

The importance of MIR-4328 gene mutations in Acute Promyelocytic Leukemia

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Abstract

Introduction: miRNAs are involved in the pathogenesis of neoplastic syndromes by silencing target genes. As previously shown, hsa-mir-4328 is downregulated in Acute Promyelocytic Leukemia (APL).

Objectives: The study aims to identify the somatic mutations of the MIR-4328 gene that caused its downregulation in APL and their localization in key regions of the mature miRNA structure.

Material and methods: The study included 24 subjects: the study group (14 patients at the onset of APL, as well as at remission and relapse) and the control group (10 apparently healthy patients). High-Resolution Melting (HRM) was used for genotyping.

Results: As MIR-4328 gene has not been studied before, a structure design of the coding region was performed to better understand the impact of somatic mutations on the mature miRNA. Following HRM, 2 mutant genotypes were identified, different from the wild-type (WT) genes.

Conclusions and discussion: Due to the major deviation of the mutant genotypic curves compared to WT, the existence of major mutations (probably insertions/deletions) can be assumed. If these mutations are located in the seed region of the gene, attachment to exon 3 of the RARA gene is no longer possible, and therefore overexpression of the target gene occurs. Also, frameshift mutations, or substitutions that change the nucleotide sequence of the seed region, can produce a completely different mature miRNA that may have tropism for another gene. To test these hypotheses, sequencing of the entire gene is required.

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Introduction

Acute Promyelocytic Leukemia (APL) is a hematological emergency. Despite the high remission rate, APL therapy, represented by all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO), can lead to severe adverse effects and death. Considering these facts, there is a need to develop a new effective therapeutic line with reduced cytotoxicity and new biomarkers that are more sensitive for disease onset and progression (1)(2).

Small non-coding RNA molecules, such as miRNAs act as cytoplasmic regulators by complementary pairing with short nucleotide sequences of the target mRNA. The major roles of miRNAs are represented by the regulation of proliferation, differentiation, apoptosis and response to cancer treatment (3)(4). Altering the expression level of certain miRNAs can contribute to carcinogenesis (5). Therefore, miRNAs molecules can be classified as oncogenes (oncomiRs) or tumor suppressors (suppressormiRs)(6). Considering all these aspects, miRNAs have been demonstrated to be used as diagnostic and prognostic biomarkers and therapeutic targets for various types of cancer. Similar to protein-coding oncogenes/ tumor suppressor genes, somatic copy number variation of miRNA genes may be an important mechanism underlying their aberrant expression in cancer (5)(7).

In contrast to the numerous studies regarding the role of miRNA in the pathogenesis and therapy of neoplasia, very little is known about somatic mutations of miRNA genes. As we previously demonstrated, hsa-miR-4328 is downregulated in APL, suggesting that some unknown somatic mutations can occur in the coding region of this miRNA. This type of mutations can lead to alterations in the structure of both pre-miRNAs and mature miRNAs.

Objective

The purpose of this study is to confirm the existence of somatic mutations in MIR-4328, that can cause the downregulation of hsa-miR-4328 expression in APL.

Methods

Patient groups

This study was carried out in the Molecular Genetics Laboratory of Hematology Department, in "Fundeni" Clinical Institute, Bucharest, using samples collected between 2018-2021. The study included 14 patients diagnosed with APL in different stages of disease (onset,

remission and relapse) and 10 patients negative for leukemia mutations with no known comorbidities (control group). For APL diagnosis, patients underwent clinical examination and bone marrow morphological analysis. APL was molecularly confirmed by the presence of PML-RARA fusion transcripts using PCR technique. The MRD of APL was assessed for all the patients in dynamics using Quantitative Real-Time PCR for establishing the exact number of copies of PML-RARA.

This study was conducted in compliance to 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.

Blood samples and DNA extraction

DNA extraction was performed from peripheral blood, using High Pure PCR Template Preparation Kit (Roche). Total RNA concentration (11.7-270.1 ng/ μ l) and purity (A260/A230, 1.78-2.00; A260/A280, 1.68-2.36) were measured by spectrophotometry (NanoDrop™ 1000, Thermo Scientific™).

High Resolution Melting PCR

For genotyping the coding region of hsa-miR-4328 (MIR4328), we manually selected forward and reverse primers using ENSEMBL Database and Temperature Calculator (Thermo Fisher Scientific) (Table 1). Further, we established an in-house protocol for HRM, using the following reagents: 10 μ l HotStarTaq Master Mix Kit 1000 U (Qiagen), 6 μ l SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen), 5 μ l ultrapure water 2 μ l primers and 20 μ M of DNA in a 25 μ l total volume reaction. The LightCycler 480 II (Roche) platform was used with the following program: one cycle of 95°C for 15 minutes; 40 cycles of Quantification at 95°C for 30 seconds, 60°C for 1 minute (Acquisition Mode: Single), 72°C for 1 minute and a HRM step of 95°C, 1 minute; 40°C, 1 minute; 72°C, 10 minutes; 95°C (Continuous, with 25 Acquisitions per °C). The control samples used in this reaction were tested previously for quantification of hsa-mir-4328 expression. For all samples we worked in duplicate, in with 96-well opaque plates. A no-template control (NTC) was also included. To achieve an approximate standard concentration of DNA (20 μ M), dilutions were performed.

	Sequence (5'→3')	Length	Start	Stop	Tm	GC%
Forward	GCAATAATTTGGCCTCTCTGGT	22	2	23	58.97	45.45
Reverse	GGAAATTTGCCTTTGGAAATTAAGTT	26	156	131	57.83	30.77
Product length	155 nt					

Table 1. Primers sequence for MIR-4328

In silico modeling of MIR-4328 gene

For the 2D and 3D in silico modeling of MIR-4328, we used the following bioinformatic tools: SWISS Prot (Biozentrum, Germany) and Sfold software (ELIXIR, bio.tools). After obtaining the structural models, we performed the conventional annotation of MIR-4328 sequence, enhancing the most important landmarks, using miRDB.org (miRNA Database).

Results

Demographic and clinical profile of study subjects

Median age of study group was 35 years, while median age of control group was 39 years. Male to female ratio in study group was 6:8, while in group B was 5:5 male. The death occurred mainly due to differentiation syndrome, inflammatory syndrome and hemorrhagic syndrome. The clinical characteristics of the study group are detailed in Table 1.

Parameters	Total	Percent
Female	6	42,85 %
Male	8	57,14 %
Median Age	35 y	-
Deaths	6	42,85 %
PML-RARA transcripts		
Bcr1	0	-
Bcr2	9	64,28%
Bcr3	5	35,71%

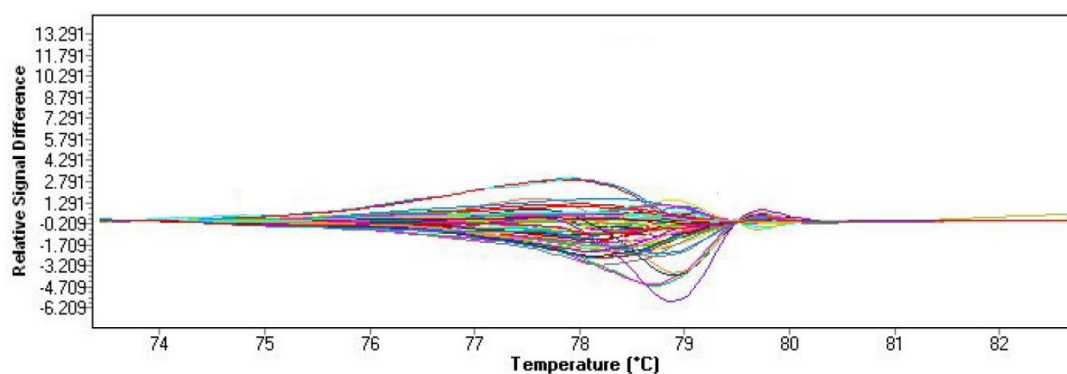
Tabel 1. Clinical characteristics of the study group

Identification of MIR-4328 genotypes

Following the High-Resolution Melting PCR, 3 different genotypes were identified by the Light Cycler 480 II Software. Remarkably, all three genotypes reflected the disease stage: genotype 1 represents all the samples at the onset of disease, genotype 2 reunited the relapse samples and the third genotype included both healthy patients and

remission stage. Due to these observations, we used control group as base line for generating the graphic of the genotypes.

Although, there are 3 identified genotypes, in the case of relapse and onset groups, those patterns are slightly different, being very similar (Figure 1).



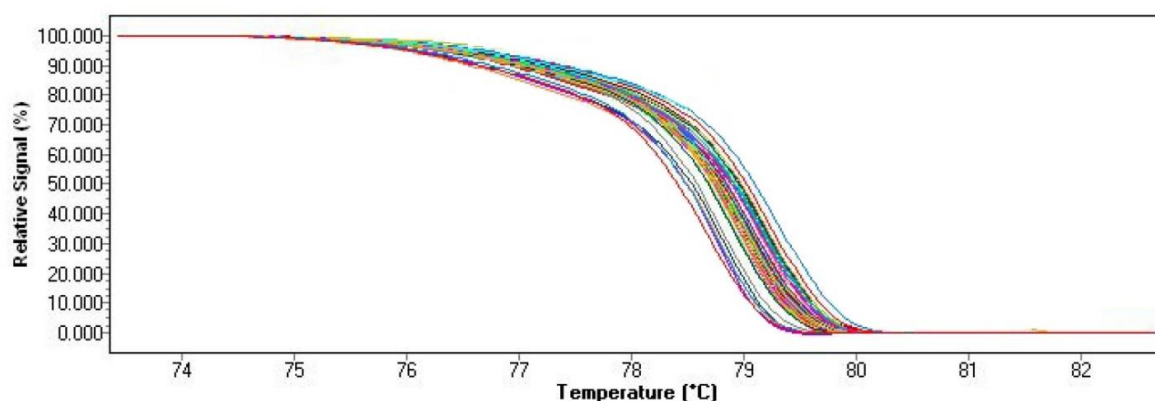


Figure 1. Genotyping of MIR-4328

In silico modeling of MIR-4328 gene

We further investigated if we could generate an *in silico* model of MIR-4328 coding gene, using bioinformatics tools. This aspect was important to establish a graphical structure that can help as gain a better understanding regarding the localization and type of the mutations. As expected, the structure of MIR-4328 gene was generated

and we could establish the seed sequence and the overall landmarks of the gene (Figure 2).

Using miRDB.org, we identified the seed sequence that targeted RARA gene: CAGUUUU. According to the canonical principals of miRNA coding region, this seed sequence is classified as 7mer-m8, realizing a perfect Watson-Crick match from nucleotides 2–8 of the miRNA seed (7).

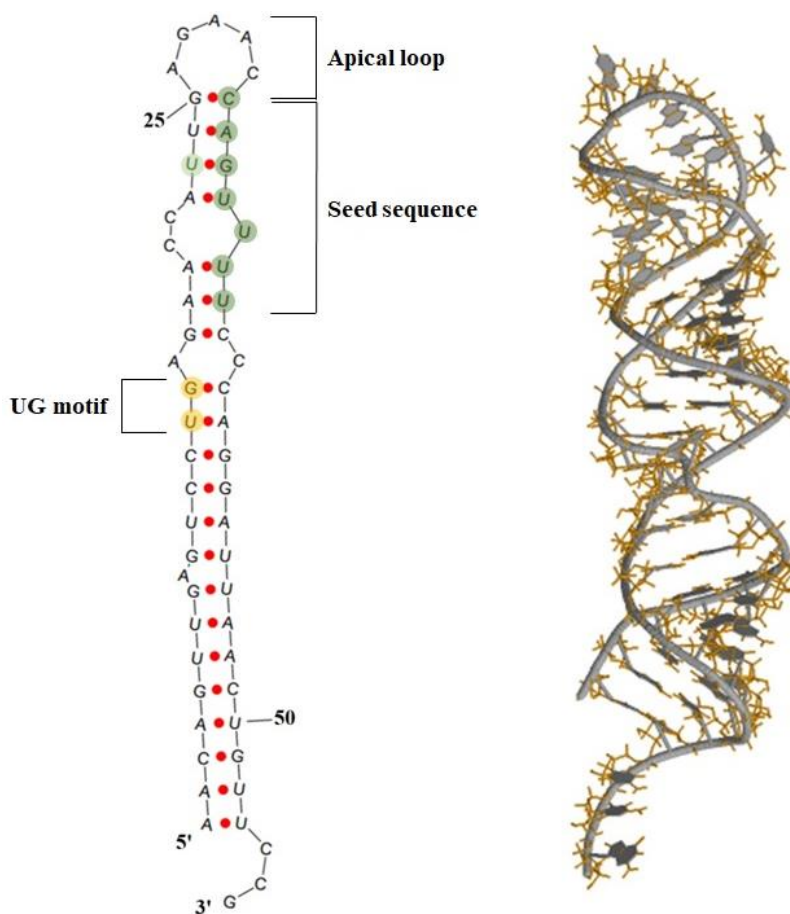


Figure 2. Gena MIR-4328 (Proiectare 2D și 3D)

Discussions

The existence of two mutant genotypes compared to the wild-type variant of the MIR-4328 gene was confirmed. Due to the major deviation of the genotypic curves compared to the control group, it can be assumed the existence of major mutations (probably insertions/deletions) that increased and respectively shortened the time required for the Melting step.

If these mutations are located in the seed region of the gene (the mRNA complementary region of the target gene), attachment to exon 3 of the RARA gene is no longer possible (8). Also, since the seed region contains a small group of nucleotides, any frameshift mutation or substitution that changes the nucleotide sequence or the reading frame of the seed region will produce a mature miRNA completely different from the original one, which may have tropism for another gene. This phenomenon of inhibition can cause the underexpression of the new target gene and the overexpression of the initial target gene (which is currently no longer inhibited). If the new gene is a tumor suppressor, blocking its expression will lead to tumor proliferation and growth, inhibiting the apoptosis process.

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Since all these aspects are speculative, and the High Resolution Melting technique is error-prone, all these hypotheses are to be confirmed by sequencing for the exact determination of mutations in the MIR-4328 gene and their impact on the coding structure of mature hsa-miR-4328.

Even if these epigenetic alterations are often involved in the pathogenesis of various neoplasias, it must be considered that the changes in the coding structure of miRNAs represent a subtle alteration that is not sufficient for the production of APL.

Given that, in the past decades, miRNA molecules are being used as biomarkers, hsa-miR-4328 can give as new perspectives regarding APL pathogeny.

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Conflict of Interest

The authors declare no conflict of interest.

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