BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2020, VOL. 77, NO. 4, 208–212
https://doi.org/10.1080/09674845.2020.1765459

Serum long noncoding RNAs FAS-AS1 & PVT1 are novel biomarkers for systemic lupus erythematous

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ABSTRACT

Background: Systemic Lupus Erythematous (SLE) is a chronic systemic autoimmune disorder whose diagnosis depends on combination of multiple factors. Circulating lncRNAs could serve as diagnostic non-invasive biomarkers for SLE. We hypothesised that serum FAS-AS1 and PVT1 are new biomarkers for SLE that relate to clinical features and laboratory markers.

Materials and Method: Measurement of serum FAS-AS1 & PVT1 by qRT-PCR, analysis of the association between two RNAs and the clinical data, activity index and laboratory markers by standard routine methods.

Results: There was a significant relative increased serum FAS-AS1 (median (IQR) 2.19 (0.13–8.62) and a significant reduced PVT1 (median (IQR) 0.52 (0.01–7.55) in SLE patients compared to controls ($P < 0.0001$ for FAS-AS1 and = 0.007 for PVT1). Serum FAS-AS1 and PVT1 were positively correlated ($r = 0.37$, $P = 0.001$). Higher FAS-AS1 was significantly linked with nephritis ($P = 0.011$), positive anti-dsDNA ($P = 0.01$) and lower serum PVT1 was significantly associated with oral ulcers ($P = 0.023$), photosensitivity ($P = 0.017$), and neurological manifestations ($P = 0.041$). Serum PVT1 negatively correlated with age ($r = -0.52$, $P < 0.0001$) and ESR level ($r = -0.29$, $P = 0.011$) in SLE patients. No correlation between disease activity and serum FAS-AS1 or PVT1 was detected.

Conclusions: Our study provides evidence that serum FAS-AS1 and PVT1 are new biomarkers for SLE.

ARTICLE HISTORY
Received 26 February 2020
Accepted 1 May 2020

KEYWORDS
FAS-AS1; PVT1; qRT-PCR; Systemic Lupus Erythematous; SLE

Introduction

Systemic Lupus Erythematous (SLE) is a long-term systemic autoimmune disorder characterized by autoreactive antibodies directed against self-antigens [1]. Up to 60% of SLE patients suffer from lupus nephritis. Renal involvement is not only the most frequent and most serious presentation of SLE, but also it adversely affects the prognosis of the disease [2]. Because of unclarified exact genetic and molecular alteration involved in SLE pathogenesis, the great heterogeneous manifestations and the unexpected disease course of SLE patients, many scientists have intended for recognition of probable new biomarkers that can predict disease susceptibility and activity [3].

Long noncoding RNAs (lncRNAs) are RNA transcripts that lack protein-coding capacity but act as regulatory molecules affecting many biological functions in cells and organs. Among these biological processes is immunological responses in which lncRNAs are implicated in the regulation of transcriptional programmes and gene expression profiles of immune cells involved in defence from pathogens and in maintaining normal health and homoeostasis [3]. Lately, some lncRNAs have been identified to be dysregulated in SLE, which suggests possible involvement in the pathogenesis of this disease [4].

Apoptosis is a fundamental pathogenic pathways likely to contribute to the pathogenesis of SLE. There are two independent apoptotic signal pathways. The extrinsic apoptosis pathway is activated by the specific death-inducing receptors on the cell membrane, such as Fas receptor, binding its particular ligand (i.e. the Fas Ligand (FasL)). The intrinsic pathway is usually initiated by DNA damage or growth factor withdrawal, regulation occuring through members of the Bcl2 family of proteins, which includes both anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax. Bcl2/Bax ratio is a crucial factor of regulation of apoptosis; a high Bcl2/Bax ratio inhibits apoptosis while low ratio induces apoptosis [5].

Previous literature has highlighted two apoptosis-related lncRNAs; FAS antisense lncRNA (FAS-AS1), also called as SAF, a recently discovered lncRNA that gained its nomenclature as it is transcribed in reverse

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orientation from the opposite strand of intron 1 of the Fas gene and was shown to modulate Fas-mediated apoptosis [6]. LncRNA plasmacytoma variant translocation 1 (PVT1) maps to the genomic locus (8q24) upstream of the well-known c-Myc oncogene, and was shown to be related to Bcl2 mediated apoptosis [7–9]. The aforementioned lncRNAs (FAS-AS1 & PVT1) are altered in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and Sjogren's syndrome [10-12] and recent evidence has confirmed that circulating cell-free lncRNAs could serve as non-invasive biomarkers for diseases including SLE [13,14].

Against this background, we hypothesised that lncRNAs FAS-AS1 and PVT1 are circulating biomarkers for SLE and that levels relate to clinical and laboratory parameters.

Materials and methods

We tested our hypotheses in a case-control study of 80 SLE patients and 80 healthy controls. Newly diagnosed SLE patients were enrolled from the Department of Internal Medicine and from the Department of Rheumatology and Rehabilitation at Fayoum University Hospital over a period from January 2019 to July 2019. They were diagnosed according to Systemic Lupus International Collaborating Clinics (SLICC) criteria revised in 2012 (SLE patients should have as a minimum 4 of 17 SLICC classification criteria, including at least one clinical and one immunologic criterion) [15]. The activity of the disease was evaluated using the SLE Disease Activity Index (SLEDAI) score which depends on the evaluation of 24 items for the nine organ systems and ranges from 0 to 105 points [16]. Exclusion criteria include the presence of concurrent autoimmune disease, malignancies, and pregnant patients. A full medical history was taken from all SLE patients, and routine laboratory investigations were performed by standard techniques. SLE nephritis was proven by biopsy. Eighty healthy individuals with no history of any disease and with matched age and sex were recruited from healthy blood donors to serve as controls. Ethical approval was obtained from the Faculty of Medicine, Fayoum University Ethics Committee. Informed consent was signed by all contributors in this study. The study was conducted in accordance with the ethical rules of the Declaration of Helsinki.

Venous blood samples were withdrawn from all participants into sterile tubes with clot activator. Samples were allowed to clot at room temperature, followed by centrifugation for 10 min at 3000 xg. Clear supernatants in the upper parts of the samples were collected into clean new tubes. Serum total RNA was extracted using the miRNeasy extraction kit (Qiagen, Valencia, CA, USA). Evaluation of RNA quantitation and purity was performed using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). RT2 first strand kit (Qiagen) was used for cDNA synthesis as per the manufacturer's protocol. GAPDH was used as housekeeping gene for both RNAs [17]. The primers for FAS-AS1 were obtained from Qiagen (Lot No 20,140,910,029, and LPH 00083A-200), the primers used to amplify the gene expression of PVT1 were forward: 5′-TGAGAACTGTCCTTACGTGACC-3′, reverse: 5′-AGAGC ACCAAGACTGGCTCT –3′ (Invitrogen). And the primer sequences of GAPDH were forward: 5′-CCCTTCATTGACCT CAACTA-3′, reverse: 5′-TGGAAGATGGTGATGGGATT-3′.

Real-time PCR was performed on a 20 µl reaction mixture using Rotor gene Q System (Qiagen) with the following conditions: 95◦C for 10 min, followed by 45 cycles at 95◦C for 15 s and 60◦C for 60 s. The $2^{-\Delta\Delta Ct}$ equation was used for the assessment of the fold change of serum and FAS-AS1 and PVT1. The cycle threshold (Ct) value is the number of qPCR cycles required for the fluorescent signal to pass a specified threshold. ΔCt was calculated by subtracting the Ct values of internal control from those of FAS-AS1 and PVT1. ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the patients' samples. For the control sample, ΔΔCt equals zero, and 2° equals one [17].

The sample size for this study was calculated by (G power version 3.0.10). The minimum sample size of patients was 64 in each group needed for a power level of 0.80, an alpha level of 0.05 (two-tailed), and a medium effect size of 0.50 for (PVT1). The calculated sample size was increased by 20% to reach 80 in each group for added confidence and to overcome any missing values. Data analysed by SPSS v22 (SPSS Inc, USA). Regards quantitative data, the mean, median, standard deviation (SD), and interquartile range (IQR) were calculated. Kolmogorov-Smirnov test (KS) test was used as a test of normality. If variables were not normally distributed, the Mann-Whitney-U test was used in comparing between the two groups. Otherwise, an independent t-test was used. Categorical data were presented as numbers and percentages. Chi-square ($\chi 2$) was used as a test of significance. Spearman correlation was performed to determine the relation of FAS-AS1 and PVT1 with study parameters. Significance was adopted at $P \leq 0.05$.

Results

Of the 80 SLE patients, 72 (90%) were females and 8 (10%) males with mean age [SD] of 29.7 [7.3] years, age of disease onset with mean [SD] of 25 [5]. The 80 healthy controls were matched for age (31.5 [6.8] years; $P = 0.106$) and gender (67 (83.8%) female and 13 (16.2%) males, $P = 0.242$). The baseline clinical and laboratory data of the patients are presented in Table 1. The relative expression level of FAS-AS1 was significantly increased with a median (IQR) fold difference of 2.19 (0.13–8.82), while the fold difference of PVT1 was significantly decreased at 0.52 (0.01–7.55) compared to

Table 1. Characteristics of 80 SLE cases.

Clinical Parameter	Data
Oral ulcers	40 (50.0%)
Arthritis	44 (55.0%)
Rash	40 (50.0%)
Photosensitivity	32 (40.0%)
Neurological symptoms	24 (30.4%)
Nephritis	61 (76.3%)
SLEDAI score	6 (3–12)
Laboratory Parameter (reference range)	
White cell count (4–11 × 10^3/mm^3)	6.3 (3.1–10.2)
Lymphocytes (20%-40%)	18 (13–38)
Platelets (150–400 × 10^3/mm^3)	247 (132–324)
Complement 3(C3) (80–160 mg/dl)	67.9 (41–78.1)
Complement 4 (C4) (16–48 mg/dl)	6.7 (5.1–17.2)
Creatinine (53–106 µmol/L)	97 (53–186)
Proteinuria (>0.5 g/24 hr urine)	1.1 (0.2–3.9)
ESR (mm/1st hr)	63.5 (25–110)
Anti-dsDNA positivity	49 (61.2%)
aCL positivity	20 (25.0%)

Clinical parameters, n (%), laboratory parameters median (inter-quartile range). SLE, systemic lupus erythematosus; TLC, total leucocytic count, Anti-dsDNA, anti–double-stranded DNA; aCL, anticardiolipin; ESR, erythrocyte sedimentation rate; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

the controls (according to the equation $2^{-\Delta\Delta Ct}$) ($P < 0.0001$ for FAS-AS1 and = 0.007 for PVT1). FAS-AS1 and PVT1 correlated significantly ($r = 0.37$, $P = 0.001$).

Table 2 shows FAS-AS1 and PVT1 according to clinical indices. Higher FAS-AS1 was significantly linked with nephritis ($P = 0.011$) and positive anti-dsDNA ($P = 0.01$), whilst lower serum PVT1 was significantly linked with oral ulcers ($P = 0.023$), photosensitivity ($P = 0.017$), and neurological manifestations ($P = 0.041$). PVT1 correlated well and inversely with age, but weakly and inversely with ESR (Table 3).

Discussion

SLE is a chronic autoimmune disease, wherein accelerated apoptosis and inefficient clearance of apoptotic cell debris are potential pathological processes [1,5]. Circulating lncRNAs are easily accessible, applicable, and accurate genetic tests for autoimmune diseases, including SLE [13,14], and in cancer [17,18]. We hypothesised that the fold change of two apoptosis-related lncRNAs, FAS-AS1 and PVT1, are biomarkers for

Table 3. Correlations of serum FAS-AS1 and PVT1 with age, clinical indices and laboratory parameters of SLE patients.

	FAS-AS1		PVT1	
Parameter	*r*	P	*r*	P
Age, years	−0.18	0.123	−0.52	<0.0001
Age of onset, years	−0.10	0.375	−0.18	0.114
White cell count	0.07	0.561	−0.1	0.390
Lymphocytes	0.04	0.762	−0.15	0.278
Platelets	−0.05	0.690	−0.17	0.142
Complement 3	0.06	0.628	−0.10	0.432
Complement 4	0.17	0.176	−0.11	0.388
Creatinine	0.13	0.265	0.13	0.251
Proteinuria	0.11	0.362	0.05	0.682
ESR (mm/1st hr)	−0.06	0.616	−0.29	0.011
SLEDAI score	−0.05	0.669	−0.15	0.197

SLE, systemic lupus erythematosus; ESR, erythrocyte sedimentation rate; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

SLE and that they are associated with SLE activity and its clinical and laboratory features. The lncRNAs were indeed altered in SLE, and PVT1 was lower in oral ulceration, in photosensitivity and neurological symptoms, whilst FAS-AS1 was higher in nephritis and in those with high anti-dsDNA antibody titres. PVT1 was inversely related to age and to ESR.

Increased FAS-AS1 is consistent with their role in regulating apoptosis: FAS-AS1 contributes to the induction of Fas-FasL mediated apoptosis through decreased serum expression levels of sFas; sFas is a soluble form of Fas protein produced by Alternative splicing (skipping of exon 6) of Fas mRNA and is competitively inhibit Fas-induced apoptosis through sequestering FasL [19].

Reduced PVT1 is consistent with its role in apoptosis. PVT1 controls the intrinsic apoptotic pathway through regulation of the Bcl2/Bax ratio; up-regulated PVT1 increases the Bcl2/Bax ratio, which inhibits apoptosis and vice versa [8,9]. These data support the view of induced apoptosis that is directly linked to lupus pathogenesis and propagation [5]. Additionally, PVT1 controls the production of inflammatory cytokines, including the levels of tumour necrosis factor-alpha, interleukin-6 (IL-6) and interleukin-1ß [20,21] which is central to the SLE pathology [22]. Moreover, some studies have revealed that the c-Myc proto-

Table 2. Links between FAS-AS1 and PVT1 serum levels and clinical parameters of SLE.

Variable		FAS-AS1	P	PVT1	P
Oral ulcers	Yes, 40 (50)	2.19 (0.29–8.55)	0.685	0.34 (0.02–6.87)	0.023
	No, 40 (50)	1.92 (0.13–8.62)		0.98 (0.01–7.55)	
Arthritis	Yes, 44 (55)	1.61 (0.29–8.62)	0.760	0.52 (0.02–7.43)	0.311
	No, 63 (45)	2.38 (0.13–8.56)		0.71 (0.01–7.55)	
Rash	Yes, 40 (50)	1.92 (0.29–8.55)	0.261	0.52 (0.02–7.55)	0.532
	No, 40 (50)	2.19 (0.13–8.62)		0.68 (0.01–7.43)	
Photosensitivity	Yes, 32 (40)	1.48 (0.29–7.56)	0.542	0.4 (0.02–6.15)	0.017
	No,48 (60)	2.57 (0.13–8.62)		1.13 (0.01–7.55)	
Neurological symptoms	Yes, 24 (30)	3.22 (0.13–8.55)	0.660	0.28 (0.01–6.43)	0.041
	No, 55 (70)	1.65 (0.17–8.62)		0.83 (0.03–7.55)	
Nephritis	Yes, 61 (76)	2.57 (0.13–8.62)	0.011	0.58 (0.02–7.55)	0.251
	No, 19 (24)	1.57 (0.17–8.55)		0.15 (0.01–1.43)	
Anti-dsDNA	Yes, 49, (61)	3.2 (0.22–8.62)	0.010	0.52 (0.01–6.87)	0.523
	No, 31(39)	1.61 (0.13–7.94)		0.52 (0.05–7.55)	
aCL	Yes, 20 (25)	1.57 (0.13–8.51)	0.162	0.68 (0.08–7.43)	0.225
	No, 60 (75)	2.19 (0.22–8.62)		0.52 (0.01–7.55)	

Anti-dsDNA, anti–double-stranded DNA; aCL, anticardiolipin; Data n (%) or median (interquartile range)

oncogene, which is the main target of PVT1 [7], is related to SLE [23]. Furthermore, PVT1 functions as a competing endogenous RNA (ceRNA) that competitively binds members of the miR-200 family, interrupting their functions [7]. Circulating miR-200 family members are biomarkers for SLE, and may participate in lupus pathogenesis by regulating the ratio of the Th17/Treg cells, which is central to lupus pathology and activity [24–26]. The variable mechanisms of action of PVT1 could be a result of different cells, tissues, and pathologies nature, or maybe due to the configuration of PVT1 itself. However, the definite role of PVT1 in the initiation, progression of lupus and the underlying mechanisms of action necessitate additional research.

Our data adds to the literature on lncRNAs in autoimmune disease. Both FAS-AS1 and PVT1 are downregulated in PBMCs obtained from multiple sclerosis patients [11], while in a study of rheumatoid arthritis patients, high expression of PVT1 was detected in the synovial tissues [27]. A significant positive correlation between the serum levels of two studies genes was obtained, however, a previous study demonstrated no significant correlation between FAS-AS1and PVT1 expression levels in the blood of multiple sclerosis patients [11]. This unexpected result could be explained by that lupus pathologies were related not only to accelerated cell apoptosis but more closely to the deficient apoptotic debris clearance [5]. There was no correlation between SLE disease activity index and levels of FAS-AS1 and PVT1 although the significant negative correlation between ESR value and serum PVT may be useful as ESR is an indirect indicator of lupus activity [28].

The major limitations of this study include the small number of patients and control groups risking false positives and false negative. Accordingly, additional studies on the exact role of these lncRNAs in SLE on a larger scale are required. Our data represent an advance in biomedical science as they point to roles for PVT1 and FAS-AS1 in SLE.

Summary

What is known about this topic

- Accelerated apoptosis and inefficient clearance of apoptotic cell debris are considered crucial processes in the pathogenesis of SLE.
- The two apoptosis related lncRNAs, FAS-AS1 and PVT1, are associated with the risk of other autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and Sjogren's syndrome.
- Circulating lncRNAs are easily accessible, applicable, and accurate genetic tests for autoimmune diseases, including SLE.

What this study adds

- Serum FAS-AS1 and PVT1 are biomarkers that discriminate patients with SLE from healthy subjects.
- PVT1 is linked to age, ESR, oral ulcers, photosensitivity and neurological symptoms, FAS-AS1 is linked to nephritis and anti-dsDNA antibodies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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