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급성골수성백혈병에서 Small RNA 차세대 유전체 시퀀싱 분석을 통한 microRNA-181, 221 연관성 규명 연구

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2018년 2월

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이 논문을 의학박사학위 논문으로 제출함

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Abstract

Small RNA sequencing profiles of mir-181 and mir-221, the most relevant microRNAs in acute myeloid leukemia

Backgound: To evaluate and select microRNAs relevant to acute myeloid leukemia (AML) pathogenesis, we analyzed differential microRNA expression by quantitative small RNA next-generation sequencing using duplicate marrow samples from individual AML patients.

Methods: For this study, we obtained paired marrow samples at two different time points (initial diagnosis and first complete remission status) in patients with AML. Bone marrow microRNAs were profiled by next-generation small RNA sequencing. Quantification of microRNA expression was performed by counting aligned reads to microRNA genes.

Results: Among 38 samples (32 paired samples from 16 AML patients and 6 normal marrow controls), 27 were eligible for sequencing. Small RNA sequencing showed that 12 microRNAs were selectively expressed at higher levels in AML patients than in normal controls. Among these 12 microRNAs, mir-181, mir-221, and

mir-3154 were more highly expressed at initial AML diagnosis as compared to first complete remission. Significant correlations were found between higher expression levels of mir-221, mir-146, mir-155, and mir-181 and higher marrow blast counts.

Conclusions: Our results demonstrate that mir-181 and mir-221 are selectively enriched in AML marrow and reflect disease activity. mir-3154 seems to be a novel candidate microRNA that is relevant to AML but needs further validation.

Keywords: microRNA; next-generation sequencing; acute myeloid leukemia

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1. Introduction

Acute myeloid leukemia (AML) is a group of hematopoietic neoplasms featuring impaired hematopoiesis and bone marrow failure caused by clonal expansion of undifferentiated myeloid precursors. Although etiological factors developing AML remain unclear, AML is often caused by karyotype abnormalities: AMLs are frequently found to have recurrent chromosomal aberrations and gene mutations [1]. Even in AML cases with normal cytogenetics, most carry driver mutations relevant to patient clinical outcomes [1]. Chromosomal aberrations and genetic mutations are associated with AML progression and progress [2,3].

In the context of posttranscriptional regulation, microRNAs, a class of small non-coding RNAs, regulate the expression of other genes by targeting over 60% of protein-coding transcripts [4]. Compelling evidence has indicated that microRNAs are key regulators of hematopoiesis [5,6]. In AML, several studies have shown that differential microRNA expression is associated with cytogenetic aberrations and impacts clinical outcomes [7-10]. In cytogenetically normal AML, Marcucci et. al. reported that microRNA signature is associated with the clinical outcome and target genes encoding proteins in specific innate-immunity pathways [8]. Regarding the role of microRNA in leukemogenesis, several findings are reported. Overexpression of microRNA-125a (miR-125a) decreases AML NB4 cell proliferation,

and resulted in inhibition of cell cycle progression and progression of cell apoptosis by targeting the ErbB pathway in AML [11]. Morris et al. identified a new role for microRNA-150 (miR-150) in myeloid differentiation. Expression of miR-150 is low or absent in AML patient samples and cell lines. MiR-150 expression was found to induce the myeloid differentiation of human acute leukemia cells and normal hematopoietic progenitors [12].

Recently, there has been growing interest in using next-generation sequencing (NGS) to profile microRNAs. Sequencing provides us with the potential to verify novel microRNAs associated with a disease of interest. However, quantitative microRNA sequencing has not been well-evaluated in AML.

In the present study, we analyzed differential microRNA expression by quantitative small RNA next-generation sequencing using duplicate marrow samples from individual AML patients, and evaluated those microRNAs relevant to AML pathogenesis. We also analyzed candidate target genes providing the groundwork for the elucidation of the mechanism of AML.

2. Methods

Patient samples

The study protocol was approved by the Institutional Review Board at Seoul National University Hospital (IRB No. 1106-092-366). All clinical samples included in this study were obtained from AML patients ≥ 20 years of age who underwent bone marrow biopsy before treatment; informed consent was obtained in accordance with the Declaration of Helsinki. Regarding paired samples, we obtained marrow samples at two different time points: initial diagnosis and first complete remission status. Control samples (normal marrow) were collected from the five patients who underwent bone marrow biopsy to rule out primary hematologic disorders and were found normal upon marrow evaluation.

Total RNA isolation

Total RNA from marrow mononuclear cells was isolated using Trizol® reagent (Invitrogen, Paisley, UK) by depleting large ribosomal RNA molecules from the total RNA samples: 1) add 0.5mL of isopropanol to the aqueous phase, per 1mL of TRIzol® reagent used for lysis, 2) incubate and centrifuge for 10 minutes, 3) discard the supernatant to obtain total RNA precipitate, 4) wash the RNA using 75% ethanol, 5) solubilize the RNA with 20-50 μL of RNase-free water, 0.1mM EDTA. We loaded each

sample on an agarose gel to estimate the quality and quantity of each RNA sample. RNA quality was also analyzed with a Bioanalyzer and RNA Nano Chips (Agilent Technologies, Santa Clara, CA, USA). Data can be displayed as a gel-like image and as electropherograms. Quantification of total RNA yield was determined using Quant-iT^{TM} RiboGreen RNA Reagent and Kit (RiboGreen; Invitrogen, Paisley, UK). Quant-iT^{TM} RiboGreen RNAReagent enables quantitation of as little as 1ng/mL RNA with a fluorescence microplate reader, standard spectrofluorometer, or filter fluorometer, using fluorescein excitation and emission wavelengths. We prepared an aqueous working solution of Quant-iT^{TM} RiboGreen reagent, and added 1.0mL of the appropriate aqueous working solution of reagent to each cuvette. We used the high-range working solution for performing the high-range assay, and use the low-range working solution or performing the low-range assay. Then, we measured the sample fluorescence using a spectrofluorometer or fluorescence microplate reader and standard fluorescein wavelengths. For a standard curve, we use 16S and 23S ribosomal RNA. To generate a standard curve of fluorescence versus RNA concentration, we subtracted the fluorescence value of the reagent blank from that of each of the samples.

Small RNA library preparation and sequencing

The library was prepared using the small RNA sample prep kit (Cat # RS200-012), a product of illumina. The table 3 shows the individual components of the small RNA sample prep kit.

Next generation sequencing (NGS) was performed by LAS Ltd (Gimpo-si, Gyeonggi-do, Korea, http://www.lascience.co.kr/). Small RNAs were sequenced using a TruSeq Small RNA Sequencing Kit (Illumina, San Diego, CA, USA) according to manufacturer instructions. All samples were sequenced on an Illumina NextSeq 500 Sequencer using a 75-cycle High Output Kit.

With the binary base call files from the sequencer, conversation into FASTQ format and de-multiplexing were performed using Illumina bcl2fastq2 software (v2.17, http://support.illumina.com/downloads/). Adaptor trimming was performed using the FASTQ Toolkit App (v1.0) of Illumna BaseSpace (http://basespace.illumina.com/apps/). Quality of the sequenced reads before and after adapter trimming was evaluated using FastQC software (v0.11.4, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cleaned sequences were aligned to the most recent mirBASE database release 21 (http://mirBase.org/[9]) using the Small RNA App (v1.0) of Illumina BaseSpace. Quantification of microRNA expression was performed by counting aligned reads to microRNA genes. Quantification of microRNA expression for each miRNA families was based on Transcripts per ten million

(TPTM = mappingCoung * 10,000,000 / totalCount).

microRNA target gene prediction

Targets genes for differentially expressed miRNAs were predicted via miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). Over 50,000 miRNA-target interactions in the miRTarBase database have been validated by experiments such as reporter assays, western blotting or microarrays experiments with overexpression or knockdown of miRNAs [14,15]. miRTarBase has accumulated more than three hundred and sixty thousand miRNA-target interactions (MTIs), which are collected by manually surveying pertinent literature after natural language processing of the text systematically to filter research articles related to functional studies of miRNAs. Generally, the collected MTIs are validated experimentally by reporter assay, western blot, microarray and next-generation sequencing experiments.

Statistical analysis

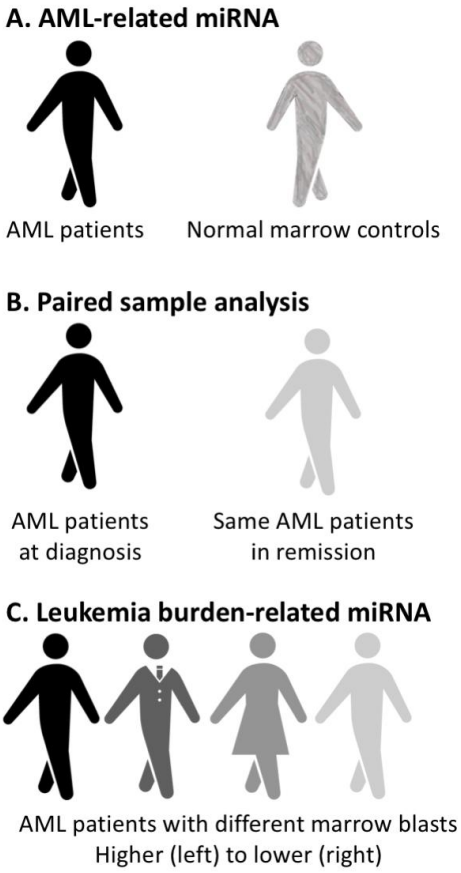
To identify potential microRNAs that were the most relevant to AML pathogenesis, a three-step approach was employed as follows:

Step 1: comparison of microRNA expression between 14 AML patients and 5 normal marrow controls (Figure 1A);

Step 2: comparison of microRNA expression using paired samples from the time of diagnosis and complete remission for each patient (Figure 1B);

Step 3: testing of the association of microRNA expression with different counts of bone marrow blasts (Figure 1C).

Figure 1. **Preplanned comparison for differential miRNA expression**



Statistical analyses were completed using Stata 12.0 software (Stata Corp LP, College Station, TX, USA). A Student's t test was used to determine statistically significant differences between comparison groups. P-values of less than .005 were considered statistically significant. Pearson's correlation coefficient was calculated and analyzed to evaluate the linear relationship between the expression of a specific microRNA and disease burden (the lowest and the highest blast counts in bone marrow).

3. Results

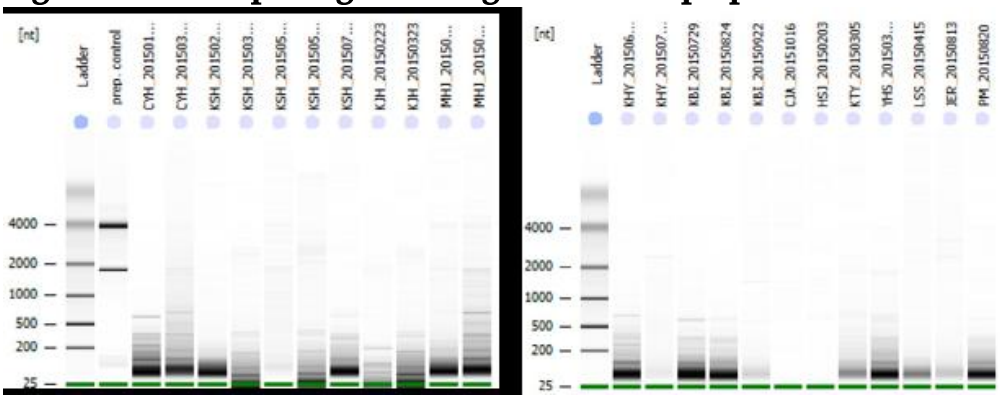
From March 2013 to August 2015, we initially collected 44 samples (32 samples from 16 AML patients with paired-samples; 6 samples from 6 AML patients with single sample at diagnosis; 6 samples from patients without marrow disorders). After RNA preparation, we enrolled 16 out of 22 AML patients whose paired samples from the time points of diagnosis and complete remission were available as well as six patients who were confirmed to have normal marrow. Among the 16 paired samples from AML marrow, eight were eligible for analysis after evaluating the quality of extracted RNA. Of the remaining eight paired samples, six single samples obtained at diagnosis were included in further analyses. Among the six samples from normal marrow, five were enrolled as normal controls except one which RNA was not qualified for sequencing; four patients were diagnosed with immune thrombocytopenia and one was diagnosed with normal reactive marrow.

The results of RNA preparation and isolation of 22 samples are shown in Table 1. The results of electrophoregram are shown in Figure 2. According to the initial Quality Control reports, all 22 samples failed to proceed to further analysis for RNA degradation. However, as for small RNA sequencing, we judged that the impact of degradation would not be significant for interpretation, and then decided to continue small RNA sequencing.

Table 1. Results of RNA preparation and isolation of 22 samples (16 paired samples + 6 single samples)

Sample QC Result										
No.	Sample ID.	Trinean			Ribogreen	Volume	Amount	Bioanalyzer		결과
		RNA Con.(ng/ul)	A260/280	A260/230	Con.(ng/ul)	(ul)	(ng)	Ratio (28S:18S)	RIN	
1	CYH_20150129	2328.75	1.29	1.13	4043.5	10.0	40434.7	0.0	2.4	Fail
2	CYH_20150309	518.94	1.83	0.39	382.4	10.0	3823.9	0.0	2.5	Fail
3	KSH_20150217	1411.09	1.88	1.11	1459.0	10.0	14590.5	0.0	2.6	Fail
4	KSH_20150324	1765.33	1.81	0.94	2009.4	10.0	20094.2	0.0	2.6	Fail
5	KJH_20150223	2235.86	1.63	1.22	2770.8	10.0	27707.9	0.0	2.6	Fail
6	KJH_20150323	2044.89	1.71	1.01	2410.1	10.0	24101.2	0.0	2.6	Fail
7	MHJ_20150223	170.58	1.71	0.26	116.9	10.0	1169.2	0.0	2.6	Fail
8	MHJ_20150428	438.80	1.82	0.52	401.2	10.0	4012.5	0.0	2.5	Fail
9	KBI_20150729	2027.40	1.66	1.20	5575.9	10.0	55759.4	0.0	2.5	Fail
10	KBI_20150824	1281.28	1.85	0.77	4960.3	10.0	49602.9	0.0	2.5	Fail
11	LHJ_20140508	706.51	1.84	0.97	694.1	15	10411.7	0.3	4.4	Fail
12	LHJ_20140610	180	1.56	0.32	113.1	10	1130.7	0.2	2.5	Fail
13	JTS_20140428	499.82	1.78	1	523.3	15	7850.2	0.5	3.4	Fail
14	JTS_20140714	72.22	1.42	0.19	10.9	8	87.1	0.5	3.4	Fail
15	KHY_20150603	1551.51	1.93	1.39	2074.0	10.0	20740.5	0.0	2.5	Fail
16	KJI_20140408	234.86	1.73	0.77	237.3	15	3559.6	1.1	4.2	Fail
17	CJA_20151016	387.99	1.45	0.26	80.7	10.0	807.1	0.0	8.3	Fail
18	HSJ_20150203	376.16	1.86	0.64	391.8	10.0	3917.6	0.0	1.0	Fail
19	KTY_20150305	226.29	1.71	0.38	168.1	10.0	1681.3	0.0	2.5	Fail
20	YHS_20150330	593.02	1.82	0.53	570.1	10.0	5701.3	0.0	2.5	Fail
21	LSS_20150415	320.87	1.49	0.23	70.2	10.0	702.1	0.0	2.6	Fail
22	PM_20150820	690.43	1.80	0.62	568.4	10.0	5683.7	0.0	2.5	Fail

Figure 2. electrophoregram image for RNA preparation



The clinical and molecular characteristics of enrolled patients are shown in Table 2. The median age of the 14 AML patients was 53 years (range, 20 to 78 years) and seven (50%) were men. Eight (57%) patients had normal karyotypes and three (21%) patients had t(8:21), and the remaining three patients had t(7:11), t(6:9), and del(9), respectively. Mean value of bone marrow blasts percentage was 51%.

Table 2. Clinical characteristics of study patients

Sample	ID	Age	Sex	Chromosomal abnormality	Bone marrow blasts, %
Paired sample of AML diagnosis and initial complete remission	1-1/1-2	55	M	t(8:21), -Y	76% / 3%
	2-1/2-2	69	F	t(7:11)	22% / 0%
	3-1/3-2	20	F	normal	69% / 1%
	4-1/4-2	49	F	t(8:21)	33% / 0%
	5-1/5-2	52	M	t(8:21)	50% / 1%
	6-1/6-2	43	M	normal	53% / 0%
	7-1/7-2	25	M	normal	71% / 4%
	8-1/8-2	60	F	t(6:9)	58% / 5%
Solitary sample of AML diagnosis	9	58	M	normal	20%
	10	65	F	del(9)(q13q22)	21%
	11	50	F	normal	22%
	12	54	M	normal	90%
	13	78	M	normal	59%
	14	45	F	normal	74%
Normal marrow control	15	69	F	immune thrombocytopenia	1%
	16	37	M	immune thrombocytopenia	3%
	17	33	F	immune thrombocytopenia	0%
	18	60	M	immune thrombocytopenia	2%
	19	63	F	normal	1%

Table 3. Components of small RNA sample prep kit.

Reagent Name16	Maker
RNA 3` Adaptor (RA3)	Illumina
Ligation Buffer (HML)	Illumina
RNase inhibitor	Illumina
T4 RNA Ligase 2 (Deletion Mutant)	Epicentre
Stop Solution	Illumina
RNA 5` Adaptor (RA5)	Illumina
ATP	Illumina
T4 RNA Ligase	Illumina
25 mM dNTP	Illumina
RNA RT Primer (RTP)	Illumina
5X First strand buffer	Invitrogen
0.1M DTT	Invitrogen
RNase inhibitor	Invitrogen
SuprerScript 2 reverse transcriptase	Invitrogen
PCR Mix (PML)	Illumina
RNA PCR primer (RP1)	Illumina

Results of library construction

In the previous gel, we confirmed that these bands really corresponded to the target size we wanted, after the bands corresponding to 140 ~ 160bp (size which considering small RNA size + the size of primer and index attached to both ends) were purified.

Sequencing results

The table 4 shows the sequencing statistics of 19 samples. Q scores are used to measure base calling accuracy, one of the most common metrics for assessing sequencing data quality. Q scores can reveal how much of the data from a given run is usable in a resequencing or assembly experiment. Our sequencing results showed that quality scores above Q30 (% >=Q30 bases) were approximately 90% in all samples, which lead to decrease false-positive variant calls.

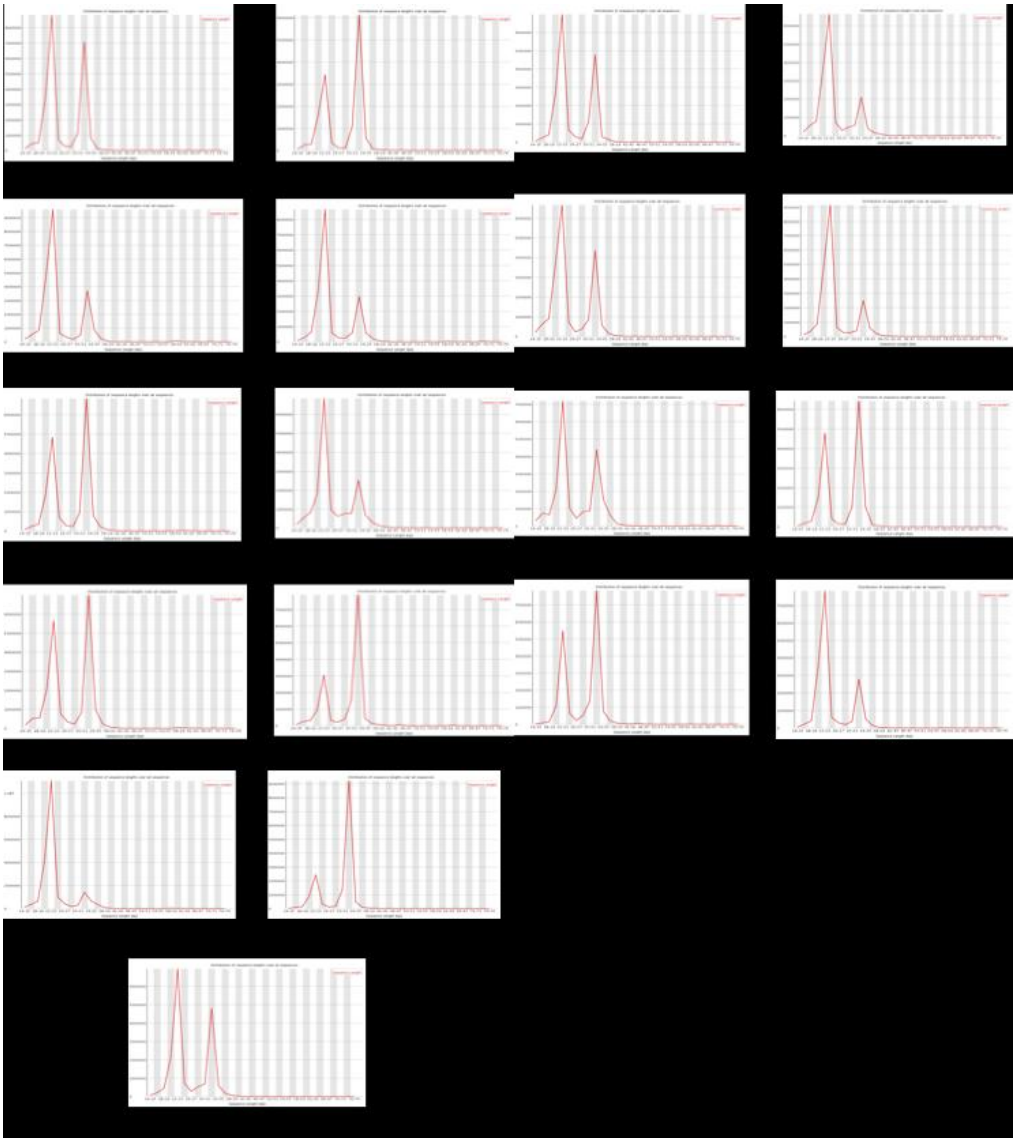
Table 4. Sequencing statistics (Shown only 19 samples in first run)

Preprocessing results

The table 5 shows the preprocessing results of 19 samples. Among total reads, we filtered reads without sequencing adapter. Then we filtered short reads (<15 nt) and long reads (>50nt) (Figure 3).

Table 5. Preprocessing results (Shown only 19 samples in first run)

Figure 3 shows the read length after cleaning (≥15nt) of 19 samples.



Descriptive statistics of microRNA differential expression

The number of reads that mapped to alleged microRNAs in the miRBase database ranged from 2,816,113 to 13,405,453. All microRNA mapping reads belonged to 511 unique microRNA families. During mapping, cleaned reads were mapping to reference genome. Then, 91 to 97% of cleaned reads were counted according to the registered human miRNA in the miRBase database. The table 6 shows the mapping results of 19 samples.

Table 6. Mapping statistics (Shown only 19 samples in first run)

	22,468,812	94%	10,234,540
	13,089,354	91%	4,413,021
	22,062,968	96%	12,887,560
	18,171,083	96%	10,000,265
	17,180,385	95%	7,995,352
	16,062,097	96%	8,780,489
	18,800,437	95%	9,206,876
	20,037,428	97%	12,661,077
	16,428,400	93%	5,897,678
	16,469,992	94%	6,458,738
	18,716,070	94%	6,543,106
	14,431,861	90%	3,222,135
	20,317,280	95%	7,611,816
	15,144,105	93%	4,596,868
	17,385,011	92%	4,371,382
	16,769,170	97%	9,966,681
	20,699,772	97%	13,405,453
	14,185,768	90%	2,816,113
	17,133,682	95%	7,766,225

Twelve microRNAs were identified as AML relevant microRNA

To establish whether the microRNA profiles of AML cells and normal controls may differ, we first compared the 14 AML samples and five normal controls (Figure 1A). Upon initial comparison, 19 microRNAs were found to be differentially expressed in AML as compared to normal controls. Twelve of these microRNAs had higher expression levels in AML than in normal controls (Table 7).

Six microRNAs were related to leukemia activity

Approximately 80% of patients with AML achieve complete remission with conventional induction chemotherapy [16]. In complete remission, the total body leukemic cell population is reduced from approximately 10^{12} to below approximately 10^{9} cells. Likewise, we hypothesized that expression of AML-relevant microRNAs could substantially decrease from the time of initial diagnosis to remission. Importantly, we assert that using a paired sample from the same patient can minimize interpersonal variation. Therefore, we compared the expression of microRNA between initial diagnosis and complete remission in eight AML paired samples (Figure 1B). Six microRNAs had higher expression levels at initial AML diagnosis as compared to initial complete remission (Table 7).

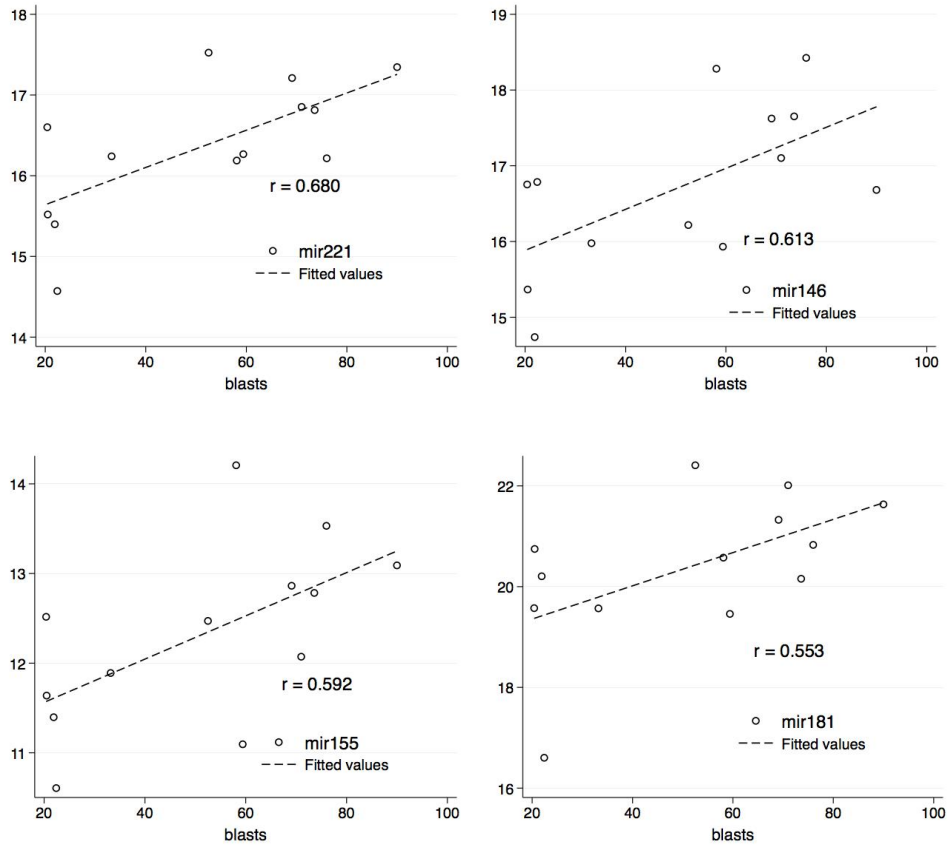
Four microRNAs according to AML tumor burden

In the context of the third comparison (Figure 1C), we assert that certain microRNAs correlated with marrow blasts counts may be relevant to AML. Indeed, we detected positive correlations between higher expression levels of mir-221, mir-146, and mir-155 with higher marrow blast counts, with Pearson's correlation coefficients of r = 0.680, r = 0.613 and r = 0.592, respectively (Table 7).

Table 7. AML-related miRNA in 3-comparison approaches

Comparison	miRNA	*p*-value
14 AML patients vs. 5 normal marrow controls AML: higher / Normal: lower expression	mir-155, mir-191, mir-221, mir-1291	$p < 0.001$
	mir-146, mir-181, mir-573, mir-935, mir-1271, mir-3154, mir-3607, mir-4791	$0.001 < p < 0.005$
In eight paired samples, initial diagnosis vs. complete remission Diagnosis: higher / Remission: lower expression	mir-181, mir-1468, mir-3154	$p < 0.001$
	mir-221, mir-577, mir-3913	$0.001 < p < 0.005$
In 14 AML patients, positive linear correlation according to blast count The higher marrow blast count, the higher miRNA expression	mir-221 mir-146 mir-155 mir-181	$r = 0.680$ ($p < 0.001$) $r = 0.613$ ($p = 0.001$) $r = 0.592$ ($p < 0.001$) $r = 0.553$ ($p = 0.002$)

Figure 4. The linear corrleation between microRNAs (mir-221, mir-146, mir-155, and mir-181) and blasts counts

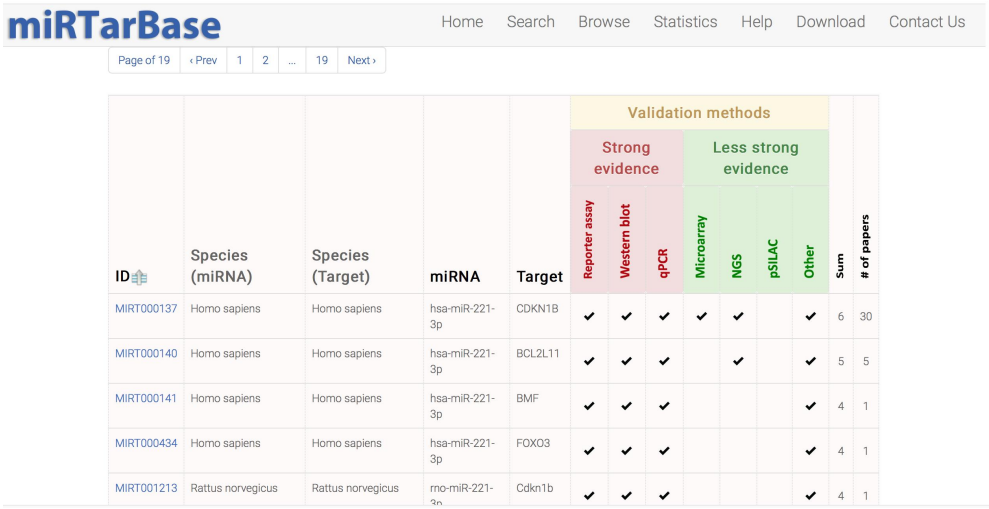


Prediction of microRNA target gene

For mir-221 and mir-181, which are selectively enriched in AML marrow and reflect disease activity, target genes were predicted via miRTarBase. We confined our results in Homo sapiens species and functional MTI (strong) support type. The number of target genes for mir-221 and mir-181 were 70 genes and 118 genes, respectively. The common target genes between mir-221 and mir-181 are *BCL2L11, CDKN1B, DTIT4, FOS, MGMT, PTEN, RUNX1, TIMP3,* and *TMED7.*

Figure 6. Process to find target genes via miRTarBase

(1) Targets of mir-221 (Shown only partial)



(2) Targets of mir-181 (Shown only partial)

4. Discussion

In the present study, we identified 12 microRNAs that were expressed more highly in AML samples than in normal controls. Among these 12 microRNAs, three were found to reflect AML stage; mir-181, mir-221, and mir-3154 were detected at significantly higher levels upon initial AML diagnosis (before chemotherapy) as compared to complete remission (after chemotherapy). We also explored microRNAs that correlated to marrow blast counts and therefore represented disease burden; high expression levels of four microRNAs (mir-221, mir-146, mir-155, and mir-181) were significantly correlated with higher marrow blast counts.

Interestingly, all comparisons revealed mir-221 expression levels to be associated with aspects of AML burden. mir-221/222 is known to inhibit normal erythropoiesis and erythroleukemic cell growth [9]. Furthermore, primary microRNA-221/222 is overexpressed in AML, and it is thus a putative oncogene [17,18]. In lymphocytic leukemia, increased expression of mir-221/222 affects the proliferation of leukemic cells [19] and is associated with poorer clinical outcome [20]. We obtained consistent results; mir-221 was expressed significantly higher in AML samples than in normal controls, and mir-221 was downregulated in patients with leukemia remission status after inhibition of proliferation.

A functional relationship exists between mir-221/222 and

p27Kip1, a key regulator of the cell cycle; a known target of mir-221/222 includes a regulator of p27Kip1, cyclin-dependent kinase inhibitor 1B (CDKN1B) [21,22]. Regulation of p27Kip1 expression by aberrant mir-221/222 expression promotes cancer cell progression [23]. Thus, the use of synthetic inhibitors of mir-221/222 as a putative oncogene might be a promising approach to cancer treatment [24-26]. Moses et al. reported that overexpression of miR-221 in acute lymphoblastic leukemia cells during bone marrow stromal cells coculture prompted cell-cycle progression and sensitization of acute lymphoblastic leukemia cells to cytotoxic agents, blunting the protective influence of the bone marrow microenvironment [26]. These novel observations indicate that bone marrow microenvironment regulation of miR-221/222 contributes to marrow niche-supported tumor cell quiescence and survival of residual cells.

The mir-181 family, which was included in comparisons 1 and 2, was the first microRNA family discovered to be specifically expressed in hematopoietic cells [27]. Accumulating data has shown that the mir-181 family plays a critical role in regulating normal cell differentiation and leukemogenesis, particularly in AML [27,28]. A number of studies have shown that mir-181 expression is correlated to cytogenetic and molecular subtypes of AML and has potential as a diagnostic marker for AML [29-31]. Moreover, mir-181 family members have been consistently reported as prognostic markers in AML patients [10, 32-34]. Based on these reports, studies on chemotherapy combined with

strategies targeting mir-181 have been conducted as a new strategy for AML therapy.

Nine genes (*BCL2L11, CDKN1B, DTIT4, FOS, MGMT, PTEN, RUNX1, TIMP3*, and *TMED7)* were targeted by both mir-221 and mir-181, meaning it had the highest connectivity of the mRNAs in the miRNA-mRNA network. A common deletion polymorphism of the gene *BCL2L11* (Bcl-2 like protein 11, *BIM)* has been reported to tyrosine kinase inhibitor resistance in several malignant rumors. However, a predictive role of *BIM* deletion was not shown in chronic myeloid leukemia [35] and AML. Further investigation of the potential function of these genes is required in AML. *DTIT4, FOS,* and *TMED7* have not been evaluated in scientific literature. *MGMT* promoter methylation is well-known predictive factor of temozolomide in the treatment of glioblastoma. Additionally, a few studies reported that a *MGMT* promoter methylation is a potential prognostic indicator for acute leukemia and is related to chemotherapy outcomes in patients with AML [36,37]. Regarding *RUNX1,* a novel fusion gene involving *RUNX1* was reported recently and its clinical implication has been studied actively [38,39]. Increasing *TIMP3* expression by hypomethylating agents facilitates NK cell-mediated immune recognition, and *TIMP3* could be a target of the demethylating treatment in AML

patients [40].

Another microRNA that was included in comparisons 1 and 2, mir-3154, has been newly reported in 2017. Chu et al. identified that transcription factor CCAAT/enhancer binding protein delta *(CEBPD)* induced miRNAs; miR-744, miR-3154 and miR-3162 could target CpG islands in the 5′-flanking region of the *CEBPD* gene, and contributed to bortezomib-induced cell death [41]. Zeng et al. reported that integrative miRNA analysis identified miR-3154, miR-7-3, and miR-600 as potential prognostic factor for cervical cancer, the most common cancer in women. miR-3154 was significantly associated with shortened survival time and more death cases in patients with cervical cancer [42].

Small microRNA sequencing by NGS has many advantages. One clear advantage is the ability to find novel microRNAs. Additionally, because microRNAs have relatively short sequence of 18 to 24 nucleotides, NGS could sequence microRNAs that are vulnerable to rapid degradation [43]]. However, there is no evidence that NGS is superior to microarray or real-time PCR [44].

Our findings are limited by selection bias with respect to a 29% sequencing failure rate and small sample sizes. Additionally, the microRNAs reported in the present study need to be validated in independent datasets, and functional characteristics must be elucidated.

Our results demonstrate that mir-221 and mir-181 are selectively enriched in AML marrow and reflect disease activity. mir-3154 seems to be a novel candidate microRNA that is relevant to AML but requires further validation.

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6. 국문초록

목적: 급성골수성백혈병은 조혈 생성 기전의 손상으로 골수 기능을 소실되는 것을 특징으로 하는 혈액암의 한 종류로, 미분화된 골수 전구체의 복제 확대로 인해 발생하는 것으로 알려져 있다. 급성골수성백혈병은 반복되는 염색체 이상과 유전자 돌연변이와 밀접한 연관이 있다는 것이 밝혀져 있는데, 최근 연구를 통해 전사 이후의 조절을 담당하는 마이크로 RNA가 유전자 돌연변이의 표현을 조절하여 암의 병태 생리에 관여한다는 사실이 보고되어 왔다. 본 연구에서는 급성골수성백혈병 환자의 골수 검체를 small RNA 차세대 유전체 분석을 통해, 급성골수성백혈병의 병태 생리와 밀접한 연관성을 보이는 마이크로 RNA를 찾아내고자 한다.

방법: 급성골수성백혈병으로 진단받은 환자의 서로 다른 두 시점 (초기 진단 및 최초 완전 관해 상태)에서 골수 샘플을 얻었다. 골수 샘플에서 RNA를 분리하고, small RNA 라이브러리를 기본으로 하여 차세대 유전체 분석을 시행하였다. 해당 마이크로 RNA 유전자에 정렬된 판독을 계수화 하여 마이크로 RNA의 서로 다른 표현양을 분석하였다.

결과: 총 16명의 급성골수성백혈병 환자에서 진단 시 및 치료 후의 서로 다른 시점에서 각각 샘플을 얻었고 (총 32개), 대조군으로 활용할 6명의 정상 골수 샘플을 합하여 총 38개의 샘플을 얻었다. 이중 차세대 유전체 분석이 가능할 정도로 RNA가 추출되었던 27개 샘플이 최종 분석 대상이 되었다. 마이크로 RNA의 유전체 분석 결과 12개의 마이크로 RNA가 정상 컨트롤에 비해 급성골수성백혈병 환자에서 선택적으로 높게 발현됨을 확인하였다. 12개의 마이크로 RNA 중에서 급성골수성백혈병 환자의 최초 완전 관해 상태 보다 처음 진단 상태에서 높게 발현된 마이크로 RNA는 mir-181, mir-221, mir-3154였다. 추가 분석을 통해 골수 내 미분화 세포가 많을수록 mir-221,

mir-146, mir-155, mir-181가 좀 더 높게 발현됨을 확인 하였다.
결**론:** 본 연구 결과 mir-221과 mir-181이 급성골수성백혈병의 골수에서 선택적으로 과발현 함을 확인 할 수 있었으며 이는 해당 마이크로RNA가 질병의 활동 상태를 반영한다는 것을 보여준다. 한편 mir-3154는 급성 백혈병과 연관이 있지만 추가적인 검증이 필요한 새로운 마이크로 RNA로 생각된다.

주요어: 마이크로 RNA, 차세대 유전체 분석, 급성골수성백혈병
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