ISTANBUL UNIVERSITY PRESS

RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1168793
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

THE EFFECT OF miR-34a-5p ON OVEREXPRESSED AML ASSOCIATED GENES

MiR-34a-5p'NİN AŞIRI İFADE EDİLEN AML İLİŞKİLİ GENLER ÜZERİNDEKİ ETKİSİ

Murat KAYA[1], İlknur SUER[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Division of Medical Genetics, Istanbul, Turkiye

ORCID IDs of the authors: M.K. 0000-0003-2241-7088; İ.S. 0000-0003-1954-4190

Cite this article as: Kaya M, Suer I. The effect of miR-34a-5p on overexpressed AML associated genes. J Ist Faculty Med 2023;86(1):59-68. doi: 10.26650/IUITFD.1168793

ABSTRACT

Objective: Acute myeloid leukemia (AML) is a deadly type of leukemia. The expression of AML-related genes may be altered not only by genetic changes but also by various epigenetic factors such as microRNAs (miRNAs). The expression levels of many genes can be altered by miRNAs. The detection of miRNA's target genes is critical for an understanding of the disease's molecular mechanism. In this study possible target genes of miR-34a-5p in AML were determined and the effect of the relationship between miR-34a-5p and target genes on the cancer process was investigated.

Materials and Methods: Leukemia Gene and Literature Database web tool (http://soft.bioinfo-minzhao.org/lgl/) includes a useful leukemia gene and literature da. There are more than 600 AML-related genes on this database. In the present study, in order to define the potential target genes of miR-34a-5p on the database, we used miRDB tool and then confirmed the findings using miRWalk, miRTarbase, Tarbase and miRNet tools. Defined miR-34a-5p AML related genes were verified by the DisGeNET platform. A Protein-Protein Interaction (PPI) network analysis of the genes was conducted using several bioinformatics tools. The effect of miR-34a-5p on cell proliferation was investigated by transfecting mimic miR-34a-5p into HL60 and NB4 cells. The mRNA expressions of *NOTCH2, IGF1R, SKP2* and *CDC25A* genes were investigated in miR-34a-5p transfected NB4 and HL60 cells and control groups.

Results: Using bioinformatics tools we determined 44 AML-related genes that could be targeted by miR-34a-5p. According to our in vitro study results statistically significant suppression of proliferation was observed in miR-34a-5p transfected cells (48h HL60 cells p=0.00011; NB4 cells p=0.0031 and 96h HL60 cells p=0.00013; NB4 p=0.00018). It was also found that *NOTCH2, IGF1R, SKP2* and *CDC25A* mRNA expressions were down-

ÖZET

Amaç: Akut miyeloid lösemi (AML) ölümcül bir lösemi türüdür. AML ilişkili genlerin ekspresyonu sadece genetik değişikliklerle değil aynı zamanda mikroRNA'lar (miRNA'lar) gibi çeşitli epigenetik faktörlerle de değiştirilebilir. MiRNA'lar birçok genin ifade seviyesini değiştirerek hücrede oldukça kritik görevler yapabilmektedir. miRNA ve hedef genleri arasındaki etkileşimin tespit edilmesi, hastalığın moleküler mekanizmasının aydınlatılması açısından oldukça önemlidir. Çalışmamızda AML dahil birçok kanserde tümör baskılayıcı role sahip miR-34a-5p'nin AML hücre proliferasyonu üzerindeki etkisi ve AML ilişkili genlerin ifade değişimindeki rolü araştırılmıştır.

Gereç ve Yöntem: Leukemia Gene and Literature Database web sitesi (http://soft.bioinfo-minzhao.org/lgl/)'de, lösemi ile ilişkili genleri içeren 600'den fazla AML ile ilgili gen bulunmaktadır. Bu web sayfasında yer alan miR-34a-5p'nin potansiyel hedef genlerini tanımlamak için yaptığımız bu çalışmada miRDB veri tabanı kullanılmıştır. Sonrasında miRWalk, miRTarbase, Tarbase ve miRNet araçlarıyla doğrulanmıştır. PPI etkileşimleri, yolak analizi, çeşitli biyoinformatik araçlar kullanılarak tanımlanmıştır. İn vitro olarak miR-34a-5p'nin AML hücreleri üzerindeki etkisi belirlenip *NOTCH2, IGF1R, SKP2* ve *CDC25A* genlerinin mimik miR-34a-5p ile transfekte edilmiş NB4 ve HL60 hücrelerinde ekspresyonu araştırılmıştır.

Bulgular: Çeşitli biyoinformatik araçlar kullanılarak miR-34a-5p tarafından hedeflenebilecek 44 AML ilişkili gen belirlenmiştir. Sonrasında yapılan in vitro çalışmada miR-34a-5p ile transfekte edilen hücrelerde proliferasyonun istatistiksel olarak anlamlı şekilde baskılandığı gözlenmiştir (48 saat HL60 hücreleri p=0,00011; NB4 hücreleri p=0,0031 ve 96 saat HL60 hücreleri p=0,00013; NB4 p=0,00018). miR-34a-5p mimik transfekte edilen NB4 ve HL60 hücrelerinde *NOTCH2, IGF1R, SKP2* ve *CDC25A* mRNA ifade seviyelerinin kontrol gruplarına göre anlamlı şekilde

Corresponding author/İletişim kurulacak yazar: Murat KAYA – kmurat@istanbul.edu.tr

Submitted/Başvuru: 31.08.2022 • **Revision Requested/Revizyon Talebi:** 05.09.2022 •
Last Revision Received/Son Revizyon: 22.11.2022 • **Accepted/Kabul:** 24.11.2022 • **Published Online/Online Yayın:** 27.01.2023

regulated in miR-34a-5p mimic-transfected HL60 cells (p=0.003; p=0.02; p=0.01; p=0.0009 respectively) and NB4 cells (p=0.02; p=0.02; p=0.01; p=0.0007 respectively) compared to the control groups.

Conclusion: miR-34a-5p may inhibit AML cell proliferation by targeting many genes like *NOTCH2, IGF1R, SKP2* and *CDC25A*. The results of our study indicate that appropriate bioinformatics tools and in vitro methods can successfully be used together when investigating the relationship between miRNAs and target genes. Further studies are required to determine the detailed relationship between these genes and miR-34a-5p.

Keywords: miR-34a-5p, AML, NB4, HL60

azaldığı tespit edilmiştir (HL60 hücrelerinde sırasıyla (p=0,003; p=0,02; p=0,01; p=0,0009) ve NB4 hücrelerinde sırasıyla p=0,02; p=0,02; p=0,01; p=0,0007).

Sonuç: miR-34a-5p; *NOTCH2, IGF1R, SKP2* ve *CDC25A* gibi birçok geni hedefleyerek AML hücre proliferasyonunu inhibe edebilir. Bu genler ile miR-34a-5p arasındaki ilişkiyi net bir şekilde belirleyebilmek için daha ileri tekniklerle farklı çalışmaların yapılmasına ihtiyaç vardır. Çalışma sonuçlarımız, miRNA-hedef gen ilişkisi araştırılırken uygun biyoinformatik araçlarla in vitro yöntemlerin birlikte başarıyla kullanılabileceğini göstermektedir.

Anahtar Kelimeler: miR-34a-5p, AML, NB4, HL60

INTRODUCTION

Acute myeloid leukemia (AML) is the most prevalent type of leukemia in adults (1). AML develops when blast cells expand clonally and invade the peripheral blood and bone marrow. Patients frequently suffer as a result of immature and ineffective erythropoiesis and bone marrow failure. Parallel to recent developments, treatment success rates have increased to 15% in individuals over 60 years of age and to around 40% in patients under the age of 60. Despite this, the prognosis remains very poor, particularly for elderly patients (2). Therefore, there is an urgent need for new research into the diagnosis, treatment, and prognosis monitoring of AML (3). MicroRNAs (miRNAs) are non-protein-coding small RNAs that are about 20 nucleotides in length (4). These small RNAs bind to the mRNAs of target genes and regulate their expression levels in the cell (5). There are over 2,000 human miRNAs and approximately 20,000 protein-coding genes (6, 7). Furthermore a miRNA may play a critical role in the regulation of numerous genes and the expression of a gene can be regulated by multiple miRNAs (8). To address this major issue various free and useful online programs based on base pairing have been developed for the detection of interactions between miRNAs and genes. Although these programs are extremely useful in miRNA-target gene research, the results of various miRNA-target gene web programs are frequently inconsistent (9, 10). As a result, when determining miRNA-target genes, it is critical to first select the genes by combining different bioinformatics tools, and then validate the findings using in vitro studies on cell lines. Therefore, we aimed to determine the miR-34a-5p target genes relationship in AML by combining the power of bioinformatics applications and in vitro methods. In the current study several bioinformatics tools were used to determine and verify the deeper associations of selected genes with AML and miR-34a-5p. The effect of miR-34a-5p on cell proliferation in HL60 and NB4 (AML cancer cell lines) was first evaluated. Then we attempted to determine the miR-34a-5p related genes involved in cancer cell proliferation.

MATERIAL AND METHODS

Bioinformatics assessment

Hundreds of AML-associated genes have been reported in the literature. A search using the Leukemia Gene and Literature Database web tool (http://soft.bioinfominzhao.org/lgl/) revealed that more than 600 genes are associated with adult AML. Potential target AML genes of miR-34a-5p in the database were identified using the miRDB tool. To verify AML-related genes, the DisGeNET platform was used. Protein-protein interaction (PPI) was demonstrated using the String tool (version 11.5) (https://string-db.org/). KEGG and GO enrichment analyses were performed using the Enrichr web server, after which miRTarbase, Tarbase, miRNet and miRWalk databases were used in order to define stronger candidate miR-34a-5p related genes. In this way, Neurogenic Locus Notch Homolog Protein 2 (*NOTCH2*), Insulin Like Growth Factor 1 Receptor (*IGF1R*), S-Phase Kinase Associated Protein 2 (*SKP2*), and Cell Division Cycle 25A (*CDC25A*) genes were selected. The GEPIA2 tool was used to define survival analysis. To uncover the relationship between the four genes and miR-34a-5p in cancers, especially in AML, the literature was searched using PubMed.

Cell line culture and miRNA mimic transfection

HL60 and NB4 AML cell lines were seeded into RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and the cells were incubated (with 37°C-5% CO_2). After 24 hours of incubation the cells were transfected with mimic miR-34a-5p for 48 hours and for 96 hours with assays. Lipofectamine 2000 reagent (Thermo Fisher Scientific Inc.) was used to transfect the cells with miR-34a-5p and the non-targeting control (nt control) with mimics (Ambion mirVana, Applied Biosystems). Transfection reagent was prepared by mixing 300 µL Opti-MEM medium with 3 µL from 10 µM stock miRNA mimic reagent and 9 µL of Lipofectamine 2000 reagent following the manufacturer's protocol. In brief, 30 pmol miR-34a-5p mimics were added to each well, and the same amount of nt control mimic was added to the control group (11).

Determination of the HL60 and NB4 cells' proliferation
Cell proliferation was defined by colorimetric cell viability (WST-8) assay using a CVDK-8 kit (EcoTech Biotechnology). In a 96-well plate, HL-60 and NB4 cell lines were seeded at around $5x10^3$ cells per well and cultured. To evaluate the effect of miR-34a-5p mimic transfection on cell proliferation, measurements were taken at 48 and 96 hours. 10 µL of CVDK-8 reagent was supplied to each well for measurement and incubated for three hours. Finally, a microplate reader (Thermo) capable of measuring absorbance at 450 nm was used to assess cell viability.

Total RNA isolation from HL60 and NB4 cells and qRT-PCR
Total RNAs from miRNA mimic transfected HL60 and NB4 cells were isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration was evaluated by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). cDNA synthesis and qRT-PCR processes were performed using a total of 1000 ng RNA obtained from both the study group and control group cells. The SCRIPT kit (Jena Bioscience) was used for cDNA synthesis and SybrMaster (Jena Bioscience) was used for qRT-PCR processes. All test steps were performed according to the kit manufacturers' protocols. *NOTCH2, IGF1R, SKP2,* and *CDC25A* gene expression levels were determined in miR-34a-5p transfected HL60 and NB4 cells as well as in the control groups. Table 1 shows the primer sequences for the genes studied.

Verification of the transfection process
TaqMan probes were used in qRT-PCR to detect the expression level of miR-34a-5p in the transfected cells. The cDNA of miRNA was constructed using 30 ng of total RNA and performed using the TaqMan miRNA reverse transcription kit (Applied Bio., Foster City, CA, USA) and miRNA RT primers according to the manufacturer's protocol. qRT-PCR was performed using TaqMan miR-34a-5p probes (Thermo Fisher Scientific Inc.), *RNU43* (control miRNA) probes (Thermo Fisher Scientific Inc.), and the TaqMan Universal Master Mix (Thermo Fisher Scientific Inc.) kit. The assay was carried out in duplicate, and the $2^{-\Delta\Delta Ct}$ method was applied for the analysis of relative quantitation. To normalize the expression of genes whose expression was investigated in the in vitro study step, *ACTB* primers were used as an internal control. Experiments were performed in duplicate. Relative quantification analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis
Analyses were performed using Student's t-test and the data were presented as mean±standard deviation. The data were considered statistically significant if the p-value was less than 0.05. Graphs were created with the GraphPad Prism 5 program and SPSS software ver.21(IBM Corp., Armonk, NY, USA). Overall survival analysis was generated using the Kaplan-Meier test via the GEPIA 2 tool. Points to consider when analyzing: Group cutoff: Median, Cutoff-High(%): 50, Cutoff-Low(%): 50, Hazards Ratio (HR): Yes, 95% Confidence Interval: Yes, Axis Units: Months

RESULTS

Bioinformatics analysis results

Identification of potential AML-related candidate genes
The miRDB database predicts 899 genes as potential targets of hsa-miR-34a-5p. We found that 44 genes overlap among 600 AML-related genes in http://soft.bioinfo-minzhao.org/lgl/ and that 899 genes are possible targets of miR-34a-5p in miRDB. The relationship between miR-34a-5p and the 44 selected genes was confirmed by miRTarbase, Tarbase, and miRNet, as presented in figure 1.

Table 1: Primers' List, that target unique sequences

Primer	Sequence	Reference
NOTCH2-F	5′-GGGACCCTGTCATACCCTCT-3′	(49)
NOTCH2-R	5′-GAGCCATGCTTACGCTTTCG-3′	
IGF1R-F	5′-TTTCCCACAGCAGTCCACCTC-3′	(50)
IGF1R-R	5′-AGCATCCTAGCCTTCTCACCC-3′	
SKP2-F	5′-ATGCCCCAATCTTGTCCATCT-3′	(51)
SKP2-R	5′-CACCGACTGAGTGATAGGTGT-3′	
CDC25A-F	5′-ATGAGGATGATGGCTTCG-3′	(52)
CDC25A-R	5′-AACACTGACCGAGTGCTG-3′	
ACTB-F	5′-GCCTCGCCTTTGCCGATC-3′	(53)
ACTB-R	5′-CCCACGATGGAGGGGAAG-3′	

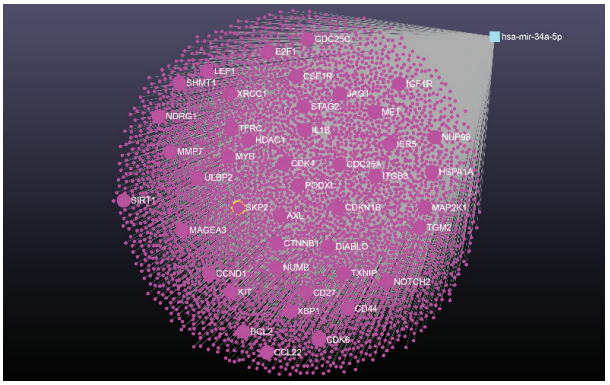

Figure 1: Forty-four AML-associated genes that are possible targets of miR-34a-5p. 5075 genes that may be targets of miR-34a-5p have been reported in the miRTarbase V8 and Tarbase V8 databases. The names of 44 genes studied in our study are shown in the figure as a larger circle. It was constructed using the miRNet tool (https://www.mirnet.ca/).

Confirmation of the selected potential AML-related genes

The association of 44 genes with AML and other cancers is shown in figure 2. Apart from 4 genes (*TGM2, ITGB3, HSPA1A1 and PODXL*), other genes were also associated with AML in the DisGeNET database. These 4 genes, which are not found in DisGeNET, were included in the study because they were found to be related to AML after the literature review (12-15). The fact that the vast majority of the 44 genes chosen from http://soft.bioinfo-minzhao.org/lgl/ database are also included in the DisGeNET database indicate that these genes could be strong AML-associated genes.

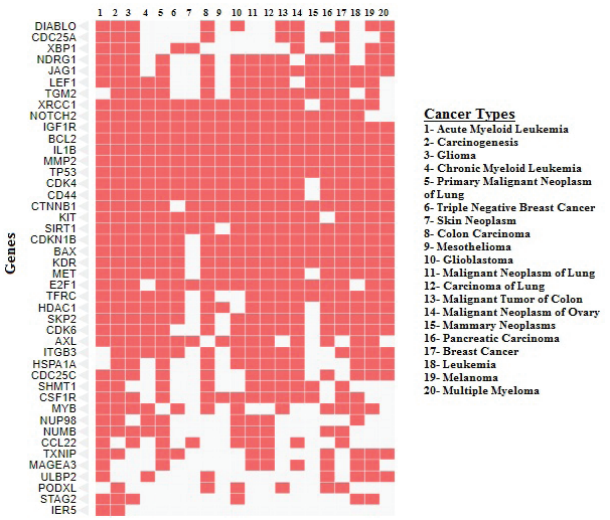

Figure 2: Forty-four genes are associated with other cancers, including AML. It was constructed via DisGeNET database (http://www.disgenet.org).

PPI of selected AML-related genes

The interaction of 44 genes suggesting there is a complex relationship between the selected genes except for the *SHMT1* gene is demonstrated in figure 3. Because this figure reveals that many of the selected genes may interact with the *TP53* gene, which is known as the genome's guardian, unraveling the interaction between the selected genes and miR-34a-5p may provide an essential clue in understanding the complicated molecular process of AML.

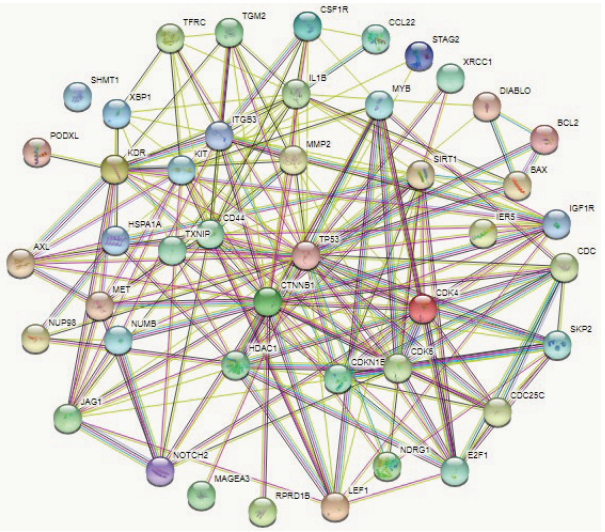

Figure 3: Using miR-34a-5p related 44 AML genes as seed, a total of 207 PPI enrichment was constructed by the STRING database (p<1.0e-16).(https://string-db.org/.)

Pathway analysis of selected AML genes

According to the results of the pathway analysis, 44 potential target genes of miR-34a-5p appear to be associated with many biological pathways. The analysis results show that one of the pathways most associated with these genes is the IGF1 pathway as seen in figure 4. In addition *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes were found to be in the IGF1 pathway as shown in figure 5. This suggests that developing a potential therapy strategy based on miR-34a-5p targeting these IGF1 pathway genes may be effective in the AML cancer process.

Investigation of the relationship between selected genes and miR-34a-5p in AML in the literature

According to our research of the literature using PubMed, we noticed that all of the *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes have critical roles in cancers including AML. However, it was observed that no study had yet been conducted showing that these genes are targeted by miR-34a-5p in AML cells.

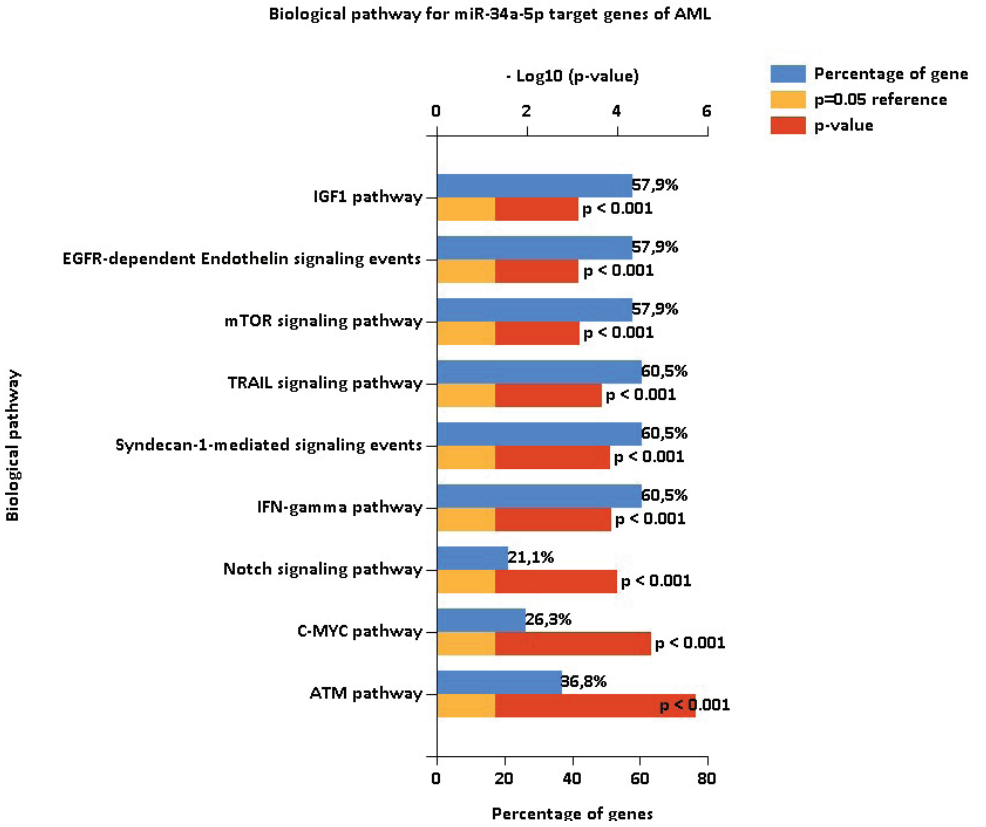

Figure 4: The defined 44 genes' pathway analysis results in AML. It was constructed using Funrich tool (http://www.funrich.org).

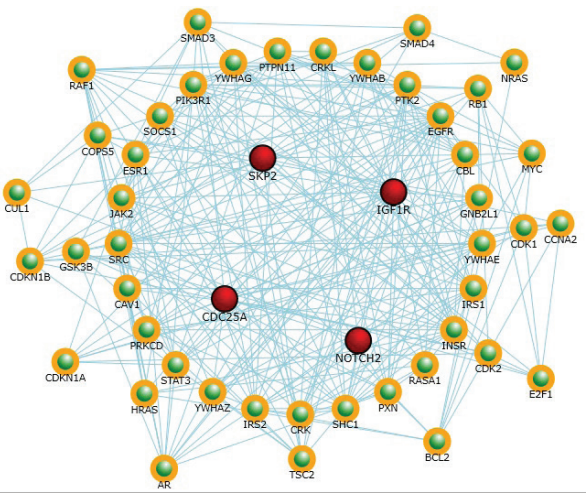

Figure 5: The *SKP2, IGF1R, CDC25A* and *NOTCH2* genes are related to the IGF1 Pathway. It was constructed via Funrich tool (http://www.funrich.org).

Identification of priority genes in elucidating AML/miR-34a-5p/target gene association

According to the TCGA data, it was detected that the *NOTCH2* gene expression level was noticeably increased in AML than in other cancer types as demonstrated in figure 6. The results of the overall survival (OS) analysis revealed that *IGF1R, SKP2*, and *CDC25A* genes had no statistically significant effect, but that the *NOTCH2* gene showed poor overall survival for AML patients as shown in figure 7. These findings suggest that *NOTCH2*, one of the 44 identified genes, is particularly significant in AML and that further research into the miR-34a-5p/*NOTCH2* axis might be beneficial.

In vitro study results

The effects of mimic transfection of miR-34a-5p on the AML cell proliferation

HL60 and NB4 cells transfected with mimic miR-34a-5p were found to have significantly higher expression of miR-34a-5p compared to the nt control group (figure 8). This indicates that mimic miR-34a-5p transfection into cells has been successfully achieved. It was determined that miR-34a-5p significantly reduced proliferation in mimic miR-34a-5p transfected HL60 and NB4 cells compared to the control group (figure 9). It was demonstrated that the values show a statistically significant decrease at both 48h measuring (HL60 p=0.00011; NB4 p=0.0031) and 96h measuring (HL60 p=0.00013; NB4 p=0.00018).

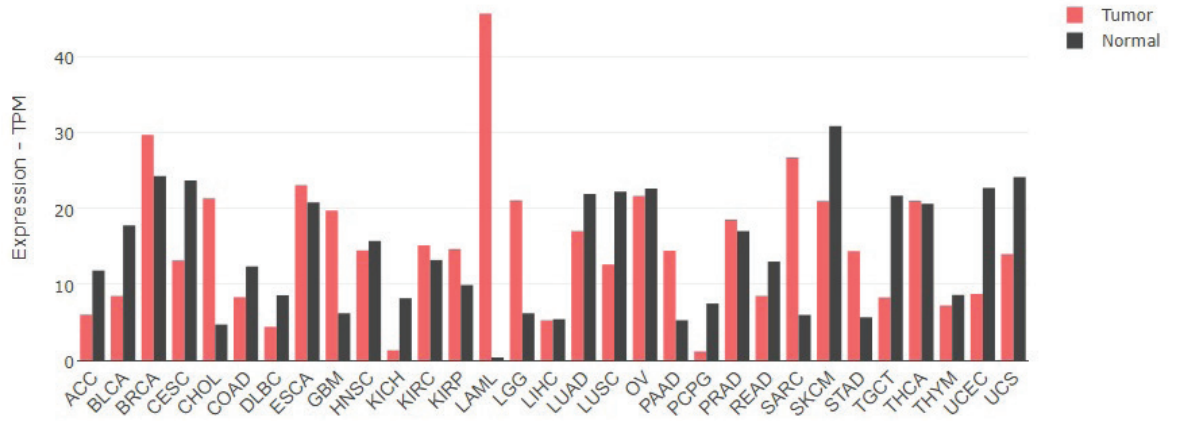

Figure 6: The expression levels of the *NOTCH2* gene in various cancer types. *NOTCH2* expression is overexpressed in many cancers, however, the increase in *NOTCH2* expression in AML is striking. LAML: Acute myeloid leukemia. TPM: Transcripts Per Million. It was constructed via GEPIA2 tool (http://gepia2.cancer-pku.cn/.).

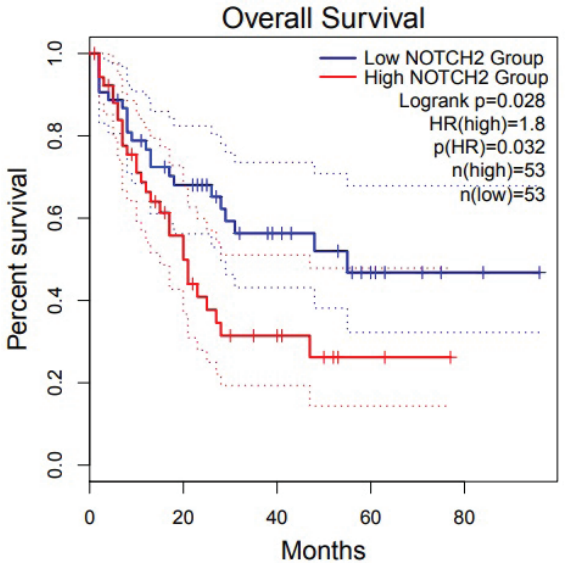

Figure 7: The impact of the *NOTCH2* gene on overall AML survival. It was created via GEPIA2 tool (http://gepia2.cancer-pku.cn/.).

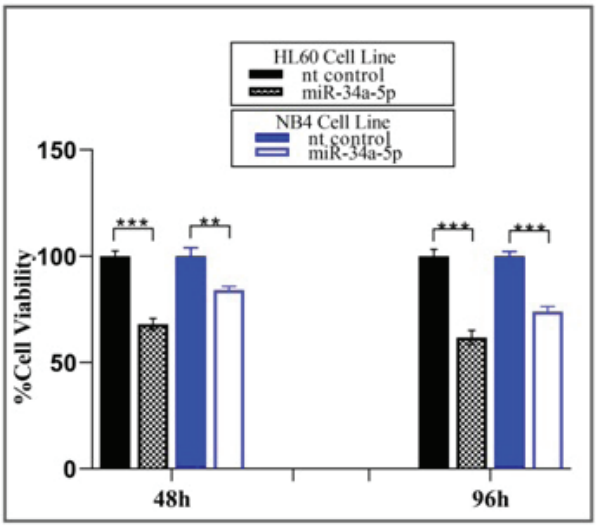

Figure 9: The effect of miR-34a-5p on cell proliferation in HL60 and NB4 cells (nt control: Non-targeting control. **p-value<0.01, ***p-value<0.001).

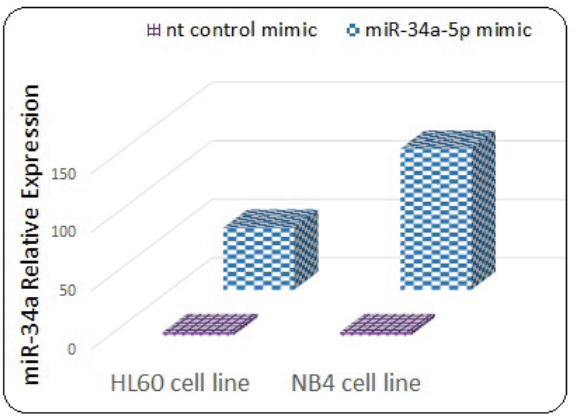

Figure 8: The transfection efficiency of miR-34a-5p in MM cells

qRT-PCR results of the selected genes

NOTCH2, IGF1R, SKP2 and *CDC25A* expression levels were detected to be statistically decreased in miR-34a-5p transfected HL60 cells (respectively p=0.003; p=0.02; p=0.01; p=0.0009) and NB4 cells (respectively p=0.02; p=0.02; p=0.01; p=0.0007) compared to the control groups (figure 10 and 11).

DISCUSSION

Over the last decade, studies have shown that miRNAs play crucial roles in nearly all biological events associated with AML, including cellular proliferation, migration, and metastasis. These findings support the idea that miRNAs could be used as biomarkers in AML. Although much has been learned about the roles of miRNAs in the initiation

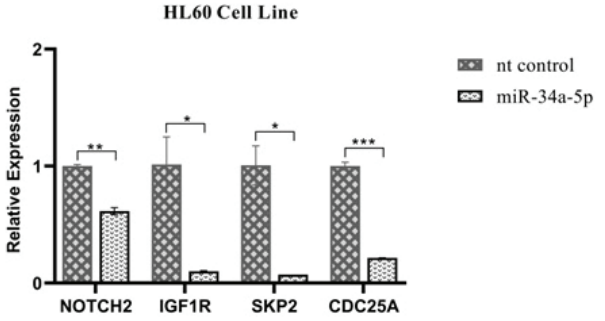

Figure 10: The expression value of selected genes in HL60 cells. *β*-actin gene expression was used for normalization (nt control: Non-targeting control, *p-value<0.05, **p-value<0.01, ***p-value<0.001).

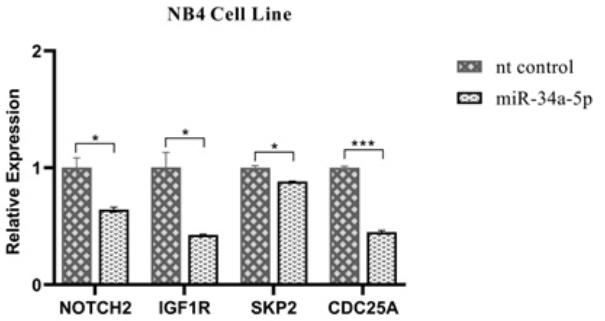

Figure 11: The expression value of selected genes in NB4 cells (nt control: Non-targeting control, *p-value<0.05, **p-value<0.01, ***p-value<0.001).

and progression of AML, many unanswered questions about the relationship between AML and miRNAs remain (16). MiR-34a-5p is a tumor suppressor, and miRNA (TsmiR) plays a vital role in oncogenesis and is essential for tumor progression inhibition (17, 18). Many studies in recent years have revealed that miR-34a-5p has a low expression level in various cancers due to the loss of its tumor suppressor effect (19-21). MiR-34a-5p is critical in the regulation of hematopoiesis. It has been reported, for instance, that miR-34a-5p can reduce mature B cells by targeting *FOXP1*, a B cell oncogene (22). MiR-34a-5p was shown to be significantly downregulated in patients with AML and cytogenetically normal-AML, and low expression of miR-34a-5p was linked with patients with intermediate/low-risk AML (23). MiR-34a-5p could influence the phenotype of leukemia cells in a variety of ways (22, 24, 25). Furthermore, miR-34a-5p is known to play a critical role in myeloid differentiation. Alteration of miR-34a-5p expression has been shown to reprogram granulocytic differentiation of blast cells with *CEBPA* gene mutations in AML (26). In the study by Liu X et al., miR-34a-5p expression was demonstrated to be downregulated in bone marrow mononuclear cells of AML patients and it was associated with tumor burden (27).

Our bioinformatics research and in vitro study results showed that *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes may be closely related to miR-34a-5p in AML. These genes have been implicated as oncogenes in a variety of cancers (28-31). For example, *IGF1R* overexpression has been shown to facilitate the progression of lung metastasis (31). Zhang et al. showed that overexpression of Skp2 in breast cancer induces breast cancer cell proliferation (32). Silencing *CDC25A* has been demonstrated to suppress the proliferation of liver cancer cells by downregulating *IL-6* (33).

It has been reported that these genes are also closely related to AML. NOTCH signaling pathway having four receptors including *NOTCH2* is important in AML cell survival, which is regulated by bone marrow stromal cells. This might be the rational evidence for novel AML eradication approaches that may become useful for diagnosis. It has been shown that chemosensitivity can be partially restored by blocking the *NOTCH2* gene in AML (34). IGF1R, another important gene in our study that may be associated with miR-34a-5p, is a tyrosine kinase transmembrane protein receptor. This gene activates signaling pathways by binding to insulin-like growth factor ligands and controls cell proliferation, differentiation and apoptosis in AML (35). The another crucial gene, *SKP2*, has been demonstrated to downregulate *C/EBPa* (CCAAT/enhancer-binding protein) expression via ubiquitin-dependent proteasome degradation, resulting in differentiation block in AML (36). Due to the increased proportion of *FLT3* mutation (25-30 percent of total of AML) and its association with poor prognosis, many *FLT3* inhibitors have been designed and tried in various clinical studies, either as a single drug or in a combination with chemotherapy. *CDC25A* is an early-stage target in *FLT3*-ITD oncogenic signaling and is a key player in proliferation and differentiation of arrest events in AML cells (37). Therefore, the relationship between miR-34a-5p and *NOTCH2, IGF1R, SKP2, CDC25A* may be of particular importance for AML.

It has been reported that these four genes are overexpressed in AML as in other cancers and are involved in the disease process (38-41). Furthermore, miR-34a-5p has been shown to directly target these genes in a variety of cancers and other diseases (42-45). A recent study, for instance, revealed that miR-34a-5p inhibits cervical tumor progression and migration by suppressing *CDC25A* (43).

The identification of genes controlled by miRNAs is a key difficulty in miRNA studies. Our study findings indicate that if proper bioinformatics tools and in vitro applications are utilized and combined, the AML/miRNA/Target genes link may be identified more accurately. The current study has filled an important gap in the literature by determining the possible target

genes of miR-34a-5p, using various bioinformatics tools, and then performing validation studies at the mRNA level in HL60 and NB4 cells. According to the pathway analysis of the present study, the majority of the 44 potential target genes of miR-34a-5p are related to the IGF1 pathway (Figure 4.) *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes, which are among the 44 genes in our study and whose expression levels decrease after miR-34a-5p transfection into HL60 and NB4 cells, are also related to the IGF1 pathway in the cell Figure 5). IGF1 signaling pathway is known to play a vital role in many important processes, including development, homeostasis, and aging. Many diseases, particularly cancers, are caused by various mutations in the genes involved in this pathway. AML has also been linked to irregularities in the IGF1 pathway. For instance, in a recent study, it was revealed that IGF1 autocrine plays a critical role in the constitutive *PI3K/AKT* activation of primary AML cells, and it was suggested that *IGF1R* could be targeted as a potential new treatment option (39). This demonstrates how important the IGF1 signaling pathway is in the development of therapeutic strategies. MiR-34a-5p has been identified in the literature as one of the miRNAs involved in the IGF1 pathway (46). Our intriguing and significant findings imply that miR-34a-5p may contribute to the cancer process in AML, particularly via the IGF1 pathway. Therefore, we suggest that the relationship between miR-34a-5p and the IGF1 pathway genes (particularly the *NOTCH2, IGF1R, SKP2,* and *CDC25A*) in AML should be studied further.

In the present study, the overall survival analysis of the *IGF1R, SKP2,* and *CDC25A* genes in AML was not found to be statistically significant, while it was demonstrated that the *NOTCH2* gene was an indicator for AML patients who had poor overall survival (Figure 7). The NOTCH pathway is a critical signaling mechanism that allows nearby cells to communicate and perform their developmental roles in a pathological environment. *NOTCH2* is one of the key players in this pathway. The *NOTCH2* gene is overexpressed in many cancers, including AML. It has been demonstrated that *NOTCH2* expression may be regulated in the cancer process by different miRNAs. For instance, Wang et al. showed that miR-181b/*NOTCH2* might overcome chemoresistance in NSCLC by modulating cancer stem cell-like characteristics (47). Jiang et al. revealed that miR-34c-3p reduced cell invasion and epithelial-mesenchymal transition in nasopharyngeal cancer by targeting *NOTCH2* (48). Our TCGA data analysis results show that *NOTCH2,* which is overexpressed in many malignancies, is much higher expressed in AML than in other cancer types (Figure 6). Literature data and our study results have revealed the fact that the miR-34a/*NOTCH2* relationship may be essential in AML.

The study's findings shed light on the relationship between miR-34a-5p and its target genes in AML. However, for more precise results, methods such as western blot and luciferase reporter assay should be used. Based on our study results, we suggest that miR-34a-5p and selected AML-related genes should be studied in more detail. Thus, the contribution of miR-34a-5p and target genes to cellular processes such as apoptosis, migration, and metastasis in AML will be determined.

Acknowledgements: The NB4 cell line was obtained from the Genetics Department of Aziz Sancar Institute of Experimental Medicine. We would like to thank Prof. Sema Sırma Ekmekci.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- M.K.; Data Acquisition- M.K., İ.S.; Data Analysis/Interpretation- M.K., İ.S.; Drafting Manuscript- M.K.; Critical Revision of Manuscript- M.K., İ.S.; Final Approval and Accountability- M.K., İ.S.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

REFERENCES

1. Shallis RM, Wang R, Davidoff A, Ma X, Zeidan AM. Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges. Blood Rev 2019;36:70-87. [CrossRef]
2. Swaminathan M, Wang ES. Novel therapies for AML: a round-up for clinicians. Expert Rev Clin Pharmacol 2020;13(12):1389-400. [CrossRef]
3. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 2010;115(3):453-74. [CrossRef]
4. Capik O, Sanli F, Kurt A, Ceylan O, Suer I, Kaya M, et al. CASC11 promotes aggressiveness of prostate cancer cells through miR-145/IGF1R axis. Prostate Cancer Prostatic Dis 2021;24(3):891-902. [CrossRef]
5. Kaya M, Karatas OF. The relationship between larynx cancer and MicroRNAs. Van medical journal 2020;27(4):535-41. [CrossRef]
6. Piovesan A, Antonaros F, Vitale L, Strippoli P, Pelleri MC, Caracausi M. Human protein-coding genes and gene feature statistics in 2019. BMC Res Notes 2019;12(1):315. [CrossRef]
7. Alles J, Fehlmann T, Fischer U, Backes C, Galata V, Minet M, et al. An estimate of the total number of true human miRNAs. Nucleic Acids Res 2019;47(7):3353-64. [CrossRef]
8. Suer I, Kaya M, Ozgur E. The effect of miR-34a-5p and miR-145-5p ectopic expression on cell proliferation and target gene expression in the MDA-MB-231 Cell Line. NKMJ 2021;9(2):166-73. [CrossRef]
9. Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB. Common features of microRNA target prediction tools. Front Genet 2014;5:23. [CrossRef]

10. Leclercq M, Diallo AB, Blanchette M. Prediction of human miRNA target genes using computationally reconstructed ancestral mammalian sequences. Nucleic Acids Res 2017;45(2):556-66. [CrossRef]
11. Chen P, Feng Y, Zhang H, Shi X, Li B, Ju W, et al. MicroRNA-192 inhibits cell proliferation and induces apoptosis in human breast cancer by targeting caveolin 1. Oncol Rep 2019;42(5):1667-76. [CrossRef]
12. Miller PG, Al-Shahrour F, Hartwell KA, Chu LP, Järås M, Puram RV, et al. In Vivo RNAi screening identifies a leukemia-specific dependence on integrin beta 3 signaling. Cancer Cell 2013;24(1):45-58. [CrossRef]
13. Pierce A, Whetton AD, Meyer S, Ravandi-Kashani F, Borthakur G, Coombes KR, et al. Transglutaminase 2 expression in acute myeloid leukemia: association with adhesion molecule expression and leukemic blast motility. Proteomics 2013;13(14):2216-24. [CrossRef]
14. Guzman ML, Yang N, Sharma KK, Balys M, Corbett CA, Jordan CT, et al. Selective activity of the histone deacetylase inhibitor AR-42 against leukemia stem cells: a novel potential strategy in acute myelogenous leukemia. Mol Cancer The 2014;13(8):1979-90. [CrossRef]
15. Favreau AJ, Cross EL, Sathyanarayana P. miR-199b-5p directly targets PODXL and DDR1 and decreased levels of miR-199b-5p correlate with elevated expressions of PODXL and DDR1 in acute myeloid leukemia. Am J Hematol 2012;87(4):442-6. [CrossRef]
16. Wallace JA, O'Connell RM. MicroRNAs and acute myeloid leukemia: therapeutic implications and emerging concepts. Blood 2017;130(11):1290-301. [CrossRef]
17. Singh G, Sharma SK, Singh SK. miR-34a negatively regulates cell cycle factor Cdt2/DTL in HPV infected cervical cancer cells. BMC Cancer 2022;22(1):777. [CrossRef]
18. Roy S, Levi E, Majumdar AP, Sarkar FH. Expression of miR-34 is lost in colon cancer which can be re-expressed by a novel agent CDF. J Hematol Oncol 2012;5:58. [CrossRef]
19. Kalfert D, Ludvikova M, Pesta M, Ludvik J, Dostalova L, Kholová I. Multifunctional roles of miR-34a in cancer: a review with the emphasis on head and neck squamous cell carcinoma and thyroid cancer with clinical implications. Diagnostics (Basel) 2020;10(8):563. [CrossRef]
20. Xiong S, Hu M, Li C, Zhou X, Chen H. Role of miR-34 in gastric cancer: From bench to bedside (Review). Oncol Rep 2019;42(5):1635-46. [CrossRef]
21. Slabáková E, Culig Z, Remšík J, Souček K. Alternative mechanisms of miR-34a regulation in cancer. Cell Death Dis 2017;8(10):e3100. [CrossRef]
22. Rao DS, O'Connell RM, Chaudhuri AA, Garcia-Flores Y, Geiger TL, Baltimore D. MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. Immunity 2010;33(1):48-59. [CrossRef]
23. Huang Y, Zou Y, Lin L, Ma X, Chen H. Identification of serum miR-34a as a potential biomarker in acute myeloid leukemia. Cancer Biomark 2018;22(4):799-805. [CrossRef]
24. Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. Leukemia 2009;23(6):1159-63. [CrossRef]
25. Li WJ, Wang Y, Liu R, Kasinski AL, Shen H, Slack FJ, et al. MicroRNA-34a: potent tumor suppressor, cancer stem cell inhibitor, and potential anticancer therapeutic. Front Cell Dev Biol 2021;9:640587. [CrossRef]
26. Pulikkan JA, Peramangalam PS, Dengler V, Ho PA, Preudhomme C, Meshinchi S, et al. C/EBPα regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with CEBPA mutations. Blood 2010;116(25):5638-49. [CrossRef]
27. Liu X, Li H. Diagnostic Value of miR-34a in Bone Marrow Mononuclear Cells of Acute Myeloid Leukemia Patients. Clin Lab 2020;66(3). [CrossRef]
28. Ray D, Kiyokawa H. CDC25A phosphatase: a rate-limiting oncogene that determines genomic stability. Cancer Res 2008;68(5):1251-3. [CrossRef]
29. Xiu MX, Liu YM. The role of oncogenic Notch2 signaling in cancer: a novel therapeutic target. Am J Cancer Res 2019;9(5):837-54.
30. Bretones G, Acosta JC, Caraballo JM, Ferrándiz N, Gómez-Casares MT, Albajar M, et al. SKP2 oncogene is a direct MYC target gene and MYC down-regulates p27(KIP1) through SKP2 in human leukemia cells. J Biol Chem 2011;286(11):9815-25. [CrossRef]
31. Alfaro-Arnedo E, López IP, Piñeiro-Hermida S, Canalejo M, Gotera C, Sola JJ, et al. IGF1R acts as a cancer-promoting factor in the tumor microenvironment facilitating lung metastasis implantation and progression. Oncogene 2022;41(28):3625-39. [CrossRef]
32. Zhang W, Cao L, Sun Z, Xu J, Tang L, Chen W, et al. Skp2 is over-expressed in breast cancer and promotes breast cancer cell proliferation. Cell Cycle 2016;15(10):1344-51. [CrossRef]
33. Chen S, Tang Y, Yang C, Li K, Huang X, Cao J. Silencing CDC25A inhibits the proliferation of liver cancer cells by downregulating IL-6 in vitro and in vivo. Int J Mol Med 2020;45(3):743-52. [CrossRef]
34. Takam Kamga P, Bassi G, Cassaro A, Midolo M, Di Trapani M, Gatti A, et al. Notch signalling drives bone marrow stromal cell-mediated chemoresistance in acute myeloid leukemia. Oncotarget 2016;7(16):21713-27. [CrossRef]
35. Ye Q, Li N, Zhou K, Liao C. Homo sapiens circular RNA 0003602 (Hsa_circ_0003602) accelerates the tumorigenicity of acute myeloid leukemia by modulating miR-502-5p/IGF1R axis. Mol Cell Biochem 2022;477(2):635-44. [CrossRef]
36. Thacker G, Mishra M, Sharma A, Singh AK, Sanyal S, Trivedi AK. CDK2-instigates C/EBPα degradation through SKP2 in Acute myeloid leukemia. Med Oncol 2021;38(6):69. [CrossRef]
37. Bertoli S, Boutzen H, David L, Larrue C, Vergez F, Fernandez-Vidal A, et al. CDC25A governs proliferation and differentiation of FLT3-ITD acute myeloid leukemia. Oncotarget 2015;6(35):38061-78. [CrossRef]
38. Takam Kamga P, Dal Collo G, Resci F, Bazzoni R, Mercuri A, Quaglia FM, et al. Notch Signaling Molecules as Prognostic Biomarkers for Acute Myeloid Leukemia. Cancers (Basel) 2019;11(12). [CrossRef]
39. Chapuis N, Tamburini J, Cornillet-Lefebvre P, Gillot L, Bardet V, Willems L, et al. Autocrine IGF-1/IGF-1R signaling is responsible for constitutive PI3K/Akt activation in acute myeloid leukemia: therapeutic value of neutralizing anti-IGF-1R antibody. Haematologica 2010;95(3):415-23. [CrossRef]
40. Dan W, Zhong L, Zhang Z, Wan P, Lu Y, Wang X, et al. RIP1-dependent apoptosis and differentiation regulated by Skp2 and Akt/GSK3β in acute myeloid leukemia. Int J Med Sci 2022;19(3):525-36. [CrossRef]

41. Sueur G, Boutet A, Gotanègre M, Mansat-De Mas V, Besson A, Manenti S, et al. STAT5-dependent regulation of CDC25A by miR-16 controls proliferation and differentiation in FLT3-ITD acute myeloid leukemia. Sci Rep 2020;10(1):1906. [CrossRef]
42. Yamamura S, Saini S, Majid S, Hirata H, Ueno K, Chang I, et al. MicroRNA-34a suppresses malignant transformation by targeting c-Myc transcriptional complexes in human renal cell carcinoma. Carcinogenesis 2012;33(2):294-300. [CrossRef]
43. Jiang T, Cheng H. miR-34a-5p blocks cervical cancer growth and migration by downregulating CDC25A. J buon 2021;26(5):1768-74.
44. Fan F, Zhuang J, Zhou P, Liu X, Luo Y. MicroRNA-34a promotes mitochondrial dysfunction-induced apoptosis in human lens epithelial cells by targeting Notch2. Oncotarget 2017;8(66):110209-20. [CrossRef]
45. Kwon H, Song K, Han C, Zhang J, Lu L, Chen W, et al. Epigenetic silencing of miRNA-34a in human cholangiocarcinoma via EZH2 and DNA methylation: impact on regulation of notch pathway. Am J Pathol 2017;187(10):2288-99. [CrossRef]
46. Jung HJ, Suh Y. Regulation of IGF -1 signaling by microRNAs. Front Genet 2014;5:472. [CrossRef]
47. Wang X, Meng Q, Qiao W, Ma R, Ju W, Hu J, et al. miR-181b/Notch2 overcome chemoresistance by regulating cancer stem cell-like properties in NSCLC. Stem Cell Res The 2018;9(1):327. [CrossRef]
48. Jiang J, Zhou X, Zhu Y, Mao Y, Wang L, Kuang Y, et al. MiR-34c-3p targets Notch2 to inhibit cell invasion and epithelial-mesenchymal transition in nasopharyngeal carcinoma. Food Sci Technol 2022;42(3):48-58. [CrossRef]
49. Wang C, Zhang W, Zhang L, Chen X, Liu F, Zhang J, et al. miR-146a-5p mediates epithelial-mesenchymal transition of oesophageal squamous cell carcinoma via targeting Notch2. Br J Cancer 2018;118(6):e12. [CrossRef]
50. Guo B, Zhao Z, Wang Z, Li Q, Wang X, Wang W, et al. MicroRNA-302b-3p suppresses cell proliferation through AKT pathway by targeting IGF-1R in human gastric cancer. Cell Physiol Biochem 2017;42(4):1701-11. [CrossRef]
51. Zhao H, Pan H, Wang H, Chai P, Ge S, Jia R, et al. SKP2 targeted inhibition suppresses human uveal melanoma progression by blocking ubiquitylation of p27. Onco Targets Ther 2019;12:4297-308. [CrossRef]
52. Feng X, Wu Z, Wu Y, Hankey W, Prior TW, Li L, et al. Cdc25A regulates matrix metalloprotease 1 through Foxo1 and mediates metastasis of breast cancer cells. Mol Cell Biol 2011;31(16):3457-71. [CrossRef]
53. Suer I, Karatas OF, Yuceturk B, Yilmaz M, Guven G, Buge O, et al. Characterization of stem-like cells directly isolated from freshly resected laryngeal squamous cell carcinoma specimens. Curr Stem Cell Res The 2014;9(4):347-53. [CrossRef]