Circulating microRNAs in patients with immune thrombocytopenia before and after treatment with thrombopoietin-receptor agonists

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs involved in the regulation of gene expression. Dysregulated expression of several miRNAs has been found in primary immune

thrombocytopenia (ITP) suggesting that miRNAs are likely involved in the pathogenesis of ITP. We aimed to explore the differential expression of miRNAs in patients with ITP before and after starting treatment with thrombopoietin-receptor agonists (TPO-RAs) to clarify their roles in the pathophysiology of ITP, and as potential diagnostic and prognostic markers of this disorder.

We performed a profiling study where 179 miRNAs were analyzed in eight ITP patients before and during treatment with TPO-RAs and in eight controls using miRNA PCR panel; 81 miRNAs were differentially expressed in ITP patients compared to controls, and 14 miRNAs showed significant changes during TPO-RA-treatment. Ten miRNAs were selected for validation that was performed in 23 patients and 22 controls using droplet digital PCR. Three miRNAs were found to be differentially expressed in ITP patients before TPO-RA-treatment compared to controls: miR-199a-5p was down-regulated ($p=0.0001$), miR-33a-5p ($p=0.0002$) and miR-195-5p ($p=0.035$) were up-regulated. Treatment with TPO-RAs resulted in changes in six miRNAs including miR-199a-5p ($p=0.001$), miR-33a-5p ($p=0.003$), miR-382-5p ($p=0.004$), miR-92b-3p ($p=0.005$), miR-26a-5p ($p=0.008$) and miR-221-3p ($p=0.023$); while miR-195-5p remained unchanged and significantly higher than in controls, despite the increase in the platelet count, which may indicate its possible role in the pathophysiology of ITP. Regression analysis revealed that pre-treatment levels of miR-199a-5p and miR-221-3p could help to predict platelet response to TPO-RA-treatment. ROC curve analysis showed that the combination of miR-199a-5p and miR-33a-5p could distinguish patients with ITP from controls with AUC of 0.93.

This study identifies a number of differentially expressed miRNAs in ITP patients before and after initiation of TPO-RAs with potential roles in the pathophysiology, as well as with a possible utility as diagnostic and prognostic biomarkers. These interesting findings deserve further exploration and validation in future studies.

Keywords: ITP, microRNAs, TPO-RAs.

Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs of ~22 nucleotides involved in post-transcriptional regulation of gene expression by inducing mRNA degradation or translation inhibition [1]. Growing evidence has shown that miRNAs are key molecules in many physiological processes [2]. Dysregulated expression of miRNAs has been found to be associated with different diseases such as cancer [3], atherosclerosis [4], and autoimmune disorders [5]. They have been identified in blood cellular components [6], and different body fluids [7]. Although platelets are anucleated blood cells, they retain megakaryocyte-derived cytoplasmic mRNAs and the ability for protein biosynthesis [8, 9]. Furthermore, platelets contain high levels of miRNAs and a substantial fraction of circulating miRNAs originates from platelets [6]. Circulating miRNAs are easily accessible and very resistant to degradation and extreme physicochemical conditions [10]. These properties make them potential biomarkers [11]. Droplet digital PCR, a method that allows for absolute quantification of miRNAs without the need of a calibrator, is a promising approach for molecular biomarkers in cell-free body fluids [12, 13].

The exact etiology of primary immune thrombocytopenia (ITP) is still unknown, but the development of autoantibodies against platelets and megakaryocytes resulting in increased platelet destruction and impaired platelet production remains central to the pathophysiology [14]. However, in recent years, various T-cell abnormalities have been identified including skewing towards Th1/Th17 phenotype; activation of cytotoxic T cell-mediated destruction of platelets and megakaryocytes; and reduction/dysregulation in the number and function of regulatory T-cells, indicating that T-cells may play a major role in the pathogenesis of ITP [15, 16].

There is no specific diagnostic test for ITP, thus it remains a disease of exclusion. Romiplostim and eltrombopag are two thrombopoietin receptor agonists (TPO-RAs) which have been licensed to treat ITP for the past 10 years. These agents bind the thrombopoietin receptor and activate intracellular signaling pathways (JAK–STAT, AKT, and MAP kinase pathways) to stimulate megakaryocyte growth and platelet production [17]. TPO-RAs increase platelet counts in 40-85% of treated ITP patients depending upon the definition of response [18-20].

Despite many studies and advances in ITP during past years, beyond the platelet count itself, there are no biomarkers that can be used to monitor disease severity and/or predict response to treatment. Recently, three studies have explored the potential role of miRNAs as biomarkers

in ITP [21-23]; however, none of these studies has addressed changes in specific miRNA expression during TPO-RAs therapy, nor whether selected miRNA profiles might be predictive of responses to this therapy.

This study explored the differential expression of plasma miRNAs in patients with ITP before and after initiating treatment with TPO-RAs to potentially clarify the roles of these small regulatory molecules in the pathophysiology of ITP, and as potential diagnostic and prognostic markers of this disorder.

Materials and Methods

Patients and study design

Thirty-one patients from Norway and USA with primary ITP who were planned to start with TPO-RAs were recruited into the study. The indication for treatment with TPO-RAs was decided by the treating hematologist and according to the label. Samples were collected after obtaining informed consent. The study comprises two parts: an initial profiling part and a subsequent validation part. The profiling was performed with plasma miRNA PCR panel in eight patients before, two, six and twelve weeks after treatment with TPO-RAs and in eight controls. Validation of the ten selected miRNAs was performed in a second cohort of 23 ITP patients, before, two and six weeks after TPO-RAs and in 22 controls.

The study was approved by the Institutional Review Board at Weill Medical College of Cornell University and the South-Eastern Norway Regional Committee for Medical and Health Research Ethics (2011/1747a).

Blood sampling

Venous blood samples were collected in five mL tubes (Becton-Dickinson, Plymouth, UK) containing 3.2% sodium citrate (0.109 mol/L) and was centrifuged within 1 hour at 2000g for 20 minutes. Plasma was pipetted and stored in aliquots at -80 °C until use.

RNA isolation and cDNA synthesis for miRNA profiling PCR panel

RNA was extracted from 200 µL plasma using miRNeasy Serum/Plasma Kit (Qiagen, Germany). The efficiency of RNA extraction was monitored by three synthetic RNA spike-ins (UniSp2, UniSp5 and UniSp6) according to manufacturer's instructions. RNA was stored at -80 °C until use. cDNA synthesis was performed using Universal cDNA Synthesis Kit II (Exiqon, Vedbæk, DK). Two RNA spike-ins (UniSp6 and cel-miR-39-3p) were added to monitor the reverse transcription reactions and to assess the presence of PCR inhibitors. Synthesized cDNA was stored at -20 °C until use.

RNA isolation and cDNA synthesis for validation by Droplet Digital PCR

RNA was extracted from 200 μ L plasma using the miRCURY™ RNA isolation Kit for Biofluids (Exiqon) according to the manufacturers' protocol. RNA was eluted in 50 μ L nuclease-free water and stored at -80 °C. Reverse-transcription was performed using the Universal cDNA Synthesis Kit II and cDNA was stored at -20 °C until use.

Plasma miRNA profiling by Serum/Plasma Focus microRNA PCR panel

Screening of 179 miRNAs was performed using Serum/Plasma Focus microRNA PCR panel, V4 (Exiqon) with the ExiLent SYBR®Green Master Mix according to manufacturer's instructions. All samples passed the requirement for hemolysis. The PCR was performed on Aria MX cycler (Agilent Technologies, Santa Clara, CA, US).

Validation/quantification of miRNA by Droplet Digital PCR

Ten miRNAs were selected for validation from the profiling part of the study (miR-423-5p, miR-16-2-3p, miR-590-5p, miR-199a-5p, miR-382-5p, miR-92b-3p, miR-221-3p, miR-33a-5p, miR-195-5p and miR-26a-5p). Eight of the ten miRNAs were chosen for both being differentially expressed in ITP and showing changes during treatment. The remaining two were selected for being involved in vascular events [24], and showing changes during treatment (miR-195-5p), or for being differentially expressed in ITP patients compared with healthy controls in the screening study and for being among the abundantly expressed miRNAs in platelets (miR-26a-5p). Validation was performed using QX200™ Droplet Digital™ PCR with miRCURY LAN PCR primer sets (Exiqon) and Eva Green dye-based assays. Samples were tested for hemolysis using qPCR; only in one case, a new sample was required for this reason. A non-template control (NTC) was included for each reverse transcription and additional 3NTCs were included for each assay to serve as negative controls. Droplets were read in the QX200™ droplet reader (Bio-Rad) and the amount of miRNA (copies/ μ L) was generated by the QuantaSoft software (Bio-Rad).

Droplet digital PCR, Data Analysis, Pathway Analysis, and Statistics were performed as described in Supplementary Methods.

Results

Patients characteristics

Patient characteristics, in both the initial profiling and the validation studies, are shown in Table 1. Eight patients and eight controls were included in the initial profiling study, four males and four females in each group. The mean age of the patients was 47.4 years and 49.1

years for the controls. Mean platelet counts for patients and controls were $30 \cdot 10^9/L$ and $236 \cdot 10^9/L$, respectively. Six patients received romiplostim, one of whom switched to eltrombopag after one week, the other two received eltrombopag.

Twenty-three patients with primary ITP and 22 controls were enrolled in the validation study, mean age of patients was 58.5 years and of controls 47.4 years; 13 of the 23 patients and 13 of the 22 controls were females. In this validation cohort, 15 patients received eltrombopag and eight romiplostim. All patients had failed to respond to at least one type of treatment for ITP. All patients suffered from chronic ITP except two, one had persistent ITP and the other newly diagnosed ITP. Only four patients were categorized as non-responders at six weeks after the initiation of treatment with TPO-RAs; a response is defined as platelet count $> 30 \cdot 10^9/L$ and doubling from baseline.

miRNA profiling using microRNA PCR panel

ITP patients versus controls

Among the 179 miRNAs included in the Serum/Plasma Focus miRNA PCR panel, two (miR-208a-3p and miR-200-3p) were excluded since they showed less than 60% valid data. Among the remaining 177 miRNAs, 81 were differentially expressed in ITP patients compared to controls with fold change (FC) > 1.5 and $p < 0.05$ (Supplemental Table 1). Of those, 17 miRNAs were statistically significant after Bonferroni correction (nominal $p < 0.00027$, adjusted $p < 0.05$); 11 miRNAs were down-regulated (miR-374b-5p, miR-26a-5p, miR-107, miR-766-3p, miR-191-5p, miR-339-5p, miR-223-3p, miR-199a-5p, miR-26b-5p, miR-103a-3p and miR-30b-5p) and 6 up-regulated (miR-486-5p, miR-629-5p, miR-222-3p, miR-1260a, miR-423-5p, and miR-378a-3p) (Supplemental Table 2).

ITP patients before and during TPO-RA-treatment

Of the 177 evaluated miRNAs, 14 miRNAs showed nominal significant changes during treatment with TPO-RAs ($p < 0.05$): miR-199a-5p, miR-423-5p, miR-16-2-3p, miR-136-3p, miR-195-5p, miR-590-5p, miR-33a-5p, miR-221-3p, miR-382-5p, miR-141-3p, miR-92b-3p, miR-584-5p, miR-338-3p, and miR-194-5p.

Validation of miRNA by ddPCR

ITP patients prior to TPO-RA-treatment versus controls

Of the ten miRNAs selected, according to the criteria stated under materials and methods, for further validation with ddPCR, three miRNAs (miR-199a-5p, miR-33a-5p, and miR-195a-5p) were found to be differentially expressed in ITP patients prior TPO-RA-treatment compared to controls ($p = 0.0001$, $p = 0.0002$ and 0.035 respectively) (Figure 1A). Of these three miRNAs,

miR-195a-5p did not change during treatment with TPO-RAs and remained significantly higher than controls six weeks after treatment ($p=0.009$).

To determine the discriminative ability of these three miRNAs between ITP patients and controls, we performed receiver operating characteristic (ROC) curve analysis. ROC curve analysis showed an area under the curve (AUC) for miR-199a-5p of 0.814 (Figure 2A), and an AUC of 0.799 (95% CI 0.681 to 0.947) for miR-33a-5p (95% CI 0.666 to 0.932) (Figure 2B). miR-195-5p showed a moderate ability to differentiate patients and normal controls, with AUC of 0.66 (95% CI 0.495 to 0.817). Using logistic regression to determine the best combination of the three miRNAs to distinguish ITP from normal controls, we found that the combination of miR-33a-5p and miR-199a-5p had a significantly increased AUC of 0.929 (95% CI 0.860 to 0.998) (Figure 2C); the combination provided a sensitivity of 91% and specificity of 77% for ITP, at a threshold of 0.40.

ITP patients before and after TPO-RA-treatment

Using Friedman test, six miRNAs showed significant changes during treatment with TPO-RAs: miR-199a-5p ($p=0.001$), miR-33a-5p ($p=0.003$), miR-382-5p ($p=0.004$), miR-92b-3p ($p=0.005$), miR-26a-5p ($p=0.008$) and miR-221-3p ($p=0.023$). Further, we used Wilcoxon signed-rank test to compare the levels of these miRNAs at two and six weeks after treatment with the levels before the initiation of treatment with TPO-RAs (Figure 1B). Five of six miRNAs (except miR-33a-5p) increased two weeks after TPO-RAs; miR-33a-5p showed a decrease at six weeks after TPO-RAs compared to pre-treatment levels.

Levels of miR-199a-5p and miR-221-3p at six weeks after treatment with TPO-RAs showed good correlations with platelet count at six weeks ($r=0.67$, $p=0.0007$ and $r=0.55$, $p=0.009$, respectively). This was particularly evident in patients who achieved complete response (Figure 3). In addition, miR-382-5p and miR-590-5p levels showed a moderate correlation with platelet counts ($r=0.43$, $p=0.049$ and $r=0.48$, $p=0.02$, respectively).

Using linear regression, we found that pre-treatment levels of miR-199a-5p and miR-221-3p independently could help to predict platelet counts at six weeks after treatment ($p=0.002$, $r^2=0.40$ and $p=0.015$, $r^2=0.26$ respectively).

Prediction of potential biological pathways

The results of signaling pathway analysis are dependent on the miRNAs selected for validation. Because of the small number of patients in the profiling cohort we chose to select the miRNAs for the pathway analysis from the validation study. The signaling pathways

targeted by the three differentially expressed miRNAs in ITP patients before treatment with TPO-RAs vs. controls (miR-199a-5p, miR-33a-5p, and miR-195a-5p) were analyzed by functional pathway analysis to clarify their biological roles. Bioinformatics analysis results demonstrated that the target genes were enriched mainly in the following pathways: Insulin/IGF pathway, axon guidance mediated by netrin, and hypoxia response via HIF activation (Supplemental Table 3). Subsequently, the target pathways of the six miRNAs that showed significant changes during treatment with TPO-RA (miR-199a-5p, miR-382-5p, miR-92b-3p, miR-221-3p, miR-26a-5p, and miR-33a-5p) were similarly assessed. The target genes of these miRNAs were primarily involved in the hedgehog signaling pathway, insulin/IGF pathway, and axon guidance mediated by netrin (Supplemental Table 4).

There were many overlapping signaling pathways between the two groups: ITP patients vs. controls, and ITP patients before and under TPO-RAs treatment. The Venn diagram shows the shared and unique signaling pathways in each group (Figure 4). Twenty-two overlapping signaling pathways were enriched in both comparison groups, being presumably determined at least in part by differences in platelet mass. The non-overlapping signaling pathways targeted by the three differentially expressed miRNAs (miR-199a-5p, miR-33a-5p, and miR-195a-5p) in ITP patients vs. controls included: hypoxia response via HIF activation, T cell activation and interleukin signaling pathway, p53 and Ras pathways, and Parkinson and Huntington diseases, while in ITP patients before and after TPO-RAs treatment were: p38 MAPK pathway, hedgehog signaling pathway, and muscarinic acetylcholine and ionotropic glutamate receptor pathways.

Discussion

Although miRNA studies in ITP are very limited, dysregulated expression of several miRNAs in ITP has been identified suggesting that miRNAs are regulatory molecules likely involved in the pathogenesis of ITP [22, 25, 26]. No studies have addressed changes in the expression of specific miRNAs during TPO-RA therapy nor whether specific miRNAs might be predictive of responses to these agents.

In our profiling study, we identified 81 miRNAs that were differentially expressed in ITP patients and 14 miRNAs that showed significant changes during treatment with TPO-RAs. Validation of ten miRNAs in a new and larger cohort of ITP patients showed three differentially expressed miRNAs, miR-199a-5p was down-regulated, while miR33a-5p and miR-195-5p were up-regulated in ITP patients before treatment with TPO-RAs compared to controls. Treatment with TPO-RAs was associated with significant changes in six miRNAs

including miR-199a-5p and miR-33a-5p, miR-382-5p, miR-92b-3p, miR-26a-5p and miR-221-3p.

We found that TPO-RA administration was associated with significant changes in a number of miRNAs. Some of these miRNAs, such as miR-92b-3p, miR-26a-5p and miR-221-3p, are highly expressed in the platelets [6, 27]; therefore, the increase in platelet mass and platelet turnover following treatment with TPO-RAs, may contribute to the increased levels of these miRNAs. Of the miRNAs that showed significant changes after TPO-RAs, only pre-treatment levels of miR-199a-5p and miR-221-3p were associated with increased platelet count at six weeks after treatment which was particularly evident in patients who achieved complete response. Therefore, these two miRNAs may help to predict response to TPO-RAs.

By using ROC curve analysis, our study showed that the combination of miR-199a-5p and miR-33a-5p could distinguish ITP patients from normal controls with good sensitivity and moderate specificity, and might therefore be of a potential diagnostic utility. Recent data indicate that miR-33a-5p enhances pro-inflammatory cytokine induction [28], while miR-199a-5p inhibits IL-6 and TNF- α production [29]. Therefore, an imbalance between these two miRNAs could promote inflammatory cytokine release and contribute to sustained inflammation which is a hallmark of ITP and may be central to its autoimmune etiopathogenesis [30]. However, these findings need to be validated in a larger ITP cohort, as well as in patients with other causes of thrombocytopenia, to confirm their potential diagnostic and prognostic utilities in the setting of ITP.

In the validation study, the up-regulated miR-195-5p in ITP patients was not influenced by the increase in platelet mass as a consequence of response to treatment with TPO-RAs. This could raise the possibility of the involvement of this miRNA in the pathogenesis of ITP. Several of the pathways that were enriched in ITP patients compared to controls, such as hypoxia response via hypoxia-inducible factor-1 α (HIF-1 α) activation, Ras, p53 and T cell activation and interleukin signaling, share the same target, AKT3, that is potentially regulated by miR-195-5p [31].

HIF-1 expression, which is regulated by miR-195-5p [32], activates a broad range of genes protecting cells against hypoxia. Furthermore, thrombopoietin (TPO), the primary regulator of platelet production, increases HIF-1 [33]. The subsequent elevation of HIF-1 mediates survival of hematopoietic stem cells [34, 35], and influences the course of T cell-mediated autoimmune diseases [36]. In addition, a previous study has shown that megakaryopoiesis is potentiated by HIF-1 activation, whereas inhibition of HIF-1 suppresses megakaryocyte maturation [37].

Interestingly, the three differentially expressed miRNAs, miR-195-5p, miR-199a-5p, and miR-33a-5p, are among the miRNAs that have previously been shown to modulate HIF expression [38], and to directly bind HIF-1 mRNA. [39-41]. Further validation in experimental studies is needed.

Although a number of studies have investigated circulating miRNAs in ITP patients, the quality of evidence on the differential expression of miRNAs in ITP is somewhat limited and inconsistent. A recent study in which miRNA profiling was performed in pooled plasma from ITP patients using miRNA microarray followed by validation by quantitative PCR, suggested a panel of four miRNAs (miR-144-3p, miR-1275, miR-3141 and miR-3162-3p) to have a diagnostic value in ITP [21]. Of these four miRNAs, only miR-144-3p was included in our screening panel and found to be among the differentially expressed miRNAs in our screening study; however, miR-144-3p was not selected for validation in our study as other miRNAs showed greater statistical significance. A second study explored miRNA expression in ITP patients before and after a Mongolian natural medicine for ITP (Qishunbaolier) using deep sequencing [23]. They found six differentially expressed miRNAs in ITP including miR-4482-3p, miR-1299, miR-412-5p, miR-204-5p, miR-199a-5p and miR-126-5p. Of those, only miR-199a-5p was among the three differentially expressed miRNAs found in our study. A third study performed in paediatric patients using qPCR showed that seven miRNAs were differentially expressed (miR-302c-3p, miR-483-5p, miR-410, miR-544^a, miR-302^a-3p, miR-223-3p and miR-597) [21, 22], with none but miR-223-3p being also found in our study. In addition to these three being relatively small studies, these differences could be due to selection of the sets of miRNAs included in the screening and validation, different PCR methods used, and different ethnicities of patients including one study of children.

One limitation of our study is the small number of ITP patients included. Another limitation is that several of the patients were on steroids, and although steroid dosages were kept unchanged until achieving response, we cannot exclude that steroids may have contributed to changes in the levels of miRNAs. Also, no comparison was made between ITP patients and patients with other types of thrombocytopenias, e.g. chemotherapy-induced thrombocytopenia, inherited thrombocytopenia, or secondary ITP. Finally, the mean age of the controls in the validation part of the study was lower than the mean age in ITP patients due to difficulty in recruiting elderly healthy hospital employees as controls. However, this would unlikely affect the results since the difference in age is relatively small. Strengths of the study are that we had measurements at three timepoints for each patient, and that we validated our findings, initially obtained with the less precise relative quantitative methods (real-time PCR), by using an absolute quantitative PCR method (ddPCR) yielding an absolute count of

miRNAs. Quantitation of circulating miRNAs potentially specific to ITP is a promising approach for the establishment of biomarkers in the diagnostic work-up of this disease.

In conclusion, our study generates several hypotheses that might have important diagnostic and prognostic values, as well as may give new insight into the pathophysiology of ITP. We propose novel miRNAs that may play important roles in ITP including miR-199a-5p and miR-221-3p that may have prognostic value in predicting response to TPO-RAs in patients with ITP, and the combination of miR-199a-5p and miR-33a-5p that may have diagnostic utility in ITP. However, larger studies are needed to confirm these findings.

Statement of Contribution

L.G. participated in study conception, design, data collection and sample analysis, statistical analysis and interpretation, and wrote and drafted the manuscript; W.G. participated in study design, patient recruitment and data analysis; A.R. and C.F.N. performed sample analysis and participated in statistical analysis; R.T-M., C.M. and ML.L. participated in pathway analysis, drafting and interpretation of the results; J.B.B. participated in patient inclusion and data collection; PM.S. participated in result interpretation; C.M.J. designed the miRNA detection part of the study and participated in statistical analysis and interpretation. All authors have reviewed and approved its final version.

Declaration of Interest

W.G. has received research support from Novartis, and lecture honoraria from Amgen and Novartis. ML.L. has received research support from Amgen, and lecture honoraria from Amgen and Novartis. J.B.B. has received research support from Amgen and Novartis, has been a consultant at Novartis, and has participated in the advisory board for Amgen and Novartis.

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Table 1: Characteristics of the study populations

Profiling/screening	Patients (n = 8)	Controls (n = 8)
Age in years, mean (SEM)	47.5(6.4)	49.1 (6.0)
Females, n	4	4
Males, n	4	4
Splenectomy, n (%)	6 (75)	0
Platelet count ($\cdot 10^9/L$), mean (SEM)	30 (11.5)	236 (15)
Romiplostim, n (%)	6 (75)	
Eltrombopag n (%)	2 (25)	
Concomitant ITP Therapy at inclusion, (n)		
-Steroids	1	
-Intravenous immunoglobulin	1	
-Immune suppressants	2	
Validation	Patients (n = 23)	Controls (n = 22)
Age in years, mean (SEM)	58.5 (3.5)	47.4 (2.5)
Females, n	13	13
Males, n	10	9
Splenectomy, n (%)	5 (21.8)	0
Platelet count ($\cdot 10^9/L$), mean (SEM)	39 (10.3)	233 (11.7)
Romiplostim, n (%)	8 (34.8)	
Eltrombopag, n (%)	15 (65.2)	
Concomitant ITP Therapy at inclusion (n)		
-Steroids	9	
-Intravenous immunoglobulin	3	
-Immune suppressants	3	

Figure 1

A

B

Figure 2

Figure 3

Supplemental Table 1: Differentially expressed miRNAs in ITP patients in comparison to controls in the profiling study

miRNAs	Fold change	p-Value
hsa-miR-374b-5p	-6,06289	0.000005536
hsa-miR-26a-5p	-4,4328	0.00001026
hsa-miR-107	-2,85058	0.000032711
hsa-miR-766-3p	-3,966	0.000035415
hsa-miR-191-5p	-2,74259	0.000041586
hsa-miR-339-5p	-3,40254	0.000048708
hsa-miR-486-5p	3,75111	0.000049864
hsa-miR-629-5p	4,10173	0.000056974
hsa-miR-223-3p	-1,69245	0.000067143
hsa-miR-222-3p	1,88067	0.000073451
hsa-miR-199a-5p	-7,55819	0.000078417
hsa-miR-26b-5p	-3,10204	0.000091002
hsa-miR-103a-3p	-3,15783	0.000124252
hsa-miR-1260a	2,6978	0,000133446
hsa-miR-423-5p	2,46442	0.000175675
hsa-miR-30b-5p	-2,29031	0.000226734
mmu-miR-378a-3p	3,0258	0.000238977
hsa-miR-151a-5p	-2,93835	0.000322222
hsa-miR-324-5p	-2,45864	0.000333085
hsa-miR-331-3p	-2,01781	0.000356624
hsa-miR-335-3p	-9,14685	0.000506967
hsa-miR-320b	2,10411	0.000579344
hsa-let-7d-5p	-3,35279	0.000652767
hsa-miR-18a-5p	-3,1637	0.000813198
hsa-miR-532-3p	2,81132	0.000870833
hsa-miR-320a	2,06208	0.001024857
hsa-miR-142-5p	-2,01616	0.001110752
hsa-miR-28-5p	-4,70531	0.001201727
hsa-miR-140-3p	2,50258	0.00134344
hsa-miR-199a-3p	-2,25626	0.001347707
hsa-miR-15b-5p	-1,8717	0.001381635
hsa-miR-301a-3p	-6,33445	0.001503588
hsa-miR-127-3p	-2,61007	0.00157194
hsa-miR-27a-3p	-1,86446	0.001767358
hsa-miR-660-5p	2,48966	0.002055184
hsa-let-7b-3p	2,45133	0.002092278
hsa-miR-374a-5p	-2,67267	0.002223502
hsa-miR-18b-5p	-2,15927	0.002460224
hsa-miR-16-2-3p	2,30137	0.002843992
hsa-miR-92a-3p	2,07104	0.002994123
hsa-miR-22-3p	2,00495	0.003207889
hsa-miR-132-3p	1,98488	0.003214071
hsa-miR-25-3p	2,01042	0,003791702
hsa-miR-27b-3p	-1,64841	0.00383061

hsa-miR-365a-3p	2,82387	0.004943076
hsa-miR-23b-3p	-1,69748	0.005328816
hsa-let-7f-5p	-2,47636	0.006014747
hsa-miR-148a-3p	2,02716	0.006134752
hsa-miR-363-3p	2,414	0.006320249
hsa-miR-590-5p	1,7768	0.008642941
hsa-miR-142-3p	-1,84677	0.00992511
hsa-miR-140-5p	-2,18887	0.010417673
hsa-let-7d-3p	1,68012	0.012343398
hsa-miR-505-3p	2,13269	0.014067643
hsa-miR-320d	1,86976	0.014757119
hsa-miR-485-3p	-2,23323	0.014888664
hsa-miR-151a-3p	-2,08339	0.014966233
hsa-miR-92b-3p	2,25823	0.019428664
hsa-miR-532-5p	1,71203	0.019824616
hsa-miR-29a-3p	1,77724	0.020426995
hsa-miR-874-3p	2,11612	0.02081456
hsa-miR-33a-5p	-2,82949	0.022277307
hsa-miR-30c-5p	-1,8947	0.023386556
hsa-miR-424-5p	2,01017	0.023642073
hsa-let-7c-5p	-2,07875	0.024636688
hsa-miR-193a-5p	2,34334	0.02710825
hsa-miR-223-5p	1,96272	0.031702581
hsa-miR-125b-5p	1,69747	0.032863323
hsa-miR-652-3p	-1,71799	0.034648075
hsa-miR-136-3p	-2,48907	0.03469106
hsa-miR-376c-3p	-2,68748	0.035886611
hsa-miR-543	-3,5483	0.036610469
hsa-miR-30e-5p	-1,52948	0.038205483
hsa-miR-106b-3p	-2,29839	0.038751542
hsa-miR-126-3p	-1,48931	0.038883884
hsa-miR-221-3p	-1,811	0.039455599
hsa-miR-144-3p	2,37047	0.04207841
hsa-miR-484	1,78164	0.042240603
hsa-miR-342-3p	1,6529	0.042494778
hsa-miR-502-3p	2,47358	0.042660115
hsa-miR-382-5p	-1,79631	0.046612355

Supplemental Table 2: Top 17 differentially expressed miRNAs in ITP patients in comparison to controls in the profiling study with relative quantification (PCR panel)

Down-regulated miRNAs		
	Fold change	p-Value
miR-374b-5p	-6.0629	0.00000553
miR-26a-5p	-4.4328	0.00001026
miR-107	-2.8506	0.00003271
miR-766-3p	-3.9660	0.00003541
miR-191-5p	-2.7426	0.00004159
miR-339-5p	-3.4025	0.00004871
miR-223-3p	-1.6924	0.00006714
miR-199a-5p	-7.5582	0.00007842
miR-26b-5p	-3.1020	0.00009100
miR-103a-3p	-3.1578	0.00012425
miR-30b-5p	-2.2903	0.00022673
Up-regulated miRNAs		
	Fold change	p-Value
miR-486-5p	3.7511	0.00004986
miR-629-5p	4.1017	0.00005697
miR-222-3p	1.8807	0.00007345
miR-1260a	2.6978	0.00013345
miR-423-5p	2.4644	0.00017567
miR-378a-3p	3.0258	0.00023898

Supplemental Table 3: Pathways overrepresentation analysis in ITP patients vs. Controls (miR-199a-5p, miR-33a-5p & miR-195-5p). Pathways are sorted by fold enrichment.

PANTHER Pathways	# Reference genes	# Input genes	# Genes expected	Fold Enrichment	rawp-value	p-value FDR
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	33	21	5.85	3.59	1.70E-05	2.78E-04
Axon guidance mediated by netrin (P00009)	35	18	6.21	2.90	6.61E-04	5.13E-03
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	41	20	7.27	2.75	4.59E-04	3.94E-03
Hypoxia response via HIF activation (P00030)	33	16	5.85	2.73	1.92E-03	1.30E-02
Alzheimer disease-amyloid secretase pathway (P00003)	69	33	12.23	2.70	1.01E-05	2.06E-04
FGF signaling pathway (P00021)	123	54	21.81	2.48	2.14E-07	5.82E-06
Angiogenesis (P00005)	173	73	30.67	2.38	4.83E-09	2.62E-07
B cell activation (P00010)	69	29	12.23	2.37	2.97E-04	3.02E-03
PI3 kinase pathway (P00048)	55	23	9.75	2.36	1.18E-03	8.77E-03
Gonadotropin-releasing hormone receptor pathway (P06664)	237	99	42.02	2.36	1.56E-11	1.27E-09
Alzheimer disease-presenilin pathway (P00004)	123	51	21.81	2.34	1.77E-06	4.12E-05
VEGF signaling pathway (P00056)	68	28	12.06	2.32	4.55E-04	4.12E-03
Metabotropic glutamate receptor group III pathway (P00039)	69	28	12.23	2.29	5.00E-04	4.08E-03
Ras Pathway (P04393)	74	30	13.12	2.29	2.87E-04	3.12E-03
Endothelin signaling pathway (P00019)	85	34	15.07	2.26	1.50E-04	1.89E-03
CCKR signaling map (P06959)	174	69	30.85	2.24	1.03E-07	3.34E-06
T cell activation (P00053)	91	36	16.14	2.23	1.44E-04	1.95E-03
p53 pathway feedback loops 2 (P04398)	51	20	9.04	2.21	4.15E-03	2.60E-02
EGF receptor signaling pathway (P00018)	140	53	24.82	2.14	1.02E-05	1.84E-04
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	61	22	10.82	2.03	7.97E-03	4.33E-02
TGF-beta signaling pathway (P00052)	97	34	17.20	1.98	1.26E-03	8.90E-03
PDGF signaling pathway (P00047)	150	52	26.60	1.96	6.75E-05	1.00E-03
Wnt signaling pathway (P00057)	312	106	55.32	1.92	3.65E-08	1.49E-06
Interleukin signaling pathway (P00036)	89	29	15.78	1.84	6.32E-03	3.68E-02
Cadherin signaling pathway (P00012)	158	51	28.02	1.82	4.17E-04	4.00E-03
Parkinson disease (P00049)	103	33	18.26	1.81	5.24E-03	3.16E-02
Apoptosis signaling pathway (P00006)	120	38	21.28	1.79	3.42E-03	2.23E-02
Integrin signalling pathway (P00034)	190	60	33.69	1.78	1.74E-04	2.03E-03
Huntington disease (P00029)	145	42	25.71	1.63	7.26E-03	4.08E-02

Supplemental Table 4: Pathways overrepresentation analysis in ITP patients before and after TPO-RA (miR-92b-3p, miR-221-3p, miR-26a-3p, miR-382-5p, miR-199a-5p & miR-33a-5p). Pathways are sorted by fold enrichment.

PANTHER Pathways	# Reference genes	# Input genes	# Genes expected	Fold Enrichment	raw p-value	p-value FDR
Hedgehog signaling pathway (P00025)	23	11	3.91	2.82	6.74E-03	4.23E-02
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	41	19	6.96	2.73	6.61E-04	8.98E-03
Axon guidance mediated by netrin (P00009)	35	16	5.94	2.69	2.05E-03	1.76E-02
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	33	15	5.60	2.68	3.00E-03	2.45E-02
Gonadotropin-releasing hormone receptor pathway (P06664)	237	97	40.24	2.41	6.33E-12	5.16E-10
p38 MAPK pathway (P05918)	42	17	7.13	2.38	4.48E-03	3.18E-02
PI3 kinase pathway (P00048)	55	22	9.34	2.36	1.62E-03	1.76E-02
Ionotropic glutamate receptor pathway (P00037)	50	20	8.49	2.36	1.99E-03	1.80E-02
Metabotropic glutamate receptor group III pathway (P00039)	69	27	11.72	2.30	6.48E-04	9.60E-03
CCKR signaling map (P06959)	174	67	29.55	2.27	9.42E-08	5.12E-06
VEGF signaling pathway (P00056)	68	25	11.55	2.17	1.69E-03	1.72E-02
Alzheimer disease-presenilin pathway (P00004)	123	45	20.89	2.15	3.56E-05	8.29E-04
Alzheimer disease-amyloid secretase pathway (P00003)	69	25	11.72	2.13	1.88E-03	1.81E-02
TGF-beta signaling pathway (P00052)	97	35	16.47	2.12	2.77E-04	4.52E-03
Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)	63	22	10.70	2.06	5.14E-03	3.49E-02
B cell activation (P00010)	69	24	11.72	2.05	4.44E-03	3.29E-02
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	61	21	10.36	2.03	7.16E-03	4.33E-02
Integrin signalling pathway (P00034)	190	65	32.26	2.01	3.92E-06	1.28E-04
Angiogenesis (P00005)	173	59	29.38	2.01	1.28E-05	3.47E-04
PDGF signaling pathway (P00047)	150	49	25.47	1.92	1.58E-04	3.21E-03
Apoptosis signaling pathway (P00006)	120	39	20.38	1.91	9.20E-04	1.07E-02
Endothelin signaling pathway (P00019)	85	27	14.43	1.87	6.67E-03	4.35E-02
Cadherin signaling pathway (P00012)	158	50	26.83	1.86	2.32E-04	4.20E-03
EGF receptor signaling pathway (P00018)	140	44	23.77	1.85	6.91E-04	8.67E-03
Wnt signaling pathway (P00057)	312	98	52.98	1.85	4.04E-07	1.65E-05
FGF signaling pathway (P00021)	123	37	20.89	1.77	3.25E-03	2.52E-02

Supplementary Methods

Droplet digital PCR

Total RNA was extracted from 200 μ l plasma using the miRCURY™ RNA isolation Kit for Biofluids (Cat no 300112, Exiqon) according to the manufacturers' protocol. Synthetic spike-ins were included to monitor RNA extraction efficiency. RNA was eluted in 50 μ l nuclease-free water and stored at -80 °C until use.

Following the protocol for microRNA PRC profiling with the QX200™ Droplet Digital™ PCR system from Exiqon, reverse transcription was performed using the Universal cDNA Synthesis Kit II. The resulting cDNA was diluted 1:50 for all assays before droplet generation and amplification for all miRNA assays. Each PCR assay was mixed in a 20 μ l volume containing 10 μ l 2X Eva Green supermix (Cat no 1864034; Bio-Rad Laboratories GmbH, Munich, Germany), 8 μ l diluted cDNA and 1 μ l of one of the miRCURY LAN PCR primer sets (Exiqon) (Supplementary Table 1). Droplets were generated using the QX200™ Automated droplet generator (Bio-Rad), and PCR was performed with the following thermal cycling conditions: 95°C for 5 min, 40 cycles of 95°C for 30 seconds, and 58 °C for 1 min (Ramp-rate 1,6°C/s), followed by three final steps; 4°C for 5 minutes, 90°C for 5 minutes and a 4 °C indefinite hold. Droplets were read in the QX200™ droplet reader (Bio-Rad) and the amount of miRNA (miRNA copies/ μ l) was generated by the Quanta Soft software (Bio-Rad).

Data analysis

The relative quantitative data generated from the profiling PCR panel were processed and analyzed using GenEX v.6 software (MultiD, Gothenburg, Sweden) according to the “Data Analysis Guide” (version3, Feb 2014). Global mean normalization was used¹. Changes in relative quantification of miRNAs using the comparative cycle threshold (Ct) values by qPCR were calculated and expressed as fold-change (FC) between the groups (prior to treatment, 3 time points post initiation of treatment, and control group).

QuantaSoft™ software was used for the analysis of ddPCR data (absolute quantitative data) generated from droplet reader (Bio-Rad). The threshold was manually set between the positive and negative droplet populations, and varied between the different miRNAs. The target miRNA concentrations were calculated using the Poisson statistics. All samples with a concentration higher than the upper 95% Confidence Interval for the merged NTCs were considered positive. The absolute transcript levels were computed in copies/20 μ L, results presented in this study are copies/ μ L plasma.

Pathway analysis

The differentially expressed miRNAs identified in the evaluation of ITP patients *vs.* controls, and in the assessment of ITP patients before and after TPO-RA treatment were analyzed using mirDIP, which integrates several miRNA target databases, to determine miRNA target genes with high confidence². For increased stringency, the analysis was performed by focusing only on top 1% targets, that were further analyzed by Panther (Annotation Version and Release Date: PANTHER version 13.1 Released 2018-02-03) to identify enriched biological pathways. The comparative analysis was performed by a Fisher's Exact Test in combination with a robust False Discovery Rate (FDR) correction for multiple testing.

Statistics

Statistical analysis for the relative quantitative screening data were performed using GenEX v.6 software (MultiD) and SPSS 23.0 software. T-test was used to assess the differentially expressed miRNAs in ITP patients before TPO-RA treatment compared to matched controls, and paired t-test was used for comparison between two time points (2 weeks, 6 weeks and 12 weeks after treatment) with pre-treatment values as reference level. Repeated measures one-way ANOVA was used to evaluate the significance of the changes in the sequential samples. For the absolute quantitative validation data (non-parametric data), Mann-Whitney test was used to evaluate the differences between ITP patients and controls using SPSS 23.0 software. Friedman test was used to evaluate the significance of the changes before and sequentially after the initiation of treatment and Wilcoxon signed-rank test was run thereafter to find where significant changes were. Spearman correlation and linear regression were used to evaluate the correlation between levels of miRNAs and platelet count using GraphPad prism 7.0 which also was used to create figures and plots.

For identification of potential diagnostic miRNA for ITP, Receiver-operator characteristic (ROC) curves were generated by R (pROC Package)³ using the absolute quantitative levels of the three differentially expressed miRNAs (miR-195a-5p, miR-33a-5p, and miR-199a-5p). Area Under Curve (AUC) was used to determine the sensitivity and specificity of these three individual miRNAs in discriminating ITP patients prior to treatment from healthy controls. Logistic regression analysis was used to find the best combination of miRNAs to generate ROC curves with highest discrimination ability between disease and controls.

Supplementary References

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