Microarray analysis of circulating microRNAs in familial Mediterranean fever

著者	Wada Taizo, Toma Tomoko, Matsuda Yusuke, Yachie Akihiro, Itami Saori, Taguchi Y-h, Murakami Yoshiki
journal or publication title	Modern Rheumatology
volume	27
number	6
page range	1040-1046
year	2017-11-02
URL	http://doi.org/10.24517/00042075

doi: 10.1080/14397595.2017.1285845

Original Article

Microarray analysis of circulating microRNAs in familial Mediterranean fever

Taizo Wada,[1*] Tomoko Toma,[1] Yusuke Matsuda,[1] Akihiro Yachie,[1] Saori Itami,[2] Y-h Taguchi,[3] and Yoshiki Murakami[2]

Institutional affiliations:
[1]Department of Pediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan; [2]Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan; and [3]Department of Physics, Chuo University, Tokyo, Japan.

Correspondence to: Taizo Wada, MD, PhD
Department of Pediatrics, School of Medicine,
Institute of Medical, Pharmaceutical and Health Sciences,
Kanazawa University
13-1 Takaramachi, Kanazawa 920-8641, Japan
Phone: +81-76-265-2313
Fax: +81-76-262-1866
E-mail: taizo@staff.kanazawa-u.ac.jp

Key words: autoinflammatory disease, familial Mediterranean fever, MEFV, microRNAs

Abstract

Objectives: Familial Mediterranean fever (FMF) is an autoinflammatory disease caused by mutations in *MEFV*. Mutations in exon 10 are associated with typical FMF phenotypes, whereas the pathogenic role of variants in exons 2 and 3 remains uncertain. Recent evidence suggests that circulating microRNAs are potentially useful biomarkers in several diseases. Therefore, their expression was assessed in FMF. ***Methods:*** The subjects were 24 patients with FMF who were between attacks: 8 with exon 10 mutations (group A), 8 with exon 3 mutations (group B), and 8 without exon 3 or 10 mutations (group C). We also investigated 8 cases of PFAPA as disease controls. Exosome-rich fractionated RNA was subjected to microRNA profiling by microarray. ***Results:*** Using the expression patterns of 26 microRNAs, we classified FMF (groups A, B, and C) and PFAPA with 78.1% accuracy. In FMF patients, groups A and B, A and C, and B and C were distinguished with 93.8, 87.5, and 100% accuracy using 24, 30, and 25 microRNA expression patterns, respectively. ***Conclusions:*** These findings suggest that expression patterns of circulating microRNAs differ among FMF subgroups based on *MEFV* mutations between FMF episodes. These patterns may serve as a useful biomarker for detecting subgroups of FMF.

Introduction

Familial Mediterranean fever (FMF) is an inherited autoinflammatory disease that is characterized by recurrent episodes of fever with serositis, synovitis, or skin rash [1]. FMF is caused by autosomal recessive mutations in the *MEFV* gene, which has 10 exons encoding a 781-amino acid protein called pyrin [1]. Pyrin is expressed in innate cells, including granulocytes, cytokine-activated monocytes, dendritic cells, and synovial and peritoneal fibroblasts. This protein appears to act as a pivotal regulator of inflammation and apoptosis, and mutated pyrin leads to aberrant production of interleukin-1β in FMF [2]. FMF was originally described in countries around the Mediterranean basin; however, the availability of genetic testing has broadened the clinical and ethnic spectrum of the disease. Pathogenic mutations of *MEFV* are concentrated in exon 10. Clinical diagnosis of FMF is based on the presence of short (12 hours to 3 days), recurrent episodes of fever with painful manifestations in the abdomen, chest, joints, or skin, with no discernible infectious cause [3]. The Tel Hashomer criteria are the most widely used to establish a diagnosis; however, milder cases without exon 10 mutations sometimes pose diagnostic problems in Western nations and in Japan [4].

MicroRNAs (miRNAs) are small, non-coding RNAs that are involved in post-transcriptional regulation of gene expression and play important roles in control of many biological processes, such as cellular development, differentiation, proliferation, apoptosis, and metabolism [5, 6]. These molecules can be detected in serum and plasma, and aberrant expression profiles of circulating miRNAs have been increasingly described in many pathological conditions, including cancer and autoimmune diseases [7, 8]. In this study, we analyzed the expression profiles of

circulating miRNAs in subgroups of FMF that were formed based on *MEFV* mutations, and we discuss the potential usefulness of these patterns as a novel biomarker in FMF.

Materials and Methods

Patients

The subjects were 24 patients affected with FMF (Table 1). A diagnosis of FMF was made on the basis of the Tel Hashomer criteria [9]. A typical FMF attack was defined as recurrent episodes lasting 12 hours to 3 days with fever of 38°C or higher. An attack was considered 'atypical' if it differed from the definition of a typical attack in only 1 or 2 of the following features: temperature less than 38°C, an attack duration longer or shorter than specified (but not shorter than 6 hours or longer than a week), no signs of peritonitis during an abdominal attack, localized abdominal attacks, or an unusual distribution of arthritis. The patients were divided into 3 subgroups based on *MEFV* mutations. Patients carrying exon 10 mutations, such as M694I, M680I, and V726A, were classified as group A. Patents without exon 10 mutations were classified into two groups based on exon 3 variants: those with P369S/R408Q were defined as group B, and those without exon 3 mutations as group C. Exon 2 variants such as E148Q were not used for classification because these mutations frequently occur in parallel with other mutations, including those in exon 10. There were no patients with systemic amyloidosis. Eight patients with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) were evaluated as disease controls. The diagnosis of PFAPA was determined clinically [10]. All patients were of Japanese ancestry, except for one FMF patient with M680I/V726A mutations. There was no consanguinity in any of the families. Blood samples were collected during a clinically stable, afebrile phase in all patients with FMF and PFAPA. Approval for the study was obtained from the Human Research Committee

of Kanazawa University Graduate School of Medical Science, and informed consent was provided according to the Declaration of Helsinki.

Mutation analysis

DNA was extracted from blood samples using a standard method. Direct sequencing of the *MEFV* gene was performed as described previously [11].

RNA preparation and miRNA microarray

Exosome-rich fractionated RNA was prepared using Exoquick (System Biosciences, CA, USA) [12]. Briefly, 900 µl of serum or plasma was mixed with 250 µl of Exoquick and incubated for 12 h at 4°C. The tubes were centrifuged at 1500 g for 30 min at room temperature and then supernatant was discarded. The pellet was dissolved in 200 µl of PBS with vigorous vortex. RNA was extracted using a miRNeasy mini kit (Qiagen, Hilden, Germany). Expression of miRNAs was assessed by microarray (Agilent human microRNA microarray release 14.0) [12]. Hybridization signals were detected with a DNA microarray scanner G205B (Agilent Technology) and the scanned images were analyzed using Agilent feature extraction software (v10.10.1.1). Raw data (gProcessedSignal) were normalized so that each expression level had a mean of zero and a sample variance of one. Principal component (PC) analysis (PCA), as well as categorical regression analysis to confirm significant differences between selected components, were performed [12-17]. Accuracy was assessed using leave-one-out cross-validation [18].

Statistical Analysis

PCA-based unsupervised feature extraction (FE) was used to identify miRNAs for discrimination [12-17]. The mathematical details are in the supplementary material. Briefly, in this methodology, in contrast to normal use of PCA, miRNAs (rather than samples) are embedded into low dimensional space. Then, PC scores are attributed to each miRNA. After identifying PC loadings attributed to samples for miRNA selection, outlier miRNAs along identified PCs with assumed multiple Gaussian distributions for PC scores (adjusted $P<0.01$) are selected. The selected miRNAs are further re-embedded into low dimensional space with PCA, and PC loadings are re-attributed to samples and used for discrimination between samples using linear discriminant analysis.

Results

Patient characteristics

Clinical and sequencing data for the patients are shown in Table 1. A heterozygous M694I mutation was found in 7 of the 8 patients in group A. The other patient had a compound heterozygous M680I/V726A mutation in exon 10 of *MEFV*. Consistent with a previous report [19], P369S and R408Q, both of which are located in exon 3, were found in *cis* in all 8 patients in group B, and were not detected in group A. The E148Q variant in exon 2 was found frequently in groups A (7/8) and B (6/8). Other variants, such as E84K in exon 1, L110P in exon 2, R202Q in exon 2, G304R in exon 2, and S503C in exon 5, were occasionally observed in each group. No *MEFV* variant was found in 4 of the 8 patients in group C. Five patients with PFAPA showed sequence variants in exon 2 or 3 of *MEFV*, but exon 10 mutations were not detected in any patients with PFAPA.

Typical FMF attacks were observed in all patients in group A and most patients in group C (7/8), whereas most patients in group B had atypical attacks, in line with previous findings [19]. All patients showed FMF symptoms and elevated levels of acute-phase reactants, including C-reactive protein (CRP), during attacks, regardless of *MEFV* mutations (data not shown), and all were well and had neither leukocytosis nor elevated levels of CRP between attacks (Table 1). No patient with PFAPA had a FMF attack, despite some carrying *MEFV* variants.

Unique expression patterns of miRNAs in FMF

To discriminate among the three FMF groups and the PFAPA controls, expression of 887 human miRNAs was assessed by microarray. We chose 26 miRNA

expression patterns for classification among these four groups by PCA (Fig. 1 and Table 3). The accuracy of diagnosis was 68.75% by leave-one-out cross-validation (Table 2). Thirty-one miRNA expression patterns according to subtype are shown in Fig. 2. Categorical regression analysis showed that each selected PC was significantly distinct among the four groups (Fig. 2).

Next, we attempted to discriminate between two arbitrary groups. The accuracies of diagnoses were 78.1% between all FMF cases and PFAPA cases using 26 miRNA expression patterns, 93.8% between FMF groups A and B using 24 miRNA expression patterns, 87.5% between FMF groups A and C using 30 miRNA expression patterns, 100% between FMF groups B and C using 25 miRNA expression patterns, 68.8% between FMF group A and PFAPA using 26 miRNA expression patterns, 81.3% between FMF group B and PFAPA using 20 miRNA expression patterns, and 75% between FMF group C and PFAPA using 21 miRNA expression patterns (Tables 2 and 3).

Discussion

Diagnosis of FMF is still based on clinical criteria, despite improved recognition of a number of *MEFV* gene variants. More than 310 *MEFV* sequence variants are now listed in the Infevers online database (http://fmf.igh.cnrs.fr/ISSAID/infevers/). Most disease-associated mutations are missense substitutions clustered in exon 10, such as M694V, V726A, M680I, and M694I [4]. Exons 2 and 3 include various missense changes that are considered to be benign polymorphisms or of unknown pathogenic significance. On the other hand, approximately 30% of patients with a typical clinical presentation of FMF have only one demonstrable mutation, although FMF is an autosomal recessive disease [1], and no mutation is found in 10% of patients [1]. Recent nationwide surveys in patients with FMF in Japan have indicated allele frequencies of M694I, R408Q, P369S, G304R, R202Q, E148Q, and E84K of 21.7%, 5.9%, 6.1%, 1.6%, 1.8%, 35.4%, and 2.4%, respectively, and only 3.2% of patients were homozygous for exon 10 mutations or were compound heterozygotes carrying two different exon 10 mutations [20, 21]. These Japanese data further support the consensus that genetic testing can support diagnosis of FMF, but not necessarily exclude it [4]. Thus, interpretation of genetic testing of *MEFV* is complicated. In addition to the genotypic variability, FMF is phenotypically variable [22]. Patients with exon 10 mutations generally have a typical FMF phenotype, including a favorable response to colchicine, whereas clinical phenotypes of individuals carrying *MEFV* variants in exon 2 or 3 range from the typical FMF phenotype to no overt signs of disease [23]. Therefore, a novel biomarker to distinguish subgroups of FMF is highly desirable.

Accumulating evidence has suggested potential roles of expression profiles of circulating miRNAs as diagnostic and prognostic biomarkers in several human diseases, including inflammatory disorders [8]. miRNAs regulate gene expression at a posttranscriptional level by degrading mRNA molecules or blocking their translation, and thus miRNAs may contribute to disease pathogenesis. In fact, characteristic miRNA expression patterns have been described in autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and Sjögren syndrome [8, 24, 25], as well as in autoinflammatory diseases, including tumor necrosis factor receptor-associated periodic syndrome and systemic onset juvenile idiopathic arthritis [26, 27]. Therefore, we examined if expression levels of circulating miRNAs can distinguish among three subgroups of FMF: typical cases carrying exon 10 mutations; cases without exon 10 mutations but with the P369S/R408Q variant in exon 3, which is infrequently associated with a typical phenotype; and cases with a typical phenotype despite having no exon 10 mutation.

In this study, we were able to show that three subgroups of FMF and a disease control (PFAPA) were clearly distinguishable from each other by leave-one-out cross-validation using selected miRNA expression profiles. We did not find a particular miRNA with the capability of classifying the FMF subgroups, indicating no evidence for preferential expression of a specific miRNA in each subgroup. However, an expression pattern of multiple miRNAs represents a novel and valuable biomarker to distinguish the subgroups. In particular, FMF patients with an exon 10 mutation in *MEFV* (group A) were clearly distinguishable from those without an exon 10 mutation (groups B or C) with high accuracy (93.8% and 87.5%, respectively). For patients exhibiting a typical FMF phenotype, it is clinically difficult to differentiate between those with and without exon 10 mutations, and there are also no current

biomarkers that differentiate these cases. Most patients in group C showed a typical FMF phenotype, and thus our results indicate that expression profiles of circulating miRNAs are useful biomarkers to differentiate between subgroups of FMF patients with a typical phenotype with or without an exon 10 mutation. Conversely, there are no biomarkers that distinguish among subgroups of FMF patients without exon 10 mutations. The pathogenic significance of most variants of exons 2 and 3 remains debatable, but our results distinguished group B from group C with 100% accuracy. However, it is unclear whether the different expression pattern of circulating miRNAs was a consequence of the presence or absence of the P369S/R408Q variant because patients in group B tended to exhibit an atypical FMF phenotype, unlike those in group C. The disease control group (PFAPA) was well differentiated from all FMF patients and from each FMF subgroup. Because of possible age-dependent changes in some circulating miRNAs [28] and younger age in patients with PFAPA compared to those with FMF, it is necessary to confirm this difference in age-matched samples. Taken together, this approach may be valuable for distinguishing among subgroups of FMF.

Circulating miRNAs examined in this study were prepared from serum or plasma between FMF attacks, at a time when all patients were afebrile and felt completely well. White blood cell counts, acute phase reactants, and serum cytokines usually normalize during this clinically stable, afebrile phase in patients with FMF, regardless of genotypic and phenotypic variations [29]. The characteristic expression patterns of circulating miRNAs in FMF patients between attacks may suggest the presence of persistent subclinical inflammation, which could lead to the next FMF attack. Further studies are required to assess whether altered expression profiles of miRNAs have a role in the pathogenesis of FMF. There is also a need to evaluate

circulating miRNAs during a FMF attack and miRNAs from cells expressing the *MEFV* product, pyrin, such as monocytes. Other limitations of this study include the small number of patients, the ethnically homogeneous samples, and the absence of quantitative analysis of miRNAs. In addition, this study did not take into account from which circulating miRNA were generated. While plasma and serum generally have similar miRNA expression patterns, considerable differences in their miRNA content could occur due to the presence or absence of factors including anticoagulants, platelets, and a clotting process [30, 31]. Moreover, additional disease controls including other autoinflammatory diseases and infectious diseases are required to assess whether expression patterns of miRNAs represent a biomarker for diagnosis of FMF.

Biological functions of most of the selected miRNAs for classifying FMF subgroups remain to be determined. However, miR-320 is known to have a wide range of biological effects [32, 33]. In particular, miR-320 regulates the nucleotide-binding oligomerization domain 2 (NOD2), an intracellular pathogen recognition sensor, whose deficiency leads to upregulation of the activity of NF-kB and transcription of downstream proinflammatory cytokines [34]. Higher expression of miR-320 in group A compared to other groups may support a possible role of miR-320 in the compensatory inhibition of chronic inflammation caused by exon 10 mutations of *MEFV*. Further studies are necessary to assess putative pathways and target genes of the selected miRNAs.

In summary, our results show that expression patterns of circulating miRNAs in patients between FMF episodes differ among FMF subgroups based on *MEFV* mutations. These patterns may serve as useful biomarkers and diagnostic tools for identifying subgroups of FMF.

Acknowledgments

We thank Ms. Harumi Matsukawa and Ms. Kaori Ishihara for their excellent technical assistance.

Conflict of interest

None.

References

1. Padeh S, Berkun Y. Familial Mediterranean fever. Curr Opin Rheumatol. 2016;28:523-9.
2. Manukyan G, Aminov R. Update on Pyrin Functions and Mechanisms of Familial Mediterranean Fever. Front Microbiol. 2016;7:456.
3. Berkun Y, Eisenstein EM. Diagnostic criteria of familial Mediterranean fever. Autoimmun Rev. 2014;13:388-90.
4. Giancane G, Ter Haar NM, Wulffraat N, Vastert SJ, Barron K, Hentgen V, et al. Evidence-based recommendations for genetic diagnosis of familial Mediterranean fever. Ann Rheum Dis. 2015;74:635-41.
5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-97.
6. Ambros V. The functions of animal microRNAs. Nature. 2004;431:350-5.
7. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 2010;101:2087-92.
8. Singh RP, Massachi I, Manickavel S, Singh S, Rao NP, Hasan S, et al. The role of miRNA in inflammation and autoimmunity. Autoimmun Rev. 2013;12:1160-5.
9. Livneh A, Langevitz P, Zemer D, Zaks N, Kees S, Lidar T, et al. Criteria for the diagnosis of familial Mediterranean fever. Arthritis Rheum. 1997;40:1879-85.
10. Vigo G, Zulian F. Periodic fevers with aphthous stomatitis, pharyngitis, and adenitis (PFAPA). Autoimmun Rev. 2012;12:52-5.
11. Tone Y, Toma T, Toga A, Sakakibara Y, Wada T, Yabe M, et al. Enhanced exon 2 skipping caused by c.910G>A variant and alternative splicing of MEFV genes

in two independent cases of familial Mediterranean fever. Mod Rheumatol. 2012;22:45-51.

12. Murakami Y, Toyoda H, Tanahashi T, Tanaka J, Kumada T, Yoshioka Y, et al. Comprehensive miRNA expression analysis in peripheral blood can diagnose liver disease. PLoS One. 2012;7:e48366.
13. Taguchi YH, Murakami Y. Principal component analysis based feature extraction approach to identify circulating microRNA biomarkers. PLoS One. 2013;8:e66714.
14. Murakami Y, Kubo S, Tamori A, Itami S, Kawamura E, Iwaisako K, et al. Comprehensive analysis of transcriptome and metabolome analysis in Intrahepatic Cholangiocarcinoma and Hepatocellular Carcinoma. Sci Rep. 2015;5:16294.
15. Taguchi YH, Murakami Y. Universal disease biomarker: can a fixed set of blood microRNAs diagnose multiple diseases? BMC Res Notes. 2014;7:581.
16. Tamori A, Murakami Y, Kubo S, Itami S, Uchida-Kobayashi S, Morikawa H, et al. MicroRNA expression in hepatocellular carcinoma after the eradication of chronic hepatitis virus C infection using interferon therapy. Hepatol Res. 2016;46:E26-35.
17. Murakami Y, Tanahashi T, Okada R, Toyoda H, Kumada T, Enomoto M, et al. Comparison of hepatocellular carcinoma miRNA expression profiling as evaluated by next generation sequencing and microarray. PLoS One. 2014;9(9):e106314.
18. Dupuy A, Simon RM. Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. J Natl Cancer Inst. 2007;99:147-57.

19. Ryan JG, Masters SL, Booty MG, Habal N, Alexander JD, Barham BK, et al. Clinical features and functional significance of the P369S/R408Q variant in pyrin, the familial Mediterranean fever protein. Ann Rheum Dis. 2010;69:1383-8.
20. Migita K, Uehara R, Nakamura Y, Yasunami M, Tsuchiya-Suzuki A, Yazaki M, et al. Familial Mediterranean fever in Japan. Medicine (Baltimore). 2012;91:337-43.
21. Migita K, Agematsu K, Yazaki M, Nonaka F, Nakamura A, Toma T, et al. Familial Mediterranean fever: genotype-phenotype correlations in Japanese patients. Medicine (Baltimore). 2014;93:158-64.
22. Demirkaya E, Acikel C, Hashkes P, Gattorno M, Gul A, Ozdogan H, et al. Development and initial validation of international severity scoring system for familial Mediterranean fever (ISSF). Ann Rheum Dis. 2016;75:1051-6.
23. Moradian MM, Sarkisian T, Ajrapetyan H, Avanesian N. Genotype-phenotype studies in a large cohort of Armenian patients with familial Mediterranean fever suggest clinical disease with heterozygous MEFV mutations. J Hum Genet. 2010;55:389-93.
24. Perez-Hernandez J, Forner MJ, Pinto C, Chaves FJ, Cortes R, Redon J. Increased Urinary Exosomal MicroRNAs in Patients with Systemic Lupus Erythematosus. PLoS One. 2015;10:e0138618.
25. Wu M, Barnard J, Kundu S, McCrae KR. A novel pathway of cellular activation mediated by antiphospholipid antibody-induced extracellular vesicles. J Thromb Haemost. 2015;13:1928-40.
26. Lucherini OM, Obici L, Ferracin M, Fulci V, McDermott MF, Merlini G, et al. First report of circulating microRNAs in tumour necrosis factor receptor-associated periodic syndrome (TRAPS). PLoS One. 2013;8:e73443.

27. Kamiya Y, Kawada J, Kawano Y, Torii Y, Kawabe S, Iwata N, et al. Serum microRNAs as Potential Biomarkers of Juvenile Idiopathic Arthritis. Clin Rheumatol. 2015;34:1705-12.
28. Noren Hooten N, Fitzpatrick M, Wood WH, 3rd, De S, Ejiogu N, Zhang Y, et al. Age-related changes in microRNA levels in serum. Aging (Albany NY). 2013;5:725-40.
29. Koga T, Migita K, Sato S, Umeda M, Nonaka F, Kawashiri SY, et al. Multiple Serum Cytokine Profiling to Identify Combinational Diagnostic Biomarkers in Attacks of Familial Mediterranean Fever. Medicine (Baltimore). 2016;95:e3449.
30. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. J Cell Mol Med. 2014;18:371-90.
31. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. PLoS One. 2012;7:e41561.
32. Bronisz A, Godlewski J, Wallace JA, Merchant AS, Nowicki MO, Mathsyaraja H, et al. Reprogramming of the tumour microenvironment by stromal PTEN-regulated miR-320. Nat Cell Biol. 2011;14:159-67.
33. Tao L, Bei Y, Zhou Y, Xiao J, Li X. Non-coding RNAs in cardiac regeneration. Oncotarget. 2015;6:42613-22.
34. Pierdomenico M, Cesi V, Cucchiara S, Vitali R, Prete E, Costanzo M, et al. NOD2 Is Regulated By Mir-320 in Physiological Conditions but this Control Is Altered in Inflamed Tissues of Patients with Inflammatory Bowel Disease. Inflamm Bowel Dis. 2016;22:315-26.

Supporting Information

S1 File. Details of PCA based unsupervised FE.

Table 1. Patient characteristics.

	FMF patients			PFAPA
	with exon 10 mutations (Group A)	with exon 3 mutations (Group B)	without exon 3 or 10 mutations (Group C)	patients
n =	8	8	8	8
Age (y)[a]	31.6 ± 20.2	21.6 ± 10.2	16.1 ± 6.7	10.0 ± 9.1
Male/Female	2/6	3/5	3/5	3/5
Typical FMF attacks	8/8	2/8	7/8	0/8
Atypical FMF attacks	0/8	6/8	1/8	0/8
WBC*	7410 ± 1270	6820 ± 3550	5690 ± 1230	7330 ± 2190
CRP*	0.08 ± 0.07	0.57 ± 0.84	0.36 ± 0.28	0.35 ± 0.79
MEFV mutations				
M694I/E148Q	4/8			
M694I/L110P/E148Q	3/8			
M680I/V726A	1/8			
P369S/R408Q		2/8		
P369S/R408Q/E148Q		4/8		1/8
P369S/R408Q/E148Q/R202Q			1/8	
P369S/R408Q/E148Q/G304R			1/8	
E84K/wt			1/8	
L110P/E148Q			1/8	2/8
E148Q/wt				2/8
R202Q/wt			1/8	
S503C/wt			1/8	
wt/wt			4/8	3/8

[a] Data at sample collection. WBC, white blood cells; CRP, C-reactive protein; wt, wild type.

Table 2. List of miRNAs that were used for discrimination.

miRNA	FMF-A/ FMF-B/ FMF-C/ PFAPA	FMF (A, B, C)/ PFAPA	FMF-A/ FMF-B	FMF-A/ FMF-C	FMF-B/ FMF-C	FMF-A/ PFAPA	FMF-B/ PFAPA	FMF-C/ PFAPA
hsa-miR-1225-5p	*	*	*	*		*		
hsa-miR-1915-3p					*			
hsa-miR-2861	*	*		*	*	*	*	*
hsa-miR-320b	*	*	*	*		*		
hsa-miR-320c	*	*	*	*	*	*	*	*
hsa-miR-320d	*	*	*	*	*	*	*	
hsa-miR-320e	*	*	*	*	*	*	*	
hsa-miR-3665				*	*			
hsa-miR-3960	*	*	*	*	*	*	*	*
hsa-miR-4270			*	*		*		
hsa-miR-4281	*	*	*	*	*	*	*	*
hsa-miR-4485-5p	*	*	*	*		*		
hsa-miR-4516	*	*	*	*	*	*	*	*
hsa-miR-451a	*	*	*	*	*	*	*	*
hsa-miR-6087	*	*	*	*	*	*	*	*
hsa-miR-6088	*	*	*	*	*	*	*	*
hsa-miR-6089	*	*	*	*	*	*	*	*
hsa-miR-6090	*	*	*	*	*	*	*	*
hsa-miR-6125	*	*	*	*	*	*	*	*
hsa-miR-638	*	*		*	*			*
hsa-miR-6510-5p	*	*	*	*		*	*	*
hsa-miR-6800-5p	*	*	*	*	*	*	*	*
hsa-miR-6821-5p				*	*			*
hsa-miR-6869-5p	*	*	*	*	*	*		*
hsa-miR-6891-5p	*	*	*	*	*	*	*	*
hsa-miR-7107-5p	*	*	*	*	*	*	*	*
hsa-miR-7110-5p			*	*		*		
hsa-miR-7150	*	*	*	*	*	*	*	*
hsa-miR-7704	*	*		*	*			*
hsa-miR-7975	*	*	*	*	*	*	*	
hsa-miR-8069	*	*		*	*	*	*	*

FMF-A, FMF group A; FMF-B, FMF group B; FMF-C, FMF group C.

Table 3. Classification of 3 subgroups of FMF and PFAPA by leave-one-out cross-validation.

a. Classification among 3 subgroups of FMF and PFAPA

	FMF-A	FMF-B	FMF-C	PFAPA	
FMF-A	6	1	0	0	
FMF-B	2	7	0	0	
FMF-C	0	0	5	4	accuracy
PFAPA	0	0	3	4	68.8%

b. Classification between pairs of subgroups

	FMF (A, B, and C)	PFAPA	
FMF (A, B, and C)	18	1	accuracy
PFAPA	6	7	78.1%

	FMF-A	FMF-B	
FMF-A	7	0	accuracy
FMF-B	1	8	93.8%

	FMF-A	FMF-C	
FMF-A	7	1	accuracy
FMF-C	1	7	87.5%

	FMF-B	FMF-C	
FMF-B	8	0	accuracy
FMF-C	0	8	100%

	FMF-A	PFAPA	
FMF-A	5	2	accuracy
PFAPA	3	6	68.8%

	FMF-B	PFAPA	
FMF-B	7	2	accuracy
PFAPA	1	6	81.3%

	FMF-C	PFAPA	
FMF-C	5	1	accuracy
PFAPA	3	7	75.0%

Figure Legends

Fig. 1. Principal component analysis of miRNA expression analysis.

The horizontal and vertical axes are the first and second principal components, respectively.

Fig. 2. Boxplots for selected miRNAs.

Thirty-one miRNA expression patterns were used for discrimination among 3 subgroups of FMF and PFAPA. The vertical axis shows the relative expression level of miRNA. Dots are the relative expression level in individuals. P-values were assessed by categorical regression analysis.

Figure 1

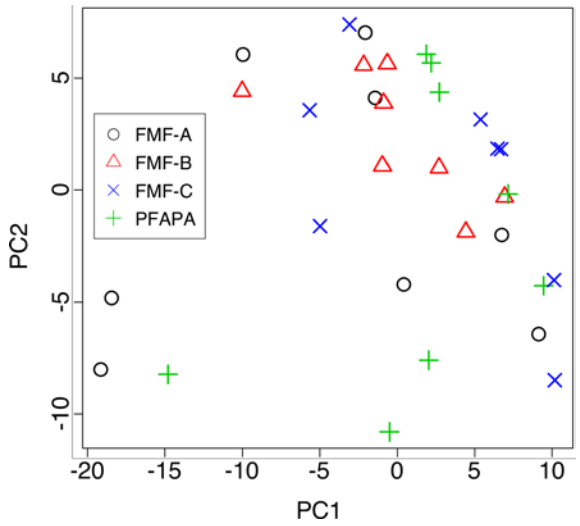
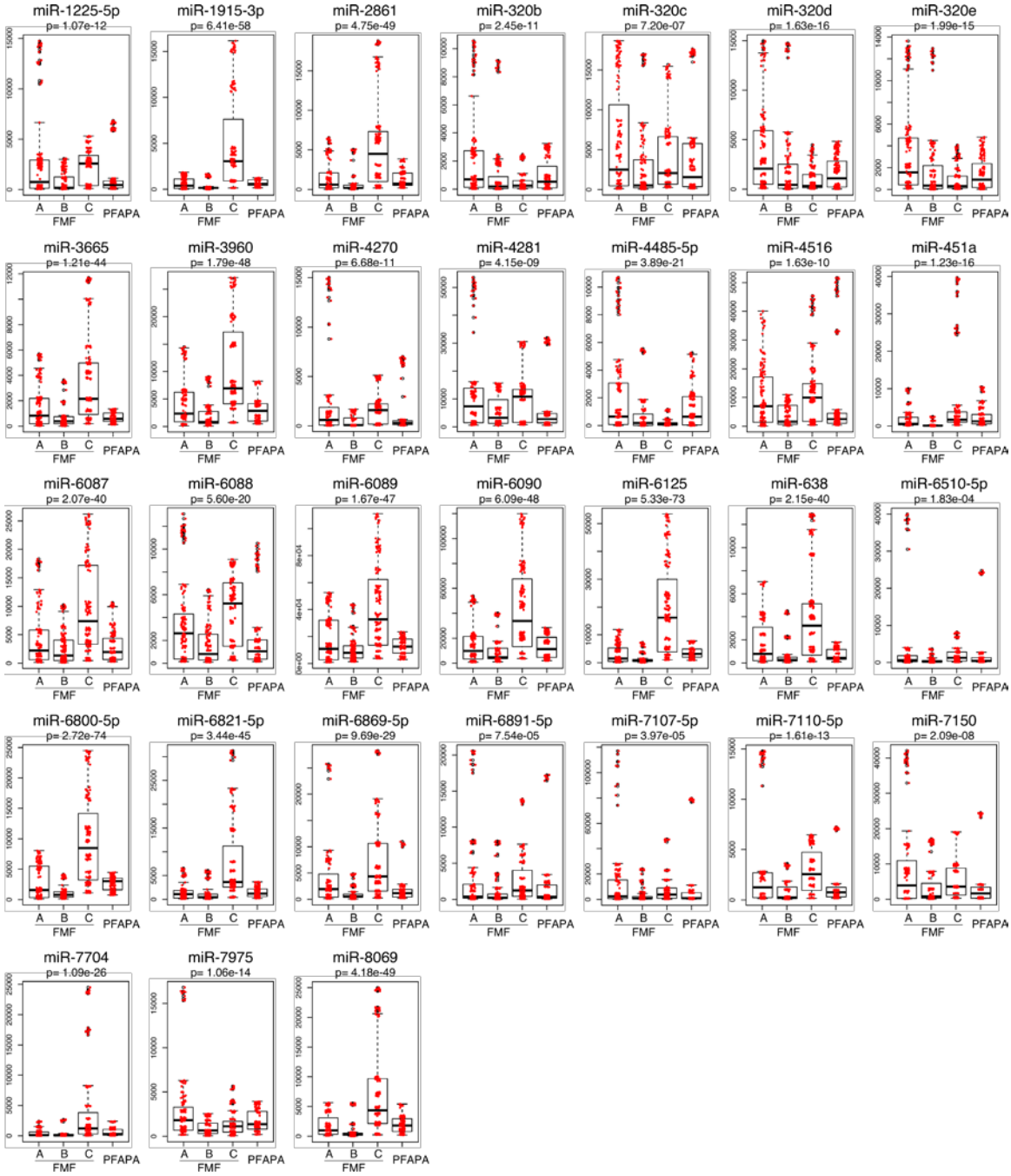


Figure 2


miR-1225-5p
p= 1.07e-12
miR-1915-3p
p= 6.41e-58
miR-2861
p= 4.75e-49
miR-320b
p= 2.45e-11
miR-320c
p= 7.20e-07
miR-320d
p= 1.63e-16
miR-320e
p= 1.99e-15
miR-3665
p= 1.21e-44
miR-3960
p= 1.79e-48
miR-4270
p= 6.68e-11
miR-4281
p= 4.15e-09
miR-4485-5p
p= 3.89e-21
miR-4516
p= 1.63e-10
miR-451a
p= 1.23e-16
miR-6087
p= 2.07e-40
miR-6088
p= 5.60e-20
miR-6089
p= 1.67e-47
miR-6090
p= 6.09e-48
miR-6125
p= 5.33e-73
miR-638
p= 2.15e-40
miR-6510-5p
p= 1.83e-04
miR-6800-5p
p= 2.72e-74
miR-6821-5p
p= 3.44e-45
miR-6869-5p
p= 9.69e-29
miR-6891-5p
p= 7.54e-05
miR-7107-5p
p= 3.97e-05
miR-7110-5p
p= 1.61e-13
miR-7150
p= 2.09e-08
miR-7704
p= 1.09e-26
miR-7975
p= 1.06e-14
miR-8069
p= 4.18e-49
A
B
C
FMF
PFAPA