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Research Article

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Downregulation of hsa-miR-4328 and target gene prediction in Acute Promyelocytic Leukemia

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Abstract

***Introduction**: Acute promyelocytic leukemia (APL) is defined by the PML-RARA fusion gene. APL treatment can have significant side effects, therefore the development of optimal therapeutic options is crucial. Although the study of miRNAs is still in its infancy, it has been shown that these molecules are involved in the pathogenesis of neoplasms by modulating the expression of target genes. miRNAs can be considered possible biomarkers in APL and can be used as therapeutic targets or as markers for the therapeutic response. **Objectives**: The purpose of this study was to determine whether differentially expressed putative miRNAs that have RARA as a target gene could be considered reliable biomarkers for APL. **Methods**: Using bioinformatics tools, a panel of 6 miRNAs with possible tropism for the RARA gene was selected from miRDB. We evaluated their expression levels in samples from patients with APL (n=20) or from healthy subjects without mutations in genes associated with leukemia or myeloproliferative diseases (n=21). **Results**: All 6 putative miRNAs were identified using electrophoresis (hsa-mir-4299, hsa-mir-4328, hsa-mir-7851-3p, hsa-mir-6827-5p, hsa-mir-6867-5p, hsa-mir-939-5p). Of the six miRNAs, hsa-mir-4328 is deeply downregulated in subjects diagnosed with APL compared to healthy subjects, whereas hsa-mir-4299 and hsa-mir-7851-3p show small differences in expression between the two study groups, but without statistical significance. Our results suggest that hsa-mir-4328 may have a role in the pathogenesis of APL and may represent a new biomarker for this type of leukemia. Keywords: miRNA, APL, leukemia, bioinformatics.*

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Introduction

Acute promyelocytic leukemia (APL) is a hematological neoplasm accounting for 10-15% of cases of acute myeloid leukemia (AML). Clinical presentation includes abnormal white blood cell (WBC) levels, low platelets, coagulopathy and anemia. Early death can occur in the first 30 days due to bleeding (1). APL is defined by a balanced translocation, t(15;17)(q22;q12-21), which causes the fusion of the promyelocytic leukemia (PML) gene with the alpha retinoic acid receptor (RARA) gene. This translocation can be identified using in situ fluorescent hybridization (FISH), while the PML-RARA fusion gene can be detected by PCR (transcripts bcr1, bcr2, bcr3). The PML gene is involved in apoptosis and tumor suppression, while the RARA gene is responsible for regulating gene expression related to myeloid differentiation. Consequently, in APL, myeloid cells are not able to differentiate beyond the stage of promyelocytes and accumulate in the peripheral blood and bone marrow (2) (3). APL treatment includes agents such as all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), with good remission and survival rates. ATRA and ATO have redefined APL from a frequently fatal disease to a curable disease and have improved survival rates by at least 80-90% (4). Although, many patients achieve complete remission, at least 10% still have high-risk of relapse (5). APL treatment can have significant side effects depending on the type of drug and the dose used, the duration of treatment, and other comorbidities. The most important side effects are differentiation syndrome and hemorrhage secondary to pancytopenia (6)(7). For this reason, development of new therapeutic management options is crucial.

miRNAs are a class of small single-stranded uncoded RNAs with a length of 19-25 nucleotides. They play key roles in the posttranscriptional regulation of cell proliferation, development, differentiation and apoptosis. miRNAs modulate a large part of the genome, their expression being dysregulated in cancers. Studies indicate that miRNAs can act as tumor suppressors or as oncogenes (8). If tumor-suppressing miRNAs lose their function, they promote the development of cancer. At the same time, overexpression of oncomiR accelerates tumor formation and development (9). Several families of miRNAs are expressed in hematopoietic tissues and have an important role in differentiating cell lines. Also, in pathological conditions, such as leukemias, miRNAs have an aberrant level of expression (10)(11). Recent studies report that miRNAs can be considered possible biomarkers in APL and can be used as therapeutic targets, predictors of response to treatment or prognostic markers (12) (13).

Most therapies involving miRNAs have been developed for solid cancers and are based on bringing miRNAs to a normal level of expression. Some of the methods based on this gene therapy are: antagomiRs, miRNA sponges and miRNA replacement therapy or mimics miRNAs (9). Because the concept of miRNA is relatively new, their study is still in its infancy.

Objective

The purpose of this study is to determine whether differentially expressed putative miRNAs that have RARA as a target gene could be reliable biomarkers for APL.

Methods

Patient groups

This study was carried out in the Molecular Genetics Laboratory of Hematology Department, Fundeni Clinical Institute, Bucharest, using samples collected between 2017-2021. The study included 2 groups: 20 patients with APL (group A) and 21 patients negative for leuke-

mia mutations (group B/control group) (Table 1). The comorbidities of subjects from group B were detailed in Annex 1. This study includes only patients at the onset of the disease, as once treatment is initiated and the period of remission of the disease begins, there is a possibility that miRNA expression may be influenced. For APL diagnosis, patients underwent clinical examination and bone marrow morphological analysis. APL was molecularly confirmed by the presence of PML-RARA fusion transcripts on PCR in peripheral blood samples. Of the 20 patients diagnosed with APL, 14 had bcr2 transcript and 6 had bcr3 transcript. All samples were tested for additional leukemia-specific mutations and fusion transcripts detailed in table 1.

This study was conducted in compliance with the principles of the Helsinki Declaration and approved by the ethics committee of the Fundeni Clinical Institute. Prior to inclusion in the study, informed written consent was obtained from all patients for the scientific use of their data.

Computational prediction of in silico miRNAs

We identified 106 in silico miRNAs targeting RARA gene using miRDB.org (miRNA Database). According to the developer recommendations, we preferentially filtered for targets with a prediction score (target score) higher than 85; resulting in 17 miRNAs with tropism for RARA gene. Their veracity was established by querying them with similar miRDB software: targetscan, mirbase and miRWalk (target score > 0.85). Finally, we selected 6 miRNAs commonly returned by all algorithms as possibly involved in APL: hsa-mir-6867-5p, hsa-mir-4328, hsa-mir-6827-5p, hsa-mir-939-5p, hsa-mir-7851-3p and hsa-mir-4299.

Table 1. Mutations excluded for group B

Acute myeloid leukemia	
AML1-ETO	t(8;21)(q22;q22)
CBFB-MYH11	inv(16)(p13q22)
PML-RARA	t(15;17)(q22;q21)
MLL-AF9	t(9;11)(p22;q23)
FLT3-ITD	internal tandem duplications
Acute lymphoblastic leukemia	
TEL-AML1	t(12;21)(p13;q22)
SIL-TAL1	del(1)(p32;p32)
E2A-PBX1	t(1;19)(q23;p13
MLL-AF4	t(4;11)(q21;q23)
BCR-ABL1	t(9;22)(q34;q11)
Chronic myelogenous leukemia	
BCR-ABL1	t(9;22)(q34;q11)
Myeloproliferative diseases	
JAK2 (V617F)	
CALR	

Blood samples and total RNA extraction

Total RNA extraction was performed from peripheral blood, using an in-house protocol. 12 ml peripheral blood and 38 ml erythrocyte lysis buffer 1x were incubated at low temperature. After supernatant removal, 1ml PBS (1x) was added. The leukocyte concentration was measured using a XN-2000 Hematology-Analyzer (Sysmex). The equivalent of $2*10^7$ leukocytes was distributed in 50 ml tubes and centrifuged (5 min, 3000 rpm, 4°C). Subsequently, 1ml of trizol (TRIzol™, Invitrogen™) was added and homogenized with a 2ml syringe, and stored for 24h at -20°C. To separate the total RNA from the trizol mixture, two washes with chloroform (200 µl) were performed, then the upper phase was extracted and transferred to a new tube. For total RNA precipitation, two consecutive washes were performed with 100% (500 µl) and 75% (1000 µl) ethanol. After incubation (30min, -20°C) and centrifugation of the samples (10 min, 10600 rpm, 4°C), the supernatant was removed. The pellet was dried and rehydrated with 35µl ultrapure water. Total RNA concentration (11.7-1270.1 ng/µl) and purity (A260/A230, 1.80-2.06; A260/A280, 1.66-2.25) were measured by spectrophotometry (NanoDrop™ 1000, Thermo Scientific™).

Reverse transcription and qRT-PCR

For miRNA detection, we used a two-step RT-PCR protocol: a Reverse Transcription PCR with stem-loop primers specific for each miRNA of interest, and qRT-PCR (TaqMan®MicroRNA Assays, Thermo Fisher Scientific) (Table 2)(14). For the reverse-transcription PCR a TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used, with a total volume of 15µL per reaction. A mixture of 7 µL Master Mix and 5 µL total RNA and 3 µL of stem-loop primer was prepared and incubated on ice for 5 min. The reverse-transcription was performed on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™), 30 min, 16°C; 30 min, 42°C; 5 min, 85°C; 10 min, 4°C.

Complementary DNA electrophoresis

DNA electrophoresis was used to verify the results of the reverse transcription reaction and to identify miRNAs of interest for qRT-PCR. A 5% agarose gel (5g agar powder, 95ml TAE, 5 µL ethidium bromide) was prepared. 10µL cDNA sample and 3µL loading dye were loaded into the wells. To identify the length of the gel migrated fragments, 5µL of molecular weight marker (Ultra Low Range DNA Ladder, Invitrogen™) was used (15). PyElph software (version 1.4) was used to estimate the size of the electrophoretic bands corresponding to the miRNAs.

Real-Time PCR

Real-Time PCR was performed with TaqMan MicroRNA Assay (20X) and TaqMan 2X Universal PCR Master Mix No AmpErase UNG (Thermo Fisher Scientific), in a 1.5 µl total volume reaction. The LightCycler 480 (Roche) platform was utilized with the following program: 95°C, 10 min (1 cycle); 95°C, 15s; 60°C, 1 min (40 cycles); 4°C, 3 min. All samples were worked in duplicate, in with 96-well opaque plates. A no-template control (NTC) was also included. The Ct values for qRT-PCR were determined using the LightCycler 480 Software (Roche) and the threshold method. To achieve the standard curve, the sample with the highest concentration of total RNA (1270.1 ng/µl) was selected. From this sample, 5 serial dilutions were performed (4 µl H2O + 4 µl sample/previous dilution). The selection of the sample was made after spectrophotometry, considering the purity of the sample expressed as a percentage of absorbance (1.98-2.04).

Statistical analysis

Statistical analysis was performed using the following software tools: R software (3.4.4 version) and GraphPad Prism version 8.3.1. The test used to determine statistical significance was unpaired two-tailed Mann-Whitney U test with a definition of statistical significance of $p<0.05$.

Table 2. Final list of the in silico miRNAs

Name	Sequence (5' – 3')	Stem - loop primer sequence	miRDB score	miRWalk score
hsa-mir-6867-5p	UGUGUGUGUAGAGGAAGAAGGGA	CCCGGUGUGUGUGUAGAGGAAGAAGGGAAGCUGGGAACCUGACUGCCUCUCCCCUCUUUACCCACUAG	98	0.923
hsa-mir-4328	CCAGUUUUCCCAGGAUU	AACAGUUGAGUCCUGAGAACCAUUGAGAACCAGUUUUCCCAGGAUUAACUGUUCCG	90	1.0
hsa-mir-6827-5p	UGGGAGCCAUGAGGGUCUGUGC	UCUGGUGGGAGCCAUGAGGGUCUGUGCUGUCUCUGAGCACCGUCUCUUCUGUUCCCCAG	87	1.0
hsa-mir-939-5p	UGGGGAGCUGAGGCUCUGGGGGUG	UGUGGGCAGGGCCCUGGGGAGCUGAGGCUCUGGGGGUGGCCGGGGCUGACCCUGGGCCUCUGCUCCCCAGUGUCUGACCGCG	87	1.0
hsa-mir-7851-3p	UACCUGGGAGACUGAGGUUGGA	UGGCUCACUGCAGCCUCCCCGCCCCCUCAGGUGAUCCUCCCACCUCAUCCUCCCAAGUAGCUGGGAAUACAGGUGUGUGCCACCAUGCUCUACAAGCUACCUGGGAGACUGAGGUUGGAAGAUUGCUUGAGCCUAGGAGGUCGAGGCGACAGUGAGCCA	86	1.0
hsa-mir-4299	GCUGGUGACAUGAGAGGC	GGGUUCUGACCAAUCAUGUUACAGUGUUUUCUCCUUUAGAGAGAGCUGGUGACAUGAGAGGCAGAAAAAGGA	86	1.0

All these tests aimed at obtaining a better veracity of the p-value and the interpretation of the Ct accordingly. The information used to calculate the p-value was represented by the raw Ct values of the two groups (mean of duplicates for each sample) and by the values obtained after applying the formula $2^{-\Delta Ct}$, where ΔCt represents the difference between the mean Ct of the target gene and the Ct of the internal control (16). As mentioned above, the sample with the highest concentration of total RNA was used to generate the standard curve and was considered as an experimental control and a calibrator for relative quantification analysis.

Results

Demographic and clinical profile of study subjects

Median age of group A was 44 years, while median age of group B was 53.3 years (p-value 0.09). Significant information from the medical history of group B subjects is detailed in Annex 1. Male to female ratio in group A was 11:9, while in group B was 15:5 male. One-year outcomes in patients who received APL treatment are: 30% of the patients achieved persistent complete remission (CR), 35% died without achieving CR and 35% relapsed. The death occurred mainly due to differentiation syndrome, inflammatory syndrome, hemorrhagic syndrome, and acute myocardial infarction.

Identification of in silico selected miRNAs

Following the 5% agarose gel electrophoresis, all 6 miRNAs of interest were identified in both groups. The size of the amplicons was measured using PyElph software, which indicated a length of 47 bp (Figure 1). This is consistent with published data suggesting the size of miRNA amplicons should be between 40-50 bp, due to the stem-loop extension resulted from reverse-transcription reaction.

Quantitative Real-Time PCR

Samples were analyzed using Real-Time PCR. Their amplification pattern was compared with a standard curve, which presented the characteristic qRT-PCR parameters within the limits recommended by the manufacturer (median efficiency 1,933; median slope -3,494; median y-intercept 44,11). Of the 6 miRNAs analyzed, expression differences were obtained between the 2 groups, in the case of hsa-mir-4328.

Data Analysis

The differential expression analysis concluded that of the six identified miRNAs, only hsa-mir-4328 reached the significant expression difference threshold (Figure 2). Statistical analysis (unpaired two-tailed Mann-Whitney U test) showed that the expression level of mir-4328 was markedly downregulated (p-value = 0.02) in the APL cohort, whereas hsa-mir-4299, hsa-mir-939-5p and hsa-mir-7851-3p were also differentially expressed between the two groups, suggesting their dysregulation in the APL patients,

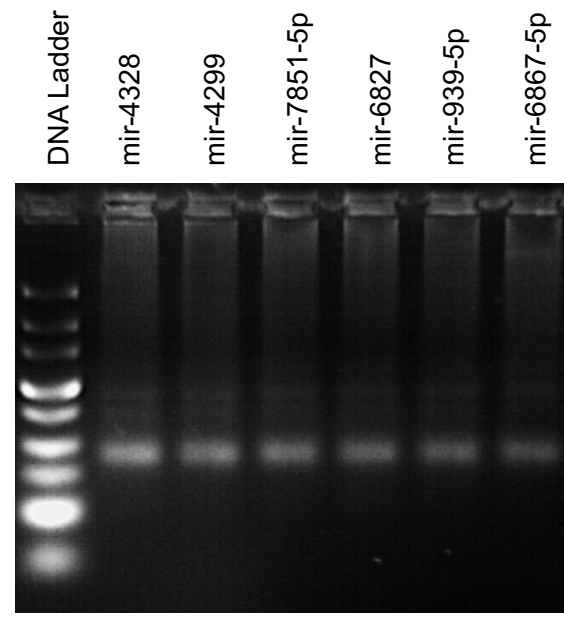

Fig. 1. Identification of miRNAs (agarose electrophoresis)

although not significant. The expression level of hsa-mir-6827 and hsa-mir-6867 was similar in both groups.

Target gene prediction and functional enrichment analysis of hsa-mir-4328

We next sought to investigate the potential genes and pathways of interest targeted by hsa-mir-4328, the candidate the most significantly downregulated in the APL patients group.

Our *in silico* prediction analysis using miRWalk (http://mirwalk.umm.uni-heidelberg.de/) with a prediction score greater than 0.95 returned 24 genes involved in AML (DOID:9119) and APL (DOID:60318) altogether and potentially modulated by hsa-mir-4328 (Figure 3A). We selected genes with hsa-mir-4328 binding sites within 5'-UTR, CDS and 3'-UTR regions of the gene. The functional enrichment analysis using miRPathDB, v2.0 (https://mpd.bioinf.uni-sb.de/) revealed 127 Gene Ontology – biological processes, 24 Gene Ontology – molecular functions pathway (Figure 4A) and 26 Reactome pathways (Figure 4B), significantly enriched for hsa-mir-4328 ($p<0.05$, over-representation analysis with Benjamini-Hochberg correction). Most of the identified pathways are involved in gene transcription and transcription regulation, suggesting that hsa-mir-4328 could modulate the transcription of RARA by interfering with nucleic acid binding and RNA polymerase action as potential mechanisms. Moreover, hsa-mir-4328 targets pathways known to be associated with AML signaling such as Receptor Tyrosin Kinases (RTKs), growth factor and interleukin signaling, MAPK pathway signaling or RUNX1 expres-

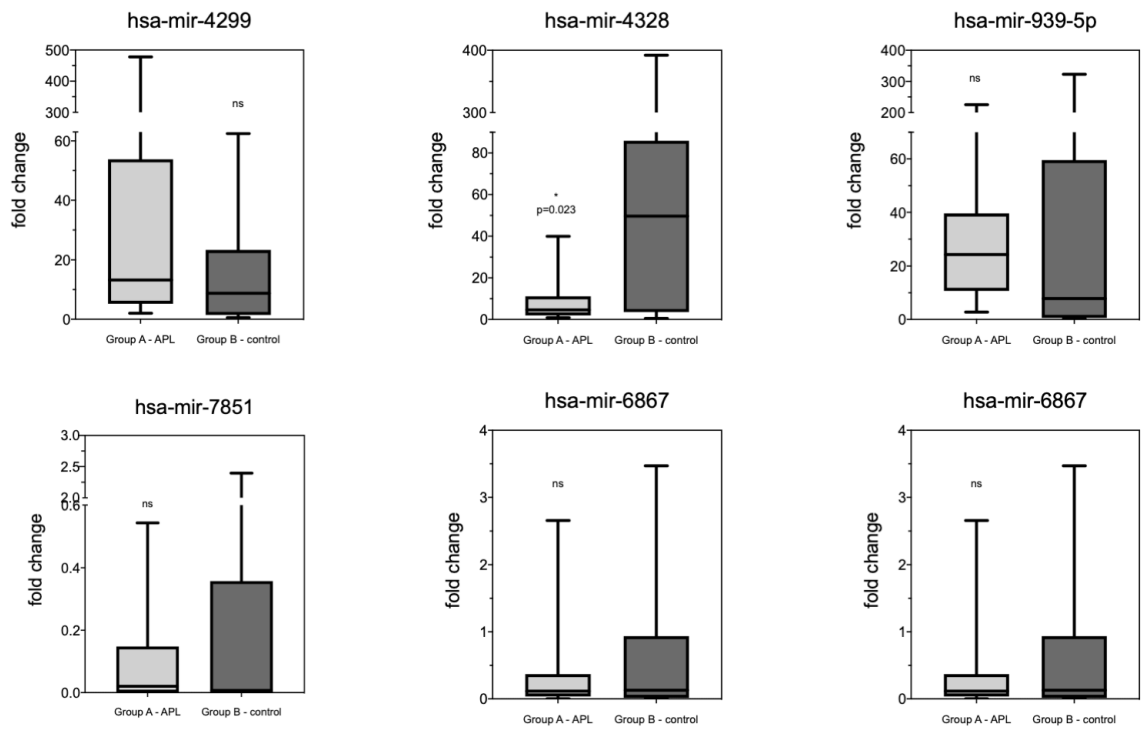

Fig. 2. Expression levels of selected miRNAs in healthy control subjects and APL patients, evaluated by $2^{-\Delta Ct}$ method. hsa-mir-4328 is significantly downregulated in the APL group (two tailed Mann-Whitney U test p-value=0.023)

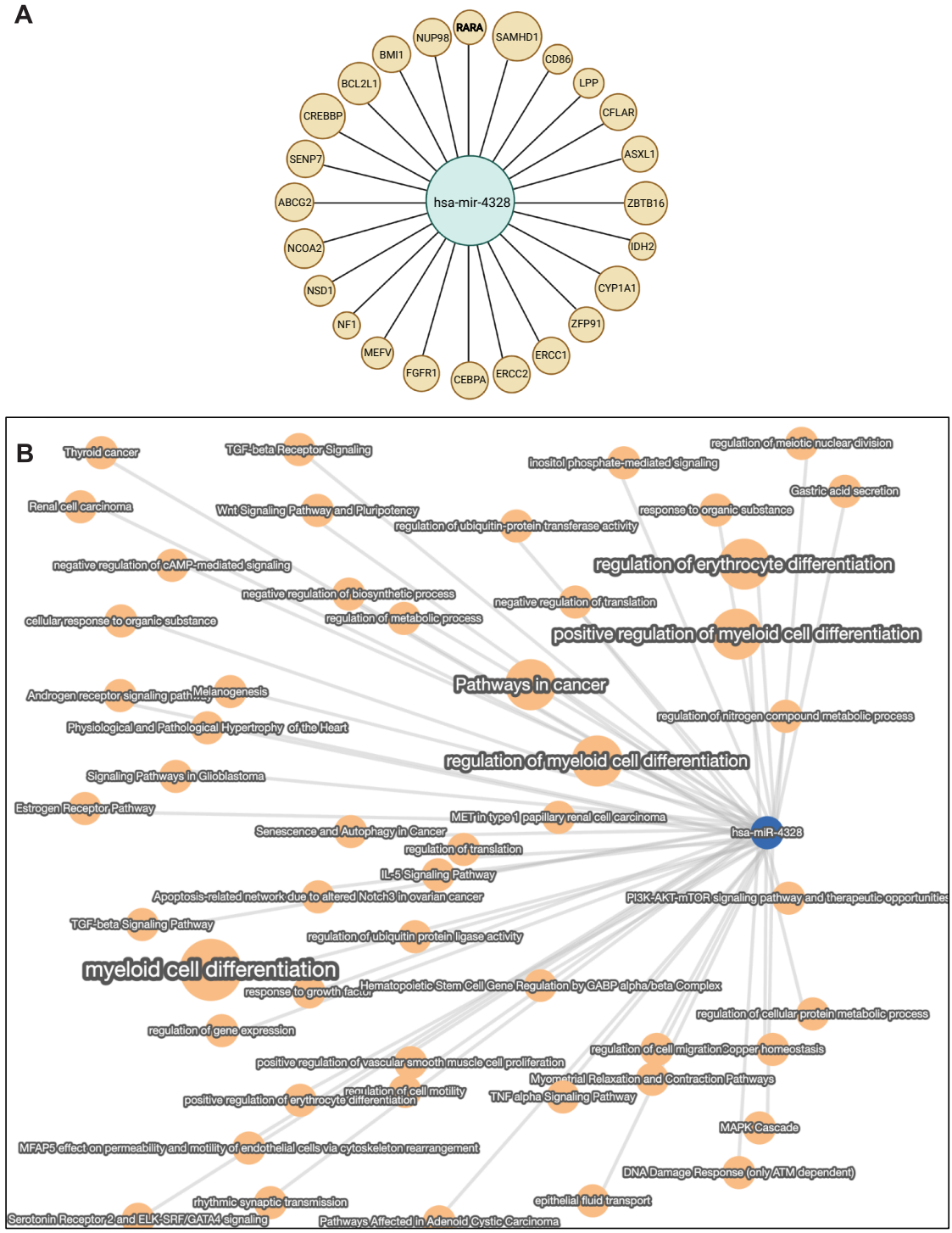

Fig. 3. Target gene prediction and pathway network of hsa-mir-4328. A: Predictive targets of hsa-mir-4328 in AML and APL identified by miRWalk; B: Random network layout representing pathway analysis of hsa-mir-4328 targets by miRTargetLink 2.0

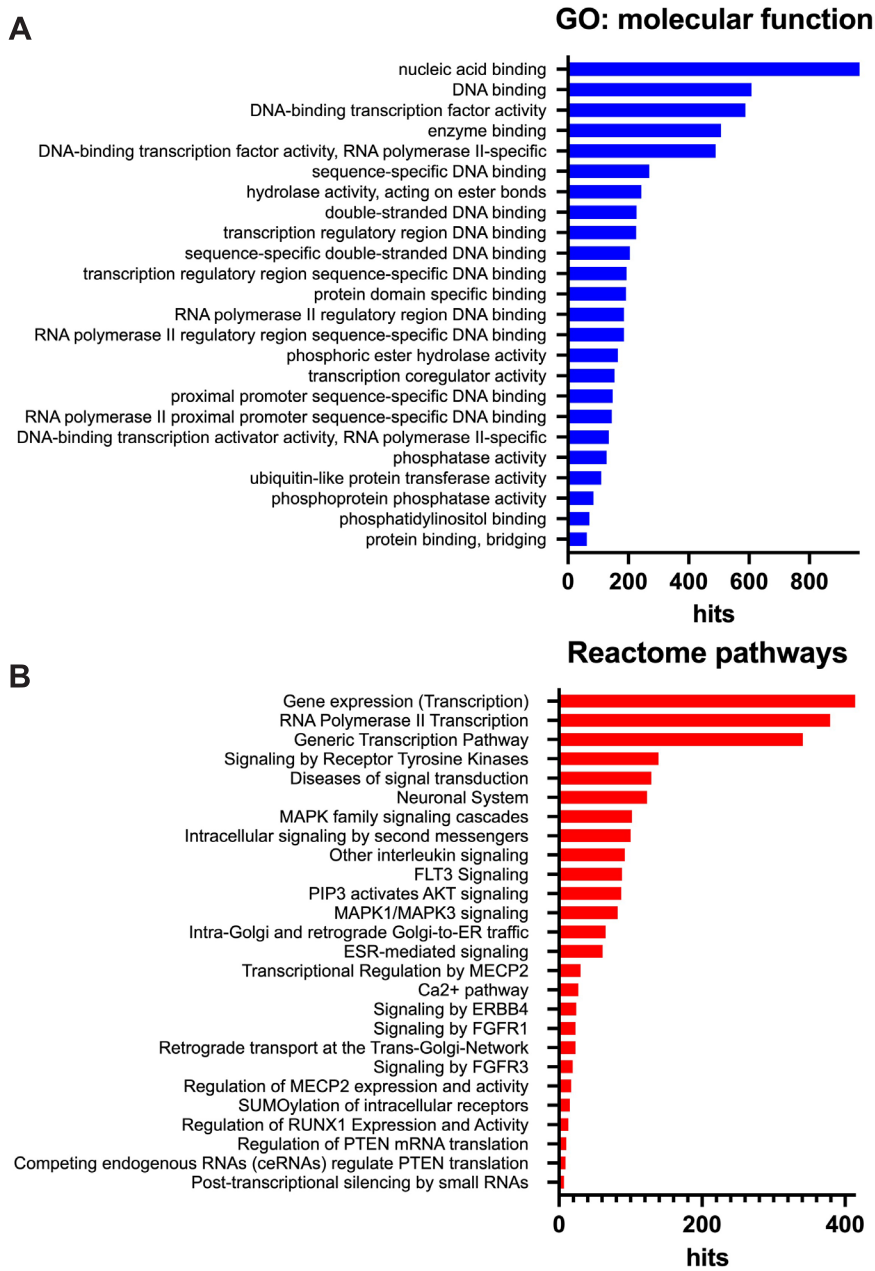

Fig. 4. Predictive pathways of hsa-mir-4328. A: Gene Ontology - Molecular function pathways enriched for hsa-mir-4328; B: Reactome pathways enriched for hsa-mir-4328

sion modulation. Remarkably, pathway analysis using miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de) of validated hsa-mir-4328 targets identified enriched pathways involved in myeloid cell differentiation, reinforcing the therapeutic potential in APL, a disease essentially characterized by a myeloid progenitors differentiation impairment.

We further investigated whether the expression of hsa-mir-4328 is influenced by the type of PML-RARA transcript (bcr2, bcr3), but no association between the type of transcript and hsa-mir-4328 expression level could be made (Spearman r=0.20, p=0.31).

Discussion

The staple of APL pathogenesis is the PML-RARA fusion gene. In this study, we investigate the expression of miRNAs that *in silico* are predicted to target RARA, in primary samples from patients with APL. We opted for RARA as target gene because, unlike PML, its size remains constant within the PML-RARA fusion gene, regardless of the transcript (bcr1, bcr2, bcr3). Although less often, RARA could also be involved in APL by fusing with other partners (PLZF, NPM, NuMA, STAT5b) (17). Considering recent literature data, we preferred to select patients at diagnosis and treatment-naïve, as the chances of a maximum load of altered-expression miRNA are much higher. Normalization of the miRNA expression profile was observed at the initiation of chemotherapy or in stages such as disease remission (18)(19).

For the subjects in the control group, the diagnosis of CML was excluded because of the possibility of transformation into AML (blastic transformation), but also due to the property of certain miRNAs to cross-target multiple oncogenic fusions such as BCR-ABL. In addition, samples from all subjects included in the control group were only included if undetectable for the fusion genes and mutations detailed in Table 1.

However, in the case of hsa-mir-4328, we observed that the amplification curves of 5 subjects from group B had a similar pattern to those in group A. Also, the threshold values of those subjects correspond to the median value of group A rather than the control group. Their medical history revealed myeloproliferative syndrome (negative for mutations in JAK2 and CALR), essential thrombocytosis, essential polycythemia, systemic lupus erythematosus, and Noonan syndrome. Because miRNAs do not have specificity for only one target gene, we hypothesize that these 5 subjects have distinct genotypic profiles in respect to hsa-mir-4328 targets. Thus, using a candidate approach from the genes known to be involved in the pathogenesis of these diseases, we queried miRDB database to look for a possible hsa-mir-4328 tropism for these genes. For all 5 subjects, 3 genes with prediction score between 77 and 89 were identified (THPO, KRAS, FCGR2B). In the case of the subject diagnosed with Noonan syndrome, which is a rare autosomal dominant RASopathy, a KRAS mutation is probable. Although bulk DNA sequencing was performed on a sample from this patient to check this hypothesis, the presence of a KRAS variant had been unknown by the time of submitting this manuscript.

For the total RNA extraction, the trizol-based method was preferred. Compared to commercially available kits, it allows to obtain significantly higher quantities and purities, a better stability of the sample over time, as well as the possibility of optimizing the method for particular cases, at each stage of the protocol (20)(21). In the reverse-transcription reaction, the use of primer pools was avoided to minimize the risk of primer-dimer formation. Stem-loop primers were chosen instead of universal ones due to their high sensitivity and specificity because they target only the amplified mature miRNAs of interest. At the same time, these primers allow immediate electrophoresis for screening purposes (14). The agarose electrophoresis method is not very efficient for miRNAs, due to their short length, with polyacrylamide electrophoresis being preferred instead. However, by optimizing the method and increasing the agarose gel concentration to 5%, the risk of certain miRNAs not being identified can be limited (15). The advan-

tage of using agar electrophoresis is to verify the efficiency of the reverse-transcription reaction and therefore saving time and resources by restricting the set of samples to be tested later by the Real-Time PCR method. The peculiarity of qRT-PCR is given by the selection of one of the samples (group B) for internal control and serial dilutions. This variant was preferred because the efficiency parameter offered by the LightCycler 480 platform has high accuracy (22). However, this method should be complemented by proteomics techniques to provide a more granular understanding of the biological processes in which selected miRNAs are involved (20).

As previously mentioned, the study of miRNAs represents a new direction of research in the pathogenesis of multifactorial diseases, especially neoplasms, due to their property to modulate the expression of target genes. According to our results, the identification of a new miRNA (hsa-mir-4328), with significant downregulated expression in APL, can represent a new starting point in gene therapy, but also in monitoring the therapeutic response.

Because the expression of this miRNA is reduced in APL at diagnosis, it would be important to observe how its expression varies dynamically through the course of the disease. Due to miRNA sensitivity, APL relapse could be anticipated by a decrease in hsa-mir-4328 expression, even before the detection of the PML-RARA fusion protein. The remission of the disease could lead to a normalized level of this miRNA. If this hypothesis is confirmed, hsa-miRNA-4328 has the potential of becoing a new marker for APL prognosis and monitoring. At the same time, the specificity of this miRNA for the RARA gene should be considered. As in the case of the 5 subjects in the control group where hsa-mir-4328 was also downregulated, these molecules show tropism for a multitude of genes. Considering this, it may be necessary to design a larger panel of miRNAs with higher specificity for APL (Annex 2).

In conclusion, the bioinformatics methods used are reliable, with a high predictive power, allowing an accurate check of the initial hypotheses. All 6 miRNAs were identified using electrophoresis, and even more, one of them (hsa-mir-4328) has a low expression in the early stage of APL, compared to the control group ($p < 0.05$). The differences in expression of this miRNA may represent a starting point in the development of a panel of miRNA for monitoring the therapeutic response of APL and for developing new gene therapies.

Abbreviations

ALL - Acute lymphocytic leukemia
AML - Acute myeloid leukemia
APL - Acute promyelocytic leukemia
ATO - Arsenic trioxide
ATRA - All-trans retinoic acid
CALR – Calreticulin
CR – Complete remission
FISH - In situ fluorescent hybridization
JAK2 – Janus Kinase 2
NPM – Nucleophosmin
NuMA - Nuclear Mitotic Apparatus
PLZF - Promyelocytic Leukemia Zinc Finger
PML - Promyelocytic Leukemia gene
RARA - Alpha retinoic acid receptor
STAT5b - Signal Transducer and Activator of Transcription 5B

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Authors' contributions

COT (Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – original draft)

PB (Writing – review & editing; Investigation; Conceptualization; Software)
AE (Writing – review & editing)
DM (Methodology; Resources; Funding acquisition)
CGD (Formal analysis; Investigation; Methodology; Writing – review & editing)
MI (Conceptualization; Software; Writing – original draft)
AS (Methodology; Resources; Funding acquisition)
CD (Project administration; Supervision; Writing – review & editing)

Conflict of Interest

The authors declare no conflict of interest.

References

1. Yilmaz M, Kantarjian H, Ravandi F. Acute promyelocytic leukemia current treatment algorithms. Blood Cancer J. 2021;11(123). DOI: 10.1038/s41408-021-00514-3
2. Cicconi L, Testi AM, Montesinos P, Rego E, Zhu HH, Takahashi H, et al. Characteristics and outcome of acute myeloid leukemia with uncommon retinoic acid receptor-alpha (RARA) fusion variants. Blood Cancer J. 2021;11(10):14-7. DOI: 10.1038/s41408-021-00561-w
3. Zhang X, Sun J, Yu W, Jin J. Current views on the genetic landscape and management of variant acute promyelocytic leukemia. Biomark Res. 2021;9(1):1-15. DOI: 10.1186/s40364-021-00284-x
4. Baljevic M, Park JH, Stein E, Douer D, Altman JK, Tallman MS. Curing All Patients with Acute Promyelocytic Leukemia: Are We There Yet? Hematol Oncol Clin North Am. 2011;25(6):1215-33. DOI: 10.1016/j.hoc.2011.10.002
5. Stahl M, Tallman MS. Acute promyelocytic leukemia (APL): remaining challenges towards a cure for all. Leuk Lymphoma. 2019;60(13):3107-15. DOI: 10.1080/10428194.2019.1613540
6. Lehmann S, Ravn A, Carlsson L, Antunovic P, Deneberg S, Möllgrd L, et al. Continuing high early death rate in acute promyelocytic leukemia: A population-based report from the Swedish Adult Acute Leukemia Registry. Leukemia. 2011;25(7):1128-34. DOI: 10.1038/leu.2011.78
7. Mi JQ, Li JM, Shen ZX, Chen SJ, Chen Z. How to manage acute promyelocytic leukemia. Leukemia. 2012;26(8):1743-51. DOI: 10.1038/leu.2012.57
8. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007;302(1):1-12. DOI: 10.1016/j.ydbio.2006.08.028
9. Ehtesham N, Sharifi M. From conventional therapy toward microRNA-based therapy in acute promyelocytic leukemia. Adv Biomed Res. 2016;5(1):187. DOI: 10.4103/2277-9175.190996
10. Nervi C, Fazi F, Rosa A, Fatica A, Bozzoni I. Emerging role for microRNAs in acute promyelocytic leukemia. Curr Top Microbiol Immunol. 2007;313:73-84. DOI: 10.1007/978-3-540-34594-7_5
11. Liu Y, Cheng Z, Pang Y, Cui L, Qian T, Quan L, et al. Role of microRNAs, circRNAs and long noncoding RNAs in acute myeloid leukemia. J Hematol Oncol. 2019;12(1):1-20. DOI: 10.1186/s13045-019-0734-5
12. Ismail I, Sulong S, Hassan R. MicroRNAs as Potential Biomarkers in Acute Promyelocytic Leukaemia. New J Sci. 2014;2014:1-6. DOI: 10.1155/2014/932342
13. Careccia S, Mainardi S, Pelosi A, Gurtner A, Diverio D, Riccioni R, et al. A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytes. Oncogene. 2009;28(45):4034-40. DOI: 10.1038/onc.2009.255
14. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33(20):1-9. DOI: 10.1093/nar/gni178
15. Lee K, Kang JH, Kim HM, Ahn J, Lim H, Lee JJ, et al. Direct electrophoretic microRNA preparation from clinical samples using nanofilter membrane. Nano Converg. 2020;7(1):1-11. DOI: 10.1186/s40580-019-0212-3
16. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3(6):1101-8. DOI: 10.1038/nprot.2008.73
17. Scaglioni PP, Pandolfi PP. The theory of APL revisited. Curr Top Microbiol Immunol. 2007;313:85-100. DOI: 10.1007/978-3-540-34594-7_6
18. Lindholm EM, Ragle Aure M, Haugen MH, Kleivi Sahlberg K, Kristensen VN, Nebdal D, et al. miRNA expression changes during the course of neoad-

juvant bevacizumab and chemotherapy treatment in breast cancer. Mol Oncol. 2019;13(10):2278-96. DOI: 10.1002/1878-0261.12561

19. Hummel R, Wang T, Watson DI, Michael MZ, Van Der Hoek M, Haier J, et al. Chemotherapy-induced modification of microRNA expression in esophageal cancer. Oncol Rep. 2011;26(4):1011-7. DOI: 10.3892/or.2011.1381
20. Rooda I, Hensen K, Kaselt B, Kasvandik S, Pook M, Kurg A, et al. Target prediction and validation of microRNAs expressed from FSHR and aromatase genes in human ovarian granulosa cells. Sci Rep. 2020;10(1):1-13. DOI: 10.1038/s41598-020-59186-x
21. Riolo G, Cantara S, Marzocchi C, Ricci C. miRNA targets: From prediction tools to experimental validation. Methods Protoc. 2021;4(1):1-20. DOI: 10.3390/mps4010001
22. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomol Detect Quantif. 2015;3:9-16. DOI: 10.1016/j.bdq.2015.01.005