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Quantitative comparison of MicroRNAs expression changes in ALL and AML cancer in HL-60 and MOLT-4 human cell lines

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Abstract

MicroRNAs (miRNAs) are trending as a hot topic among researchers in the cancer drug discovery field, as targeting these molecules with therapeutic agents or measuring their expression levels as markers to determine the disease development or chance of recurrency showed promising results. In this study, our primary goal was to investigate the expression level of 2 miRNAs, miR-143 and miR-128a, in leukemia cancer cell lines (Molt-4 and HL-60) compared to normal, healthy cells. Previous studies projected the involvement of these two miRNAs in cancer development/suppression. To achieve this, we evaluated the expression level of these miRNAs in leukemia cancer cells- taken from patients- and normal cells using the real-time polymerase chain reaction (RT-PCR) method. By doing so, we aimed to gain insights into how these specific miRNAs may be involved in leukemia development and their usage in guiding future diagnostic and therapeutic strategies for this disease.

Keywords: MicroRNA, RT-PCR, Leukemia Cancer, miR-143, miR-128a.

1. INTRODUCTION

In the realm of cancer therapy advancements, RT-PCR plays a pivotal role in identifying the genes associated with cancer progression as potential targets for diagnosis or treatment. The real-time monitoring feature enabled RT-PCR to provide dynamic insights into the genes and genetic-associated factors driving cancer(1).

It also allows researchers and healthcare professionals to track the changes in gene expression in different stages of cancers in response to the therapeutic candidates to optimize a personalized care approach based on individual genetic profiles(2).

This non-invasive technique also empowers researchers to predict the probability of cancer recurrence based on ongoing evaluation of specific gene expressions post-therapeutic intervention(3). Whether employed in isolation or conjunction with other methods, RT-PCR stands out as a promising method to explore and validate the targets within the cancer drug discovery pipeline.

Among all genetic-associated molecules involved in cancer progression/ suppression, miRNAs stand out as compelling targets for cancer drug discovery researchers. These molecules carry out essential roles in the regulation of genes associated with cancer, functioning as either promoters or inhibitors.

For instance, specific miRNAs act as tumor suppressors by silencing genes promoting oncogenesis, while others enhance the oncogenic gene activity involved in processes like angiogenesis, metastasis, drug resistance, and more. The diverse and broad functions, either as an accelerator or brake, of miRNAs in the complex journey of cancer development offer a promising opportunity for intervention and novel therapeutic approaches to combat this malicious disease.(4, 5)

Inspired by the previous research about the role of two miRNAs, including miR-143 and miR-128a, respectively in cancer suppression(6) and promotion(7), for the first time, we evaluated and reported the difference between the level of expression of miR-143 and miR-128a in acute lymphoblastic leukemia (ALL) Molt-4 cell line compared to the control group. We also evaluated the mentioned miRNAs in the acute promyelocytic leukemia (AML) cell line HL-6 compared to the control. Herein, we report our findings obtained by the RT-PCR technique.

2. MATERIAL AND METODS

In this study, the HL-60 cell line (ATCC Number: CCL-240, http://fa1.pasteur.ac.ir/visitLink.aspx?id=C217) and MOLT-4 cell line (ATCC Number: CRL-1582, http://fa1.pasteur.ac.ir/visitLink.aspx?id=C149) were purchased from Pasteur Institute of Iran.

These cell lines were cultured in RPMI 1640 media with 10% FBS for molecular studies. After the total RNA extraction and cDNA synthesis, alterations in the expression of miR-128a and miR-143 were analyzed using the Real-Time PCR technique.

Prediction of essential microRNAs in ALL and AML:

The following software was used to predict miRNAs that target the key gene in the signaling pathway of this disease.

1- Pictar software (http://pictar.mdc-berlin.de/)

2- Microcosm software (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5)

3- Target Scan software (http://www.targetscan.org/ Target Scan release5/2)

4- miRWalk software (http://www.microrna.org)

5- miRanda software (http://www.microrna.org)

6- Diana- microT software

7- DAVID website (http://david. Abcc.ncifcrf.gov)

8- KEGG website (http://www.genome.jp/kegg)

This website illustrates intracellular signaling pathways in various diseases.

According to the significant and numerous roles of miR-143 and miR-128a in acute lymphoblastic and myeloid leukemia, these two microRNAs were selected for this study.

Primer Design:

To identify microRNAs, the stem-loop structure was utilized. Design tools such as oligo6, GeneRunner, AlleleID6, and mfold (for secondary structure verification) were employed. Specific sense primers were developed for each miRNA, complementary to its nucleotide sequence (excluding nucleotides added at the 5′ end for Tm adjustment). Additionally, an antisense primer was crafted on the stem of the stem-loop structure to facilitate the detection of short miRNA molecules (table 1).

Homo sapiens microRNA	Sequence (5'-3')	Length (bp)	GC (%)	Tm (°C)
Homo sapiens microRNA-128a	AATATTACGGGGCCGTAGCACTGTCTGAGAGTCGTATCCAGTGCGAATACCTCGG ACCCTGCACTGGATACGAC	74		
MiR-128a Forward Primer	AATATTACGGGGCCGTAGCA	20	50	59
MiR-128a Reverse Primer	GTCGTATCCAGTGCAGGGT	19	58	59
Homo sapiens microRNA -143	AACACGCTGAGATGAAGCACTGTAGCTCGTCGTATCCAGTGCGAATACCTCGGAC CCTGCACTGGATACGAC	72		
MiR-143 Forward Primer	AACACGCTGAGATGAAGCAC	20	50	59
MiR-143 Reverse Primer	GTCGTATCCAGTGCAGGGT	19	58	59
RTprimer(specific)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAA	50	56	

Table 1: Primer Sequences Designed for miRNA Detection

We analyzed the expression levels of specific microRNAs relative to the housekeeping gene (GAPDH) to quantify their expression levels accurately.

The steps of extraction of microRNA:

One milliliter of cold RNX-Plus was added to the buffy coat (the layer containing white blood cells) and thoroughly mixed. The resulting suspension was transferred to a 1.5 ml microtube and vortexed for 1 minute. Following vertexing, the mixture was allowed to sit at room temperature for 5 minutes.

200 µl Chloroform was added to the contents of the microtube to separate cellular components based on density and the mixture was vigorously shaken for one minute. After that, it was centrifuged for 5 minutes at 4°C and 1200rpm, separating the supernatant solution (aqueous phase). This aqueous phase was then transferred to a new microtube. An equal volume of cold 100% ethanol was added to the supernatant solution.

The procedure began with gently mixing the sample, followed by overnight placement in a -20°C environment. Subsequently, centrifugation was carried out at 4°C for 45 minutes at 1200 rpm, and the supernatant was

carefully discarded. One milliliter of cold 70% ethanol was added and thoroughly vortexed to remove any sediment at the bottom. Another round of centrifugation was conducted at 4°C for 15 minutes at 1200 rpm. After discarding the supernatant, the tube was left open for 10 minutes, allowing the RNA to precipitate and dry. Approximately 30-50µl of RNase-DNase-free water was added to the tube, and the concentration of the extracted RNA was determined. Finally, the solution was transferred to a -70°C freezer for storage.

Production of cDNA:

Production of cDNA involves converting mRNA into cDNA for use in PCR reactions. To accomplish this, a reverse transcription reaction is necessary. This reaction employs a random hexamer primer, which is a blend of six-nucleotide random sequences. These primers attach to mRNA sequences as initiation points for the reverse transcriptase enzyme. Consequently, all present RNAs are transformed into cDNA.

For the reverse transcription (RT) reaction using the Add Bio Kit (Add Script cDNA Synthesis Kit), we followed these steps:

1. Prepare a 0.2 mL RNase-free microtube and add one microliter of random hexamer primer, along with total RNA (subtracting an equivalent volume of one microgram) and DEPC-distilled water to reach a total volume of 10 µl. Mix the reaction thoroughly.
2. Incubate the mixture at 65 ℃ for 5 minutes, followed by rapid cooling on ice.
3. Add 10 µl of the RT-premix and pipette the solution to ensure complete mixing. Incubate the mixture at room temperature for 10 minutes.
4. Continue the reaction by incubating the solution at 50 ℃ for 60 minutes.
5. heat the mixture at 70 ℃ for 10 minutes to stop the reaction.
6. Add 10µl of the cDNA Synthesis Mix into each RNA-primer mixture. Mix gently and centrifuge briefly. 7. Incubate at 42°C for 60 min.
8. Terminate the reaction by incubate the tubes at 85°C for 5 min. Chill the tubes on ice and collect the solution by centrifuge the tube briefly. 9. The synthesized cDNA can be directly used in PCR, by addition of 1 – 2µl of the cDNA reaction mixture to a 25µl PCR reaction.

Expression of miRNAs:

Following RNA extraction and cDNA synthesis, the stem-loop RT primer targeted each specific miRNA, incorporating its terminal region. Subsequently, real-time PCR reactions were conducted using the StepOne Plus real-time device from the American company ABI. The GAPDH gene, a short-length RNA, was employed as an internal control for result normalization (table 2,3).

Ampliqon Master Mix for probe High Rox	10 µl
Forward Primer(10pmol)	0.5
Universal Reverse Primer(10pmol)	0.5
Template (cDNA)	2 µL
diH2O	Up to volume 20

Table 2: Essential Components for Real-time PCR Reactions on the StepOne Plus ABI Device

steps	Temperature	Time	Number of cycles
Hold	95°C	10min	
Denaturation	95°C	30sec	
Annealing	58°C	35sec	45
Elongation	72°C	45sec	
Melt Cure	50-90°C		

Table 3: Real-time PCR Reaction Schedule for miRNAs

The expressions of miR-128a and miR-143 were assessed in Molt-4 and HL-60 cell lines representing cancer samples, while PBMC samples were utilized as healthy controls. The obtained data were then analyzed using GraphPad Prism.

3. Results

The data obtained indicates increased expression of miR-128a and miR-143 in Acute Lymphoblastic Leukemia (ALL) and increased expression of miR-128a and miR-143 in Acute Myeloid Leukemia (AML) (Figure 1).

This suggests that reducing the expression of these two microRNAs could potentially lead to a decrease in cell proliferation associated with these diseases (Figure 2). Further investigation is necessary to identify the target genes of these microRNAs and conduct a thorough exploration of their involvement in this disease.

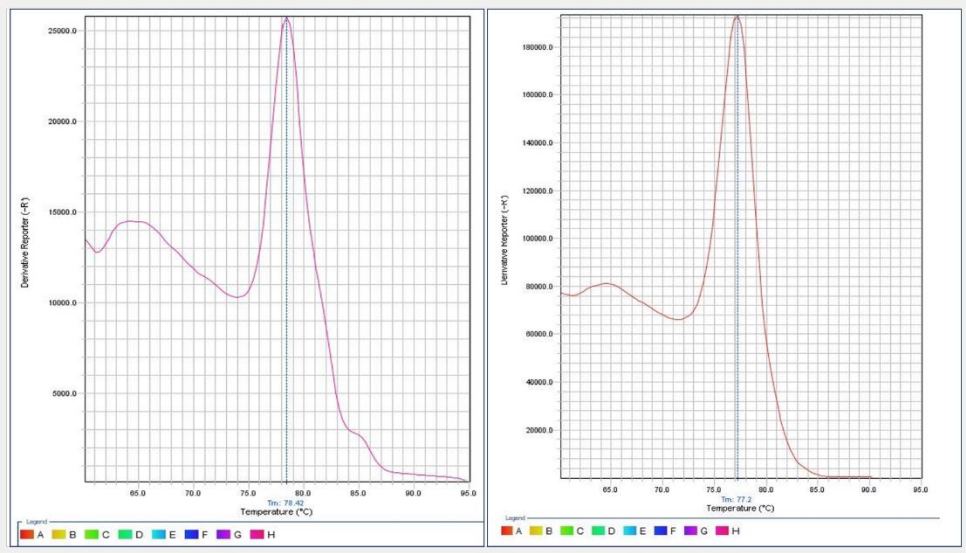

Figure 1: a) Real-time Melting Curve Analysis for miR-128a in MOLT-4 Cell Line b) Real-time Melting Curve Analysis for miR-128a in HL-60 Cell Line

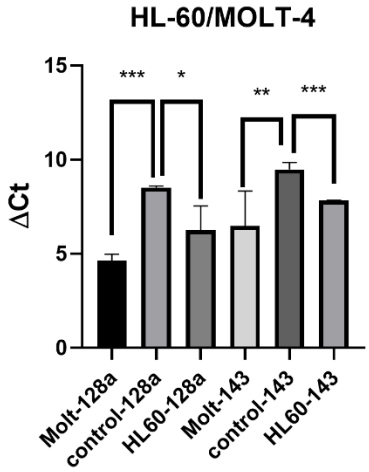

Figure 2: Significant Differential Expression of miR-M143 and miR-128a Between Samples and Control (GAPDH) in HL-60 and MOLT-4 Cell Lines

Results of Real-Time PCR:

According to Tables 4 and 5, the Real-Time PCR results revealed elevated expression levels of miR-128a and miR-143 in the MOLT-4 cancer cell line when compared to the control group, indicating increased expression for both miRNAs.

Additionally, Image 1 illustrates Real-time Melting Curve Analysis for miR-128a in the MOLT-4 Cell Line, while Image 2 displays Real-time Melting Curve Analysis for miR-128a in the HL-60 Cell Line.

Furthermore, the expression levels of these miRNAs in the HL-60 cancer cell line significantly exceeded those in the control group, demonstrating elevated expression for both miRNAs.

The GAPDH gene served as the internal control throughout this study. Finally, Figure 3 depicts the Significant Differential Expression of miR-M143 and miR-128a Between Samples and Control (GAPDH) in HL-60 and MOLT-4 Cell Lines).

Sample Name	Target Name	$\Delta CT\ Sample$	$\Delta CT\ Control$	Pvalue
M128a-1	HL-60	6.01	8.42	
M128a-2	HL-60	6.18	8.61	0.0127
M128a-3	HL-60	6.58	8.51	
M128a-1	MOLT-4	4.98	8.42	<0.0003
M128a-2	MOLT-4	4.25	8.61	
M128a-3	MOLT-4	4.61	8.51	

Table 4: Comparative Analysis of ΔCT Values for miR-128a Expression in HL-60 and MOLT-4 Cell Lines, with Control (GAPDH)

Sample Name	Target Name	$\Delta CT\ Sample$	$\Delta CT\ Control$	Pvalue
M143-1	HL-60	7.82	9.76	<0.0008
M143-2	HL-60	7.85	9.63	
M143-3	HL-60	7.83	9.055	
M143-1	MOLT-4	7.07	9.76	0.0021
M143-2	MOLT-4	7.96	9.63	
M143-3	MOLT-4	7.405	9.055	

Table 5: Comparative Analysis of ΔCT Values for miR-M143 Expression in HL-60 and MOLT-4 Cell Lines, with Control (GAPDH)

* RT-qPCR has performed for three samples of each cell culture and the numbers indicate corresponding sample.
** PBMCs from a normal donor were used as the control for comparing the expressional changes by ΔCT method
*** P-values < 0.05 are considered meaningful
SYBR has applied as the reporter.

4. Discussion

Leukemia cancer was responsible for more than 300,000 deaths worldwide in 2020(8), and this number is on the rise since the resistance to the current standard therapeutic agents is growing.(9) It's crucial to discover new molecules and molecular pathways involved in leukemia cancer development that can be targeted to break or slow down the disease's progression. In this study, we sought to investigate the expression levels of specific miRNAs, miR-143 and miR-128a, in two different leukemia cancer cell lines (Molt-4 and HL-60) derived from patients by PBMC method, compared to the normal cells. We observed heightened expression of both miRNAs in both leukemia cancer cell lines compared to the normal cells. MiR-143 and miR-128a expression levels in Molt-4 and HL-60 were 0.29 ,0.21 and 0.35 ,0.59 respectively, higher than normal cells.

We hypothesized that understanding the expression pattern of these miRNAs in leukemia cancer cells is essential since the previous research showed their importance in the suppression and progression of different cancer types(10, 11). Our findings suggest that based on the obtained explicit miR-143 and miR-1281 expression patterns in Molt-4 and HL-60, these two miRs can be considered as novel potential targets in leukemia cancer therapy or as potential markers in leukemia cancer diagnosis. Targeting these miRNAs and their associated molecular pathways can open new gates for developing innovative treatments, as manipulating their expression level might help suppress the disease progression and potentially lead to better outcomes for leukemia patients.

MicroRNA-143, a multifaceted molecule, plays a dual role in cancer, acting both as a tumor suppressor and an oncogene (12). Esquela-Kerscher and Slack (2006) introduced the concept of oncomirs, highlighted the microRNAs' involvement in cancer, including the intricate role of miR-143 (13).

This molecule, known for its tumor-suppressive properties, has been shown to inhibit cancer cell proliferation and metastasis, acting as a guardian against tumorigenesis (14). On the other hand, Kent and Mendell discussed the paradoxical nature of microRNAs, emphasizing how miR-143 can also function as an oncogene, promoting tumor growth and progression in certain contexts (15).

miR-128, a microRNA, exhibits differential expression across various tumor types, with aberrant levels detected in gastric carcinoma, lung cancer, pancreatic cancer, and hepatocellular carcinoma, among others. Its dysregulation is associated with tumorigenesis and metastasis (16,17). While some studies indicate miR-128's tumor-suppressive role, evidenced by its ability to reduce cell motility and invasiveness in neuroblastoma and curb proliferation and invasion in prostate cancer, it also targets vascular endothelial growth factors, inhibiting tumorigenesis, angiogenesis, and lymphangiogenesis (18,19).

Conversely, miR-128 can act as an oncogene; for instance, it targets the PHF6 tumor suppressor gene in T-cell acute lymphoblastic leukemia (20).

Moreover, its up-regulation in hepatocellular carcinoma tissues compared to adjacent non-tumor tissues suggests an oncogenic role (21). Its involvement in breast cancer progression has been noted, although its specific expression pattern and underlying molecular mechanisms remain incompletely understood.

Further experiments replicating these findings across different leukemia cancer cell lines and exploring the impact of targeting these molecules with active compounds through in vitro methods would be beneficial. This could provide deeper insights into the expression patterns of these miRNAs and their potential role in suppressing leukemia cancer cell proliferation. In conclusion, our study highlights the altered expression levels of specific miRNAs in leukemia cancer cell lines, offering promising avenues for future research on other leukemia cancer lines and the development of targeted therapies against these miRNAs.

Conclusion

The results of our investigation suggest that applying zinc nanoparticles and the antibiotic ciprofloxacin at the same time may prevent the expression of pump genes linked to bacterial resistance to fluoroquinolone drugs.

As a result, using these substances is suggested as a possible tactic for managing or avoiding bacterial resistance. To determine whether it would be feasible to use these substances in place of or in addition to antibiotics, more research is necessary. Furthermore, comparison analyses with different antibiotics under different situations should be taken into account. To create nanoparticles of the highest purity and maximize desired results while limiting nanoparticle concentration requirements, a variety of nanoparticle synthesis techniques should be investigated.

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