doi:10.4149/neo_2017_411

Microarray profiling defines circulating microRNAs associated with myelodysplastic syndromes

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Received November 7, 2016 / Accepted January 31, 2017

Circulating microRNAs (miRNAs) are non-coding RNAs secreted into body fluids, and aberrant levels of these miRNAs correlate with diseases of various origins, making them highly potential clinical biomarkers. We investigated the spectrum of circulating miRNAs in the plasma of myelodysplastic syndrome (MDS) patients to identify miRNAs showing discriminatory levels in the patients with different prognosis. Plasma samples were analyzed with microarrays to define miRNA profiles, and the deregulated miRNAs were further studied using droplet digital PCR. With regard to the prognosis, the levels of miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p and miR-451a were reduced in higher-risk MDS. Multivariate analysis indicated miR-451a level as an independent predictor of progression-free survival (HR = 0.072, $P = 0.006$) and revealed a significant association of miR-223-3p level with overall survival (HR = 0.039, $P = .032$). Our data demonstrate that plasma levels of specific miRNAs are associated with MDS patient outcome and may add information beyond the currently used scoring systems.

Key words: circulating miRNA, myelodysplastic syndromes, plasma, progression, microarray

Myelodysplastic syndromes (MDS) are a heterogeneous group of malignant hematopoietic stem cell (HSC) disorders characterized by cytopenias, inefficient hematopoiesis, dysplasia in one or more myeloid cell lineages and an increased risk of development to acute myeloid leukemia (AML). Patient prognosis is predicted using the International Prognostic Scoring System (IPSS), which takes into account peripheral cytopenias, blast percentage, and cytogenetic aberrations. However, due to the high heterogeneity of MDS, development of molecular tools that can predict patient prognosis and outcomes is still necessary.

Recently, it was discovered that microRNA (miRNA) molecules are not only present in the cellular environment but also circulate in a wide variety of body fluids, including blood plasma. These extracellular molecules are not mere remnants of cellular degradation lacking specific functions, as the active transport of miRNAs between distant cells can play an important role in long distance cell-to-cell communication [1-3].

In cancers, monitoring of plasma miRNAs has the potential to allow earlier diagnosis and refine a prognosis or predict the response to therapy. To date, only a few studies have investigated circulating miRNAs in MDS [4-6]. Two papers [4, 5] focused on specific circulating miRNAs (miR-21, let-7a, and miR-16) that were preselected based on information about their deregulation in blood cells and their importance in similar diseases. Researchers monitored the plasma/serum levels of these miRNAs and showed that their levels could serve as prognostic markers for MDS [4, 5]. A recently published study by Zuo *et al.* [6] is the only available study investigating the miRNA profile in MDS plasma thus far. This study profiled the expression of 800 human miRNAs in MDS plasma and identified a 7-miRNA signature (let-7a, miR-144, miR-16, miR-25, miR-451, miR-651, and miR-655) as an independent predictor of survival in MDS patients with normal karyotypes.

To extend the panel of molecular markers for the prediction of MDS patient outcomes, we studied the changes in the plasma miRNAome in MDS patients, irrespective of cytogenetic findings, using arrays covering more than 2,000 human miRNAs. We determined miRNAs whose levels discriminated lower-risk patients from those with higher-risk MDS and evaluated their prognostic significance in predicting patient outcomes.

Patients and methods

Patients. The study included 40 peripheral blood plasma samples collected from patients with primary MDS with no known history of previous malignancy, chemotherapy or radiation therapy from the Institute of Hematology and Blood Transfusion. The patient's diagnosis was assessed according to the WHO classification criteria. None of the patients had received therapy for their disease or HSC transplantation (HSCT) prior to blood collection. Blood plasma samples from 20 age-matched healthy donors with no adverse medical histories were used as controls. Written informed consent was obtained from all tested subjects in accordance with the approval from the Institutional Review Board.

RNA extraction. Peripheral blood was collected in EDTA tubes, blood plasma was separated from peripheral blood by centrifugation, and the samples were stored at -80 °C. The absence of hemolysis in the plasma samples was confirmed spectrophotometrically by measuring the free hemoglobin (oxyhemoglobin absorbance at 414 nm) [7]. Plasma samples were further centrifuged at 12,000 g, 4 °C for 10 minutes to dispose of cell debris. Total RNA was extracted from individual samples using a modified phenol-chloroform extraction method with Trizol LS reagent (Invitrogen, Basel, Switzerland) according to the procedure of Filková *et al.* (2014) [8] and was quantified with a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Patient characteristics.

Number of patients		40
Gender (male/female)		30/10
Age; mean (range)		66 (40-85)
Diagnosis (RCUD/RARS/RCMD/RAEB1/RAEB2)		2/3/17/4/14
IPSS category (low/intermediate-1/intermediate-2/high)		4/20/10/6
IPSS karyotype (good/intermediate/poor)		25/9/6
Cytogenetic features		
normal karyotype		23
isolated del(20q)		2
isolated +8		2
complex		6
other		7
Marrow blasts [%]: mean (range)		6.1 (0.0-19.6)
Hemoglobin (g/L): mean (range)		100 (51-138)
Neutrophils (x10^9/L): mean (range)		1.9 (0.2-8.6)
Platelets (x10^9/L): mean (range)		123 (13-528)
Follow-up	number of patients	38
	mean follow-up [months] (range)	22.7 (2.3-78.5)
i. stable disease	number of patients	16
	mean follow-up [months] (range)	27.7 (3.2-78.5)
ii. progression	number of patients	22
	mean time from diagnosis [months] (range)	14.3 (2.3-58.0)
iii. HSCT	number of patients	7
	mean time from diagnosis [months] (range)	6.7 (2.3-13.4)
iv. exitus	number of patients	11
	mean time from diagnosis [months] (range)	18.5 (3.6-46.7)

miRNA microarray profiling. Genome-wide miRNA profiles were determined using Agilent Human miRNA Microarrays (Sure Print G3 Unrestricted miRNA 8x60K, Release 19.0, Agilent Technologies, Santa Clara, CA, USA) in a discovery cohort (14 MDS patients and 7 healthy donors). The sample input was 350 ng of total RNA. The samples were processed individually according to the manufacturer's protocol, and scanning was performed on an Agilent Microarray Scanner.

Quality control and background subtraction of raw microarray data were performed using Feature Extraction Software. Only those miRNAs whose signals were detected in more than 4 samples were included for further analysis. Quantile normalization and differential gene expression were performed in the R statistical environment (www.r-project.org) using standard statistical functions and the gtools package (|FC| and its modifications). Subsequent analysis of the microarray data was performed using MeV v4.8.1 software [9]. The Welch approximate t-test was applied to determine miRNAs differentially expressed between two groups of samples: (i) patients and controls and (ii) lower- and higher-risk patients. The complete raw and normalized data have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible through GEO Series accession number GSE76775.

Droplet digital PCR. The quantity of miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a was verified in the validation cohort (all 40 patients) via droplet digital PCR (ddPCR) using QX200 ddPCR system (Bio-Rad, Hercules, CA, USA). Firstly, miRNAs were reverse-transcribed by TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) following manufacturer´s instruction. Subsequently, PCR reaction was done with 2X ddPCR supermix for probes (Biorad) and 20X Taqman miRNA assay (Life Technologies). Droplets were prepared using QX200 Droplet Generator (Biorad) and PCR was performed under following conditions: 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, followed by enzyme deactivation at 98 °C for 10 minutes. Droplets with signal were counted by QX200 Droplet Reader (Biorad) and QuantaSoft software v. 1.6.6 (Biorad). A no template control and a negative control for each reverse transcription reaction were included in every assay.

Data analysis. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and SPSS (SPSS Inc., Chicago, IL) software. A Mann-Whitney test was used to compare levels of circulating miRNAs between different sample groups. The sensitivity and specificity of the optimum cut-off points were defined as the values that maximized the area under the ROC curve (AUC). The progression-free survival (PFS) and overall survival (OS) curves were generated by the Kaplan-Meier method, and the differences between groups were assessed by the log-rank test. Multivariate analysis was performed using the Cox proportional regression model. Pearson correlation analysis was

performed to identify possible dependence between miRNA levels and blood count data.

Results

Characteristics of the study cohort. The study cohort included 40 plasma samples (30 males and 10 females) from primary MDS patients. The detailed clinical characteristics of all patients are summarized in Table 1. Patients' ages ranged between 40 and 85 years (mean 66 years), and their diagnoses according to the WHO criteria were as follows: refractory cytopenia with unilineage dysplasia (RCUD; 5%; N = 2), refractory anaemia with ringed sideroblasts (RARS; 7.5%; N = 3), refractory cytopaenia with multilineage dysplasia (RCMD; 42.5%; N = 17), refractory anaemia with excess blasts-1 (RAEB-1; 10%; N = 4), and RAEB-2 (35%; N = 14). Karyotyping revealed normal karyotypes in 23 patients (57.5%). Various chromosomal abnormalities were found in 17 patients (42.5%; e.g., isolated del(20q) – 2 patients, trisomy 8 – 2 patients, complex karyotype – 6 patients). According to the IPSS scoring system, the cytogenetic risk was good in 62.5% (N = 25), intermediate in 22.5% (N = 9), and poor in 15% (N = 6) cases, and patients were stratified into low (10%, N = 4), intermediate-1 (50%, N = 20), intermediate-2 (25%, N = 10), and high (15%, N = 6) IPSS categories. For expression analyses, the patients were further divided into a lower-risk MDS group (low /intermediate-1 IPSS categories, N = 24) and a higher-risk MDS group (intermediate-2/high IPSS categories, N = 16).

Table 2. The most abundant miRNAs in plasma of MDS patients and healthy donors

	MDS patients	Signal intensity (mean)	Healthy donors	Signal intensity (mean)
1	miR-6089	25625	miR-4454	23779
2	miR-4454	11048	miR-6089	14383
3	miR-6125	9097	miR-451a	6069
4	miR-4516	4895	miR-6090	5327
5	miR-6090	4797	miR-223-3p	4575
6	miR-2861	4414	miR-3960	3652
7	miR-3960	3892	miR-6125	3605
8	miR-638	3724	let-7b-5p	3151
9	miR-4459	2711	miR-4516	2510
10	miR-3665	1805	miR-21-5p	2494
11	miR-1234-5p	1775	miR-2861	1876
12	miR-6068	1774	miR-5100	1788
13	miR-4281	1698	miR-16-5p	1678
14	miR-1915-3p	1622	let-7a-5p	1641
15	miR-630	1579	miR-4459	1407
16	miR-371b-5p	1533	miR-4281	1271
17	miR-451a	1351	miR-6087	1205
18	miR-6087	1309	miR-638	1162
19	miR-5703	1267	let-7f-5p	1129
20	miR-5100	1207	miR-92a-3p	1092

Expression profiling of circulating miRNAs in MDS plasma. Genome-wide miRNA profiling was performed in the discovery cohort, and among the 2,006 miRNAs present on the array, 207 and 201 miRNAs were detectable in the MDS and control samples, respectively. The miRNAs that showed the highest expression levels in patients and healthy donors are listed in Table 2. Comparative analysis of the patient and control groups of samples identified 48 miRNAs showing significantly ($P < .01$) altered levels in the plasma of MDS patients (irrespective of their clinical parameters) compared to their expression levels in control plasma (Figure 1 and Suppl

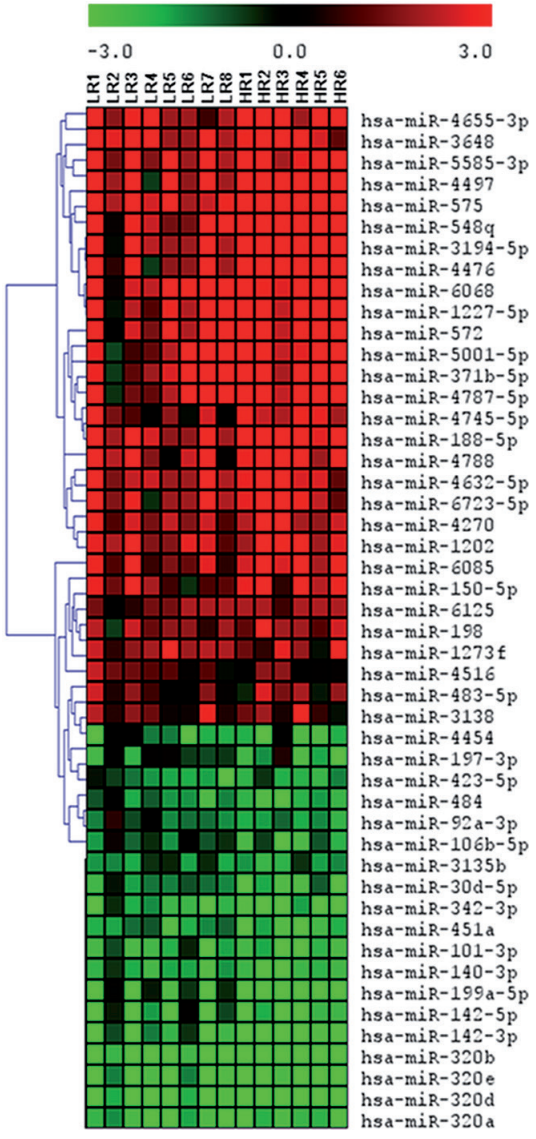

Figure 1. MiRNAs deregulated in MDS plasma compared to controls. Heatmap shows only the differentially expressed plasma miRNAs ($P < .01$) in all MDS patients compared to controls. Color gradient intensity scale indicates fold change (binary logarithm) of signal intensities compared with the mean signal intensity of controls. red – increase, green – decrease, LR – lower-risk MDS patient, HR – higher-risk MDS patient.

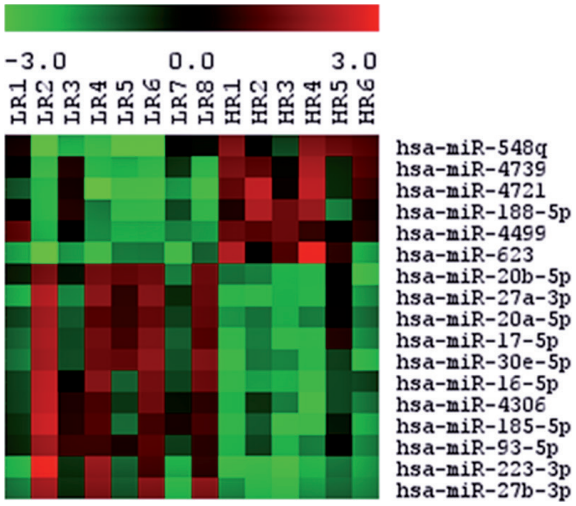

Figure 2. MiRNAs altered between lower-risk and higher-risk MDS. Heatmap shows differentially expressed plasma miRNAs ($P < .01$) between lower-risk and higher-risk MDS patients. Color gradient intensity scale indicates fold change (binary logarithm) of signal intensities compared with the mean signal intensity for all patient samples. red – increase, green – decrease, LR – lower-risk MDS patient, HR – higher-risk MDS patient.

Table 1). Among those miRNAs, 19 were downregulated (e.g., miR-451a, miR-92a-3p, miR-320a/b/d/e, and miR-142-3p/5p), and 29 were upregulated (e.g., miR-150-5p, miR188-5p, and miR-371b-5p) in the patients.

The patients were further divided into a lower-risk category (N = 8) and a higher-risk category (N = 6). Statistical testing was performed to compare expression profiles of plasma miRNAs between these two groups, and 17 miRNAs were identified as differently expressed (e.g., miR-16-5p, miR-17-5p, miR-27a-3p/b-3p, miR-223-3p were downregulated and miR-188-5p, miR-623 were upregulated in higher-risk MDS compared to lower-risk MDS) (Figure 2 and Suppl Table 2).

Quantification of individual circulating miRNAs by droplet digital PCR. Based on the microarray results, we selected six hematopoiesis- and/or oncology-related miRNAs (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a) that showed deregulated levels in MDS plasma and quantified their levels via ddPCR in the validation cohort. In concordance with the microarray data, plasma miR-150-5p was increased and miR-16-5p, miR-27a-3p, miR-199a-5p, and miR-451a were reduced in MDS patients compared to healthy controls. Comparison of miRNA levels between higher-risk and lower-risk groups revealed significantly ($P < .05$) lower levels of miR-27a-3p, miR-199a-5p, and miR-223-3p in higher-risk disease (Figure 3).

Correlation of miRNA levels with clinical variables. A series of univariate analyses was performed for various clinical variables and for each of the selected miRNAs to evaluate whether their plasma levels were potential predictors of patient outcomes. The mean patient follow-up was 22.7 months, and within this time period, 16 patients exhibited stable disease; 22 patients progressed; and 11 patients died. The univariate analysis indicated significantly different PFS ($P < .05$) associated with the following parameters: IPSS karyotype, IPSS score, amount of cytopenias, WHO-based diagnosis, and the levels of five miRNAs (miR-27a-3p, miR-

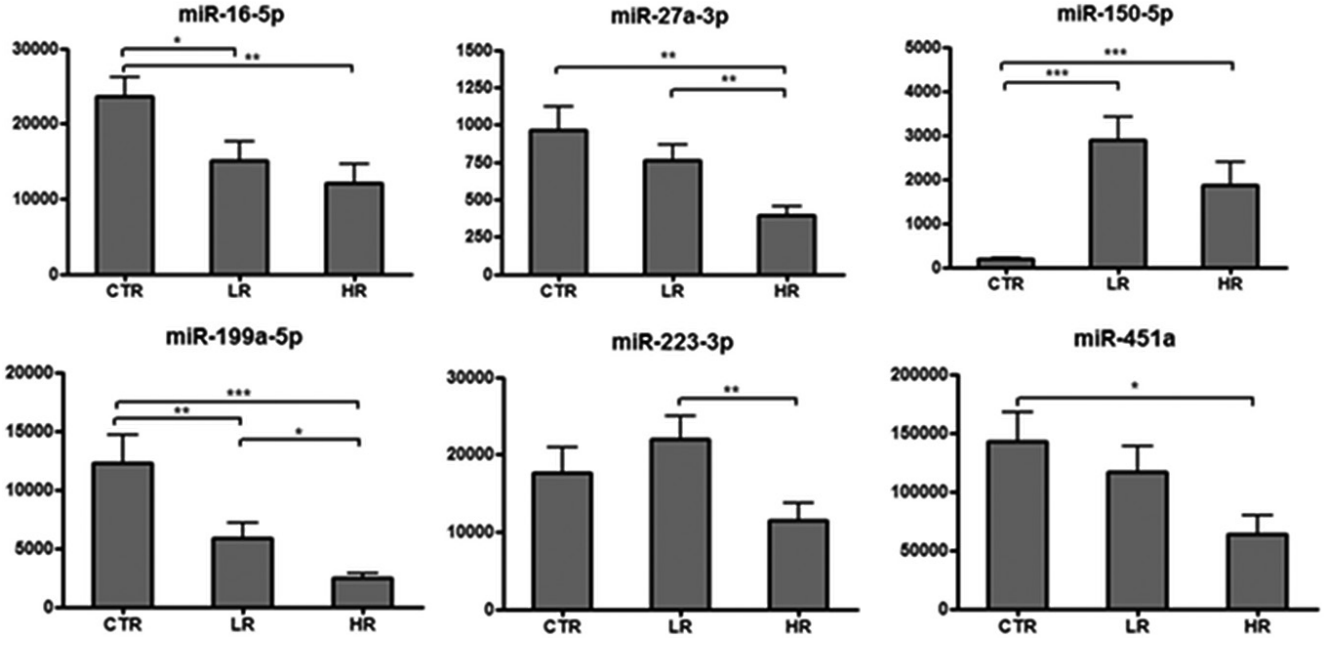

Figure 3. Level of plasma miRNAs. Absolute amounts of plasma miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a were quantified via ddPCR. CTR – control, LR – lower-risk, HR – higher-risk MDS, * $P < .05$, ** $P < .01$, *** $P < .001$.

150-5p, miR-199a-5p, miR-223-3p, and miR-451a). The univariate analysis for OS showed distinct stratification of MDS patients based on miR-27a-3p and miR-223-3p plasma levels. The cut-off values for each miRNA assessed based on ROC curve analysis, mean PFS and OS values, 95% confidence intervals (CIs) and *P* values are listed for each of the tested variables in Table 3. Multivariate Cox analyses confirmed that the miR-451a plasma level (cut off $100x10^4$ copies/µl of plasma) and amount of cytopenias were associated with PFS and that the plasma level of miR-223-3p (cut off $17x10^4$ copies/µl of plasma) was associated with OS (Table 4). Kaplan-Meier curves showing that patients with low miR-451a expression exhibited significantly decreased PFS (25.7 months, 95% CI 10.4 to 41.1) compared with patients with high expression (41.5 months, 95% CI 29.1 to 54.0) and that patients with low miR-223-3p expression presented significantly decreased OS (27.7 months, 95% CI 13.4 to 41.9 months) compared with patients with high expression (70.6 months, 95% CI 57.1 to 84.1 months) are included in Figure 4.

To examine the possible origin of circulating miRNAs in different blood cell populations, we performed a correlation analysis for the six selected miRNAs with blood count data (numbers of erythrocytes, platelets, neutrophils, monocytes, leukocytes, and lymphocytes; hemoglobin concentration; and percentage of blasts in bone marrow). However, we did not detect significant correlations between the levels of any of the selected miRNAs and blood counts of the tested lineages.

Table 3. Univariate analysis for progression-free survival and overall survival.

Variable		Progression-free survival			Overall survival		
		Mean est. (mo)	95% CI	*P* value	Mean est. (mo)	95% CI	*P* value
Age	< 65 years	26.0	9.5-42.4	0.155	52.1	29.9-74.3	0.739
	≥ 65 years	35.9	23.3-46.6		42.6	27.6-57.6	
Gender	male	36.8	23.3-50.4	0.375	46.3	33.0-65.6	0.507
	female	23.8	8.6-39.1		49.3	29.4-63.1	
Diagnosis	RCUD/ RARS	41.7	19.5-63.9	**0.038**	41.7	19.5-63.9	0.958
	RCMD	41.2	24.6-57.8		50.6	32.4-68.9	
	RAEB-1/ RAEB-2	13.2	7.5-18.9		26.8	20.1-33.5	
IPSS category	lower-risk	42.2	27.9-56.4	**0.013**	50.8	35.2-66.5	0.709
	higher-risk	12.7	6.9-18.6		26.3	19.2-33.5	
IPSS karyotype	good	42.5	27.9-57.2	**0.005**	53.6	36.8-70.5	0.082
	intermediate	24.7	10.6-38.9		42.0	30.7-53.3	
	poor	6.4	3.3-9.6		10.2	6.4-13.9	
Blasts	< 5%	38.5	24.0-53.0	0.191	45.3	28.9-61.7	0.314
	≥ 5%	22.0	8.8-35.1		47.6	28.4-66.8	
Hemoglobin	< 100 g/l	26.2	12.9-39.5	0.082	48.5	29.5-67.5	0.699
	≥ 100 g/l	37.0	22.7-51.2		42.3	27.7-57.0	
Neutrophils	$< 1.5x10^9$/l	29.4	15.1-43.7	0.467	40.7	24.1-57.3	0.183
	$\geq 1.5x10^9$/l	34.5	20.7-48.3		59.7	46.9-72.4	
Platelets	$< 150x10^9$/l	27.3	15.0-39.5	0.126	43.8	26.7-60.7	0.179
	$\geq 150x10^9$/l	38.8	23.2-54.4		49.6	32.4-66.8	
Amount of cytopenia	0-1 lineage	46.8	31.0-62.5	**0.024**	59.0	49.2-68.7	0.084
	2-3 lineages	24.9	13.7-36.1		40.0	23.8-56.2	
miR-16-5p plasma level	$< 12x10^4$ copies/µl of plasma	25.6	11.4-39.8	0.338	49.8	29.1-70.4	0.769
	$\geq 12x10^4$ copies/µl of plasma	33.6	20.3-47.0		41.8	25.7-58.0	
miR-27a-3p plasma level	$< 2.7x10^3$ copies/µl of plasma	11.2	6.2-16.2	**0.005**	18.0	11.8-24.1	**0.001**
	$\geq 2.7x10^3$copies/µl of plasma	41.9	28.0-55.8		60.7	45.7-75.6	
miR-150-5p plasma level	$< 25x10^3$ copies/µl of plasma	26.9	14.0-40.0	**0.038**	44.9	27.6-62.2	0.089
	$\geq 25x10^3$ copies/µl of plasma	50.1	36.0-64.1		60.0	43.4-76.4	
miR-199a-5p plasma level	$< 2.6x10^3$ copies/µl of plasma	21.9	8.9-34.9	**0.048**	37.9	19.4-56.5	0.100
	$\geq 2.6x10^3$ copies/µl of plasma	37.4	24.3-50.5		47.6	30.9-64.4	
miR-223-3p plasma level	$< 17x10^4$ copies/µl of plasma	18.3	9.0-27.7	**0.008**	27.7	13.4-41.9	**0.001**
	$\geq 17x10^4$ copies/µl of plasma	49.6	32.1-67.2		70.6	57.1-84.1	
miR-451a plasma level	$< 100x10^4$ copies/µl of plasma	25.7	10.4-41.1	**0.029**	56.3	38.6-74.1	0.709
	$\geq 100x10^4$ copies/µl of plasma	41.5	29.1-54.0		47.6	33.3-61.9	

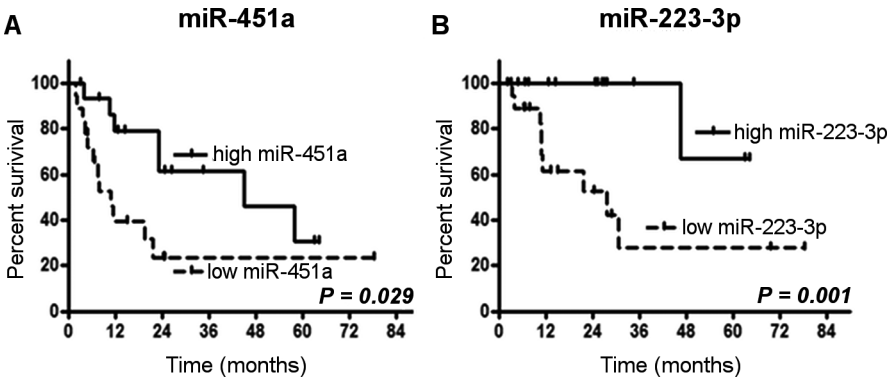

Figure 4. Patient survival according to miRNA levels. Kaplan-Meier curves for progression-free survival based on miR-451a plasma level (A) and overall survival according to miR-223-3p plasma level (B).

Expression of the selected miRNAs in CD34+ cells. Previously, we assessed miRNA signatures in CD34+ MDS cells through microarray profiling [10]. Here, we employed these data to evaluate the cellular levels of the plasma-related miRNAs (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a) with regard to patient prognosis. We observed some expression changes between controls, higher-risk, and lower-risk patient subgroups (Suppl Figure 1); however, we did not found any apparent relations of intra- and extracellular miRNA levels.

Discussion

Due to stability and accessibility, circulating miRNAs represent promising non-invasive cancer biomarkers. Numerous studies have demonstrated their ability to accurately classify discrete tumor types and disease stages. In this study, we defined specific miRNA profiles in MDS plasma and demonstrated that extracellular miRNAs may also be used for effective stratification of MDS patients with different prognoses.

To address the association of plasma miRNAs with patient prognosis, we assayed the levels of MDS-related miRNAs (miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a) selected based on our microarray results. Comparison of the miRNA plasma levels between higher-risk and lower-risk groups of patients suggested an association of the decreased levels with adverse prognosis. With respect to the premise that circulating miRNAs serve as active extracellular cell-to-cell communicators, reduction of multiple hematopoiesis-related miRNAs in plasma should presumably contribute to a more progressive failure of the entire hematopoietic process.

Using ROC curve analysis, we defined optimal cut-off points for each of these miRNAs and evaluated their prognostic value for PFS and OS. We showed that the low level of miR-451a is an independent predictor of reduced PFS, and the low level of miR-223-3p associates significantly with reduced OS. These results indicate that the levels of specific plasma miRNAs may predict MDS patient outcomes independent of other criteria.

The physiological roles of circulating miRNAs and the reasons of their deregulation in MDS are unclear. However, it is known that cellular miR-451a and miR-223 are strong regulators of the hematopoiesis and target differentiation of blood

Table 4. Multivariate Cox analysis for progression-free survival and overall survival.

	Progression-free survival			Overall survival		
Variable	HR	95% CI	P value	HR	95% CI	P value
Diagnosis	0.512	0.040-6.488	0.300	0.923	0.148-5.744	0.932
IPSS category	6.398	0.835-49.032	0.074	1.089	0.089-13.369	0.947
IPSS karyotype	1.207	0.358-4.064	0.762	0.151	0.014-1.693	0.125
Amount of cytopenia	12.647	1.578-101.358	**0.017**	2.128	0.172-26.287	0.556
miR-27a-3p	0.177	0.026-1.201	0.076	0.682	0.103-4.511	0.691
miR-150-5p	2.983	0.376-23.685	0.301	n.a.	n.a.	n.a.
miR-199a-5p	4.133	0.527-32.404	0.177	n.a.	n.a.	n.a.
miR-223-3p	0.739	0.155-3.513	0.704	0.039	0.002-0.856	**0.032**
miR-451a	0.072	0.011-0.467	**0.006**	n.a.	n.a.	n.a.

HR – hazard ratio, CI – confidence interval, n.a. – not analyzed

cells as well as their proliferation and apoptosis in many ways. miR-451 is a positive regulator of erythroid cell maturation [11], and miR-223 induces myeloid differentiation [12]. Both miR-451 and miR-223 are thought to act as tumor suppressors, and the low plasma levels of these miRNAs in higher-risk MDS patients may contribute to the promotion of leukemic cell growth. In AML, Gentner *et al.* found significantly higher miR-223 levels in blasts from patients with a favorable prognosis, whereas patients with low miR-223 expression showed worse outcomes [13]. Notably, we previously detected overexpression of cellular miR-451 and miR-223 in CD34+ cells in MDS patients with del(5q) [14], supporting our finding that high expression of these miRNAs is associated with a favorable prognosis in MDS.

There is a great interest in revealing the origin of circulating miRNAs in hematopoietic diseases. For example, Stamatopoulos *et al.* [15] showed that high levels of serum miR-150 in chronic lymphocytic leukemia (CLL) are linked to lymphocytosis and that cellular miR-150 could be regulated by its release from leukemic cells into the extracellular space. However, they could not definitively demonstrate the correlation between cellular and serum miR-150 levels, suggesting that multiple cells might release miR-150. We also attempted to trace the origin of deregulated miRNAs and tested whether cytopenia (one of the main features of MDS) is linked to the reduction of plasma levels of the miRNAs enriched in the cytopenic lineages (e.g., miR-451a is enriched in erythrocytes [11], miR-223 in myeloid cells [12], miR-27 in granulocytes [16], and miR-199 in platelets [17]). However, we did not observe any correlation between miRNA plasma levels and blood count values. Furthermore, we tested the myeloblastic cell population as another source of circulating miRNAs in MDS. We previously measured miRNA expression profiles in CD34+ MDS cells [10] (the majority of MDS myeloblasts are CD34+ [18]) and here we used these data to assess the correlation between cellular and plasma miRNA levels. However, the levels of plasma miRNAs did not reflect their levels in CD34+ cells. To sum up, these data suggest that miRNA release into MDS plasma is probably not a cell-specific but rather a miRNAs-specific process. In this context, Pigali *et al.* [19] previously reported that released miRNAs may not reflect the abundance of miRNAs in the cell of origin, suggesting that miRNAs are retained or released selectively and that extracellular miRNAs should be considered independent of cellular miRNA abundance when considering diagnostic markers.

In conclusion, the miRNA profiling performed on MDS plasma provides new information about the phenomenon of circulating miRNAs in MDS pathophysiology. Our data indicate that the specific miRNA signature in MDS plasma may predict patient outcomes independent of the currently used scoring systems. In particular, decreases in the levels of miR-451a and miR-223-3p appear to be potential indicators of an adverse prognosis in MDS. Further investigations to examine the plasma levels of these miRNAs in an independent series of patients and to monitor them over the course of disease therapy are needed to evaluate the applicability of these molecules in MDS prognostication.

Supplementary information is available in the online version of the paper.

Acknowledgements: The authors thank Prof Kyra Michalova (Center of Oncocytogenetics, Faculty Hospital and First Faculty of Medicine, Charles University, Prague) for the cytogenetic data. This work was supported by grant 16-33617A from the Ministry of Health of the Czech Republic.

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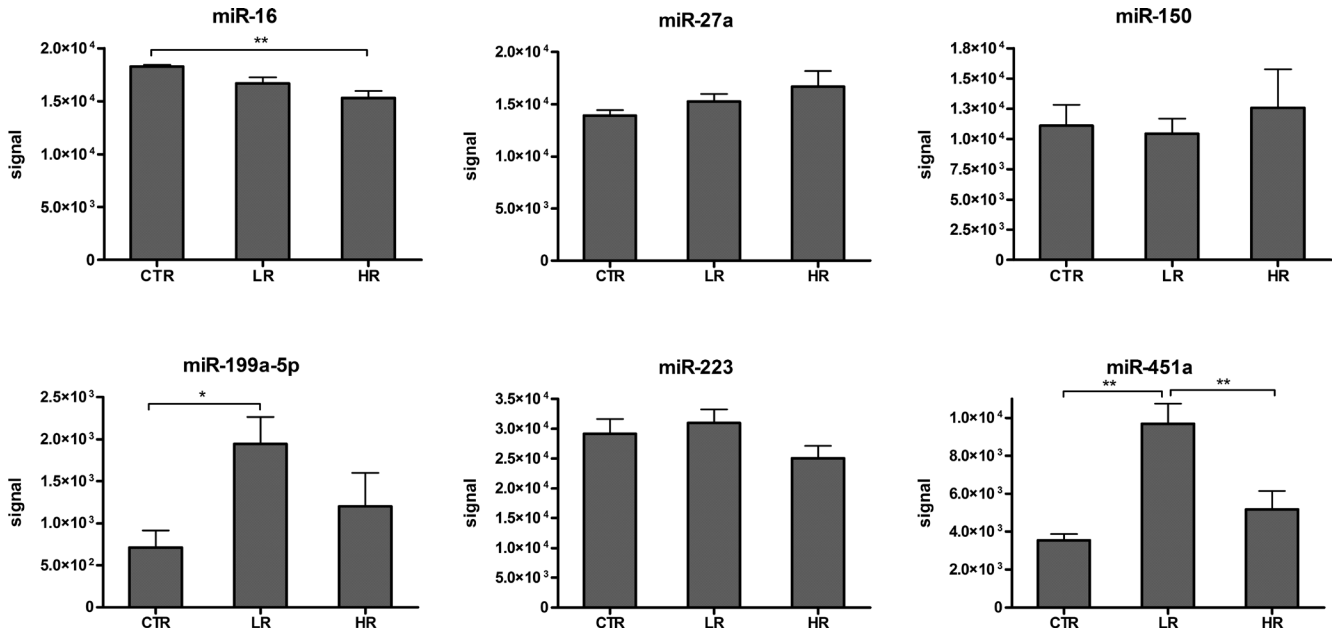

Supplementary Figure 1. MiRNA level in CD34+ MDS cells. Relative expression of miR-16-5p, miR-27a-3p, miR-150-5p, miR-199a-5p, miR-223-3p, and miR-451a in CD34+ MDS cells were calculated based on microarray data [10]. CTR – controls, LR – lower-risk, HR – higher-risk MDS patients, * $P < .05$, ** $P < .01$, *** $P < .001$.

SI Table 1. MiRNAs significantly deregulated ($P < .01$) in MDS plasma compared to controls.

miRNA	Signal intensity (mean)		Fold change	*P* value
	CTR	MDS		
miR-4655-3p	4.2	39.1	9.41	.00004
miR-3648	4.2	58.4	14.05	.00001
miR-5585-3p	4.2	53.7	12.92	.00002
miR-4497	6.9	92.9	13.39	.0003
miR-575	98.0	646.4	6.59	.0022
miR-548q	8.9	96.6	10.86	.0004
miR-3194-5p	4.2	40.0	9.63	.00004
miR-4476	6.5	40.3	6.23	.0019
miR-6068	393.5	1774.3	4.51	.0048
miR-1227-5p	137.3	750.8	5.47	.0039
miR-572	92.2	519.7	5.64	.0033
miR-5001-5p	192.5	669.7	3.48	.0084
miR-371b-5p	363.4	1532.5	4.22	.0061
miR-4787-5p	207.2	814.6	3.93	.0057
miR-4745-5p	15.7	59.7	3.80	.0061
miR-188-5p	9.7	35.7	3.68	.0068
miR-4788	18.3	71.1	3.89	.0060
miR-4632-5p	5.3	22.4	4.20	.0013
miR-6723-5p	4.2	24.0	5.78	.0003
miR-4270	120.7	306.3	2.54	.0092
miR-1202	238.9	660.7	2.77	.0087
miR-6085	83.2	228.3	2.74	.0099
miR-150-5p	17.0	90.3	5.30	.0005
miR-6125	3605.0	9097.4	2.52	.0094
miR-198	4.9	12.4	2.54	.0064
miR-1273f	4.2	15.6	3.76	.0023
miR-4516	2510.0	4894.8	1.95	.0083
miR-483-5p	9.2	26.9	2.92	.0047
miR-3138	8.6	24.9	2.88	.0041
miR-4454	23778.6	11048.1	0.46	.0081
miR-197-3p	126.8	70.7	0.56	.0045
miR-423-5p	150.6	56.8	0.38	.0040
miR-484	36.8	11.2	0.31	.0016
miR-92a-3p	1092.1	410.1	0.38	.0018
miR-106b-5p	157.3	70.4	0.45	.0062
miR-3135b	352.4	120.3	0.34	.0006
miR-30d-5p	469.8	57.1	0.12	.0018
miR-342-3p	58.9	11.2	0.19	.0020
miR-451a	6068.9	1351.3	0.22	.0065
miR-101-3p	34.9	10.6	0.30	.0059
miR-140-3p	37.7	9.3	0.25	.0024
miR-199a-3p	155.2	49.1	0.32	.0094
miR-142-5p	96.7	28.6	0.30	.0064

miR-142-3p	466.4	112.2	0.24	.0086
miR-320b	399.7	45.2	0.11	.0005
miR-320e	520.3	55.1	0.11	.0005
miR-320d	768.8	67.8	0.09	.0006
miR-320a	153.9	23.8	0.15	.0014
miR-4655-3p	4.2	39.1	9.41	.00004
miR-3648	4.2	58.4	14.05	.00001
miR-5585-3p	4.2	53.7	12.92	.00002

SI Table 2. MiRNAs significantly altered ($P < .01$) between lower-risk and higher-risk MDS.

miRNA	Signal intensity (mean)		Fold change	*P* value
	Lower-risk MDS	Higher-risk MDS		
miR-548q	49.1	160.1	3.26	.0069
miR-4739	70.8	193.3	2.73	.0026
miR-4721	21.7	90.3	4.16	.0005
miR-188-5p	21.6	54.6	2.53	.0062
miR-4499	18.1	36.2	2.00	.0028
miR-623	7.7	34.4	4.44	.0095
miR-20b-5p	45.1	17.9	0.40	.0064
miR-27a-3p	30.4	4.7	0.15	.0084
miR-20a-5p	180.6	85.3	0.47	.0060
miR-17-5p	102.7	41.8	0.41	.0081
miR-30e-5p	36.0	11.8	0.33	.0020
miR-16-5p	744.0	255.9	0.34	.0018
miR-4306	127.0	49.6	0.39	.0030
miR-185-5p	96.6	31.8	0.33	.0014
miR-93-5p	59.1	26.5	0.45	.0025
miR-223-3p	1521.8	339.8	0.22	.0091
miR-27b-3p	311.9	74.0	0.24	.0097
miR-548q	49.1	160.1	3.26	.0069
miR-4739	70.8	193.3	2.73	.0026