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ORIGINAL ARTICLE

MicroRNA Expression Analysis in Patients with Primary Myelofibrosis, Polycythemia vera and Essential Thrombocythemia

Anil Tombak · Ozlem Izci Ay · Mehmet Emin Erdal · Mehmet Ali Sungur · Mehmet Ali Ucar · Aydan Akdeniz · Eyup Naci Tiftik

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Abstract MicroRNAs (miRNA) are small non-coding RNA molecules that play critical roles in cell differentiation, proliferation and apoptosis and thus regulate haematopoietic stem cells and committed progenitor cells. We analyzed expressions of miRNAs associated with hematopoietic transformation of myeloid, erythroid and megakaryocytic progenitor cells during haematopoiesis (mir155, mir181a, mir221, mir222, mir223, mir451), in patients with primary myelofibrosis (PMF) ($n = 22$), polycythemia vera (PV) ($n = 33$), essential thrombocythemia (ET) ($n = 49$) and in healthy controls ($n = 40$) by quantitate/real time polymerase chain reaction. RT-PCR testing was negative for *BCR–ABL1* fusion gene in all the patients. Mir155 was expressed in higher levels in all 3 disorders ($p < 0.05$). Mir221 was higher especially in ET and PMF group ($p < 0.05$). Mir222 expression was lower in PV patients ($p < 0.05$) and higher in ET and PMF patients compared to control group. Mir223 expression was higher in ET and PMF group than control group ($p > 0.05$). Mir451 levels were lower in all three groups compared to control group ($p < 0.05$). There was no difference in expression levels of mir181a between groups. *JAK2V617F* positivity, co-morbidities, drugs, and gender did not affect miRNA expressions. This study holds promise for the future application of these molecules for differential diagnosis and as therapeutic targets in Philadelphia chromosome negative myeloproliferative neoplasms.

Keywords MicroRNA · Myeloproliferative disorder · Polycythemia vera · Essential thrombocythemia · Myelofibrosis

A. Tombak (✉) · M. A. Ucar · A. Akdeniz · E. N. Tiftik
Department of Hematology, Mersin University Faculty of Medicine, Mersin, Turkey
e-mail: aniltombak@mersin.edu.tr

M. A. Ucar
e-mail: mucardr@hotmail.com

A. Akdeniz
e-mail: akdenizdr@hotmail.com

E. N. Tiftik
e-mail: tiftiknaci@mersin.edu.tr

O. I. Ay · M. E. Erdal
Department of Genetics, Mersin University Faculty of Medicine, Mersin, Turkey
e-mail: ozlemay@mersin.edu.tr

M. E. Erdal
e-mail: merdal36@hotmail.com

M. A. Sungur
Department of Biostatistics, Mersin University Faculty of Medicine, Mersin, Turkey
e-mail: malisungur@yahoo.com

Introduction

MicroRNAs (miRNA) are 18–24 nucleotide single stranded non-protein-coding RNAs. They function as gene repressors by binding to target messenger RNAs (mRNA) to regulate gene expression and protein expression by repressing translation or destabilizing mRNA target at posttranscriptional level and have critical roles in regulation of cell differentiation, proliferation, apoptosis [1, 2]. MiRNAs are tissue-specific; different miRNAs are expressed in different cell types and they can be detected in peripheral blood.

MiRNAs regulate normal haematopoiesis in both haematopoietic stem cells and committed progenitor cells [3], but they also play a role in the pathogenesis of some acquired hematologic neoplasms [4]. Deregulated

expression of miRNAs is associated with neoplasia through tumor suppressor gene down-regulation or oncogene overexpression [5–7].

Myeloproliferative neoplasms (MPN); polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) originate from deregulated proliferation of hematopoietic stem cells [8]. A *JAK2*V617F mutation in negative regulatory domain of the tyrosine kinase Janus Kinase 2 can be detected in >95 % of PV, 50–60 % of ET and PMF patients [9]. *JAK2*V617F mutation causes activation of JAK/STAT pathway and is able to cause MPNs. However, there is significant heterogeneity in disease phenotypes between different murine lines, suggesting that disease phenotype is affected by other unknown genetic or epigenetic factors [10]. Recent progress suggests that deregulated miRNAs may contribute to MPN pathogenesis. Determination of expression patterns of related miRNAs in pathogenesis of MPNs may lead to the development of new drugs in this area and they can be used for differential diagnosis of MPNs.

In this study, we investigated the expressions of miR155, miR181a, miR221, miR222, miR223, miR451 in patients with PV, ET and PMF, which have roles in both normal and malignant haematopoiesis.

Materials and Methods

Study Participants

Patients with PV, ET, PMF diagnosed according to 2008 World Health Organization criteria [11] and healthy subjects as control group were included into the study. Peripheral blood samples of 33 PV, 49 ET, 22 PMF patients and 40 healthy subjects were obtained by venipuncture in tubes containing 2 % EDTA with approval of local Ethics Committee of Mersin University Medical Faculty. Informed consent was obtained in accordance with the Declaration of Helsinki. Real time polymerase chain reaction (RT-PCR) testing was negative for *BCR–ABL1* fusion gene in all the patients. None of the cases were in blastic transformation and none of the PV/ET patients were in fibrotic phase. Ages, genders, drugs used for the MPNs [hydroxyurea (n:44), anagrelide (n:8), and interferon-α2a (n:3)] and co-morbidities [hypertension (n:14), type 2 diabetes mellitus (n:9), coronary heart disease (n:5), cardiac failure (n:3), chronic renal disease (n:3), hepatitis C virus (HCV) carriers (n:2), Parkinson's disease (n:2), liver failure (n:1), pemphigus (n:1)] were noted. All patients on any drugs for MPNs were getting them since diagnosis and there was no patient using oral anticoagulants for thrombosis. Summary of demographic and clinical characteristics of cases is shown in Table 1. The medians and ranges of duration of administration of the drugs since diagnosis were also displayed in Table 1. Since mir155, mir181a, mir221, mir222, mir223, mir451 have critical roles in both normal and malignant haematopoiesis [3, 4], these 6 miRNAs were selected. Expressions of the selected miRNAs in blood samples of all participants were analyzed by quantitate/real time polymerase chain reaction (qRT-PCR) and *JAK2V617F* status of patients was analyzed by RT-PCR.

RNA Extraction and Reverse Transcriptase PCR Reactions (RT-PCR)

Total RNA was extracted from peripheral whole blood using Tri-Reagent (Sigma). Reverse transcriptase reactions contained 5 µl of extracted total RNA, 50 nM stem–loop RT primer, 1× RT buffer, 0.25 mM each of dNTPs, 50 units of modified M-MuLV Reverse Transcriptase (Thermo Scientific, Vilnius, Lithuania), 25 units of RiboLock RNase inhibitor (Thermo Scientific, Vilnius, Lithuania) and nuclease-free water to a total reaction volume of 15 µl. The reaction was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). RT-PCR conditions for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4 °C.

Quantitative-Comparative CT ($\Delta\Delta C_T$) Real-time PCR

Quantitative-Comparative C_T ($\Delta\Delta C_T$) Real-time PCR was performed in ABI Prism 7500 RT-PCR System using SDS 2.0.6 software (Applied Biosystems). Specific primers and fluorogenic ZNA™ probes (Metabion International AG) for miRNAs were designed using Primer Express 3.0 software (Applied Biosystems) and are listed in Table 2. The hsa-miR-26b-5p was used as endogenous control miRNA. TaqMan Control, Human RNA 50 ng/µl (Applied Biosystems) was used as a Reference RNA sample. Primers and probes were purchased from Metabion International AG, D-82152 Martinsried/Deutschland. The 25 µl PCR included 3 µl RT-PCR products, 12.5 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nmol of each primer (Primer F and Universal Primer R) and 200 nmol TaqMan® probe. Reactions were incubated in a 96-well plate of preincubation at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 90 s. All reactions were run in triplicate.

Statistical Analysis

Data were processed and analyzed using statistical package SPSS-11.5 for Windows and Statistica 6.1. Normality assumption of $2^{-\Delta\Delta CT}$ values was checked by Shapiro–

Table 1 Summary of the demographic and clinical characteristics of cases (*PV* polycythemia vera, *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *N/N* no mutation, *N/M* heterozygote mutant)

	Mean	Number of participants *n* (%)
Total number of patients		104
Patients with ET		49 (47.1)
Age (years)	60.9 ± 15,32 (45–76)	
Sex		
Male		23 (46.9)
Female		26 (53.1)
Patients with co-morbidities		17 (34.7)
Drugs		
None		28 (57.1)
Hydroxyurea (median: 22 months, range: 2–60 months)		13 (26.5)
Anagrelide (median: 18 months, range: 5–25 months)		7 (14.3)
Interferon-α2a (median: 1 month, range: 1 month)		1 (2.0)
JAK2		
Not tested		5 (10.2)
N/N		27 (55.1)
N/M		17 (34.7)
Patients with PV		33 (31.7)
Age (years)	59.88 ± 14,74 (45–75)	
Sex		
Male		14 (42.4)
Female		19 (57.6)
Patients with co-morbidities		15 (45.5)
Drugs		
None		8 (24.2)
Hydroxyurea (median: 32 months, range: 6–75 months)		23 (69.7)
Anagrelide		0 (0)
Interferon-α2a (median: 5 months, range: 3–7 months)		2 (6.1)
JAK2		
N/N		14 (42.4)
N/M		19 (57.6)
Patients with PMF		22 (21.2)
Age (years)	54.82 ± 16,56 (38–72)	
Sex		
Male		9 (40.9)
Female		13 (59.1)
Patients with co-morbidities		8 (34.6)
Drugs		
None		13 (59.1)
Hydroxyurea (median: 13 months, range: 6–26 months)		8 (36.4)
Anagrelide (median: 4 months, range: 4 month)		1 (4.5)
Interferon-α2a		0 (0)
JAK2		
Not tested		4 (18.2)
N/N		12 (54.5)
N/M		6 (27.3)
Total number of healthy participants (control group)		40
Age (years)	49.45 ± 9,33 (40–59)	

Table 1 continued

	Mean	Number of participants *n* (%)
Sex		
Male		20 (50)
Female		20 (50)
Cases with co-morbidities		0 (0)
Drugs		
None		0 (0)

Table 2 Primer/probe sequences of the miRNA analyzed by quantitative RT–PCR

miRNA name	Gene ID[a]	NCBI reference sequence number[b]	Primer/probe sequence[c]
hsa-miR-26b-5p	407017	NR_029500.1	hsa-miR-26b-5p-RT, 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCG CACTGCATACGACACCTAT-3′
			hsa-miR-26b-5p-F, 5′-GCCGCTTCAAGTAATTCAGG-3′
			hsa-miR-26b-5p-PR, 5′-FAM-TG(pdC)ATA(pdC)GA(pdC)A(pdC)CTATCC-ZNA4-BHQ-1-3′
hsa-miR-9-5p	407046	NR_029691.1	hsa-miR-9-5p-RT, 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACTCATAC-3′
			hsa-miR-9-5p-F, 5′-GCCGCTCTTTGGTTATCTAGCT-3′
			hsa-miR-9-5p-PR, 5′-FAM-TG(pdC)ATA(pdC)GA(pdC)T(pdC)ATA(pdC)AG-ZNA4-BHQ1-3′
hsa-miR-155-5p	406947	NR_030784.1	hsa-miR-155-5p-RT 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGC ATACGAC ACCCCT-3′
			hsa-miR-155-5p-F 5′-GCCGCTTAATGCTAATCGTGAT-3′
			hsa-miR-155-5p-PR 5′-FAM-TG(pdC)ATA(pdC)GA(pdC) A(pdC)C(pdC) (pdC)TAT -BHQ-1-3′
hsa-miR-181a-5p	406954	NR_029611.1	hsa-miR-181a-5pRT 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGC ATACGAC ACTCAC-3′
			hsa-miR-181a-5pF 5′-GCCGCAACATTCAACGCTGTCG-3′
			hsa-miR-181a-5p-PR 5′-FAM-TG(pdC)ATA(pdC)GA(pdC) ACT(pdC)ACCGA-BHQ-1-3′
hsa-miR-221-5p	407006	NR_029635.1	hsa-miR-221-5p RT 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGC ATACGAC GAAACC-3′
			hsa-miR-221-5p F 5′-GCCGCAGCTACATTGTCTGCTG-3′
			hsa-miR-221-5p PR5′-FAM-TG(pdC)ATA(pdC)GA(pdC) GAAA(pdC)CCA-BHQ-1-3′
hsa-miR-222-5p	407007	NR_029636.1	hsa-miR-222-5p RT 5′-GTCTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGAC ACCCAG-3′
			hsa-miR-222-5p F 5′-GCCGCAGCTACATCTGGCTA-3′
			hsa-miR-222-5p PR5′-FAM-TG(pdC)ATA(pdC)GA(pdC) ACC(pdC)AGTAGC-BHQ-1-3′
hsa-miR-223-3p	407008	NR_029637.1	hsa-miR-223-5p-RT 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGC ATACGAC TGGGGT-3′
			hsa–hsa-miR-223-5p-F 5′-GCCGCTGTCAGTTTGTCAAAT-3′
			hsa-miR-223-3p-PR 5′-FAM-TG(pdC)ATA(pdC)GA(pdC) TGGGGTAT-ZNA4-BHQ-1-3′
hsa-miR-451a	574411	NR_029970.1	hsa-miR-451a-RT 5′-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGC ATACGAC AACTCA-3′
			hsa-miR-451a-F 5′-GCCGCAAACCGTTACCATTAC-3′
			hsa-miR-451a-PR5′-FAM-TG(pdC)ATA(pdC)GA(pdC) AA(pdC)TCAGT-ZNA4-BHQ-1-3′
			miR-Universal-R, 5′-GTGCAGGGTCCGAGGTAT-3′

[a] http://www.ncbi.nlm.nih.gov/gene

[b] http://www.ncbi.nlm.nih.gov/RefSeq/

[c] *pdC* substitution of C-5 propynyl-dC (pdC) for dC is an effective strategy to enhance base pairing. Using these base substitutions, duplex stability and melting temperatures are raised by C-5 propynyl-C 2.8° per substitution. *Zip nucleic acids* (ZNA) ZNA probes provide broad flexibility in assay design and represent an effective alternative to minor groove binder (MGB)- and locked nucleic acid (LNA)-containing oligonucleotides [12]

Wilk test. Since the assumption of normality was not met, $2^{-\Delta\Delta CT}$ values were expressed as median, first quartile (25th percentile), third quartile (75th percentile), and the comparison between groups was performed using the Kruskal–Wallis and Dunn post hoc tests. Mann–Whitney test was used for comparing *JAK2V617F* status of all patient groups and Kruskal–Wallis test was used for comparing drug usage of all patient groups. Box-plot graph was used to represent data distribution of hsa-miR-155-5p, hsa-miR-181a-5p, hsa-miR-221-5p, hsa-miR-222-5p, hsa-miR-223-5p, hsa-miR-451a variables according to groups. Differences (two-tailed p) less than 0.05 were regarded as significant.

Results

Expression of mir155 was significantly higher in patients with ET, PV, PMF than control group ($p = 0.001$, 0.006 and 0.001 respectively) (Table 3). However, its expression was similar between three disease groups.

Higher expressions of mir181a in all patient groups compared to control were determined; however, the differences did not reach statistical significance ($p = 0.932$) (Table 3).

A difference was determined in expression of mir221 as compared to control group ($p = 0.002$). Mir221 expression was higher in three patient groups; however, there was a significant difference between ET patients with controls ($p = 0.004$) and between PMF patients with controls ($p = 0.030$), but no significant difference was determined between PV patients and controls, statistically (Table 3).

We found a difference in expression levels of mir222 between groups ($p = 0.001$); expression was significantly higher in ET and lower in PV patients compared to controls. Therefore, a statistically significant difference occurred between ET-PV patients ($p = 0.001$). Higher expression of mir222 in PMF patients compared to controls was also determined, which did not reach statistical significance (Table 3).

Different expression of mir223 was found between groups ($p = 0.001$); expression was highest in PMF patients ($p = 0.001$) and was significantly higher in ET patients ($p = 0.004$). Lower expression in PV patients compared to controls was also determined, which didn't create a statistically significant difference (Table 3).

A difference was found in expression of mir451 as compared to control group ($p = 0.001$). Mir451 was significantly down-regulated in ET, PV, PMF patients compared to controls ($p = 0.001$ for all three comparisons) (Table 3).

Box-plot graphs of miRNA expressions in PV, ET, PMF, and healthy control groups were shown in Fig. 1.

When we compared the miRNA expressions by gender and co-morbidities, no difference was determined between groups. Drugs (hydroxyurea, anagrelide and interferon-α2a) and *JAK2V617F* mutation status also did not affect expressions (Tables 4, 5).

Table 3 MiRNA expressions in PV, ET, PMF, and healthy control groups analyzed by qRT-PCR

Disease		mir155	mir181a	mir221	mir222	mir223	mir451
Control	n	40	40	40	40	40	40
	25 %	0.00019	0.11933	0.86799	0.01627	5.14917	1490195.13331
	Median	0.00037	0.25122	2.42670	0.02892	23.23962	5020982.30299
	75 %	0.00064	0.82563	8.87502	0.07839	89.72211	42569739.79780
PV	n	33	31	29	26	27	33
	25 %	0.00036	0.13490	1.75430	0.00593	2.73777	7017.45668
	Median	0.00126*	0.30166	10.32450	0.01972#	13.00505	78532.38002*
	75 %	0.00365	0.84206	71.99002	0.03853	39.50600	519464.01602
ET	n	49	49	42	48	44	46
	25 %	0.00073	0.09216	1.40092	0.03147	28.03377	7689.33057
	Median	0.00187*	0.33681	22.32815*	0.10972[#]	87.81531[¢]	73275.06169*
	75 %	0.00782	1.60078	140.63741	1.88131	1593.01057	578232.88609
PMF	n	19	19	15	17	18	17
	25 %	0.00061	0.09929	3.49429	0.01447	42.00267	27086.65632
	Median	0.00358*	0.45470	16.98277*	0.04694	385.39057[¢]	538654.24585*
	75 %	0.05991	1.31859	51.95862	2.63616	22702.64884	4604225.53575

PV polycythemia vera, *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *n* number

* There is a statistical significance in the expression of miRNAs between MPN versus controls [$p < 0.05$]

[#] There is a statistical significance in the expression of miR222 between PV and ET patients [$p < 0.05$]

[¢] There is a statistical significance in the expression of miR223 between ET and PMF patients [$p < 0.05$])

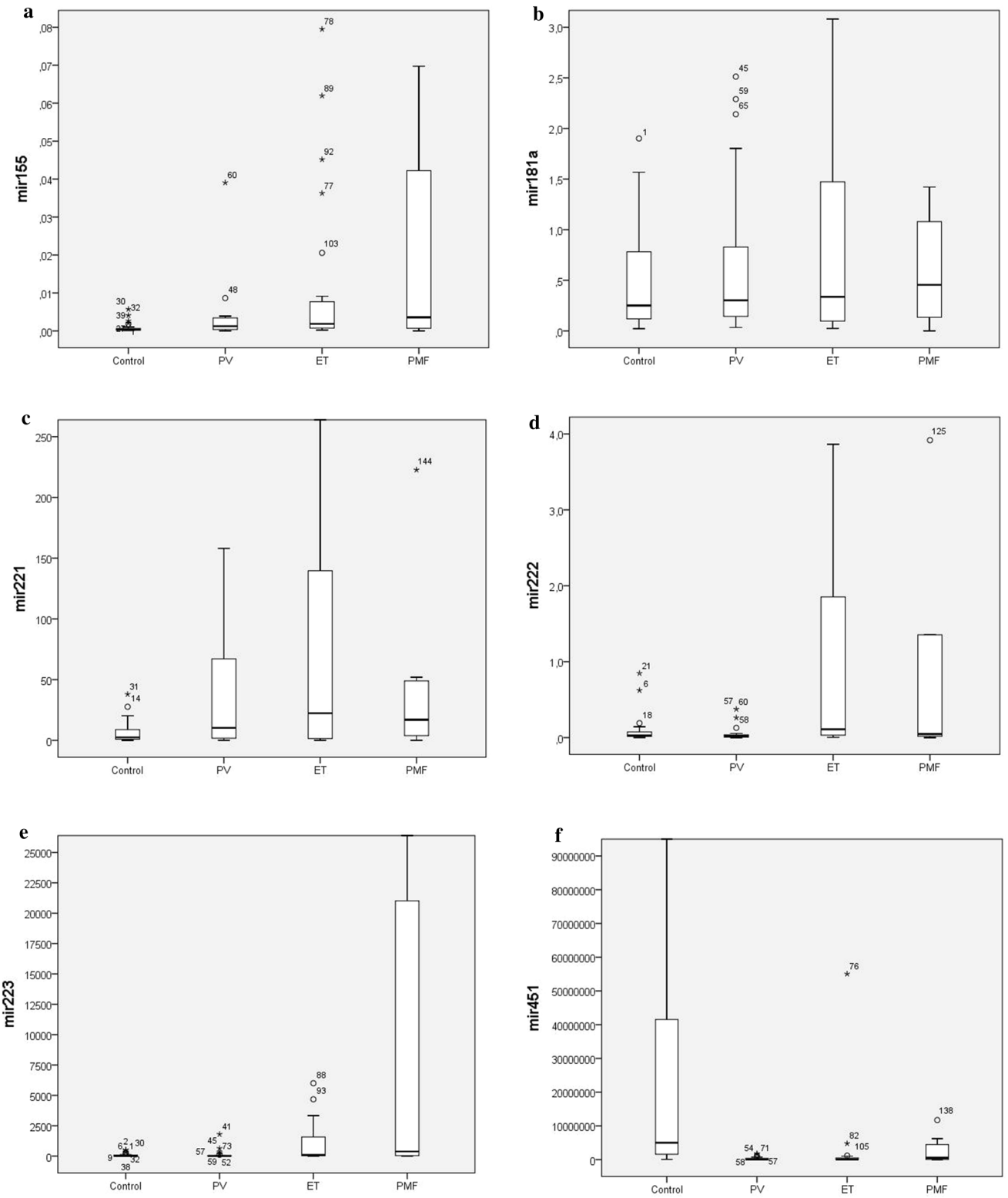

Fig. 1 Box-plot graphs of miRNA expressions in PV, ET, PMF, and healthy control groups analyzed by qRT-PCR

Table 4 The effects of drugs on miRNA expressions

Disease	Drug		mir155	mir181a	mir221	mir222	mir223	mir451
PV	No drug	n	8	7	7	7	7	8
		Median	.00182	.16346	3.27842	.02820	103.25015	235369.58329
	Hydroxyurea	n	23	22	20	18	19	23
		Median	.00095	.35243	10.84662	.01151	7.61582	78532.38002
	Interferon-α2a	n	2	2	2	1	1	2
		Median	.00165	.32181	77.20719	.03492	.76154	6845.21044
ET	No drug	n	28	28	23	27	25	25
		Median	.00249	.24292	16.98277	.08870	71.60557	97424.70247
	Hydroxyurea	n	13	13	12	13	11	13
		Median	.00152	.37736	34.46876	.11352	75.42652	191236.71456
	Interferon-α2a	n	1	1	1	1	1	1
		Median	.00063	1.47223	14.20178	.04934	845239.11112	164189.23330
	Anagrelide	n	7	7	6	7	7	7
		Median	.00060	.90963	120.53587	.24827	217.82111	13786.79984
PMF	No drug	n	10	10	10	9	9	9
		Median	.00229	.36666	29.19772	.04694	104.69146	1015669.78639
	Hydroxyurea	n	8	8	4	7	8	7
		Median	.01141	.38167	28.12615	.02217	2953.65396	538654.24585
	Anagrelide	n	1	1	1	1	1	1
		Median	.00040	.47797	3.49429	.13985	9.93831	5545.10860

PV polycythemia vera, *ET* essential thrombocythemia, *PMF* primary myelofibrosis, *n* number1

Table 5 MiRNA expressions according to *JAK2* status

Disease	JAK2		mir155	mir181a	mir221	mir222	mir223	mir451
PV	N/N	n	14	13	13	13	13	14
		25 %	.00034	.10784	.95286	.00430	2.30812	12223.76574
		Median	.00075	.49141	11.36874	.01066	7.61582	91042.50528
		75 %	.00246	1.97154	71.99002	.08263	66.89901	693841.58107
	N/M	n	19	18	16	13	14	19
		25 %	.00035	.14085	2.20276	.01168	2.32614	1568.31539
		Median	.00165	.18829	8.53751	.02768	23.05051	67471.93891
		75 %	.00865	.56580	107.32150	.03940	73.92229	395686.96109
ET	N/N	n	27	27	23	27	25	25
		25 %	.00077	.11859	.29463	.00858	29.36324	35961.42677
		Median	.00216	.49655	20.82147	.11352	85.74615	191236.71456
		75 %	.00794	3.08075	143.69502	.51513	1517.89727	2986765.08176
	N/M	n	17	17	16	17	16	17
		25 %	.00050	.08911	4.10464	.02139	8.18915	3987.74619
		Median	.00104	.32534	17.68097	.08184	73.51605	72767.16580
		75 %	.00546	1.19152	153.65810	.97912	1564.75536	632327.93305
PMF	N/N	n	10	10	8	8	10	10
		25 %	.00049	.25414	3.96522	.01431	29.33003	302647.05392
		Median	.00097	.50378	29.70620	.03398	95.15941	955171.09772
		75 %	.07623	4.74467	213396737.23892	.18863	96579.14033	5102052.22745
	N/M	n	5	5	3	5	4	5
		25 %	.00199	.24834	2.36199	.00027	85.10836	27086.65632
		Median	.00546	1.31859	3.49429	.01725	385.39057	466979.59947
		75 %	5.10756	4.19758	41.41266	.12024	1535.32345	5136637.97809

N/N no mutation, *N/M* heterozygote mutant

Discussion

This study was designed to investigate the expression profiles of miR155, miR181a, miR221, miR222, miR223 and miR451 in the pathogenesis of PV, ET and PMF, and to explore their potential as future therapeutic targets.

Mir155 controls differentiation of B and T lymphocytes in normal haematopoiesis. High levels of mir155 are present in activated B and T cells and activated monocytes [13]. Mir155 also regulates germinal center reaction, T-helper differentiation by affecting cytokine production [14]. In addition, erythroid and myeloid colony formation are reduced by mir155 [15]. During normal erythropoiesis, mir155 is progressively down-regulated with mir150, mir221 and mir222 [16]. In a study; mice transplanted with multipotent progenitor cells that over-express mir155 developed a MPN with decreased erythroid/megakaryocytic lineage in bone marrow [17]. Transgenic mice studies suggest that both stem cell deregulation and inflammatory stress signaling are important in MPN pathogenesis [18]. Inflammatory stress has a clear impact on blood cell development and blood diseases via deregulated production of various cytokines and growth factors [19]. Elevated circulating cytokine levels have been reported in PV, PMF patients [20] and expression of mir155 increases by inflammatory stimuli [17].

In our study, we found a difference in expression of mir155. Expression of mir155 was significantly higher in patients with ET, PV, PMF—but similar between three disease groups—than the control group (Table 3). Probably, excessive mir155 expression triggered by elevated circulating inflammatory cytokines [17, 19, 20] takes an important role in pathogenesis of MPNs and thus, mir155 can be a target molecule in treatment of MPNs.

Mir181 is preferentially expressed in thymus, bone marrow and spleen. It blocks the differentiation of haematopoietic progenitor cells, while is up-regulated during B cell differentiation [15, 21]. Mir181a modulates T cell receptor signalling, regulates T cell sensitivity and selection [22]. In a miRNA profiling study in *JAK2V617F*-mutated SET2 cell line, Bortoluzzi et al. revealed that 21 miRNAs including mir181a were highly expressed [23]. Mir181a has not been studied in human patients with MPNs before and in our study, we revealed higher expression levels of mir181a in patients with ET, PV, PMF compared to the control group; however no difference was determined statistically (Table 3).

During erythroid differentiation, mir221, mir222, mir223 are downregulated. Mir221 and mir222 block erythroid differentiation by targeting *c-KIT,* mir223 blocks differentiation by targeting *LMO2* [24, 25]. However, mir223 is up-regulated during granulocyte differentiation [26]. Bruchova et al. showed decreasing levels of mir221 and mir222 during erythropoiesis in $CD34^+$ peripheral blood cells of healthy subjects and PV patients in vitro. Although these reduced expressions, mir221 expression was higher in PV group than control group, and mir222 expression was lower at 7–9th days of incubation, was higher at 11–21th days of incubation [16]. In another study conducted by Bruchova et al. the higher expression of mir223 in peripheral blood mononuclear cells of PV, ET, PMF patients was determined compared to control group [27].

Mir222 and mir223 normally suppress erythropoiesis and detection of lower levels of these miRNAs in PV than the control group may be responsible for developing polycythemia. In the study conducted by Bruchova et al. expressions were determined in special cell cultures and this may explain the different expression levels of these miRNAs, since we investigated the expressions in peripheral blood [16]. However, it is also evident that we need standardized methods for the research of miRNA expressions. New therapies targeting to increase expressions of mir222 and mir223 may prevent polycythemia in PV patients by suppressing the erythropoiesis, but more research is needed.

Higher expressions of mir221, mir222, mir223 in ET and PMF patients which normally suppress erythropoiesis in normal haematopoiesis probably result from excessive cell proliferation. Very high levels of mir221 and mir223 may contribute to anemia in PMF by suppressing erythropoiesis. Thus, these miRNAs can be used as therapeutic targets for treating anemia in PMF, and also mir223 can be used in differential diagnosis of patients with early phase PMF from patients of ET, since mir223 is expressed in reasonably higher levels in PMF compared to ET.

Mir451 was found to be erythroid specific and is up-regulated with mir144 during normal erythropoiesis [28]. Bruchova et al. showed the gradual increase of mir451 in $CD34^+$ peripheral blood cells of both healthy and PV subjects during erythroid differentiation in vitro. In that study; it was determined that the expression of mir451 was higher in PV samples compared to controls during early incubation period of cells (1–11th days), but was higher in controls after day 11 (differentiation period) with the presence of erythropoietin (EPO). Accordingly, mir451 expression is higher in early, EPO independent PV progenitors compared to healthy progenitors, and PV progenitors differentiate faster than healthy erythroid progenitors [16]. Zhan et al. showed higher expression of mir451 in $CD34^+$ peripheral blood cells of PV patients compared to healthy subjects [28]. When they examined nucleated erythroid cells of the Burst Forming Unit-Erythroid (BFU-E) colonies, there appeared a greater mir451 expression in PV BFU-E colony cells than in ET (both *JAK2V617F* positive and negative) BFU-E colony cells [28]. In two recent reports, enforced expression of mir451 promoted erythroid differentiation of K562 cells [29] and mice deficient mir451 had impaired late erythroblast maturation, erythroid hyperplasia, splenomegaly and mild anemia [30].

Unlike other studies, determination of lower expression level of mir451 in our study in especially PV and ET group compared to healthy subjects may be a reason of the analyzed sample that we used peripheral blood rather than the specific cell culture. In other words, up-regulated mir451 expression in progenitor cells cannot be adequately detected in peripheral blood. It is evident that we need more research in this era and standardized measurement methods for expression levels. Whereas, mir451 can be used for the differential diagnosis of early phase PMF patients especially from ET patients, since median mir451 expression was very high in PMF group (538654.24585) compared to ET and PV groups (73275.06169 and 78532.38002, respectively). In addition, down-regulated mir451 in PMF patients compared to control can contribute to anemia and massive splenomegaly in PMF patients as determined in mir451 deficient mice model [30].

When we compared miRNA expressions by gender, we did not determine a difference between groups. This result shows that sex doesn't affect miRNA expression. Co-morbidities also did not affect miRNA expressions. We think that miRNAs investigated in this study are haematopoiesis-specific and expression levels are probably not affected by diseases affecting other organ systems than the haematopoietic system. Drugs (hydroxyurea, anagrelide and interferon-α2a) also did not affect miRNA expressions. This may be a reason of small number of patients using drugs. However, these drugs just make cytoreduction in MPNs, but do not cure existing genetic disorder. Because of the reason that MPNs are caused by genetic disorders and these mutations are not fixed by these drugs, miRNA expressions may not be affected by these drugs.

How *JAK2V617F* mutation could be responsible for three different MPN phenotypes has not been explained. It is possible that disease-specific miRNA deregulations can contribute to specific phenotypes of PV, ET, PMF and can be used to differentiate 3 disorders. Some deregulated miRNA expressions were reported to correlate with *JAK2V617F* allele burden or JAK2 activity, while many miRNA deregulations do not [28]. Girardot et al. showed that mir28 is up-regulated in 30 % of patients with PV, ET, PMF, and it suppresses the megakaryocytic differentiation in $CD34^+$ cells. They also commented that expression level of mir28 may be related with the *JAK2V617F* allele burdens [31]. In the study conducted by Zhan et al. in PV patients, no correlation was determined between miRNA expression levels and $CD34^+$ cell *JAK2V617F* allele burdens [28]. In our study, we didn't find a correlation between *JAK2* mutation status and miRNA expressions (Table 3). Probably many deregulated miRNAs act independently from JAK2 signaling in pathogenesis of MPNs. Investigation of common and different miRNAs in *JAK2V617F* positive–negative disorders may reveal some new evidences in development of MPNs.

The miRNA analysis in this study was performed by using the peripheral blood samples of the patients that means we cannot tell if the difference of miRNA expressions is caused from the mutated clone of MPNs or the entire peripheral blood cells. In addition, we cannot also tell if the difference of miRNA expression is caused from the other sub-populations like endothelial cells in bone marrow niche. Whereas the microenvironment has a great part in the pathogenesis of hematological neoplasms. Wang et al. showed that loss of Notch signaling in the bone marrow niche alters hematopoietic homeostasis and leads to lethal myeloproliferative-like disease [32]. Accordingly, loss of Notch/RBPJ signaling up-regulates mir155 in bone marrow endothelial cells, leading to increased proinflammatory cytokine production. In future miRNA studies, it should be considered that not only the mutated cell clone, but also the cells in the bone marrow niche play role in the pathogenesis of MPNs and miRNA expressions.

Conclusion

We showed the different expression levels of selected miRNAs in peripheral blood samples of PV, ET, PMF patients. Expression levels of these haematopoiesis-specific miRNAs are not affected by co-morbidities, drugs and *JAK2V617F* status. However, we need more research for understanding the pathogenesis of these disorders in current era.

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