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Original Article

Analysis of Plasma microRNA Profiles in Essential Thrombocythemia Using a Novel Multi-Gene Detection Platform

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SUMMARY

Background: A novel multi-gene detection platform, PanelChip™ Analysis System, was developed for the analysis of microRNA levels in the blood. This novel platform has been successfully used to screen for potential diagnostic biomarkers in the plasma of patients with solid malignancy. Because microRNA deregulation has been proposed to play roles in the pathogenesis and disease phenotype in essential thrombocythemia (ET), we aimed to investigate the plasma microRNA profiles in ET patients using this novel platform with a focus on elderly patients.

Materials and methods: Fifty-four ET patients and 8 healthy adults were enrolled. Plasma microRNA profiling was carried out on PanelChip™ Analysis System using a customized microRNA panel including 165 microRNAs called mirSCAN™ PanCancer Chips 1 & 2. KEGG pathways that microRNAs were associated with were analyzed using DIANA TOOLS – mirPath v.3.

Results: Elderly ET patients had significantly higher white blood cell count at diagnosis, and had significantly inferior overall survival compared with young patients (median, 8.7 years vs. not reach, respectively, p = 0.008). Four miRNAs (miR-451a, miR-486-5p, miR-224-5p and miR-34a-5p) had significantly higher and 1 miRNA (miR-760) had significantly lower expression levels in elderly ET patients, respectively. A total of 40 differentially expressed microRNAs were identified. The putative target genes of these 40 differentially expressed microRNAs were enriched in PI3K-Akt, Ras, and Rap1 signaling pathway.

Conclusion: The use of PanelChip™ platform was feasible and distinct plasma microRNA profiles correlated with age and genotypes in ET patients. A larger study is warranted to validate our observation in ET patients.

1. Introduction

Thrombocytosis is a common hematologic abnormality in clinical practice and the prevalence of thrombocytosis in patients who were 65 years of age or older was 1.9%.[1] The causes of thrombocytosis can be primary (clonal) or secondary. In a retrospective study on adult patients with thrombocytosis, primary thrombocytosis was observed in 5.2% of patients and essential thrombocythemia (ET) accounted for 52.4% of the primary thrombocytosis cases.[2] In the majority of cases, thrombocytosis can be attributed to secondary causes including non-infectious (50.6%, such as tissue damage, malignancy, and iron-deficiency anemia) and infectious causes (47.9%, such as soft tissue, pulmonary, and gastrointestinal infection).

ET is one of the classic *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs) which also include polycythemia vera (PV) and primary myelofibrosis (PMF).[3] The histopathological and clinical features of ET include an increased number of mature megakaryocytes in the bone marrow and sustained thrombocytosis in the peripheral blood. The annual incidence rate for ET is 1.03 per 100,000.[4] Although ET can occur in people of any age, it is more commonly diagnosed in the elderly population, and the median age at diagnosis is estimated at 71 years.[5] Most ET patients can have a normal life expectancy, but some may suffer from serious complications due to an increased risk of thrombotic and hemorrhagic complications. In addition, ET also has the risk of transformation to post-ET myelofibrosis or secondary acute myeloid leukemia.[6]

Since 2005, three driver mutations in MPNs are discovered: *JAK2* (Janus kinase 2) V617F, *CALR* (Calreticulin) exon 9, and *MPL* (receptor for thrombopoietin) exon 10 mutations.[7,8] All these driver mutations have been found to activate JAK-signal transducer and activator of transcription (STAT) signaling pathway leading to myeloproliferation,[9,10] and they also play important diagnostic, therapeutic, and prognostic roles in MPNs. MPN patients that are negative for all the three driver mutations are called triple-negative MPNs, and other mutations have been reported in this group of patients.[11,12] In ET, the frequency of *JAK2* V617F mutation is about 60%, 22% *CALR* exon 9 mutations, and 3% MPL mutation.[13–15] We and others have reported that *CALR* mutations are associated with distinct clinical

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characteristics including higher platelet counts, lower leukocyte counts and hemoglobin levels, and a lower thrombosis risk when compared to *JAK2*-mutated ET patients.[14–17] Besides, other non-driver mutations such as *LNK*, *TET2* and *DNMT3A* have also been detected in patients with ET. However, they are not mutually exclusive with *JAK2*, *CALR* and *MPL* mutations and not specific to patients with MPN.[18,19]

In addition to genetic mutations, microRNAs (miRNA) deregulation has been proposed to play roles in the molecular pathogenesis and disease phenotype in ET patients.[20,21] MiRNA are small 18–24 nucleotide single stranded non-coding RNAs that regulate gene expression by acting as negative regulators of mRNA translation and/or stability.[22,23] The role of miRNAs in *JAK2*-mutated ET had been studied after the discovery of *JAK2*V617F mutation.[24] However, miRNA deregulation in *CALR*-mutated ET is less well explored and characterized.[25] We assumed that miRNA in the plasma of ET patients might predict clinical characteristics and outcomes. Recently, a novel multi-gene detection platform, PanelChip™ Analysis System, was developed for the analysis of miRNA levels in the blood.[26] This novel platform has been successfully used to screen for potential diagnostic biomarkers in the plasma of patients with oral cavity squamous cell carcinoma. All the 165 miRNAs included in the PanelChip™ Analysis System are oncomiR that might be found in both solid and hematologic neoplasms. The aims of this study were to investigate the plasma miRNA profiles in ET patients by using this novel multi-gene detection platform and its correlation with clinical and prognostic features with a focus on the elderly patients.

2. Materials & methods

2.1. Patients and samples

ET patients with archived plasma samples at the MacKay Memorial Hospital were enrolled into this study. The screening for mutations in patients with hematologic neoplasms was approved by the Institutional Review Board of MacKay Memorial Hospital (09MMH IS157 and 12MMHIS034). Fifty-four adult Taiwanese ET patients were enrolled and written informed consent was obtained. The clinical and laboratory characteristics at the time of diagnosis or referral were determined retrospectively by chart review. Total plasma miRNA was derived from peripheral blood in patients or healthy adults. Plasma samples were stored at -80 °C. Collection, processing and storage of venous blood samples were performed under standardized conditions. Mutational status of *JAK2*V617F, *CALR* and *MPL* were detected by Sanger sequencing and/or allele-specific PCR as described previously.[16,27]

2.2. Plasma preparation and miRNA extraction

Plasma was isolated by centrifugation at 400 × g at room temperature for 10 min in a swing bucket rotor. Supernatant was transfer to a new labeled 1.5 ml tube without disturbing the buffy coat. Plasma samples with a concentration of hemoglobin ≤ 50 mg/dL was then centrifuged at 1,000 × g at 4 °C for 20 min. Supernatant was transferred to a new labeled 1.5 ml tube without disturbing the sediment at the bottom of the tube and stored at -80 °C immediately. miRNA extraction and quantification were performed as previously described.[26]

2.3. cDNA synthesis

2 ng of total miRNAs from patient and healthy donor samples were used to synthesize cDNA in 20 μl reverse transcription reactions. QuarkBio MicroRNA Reverse Transcription kit (Quark Biosciences, Inc.) was used for cDNA synthesis.

2.4. Plasma miRNA profiling on PanelChip™ Analysis System and mirSCAN PanCancer Chips 1 & 2

Plasma miRNA profiling was carried out on PanelChip™ Analysis System (Quark Biosciences, Taiwan) using a customized miRNA panel including 165 miRNAs called mirSCAN™ PanCancer Chips 1 & 2 (Supplementary Table S1. List of mirSCAN 165 miRNAs).[26] Q-Chip™ containing multiple clusters are called PanelChip™. PanelChipTM was run on PanelStation™ platform as previously described.[26] miPrimer™ was used to design primers.[28]

2.5. Statistical analysis

Data obtained from mirSCAN™ PanCancer Chips 1 & 2 were preprocessed and modeled. Analyses were based on the data of 54 ET patients and 8 healthy adults. A total of 80 mirSCAN™ PanCancer profiles were normalized using the miRNA RT and qPCR controls. The miRNA RT control and qPCR control were spiked in during reverse transcription and qPCR, respectively, as process controls.[26] For subsequent data preprocessing, the missing miRNA values for the individual profile were replaced with the maximum ΔCq of the entire profile. KEGG pathways that miRNAs were associated with were analyzed using DIANA TOOLS – mirPath v.3.[29]

Differentially expressed miRNAs were identified by student t-test or Mann-Whitney U test ($p < 0.05$) and significantly affected miRNAs in ET were identified by a change in expression of two-fold or more compared to the expression in healthy adults. The chi-square test or Fisher's exact test was used to calculate the correlation between elderly (age ≥ 65 years) and young ET patients, mutational groups and clinical characteristics. Spearman's rank correlation coefficient was used to evaluate the relationship between two continuous variables. Overall survival was calculated using the Kaplan-Meier analysis. Statistical analysis was performed using SPSS (IBM, New York, USA). A two-side p value of less than 0.05 was considered as significant difference. A total of 40 differentially expressed miRNAs were identified and are displayed in the heatmap.

3. Results

3.1. Patients

We used 8 healthy adults as control and enrolled 54 patients with ET in this study. Table 1 showed the clinical and laboratory characteristics at diagnosis of the patients studied according to age groups and their genotypes. The molecular subgroups in ET included 35 (64.8%) patients with *JAK2*V617F mutation, 8 (14.8%) with *CALR* mutation [4 type 1 (p.L367fs*46), 2 type 2 (p.K385fs*47), 1 type 34 (p.K385fs*47), and 2 other types (p.E369fs*50, and p.L367fs*43)], 2 (3.7%) with *JAK2*/*CALR* co-mutation, and 9 (16.7%) triple-negative for *JAK2*, *CALR* and *MPL* mutations. No patient harbored *MPL* mutation. Elderly ET patients had significantly higher white blood cell count at diagnosis compared with young patients. The incidences of myelofibrosis and leukemic transformation, second solid cancers, hemorrhage and thrombosis were not different between elderly and young ET patients. *JAK2*V617F-mutated ET patients had significantly higher hemoglobin level at diagnosis and higher incidence of thrombosis compared with ET patients with other genotypes.

Table 1
Clinical and laboratory characteristics in healthy adults and patients with essential thrombocythemia stratified by age (≥ 65 years) and genotypes.

Variables	ET (n = 54)*	Elderly ET (n = 20)	*JAK2* mutation (n = 35)	*CALR* mutations (n = 8)	TN (n = 9)	Elderly vs. young ET	*JAK2* mutation vs. other genotypes
						p value	*p* value
Male/female gender, n (%)	22/32 (41/59)	11/9 (55/45)	15/20 (43/57)	5/3 (62.5/37.5)	1/8 (11/89)	NS	NS
Age at diagnosis (y), median (range)	60 (25–83)	74 (65–83)	62 (25–83)	58 (25–76)	50 (35–81)	< 0.001	NS
Follow-up (y), median (range)	6.2 (0–27.8)	5.2 (0.4–10.6)	5.5 (0–27.8)	6.9 (2.81–13.82)	4.9 (0.06–15.27)	NS	NS
Hemoglobin at diagnosis (g/dL), median (range)	13 (5.8–18)	13.0 (7.7–18)	13.4 (8.2–18)	11.0 (8.4–15)	12.4 (5.8–13.7)	NS	0.02
WBC at diagnosis (x10^9/L), median (range)	10.5 (4.6–29.9)	13.1 (4.6–29.9)	11.8 (4.8–29.9)	8.0 (7.1–14.4)	10.5 (4.9–23.2)	0.02	NS
Platelet at diagnosis ($\times10^9$/L), median (range)	896 (58–2834)	890 (58–1496)	890 (514–1931)	1200.5 (301–2834)	738 (532–1020)	NS	NS
Cytoreductive therapy with hydroxyurea, n (%)	46 (85.2)	18 (90)	32 (91.4)	7 (87.5)	5 (55.6)	NS	NS
Splenomegaly, n (%)	16 (29.6)	6 (30)	10 (28.6)	2 (25.0)	2 (22.2)	NS	NS
Second solid cancer, n (%)	4 (7.4)	2 (10)	2 (5.7)	2 (25)	0	NS	NS
Myelofibrosis transformation, n (%) (n = 53)	2 (3.8)	1 (5)	0	1 (12.5)	0	NS	NS
Acute leukemia transformation, n (%)	2 (3.7)	2 (10)	1 (2.9)	0	0	NS	NS
History of hemorrhage, n (%)	18 (33.3)	6 (30)	11 (31.4)	4 (50)	2 (22.2)	NS	NS
History of thrombosis, n (%) (n = 53)	16 (30.2)	8 (42.1)	14 (40)	1 (12.5)	1 (11.1)	NS	0.03

Abbreviations: ET, essential thrombocythemia; HA, healthy adults; n, number; NS, not significant; TN, triple-negative; WBC, white blood cell; y, year.
* Included 2 patients with *JAK2* and *CALR* co-mutations.

3.2. MicroRNA expression profiles of ET patients and healthy adults

Forty differentially expressed miRNAs were identified and were displayed in the heatmap (Figure 1 left axis). Among these 40 miRNAs, 4 miRNAs (hsa-miR-940, hsa-miR-411-5p, hsa-miR-596 and hsa-miR-376c-3p) were found to have higher expression levels in ET patients when compared to healthy adults (Supplementary Table S2. MicroRNA expression profile in ET). ET patients were clustered to either low or high expression groups shown at the top of the heatmap according to the expression profiles of these 40 miRNAs. Whereas, all healthy adults clustered in the high expression group. Most *JAK2*-mutated ET patients clustered in the high expression groups. *CALR*-mutated and triple-negative ET patients were clustered in both low and high expression groups.

3.3. KEGG pathways analysis

One of the miRNA hsa-miR-320a-3p was not included in DIANA-mirPath v.3 database and was excluded from our query. The pu-

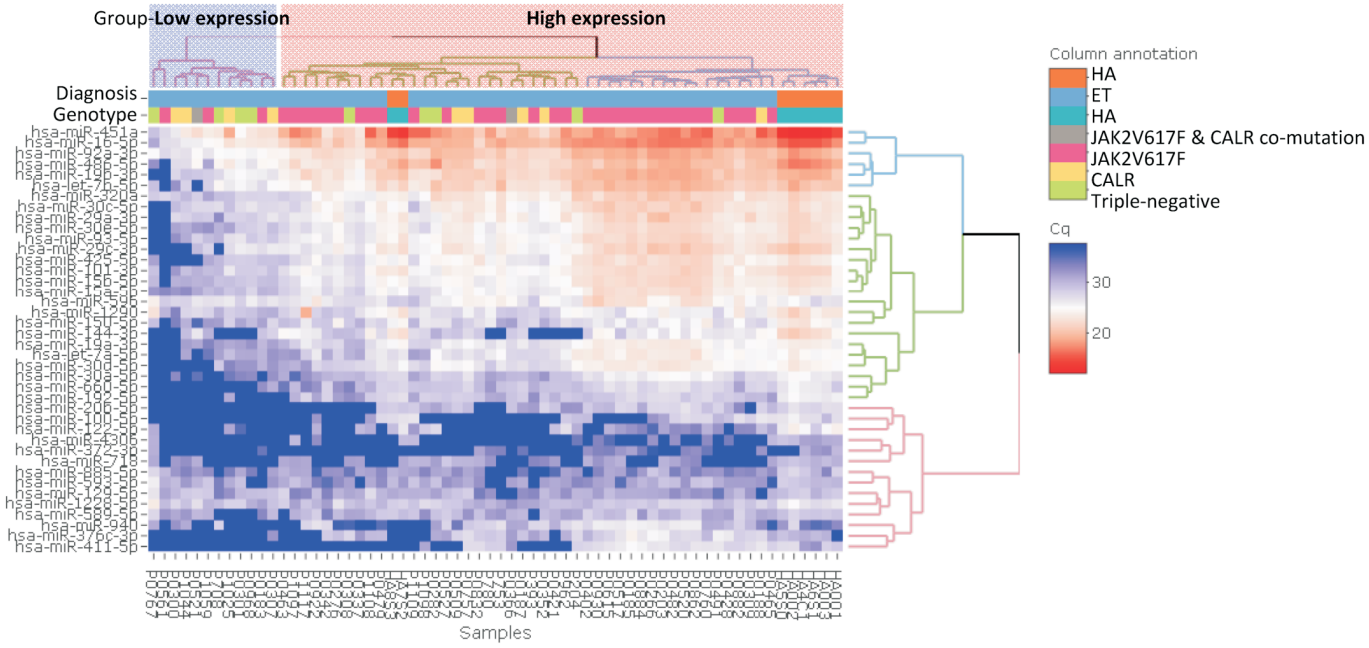

Figure 1. Heatmap of the microRNA expression profiles of ET patients and healthy adults. A total of 40 differentially expressed miRNAs were identified and are displayed on the left axis. The individual patient sample was displayed at the bottom. According to the expression levels of these 40 miRNAs, samples were grouped into two clusters: low expression group and high expression group, respectively. HA, healthy adults.

tative target genes of the remaining 39 differentially expressed miRNAs were enriched in signaling pathway including PI3K-Akt signaling pathway (p = 0.0003), Ras signaling pathway (p = 0.01), Rap1 signaling pathway (p = 0.004), cAMP signaling pathway (p = 0.004), and cGMP-PKG signaling pathway (p = 0.0006) listed according to the numbers of genes and miRNAs involved in these pathways (Table 2).

3.4. Clinical correlation of miRNA profiles in ET

According to the expression levels of these 40 miRNAs, samples were grouped into two clusters, i.e. low expression group and high expression group (Figure 1). ET patients with *JAK2*V617F were significantly associated with high expression of 40 miRNAs (n = 31 of 34, 91%) when compared with triple-negative ET patients (n = 5 of 9, 56%) (p = 0.03). However, the expression levels of these 40 miRNAs when grouped into two clusters were not statistically correlated with clinical features including hemogram, secondary solid cancer, myelofibrosis and leukemic transformation, or thrombotic/hemorrhagic events. We also analyzed the correlation of clinical characteristics of the patients (age, platelet count, hemoglobin levels and leukocyte count) with each of the 40 miRNA expression levels (ct value) by the Spearman's rank correlation coefficient. Only miRNA-100-5p expression level was significantly correlated with platelet count (ρ = 0.518, p = 0.006) (Figure 2). Besides, another miRNA-20b-5p expression level was found to significantly correlate with hemoglobin level (ρ = 0.4708, p = 0.002) (Figure 2). No correlation with leukocyte count and age was observed (data not shown).

We used non-parametric Mann-Whitney U test to identify miRNAs differentially expressed in ET patients according to age and mutational status. Four miRNAs (hsa-miR-451a, hsa-miR-486-5p, hsa-miR-224-5p and hsa-miR-34a-5p) had significantly higher and 1 miRNA (hsa-miR-760) had significantly lower expression levels in elderly ET patients, respectively (Table 3a). Interstingly, higher miR-451a expression levels was significantly associated with ET patients with other genotypes (73.7% vs. 26.3%, p = 0.018). All these 5 miRNAs did not correlated with overall survival, secondary solid cancer, myelofibrosis and leukemic transformation, or thrombotic/hemorrhagic events in this study. Furthermore, 1 and 63 miRNAs had significantly higher and lower expression levels in *JAK2* V617F-mutated ET patients, respectively (Table 3b). In addition, elderly ET patients had significantly inferior overall survival compared with young patients (median, 8.7 years vs not reached, respectively, p = 0.008). Because many samples were obtained after cytoreductive treatment had been commenced, we also analyzed the effects of cytoreductive treatment on the expression levels of miRNAs. Our results showed that only 6 (3.9%) of the 155 miRNAs analyzed (hsa-

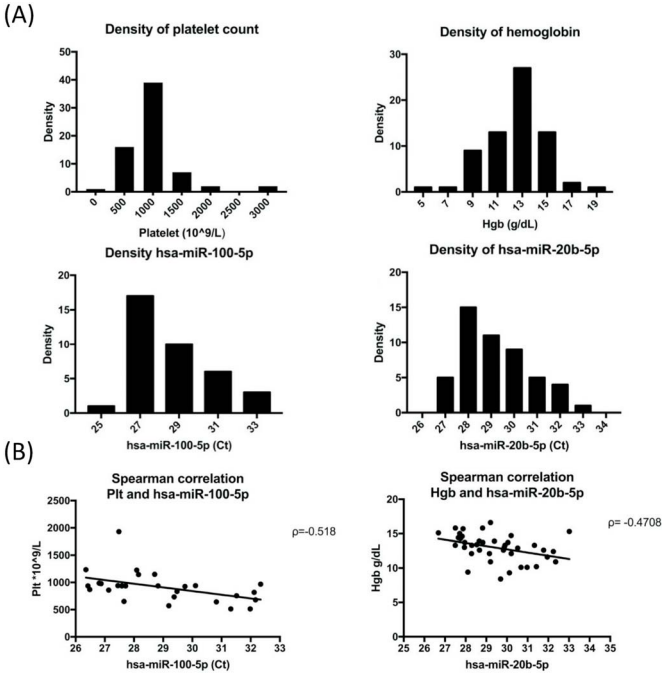

Figure 2. Correlation of clinical characteristics with miRNA expression levels. (A) The upper panel showed the density of platelet counts and hemoglobin. The lower panel showed the expression levels of hsa-miR-100-5p and hsa-miR-20b-5p, respectively. (B) The histogram showed the Spearman correlation of hsa-miR-100-5p expression and platelet counts, and hsa-miR-20b-5p expression and hemoglobin levels. Hgb, hemoglobin; Plt, platelet.

Table 2
The results of 39 miRNAs functional analyses using KEGG annotations based on DIANA-mirPath v.3 database.

KEGG pathway	*p* value	Number of genes	Number of miRNAs
Pathways in cancer	1.04E-07	228	38
PI3K-Akt signaling pathway	0.00026869	189	36
Ras signaling pathway	0.00990842	122	36
Rap1 signaling pathway	0.00351888	116	35
cAMP signaling pathway	0.00351888	112	36
cGMP-PKG signaling pathway	0.00055746	98	36
Oxytocin signaling pathway	0.00055746	94	34
Signaling pathways regulating pluripotency of stem cells	1.69E-05	87	37
Hippo signaling pathway	0.00351888	86	35
Wnt signaling pathway	0.00068894	82	35
FoxO signaling pathway	0.00728526	75	35
AMPK signaling pathway	0.00711077	73	35
Platelet activation	0.02172656	71	35
Thyroid hormone signaling pathway	7.37E-05	70	35
Sphingolipid signaling pathway	0.0009557	69	33
Retrograde endocannabinoid signaling	7.37E-05	65	34
HIF-1 signaling pathway	0.02686069	61	33
ErbB signaling pathway	9.54E-06	59	37
Estrogen signaling pathway	0.00399181	55	35
TGF-beta signaling pathway	2.03E-05	49	30
Prolactin signaling pathway	0.00156454	44	32
p53 signaling pathway	0.02880884	42	30
mTOR signaling pathway	0.00639454	39	34

Table 3
MicroRNAs (miRNAs) with significantly different expression levels in patients with essential thrombocythemia (ET) stratified by age (≥ 65 years) and genotypes.

a. Five microRNAs with significantly different expression levels stratified by age (= 65 years).

MiRNA	Total (n = 54)	Elderly ET (n = 20)	Young ET (n = 34)	*p* value
hsa-miR-451a	19.0 (15.6–29.4)	21.1 (17.5–29.4)	18.8 (15.6–24.9)	0.008
hsa-miR-486-5p (n = 53)	21.7 (17.7–32.5)	22.5 (19.4–32.5)	20.6 (17.7–26.8)	0.019
hsa-miR-224-5p (n = 38)	29.2 (25.4–33.1)	30.7 (25.6–33.1)	28.7 (25.4–32.1)	0.025
hsa-miR-34a-5p (n = 44)	29.0 (25.4–33.1)	29.6 (26.9–32.4)	28.5 (25.1–33.0)	0.023
hsa-miR-760 (n = 41)	31.1 (27.2–34.7)	29.8 (27.2–32.9)	31.2 (27.3–34.7)	0.026

b. Sixty-four microRNAs with significantly different expression levels stratified by genotypes.

No.	MiRNA	No. of patients in each testing	Total (n = 54)	*JAK2* V617F mutation (n = 35)	Other genotypes (n = 19)	*p* value
			miRNA expression levels (median)			
1	hsa-miR-198	15	31	31.3	29.8	0.010
2	hsa-let-7a-5p	50	26.5	25.5	27.7	0.040
3	hsa-let-7b-5p	51	22	21.6	23.2	0.020
4	hsa-let-7d-3p	54	29.2	27.8	29.9	0.020
5	hsa-let-7d-5p	48	24.3	23.9	25.6	0.030
6	hsa-miR-101-3p	53	24.6	24	26.1	< 0.001
7	hsa-miR-103a-3p	51	23.2	22.1	24.7	0.010
8	hsa-miR-126-3p	53	23.1	22.7	25.1	0.030
9	hsa-miR-1290	54	27.2	26.4	28.3	0.010
10	hsa-miR-142-3p	54	22.7	21.4	25.2	< 0.001
11	hsa-miR-143-3p	46	27.9	27.3	29.1	< 0.001
12	hsa-miR-145-5p	52	26.4	25.4	27.3	0.010
13	hsa-miR-146a-5p	52	24.5	23.3	25.7	0.010
14	hsa-miR-146b-5p	47	27.9	26.5	29.3	< 0.001
15	hsa-miR-150-5p	52	27.7	27	28.7	< 0.001
16	hsa-miR-151a-3p	52	28.5	27.2	29.4	0.040
17	hsa-miR-152-3p	44	29.5	28.8	30.9	0.020
18	hsa-miR-155-5p	32	30.8	30.5	32.1	0.040
19	hsa-miR-15a-5p	54	25.7	24.8	27.5	0.010
20	hsa-miR-15b-5p	53	25.3	24.6	25.8	0.010
21	hsa-miR-16-5p	54	19.5	18.8	20.4	< 0.001
22	hsa-miR-17-3p	42	31.1	29.9	31.7	0.020
23	hsa-miR-181a-5p	50	27.6	26.6	29.2	0.010
24	hsa-miR-18a-5p	50	25.4	24.4	26.6	0.020
25	hsa-miR-191-5p	53	23.7	22.5	25.8	0.010
26	hsa-miR-195-5p	45	27.7	27.1	28.3	0.030
27	hsa-miR-199a-3p	50	24.3	22.8	25.2	0.010
28	hsa-miR-199a-5p	49	26	24.7	27	0.010
29	hsa-miR-19a-3p	50	26	25.8	27.4	< 0.001
30	hsa-miR-19b-3p	52	21.5	20.9	22.9	< 0.001
31	hsa-miR-210-3p	41	26.7	26.3	27.9	0.020
32	hsa-miR-21-5p	54	22.7	21.1	25	< 0.001
33	hsa-miR-221-3p	50	24.1	23.2	25.6	0.010
34	hsa-miR-222-3p	54	26.3	25.1	27.2	0.020
35	hsa-miR-223-3p	54	20.3	19.8	22.1	0.010
36	hsa-miR-22-3p	54	22.4	21.6	24.1	0.010
37	hsa-miR-23a-3p	54	21.4	20.6	23.1	0.010
38	hsa-miR-24-3p	54	21.9	21	23.3	0.010
39	hsa-miR-26a-5p	53	23.4	22.5	25	0.010
40	hsa-miR-27a-3p	54	22	21.4	23	0.020
41	hsa-miR-29a-3p	52	24.1	23.3	25.9	< 0.001
42	hsa-miR-29a-5p	34	31.1	30.7	31.8	< 0.001
43	hsa-miR-29b-3p	49	26	25.1	28.2	0.010
44	hsa-miR-29c-3p	49	24.2	23.5	25.4	0.050
45	hsa-miR-30a-5p	48	29.3	28.4	30	0.010
46	hsa-miR-30b-5p	53	25.6	24.4	26.9	0.010
47	hsa-miR-30c-5p	51	23.8	23.1	25.9	0.010
48	hsa-miR-30d-5p	48	26.4	25.4	27.3	0.040
49	hsa-miR-30e-5p	51	25.2	24.2	26.5	< 0.001
50	hsa-miR-320a	54	23.7	23.2	25	0.040
51	hsa-miR-330-5p	19	27.9	27.8	31.4	< 0.001
52	hsa-miR-335-5p	48	28.4	27	29.8	0.010
53	hsa-miR-361-5p	50	28.1	27.4	28.9	0.020
54	hsa-miR-423-3p	53	25.3	24.7	27.6	0.010

Table 3. Continued

No.	MiRNA	No. of patients in each testing	Total (n = 54)	*JAK2* V617F mutation (n = 35)	Other genotypes (n = 19)	*p* value
			miRNA expression levels (median)			
55	hsa-miR-423-5p	54	25.5	24.9	26.8	0.020
56	hsa-miR-425-5p	50	24.8	24.2	26.5	0.010
57	hsa-miR-429	53	28.7	28.4	29.2	0.040
58	hsa-miR-451a	54	19	18.5	19.9	0.020
59	hsa-miR-484	53	24.3	23.6	26.4	0.010
60	hsa-miR-625-5p	40	28.1	27.8	29.3	0.030
61	hsa-miR-652-3p	52	25.2	24.6	27.7	0.020
62	hsa-miR-92a-3p	54	21.5	20.8	22.3	0.040
63	hsa-miR-93-5p	51	24.6	23.7	25.2	0.010
64	hsa-miR-940	40	27.8	27.6	31	< 0.001

miR-141-3p, hsa-miR-144-3p, hsa-miR-198, hsa-miR-34a-5p, hsa-miR-589-5p and hsa-miR-601) had significant differentially expressed levels (data not shown).

4. Discussion

Deregulated miRNAs have been reported to play a role in hematopoietic malignancies including MPNs, and can function either as oncogenes or as tumor suppressor genes. Some studies have evaluated deregulated miRNA profiles in MPNs using primary patient samples including peripheral blood mononuclear cells, granulocytes, platelets and bone marrow cells.[25,30–34] In this study, we have successfully analyzed the plasma miRNA expression profiles in patients with ET using a novel multi-gene detection platform called PanelChip™ Analysis System (Quark Biosciences, Taiwan) which is a customized miRNA panel including 165 miRNAs called mirSCAN™ PanCancer Chips 1 & 2. Cell-free miRNAs have been found to be remarkably stable in plasma, making them an invaluable source for clinical testing because blood samples can be collected quite easily from patients and healthy individuals. In addition, the PanelChip™ Analysis System has been demonstrated to be capable of separating healthy subjects and patients with oral cavity squamous cell carcinoma using plasma miRNA expression profiles.[26] In this study, our results provided further evidence that the PanelChip™ Analysis System is a reliable platform for the testing of plasma miRNA profiles in patients with hematologic neoplasm.

In our study, 40 differentially expressed miRNAs were identified in ET patients when compared to healthy control. In addition, ET patients were clustered to either low or high expression groups according to the expression profiles of these 40 miRNAs. Interestingly, all healthy adults clustered in the high expression group. Furthermore, the expression pattern of *JAK2*-mutated ET patients was different from *CALR*-mutated and triple-negative ET patients suggesting that different driver mutations in ET patient might affect the expression profiles of these 40 miRNAs. However, the expression levels of these 40 miRNAs were not statistically correlated with other clinical features. Our study is limited by small number of ET patients and larger study is warranted to evaluate the correlation of miRNA expression profiles with clinical features in ET.

Mutations in *JAK2*, *CALR*, and *MPL* all activate the JAK-STAT signaling pathway in MPNs including ET. The JAK-STAT signaling pathway not only play an important role in the molecular pathogenesis but also has therapeutic implications in MPNs. However, the phenotypic variation in MPNs could not be satisfactorily explained by the 3 driver mutations. It has been proposed that disease-specific miRNA deregulations could contribute to the specific phenotypes of MPNs. We have used DIANA-mirPath to analyze the putative target genes of the 40 differentially expressed miRNAs discovered by PanelChip™ Analysis System. We found that the putative target genes were enriched in signaling pathway including PI3K-Akt signaling pathway, Ras signaling pathway, Rap1 signaling pathway, cAMP signaling pathway, and cGMP-PKG signaling pathway. It is possible that these signaling pathway may involve in the pathogenesis, clinical phenotype, and prognosis in MPNs especially ET.

We have also found 5 differentially expressed miRNAs in elderly ET patients. The biological roles of these 5 miRNAs in elderly ET patients are not yet clear at this time. It is noteworthy that the expression levels of hsa-miR-451a were associated with genotypes in ET patients. MiR-451a has been found to correlate with cancer persistence and recurrence.[35] On the other hand, miR-451a could decrease the expression of multi drug resistance protein 1 and induced abrogation of chemoresistancein FLT3-ITD-positive acute myeloid leukemia cells.[36] In view of these conflicting findings, the role of miR-451a in ET deserves further investigation. Although the expression levels of the above-mentioned 5 miRNAs did not correlate with survival, elderly ET patients had significantly inferior overall survival compared with young patients. Current treatment guidelines recommend the use of cytoreductive therapy such as hydroxyurea and aspirin in elderly ET patients due to a higher risk of thrombotic complications in these patients.[37]

5. Conclusions

In conclusion, the use of PanelChip™ platform in ET patients was feasible, and distinct plasma miRNA profiles correlated with age and genotypes in ET patients. A larger cohort is warranted to validate our findings in ET patients. Future studies on other MPN subtypes such as PV and PMF may provide further insight into the role of miRNAs in the pathogenesis and prognosis of these diseases.

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Supplementary materials

Supplementary materials for this article can be found at

http://www.sgecm.org.tw/ijge/journal/view.asp?id=27.

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