constitute 1-3% of the human genome (8, 9). miRNAs are involved in many processes in cancer biology, such as apoptosis, metastasis, and proliferation (10, 11). Therefore, because miRNA expression can be associated with cancer type, cancer stage, patient response to treatment, and clinical changes, studies on the use of miRNAs as biomarkers, especially for cancer diagnosis and prognosis, have increased in recent years (12).

Studies have shown that miR-638-5p may have a significant role in many malignancies, including hepatocellular carcinoma (13), gliomas (14), lung cancer (15), breast cancer (7), stomach cancer (16), and leukemia (17). In these studies, decreased miR-638-5p levels were observed, and decreased miR-638-5p was associated with metastasis, poor prognosis, and a low survival rate for these disease types. miR-638-5p, which may have different target genes for each disease, causes a decrease in the expression of target genes by targeting oncogenes and thus acts as a tumor suppressor in the cell. Because of this feature, miR-638-5p, which has a tumor suppressor effect on cells, has been determined as a suitable candidate for therapeutic studies in various types of cancer and solid tumors (18).

Only one study has investigated the relationship between AML and miR-638-5p, which showed that decreased miR-638-5p was associated with deterioration of disease status, poor prognosis, and increased metastasis for AML (19). The effect of the association between miR-638-5p and its potential target genes on AML processes requires elucidation. Therefore, the present study investigated the function of miR-638-5p and potential target genes in AML cells. Initially, potential target genes of miR-638-5p were identified using *in silico* tools. The functional effects of ectopic miR-638-5p on AML cells were assessed, and the expression levels of possible target genes of miR-638-5p in AML cells were examined utilizing *in vitro* methods. The protein-protein interactions (PPI) association between possible target genes of miR-638-5p and their impact on AML survival was examined using *in silico* databases.

MATERIAL AND METHODS

Proliferation, Passage, and Cryopreservation of Cells: The HL-60 and NB-4 cells were cultured in RPMI-1640 medium containing 10% FBS and 1% antibiotic. Cells were cultured in an incubator at 37°C temperature condition with 5% CO_2 and passaged as needed. Cells were frozen and stored at -80°C in RPMI-1640 freezing medium containing 10% DMSO for use in other experimental stages.

miR-638-5p and Non-Targeting miRNA Mimic Transfection: Cells were counted, and 4x105 cells were placed in each well of a 6-well plate. RPMI-1640 medium containing 10% FBS without antibiotics was used for transfection. 24 hours after transplantation, miR-638-5p mimic transfection, and non-targeting miRNA mimic transfection were performed according to the Lipofectamine 2000 reagent protocol (Invitrogen, Carlsbad, CA, USA).

Observation of the Effect of miR-638-5p on Cellular Processes: The CVDK-8 kit (EcoTech Biotechnology) was used to examine the proliferation changes in cells transfected with the miR-638-5p mimic and non-targeting miRNA mimic via the WST8 method. The Caspase-3 Assay was used for the evaluation of the effects of miR-638-5p on apoptosis.

Identification of Potential Target Genes of miR-638-5p: In this study, some criteria were considered while selecting potential target genes of miR-638-5p; a) Since miR-638-5p is a tumor suppressor miRNA, genes reported to be overexpressed in cancers were selected, b) Genes targeted by miR-638-5p in at least one of the *in silico* databases miRWalk, miRDB, TargetScan, and miRTarBase were selected, c) Each of the selected genes has been shown to contribute to the cancer process by being targeted by miR-638-5p in cancer types other than AML.

Determination of the Effect of Potential Target Genes of MiR-638-5p on AML Overall Survival: The BloodSpot is a useful database that includes information about gene expression from AML patients that is crucial to the formation and maturation of blood cells. This tool has facilitated the rapid acquisition of an overview of gene expression patterns in healthy and malignant hematopoiesis for many researchers (20). By the

BloodSpot database, the effects of potential target genes of miR-638-5p on the overall survival (OS) rate of AML patients were explored in this research.

Enrichment Analysis: KEGG pathways and hub genes associated with the selected potential target genes of miR-638-5p were investigated using the Enrichr (21) tool, and correlation analysis was performed using the Correlation AnalyzeR (22) tool.

RNA Isolation: Total RNA was isolated 24 h after mimic transfection using TRIzol (Invitrogen). NanoDrop ND-2000c spectrophotometer (Thermo Fisher) was used to measure the concentration and purity of the isolated RNAs. The isolated RNAs were stored at -80 °C to be used in the necessary stages of the study.

cDNA Synthesis and qRT-PCR Analysis: The expression level of miR-638-5p was analyzed to determine the transfection level in HL-60 and NB4 cell lines. For this purpose, the synthesized cDNA samples were used in qRT-PCR analysis. qRT-PCR experiment was performed using TaqMan Universal Master Mix (Thermo Fisher), TaqMan miRNA probes (Thermo Fisher) and TaqMan control probes (RNU43) on the LightCycler480 (Roche). cDNA synthesis was performed with the SCRIPT Reverse Transcriptase kit and the β-actin gene was used in normalization as a housekeeping gene. The 5x HOT FIREPol EvaGreen qPCR Supermix (Solis Bio Dyne) was used in accordance with the manufacturer's protocol. Expression analysis of selected genes as candidate targets of miR-638-5p was performed.

Statistical Analysis: SPSS 28 software was used for the statistical analysis of the study data. Student's t-test was used to evaluate statistical significance, and according to the test results, data with a p-value less than 0.05 were considered significant. The $2^{-\Delta\Delta Ct}$ method was used for relative quantitation analysis of the qRT-PCR results. GraphPad Prism 9.3 software was used to create figures.

RESULTS

Ectopic expression of miR-638-5p inhibits AML Cell Proliferation: qRT-PCR analysis revealed that miR-638-5p levels were substantially elevated in transfected cells in comparison to control groups, indicating that transfection had occurred as expected (Figure 1). Cell viability was considerably reduced in HL-60 and NB4 cells transfected with mimic miR-638-5p, as assessed by the WST-8 method at 48 and 72 hours ($p<0,05$) (Figure 2A).

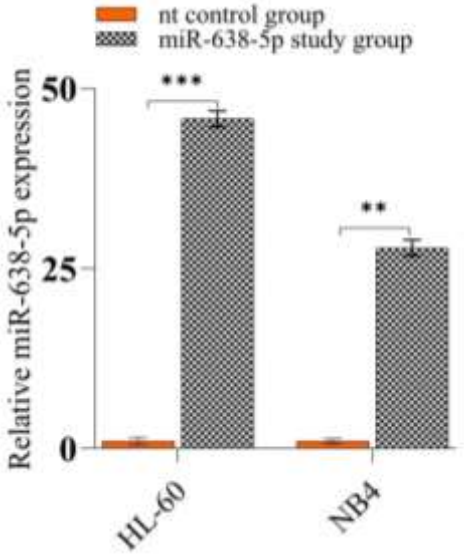

Figure 1. Validation of miR-638-5p mimic transfection efficiency (**p <0.01, ***p <0.001).

Overexpression of miR-638-5p Induces Apoptosis in AML Cells: After miR-638-5p transfection into NB4 cells, the amount of Caspase 3 showed a statistically significant increase compared to the control group (Figure 2B). The significant elevation of Caspase 3, recognized as an apoptosis marker, in miR-638-5p transfected cells relative to the control group suggests that miR-638-5p may induce apoptosis in AML cells.

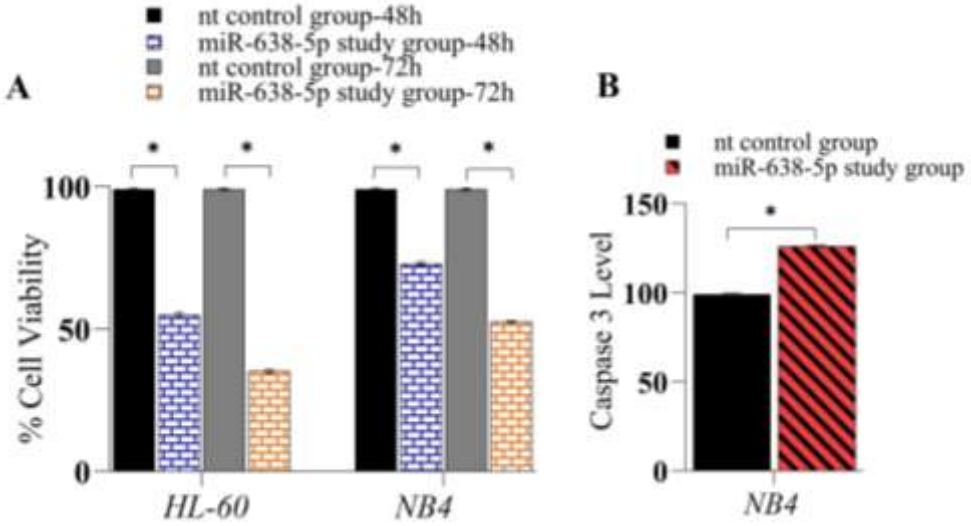

Figure 2. miR-638-5p mimic transfection A) inhibited proliferation and B) elevated apoptosis of the acute myeloid leukemia cells (**p <0.01, ***p <0.001).

miR-638-5p may Affect AML Prognosis via Potential Target Genes: According to the determined criteria; the *MECP2, PIM1, MEF2C, PGK1,* and *SPAG1* genes were selected as the targets of miR-638-5p for *in vitro* study. *In silico* investigations revealed that *PIM1* expression was elevated in complex cytogenetic anomalies, a poor prognostic indicator in AML (Figure 3). Furthermore, the results of the overall survival (OS) investigation showed that overexpression of *MECP2, MEF2C,* and *PGK1* does not affect the survival of AML patients; however, overexpression of *SPAG1* and *PIM1* has a detrimental effect on AML survival (Figure 4). Enrichment analysis results showed a positive correlation between *PIM1* and *PGK1* genes (Figure 5).

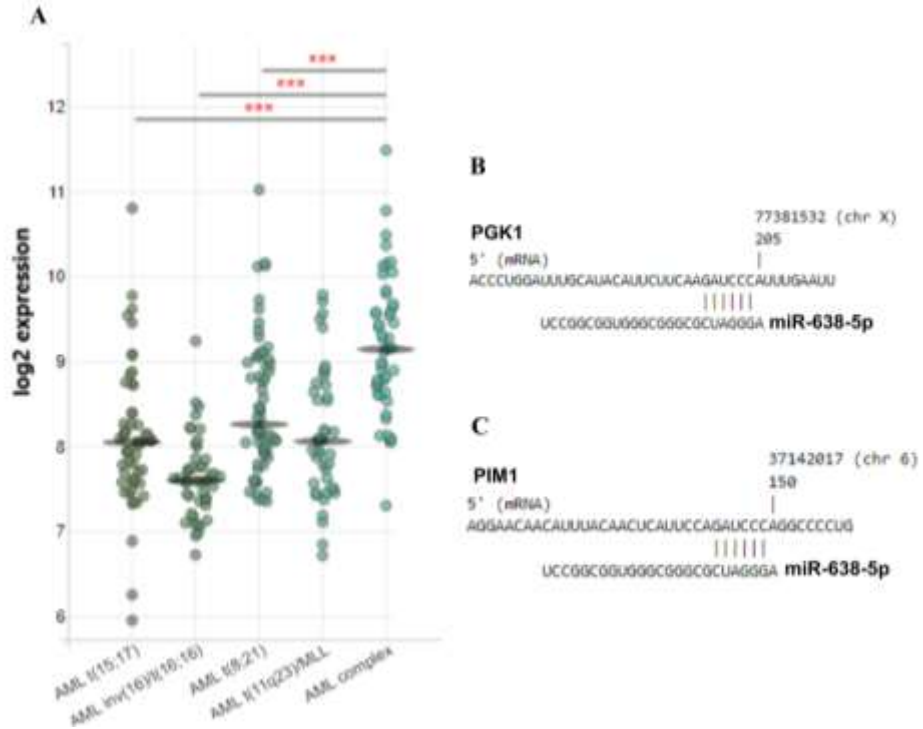

Figure 3. Analysis of the relationship between miR-638-5p, a selected target gene, and AML *in silico* A) Comparison of *PIM1* gene expression in cases of prevalent cytogenetic abnormalities associated with AML cases, B) Base pairing between miR-638-5p and *PGK1* and *PIM1*.

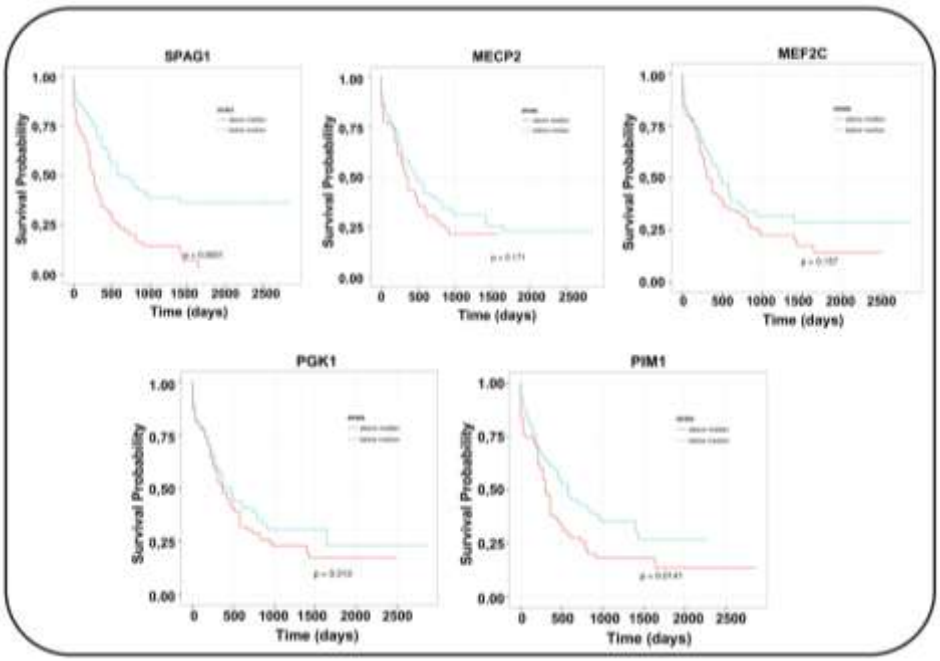

Figure 4. Determination of the impact of the potential target genes of miR-638-5p on the survival of AML patients. The overexpression of *MECP2, MEF2C,* and *PGK1* does not influence the survival of AML patients; conversely, the overexpression of *SPAG1* and *PIM1* adversely impacts AML survival.

Evaluation of the Expression Levels of Target Genes: The selected genes' (*MECP2*, *PIM1*, *MEF2C*, *PGK1*, and *SPAG1*) primer sequences were added to Table 1. *PIM1* and *PGK1* gene expressions were detected to be decreased in the miR-638-5p transfected group compared to the control group in both HL-60 (p=0.019 and p=0.003) and NB4 (p=0.010 and p=0.016) cell lines. On the other hand, it was determined that the expressions of *MECP2*, *MEF2C*, and *SPAG1* genes did not show a statistically significant change (Figure 5, Figure 6).

Table 1. Primer sequences used in qRT-PCR

	Primer Sequence	Reference
PIM1-F	5′- CCGTCTACACGGACTTCGAT-3′	(50)
PIM1-R	5′- CTGGCCCCTGATGATCTCTT-3′	
PGK1-F	5'-TCACTCGGGCTAAGCAGATT-3	(51)
PGK1-R	5'-CAGTGCTCACATGGCTGACT-3'	
MECP2-F	5'-ACTTCTGGCCCTGGTTAGGT-3'	(52)
MECP2-R	5'-CCGTGACCGAGAGAGTTAGC-3'	(52)
MEF2C-F	5'-GCACCAACAAGCTGTTCCAG-3'	(53)
MEF2C-R	5'-TGTCTGAGTTTGTCCGGCTC-3'	
SPAG1-F	5'-CCGCAGTGGTATAGCAACAG-3'	(54)
SPAG1-R	5'-GGCTTTCACGTTCCCATCAG-3'	
β-actin-F	5'-GCCTCGCCTTTGCCGATC-3'	(55)
β-actin-R	5'-CCCACGATGGAGGGGAAG-3'	

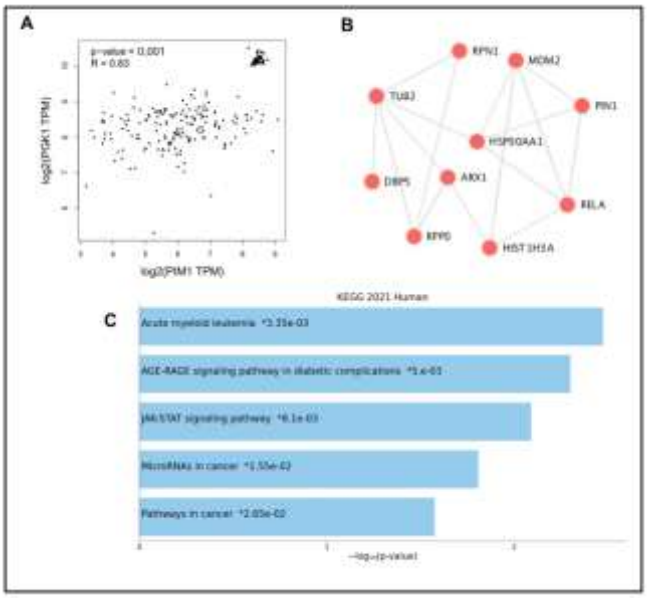

Figure 5. Enrichment analysis results of *PIM1*, a potential target gene of miR-638-5p. A) Positive correlation between *PIM1* and *PGK1* genes, potential target genes of miR-638-5p, B) 10 hub genes of *PIM1*, C) Pathways associated with *PIM1* according to KEGG 2021.

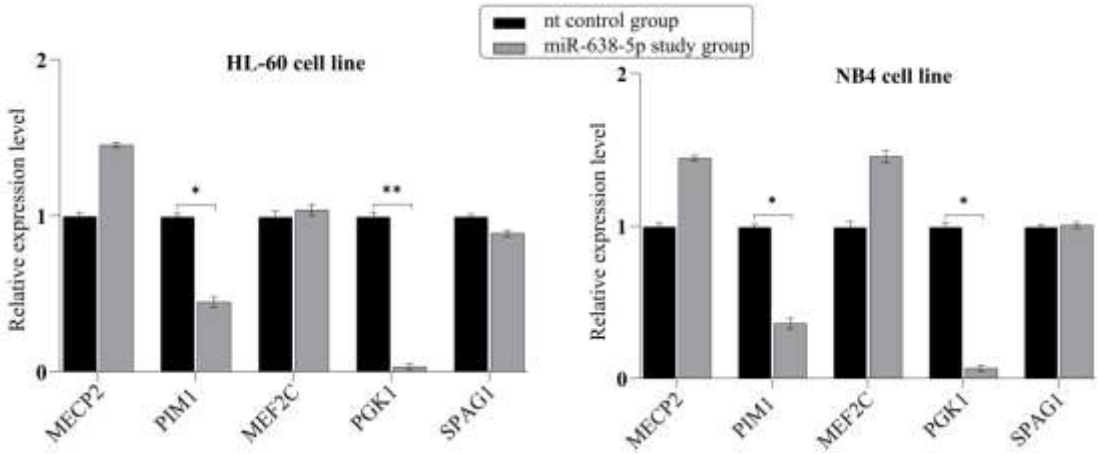

Figure 6. The relative expression level of potential target genes of miR-638-5p selected via *in silico* approaches A) HL-60 cells, B) NB4 cells (*p <0.05, **p <0.01).

DISCUSSION

Acute Myeloid Leukemia (AML) is a highly aggressive cancer, especially prevalent in the elderly population, characterized by poor therapeutic response, therefore presenting a substantial health challenge (23). Recent findings indicate that dysregulated miRNAs significantly influence AML pathogenesis by controlling cell differentiation, proliferation, and apoptosis. For instance, overexpression of miR-155 is frequently seen in AML and is associated with poor prognosis (24). The miR-181 family members are often downregulated in AML. Their inhibition results in increased expression of oncogenes and facilitates leukemic transformation (25). MiRNAs may serve as biomarkers for diagnosing AML and differentiating it from ALL. Garzon et al. showed that overexpression of the miR-20a, miR-25, miR-191, miR-199a, and miR-199b in AML were associated with clinical outcomes of AML patients such as poor prognosis and low survival rate (26).

miR-638-5p is one of the miRNAs that is associated with many diverse types of cancer and has been reported to play a role in the cancer formation process. miR-638-5p, which has been found to support tumor formation in cancers such as lung cancer (27), breast cancer (28), and melanoma (29), has shown tumor suppressor properties in types such as stomach cancer (16), colorectal cancer (30), and leukemia (31). In breast cancer and osteosarcoma, it can act both as a promoter of tumor formation and a suppressor of tumor formation by targeting different genes (32). Few studies have examined the relationship between miR-638-5p and AML (17-19). In one of these studies, miR-638-5p expression was found to be repressed in primary myeloid cells (19).

In this study, miR-638-5p transfection reduced the proliferation of both NB4 and HL-60 cells. This result was confirmed by data obtained from the measurement of apoptosis in NB4 cells. The data obtained were compatible with literature data, and miR-638-5p was observed to reduce cell viability and increase apoptosis in AML cells, and it was confirmed that it had a tumor suppressor effect on AML (14, 33, 34).

According to our gene expression results, a statistically significant decrease in the expression of *PIM1* and *PGK1* was observed in both NB4 and HL-60 cell lines in the miR-638-5p transfected group compared to the control group. On the other hand, no significant change was observed in the expression of *MECP2, MEF2C,* and *SPAG1* among the target genes in either cell line.

In our study *in silico* analyses showed that *PIM1* overexpression may be associated with poor survival in AML patients. Correlation analysis results revealed a significant positive correlation between *PIM1* and *PGK1*. Based on the findings of enrichment analysis, it seems that the *PIM1* gene may significantly contribute to the development of several malignancies, including AML. As a result of hub gene analysis, many of the 10 genes identified to be associated with *PIM1* were determined to be among the genes that play an important role in the AML process. For instance, *MDM2* serves as a negative regulator of the tumor suppressor *p53* and is often overexpressed in acute myeloid leukemia (AML) and many solid malignancies (35). *RPN1*, one of the *PIM1* hub genes, is crucial in the progression of many malignancies (36). *EVI1* is a low-expressed gene in normal hematopoietic cells; nevertheless, AML-characterized t(3;3), inv(3), or ins(3;3) chromosomal abnormalities lead to the juxtaposition of the promoter for the housekeeping gene *RPN1*, situated upstream of the *EVI1*, causing significant upregulation of *EVI1* (37). These examples as well as additional studies in the literature suggest that *PIM1* may be essential to AML processes via several AML-associated genes.

Studies show that miR-638-5p may inhibit cancer cell proliferation via *PIM1* in some cancers. For example, in a study by Wang et al., *PIM1* expression was increased in osteosarcoma cells, and it was concluded that it was associated with a low survival rate for this type of cancer, and increased miR-638-5p expression was reported to play a tumor suppressor role by inhibiting *PIM1* gene expression (38). On the other hand, the relationship between miR-638-5p and *PIM1* has not been investigated in AML as of yet. *PGK1* is a proto-oncogene associated with many types of cancer because it participates in DNA repair and angiogenesis. Increased *PGK1* expression in solid tumors, such as stomach cancer (39), ovarian cancer (40), brain tumors (41, 42), breast cancer (43), hepatocellular carcinoma (44), and prostate cancer (45), has been associated with tumor development and metastasis (46). miR-638-5p has been shown to contribute to the disease process via *PGK1* in human aortic endothelial cells (47). However, the miR-638-5p/*PGK1* axis has not been investigated in AML.

The current study investigated the role of ectopic miR-638-5p expression in AML cells, revealing that miR-638-5p may suppress AML cells via the *PIM1* and *PGK1* genes. We conclude that PIM1 and PGK1 genes may have the potential to play a key role in the pathogenesis of AML. The results of our study will contribute to the literature by demonstrating miR-638-5p/*PGK1* and miR-638-5p/*PIM1* relationships in AML cells.

Studies using mimic and inhibitor transfection approaches to investigate the functions and mechanisms of miRNAs have made it possible to investigate relevant miRNA-disease relationships *in vitro*. However, miRNA-mediated approaches are disadvantaged by the *in vivo* stability and availability of these molecules, which unfortunately limits the clinical applicability of miRNAs. Current studies have focused on alternative techniques such

as nanoparticle-mediated methods for the enhancement of miRNA stability and targeted delivery. Thus, miRNAs can be much more efficient in clinical applications in the future by overcoming these issues (48, 49).

Our study results indicate that miR-638-5p may play a role as a tumor suppressor in AML cells via the *PGK1* and *PIM1* genes and may be a candidate therapeutic miRNA for future studies. Even though these data at the mRNA level require confirmation with further studies. As a further study, the protein levels of *PIM1* and *PGK1* after miR-638-5p transfection can be investigated by western blot. Confirming whether miR-638-5p targets the 3' UTR of these target genes using the luciferase reporter assay method is another possibility for further investigation. It is particularly important to confirm the findings obtained in cell lines in the peripheral blood and bone marrow samples of patients with AML. Overall, these data will be valuable for the design of future *in vivo* animal experiments.

CONCLUSION

miR-638-5p transfection reduced proliferation, increased apoptosis, and targeted *PIM1* and *PGK1* oncogenes, reducing their expression in two AML cell lines, HL-60 and NB4. The results obtained in this study are especially important as preliminary data for future studies that will investigate the potential of miR-638-5p as a therapeutic biomarker in AML by targeting the *PIM1* and *PGK1* genes.

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Conflict of Interest: There is no conflict of interest.

Ethics Committee Approval and Informed Consent: Ethics committee approval is not required, patient samples were not incorporated, and only cell lines were included in this study.

Authorship Contributions: SOO (conception and design, or analysis and interpretation of the data; drafting the article or revising it critically for important intellectual content and approval of the final version), IS (conception and design, or analysis and interpretation of the data; drafting the article or revising it critically for important intellectual content and approval of the final version), MK (drafting the article or revising it critically for important intellectual content and approval of the final version), SO (conception and design, or analysis and interpretation of the data and approval of the final version).

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