CLINICAL TRIAL

JBMR®

A Signature of Circulating miRNAs Associated With Fibrous Dysplasia of Bone: the mirDys Study

Mélanie A Legrand,[1,2] Marjorie Millet,[2] Blandine Merle,[2] Jean-Charles Rousseau,[2] Anaelle Hemmendinger,[3] Evelyne Gineyts,[2] Elisabeth Sornay-Rendu,[2] Pawel Szulc,[2] Olivier Borel,[2] Martine Croset,[2] and Roland Chapurlat[1,2]

[1]Department of Rheumatology, Edouard Herriot University Hospital, Lyon, France
[2]INSERM UMR 1033, Université de Lyon, Lyon, France
[3]The Lymphoma Academic Research Organization (LYSARC), Pierre-Bénite, France

ABSTRACT

Fibrous dysplasia (FD) is a rare bone disease caused by activating mutations of *GNAS* encoding the Gsα protein, enhancing cyclic adenosine monophosphate (cAMP) production by overstimulation of adenylyl cyclase and impairing osteoblastic differentiation. The clinical presentation ranges from asymptomatic to polyostotic forms with severe disability, explained by the mosaic distribution of the GNAS mutation. Physicians have to deal with the gap of knowledge in FD pathogenesis, the absence of prognostic markers and the lack of specific treatment. The identification of specific biomarkers for FD is an important step to improve the clinical and therapeutic approaches. An epigenetic regulation driven by microRNAs (miRNAs), known as promising biomarkers in bone disease, could be involved in FD. We have sought circulating miRNAs that are differentially expressed in FD patients compared to controls and would reflect dysregulations of osteogenesis-related genes and bone disorder. The global miRNA profiling was performed using Next Generation Sequencing in patient serum collected from a discovery cohort of 20 patients (10 polyostotic and 10 monostotic) and 10 controls. From these, we selected 19 miRNAs for a miRNA validation phase from serum of 82 patients and 82 controls, using real-time qPCR. Discovery screening identified 111 miRNAs differentially expressed in patient serum, after adjusting for the false discovery rate (FDR). Among the 82 patients, 55% were polyostotic, and 73% were women with a mean age of 42 years. Six miRNAs (miR-25-3p, miR-93-5p, miR-182-5p, miR-324-5p, miR-363-3p, and miR-451a) were significantly overexpressed in serum, with FDR <0.05. The expression level of these six miRNAs was not associated with the FD severity. In conclusion, we identified a signature of circulating miRNAs associated with FD. These miRNAs are potential negative regulators of gene expression in bone cell progenitors, suggesting their activity in FD by interfering with osteoblastic and osteoclastic differentiation to impair bone mineralization and remodeling processes. © 2020 American Society for Bone and Mineral Research.

KEY WORDS: MCCUNE-ALBRIGHT SYNDROME; FIBROUS DYSPLASIA OF BONE; GNAS; EPIGENETIC; MICRORNAS

Introduction

Fibrous dysplasia of bone (FD) is a rare congenital bone disease, characterized by bone pain, bone deformities and fracture, involving one or several bones.[1] FD is caused by postzygotic activating mutation of GNAS, coding for the α-subunit of a G-protein (Gs), that results in the excess of cyclic adenosine monophosphate (cAMP) production.[2] The mutation-induced postzygotic events occurring in pluripotent stem cells at various stages of development lead to phenotypic variability and a broad spectrum of skeletal and extraskeletal mosaic disorders.[3] At the level of skeletal progenitors, this mutation is responsible for a defect in osteoblast differentiation and an increase in osteoblastic proliferation.[4] Sick osteoblasts produce abnormal fibrous bone matrix, characteristic of FD lesions. There is also an excess of osteoclastogenesis in bone lesions and normal bone, due to the secretion of IL6 and RANKL by the mutated cells.[5,6] Among patients, the clinical presentation of FD is extremely variable, from asymptomatic forms to polyostotic forms with severe disability, explained by the mosaic distribution of the GNAS mutation. Because of the lack of predictive markers of the severity and evolution of this disease, the prognosis remains challenging.

Patients with polyostotic FD or renal phosphate wasting have an higher risk of fractures.[7] A scintigraphy skeletal burden score based on the initial spread of the disease has been developed.

Received in original form February 1, 2020; revised form May 5, 2020; accepted June 7, 2020. Accepted manuscript online June 11, 2020.
Address correspondence to: Roland Chapurlat, MD, PhD, Edouard Herriot Hospital - Pav. F, 5 place d'Arsonval, 69003, Lyon, France. E-mail: roland.chapurlat@inserm.fr
Additional Supporting Information may be found in the online version of this article.
Public clinical trial registration: http://clinicaltrials.gov/show/NCT03838991. Epigenetic Regulation of Activity and Severity of Fibrous Dysplasia in Bone: mirDYS Study.
Journal of Bone and Mineral Research, Vol. 35, No. 10, October 2020, pp 1881–1892.
DOI: 10.1002/jbmr.4111

This tool is able to predict functional outcome but is not proposed as a monitoring tool.[8] Recently, higher levels of serum periostin, an extracellular matrix protein, were found in the polyostotic form and those with prevalent fracture, but it remains unclear whether this marker can predict incident fracture.[9]

It is presently unknown why patients sharing the same genotype are so unequal as to disease presentation and phenotype. The activating mutation of *GNAS* might induce underlying epigenetic regulations, especially in skeleton stem cells, which could explain heterogeneity of phenotype and prognosis in FD. MicroRNAs (miRNAs) are small noncoding RNAs of 22 to 28 nucleotides in length and are key epigenetic regulators of many physiological and pathophysiological processes by regulating gene expression.[10] By binding to their target genes, they can promote their degradation or inhibit their translation, resulting in repression of gene expression.[11,12] miRNAs control bone development in embryogenesis and bone homeostasis through their activity in osteoblastogenesis and osteoclastogenesis.[13–17] Because of their remarkable physicochemical stability and their role in osteogenesis, circulating miRNAs appear as promising noninvasive biomarkers in bone disease. They have been studied in various bone pathologies (eg, bone metastasis,[18–20] osteoporosis[21–24]) but they have never been studied in FD. miRNAs could regulate pathophysiological pathways of FD and/or they also could be released from altered bone FD tissue in blood. Thus, miRNAs could be biomarkers of prognosis and also point to new pathophysiological mechanisms in this disease.

We conducted a case-control study to assess whether some miRNAs released in blood could be dysregulated in FD patients compared with controls, whether this dysregulation differs depending on the disease severity, and we also investigated their association to new pathophysiological pathways.

Patients and Methods

Recruitment of patients and control subjects

Patients

For the screening phase, we recruited 10 patients with the monostotic form and 10 patients with the polyostotic form of FD. Patients were included in the national reference center of fibrous dysplasia of bone in Edouard Herriot Hospital (Lyon, France), from January to March 2019. The recruitment was conducted either during a hospitalization or during a mere consultation. The inclusion criteria were as follows: patient age ≥18 years, with FD diagnosed by an expert rheumatologist. Patients were excluded if they were age <18 years, with physical conditions known to interfere with bone turnover (other bone diseases: Paget, osteogenesis imperfecta, osteoporosis; inflammatory rheumatic diseases; cancer or bone metastases; long-term glucocorticoids treatment; intestinal malabsorption; severe renal impairment; or hyperthyroidism), pregnant, or had a severe psychiatric condition impeding consent, lack of consent, or refusal of participation. Written informed consent was obtained from each participant and the study was approved by a French ethics committee (CPP Ouest 6, registration N°:2018-AO2370-55). The study is registered under the ClinicalTrials.gov Identifier: NCT03838991.

In the validation phase, we included 82 patients with monostotic and polyostotic FD. 20 patients from the screening phase and 62 from the Periosdys FD cohort[9] were included. Briefly, the recruitment was conducted either during hospitalization or during a consultation. The inclusion criteria for the Periosdys study were as follows: patients aged ≥18 years, with FD. The exclusion criteria were as follows: age <18 years, absence of consent, pregnancy, or physical conditions known to be associated with elevated level of periostin (metastatic cancer, severe allergic asthma, systemic scleroderma, hyper IgG4 syndrome, spinal fibrosis). At time of recruitment, each patient was classified by an expert physician as monostotic or polyostotic, according to the number of bones affected with the disease on imaging (standard radiography, computed tomography, and bone scintigraphy). The diagnosis of FD was made by an expert rheumatologist of the FD reference center, based on clinical and radiologic arguments (including conventional radiographs, computed tomography, and magnetic resonance imaging), in order to differentiate FD from other fibro-osseous lesions.[25,26] Bone biopsy with GNAS mutation analysis was performed only in case of diagnostic doubt, as recommended,[26] or if patient underwent surgery for their disease.

No fracture had occurred in the year preceding the blood sampling.

Control subjects

Controls were healthy individuals matched for age and sex recruited from the Ofely, Modam, and Strambo population-based cohorts,[27–30] at INSERM UMR 1033 (Lyon, France). Ofely (Os des Femmes de Lyon)[27,28] is a prospective cohort of 1089 healthy women between 31 and 89 years old, recruited from 1992 to study the determinants of bone fragility. Modam[29] is a prospective cohort of mothers and their daughters, between 20 and 97 years old, recruited from 2010. Ofely has regular follow-ups concerning bone fragility and fracture risk, including blood sampling. Strambo (the structure of the aging men's bones)[30] is a cohort of 1169 healthy men, between 20 to 85 years old, recruited between 2006 and 2016, in Lyon.

The participant distribution and the study design are displayed in Fig. 1.

Blood sample collection

Blood samples were collected preferentially in a fasting state in the morning at the medical encounter, aliquotted, and stored at −80°C until assayed. A macroscopic visual analysis of the blood samples was carried out to assess the quality of the serum; ie, presence of hemolysis and/or fibrin.

Bone turnover measurements

Bone turnover was evaluated by serum CTX and bone alkaline phosphatase levels only for subjects who were in morning fasting. Serum CTX was measured with electrochemiluminescence (COBAS E411 ROCHE®; Roche Diagnostics, Mannheim, Germany) and bone alkaline phosphatase was measured with automated chemiluminescence (Liaison XL®; DiaSorin, Stillwater, MN, USA). Standards used to define high/low bone remodeling were those of the laboratory, depending on the year and the gender.

Severity and fracture evaluation

Severity of FD was defined by: polyostotic form and/or high bone turnover (elevation of CTX or bone alkaline phosphatase above laboratory upper standard values), and/or prevalent fractures

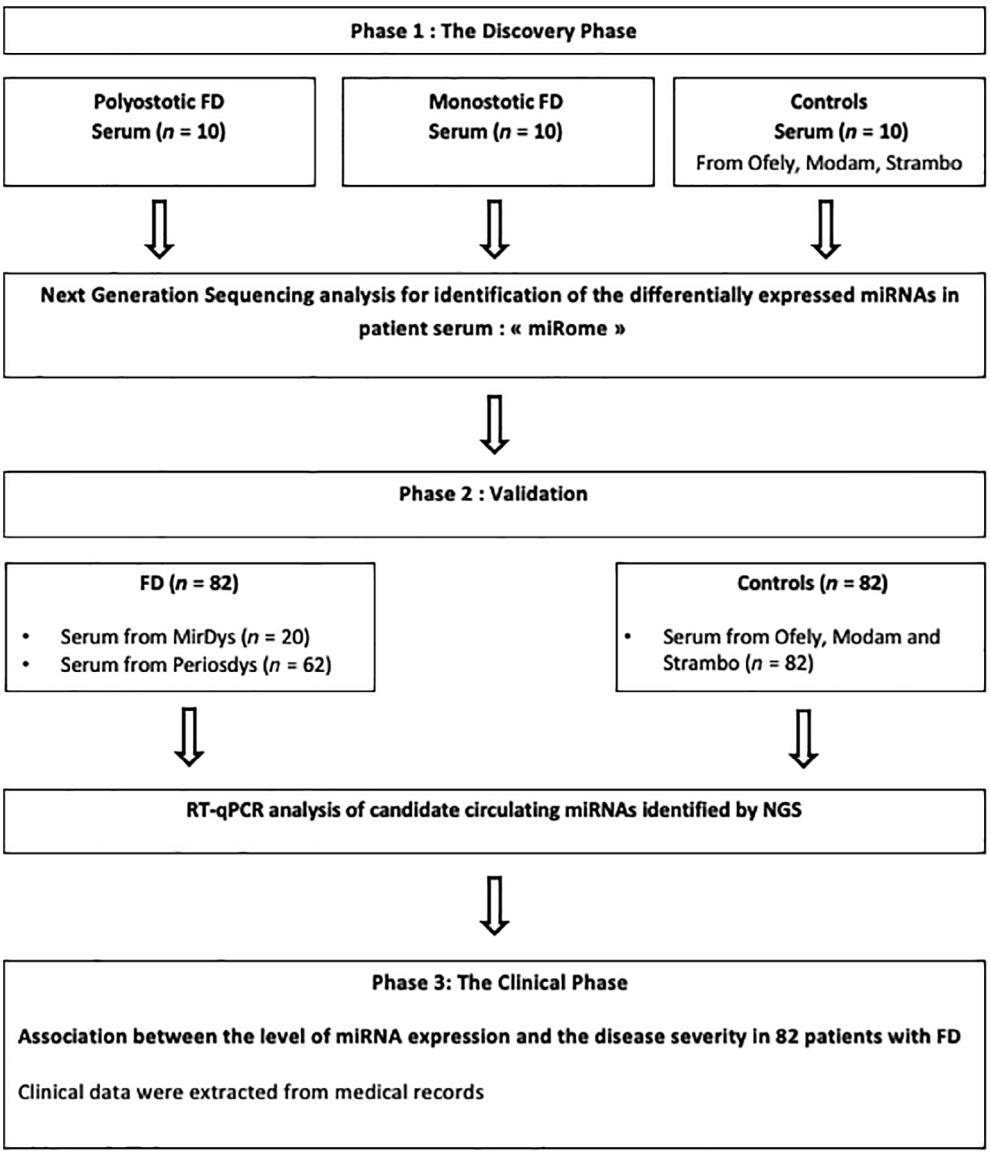

Fig 1. Flowchart of the MirDys study. The study was conducted in a French national reference center of fibrous dysplasia of bone in Edouard Herriot Hospital. The initial screening phase identified potential miRNAs that were quantified in larger groups of subjects in the validation phase to investigate the potential associations of circulating miRNAs with the disease.

(two or more prevalent fractures), bone pain (visual analogue scale [VAS] >3/10 and current or prior treatment by bisphosphonates) and/or McCune-Albright syndrome (MAS). Prevalent fractures due to FD lesions were reported at inclusion, using patient questionnaires and medical records. All fractures were confirmed by radiographs or surgical reports. We have included only low-trauma fractures localized at the site of FD lesions.

The miRome analysis by Next Generation Sequencing

A screening phase with analysis of miRNA expression profile (miRome) was performed by Next Generation Sequencing (NGS) analysis after total RNA extraction, library preparation, and quantification, followed by the miRNA sequencing (Illumina platform; QIAGEN Genomic Services, Germantown, MD, USA). Total RNA (5 μL) extracted from 400 μL of serum with the miRNAeasy/plasma (QIAGEN) was converted into miRNA NGS libraries using the QIAseq miRNA library kit (QIAGEN). The cDNA was preamplified prior to the library purification by gel electrophoresis and analyzed to perform quality control (QC). After quantification by qPCR, optimal concentration of the library pools was used to generate the clusters on the flow cell surface before sequencing on a NextSeq500 sequencing instrument (Illumina, San Diego, CA, USA). To ensure the quality of serum enzymatic reactions and/or amplification, the expression level of exogenous spike-in and endogenous control miRNAs was analyzed by qPCR prior to NGS analysis (Supplementary Fig. S1*A*–*C*) (for detailed procedures of library preparation, normalization, and QC, see the Supplementary Information). Measurements were expressed as counts and normalized by dividing the count for a miRNA by the total count in a sample multiplied by 10^6. The miRNA level was further normalized by the trimmed mean of M-

values (TMM) method and compared between groups by the TMM ratio expressed as relative data as log_2(fold change) (LogFC).[31]

The miRNA analysis by real-time qPCR

Total RNA was extracted from 200 μL serum with the Nucleospin miRNA plasma kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Samples were thawed on ice and centrifuged at 3000*g* for 5 min. A lysis buffer solution containing 1 μL of glycogen as RNA carrier and a synthetic spike-in control RNA (cel-miR-39-3p) as exogenous control were added to the serum. After cellular lysis, protein precipitation, and washes, RNA was eluted from a silica microcolumn with 40 μL RNAse/DNAse-free H_2O and stored at −80°C. The miRNAs were quantified using TaqMan Advanced miRNA technology (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). The miRNAs were reverse-transcribed (RT) to prepare cDNA from 2 μL of total RNA using a TaqMan Advanced miRNA cDNA synthesis kit. The qPCR amplification was performed on cDNA obtained by miR-Amp reaction on the RT samples, using the 2X Fast Advanced Master Mix and the 20X TaqMan Advanced miRNA Assays. Then the miRNAs were quantified using TaqMan low-density microRNA arrays (TLDA) and TaqMan Fast Advanced Mastermix (Applied Biosystems, ThermoFisher Scientific). The TLDA were designed for the real-time PCR quantification in duplicate of 10 miRNAs (six candidate miRNAs, three endogenous controls, miR-191-5p, miR-222-3p, and miR-361-5p, and the exogenous control) on a QuantStudio® 7 flex (Applied Biosystems) (Supplementary Table S1A,B) (for detail procedures, see the Supplementary Information). The threshold cycle (C_T) value was recorded as the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal significantly above the ROX™ fluorescence background recorded in each sample. We used the software Expression Suite (Applied Biosystems) to express the miRNA level as relative quantification (RQ). The C_T values of each miRNA were normalized with the mean of expression level of the three endogenous miRNAs (miR-191-5p, miR-222-3p, and miR-361-5p) that are ubiquitously expressed and unrelated to the metabolism of bone and joint tissues. RQ was calculated as $2^{-\Delta\Delta CT}$, with $\Delta C_T = (C_T$ miRNA − C_T mean of the three endogenous controls) and $\Delta\Delta C_T = (\Delta C_T$ of the miRNA − ΔC_T mean of the miRNA through all samples) and converted as fold change (FC) = $\log2(2^{-\Delta\Delta CT})$. The exogenous spike-in cel-miR-39-3p was used as a qPCR quality control.[32,33]

Statistical analysis

The characteristics of patients and controls are presented as mean ± SD for continuous variables and number (%) for categorical variables. Controls were matched to patients for age, sex, and menopausal status. The NGS step and differential expression analysis of circulating miRNAs were performed by Qiagen using the EdgeR software package. Differential expression was assessed for each gene using an exact test analogous to Fisher's exact test, but adapted for overdispersed data.[34] Relative quantification for RT-qPCR was calculated with ExpressionSuite Software. The Wilcoxon test was used to examine the significance of the difference between serum miRNAs levels in patients in comparison with controls. Values of *p* were corrected for the false discovery rate (FDR) by the Benjamini Hochberg method and were considered significant for values <.05. Significant associations were then adjusted for age, phosphate wasting, and number of involved bones with partial regression. Association of the miRNA levels with the severity of the disease was evaluated with an unpaired *t* test and a Mann-Whitney rank sum test. All the analyses were performed with R software, v3.5 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/). Graphics and figures were done with Stata software, v15.1 (Stata Corporation, Inc., College Station, TX, USA).

Results

The discovery phase for the identification of differentially expressed circulating miRNAs in FD by NGS analysis

Subject characteristics

The expression level of miRNAs was measured in the serum of 10 patients with monostotic and 10 patients with polyostotic FD and compared with expression levels in 10 control subjects. Patients were matched for age, gender, and menopausal status with the control subjects. Mean age of patients was 42 ± 14 years and there were 16 women (80%) and four men (20%). Among patients, 14 (70%) had bone pain and six (30%) had prevalent fractures. A total of 15 (75%) patients—80% polyostotic and 70% monostotic—had current or a history of bisphosphonate treatment (Table 1).

Serum miRNA profiling of patients and control subjects in the discovery phase

The screening phase carried out by NGS identifies 164 miRNAs present in all samples with an expression level >10 TPM (tags per million) and 250 miRNAs present in all samples with an expression level >1 TPM. Comparison of serum miRNA profiling in patients with polyostotic and monostotic disease with controls identified 111 miRNAs that were differentially expressed in patients, with Benjamini-Hochberg FDR-corrected *p* values

Table 1. Baseline Characteristics of Patients and Controls Recruited for the Screening Phase

Characteristic	FD patients	Controls	*p**
n	20	10	
Form, *n*			
Monostotic	10		
Polyostotic	10		
Women, *n* (%)	16 (80)	8 (80)	1
Age (years), mean ± SD	42 ± 14	42 ± 14	.993
Postmenopausal women, *n* (%)	7 (44)		
BMI (kg/m^2), mean ± SD	25.7 ± 4.9	23.3 ± 3.5	.185
Patients with pain, *n* (%)	14 (70)		
VAS, mean ± SD	5.2 ± 1.63		
McCune Albright syndrome, *n* (%)	1 (5)		
Prevalent fracture, *n* (%)	6 (30)		
Bisphosphonate use, *n* (%)	15 (75)		
Monostotic	7 (70)		
Polyostotic	8 (80)		

BMI = body mass index; VAS = visual analogue pain scale on 10-cm line.
*Significant difference for *p* value < .05.

Table 2. Selection of the 19 miRNAs for Further RT-qPCR Analysis in the Validation Phase

miRNAs	log_2(FC)	*p*	FDR correction	Patient TMM	Control TMM	Type of FD
miR-182-5p	1.80	****	****	2567.19	739.5	Monostotic and polyostotic
miR-183-5p	1.60	****	****	1444.07	477.79	Monostotic and polyostotic
miR-324-5p	1.17	****	****	421	188.18	Monostotic and polyostotic
miR-15b-5p	1.05	****	****	1662.97	804.13	Monostotic and polyostotic
miR-15a-5p	1.19	****	****	984.33	431.17	Monostotic and polyostotic
let-7d-5p	1.00	****	****	2059.58	1031.44	Monostotic and polyostotic
miR-374a-5p	1.15	****	****	155.25	70.94	Monostotic and polyostotic
miR-363-3p	1.46	****	****	530.9	193.02	Monostotic
miR-451a	1.51	****	****	18728.05	6580.03	Monostotic and polyostotic
miR-454-3p	1.41	****	****	529.9	200.21	Monostotic and polyostotic
miR-96-5p	1.43	****	****	418.42	156.41	Monostotic and polyostotic
miR-107	1.07	****	****	621.79	296.51	Monostotic and polyostotic
miR-32-5p	1.02	****	****	179.82	90.06	Monostotic and polyostotic
miR-103a-3p	0.88	****	****	7446.65	4061.43	Monostotic and polyostotic
miR-93-5p	0.88	****	****	11552.95	6293.92	Monostotic
miR-20b-5p	1.21	****	****	286.11	124.37	Monostotic and polyostotic
let-7i-5p	0.76	****	****	17208.91	10165.97	Polyostotic
miR-19b-3p	0.95	****	***	969.58	503.67	Monostotic and polyostotic
miR-25-3p	0.94	****	***	26769.69	13999.05	Monostotic

The 19 candidate miRNAs were selected on the basis of their highly significant upregulated level in both polyostotic and monostotic FD (FDR-corrected *p* values <.0005) and of their high NGS reads (TMM).

***$p < .005$.

****$p < .001$.

<.05. Eighty miRNAs were overexpressed and 31 were underexpressed in the serum of patients, compared with controls (Supplementary Table S2A,B). Among these, 19 miRNAs were selected for further analysis in the validation phase, on the basis of their highly significant upregulated level in both polyostotic and monostotic FD (FDR corrected *p* values <.0005) and of their high NGS reads (Table 2). Indeed, we did not select any downregulated miRNAs for further analysis because most of them had a weaker level of statistical significance and/or a lower expression level (Supplementary Table S2B).

The validation phase of the miRNAs differential expression by real-time qPCR analysis

Subject characteristics

For the validation phase of candidate miRNAs, we selected 82 patients and 82 controls that were matched for age (mean 42 years) and sex (60% women) (Table 3). Among patients, 45 (55%) were polyostotic, 59 patients (73%) had bone pain, with a 3.33 mean value on the VAS (10-point scale), 25 patients (43%) had prevalent fractures. A total of 63 (77%) patients—71% polyostotic and 84% monostotic—had current or history of bisphosphonate treatment (Table 3).

Expression level of miRNAs in serum of patients and control subjects in the validation phase

The 19 miRNAs selected from the screening phase were analyzed by RT-qPCR in the serum of 164 subjects and their level was expressed by relative comparison with the miRNA median of expression through all samples. We found that 14 miRNAs were significantly differentially expressed in the serum of patients versus controls by comparing their relative level by the Wilcoxon test; however, these values remained significant for only six miRNAs after FDR correction (Table 4). The median level of these six circulating miRNAs (miR-25-3p, miR-93-5p, miR-182-5p, miR-324-5p, miR-363-3p, and miR-451a) was significantly higher in patients compared with controls (Fig. 2).

Table 3. Baseline Characteristics of Patients and Control Recruited for the Validation Phase

Characteristic	FD patients	Controls	*p**
n	82	82	
Form, *n* (%)			
Monostotic	37 (45)		
Polyostotic	45 (55)		
Gender, *n* (%)			
Women	60 (73)	60 (73)	1
Men	22 (27)	22 (27)	1
Postmenopausal women, *n* (%)	19 (32)	17 (28)	0.84
Age (years), mean ± SD	42 ± 14.6	43 ± 14	0.87
BMI (kg/m^2), mean ± SD	27.2 ± 5.9	26.6 ± 4.3	0.48
Patients with pain, *n* (%)	59 (73%)		
VAS, mean ± SD	3.33 ± 2.6		
McCune Albright syndrome, n (%)	11 (13)		
Bisphosphonate treatment, *n* (%)	63 (77)		
Monostotic	31 (84)		
Polyostotic	32 (71)		
Prevalent fractures, n (%)	25/92 (30)		
Phosphate wasting, n (%)	4 (5)		
Mazabraud syndrome, n (%)	2 (2)		
Number of bones affected, mean ± SD	3.4 ± 4.28		

BMI = body mass index; VAS = visual analogue pain scale on 10-cm line.
*Significant difference for *p* value <.05.

Table 4. Comparison of the miRNAs Levels (Medians) in Serum of Patients and Controls

miRNAs	Relative levels (medians) Patients (n = 82)	Controls (n = 82)	p	After FDR
let-7d-5p	1.1	0.8	.11	>0.05
let-7i-5p	1.3	0.7	.013	0.25
miR-103a-3p	1.4	0.8	.36	>0.05
miR-107	1.8	0.8	.018	0.34
miR-15a-5p	1.3	1.0	.032	0.61
miR-15b-5p	1.1	1.2	.82	>0.05
miR-182-5p	1.7	0.6	<.0001	0.001*
miR-183-5p	2.2	0.9	.016	0.31
miR-19b-3p	1.7	0.7	.043	0.82
miR-20b-5p	1.5	1.0	.012	0.23
miR-25-3p	1.5	0.7	<.0001	<0.0001*
miR-324-5p	1.4	1.0	.0019	0.036*
miR-32-5p	1.0	2.6	.0047	0.089
miR-363-3p	1.8	0.7	.0001	0.002*
miR-374a-5p	1.2	0.8	.37	>0.05
miR-451a	1.5	0.7	<.0001	0.0007*
miR-454-3p	1.2	0.8	.53	>0.05
miR-93-5p	1.3	0.8	.0002	0.004*
miR-96-5p	1.5	0.7	.004	0.08

The 19 candidate miRNAs were analyzed by RT-qPCR in the serum of the 82 patients and their 82 age- and sex-matched controls. After normalization, miRNAs levels were expressed in relative values and a comparison of medians was performed between the two groups (Wilcoxon test). The median level of 6 circulating miRNAs (miR-25-3p, miR-93-5p, miR-182-5p, miR-324-5p, miR-363-3p, and miR-451a) was significantly higher in patients in comparison with controls.

*Value of $p < .05$.

The association of the miRNA differential expression and clinicopathological parameters of FD

The influence of prognostic factors in FD on the miRNA differential expression was then investigated by multivariate analysis. We found that none of the three main factors, age, phosphate wasting, and the number of affected bones, had influence on the miRNA level (Table 5*A*). We have further looked for a potential association between the miRNA expression level and the severity of the disease (Table 5*B–E*). Indeed, the miRNA level was not higher in patients having a severe disease, defined by polyostotic form, with a high bone turnover, prevalent fractures, and bone pain. Surprisingly, the patients without MAS presented a trend toward a higher median level for five miRNAs in comparison to MAS patients, and this was slightly significant for miR-25-3p (0.03) (Supplementary Table S3).

No significant difference in miRNA levels was observed between patients receiving or not receiving bisphosphonate treatment (Table 5*F*).

The interaction of miRNAs with *GNAS* and the osteogenesis-related genes

The miRNAs miR-25-3p, miR-93-5p, miR-182-5p, miR-324-5p, miR-363-3p, and miR-451a were overexpressed in FD. To determine whether they are only biomarkers of the disease, being released from tissue after alterations of bone homeostasis, or whether they regulate biological pathways involved in bone homeostasis, we investigated the potential interaction of the seed region of the upregulated miRNAs with the genes involved in the pathophysiology of the disease, using the miRWalk prediction database (http://mirwalk.umm.uni-heidelberg.de/). We have searched for the highest probability score of interactions (100%) between the six miRNA and the nucleotide sequence of *GNAS*, the genes involved in the *GNAS* subsequent signaling pathways, and also the genes related to bone disease (Table 6*A,B*). Among the 29 genes that were predicted, we found several osteogenesis-associated genes (*Osterix* (*SP7*), *RunX2*, *WNT*, *BMP2*, *FOXO1*, *FOXO3*, *RANKL*, and *IL6ST)*. Among the 29 genes, we further selected those that had been experimentally validated in vitro and/or in vivo and reported by the miRWalk database through miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) (Table 6*B*). Interestingly, GNAS was a target only of miR-93-5p, suggesting that the miRNA overexpression does not impair cAMP increase but rather osteoblast function.

Discussion

To identify potential epigenetic regulations explaining some of the phenotypic disease heterogeneity, we performed a preliminary screening of the miRome in serum from patients and control subjects. Among the 111 miRNAs significantly differentially expressed in serum of FD patients, we have selected 19 miRNAs and confirmed by qPCR that six of them were significantly overexpressed in 82 FD patients. These miRNAs do not appear to interfere with the increase of cAMP production induced by the GNAS mutation but exhibit major activities in bone cell homeostasis. This suggests their potential involvement on the dysregulation of gene expression in bone, observed in FD disease.

In FD, the missense mutation of *GNAS* increases the cAMP level in osteoblast and results in a wide spectrum of clinical features ranging from asymptomatic to severe skeletal lesions. The current treatments are symptomatic and aim to decrease pain. Despite a number of publications reporting some beneficial

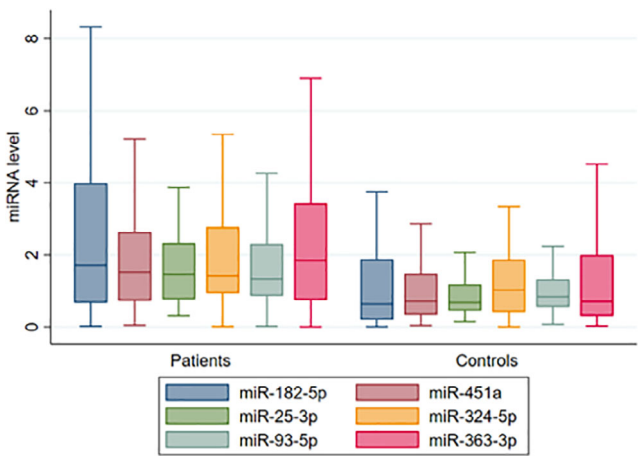

Fig 2. The relative levels of the six miRNAs that were significantly different between patients (n = 82) and control subjects (n = 82). From the screening discovery, 19 miRNAs were selected for the validation phase and further analyzed using real-time qPCR. The level of six miRNAs measured by RT-PCR and expressed as median were significantly different in the two groups of serum (Wilcoxon test).

Table 5. Association of Clinicopathological Parameters of FD and Circulating miRNA Levels

(*A*) Influence of the prognosis factors of FD and the differential expression of miRNAs			
miRNAs	Age	Phosphate wasting	Number of affected bones
miR-182-5p	0.66	0.58	0.35
miR-25-3p	0.32	0.46	0.13
miR-324-5p	0.66	0.7	0.28
miR-451a	0.86	0.88	0.98
miR-93-5p	0.94	0.47	0.16
miR-363-3p	0.89	0.69	0.8

(*B*) Comparison of the miRNA level between patients with monostotic (*n* = 37) and polyostotic (*n* = 45) forms of the disease			
miRNAs	Relative levels (medians) in monostotic FD (*n* = 37)	Relative levels (medians) in polyostotic FD (*n* = 45)	*p*
miR-182-5p	1.3	2	.25
miR-25-3p	1.6	1.4	.45
miR-324-5p	1.7	1.4	.3
miR-451a	1.4	1.5	.80
miR-93-5p	1.5	1.3	.60
miR-363-3p	1.9	1.8	.87

(*C*) Comparison of the miRNA level between patients with high (*n* = 31) or low (*n* = 35) bone turnover markers			
miRNAs	Relative levels (medians) High bone turnover markers (*n* = 31)	Relative levels (medians) Low bone turnover markers (*n* = 35)	*p*
miR-182-5p	1.7	1.3	.76
miR-25-3p	1.3	1.6	.39
miR-324-5p	1.3	1.4	.56
miR-451a	1.4	1.8	.46
miR-93-5p	1.4	1.2	.74
miR-363-3p	1.7	1.6	.21

(*D*) Comparison of the miRNA level between patients with ≥2 prevalent fractures (*n* = 12) or <2 prevalent fractures (*n* = 70)			
miRNAs	Relative levels (medians) ≥2 Prevalent fractures (*n* = 12)	Relative levels (medians) <2 Prevalent fractures (*n* = 70)	*p*
miR-182-5p	1.5	1.8	.93
miR-25-3p	1.2	1.5	.26
miR-324-5p	1.1	1.4	.17
miR-451a	1.2	1.7	.31
miR-93-5p	1.2	1.3	.60
miR-363-3p	1.1	1.8	.35

(*E*) Comparison of the miRNA level between patients having (*n* = 40) or not having (*n* = 42) bone pain			
miRNAs	Relative levels (medians) Pain (*n* = 40)	Relative levels (medians) No pain (*n* = 42)	*p*
miR-182-5p	1.3	1.8	.39
miR-25-3p	1.6	1.4	.97
miR-324-5p	1.4	1.4	.98
miR-451a	1.4	1.8	.90
miR-93-5p	1.3	1.3	.69
miR-363-3p	1.8	1.8	.31

(*F*) Comparison of the miRNA level between patients receiving (*n* = 63) and or not (*n* = 19) a bisphosphonate treatment			
miRNAs	Relative levels (medians) Bisphosphonates (*n* = 63)	Relative levels (medians) No bisphosphonates (*n* = 19)	*p*
miR-182-5p	1.34	2.30	.17
miR-25-3p	1.46	1.45	.53
miR-324-5p	1.35	1.82	.77

(Continues)

Table 5. Continued

(*F*) Comparison of the miRNA level between patients receiving (n = 63) and or not (n = 19) a bisphosphonate treatment			
miRNAs	Relative levels (medians) Bisphosphonates (n = 63)	Relative levels (medians) No bisphosphonates (n = 19)	p
miR-451a	1.38	2.07	.083
miR-93-5p	1.32	1.54	.49
miR-363-3p	1.72	1.85	.42

Value of p obtained after partial regression about main prognosis factors of FD disease.

Results were obtained using an unpaired t test and a Mann-Whitney rank sum test. Significance was defined by $p < .05$. Standards used to define high/low bone remodeling were those of the laboratory, depending on the age and the gender. Bone turnover was evaluated by CTX and bone alkaline phosphatase level. Data were available for 66 of the 82 patients (80%). Bone pain was defined by a score of VAS > 3/10 and a history of or current bisphosphonate treatment.VAS = visual analogue scale.

effects of bisphosphonates[35–37] and denosumab[38] on pain and bone turnover markers, there are no medical therapies able to alter the course of the disease. The transcriptomic regulations underlying the GNAS-activating mutations are not understood, especially those due to the miRNAs, the cellular endogenous regulators of gene expression. Here, we report the overexpression of miRNAs miR-182-5p, miR-93-5p, miR-25-3p, miR-363-3p, miR-324-5p, and miR-451a in the serum of FD patients. According to their targeted genes highlighted in the miRWalk database and by experimental data reported in literature, these miRNAs do not appear to interfere with the increase of cAMP production but exhibit major activities in bone metabolism. The miRNA cluster located in the human chromosome 7q32.2 encodes for three members (miR-183, miR-182, and miR-96) that were significantly increased in patient serum, by NGS analysis, suggesting their common regulation in FD. However, only miR-182-5p reached significance in the validation phase, probably due to its higher level of expression. miR-182-5p is consistently reported as a negative regulator of osteogenesis by interfering with osteoblastogenesis and promoting osteoclastogenesis.[39,40] The expression of miR-182 increased with the differentiation of MC3T3-E1 preosteoblasts, resulting in the inhibition of

Table 6. The miRWalk Algorithm Analysis of miRNA Interactions With *GNAS* and the Osteogenesis-Related Genes

(*A*) Interactions predicted with 100% probability between the miRNA seed region and the nucleotide sequence of *GNAS* and of osteogenesis-associated genes	
miRNAs	Genes[a]
miR-93-5p	*FOSB*; *FOXO3*; *MAML1*; *BMP2K*; *WNT7B*; *SP7*; *ESR2*
miR-182-5p	*FOSB*; *WNT10B*
miR-25-3p	*FOSL1*; *RUNX2*; *TNFSF11*; *IL6R*; *BMP2K*; *WNT9A*; *ESR2*; *SOST*
miR-324-5p	*FOSB*; *FOXO3*; *RUNX2*

(*B*) Experimentally validated interaction between miRNAs, *GNAS*, and osteogenesis-associated genes			
miRNAs	Genes[b]	Accession ID miRTarBase	Reference
miR-25-3p	*IL6ST*	MIRT216029	Hafner and colleagues (2010)[c]
miR-324-5p	*FOXO1*	MIRT557808	Hafner and colleagues (2010)[c]
miR-363-3p	*IL6ST*	MIRT216033	Hafner and colleagues (2010)[c]
miR-451a	*IL6R*	MIRT054843	Chi and colleagues (2009)[d]
miR-93-5p	*GNAS*	MIRT727561	Riley and colleagues (2012)[e]
	FOXO3	MIRT735009	Jiang and colleagues (2015)[f]
	PDGFB	MIRT492785	Whisnant and colleagues (2013)[g]
	ESR2	MIRT683400	Xue and colleagues (2013)[h]
	BMP2	MIRT480783	Whisnant and colleagues (2013)[g]

[a]Sticht C, De La Torre C, Parveen A, Gretz N. miRWalk: an online resource for prediction of microRNA binding sites. PLoS One. 2018;13(10):e0206239.

[b]Hsu SD, Lin FM, Wu WY, et al. miRTarBase: a database curates experimentally validated microRNA-target interactions. Nucleic Acids Res. 2011;39(Database issue):D163–9.

[c]Hafner M, Landthaler M, Burger L, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell. 2010;141(1):129–41.

[d]Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature. 2009;460(7254):479–86.

[e]Riley KJ, Rabinowitz GS, Yario TA, Luna JM, Darnell RB, Steitz JA. EBV and human microRNAs co-target oncogenic and apoptotic viral and human genes during latency. EMBO J. 2012;31(9):2207–21.

[f]Jiang L, Wang C, Lei F, et al. miR-93 promotes cell proliferation in gliomas through activation of PI3K/Akt signaling pathway. Oncotarget. 2015;6(10):8286–99.

[g]Whisnant AW, Bogerd HP, Flores O, et al. In-depth analysis of the interaction of HIV-1 with cellular microRNA biogenesis and effector mechanisms. mBio. 2013;4(2):e000193.

[h]Xue Y, Ouyang K, Huang J, et al. Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. Cell. 2013;152(1–2):82–96.

osteoblast proliferation and differentiation.[39] The miR-182 targets and represses FoxO1, a transcriptional factor that promotes osteogenesis.[41] In a TNFα-mediated inflammatory murine model, Miller and colleagues[42] have shown that miR-182 promotes osteoclastogenesis driven by TNFα by direct downregulation of the osteoclastogenesis repressor FoxO3 and Maml1. The NGS analysis also showed that the three members of the miR-93-25 gene cluster located in chromosome 7 (miR-93, miR-106b, and miR-25) were significantly overexpressed in patient serum. We have not quantified miR-106b in the validation phase, with regard to its low level of expression in NGS, but the RT-qPCR analysis confirmed the overexpression of miR-93 and miR-25 in FD patient serum. Both miRNAs interfere with osteoblast and/or osteoclast signaling pathways and subsequent bone mineralization.[43–48] miR-93 expression is strongly downregulated during osteoblast mineralization and its overexpression in cultured primary mouse osteoblasts impairs osteoblast mineralization through a miR-93/Sp7 regulatory feedback loop.[47] In trauma-induced osteonecrosis of the femoral head, Zhang and colleagues[48] have shown that miR-93-5p inhibits osteoblast differentiation of bone mesenchymal stem cells (bMSC) and represses osteoblastogenesis by targeting bone morphogenetic protein-2 (BMP2). Consistent with this study, our miRWalk search identified BMP2 as an experimentally validated target of miR-93-5p in bone diseases. Overall, miR-93-5p appears not only as a biomarker but also as a regulator of the disease by targeting osteogenic factors.

Resulting from this cluster expression, the amount of miR-25-3p was higher in serum, whereas its counterpart miR-25-5p was detected as a trace, probably due to its weak thermodynamic stability. Despite of the lack of literature data in support of the role of miR-25-3p in osteoblastogenesis, the in silico search suggests that miR-25-3p might be a negative regulator of osteoblastogenesis by downregulating the expression of genes involved in osteoblast differentiation (*FOSL1*, *RUNX2*, *WNT9A*, *BMP2K*). miR-25-3p could also reduce osteoclastogenesis by targeting RANKL, IL6R, and IL6ST. The Fos-related antigen Fra-1 encoded by *FOSL1* is an important transcription factor and regulates bone mass by affecting bone matrix production and maintaining osteoblast activity. Its ectopic expression in transgenic mice leads to an osteosclerotic phenotype with increased bone mass.[49] However, Huang and colleagues[46] have shown that miR-25-3p expression decreases significantly in mature osteoclasts and negatively regulates osteoclast function through nuclear factor I X (NFIX), which is consistent with the targeting and downregulation of RankL and IL6-related genes underscored in our data search. In addition, Fujiwara and colleagues[50] have demonstrated that high level of serum miR-25-3p is a diagnostic and prognostic biomarker of osteosarcoma, a known complication of FD.[51] Therefore, regarding its significant increase in patient serum, miR-25-3p appears as a biomarker of FD but its role in regulating the bone cell–related genes is presently unknown and should be studied in osteoprogenitor cell lines expressing the GNAS mutation.

The NGS analysis showed that the six miRNAs (miR-106a, miR-18b, miR-20b, miR-19b, miR-92, and miR-363) encoded by the miR-106a-363 cluster are significantly increased in the serum of FD patients as a result of a joint activation of transcription. Among the three miRNAs measured in the validation phase (miR-363-3p, miR-19b-3p, and miR-20b-5p), only miR-363-3p remained significantly increased with a Log2FC >2, whereas miR-19b-3p and miR-20b-5p did not reach a significant increase after FDR adjustment. The increase of circulating miR-363-3p level has been reported in osteoporotic patients.[52] This miRNA promoted osteoclastogenesis, by stimulating the differentiation of osteoclast precursors and inhibited osteoblast differentiation by targeting phosphatase and tensin homolog (PTEN) and activating the PIK3K/AKT signaling pathway.[52] In FD patients, we hypothesize that miR-363-3p transcription is promoted by the elevation of cAMP. Only IL6ST was found as a targeted gene that is in favor of a negative regulation of osteoclastogenesis. It appears that miR-363-3p may be a biomarker in FD but its role in the disease remains to be elucidated. Finally, miR-324-5p and miR-451a were significantly increased in the serum of the 82 patients in the validation phase. miR-324-5p is increased in end-stage osteoarthritis by regulating hedgehog signaling and osteogenesis in human MSCs[53] and it is also downregulated in the bone marrow of osteoporotic patients.[54] By searching in the miRWalk database, we have found that miR-324-5p targets three genes (FOXO1/FOXO3, FOSB, and RUNX2) that promote osteoblastogenesis and consequently would interfere negatively with osteoblast differentiation and bone mineralization. Karvande and colleagues[55] have shown that the bone formation induced by PTH therapy was related to the increase in miR-451a serum levels. In vitro, miR-451a promotes osteoblast differentiation and mineralization due to Odd skipped related 1 inhibition and to Runx2 activation. These data show that miR-451a enhances bone formation and its activity in FD remains questionable.

Overall, these miRNAs might exert an epigenetic regulation of gene expression in bone marrow cells and thus have a role in FD pathogenesis. According to literature data and our in silico search these miRNAs do not target genes that interfere with the excess of cAMP production. Rather, the cAMP-induced transcriptional activation would not only increase the miRNA circulating level but also promote their activity as negative regulators of bone cell differentiation and bone mineralization. This miRNA signature would mostly reflect the impairment of osteoblast functions for which we have identified targeted genes.

We did not find any association between the miRNA signature and FD severity. This could be explained by the age of our patients (mean age 42 years), if the most important dysregulations exist during bone growth. Indeed, FD lesions are characterized by age-related histologic, radiologic, and clinical changes.[56] Kuznetsov and colleagues[57] reported a decline in the number of mutated bMSCs in older patients and, in some cases, the histologic normalization of the bone and the bone marrow in FD lesions. This suggests that mutated bMSCs fail to self-renew with age and tend to be replaced by normal bMSCs. Natural evolution of FD also supports a model of age-related changes in disease severity. FD becomes symptomatic in early childhood, with the majority of skeletal lesions and their associated disability manifesting within the first decade of life. At NIH, 109 subjects with a spectrum of FD were studied for up to 32 years; >90% of FD lesions were established at all sites before the age of 15 years and among patients needing assistance with ambulation, 92% showed this need prior to the age of 17 years.[58] It might be relevant to assess the potential of these miRNAs as prognostic biomarkers in younger patients.

We have also considered the possibility that the absence of association of the miRNA signature with the disease severity might be explained at least in part by the mosaic state of the disease. Indeed, clinical bone FD lesions consist in a mosaic of normal and mutated stem/progenitor cells contributing to the diversity and evolution of the disease with time.[59] It has been

reported that the clinical phenotype reflects the type, number, and viability of mutated cells arising from these mutated progenitors but also from the contribution of nonmutated cells.[5] For example, the high apoptotic/senescence rate in lesions decreased the frequency of mutated clonogenic stem cells that is negatively correlated to the age of patients. The decreased prevalence of mutated stem/progenitor cells observed with skeletal maturity leads to the absence of detected mutations in older FD lesions that are also less metabolically active.[5,57] The miRNA levels in patients with or without MAS did not differ, with miR-25-3p being even significantly decreased in the serum of patient with MAS. This suggests a weak prevalence of mutated osteoprogenitors in the polyostotic lesions of patients with MAS with the contribution of the TRAP-positive nonmutated cells to the expansion of bone lesions. In this case, the activation of the miR93-25 cluster is no longer sustained by the cAMP elevation induced in osteoblasts expressing the GNAS mutation.

In the present study, we have reported prevalent fractures and not incident fractures. These fractures may be old ones and thus not reflecting the current activity of the disease.

We have also explored the possibility that the bisphosphonate treatment may have interfered with the miRNA expression in tissue and thus with the expression level of circulating miRNAs. Indeed, in a rat model of postmenopausal osteoporosis, Kocijan and colleagues[60] have shown that zoledronate treatment has a significant impact on miRNA levels in bone tissue and blood. Zoledronate treatment was able to reverse miRNAs changes in tissue and serum of untreated ovariectomized animals and to rescue the pathologic upregulation of some osteoinhibitory miRNAs. In our study, we found no difference in the miRNA level in serum from patients receiving or not a bisphosphonate treatment. Given the limited sample size, however, we cannot firmly rule out the possibility of a weak difference. Also, the rat bone differs from the human bone by lifelong modeling, whereas the human adult bone is remodeling. So, the possible effect of bisphosphonates on circulating miRNA levels may diverge in rats and humans.

Our study is the first report of a miRNA signature associated to FD and with a sizeable number of patients for this rare bone disease. The patients recruited in a French national reference center of FD had well-defined inclusion criteria and clinicopathological follow-up. The analysis was conducted as a case-control analysis, with adjustment for potential confounding factors (age, phosphate wasting, and number of involved bones). Moreover, we have conducted a screening phase to decipher the miRNA epigenetic regulation in the disease and associated these differentially overexpressed miRNAs to targeted genes and impairment in bone cell homeostasis.

Our study has several limitations. The first question is the miRNA tissue source and whether the miRNA serum concentration adequately reflects changes in miRNA bone tissue levels.[61] In the present study, the source of circulating miRNAs remains uncertain and in absence of bone tissue analysis, we cannot conclude for sure about the correlation between bone activity and circulating miRNAs levels. It is also known that the GNAS mutation in FD patients can be detected at non-affected skeletal sites. So, it may be possible that the serum levels of miRNAs were related to the number of mutated, and maybe slightly dysfunctional, osteogenic cells rather than to the number of clinically evident lesions.

Another limitation is that solely overexpressed circulating miRNAs selected by the screening phase have been quantified in the validation phase. The downregulated miRNAs were not further analyzed mainly because this decrease in expression in subject serum might be difficult to assess from an analytical point of view, limiting potential clinical settings.

In conclusion, our study has profiled circulating miRNAs in FD patients and identified six upregulated miRNAs associated with the disease that might be used as potential biomarkers. This miRNA signature would mostly reflect the impairment of osteoblastic functions for which we have identified targeted genes. The potential pathogenic role of these miRNAs in FD need to be confirmed by in vitro and in vivo studies.

Disclosures

All authors state that they have no conflict of interest.

Acknowledgments

This work was made possible by a scholar grant from the Société Française de Rhumatologie to Mélanie Legrand.

We thank INSERM U1033 (Lyon, France), Université Lyon 1 Claude Bernard (Lyon, France) and Hospices Civils de Lyon (Lyon, France) for promoting the study.

Authors' roles: study design: RC; study conducted by: ML, BM, MM, MC; data collection: ML; data analysis: ML, AH; data interpretation: ML, MM, MC, RC; drafting manuscript: ML, MC, RC; revising manuscriptcontent: ML, MM, MC, RC; approving final version of manuscript: ML, MC, MM, BM, JCR, AH, EG, ESR, OB, PS and RC. ML and RC take responsibility for the integrity of the data analysis.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1002/jbmr.4111.

References

1. Chapurlat RD, Meunier PJ. Fibrous dysplasia of bone. Baillieres Best Pract Res Clin Rheumatol. 2000;14(2):385–98.
2. Weinstein LS. G(s)alpha mutations in fibrous dysplasia and McCune-Albright syndrome. J Bone Miner Res. 2006;21(Suppl 2):P120–4.
3. Boyce AM, Collins MT. Fibrous dysplasia/McCune-Albright syndrome: a rare, mosaic disease of Gα S activation. Endocr Rev. 2020;41(2):345–70.
4. Marie P. Cellular and molecular biology of fibrous dysplasia. Ann Pathol. 2001;21(6):489–98.
5. Yamamoto T, Ozono K, Kasayama S, et al. Increased IL-6-production by cells isolated from the fibrous bone dysplasia tissues in patients with McCune-Albright syndrome. J Clin Invest. 1996;98(1):30–5.
6. de Castro LF, Burke AB, Wang HD, et al. Activation of RANK/RANK-L/OPG pathway is involved in the pathophysiology of fibrous dysplasia and associated with disease burden. J Bone Miner Res. 2019;34(2):290–4.
7. Benhamou J, Gensburger D, Messiaen C, Chapurlat R. Prognostic factors from an epidemiologic evaluation of fibrous dysplasia of bone in a modern cohort: the FRANCEDYS study. J Bone Miner Res. 2016;31(12):2167–72.
8. Collins MT, Kushner H, Reynolds JC, et al. An instrument to measure skeletal burden and predict functional outcome in fibrous dysplasia of bone. J Bone Miner Res. 2005;20(2):219–26.
9. Guerin Lemaire H, Merle B, Borel O, Gensburger D, Chapurlat R. Serum periostin levels and severity of fibrous dysplasia of bone. Bone. 2019;121:68–71.

10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281–97.
11. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci U S A. 2007;104(23):9667–72.
12. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet. 2012;13(4): 271–82.
13. Bernstein E, Kim SY, Carmell MA, et al. Dicer is essential for mouse development. Nat Genet. 2003;35(3):215–7.
14. Sugatani T, Hruska KA. Impaired micro-RNA pathways diminish osteoclast differentiation and function. J Biol Chem. 2009;284(7):4667–78.
15. Gaur T, Hussain S, Mudhasani R, et al. Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. Dev Biol. 2010;340(1):10–21.
16. Mizoguchi F, Izu Y, Hayata T, et al. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption. J Cell Biochem. 2010; 109(5):866–75.
17. Gennari L, Bianciardi S, Merlotti D. MicroRNAs in bone diseases. Osteoporos Int. 2017;28(4):1191–213.
18. Croset M, Kan C, Clézardin P. Tumour-derived miRNAs and bone metastasis. Bonekey Rep. 2015;4:688.
19. Puppo M, Taipaleenmäki H, Hesse E, Clézardin P. Non-coding RNAs in bone Remodelling and bone metastasis: mechanisms of action and translational relevance. Br J Pharmacol Forthcoming. Epub. 2019 Aug 18. https://doi.org/10.1111/bph.14836.
20. Hesse E, Taipaleenmäki H. MicroRNAs in bone metastasis. Curr Osteoporos Rep. 2019;17(3):122–8.
21. Ji X, Chen X, Yu X. MicroRNAs in osteoclastogenesis and function: potential therapeutic targets for osteoporosis. Int J Mol Sci. 2016;17 (3):349.
22. Suttamanatwong S. MicroRNAs in bone development and their diagnostic and therapeutic potentials in osteoporosis. Connect Tissue Res. 2017;58(1):90–102.
23. Seeliger C, Karpinski K, Haug AT, et al. Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. J Bone Miner Res. 2014;29(8):1718–28.
24. Feurer E, Kan C, Croset M, Sornay-Rendu E, Chapurlat R. Lack of association between select circulating miRNAs and bone mass, turnover, and fractures: data from the OFELY cohort. J Bone Miner Res. 2019;34 (6):1074–85.
25. Chapurlat RD, Orcel P. Fibrous dysplasia of bone and McCune-Albright syndrome. Best Pract Res Clin Rheumatol. 2008;22(1):55–69.
26. Javaid MK, Boyce A, Appelman-Dijkstra N, et al. Best practice management guidelines for fibrous dysplasia/McCune-Albright syndrome: a consensus statement from the FD/MAS international consortium. Orphanet J Rare Dis. 2019;14(1):139.
27. Arlot ME, Sornay-Rendu E, Garnero P, Vey-Marty B, Delmas PD. Apparent pre- and postmenopausal bone loss evaluated by DXA at different skeletal sites in women: the OFELY cohort. J Bone Miner Res. 1997;12(4):683–90.
28. Sornay-Rendu E, Boutroy S, Munoz F, Delmas PD. Alterations of cortical and trabecular architecture are associated with fractures in postmenopausal women, partially independent of decreased BMD measured by DXA: the OFELY study. J Bone Miner Res. 2007;22(3): 425–33.
29. Nagy H, Sornay-Rendu E, Boutroy S, Vilayphiou N, Szulc P, Chapurlat R. Impaired trabecular and cortical microarchitecture in daughters of women with osteoporotic fracture: the MODAM study. Osteoporos Int. 2013;24(6):1881–9.
30. Chaitou A, Boutroy S, Vilayphiou N, et al. Association between bone turnover rate and bone microarchitecture in men: the STRAMBO study. J Bone Miner Res. 2010;25(11):2313–23.
31. Garmire LX, Subramaniam S. Evaluation of normalization methods in mammalian microRNA-Seq data. RNA. 2012;18(6):1279–88.
32. Huggett JF, Foy CA, Benes V, et al. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin Chem. 2013;59(6):892–902.
33. Pickering M-E, Millet M, Rousseau J-C, et al. Selected serum microRNA, abdominal aortic calcification and risk of osteoporotic fracture. PLoS One. 2019;14(5):e0216947.
34. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139–40.
35. Liens D, Delmas PD, Meunier PJ. Long-term effects of intravenous pamidronate in fibrous dysplasia of bone. Lancet. 1994;343(8903): 953–4.
36. Chapurlat RD, Hugueny P, Delmas PD, Meunier PJ. Treatment of fibrous dysplasia of bone with intravenous pamidronate: long-term effectiveness and evaluation of predictors of response to treatment. Bone. 2004;35(1):235–42.
37. Majoor BC, Appelman-Dijkstra NM, Fiocco M, van de Sande MA, Dijkstra PS, Hamdy NA. Outcome of long-term bisphosphonate therapy in McCune-Albright syndrome and polyostotic fibrous dysplasia. J Bone Miner Res. 2017;32(2):264–76.
38. Boyce AM, Chong WH, Yao J, et al. Denosumab treatment for fibrous dysplasia. J Bone Miner Res. 2012;27(7):1462–70.
39. Kim KM, Park SJ, Jung S-H, et al. miR-182 is a negative regulator of osteoblast proliferation, differentiation, and skeletogenesis through targeting FoxO1. J Bone Miner Res. 2012;27(8):1669–79.
40. Inoue K, Deng Z, Chen Y, et al. Bone protection by inhibition of microRNA-182. Nat Commun. 2018;9(1):4108.
41. Rached M-T, Kode A, Xu L, et al. FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. Cell Metab. 2010;11(2):147–60.
42. Miller CH, Smith SM, Elguindy M, et al. RBP-J-regulated miR-182 promotes TNF-α-induced osteoclastogenesis. J Immunol. 2016;196(12): 4977–86.
43. Fang T, Wu Q, Zhou L, Mu S, Fu Q. miR-106b-5p and miR-17-5p suppress osteogenic differentiation by targeting Smad5 and inhibit bone formation. Exp Cell Res. 2016;347(1):74–82.
44. Liu K, Jing Y, Zhang W, et al. Silencing miR-106b accelerates osteogenesis of mesenchymal stem cells and rescues against glucocorticoid-induced osteoporosis by targeting BMP2. Bone. 2017;97:130–8.
45. Li X, Ji J, Wei W, Liu L. MiR-25 promotes proliferation, differentiation and migration of osteoblasts by up-regulating Rac1 expression. Biomed Pharmacother. 2018;99:622–8.
46. Huang Y, Ren K, Yao T, et al. MicroRNA-25-3p regulates osteoclasts through nuclear factor I X. Biochem Biophys Res Commun. 2020; 522(1):74–80.
47. Yang L, Cheng P, Chen C, et al. miR-93/Sp7 function loop mediates osteoblast mineralization. J Bone Miner Res. 2012;27(7):1598–606.
48. Zhang Y, Wei Q-S, Ding W-B, et al. Increased microRNA-93-5p inhibits osteogenic differentiation by targeting bone morphogenetic protein-2. PLoS One. 2017;12(8):e0182678.
49. Eferl R, Hoebertz A, Schilling AF, et al. The Fos-related antigen Fra-1 is an activator of bone matrix formation. EMBO J. 2004;23(14):2789–99.
50. Fujiwara T, Uotani K, Yoshida A, et al. Clinical significance of circulating miR-25-3p as a novel diagnostic and prognostic biomarker in osteosarcoma. Oncotarget. 2017;8(20):33375–92.
51. Ruggieri P, Sim FH, Bond JR, Unni KK. Malignancies in fibrous dysplasia. Cancer. 1994;73(5):1411–24.
52. Li M, Luo R, Yang W, Zhou Z, Li C. miR-363-3p is activated by MYB and regulates osteoporosis pathogenesis via PTEN/PI3K/AKT signaling pathway. In Vitro Cell Dev Biol Anim. 2019;55(5):376–86.
53. Woods S, Barter MJ, Elliott HR, et al. miR-324-5p is up regulated in end-stage osteoarthritis and regulates Indian Hedgehog signalling by differing mechanisms in human and mouse. Matrix Biol. 2019; 77:87–100.
54. Gu H, Wu L, Chen H, et al. Identification of differentially expressed microRNAs in the bone marrow of osteoporosis patients. Am J Transl Res. 2019;11(5):2940–54.
55. Karvande A, Kushwaha P, Ahmad N, et al. Glucose dependent miR-451a expression contributes to parathyroid hormone mediated osteoblast differentiation. Bone. 2018;117:98–115.

56. Robinson C, Collins MT, Boyce AM. Fibrous dysplasia/McCune-Albright syndrome: clinical and translational perspectives. Curr Osteoporos Rep. 2016;14(5):178–86.
57. Kuznetsov SA, Cherman N, Riminucci M, Collins MT, Robey PG, Bianco P. Age-dependent demise of GNAS-mutated skeletal stem cells and "normalization" of fibrous dysplasia of bone. J Bone Miner Res. 2008;23(11):1731–40.
58. Hart ES, Kelly MH, Brillante B, et al. Onset, progression, and plateau of skeletal lesions in fibrous dysplasia and the relationship to functional outcome. J Bone Miner Res. 2007;22(9):1468–74.
59. Riminucci M, Robey PG, Saggio I, Bianco P. Skeletal progenitors and the GNAS gene: fibrous dysplasia of bone read through stem cells. J Mol Endocrinol. 2010;45(6):355–64.
60. Kocijan R, Weigl M, Skalicky S, et al. MicroRNA levels in bone and blood change during bisphosphonate and teriparatide therapy in an animal model of postmenopausal osteoporosis. Bone. 2019;131:115104.
61. Hackl M, Heilmeier U, Weilner S, Grillari J. Circulating microRNAs as novel biomarkers for bone diseases - complex signatures for multifactorial diseases? Mol Cell Endocrinol. 2016; 432:83–95.