MicroRNAs as Potential Indicators of the Development and Progression of Uterine Leiomyoma

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Abstract

Recent studies demonstrated a significant role of several microRNAs (miRs) in the development of leiomyoma. Here, we investigated miR expression profiles using microarray and found a significantly higher expression of miRs in leiomyoma than in adjacent myometrium. We also confirmed the upregulation of five selected miRs including miR-181a-5p, 127-3p, 28-3p, 30b-5p and let-7c-5p in cellular proliferation, extracellular matrix turnover, and angiogenesis by RT-qPCR. Interestingly, the miRs showed a higher expression in cases of large leiomyoma or in patients with a history of transfusion due to anemia. We then analyzed the expression of the miR target molecules including TGFBR2 and IGF2BP1 via immunohistochemistry. TGFBR2 and IGF2BP1 were positively stained in 81% and 62.5% of leiomyoma tissues but not in adjacent myometrium. Both were more frequently positive in patients with ≥ 6 cm leiomyoma and mass effect. The mean expression levels of miR-181a-5p, 127-3p, 28-3p, 30b-3p and let-7c-5p were higher in cases with TGFBR2 and IGF2BP1 positive leiomyoma. These results provide insight into the role of miRs in the development and progression of leiomyoma and underscore the need to validate their utility as diagnostic or therapeutic targets.

Introduction

Uterine leiomyoma is the most common benign tumor in women of reproductive age and represent an important public health issue. Patients usually present with abnormal uterine bleeding and sometimes complain of abdominal discomfort due to compression by the mass. The manifestations change throughout lifetime such as continuously growing or decreasing mass, irregular bleeding and rarely malignant transformation. Several treatment options exist ranging from observation or medical therapy including iron supplementation, oral contraceptive, and progesterone, uterine artery embolization and focused ultrasound, hysteroscopy or laparoscopic myomectomy to hysterectomy. Therefore, precise diagnosis, regular follow-up, and adequate treatment are important especially when patients show symptomatic changes related to uterine leiomyoma[1]. Clinicians should evaluate not only the symptoms but also associated conditions such as anemia, fertility preservation, and the possibility of malignancy before performing hysterectomy.

Although uterine leiomyoma is the most common cause of hysterectomy, its pathogenesis remains unclear, and hence the limited number of treatment options available for symptomatic patients. Recent studies compared the microRNA (miR) expression of uterine leiomyoma and myometrium and reported that a few specific miRs such as miR − 150-5P[2], miR-29 family[3], and miRNAs let-7[4] played a pathogenic role in the development of leiomyoma. However, their clinical impact, specifically on disease severity, is not fully understood.

In our study, we analyzed miR in a sample set of 19 patients with leiomyoma using microarrays and real-time quantitative PCR (RT-qPCR). We observed distinct miR expression profiles between leiomyoma and adjacent myometrium and selected miRs showing prominent differences. We then selected two target molecules of selected miRs, TGFBR2 and IGF2BP1, for immunohistochemical analysis of corresponding

tissue samples. The reported findings validate the role of miRs in mediating changes associated with the development and severity of leiomyoma.

Results

Patient's characteristics. The clinical characteristics of 19 participants are summarized in Table 1. The median age was 46 years and BMI was 22.9 ± 2.7 kg/m^2. Irregular menstruation and heavy menstrual bleeding were reported by six (31.6%) and eight (42.1%) participants, respectively. Indications for surgery were mass effects including pressure symptoms and/or palpable mass (n = 8), heavy menstrual bleeding (n = 8) and both symptoms (n = 3). The mean size of the uterine leiomyoma was 7.3 ± 3.7 cm (2–15 cm) in its largest diameter. Eight patients (42.1%) received oral iron supplementation and three patients (15.8%) had a history of transfusion of packed red cells (PRC) due to anemia. Total laparoscopic hysterectomy was performed in 18 patients and myomectomy was conducted in 1 patient.

Table 1
Patient characteristics

Variables	n = 19
Age (years)	46 (40–50)
Height (cm)	158.0 ± 4.8
Weight (kg)	57.5 ± 7.6
BMI (kg/m^2)	22.9 ± 2.7
Parity	2 (0–3)
Irregular menstruation	31.6% (n = 6)
Dysmenorrhea	
No	3 (15.8%)
Mild	10 (52.6%)
Moderate	5 (26.3%)
Severe	1 (5.3%)
Symptom of uterine leiomyoma	
Heavy menstrual bleeding	8 (42.1%)
Mass effect (palpable mass)	8 (42.1%)
Both symptoms	3 (15.8%)
Size of leiomyoma (cm)	7.3 ± 3.7 (2–15)
Hematologic test	
Hgb (g/dL)	11.3 ± 2.5 (4.9–14.3)
Hct (%)	34.4 ± 6.8 (18.8–43.2)
Oral iron supplement	42.1% (n = 8)
History of transfusion of PRC	15.8% (n = 3)
Data are expressed as median (range) or mean ± standard deviation.	
BMI, body mass index; Hgb, hemoglobin; Hct, hematocrit; PRC, packed red cells.	

miR microarray analysis. Hierarchical clustering analysis [Euclidean method, complete linkage] was performed using the miR profiles of 10 leiomyomas and 6 samples of myometrium. MiR profile distinguished leiomyoma from myometrium and both were divided into two subcategories (Fig. 1). A volcano plot was generated to visualize the differential expression of the two conditions and a multidimensional scaling plot was generated to represent the relationship between objects differing in

miR data, as shown in Fig. 2. To identify the most significant candidates, miRs with at least 3.0-fold expression variation were selected (Supplementary Table 2). A total of 89 miRs were expressed differentially between leiomyoma and myometrium ($P < 0.05$) and among them 43 miRs showed FDR < 0.05. Compared with the myometrium, the leiomyoma showed that 26 of the 43 miRs were up-regulated and 17 miRs were down-regulated, according to the criteria. The miR DB expressed differentially between leiomyoma and myometrium included 14 up-regulated miRs including hsa-miR-181a-5p, hsa-miR-4429, hsa-miR-423-5p, hsa-miR-7110-5p, hsa-miR-320e, hsa-miR-877-5p, hsa-let-7i-5p, hsa-let-7b-5p, hsa-miR-130a-3p, hsa-let-7f-5p, hsa-let-7c-5p, hsa-miR-100-5p, hsa-miR-199a-3p and hsa-miR-199b-3p, and 14 down-regulated miRs including hsa-miR-4270, hsa-miR-4739, hsa-miR-6775-5p, hsa-miR-4687-3p, hsa-miR-4530, hsa-miR-320e, hsa-miR-3141, hsa-miR-7847-3p, hsa-miR-4459, hsa-miR-4298, hsa-miR-6722, hsa-miR-7162-3p, hsa-miR-8075 and hsa-miR-6732-5p.

RT-qPCR of miRNA expression. Five selected miRs (miR-181a-5p, miR-127-3p, miR-28-3p, miR-30b-3p, and miR-let-7c) were expressed higher in leiomyoma than in myometrium. However, no statistically significant difference existed between the two groups due to high variation among samples (Supplementary Fig. 1). We then compared the expression according to clinical characteristics including size of leiomyoma, delivery type, history of transfusion due to anemia, and iron supplementation therapy. Their relative expression levels were correlated with the size of leiomyoma ($P < 0.05$, Supplementary Table 3). Leiomyoma samples from patients with a history of PRC transfusion due to anemia showed higher expression levels of the five miRs ($P < 0.05$; Fig. 3a).We also found that miR-181a-5p, 127-3p, 28-3p, 30b-3p and let-7c-5p levels were significantly higher in large leiomyoma (≥ 11 cm) than in leiomyoma measuring less than 11 cm ($P < 0.05$, Fig. 3b).

Clinical significance of TGFBR2 and IGF2BP1 expression. We identified the predicted target molecules via web-based computational approaches (miRDB; http://mirdb.org, ver 4.0, miRBase rel.18.0). TGFBR2 and IGF2BP1 were selected as the common target genes of differentially expressed miRs using available antibodies in IHC. IHC was successfully performed for two targets in 16 samples. We found that TGFBR2 and IGF2BP1 were positively stained in 13 (81%) and 10 (62.5%) leiomyoma tissues; however, neither of them was stained in the adjacent myometrium (Supplementary Fig. 2). IGF2BP1 was more frequently positive in patients with ≥ 6 cm leiomyoma, irregular menstruation and mass effect. TGFBR2 was more frequently positive in patients with ≥ 6 cm leiomyoma and mass effect (Table 2).The mean expression levels of miR-181a-5p, 127-3p, 28-3p, 30b-3p and let-7c-5p were higher in patients with IGF2BP1- and TGFBR2-positive leiomyoma. However, no statistical significant difference was found between the two groups (Supplementary Fig. 3).

Table 2
Comparison of IGF2BP1 and TGFBR2 expression

	IGF2BP1			TGFBR2		
	Positive	**Negative**	P	**Positive**	**Negative**	P
No. patients	10	6		13	3	
Leiomyoma size (≥ 7 cm)	80%	16.7%	0.013	69.2%	0%	0.029
Irregular menstruation	50.0%	0%	0.037			
Mass effect	80.0%	16.7%	0.013	69.2%	0%	0.029
Data describe median (range) or mean ± standard deviation.						
No., number; Hgb, hemoglobin; PRC, packed red cells.						

Discussion

We investigated the pathogenesis of leiomyoma based on miR expression profile and identified several significant miRs and their association with clinical findings. Differentially expressed miRs in leiomyoma were selected compared with those in adjacent myometrium in order to provide additional evidence of pathogenesis. Previous studies also revealed that a subset of miRs were significantly dysregulated in leiomyomas compared with matched myometrial samples[5–7] and many of these miRs were also associated with other neoplasms, indicating their general role in tumorigenesis[8]. These miRs are crucial regulators of physiological events including cellular proliferation, apoptosis, extracellular matrix turnover, angiogenesis and inflammation of uterine leiomyoma[9,10]. Recent studies reported that these miRs act as tumor suppressors and anti-apoptotic mediators (let-7 family, miR-17-92 cluster, miR-372-373, miR155-BIC, miR 15/16, miR-20, miR-21, miR-26a), and play a role in differentiation and hypertrophy (let-7 family, miR-181b, miR-21), inflammation (miR-125, miR-155) and in tissue remodeling/extra-ECM turnover (miR-21, miR-192, miR-206, miR-1, miR-133a) [2,4,9,10].

We measured the expression of 5 up-regulated miRs in leiomyoma including miR-181a-5p, 127-3p, 28-3p, 30b-5p, and let-7c-5p using RT-qPCR, and found their association with the size of leiomyoma. The expression of miR-181a-5p and 127-3p strongly up-regulated during myoblast differentiation[11–13]. miR-181a is recognized as a senescence-associated miR and a potent negative regulator of Akt pathway[14]. It also affected the expression of insulin-like growth factor binding proteins (IGFBP), which might be of some importance in leiomyomas[15]. miR-127-3p is expressed at significantly higher level in proliferating rather than differentiating cells[16]. The miR-28-3p is a crucial regulator of myogenic differentiation of rhabdomyosarcoma cells and myoblasts[17]. The expression of miR-30b-5p was associated with differentiation of both skeletal and vascular smooth muscles via interaction with MBNL1 and/or MBNL2 [18,19]. let-7 is one of the most widely studied miRNAs and let-7c-5p is important for cell growth,

proliferation and inflammation[20]. let-7 expression was significantly decreased in leiomyosarcoma tissues, which may contribute to the malignant transformation of leiomyoma accompanied by overexpression of HMGA2 [21,22].Therefore, we postulated that the up-regulated miRs modify target molecules in addition to proliferation and differentiation of muscle cells and were associated with the development and growth of leiomyoma.

We investigated the expression of two target molecules of those miRs via IHC. Leiomyoma tissues but not the adjacent myometrium stained positive for TGFBR2 and IGF2BP1. TGFBR2 is a receptor of a secreted polypeptide TGF-β2, which regulates leiomyoma growth [23].The IGF2BP1, an oncofetal RNA-binding protein, has been identified to play an important role in cell proliferation and growth of normal tissues and tumor tissues, as well as tumor cell adhesion, apoptosis, migration, and invasion[24]. Studies have demonstrated that fibroblasts and smooth muscle cells from leiomyoma tissue not only increase the expression and secretion of growth factors including TGF-β and IGF, but also up-regulate their corresponding receptors[25–27]. Notably, the overexpression of both proteins was associated with leiomyoma size and mass effect. Therefore, we strongly suspected that such changes in miR up-regulated TGFBR2 and IGF2BP1 in leiomyoma tissue and affected its growth.

Although we have investigated the pathogenic role of miRs in leiomyoma, the study has some limitations. First, the number of cases was relatively small to demonstrate the clinical significance other than leiomyoma size. Second, no direct association between miRs and protein expression was identified. Previous studies suggested that increased miRs such as miR-181a-5p, 28-3p and let-7 family may represent new therapeutic targets in various diseases[17,28,29]. Further studies are needed to validate the significance of the miRs as diagnostic or therapeutic targets.

Taken together, the results demonstrated that miRs played a significant role in the pathogenesis of leiomyoma. Particularly, the five miRs including miR-181a-5p, 127-3p, 28-3p, 30b-3p and let-7c-5p, and their target molecules including TGFBR2 and IGF2BP1, were up-regulated in leiomyoma tissues. Additionally, their expression was associated with leiomyoma size and size effect. These findings are significant in understanding of miRs and their role as a potential indicator of leiomyoma development and disease progression.

Methods

Study Subjects and Tissue Specimens. The study enrolled 19 women who received surgical management for uterine leiomyoma (heavy menstrual bleeding, palpable mass) from May, 2018 to February, 2019 in a university hospital. The study received institutional review board approval from Bucheon St. Mary's Hospital, The Catholic University of Korea (HCI8SESI0037), and all participants provided written informed consent. We conducted this study under the amended Declaration of Helsinki on the ethical conduct of research involving human subjects. Clinical data were analyzed through chart review, and the association between miR and cytokine was investigated.

Uterine tissues were collected from leiomyoma mass and adjacent (within 2 cm) myometrial tissues within 1 hour of surgery. MiR was extracted from RNA in later-preserved tissues using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) at -80ºC, following the manufacturer's protocol. RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

miR profiling using array. The miRs of 10 leiomyoma masses and 6 myometrial tissues were profiled using the Affymetrix Genechip miR 4.0 array according to the manufacturer's protocol. RNA samples (130 ng) were labeled with the FlashTag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA). The labeled RNA was quantified, fractionated and hybridized to the miR microarray according to the standard procedures provided by the manufacturer. The labeled RNA was heated to 99°C for 5 min and then to 45°C for 5 min. RNA-array hybridization was performed via agitation at 60 rotations per min for 16 h at 48°C on an Affymetrix Hybridization oven. The chips were washed and stained using a Genechip Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA). The chips were then scanned with an Affymetrix GCS 3000 scanner (Affymetrix). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Raw data were extracted automatically via Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip® Command Console® Software (AGCC). The CEL files were imported and miRNA level RMA + DABG were analyzed. All analyses and results were exported using Affymetrix® Power Tools (APT) Software. After normalization, significant differentially expressed miRNAs were identified through Volcano Plot filtering between the two experimental groups. Finally, hierarchical clustering was performed for distinct miR expression profiling of the samples. The differentially expressed miRNAs were identified by a fold change of ≥ 3.0.

miR quantification using reverse transcription quantitative PCR (RT-qPCR). RT-qPCR was performed to validate the expression of miRs using 38 samples including 19 pairs of myometrium and leiomyoma. After a database search and literature review, miR-181a-5p, miR-127-3p, miR-28-3p, miR-30b-5p and let-7c-5p were selected for further validation (Supplementary Table 1). MicroRNA TaqMan® Reverse Transcription Kit and TaqMan MicroRNA Assays were used (Applied Biosystems, Foster City,CA, USA). U6 small nuclear 2 (RNU6b) was selected to normalize miR expression levels. RT-qPCR was performed using the Lightcycler 480 PCR system (Roche, Basel, Switzerland). PCR reaction mixtures containing 10 ng DNA, 20X Taqman microRNA assay, 2X Taqman master mix (Applied Biosystems) were prepared and reactions were performed under the following conditions: 1 cycle of polymerase activation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec, followed by annealing/extension at 60°C for 1 min. Results were analyzed using Lightcycler 480 instrument software 1.2 (Roche). Each experiment was performed in duplicate.

Immunohistochemistry (IHC) of TGFBR2 and IGF2BP1. IHC was performed to evaluate the expression of TGFBR2 and IGF2BP1 in 16 leiomyoma tissues. Tissues were fixed in formalin and embedded in paraffin, and 3-μm-thick paraffin sections were obtained. IHC was performed using an automated immunohistochemical stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's protocol. The sections were deparaffinized and pretreated with a cell-conditioning solution

(CC1, Ventana), followed by UV irradiation to abrogate the endogenous hydroperoxidase activity. The primary antibodies were diluted in Dako antibody diluent (Dako Cytomation, Glostrup, Denmark) with background-reducing components and used under the following dilutions: TGFBR2 (1:100, ab61213, Abcam, Franklin Lakes, NJ, USA) and IGF2 BP1 (1:100, ab82968, Abcam,). The sections were incubated with primary antibodies at room temperature for 32 min, and hybridized with HRP-conjugated secondary antibody (Ventana) for 8 min. The reaction was developed with diaminobenzidine (DAB; Dako) for 5 min and the slides were counterstained with hematoxylin II (Ventana) for 4 min and bluing reagent (Ventana) for 4 min. The sections were observed under light microscope (BX50, Olympus, Japan).

Statistical analysis. The comparative analysis of test and control samples was carried out using an independent t-test and fold change was determined, based on the null hypothesis stating that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting the p value using Benjamini-Hochberg algorithm. All statistical tests and visualization of differentially expressed genes were conducted using R statistical language 3.3.3.

In real-time PCR, statistical analysis of the association between miR expression and different tumor histotypes was conducted using the non-parametric Mann-Whitney test. The Tukey's multiple comparisons test was used to evaluate the differences in miR expression according to clinicopathological parameters. Pearson’s chi-square test was utilized to analyze the relationship between parametric data. Pearson’s correlation was performed for the quantitative analysis of size of leiomyoma and miR expression. All statistical analyses were performed using SPSS Statistics for Windows, Version 20.0 (IBM Corp., Amonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Declarations

Acknowledgments

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Author contributions

M.J.K conceived and designed the experiments. H.J.L, M.J.K and M.Y.K performed the experiments. M.K and M.J.K analyzed the data and revised the paper. All authors approved the final manuscript.

Competing interests

The authors have no conflicts of interest to report. The authors alone are responsible for the content and writing of this paper.

Availability of materials and data

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Figures

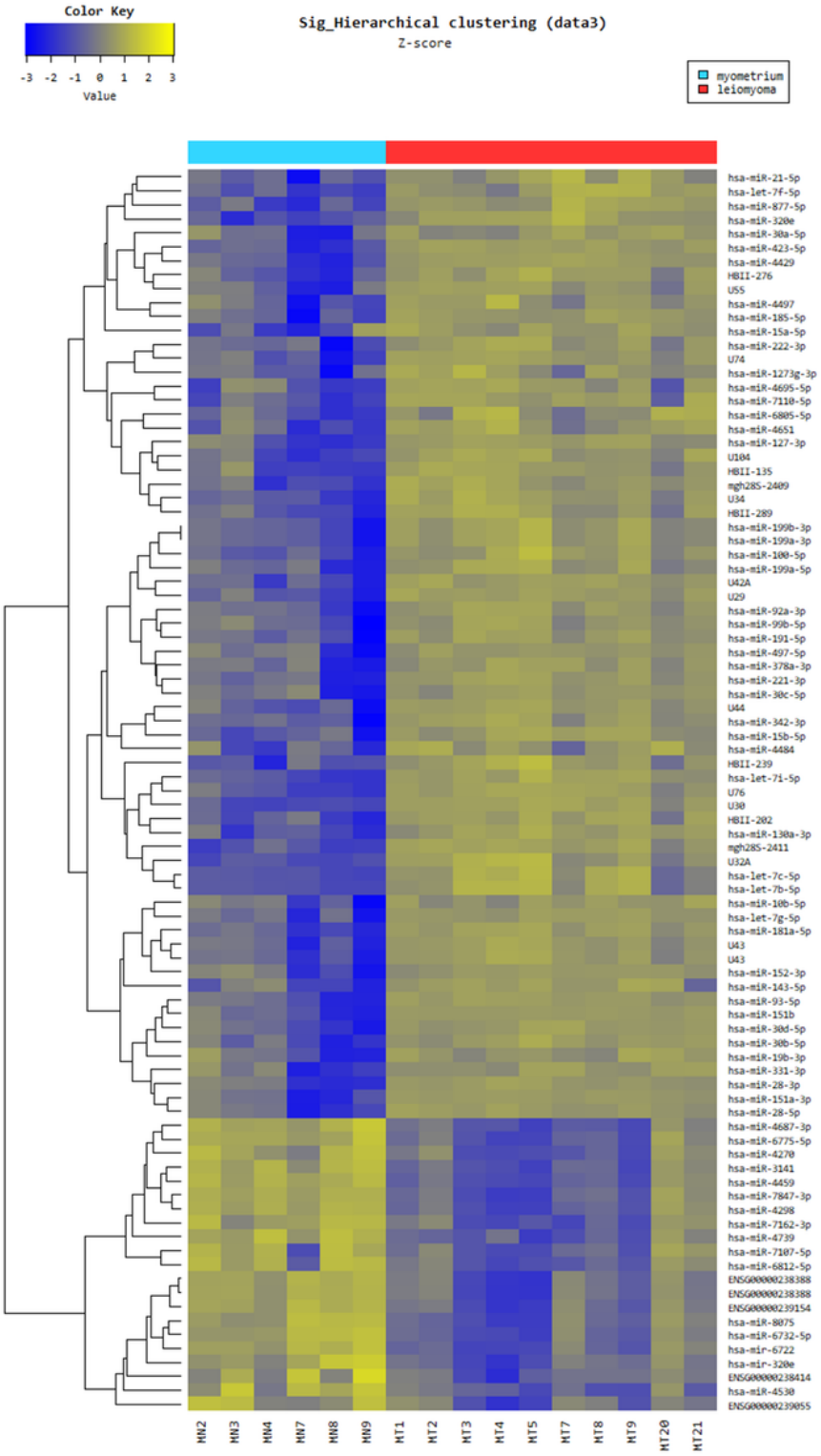

Figure 1

Differential expression of miRNAs between leiomyoma (n = 10) and myometrium (n = 6). Yellow color indicates high relative expression and blue color denotes low relative expression. miRNA with expression fold change >3.0 and P < 0.05 was considered statistically significant.

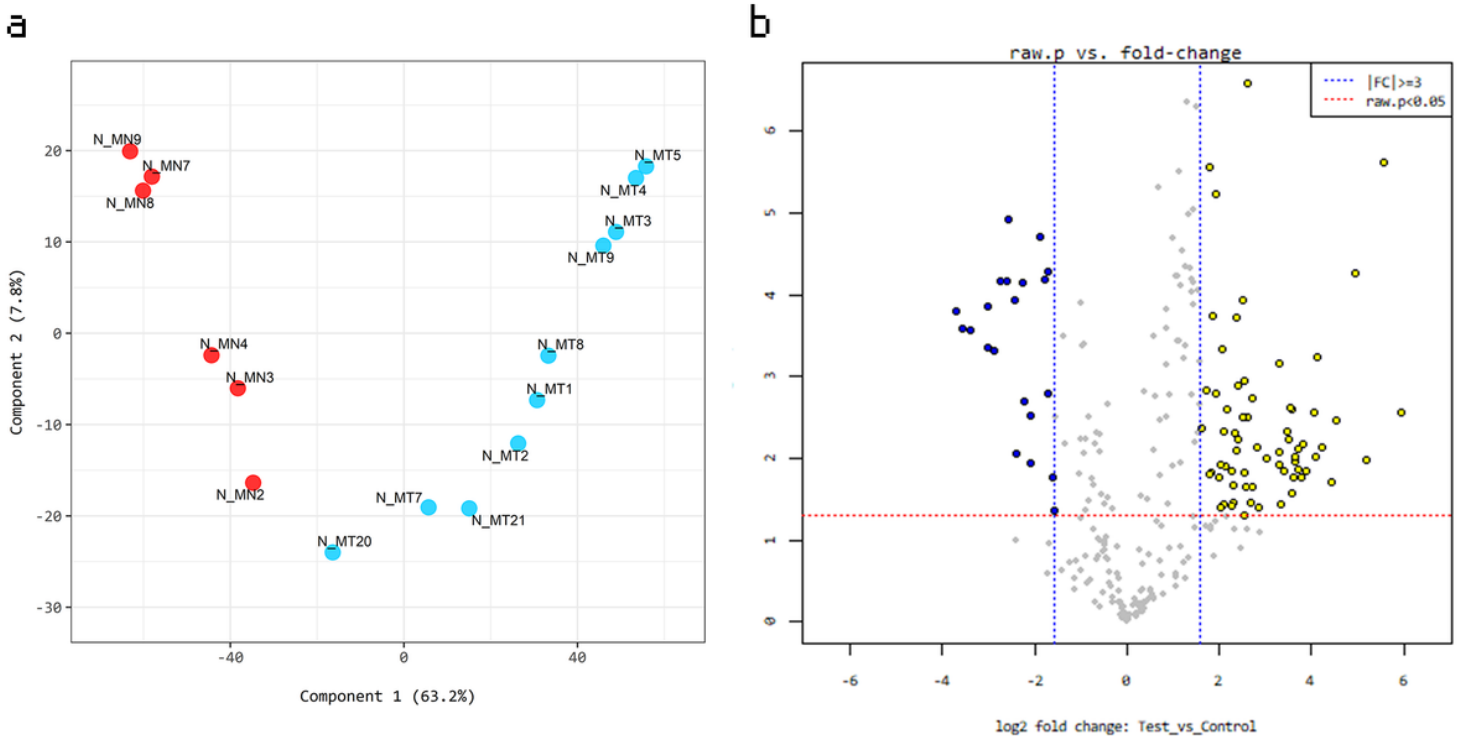

Figure 2

Multidimensional scaling (MDS) plot (a) and volcano plot (b) of the miRNA microarray analysis. (a) MDS plot shows similarity of miRNA profiles in leiomyoma (MT) and adjacent myometrium (MN). The dots represent samples colored by group. (b) Volcano plots of aberrantly expressed miRNAs in leiomyoma. The vertical lines correspond to 1.5-fold up-regulation and down-regulation, respectively, and the horizontal line represents a P value of 0.05. Abbreviation: FC, fold change.

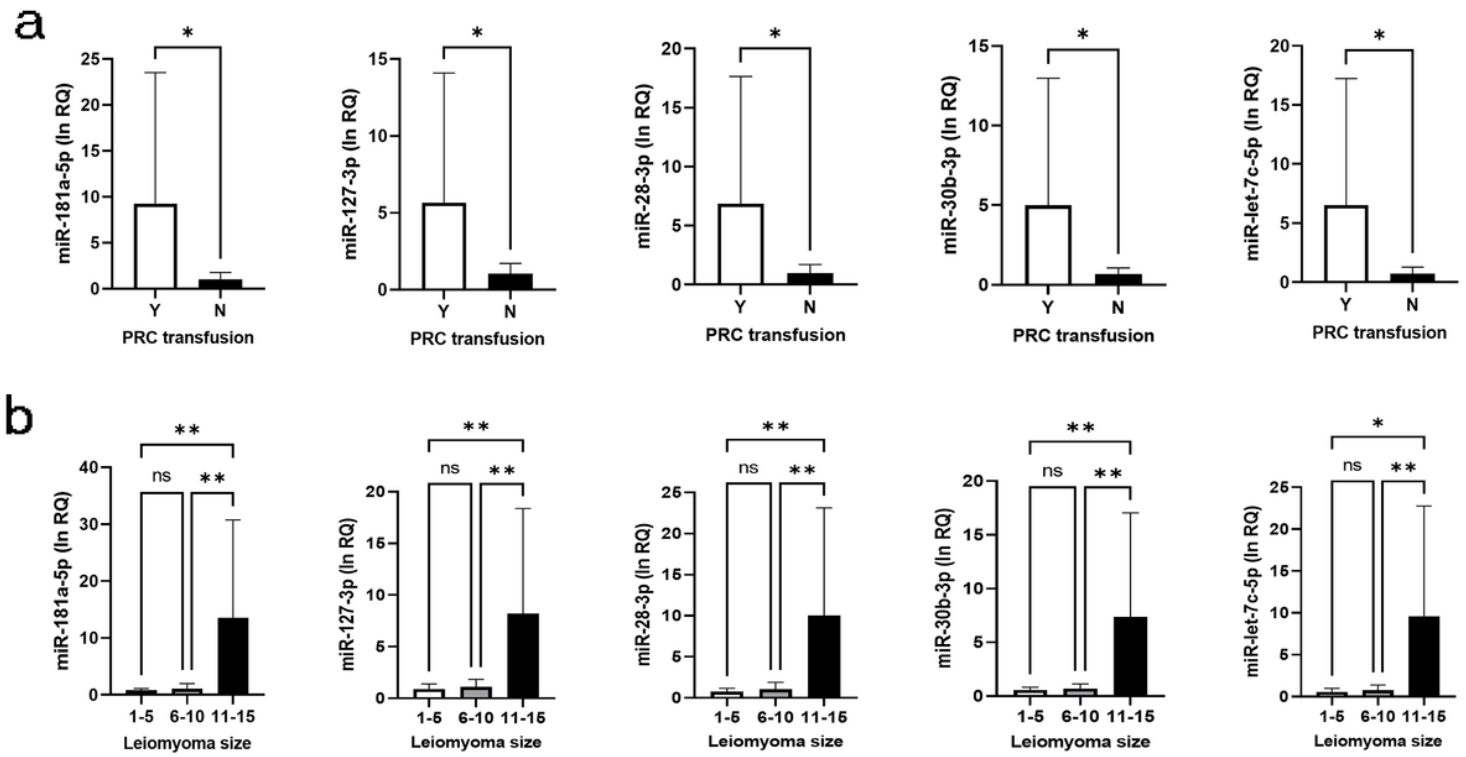

Figure 3

Comparative analysis of miRNA expression using RT-qPCR. All five miRNAs show significantly higher expression in large leiomyoma (≥ 11 cm) (a) and (b) patients who had history of PRC transfusion due to anemia (Y) compared with those without (N). Results are presented as mean ± SD calculated using data obtained from independent samples of 19 leiomyomas and adjacent myometrial tissue.

Supplementary Files

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- Supplementaryinformation0825.pdf