Expression signature of microRNA-181-a reveals its crucial role in the pathogenesis of paediatric systemic lupus erythematosus

Y.A. Lashine[1], A.M. Seoudi[1], S. Salah[2], A.I. Abdelaziz[1]

[1]The Molecular Pathology Research Group, German University in Cairo, Cairo;
[2]Department of Paediatrics, Kasr El Einy Hospital, Cairo University Medical School, Cairo, Egypt.

Abstract

Objectives

Systemic lupus erythematosus (SLE) is an autoimmune disease manifested by self-reactive antibodies due to failure of selection in both B and T lymphocytes leading to immune intolerance accompanied by increased rate of apoptosis and deficiency in the clearance of the apoptotic cells. Micro RNAs regulate posttranscriptional gene expression and have been recently identified to regulate cellular differentiation, establishing immunological tolerance and are involved in the pathogenesis of several diseases. miR-181-a, expressed in haematopoietic cell lineage, has shown to be an important modulator of B and T cell differentiation, maturation and function. This study aims to identify the expression signature of miR-181-a in the peripheral blood of paediatric SLE and its regulatory effect on the consequent expression of its target gene PCAF.

Methods

Twenty SLE paediatrics patients, 9 healthy controls and 4 FMF patients were enrolled in this study. The relative expression of miR-181-a, miR-223, PCAF and Hdm2 were performed using quantitative real time PCR.

Results

For the first time we show that miR-181-a was significantly downregulated in SLE pediatrics as compared to healthy controls. Furthermore, miR-181-a showed significant difference in its expression among groups with different SLEDAI scores. This special signature of miR-181-a expression is unique to SLE as compared to FMF samples which showed a parallel expression to healthy controls. PCAF was upregulated in SLE patients compared to healthy controls, which has an impact on the ubiquitination of Hdm2 and hence releases p53 leading to the induction of apoptosis.

Conclusion

miR-181-a plays an important role in SLE pathogenesis.

Key words

miR-181-a, paediatrics SLE, PCAF, Hdm2.

*Yasmine Adel Lashine, MD, MSc**
*Aliaa Mahmoud Seoudi, MSc**
Samia Salah, MD, PhD, Professor
Ahmed Ihab Abdelaziz, MD, PhD, Assist. Professor

**Both authors contributed equally to the study*

Please address correspondence and reprint requests to:
Dr Ahmed Ihab Abdelaziz,
The German University in Cairo – GUC,
New Cairo City,
Main Entrance Al Tagamoa Al Khames,
11835 Cairo, Egypt.
E-mail: ahmed.abdel-aziz@guc.edu.eg

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Introduction

Micro RNAs (miRNAs or miRs) were recently reported to be essential in the process of haematopoiesis, since miR-223 is distinctively expressed in myeloid cells (1, 2), and miR-150 is involved in B- and T-cell development (3). Furthermore, the selective disruption of miRNAs in regulatory T cells (T reg) has led to severe uncontrolled autoimmune disease showing the role of miRNAs in the development of autoimmunity (4). Additionally, several miRNAs have shown different expression patterns in polycythemia vera (PV) patients (5). miR-181-a is known for its involvement in haematopoiesis and was found to be expressed in brain, lungs and with the highest relative expression in the thymus; demonstrating its role in the maturation of T lymphocytes (6). miR-181-a was reported to be dynamically regulated during thymocytes differentiation showing the highest expression level in early stages of T-cell differentiation especially in both double positive (DP) and double negative (DN3) thymocytes; indicating the importance of miR-181-a in T-cell selection (7). miR-181-a was also shown to modulate TCR-mediated T cell activation, as well as augmentation of both strength and sensitivity of TCR signalling to strong and weak agonists suggesting its critical role in the development, function and maintenance of T-cell tolerance (8). miR-181-a has previously shown high expression in human B and T cells, furthermore the ectopic expression of miR-181-a in haematopoietic stem cells led to significant increase in the B-cell lineage fraction (1, 6). The hallmark in the pathogenesis of SLE is the presence of auto-antibodies as antinuclear antibodies (ANA), anti-DNA antibodies, and anti-Smith antibodies (Anti-Sm). The occurrence of these auto-antibodies is a consequence of several defects in multiple crucial checkpoints in the B- and T-cell tolerance and maturation, rendering these cells self-reactive (9). The existence of functionally and phenotypically abnormal T- helper cells in the peripheral blood of SLE patients, in addition to the decline in the T reg cells in SLE paediatric patients during disease activity, suggest the role played by the T-cell intolerance in the pathogenesis of SLE. This was further confirmed when the use of anti-CD4 antibody has shown improvement of SLE disease in murine mice (10-12). The interplay between B- and T-cell immaturity with excessive apoptosis and deficient clearance of apoptotic bodies is the chief dogma behind the pathogenesis of SLE (13). Those abnormalities in apoptosis are suspected to occur specifically during the lymphocyte maturation and differentiation stages, owing to the existence of autoreactive B and T lymphocytes (14). Increased apoptosis was attributed to the over-expression of p53 protein levels in SLE patients (15). Two main key players in the loop of control of the tumour suppressor protein p53 are p300-CBP-associated factor (PCAF) and human double minute oncoprotein (Hdm2) (16, 17). As inferred from its name, PCAF integrates with p300 and CREB binding protein (CBP) acting as a cofactor for transcription activation of a multiplicity of genes promoting cell differentiation, cell cycle arrest, inhibiting cell migration and suppressing tumourigenesis and tumour growth (18). Other than the N-acetyltransferase domain, which is vital for most of its known activities, PCAF was reported to have an intrinsic ubiquitination activity that is critical in controlling Hdm2 protein levels; therefore, it indirectly controls p53 protein levels (19). PCAF was previously identified as one of the predicted targets of miR-181-a (20). A recent study correlated miR-181-a expression and its effect on PCAF expression in the pathogenesis of multiple myeloma (MM), this study showed that miR-181-a was highly expressed in most of the MM samples and MM cell lines. PCAF expression was almost absent in most of the MM cell lines following the increase in miR-181-a expression, therefore down regulating the expression of p53 with a consequent decrease in apoptosis (20). From the above-mentioned findings it is expected that SLE and MM are inversely correlated, regarding their apoptotic behaviour. The behaviour of micro-RNAs in SLE pathogenesis was recently tested suggesting the miR-184,

miR-198 and miR-21 to be SLE specific miRNAs (21). However the miR-181-a was never mentioned to have any significant pattern of expression in SLE. This study aims to investigate the expression signatures of miR-181-a and its target gene PCAF and their roles in the pathogenesis of SLE.

Patients and methods

A total of 20 paediatric patients (2 males and 18 females) diagnosed with SLE disease were included in this study. All patients were diagnosed for SLE according to the 1982 revised criteria for the classification of systemic lupus erythematosus (22). Ten patients were maintained on corticosteroid therapy, those patients were designated "high-dose" and the other 10 patients were either on mild-dose corticosteroid therapy (≤7.5 mg/day, n=4) or recently diagnosed by the time of sample taking (n=6) and those were annotated as "low-dose" and "no treatment" respectively. Each patient was given a score according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) by the time of blood draw. All patients were further divided according to their SLEDAI scores into patients with SLEDAI score of more than or equal 10 (high SLEDAI, n=11) and patients with SLEDAI score of less than 10 (low SLEDAI, n=9). Additionally 9 age-matched healthy volunteers were used as controls and 4 Familial Mediterranean fever (FMF) paediatric patients were used for comparison. All healthy volunteers were free of any concurrent infection by the time of sample taking. A written informed consent was received from the legal guardians of all patients and controls. Patients' data are shown in Table I.

Sample collection

Blood samples from SLE and FMF patients were collected from Abou-El Reesh Hospital, Cairo University and the control samples were collected from healthy controls. Five ml of peripheral venous blood was collected in the presence of an anticoagulant (EDTA) from healthy controls and SLE patients. For isolation of peripheral blood mononuclear cells (PBMCs) the Ficoll density gradient centrifugation method was used.

Total RNA and miRNA extraction

Total RNA extraction and MicroRNA extraction were done by mirVana miRNA extraction kit and performed according to the manufacturer's protocol (Ambion Applied Biosystems, USA).

Reverse transcription of miRNA and mRNA

Five µl of the extracted miRNA sample were reverse transcribed into single-stranded complementary DNA (cDNA) using the High-capacity cDNA reverse transcription Archive kit (Applied Biosystems, USA) and 3 µl of the RT primer from each assay set were used. The miRNAs assays used were miR-181-a, miR-223 and U6 small nuclear RNA (RNU6B) served as an internal control. Ten µl of the isolated total RNA was also used to generate single stranded complementary DNA (cDNA) by the High-capacity cDNA reverse transcription Archive kit (Applied Biosystems, USA).

Expression profiling of miR-181-a, miR-223, PCAF and Hdm2 in SLE patients, FMF patients and controls

miR-181-a and miR-223 levels were relatively quantified using quantitative real time PCR, ABI Prism 7000 (Applied Biosystems, USA). Reagents used were the miR-181-a assay, the miR-223 assay and the RNU6B assay (Applied Biosystems, USA), added to the TaqMan Universal master mix (Applied Biosystems, USA). The relative expression of each sample was normalised to RNU6B. Relative expression of PCAF and Hdm2 were quantified using TaqMan Real-Time Quantitative Polymerase Chain Reaction, ABI Prism 7000 (Applied Biosystems USA). The mRNA relative quantitation of each sample was normalised to that of GAPDH. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. All PCR reactions including controls were run in duplicate reactions.

Statistical analysis

The expression of miR-181-a, miR-223, RNU6B, PCAF, Hdm2 and GAPDH were expressed in relative quantitation ($RQ=2^{-\Delta\Delta CT}$) and the data are expressed as median. Mann-Whitney U-test was used to compare miRNA and mRNA relative expression among different sample groups. Parametric student *t*-test was used to compare miR-181-a relative expression in comparison between high SLEDAI and low SLEDAI patients. A *p*-value of less than 0.05 was considered statistically significant. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and ns = statistically insignificant.

Table I. Clinical features of all the 20 paediatric SLE patients.

Characteristic	No treatment SLE patients (n= 6)	Low-dose SLE patients (n=4)	High-dose SLE patients (n=10)	FMF Patients (n=4)	Healthy controls (n=9)
Sex: Male / Female	0/6	0/4	2/8	0/4	3/6
Age (yr), mean ±SEM	9.5±1.15	10±1	9.7±1.2	11.5±1.2	12±1.4
SLEDAI , mean ±SEM	10.7 ± 2.6	12.25±1.3	7.6±2	_	_
Disease duration (yr), mean ±SEM	Freshly diagnosed	1.25 ± 0.59	1.6±0.4	1.7±0.5	_
Anti-dsDNA , +ve/ -ve	2/4	2/2	4/ 6	_	_
ANA, +ve/ -ve	6/0	4/0	10/0	_	_
Proteinuria, +ve /-ve	3/3	1/3	3/7	_	_
Cytopenia, +ve/ -ve	2/4	1/3	3/7	_	_
CNS involvement, +ve/ -ve	0/6	2/2	0/10	_	_
Prednisone intake, +ve /-ve	0/6	4/0	10/0	_	_
Hydroxychloroquine intake, +ve /-ve	0/6	3/1	10/0	_	_
Azathioprine intake, +ve/ -ve	0/6	2/2	8/2	_	_

Results

Relative expression of miRNA-181-a in peripheral blood of SLE patients and healthy controls

miR-181-a showed a marked down regulation in all SLE patients as com-

pared to controls, p=0.0004. miR-181-a showed significant downregulation in SLE patients with no treatment as compared to controls, p=0.0004. It also showed a significant downregulation in SLE patients on low-dose corticosteroid treatment as compared to controls, p=0.0196. Patients on high-dose treatment also showed marked downregulation of their miR-181-a expression as compared to controls, p=0.0057. However miR-181-a showed no significant difference between patients with no treatment *versus* patients on low-dose or high-dose treatment, p=0.3524 & p=0.9578 respectively. There was not either a significant difference between low-dose and high-dose patients in miR-181-a expression, p=0.7333 (Fig. 1).

The relative expression of miR-181-a in reference to the SLEDAI scores in SLE paediatrics

miR-181-a showed significant down regulation in SLE patients with high SLEDAI score (SLEDAI score ≥10) in comparison to normal healthy controls with a relative quantitation RQ [0.1447±0.03165 (n=11) and 1.486±0.3616 (n=13), respectively, p=0.0026]. Furthermore, miR-181-a showed significant downregulation in SLE patients with low SLEDAI score (SLEDAI score <10) in comparison to healthy controls with a relative quantitation RQ [0.3480±0.09702 (n=9) and 1.486±0.3616 (n=13) respectively, p=0.0188]. miR-181-a also showed significant downregulation in SLE paediatric patients with high SLEDAI score in comparison to SLE paediatric patients with low SLEDAI score with a relative quantitation RQ [0.1447±0.03165 (n=11) and 0.3480±0.09702 (n=9) respectively, p=0.0444] (Fig. 2).

Relative expression of miRNA-181-a in peripheral blood of SLE and FMF patients

miR-181-a showed significant down regulation in SLE patients in comparison to controls and FMF patients, p=0.0004 and p=0.0029, respectively. At the same time, FMF patients showed no significant change in miR-181-a expression as compared to controls, p=0.7105 (Fig. 3).

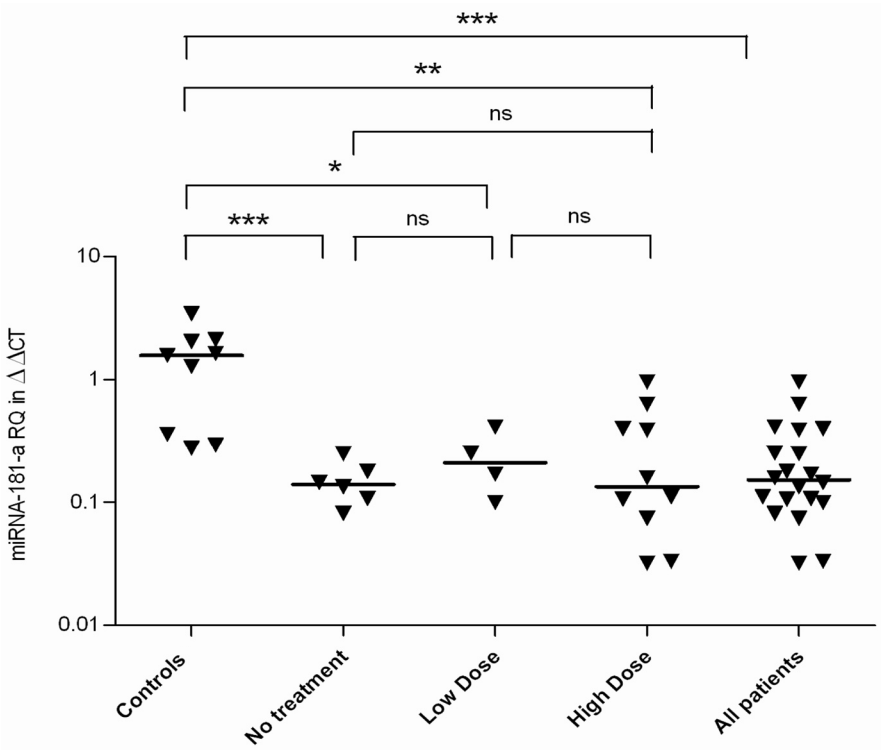

Fig. 1. Relative expression of miRNA-181-a represented by RQ in peripheral blood of SLE patients and healthy controls

The relative expression of miR-181-a in 20 SLE paediatric patients in comparison to 9 healthy controls. The 20 SLE paediatric patients were subdivided into 6 patients with no treatment, 4 patients on low-dose corticosteroid therapy and 10 patients on high-dose treatment. miR-181-a was significantly downregulated in patients on high-dose, patients on low-dose and patients with no treatment as compared to controls. Values are expressed as median.

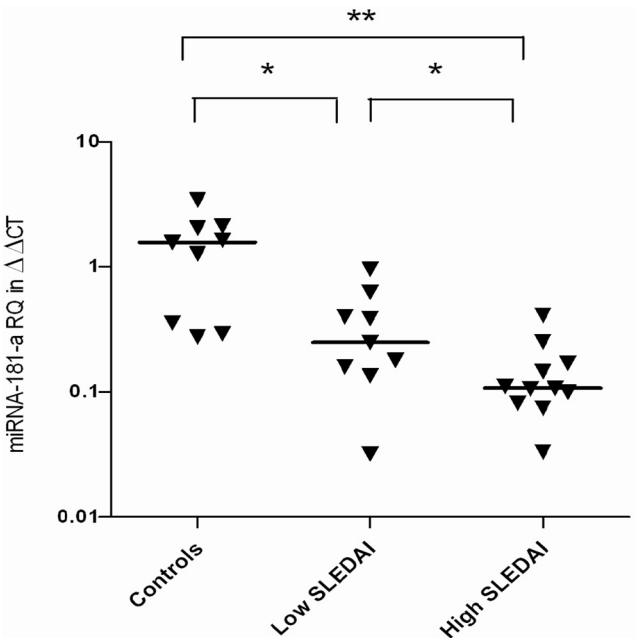

Fig. 2. The correlation between miR-181-a expression and the SLEDAI scores in SLE paediatric patients.

The relative expression of miR-181-a in 9 SLE paediatric patients with low SLEDAI score (SLEDAI <10) and 11 SLE paediatric patients with high SLEDAI score (SLEDAI ≥10) as compared to 9 healthy controls. miR-181-a was significantly downregulated in SLE patients with high SLEDAI score compared to SLE patients with low SLEDAI score. miR-181-a was significantly downregulated in SLE patients with low SLEDAI score compared to healthy controls. Values are expressed as mean ± SEM.

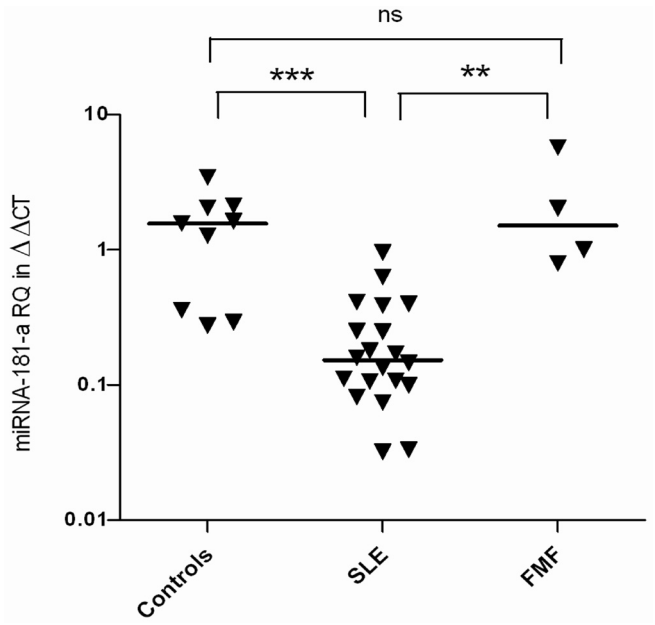

Fig. 3. Relative expression of miRNA-181-a represented by RQ in the peripheral blood of SLE and FMF patients.
The relative expressions of miR-181-a in 20 SLE paediatric patients as compared to 9 healthy controls and 4 FMF patients. miR-181-a was significantly downregulated in SLE patients in comparison to healthy controls and FMF patients. FMF patients showed a similar pattern of miR-181-a expression as compared to healthy controls. Values are expressed as median.

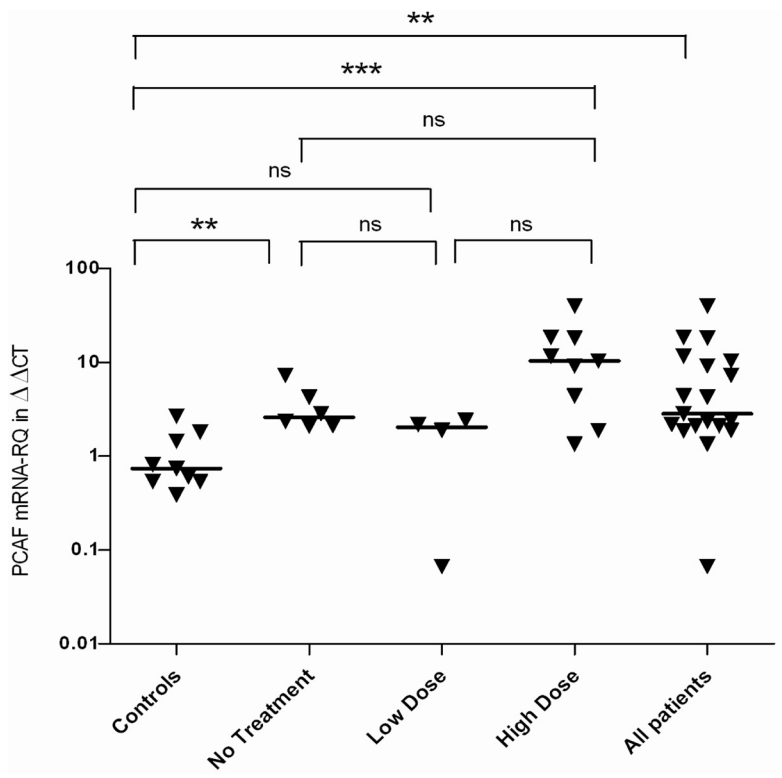

Fig. 4. Relative expression of PCAF mRNA represented by RQ in SLE patients and healthy controls.
The relative expression of PCAF mRNA in 20 SLE paediatric patients in comparison to 9 healthy controls. The 20 SLE paediatric patients were subdivided into 6 patients with no treatment, 4 patients on low-dose corticosteroid therapy and 10 patients on high-dose treatment. PCAF was significantly upregulated in patients on high-dose and patients with no treatment as compared to controls. Patients on low-dose treatment exhibited a similar pattern of PCAF expression in comparison to controls. Values are expressed as median.

Relative expression of miRNA- 223 in peripheral blood of SLE patients and healthy controls

miR-223 showed no significant change between SLE patients and healthy controls, p=0.4943. miR-223 also showed no significant change in SLE patients with no treatment as compared to controls, p=0.7756. Nor did miR-223 show a significant change in expression between SLE patients on low-dose treatment or high-dose treatment as compared to controls, p=0.7105 and p=0.4976, respectively. In addition, miR-223 showed no significant difference between low-dose and high-dose corticosteroid treatment patients, p=0.9118.

Relative expression of PCAF mRNA in SLE patients and healthy controls

PCAF showed marked upregulation in SLE patients compared to controls, p=0.0012. PCAF showed significant upregulation in SLE patients with no treatment as compared to controls, p=0.0028. PCAF also showed a significant upregulation in SLE patients on high-dose treatment as compared to controls, p=0.0005. Patients on low-dose treatment showed no significant difference in PCAF expression as compared to healthy controls, p=0.4140. Similar to the results of miR-181-a, PCAF showed no significant difference between patients with no treatment *versus* patients on low-dose or high-dose treatment, p=0.1714 and p=0.1135, respectively, and there was no significant difference between low-dose and high-dose patients in PCAF expression, p=0.0755 (Fig. 4).

Relative expression of Hdm2 mRNA in SLE patients and healthy controls

Hdm2 showed significant upregulation in SLE patients on high-dose treatment as compared to controls, p=0.0006. However, Hdm2 showed no significant difference in expression between SLE patients with no treatment or low-dose treatment as compared to controls, p=0.1447 and p=0.3301, respectively. Patients on high-dose corticosteroid treatment showed no statistically significant difference in expression of Hdm2 as compared to patients with no

treatment or on low-dose treatment, p=0.0559 and p=0.0539, respectively. Hdm2 expression did not differ significantly between patients with no treatment and patients on low-dose of treatment, p=0.2850 (Fig. 5).

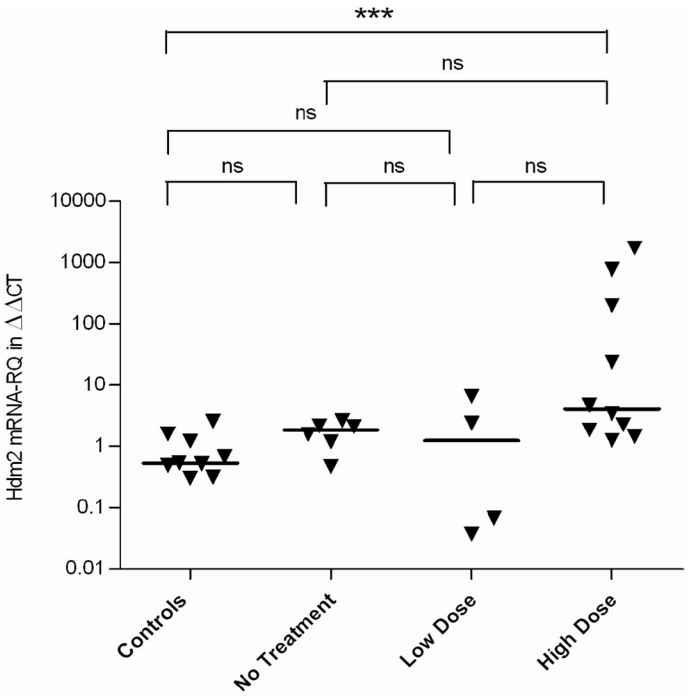

Fig. 5. Relative expression of Hdm2 mRNA represented by RQ in SLE patients and healthy controls. The relative expression of Hdm2 mRNA in 20 SLE paediatric patients in comparison to 9 healthy controls. The 20 SLE paediatric patients were subdivided into 6 patients with no treatment, 4 patients on low-dose corticosteroid therapy and 10 patients on high-dose treatment. Hdm2 was significantly upregulated in patients on high-dose treatment as compared to controls. Patients on low-dose and no treatment exhibited a similar pattern of Hdm2 expression in comparison to controls. Values are expressed as median.

Discussion

The role of miR-181-a in B and T lymphocytes maturation and function has been emphasised in previous studies (1, 6-8). miR-181-a has also shown an essential role in controlling the expression of its target gene PCAF (20), which practices chief control on apoptosis by stabilising the oncoprotein p53 through acetylating it and by prompting its negative controller –Hdm2– for ubiquitination, thus setting p53 free (19). In multiple myeloma samples miR-181-a showed significant over expression, which, in turn, downregulated PCAF, freeing Hdm2 and thus rendering p53 inactive, eventually leading to a decreased rate of apoptosis (20). Furthermore, the use of miR-181 antagomir has shown a significant decrease in the multiple myeloma tumour size (20). SLE and multiple myeloma are thought to be inversely correlated because both are the results of aberrant conduct in apoptosis. This is also in line with the fact that p53 levels were repeatedly reported to be over expressed in SLE patients, while it was downregulated in multiple myeloma (15, 20, 23). In this study, the relative quantitation of miR-181-a and its target PCAF mRNA were investigated for the first time in SLE paediatric patients in comparison to healthy controls. miR-181-a showed marked downregulation in paediatric patients with SLE disease as compared to healthy controls (Fig. 1). When the relative expression of miR-181-a in non-treated patients was compared to low-dose and high-dose corticosteroid treated patients, miR-181-a showed no significant change between these three groups of patients and this suggests that corticosteroid therapy has no effect on the relative expression of miR-181-a (Fig. 1). On the other hand, on comparison of the relative expression of miR-181-a among patients with regard to their respective SLEDAI scores, miR-181-a showed a significant downregulation in patients with high SLEDAI score in comparison to patients with low SLEDAI score, indicating the essential role of miR-181-a not only in SLE pathogenesis but also in the SLE disease activity (Fig. 2). However, the expression of miR-181-a did not correlate with the existence of target organ manifestations of SLE including cytopenia, proteinuria or CNS manifestations. The downregulation of miR-181-a was only observed in SLE patients, as paediatric FMF patients showed an analogous expression of miR-181-a as compared to healthy controls (Fig. 3). In contrast to miR-181-a, the relative expression of miR-223 in SLE patients showed no significant change when compared to its relative expression in healthy controls, which confirms the fact that miR-223 is myeloid-specific and not involved in other haematopoietic cell development or function and therefore has no share in the pathogenesis of a disease based on B- and T-cell intolerance (1, 2). Interestingly, PCAF was found to have a role in Epstein-Barr virus (EBV) infection, which represents one of the famous risk factors to the development of SLE and autoimmunity (24); where it is recruited by Epstein-Barr virus nuclear protein 2 (EBNA2) to activate cellular and viral gene expression (25). Therefore, the expression analysis of PCAF in SLE patients might be essential due to its involvement in EBV infection, in addition to being one of the target genes of miR-181-a (22). The relationship between miR-181-a down regulation and the relative expression of PCAF mRNA is shown in Figure 4 where PCAF showed significant up regulation in SLE paediatric patients as compared to healthy controls (Figs. 1, 4). This shows that miR-181-a expression might have a role in braking the expression of PCAF while its down regulation elevates PCAF, triggering the PCAF-Hdm2-p53 pathway which majorly contributes to the pathogenesis of SLE. Hdm2 showed no difference in expression between untreated patients and patients on low-dose treatment against controls. This corresponds to the mode of control of PCAF over Hdm2, as they interact on the protein

level and not the transcriptional one. The fact that patients on high-dose of treatment showed increased relative expression of Hdm2 in comparison to healthy controls as shown in Figure 5 might be attributed to the corticosteroids administered, as Hdm2 was previously proven to be a mineralocorticoid responsive gene (26). This can elucidate the mode of action of corticosteroids in lowering the rate of apoptosis and thus the disease activity.

In conclusion, the results of this study demonstrate for the first time that the peripheral blood mononuclear cells of SLE paediatric patients show significant downregulation in the relative expression of miR-181-a in comparison to healthy controls and FMF paediatric patients. In addition, the relative expression of the mRNA of PCAF was significantly upregulated, which might explain the increased ubiquitination of Hdm2 protein, hence the release of p53 and the consequent increase of apoptosis in SLE patients. Further direct studies on the protein level are required to confirm our data.

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