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Positive Correlation of STAT1 and miR-146a with Anemia in Patients with Systemic Lupus Erythematosus

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Abstract

Purpose—Anemia is one of the most common hematological manifestations in SLE patients, occurring in about 50% of active cases. STAT1 is a critical signaling molecule required for the production of type-1 interferon (I-IFN), CCL2, and CXCL10, all of which are upregulated in SLE. Overexpression of STAT1 has been described to be involved in anemia in animal models. The aim of this study is to analyze how these components are involved in SLE-associated anemia.

Methods—Blood samples were collected from 39 healthy donors and 101 SLE patients fulfilling ACR criteria. Samples were collected from a total of 180 visits (58 patients had 2 or more visits) of which 52 visits included a diagnosis of anemia. Healthy donors had only single visit. Total RNA, isolated from leukocytes, was analyzed by Taqman qPCR. Relative expression levels of I-IFN signature genes, chemokines, and miR-146a were determined by the $\Delta\Delta CT$ method. Results were correlated with clinical data and analyzed by the Wilcoxon/Kruskal-Wallis test and Fisher's exact test.

Results—Significant increases in IFN score ($p < 0.0001$), STAT1 ($p < 0.0001$), miR-146a ($p < 0.0005$), CCL2 ($p = 0.0047$), and CXCL10 ($p = 0.017$), as well as a significant decrease in pri-miR-146a ($p = 0.0002$), were detected in the anemic SLE patient visits ($n = 52$) compared to non-anemic SLE visits ($n = 128$). Regardless of disease activity, lupus nephritis, or race, anemic SLE patients displayed significantly elevated levels of STAT1 and miR-146a compared to non-anemic SLE patients.

Conclusions—STAT1 and miR-146a may be upregulated during inflammation and via proinflammatory cytokines and chemokines in SLE. Prolonged upregulation of STAT1 and miR-146a appears to play an important role in anemia in SLE patients.

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Appendix

Supplemental figure 1 through 7.

Disclosures The authors declare that they have no conflicts of interest.

Keywords

anemia; CCL2; CXCL10; miR-146a; lupus

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic systemic autoimmune disease with complex clinical manifestations that can affect multiple organs or different organs at different periods of activity. Around 50% of SLE patients suffer from anemia [1]. Commonly anemia arises from iron deficiency, but several forms of anemia can be found in SLE patients, which include anemia of chronic disease, uremic anemia, megaloblastic anemia and hemolytic anemia [1]. The most common cause of anemia in SLE [1] is anemia of chronic disease, which is result of prolonged inflammation leading to the production of hepcidin by the liver [2]. Hepcidin upregulates ferroportin causing the sequestration of iron in the bone marrow which leads to impaired erythropoiesis. Similarly, uremic anemia is a result of decreased erythropoietin caused by accumulation of nitrogenous waste such as urea due to renal failure. Megaloblastic anemia is resulted from impaired DNA synthesis due to a deficiency of vitamin B12. Hemolytic anemia is caused by the autoantibodies to erythrocytes via complement activation and hemolysis.

Environmental and genetic factors have been implicated in SLE etiopathogenesis. Type I interferon (I-IFN) expression has been discovered to play a key role in the pathogenesis of SLE [3, 4]. Due to the presence of many isoforms of IFN α , one common way to evaluate this component is to measure IFN response genes [5–7]. IFN response genes such as OAS1, MX1, and LY6E can be specific for type I IFN signaling, or can respond to multiple types of IFN signaling as STAT1 (signal transducers and activators of transcription 1). STAT1 is involved in type I, II, and III IFN signaling and has been observed to be elevated in SLE [8]. In response to type I IFN, STAT1 causes interferon receptor (IFNAR) 1 and 2 dimerization, activation and phosphorylation of IFNAR by Tyk2 and Jak1, and thus docking and phosphorylation of STAT1 and STAT2 (signal transducers and activators of transcription 2) [9]. The heterodimer STAT1–STAT2 is then translocated into the nucleus where it can bind specific promoters playing a key role in IFN signaling and production [10]. STAT1 is also involved in multiple chemokine and cytokine signaling pathways [11, 12].

Chemokines such as “C-C motif chemokine ligand 2” (CCL2) and “C-X-C motif chemokine 10” (CXCL10) have become another important topic of research in SLE in recent years [13, 14]. CCL2 is a potent recruiter of monocytes, T cells, basophils, and dendritic cells to site of infection or tissue damage [8, 15]. Roles of CCL2 have been implicated in psoriasis, rheumatoid arthritis, and multiple sclerosis [16]. In SLE, CCL2 was reported to be upregulated in the serum [13].

CXCL10, a potent attractor of monocytes, macrophages, T cells, NK cells, and dendritic cells to site of tissue damage and infection [17, 18], was identified to be upregulated in serum of SLE patients [13, 14]. Both CCL2 and CXCL10 with CCL19 were shown to be good indicators of SLE activity [13, 14].

miR-146a is a small noncoding RNA involved in the regulation of endotoxin induced tolerance and cross tolerance [19–22]. miR-146a was reported to be underexpressed in PBMCs of Chinese SLE patients resulting in deregulation of STAT1 and IRF5 that could potentially result to overexpression of IFN [23].

Our present study observes the interaction among STAT1, CCL2, CXCL10, and miR-146a in SLE patients with and without anemia, demonstrating that these biomarkers are overexpressed in anemic SLE patients and that anemia appears to be partially dependent on clinical features and lupus nephritis but not with therapy and race. This study explores the potential upregulation of STAT1 and miR-146a in SLE and the association of anemia with STAT1 and miR-146a.

Materials and Methods

Donor and SLE Patient Demographic Data

All studies have been performed in accordance with the 1964 Declaration of Helsinki and its later amendments. All human blood samples were obtained from enrolled individuals in the University of Florida Center for Autoimmune Diseases registry from 2008–2011 with the approval of institutional review board at the University of Florida. Whole blood was collected from a total of 101 SLE patients and 39 healthy controls. All persons gave their informed consent prior to their inclusion in the study. There were a total of 180 SLE visits including sequential samples collected in 58 SLE patients (43 patients with 2 visits, 10 patients with 3 visits, and 5 patients with 4 visits) and 41 patients with a single visit. Female to male ratio in SLE were ~6.6:1 by both individuals (Supplemental Fig. 1A) and visits (Supplemental Fig. 1B). All SLE patients fulfilled the American College of Rheumatology (ACR) classification criteria [24] and patients with LN met the ACR criteria for LN [25]. The ethnic profile of SLE patients and healthy controls were segregated into African Americans (AA), Asian Americans (AsA), European Americans (EA), Latino Americans (LA), and Multiethnic Americans (MA, Supplemental Fig. 1C,2A,B). Average age of SLE patient was 43 year old and the average age of HD was 33 years old (Supplemental Fig. 1D). SLE patients were active in 43/180 visits, according to the SLEDAI score greater than 4. SLE patients were receiving 0–60 mg/day of prednisone (PDN), 0–400 mg/day of hydroxychloroquine (HCQ), and 0–3500 mg/day of mycophenolate mofetil (MMF, Supplemental Fig. 2C–E) and many of them were on multiple medications (Supplemental Fig. 1D, 2C–E).

Leukocytes and RNA Purification

Peripheral blood leukocytes were collected from whole blood using Ambion LeukoLOCK kit (Ambion, Austin, TX). LeukoLOCK filters were washed twice with 3 ml of PBS and stabilized with 3 ml of RNAlater solution. Stabilized filters were stored at –80°C for a minimum of 24 h before collecting total RNA. Total RNA including small RNAs was collected using the “Alternative Protocol” (version 0602, Ambion) for the extraction of RNA from cells captured on LeukoLOCK filters using TRI reagent.

miRNA and mRNA qRT-PCR

OAS1, MX1, LY6E, CCL2, CXCL10, and miR-146a levels were analyzed by qRT-PCR. MiRNA qRT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Fast Advance PCR Master Mix, and TaqMan MicroRNA primers (Applied Biosystems, Foster City, CA). mRNA qRT-PCR was performed using the TaqMan High-Capacity cDNA Reverse Transcription Kit, TaqMan Fast Advance PCR Master Mix, and TaqMan mRNA assay primers (Applied Biosystems). All reactions were analyzed using StepOne Real-Time PCR System (Applied Biosystems).

IFN Score and SLE Activity

The expression of three known type-1 IFN signature genes, MX1, OAS1, and LY6E, were z-transformed into Interferon Scores (IFN score) as previously shown [7, 26]. The SLE

Disease Activity Index (SLEDAI) was used to classify patients into active or inactive at the time of the visit. SLE patients with a SLEDAI greater than 4 were considered active and patients with SLEDAI of 4 or less were considered to be inactive.

Anemia

Anemia was defined by hemoglobin level <12 gm/dl for women and <13 gm/dl for men. Evaluation of anemia for a few pregnant patients was excluded. Twelve patients displayed a change in anemia status between visits. Only one patient of the 51 anemic patients was diagnosed to have hemolytic anemia.

Data Analysis

The copy number of miR-146a was normalized to total RNA loaded whereas mRNA levels were normalized to 18S RNA. Copy number of miR-146a was determined using a standard curve with synthetic miR-146a (Integrated DNA Technologies Inc., Coralville, IA) [27]. Relative expression of mRNA was determined by the $\Delta\Delta C_T$ method [28] where the cycle threshold (C_T) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined for the miRNA or mRNA expression relative to untreated controls. Analyses were performed using SAS version 9.2 and JMP Genomics version 5 (SAS, Cary, NC). Wilcoxon/Kruskal-Wallis test was used to evaluate significance between groups. Wilcoxon Signed Rank test for matched pairs was used to evaluate SLE patients with two visits. Fisher's Exact Test was used to determine significance between groups in contingency tables. Data from patients with two visits showing changes in their anemia status from one visit to next were compared using paired t test. P values less than 0.05 were considered significant.

Results

Anemia and SLE biomarkers

The SLE cohort was segregated into patient visits that were clinically diagnosed with anemia (n=52, 28%) and without anemia (n=128, 72%) according to medical records. The IFN scores in anemic SLE patients were elevated compared to non-anemic SLE patients ($p<0.0001$) and healthy donors (HD, $p<0.0001$, Fig. 1A). Also the IFN score of non-anemic SLE patients was higher than HD ($p=0.017$). Similarly, STAT1 (Fig. 1B), CCL2 (Fig. 1C), CXCL10 (Fig. 1D), and miR-146a (Fig. 1E) were significantly elevated in anemic compared to non-anemic SLE patients and HD. In addition, STAT1 ($p<0.0001$), CCL2 ($p<0.0001$), and CXCL10 ($p<0.0001$, Fig. 1B–D) were elevated in non-anemic patients compared to HD. In contrast, primary microRNA-146a (pri-miR-146a) was higher in non-anemic than anemic SLE patients and HD (Fig. 1F). Primary miRNA is converted into the precursor miRNA and finally to the mature miRNA. The reverse in expression of pri-miR-146a vs miR-146a may be indicative of problems in pri-miR-146a being converted to mature miR-146a in SLE patients. Expression of TNF α , which is not an accepted biomarker for SLE, was not significantly different among all the groups (data not shown).

To rule out any potential bias in the contribution of patients with multiple visits, miR-146a and STAT1 data using only a single visit per patient were also analyzed (Supplemental Fig. 3). When data from the first visit of patients with multiple visits were selected along with patients with only a single visit, miR-146a, STAT, and SLEDAI were significantly elevated in anemic patients compared to non-anemic patients (Supplemental Fig. 3A–C). Similarly, when data from only the second visits were selected instead of the first visit, miR-146a, STAT, and SLEDAI were also significantly elevated in anemic patients compared to non-anemic patients (Supplemental Fig. 3D–F). Finally, when data from visits that were anemic were selected over non-anemic in the multiple-visit patients to enrich the population for

anemia, as seen before, miR-146a, STAT, and SLEDAI were significantly elevated in anemic patients compared to non-anemic patients (Supplemental Fig. 3G–I). These results demonstrated that considering visits rather than individual patients did not affect the observed outcome for anemia. To our knowledge, the observation that these biomarkers are significantly elevated in anemic compared to non-anemic SLE patients is a novel finding.

Relationship of Anemia and SLEDAI

To examine whether anemia has any relationship with SLE disease activity, patient's visits with and without anemia were compared to visits with 43 active and 135 inactive SLEDAI statuses of the patients. Active SLE patients were more likely to be anemic than non-anemic (anemia (–), likelihood ratio: 5.0, Fisher exact test: $p=0.020$, Fig. 2A). Additionally anemic SLE patients displayed higher SLEDAI scores than non-anemic SLE patients ($p=0.0028$, Fig. 2B).

To further investigate the interplay between anemia and SLEDAI, the expression of IFN score, STAT1, CCL2, CXCL10, miR-146a, and pri-miR-146a was analyzed in patients classified into 4 groups by a combination of anemia and SLE activity (SLEDAI >4 for SLEDAI active and SLEDAI ≤ 4 for SLEDAI inactive, Fig. 2C–H). Among the inactive patients, anemic patients displayed elevated ($p=0.021$) IFN score, STAT1, CCL2, CXCL10, and miR-146a compared to non-anemic patients (Fig. 2C–G). Active patients differed completely with only STAT1 and miR-146a significantly elevated in the anemic patients compared to non-anemic patients (Fig. 2D,G); pri-miR-146a was actually significantly higher in non-anemic than anemic patients (Fig. 2H). Even when excluding multiple visits, similar results were observed for miR-146a and STAT1 (Supplemental Fig. 4). In summary, SLE disease activity and anemia are related; STAT1 and miR-146a were exclusively elevated in anemic patients regardless of SLE disease activity.

Relationship of Anemia and Lupus Nephritis

Lupus nephritis (LN) is one of the most serious pathological manifestations in SLE. Additionally, anemia has been shown to be associated with LN as to be a good predicting factor for a nephritis flare [29, 30]. To examine the link between anemia and LN similar analysis was performed as above. It was not surprising to observe that LN patients were more likely to be anemic than non-anemic also in our cohort (likelihood ratio: 5.0, Fisher exact test: $p=0.024$, Fig. 3A). To further analyze the etiological association between anemia and LN, SLEDAI, IFN score, STAT1, CCL2, CXCL10, miR-146a, and pri-miR-146a expression were compared (Fig. 3B–H). In LN patients with anemia, SLEDAI, CCL2, and CXCL10 were elevated ($p=0.024$) when compared to LN patients without anemia; however, such difference was not observed in non-LN patients (Fig. 3B,E,F). In contrast, regardless of LN, anemic patients were significantly higher in IFN score, STAT1, and miR-146a (Fig. 3C,D,G). Unlike miR-146a, pri-miR-146a displayed higher levels in non-anemic than anemic patients (Fig. 3H). Similar to what was observed for the analysis of anemia and SLEDAI (Fig. 2), STAT1 and miR-146a levels were elevated in anemic patients regardless of LN.

Relationship of Anemia and Race

African Americans (AA) and European Americans (EA) were selected since they were the majority of SLE patients (Supplemental Fig. 1C,2A,B). Additionally, AA were significantly more likely to be anemic than EA (likelihood ratio: 8.5, $p=0.0032$, Supplemental Fig. 5A).

To better understand the effects of race on these biomarkers, AA and EA were analyzed separately into anemic and non-anemic patients. STAT1 and miR-146a were elevated in anemic compared to non-anemic regardless of ethnicity (Fig. 4A,D); however, pri-miR-146a

was elevated in non-anemic compared to anemic patients (Fig. 4E). CCL2 and SLEDAI were elevated only in EA that were anemic (Fig. 4B,F). There was no significant difference for CXCL10 (Fig. 4C). Thus, STAT1 and miR-146a association with anemia appeared largely independent of race based on our limited data.

Effects of Therapy on Anemia

The majority of SLE patients were receiving therapy in the form of PDN, HCQ, and MMF (Supplemental Fig. 2C–E). PDN is a glucocorticoid that suppresses inflammation by blocking NF- κ B. SLE patients receiving PDN (PDN+) were more likely to be anemic than non-anemic (likelihood ratio: 9.8, $p=0.0015$, Supplemental Fig. 3B). HCQ is an antimalarial drug that increases the pH of the endosomal vesicles interrupting antigen processing and the function of TLR3, 7, 8, and 9 as well as inhibiting production of interleukin-1 and -6 by macrophages [31–33]. MMF is an immunosuppressant that affects T and B cell growth by inhibiting inosine monophosphate dehydrogenase, which is critical enzyme for *de novo* synthesis of guanosine nucleotides [31].

Regardless of therapy, STAT1 and miR-146a were elevated ($p=0.048$) in anemic compared to non-anemic patients (Fig. 5A,D) while the reverse was observed for pri-miR-146a (Fig. 5E). Unlike STAT1 and miR-146a, CCL2, CXCL10, and SLEDAI were all higher in anemic vs non-anemic patients but statistical significance varied from each treatment; whether this was an influence of treatment or just limited sample size remained unclear. When patients instead of visits were analyzed, PDN, MMF, and HCQ were pooled into treated (Tx) grouping. Anemic Tx patients displayed significantly higher miR-146a and STAT1 than non-anemic Tx patients; however anemic untreated (UTX) patients were not significantly different from non-anemic UTX patients (Supplemental Fig. 6). The lack of statistical significance for UTX may be due to the small sample size.

Effects of Age and in Paired Visits

Due to age being a potential confounder, STAT1 and miR-146a were plotted against patient age (Supplemental Fig. 7). There was no significant association for the expression of STAT1 and miR-146 with patient age in the HD, non-anemic patients, and anemic patients in our cohort. Finally, patients with two visits were analyzed for changes in miR-146a and STAT1 when the patient becomes anemic (Fig. 6). There were twelve patients with two visits showing changes in anemia status from one visit to the next. Anemic patients displayed significant increase in miR-146a and STAT1 compared to the visits that they were not anemic (Fig. 6); however, IFN score, CCL2, CXCL10, and SLEDAI did not display significant change (data not shown).

Discussion

In this study, expression of previously identified SLE biomarkers was examined and correlated with demographic and clinical parameters. Anemic SLE patients displayed significantly higher levels of these biomarkers vs. non-anemic patients, particularly STAT1 and miR-146a, independent of disease activity and association with LN. A larger cohort would be needed to further substantiate the usefulness of STAT1 and miR-146a as critical biomarkers for anemia and the observation is independent from LN and therapy. In general, anemia is also considered as a good predictor of SLE flares [34]. Anemia can lead to reduced levels of oxygen even resulting in hypoxia in organs [35]. Low oxygen conditions associated with anemia can promote neutrophils, monocytes, and macrophage survival [36, 37], macrophages and dendritic cell pro-inflammatory response, and direct activation of monocytes [38–40]. Pro-inflammatory transcription factors such as NF- κ B, Ets-1, C/EBP α / β , and AP-1 are activated in hypoxic conditions [38, 39]. Since a link exists between

hypoxia, anemia, and inflammation via hepcidin [41], it is possible that hypoxia and anemia act as factors that modulate the immune response to enhance inflammation to expedite the clearance of foreign pathogens. Our study shows that anemic SLE patients display significantly higher IFN score, STAT1, CCL2, CXCL10 and miR-146a levels in anemic patients than non-anemic patients and HD. Active SLE patients with LN displayed significantly elevated STAT1 and miR-146a exclusively in those with anemia. Even the role of race did not influence the effects of anemia on STAT1 and miR-146a observed in this study.

In a study using zebra fish models for hematopoiesis, STAT1 was required for hematopoiesis and its increase was associated with increase in myeloid lineage but decrease in erythroid lineage [42]. In both humans and mice, hepcidin is primarily produced by the liver in response to IL-6 [2] but has also been shown to be produced in mouse macrophage cell line RAW264.7 when treated with IFN β or IFN γ [43]. STAT1 as well as C/EBP β were shown to be required for hepcidin induction in the RAW264.7 [43] which may indicate that macrophages contribute to anemia via production of hepcidin by IFN stimulation.

The present study shows that miR-146a can be considered as another biomarker consistently elevated in anemic SLE patients. In addition, miR-146a is transcriptionally regulated by NF- κ B, Ets-1, and PU.1 [44–46]. In zebra fish, transcriptional regulation of miR-146a by PU.1 has been shown to be critical for macrophage development and hematopoiesis and loss of miR-146 led to a loss of macrophages [47]. In mice where miR-146a was overexpressed, erythropoiesis and bone marrow reconstitution capacity decreased [48]. Activation of NF- κ B and Ets-1 under hypoxic conditions [38, 39, 49], may in part explain why miR-146a levels are elevated in anemic SLE patients, but it is unclear why elevated levels of miR-146a are not decreasing levels of IFN in SLE. TRAF6 and IRAK1/2 are known targets of miR-146a, they are critical for TNF α induction but may not be critical players in the pathogenesis of SLE as described in rheumatoid arthritis patients [50, 51]. miR-146a has been reported to target STAT1, but miR-146a was also shown to be reduced in SLE patients in the same study which is not the case in our study and may be due to differences between ethnic groups and differences between PBMCs vs mononuclear cells [23]. In SLE patients, there may be a loss of function of miR-146a that becomes unable to regulate STAT1. Furthermore, STAT1 has two isoforms (STAT1a and STAT1b). STAT1a is the long isoform which according to Targetscan is predicted to be targeted by miR-146a while the short form STAT1b is not predicted to be regulated by miR-146a. STAT1b is the same isoform that was shown to decrease erythropoiesis in zebra fish [42]. The elevated expression of STAT1 and miR-146a in anemia represents a novel observation in SLE that requires further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA African Americans

AsA	Asian Americans
CCL2	C-C motif chemokine ligand 2
CXCL10	C-X-C motif chemokine 10
EA	European Americans
HCQ	hydroxychloroquine
I-IFN	type-1 interferon
IFNAR	interferon receptor
LA	Latino Americans
LN	lupus nephritis
MA	multiethnic Americans
MMF	mycophenolate mofetil
PDN	prednisone
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
STAT1	signal transducers and activators of transcription 1

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Fig. 1.

Relationship between anemia and biomarker expression in SLE. (A–D) IFN score, STAT1, CCL2 and CXCL10 in both SLE patients with and without anemia displayed significantly higher expression than in healthy donors (HD), and they were also significantly higher in SLE patients with than without anemia. (E) miR-146a in anemic SLE patients is significantly higher than in non-anemic SLE patients and HD. (F) pri-miR-146a is significantly higher in non-anemic SLE patients than in anemic SLE patients and HD.

Fig. 2.

Differential expression of IFN score, STAT1, CCL2, CXCL10, miR-146a, and pri-miR-146a in active and inactive SLE patients with anemia. (A) A contingency plot to compare active and inactive SLE patients against anemic and non-anemic SLE patients revealed that anemic patients were more likely (likelihood ratio: 5.0) to be active patients. (B) SLEDAI scores were significantly elevated in anemic compared to non-anemic patients. (C–H) Comparison of the relative levels of listed biomarkers in non-anemic (–) vs anemic (+) patients who were either defined inactive (left panel) or active (right panel) based on their SLEDAI.

Fig. 3.

The interactions of anemia and lupus nephritis on SLE biomarkers. (A) A contingency plot to compare patients with and without lupus nephritis (LN) against anemic and non-anemic SLE patients revealed that anemic patients were more likely to have LN. (B) SLEDAI scores were significantly elevated in anemic LN patients compared to non-anemic SLE patients with LN. (C–H) Comparison of the relative levels of listed biomarkers in non-anemic (–) vs anemic (+) patients without LN (left panel) or with LN (right panel).

Fig. 4.

The relationship of race and anemia on STAT1, CCL2, CXCL10, miR-146a, pri-miR-146a and SLEDAI. (A-F) Comparison of the relative levels of listed biomarkers and SLEDAI in non-anemic (-) vs anemic (+) patients who were AA (left panel) or EA (right panel).

Fig. 5. STAT1, CCL2, CXCL10, miR-146a, and pri-miR-146a in anemic vs non-anemic SLE patients on different treatment. (A-F) Comparison of the relative levels of listed biomarkers and SLEDAI in non-anemic (-) vs anemic (+) patients who were treated with PDN, MMF, or HCQ.

Fig. 6. Changes of anemia status were correlated with miR-146a and STAT1 mRNA levels. Twelve patients with two visits where their anemia status changed from non-anemic to anemic were compared with paired t test. Both miR-146a (A) and STAT1 (B) displayed significant increase when the patients changed from non-anemic to anemic status.