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Evaluation of *ITGB2* (*CD18*) and *SELL* (*CD62L*) genes expression and methylation of ITGB2 promoter region in patients with systemic sclerosis

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Abstract

Systemic sclerosis (SSc), an autoimmune disease of connective tissue, is characterized by inflammation, fibrosis, and vessel endothelial damage. Products of *Integrin subunit beta 2* (ITGB2) and *selectin L* (SELL) genes participate in several functional pathways of immune system. The aim of this investigation was to survey the transcript level of *ITGB2* and *SELL* genes as well as methylation status of CpG sites in promoter region of differently expressed gene in PBMCs of SSc patients. PBMCs were isolated from whole blood of 50 SSc patients and 30 healthy controls. Total RNA and DNA contents of PBMCs were extracted. Gene expression was analyzed by real-time PCR using the SYBR Green PCR Master Mix. To investigate the methylation status of CpG sites, DNA samples were treated by bisulfite, amplified through nested PCR, and sequenced through Sanger difficult sequencing method. *ITGB2* gene in PBMCs of SSc patients was overexpressed significantly in comparison to healthy controls. However, no altered *SELL* expression was observed. Three CpG sites of 12, 13 and 14 were significantly hypomethylated in patients group, despite overall methylation status of *ITGB2* gene promoter revealed no significant difference between study groups. There was no statistically significant correlation between methylation status of *ITGB2* promoter and the gene expression in patients. Regarding to lack of correlation of increased expression of *ITGB2* with its promoter hypomethylation in SSc patients, our study suggests that upregulation of *ITGB2* in PBMCs from SSc patients is probably due to another mechanism other than methylation alteration.

Keywords Systemic sclerosis · ITGB2 · SELL · CpG site · DNA methylation

Introduction

Systemic sclerosis (SSc) or scleroderma, a systemic and autoimmune disease of connective tissue, is characterized by vasculopathy, fibrosis, and the production of autoantibodies to nuclear proteins [1]. SSc can lead to death by involving the internal organs such as heart, lungs, kidneys, and intestine [2]. SSc is a rare rheumatic disease, which affects approximately 250 per million persons [3]. This disease associates with early vascular changes and cellular infiltration to target tissues. Although SSc pathogenesis is not completely understood, leukocyte infiltration and immigration to involved tissues and inflammation have been observed. Alternately, the infiltrated leukocytes release cytokines, growth factors, and chemokines, resulting in fibroblast activation, excess collagen deposition, fibrosis, and inflammation. These process may need to cell–cell and cell-extra cellular matrix (ECM) interactions, which is mediated by different cell surface adhesion molecules [4]. The main clinical subsets of SSc are limited cutaneous SSc (lSSc) and diffuse cutaneous SSc (dSSc), which are distinguished by fibrosis severity and autoantibodies features. The lSSc form is identified by

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slight vascular damages with slight fibrosis of skin and internal organs, and is accompanied with low progression rate. In contrast, the dSSc patients have progressive fibrosis of skin and internal organs, leading to high mortality rate [5–11]. However, it should be noted that despite lSSc form displays mild complications and the mortality and morbidity rates are slightly lower in them in comparison to dSSc form, the mortality and morbidity rate in lSSc form are still remarkable [12].

Both genetic and epigenetic factors play important roles in SSc etiopathogenesis [13–15]. Many studies demonstrated that the epigenetic mechanisms, including CpG DNA methylation, histones modifications, and miRNAs associate with vascular disorders, immune system activation, and accumulation of excessive ECM in SSc disease [16]. DNA Methylation occurs on the C5 position on a cytosine in the DNA sequence called CpG islands. This reaction is catalyzed by DNA methyltransferase (DNMT) enzymes, which transfer methyl (CH3) group of *S*-adenosylmethionine to the 5th carbon of cytosine molecule to form the 5-methylcytosine (5mC). About 70% of genes have CpG island(s) in their promoter region [17–19]. Universally, DNA hypermethylation leads to downregulation and its hypomethylation results in the upregulation of gene expression [20].

Peripheral blood cells (PBCs) of patients with SSc display increased expression of several selectin and integrin genes such as *ITGB2*, encoding integrin β2 protein, and *SELL*, encoding selectin L protein, which mediate cell adhesion to vascular endothelium and have important roles in vasculopathy, inflammation, and fibrosis in SSc disease [21]. *ITGB2* gene is on chromosome 21 (21q22.3) and encodes integrin β2 protein (CD18) [22]. Integrin β2 family plays important roles in leukocyte adhesion, immune and inflammatory reactions, immigration through endothelial and chemotaxis [23]. Furthermore, selectin proteins family is expressed on most leukocytes such as neutrophils, monocytes, eosinophils, basophils, and many lymphocytes. *SELL* gene is located on chromosome 1 (1q23–q25) [24] and encodes L-selectin protein (CD62L), which interacts with inducible ligands on endothelium of non-lymphoid vessels, mediating first step of leucocyte immigration that called 'rolling' [4].

Due to the role of *SELL* and *ITGB2* genes in inflammation and vasculopathy, and considering the important participation of DNA methylation in the regulation of gene expression, herein we intended to look deep inside the molecular mechanisms of inflammation and vascular disorders in patients with SSc with respect to analysis of mRNA expression and promoter methylation of the mentioned genes in PBMCs from SSc patients. Furthermore, the correlation of an important clinical manifestation of SSc patients, the Rodnan score, with RNA expression and promoter methylation of *ITGB2* gene was investigated.

Materials and methods

Study participants

Fifty SSc patients (25 lSSc and 25 dSSc) were selected randomly from Rheumatology Research Center (RRC) clinic, Shariati Hospital. For all SSc patients, diagnosis of the disease were determined by American College of Rheumatology (ACR) criteria [25]. Moreover, 30 healthy controls without history of rheumatic and autoimmune diseases were selected that were matched base on gender, race and age with the patients. None of the healthy controls had rheumatic and autoimmune diseases in themselves and their close family members. The disease form was determined and Rodnan score of patients and erythrocyte sedimentation rate (ESR) for study subjects were measured. Baseline and demographic characteristics of dSSc and lSSc patients and healthy controls are listed in Table 1 with more details. The Human Research Ethics Committee of Tehran University of Medical Sciences confirmed this study. Written informed testimonial forms were obtained from all the participants. About 10 ml of blood samples was taken from SSc patients during clinical diagnosis; also, samples were taken from healthy controls.

PBMC isolation, RNA extraction and cDNA synthesis

The Ficoll-Hypaque density gradient centrifugation approach was applied to isolate PBMCs from peripheral blood of the cases. Total RNA of peripheral blood leukocytes of 80 samples were extracted by miRNeasy Mini Kit (Cat No. 217004, Qiagen, USA) according to the manufacturer's instructions. Synthesis of cDNA was also performed by miScript II RT Kit (Cat No. 218161, Qiagen, USA) according to the manufacturer's instructions. Yield and purity of extracted RNAs and synthesized cDNAs were

Table 1 Baseline and demographic characteristics of dSSc (diffuse cutaneous SSc) and lSSc (limited cutaneous SSc) patients and healthy controls

Characteristic	dSSc patients ($n=25$) Median (IQR)[a]	lSSc patients ($n=25$) Median (IQR)	Healthy subjects ($n=30$)
Male	6 (24%)	3 (12%)	6 (18%)
Female	19 (76%)	22 (88%)	24 (82%)
Age	44 (16)	44 (18)	41 (20.25)
ESR	13 (29.5)	14 (18)	13 (8.5)
Disease duration	6.5 (10.5)	5 (7)	–
Rodnan score	22.5 (10)	11 (10)	–

dSSc diffuse cutaneous systemic sclerosis, *lSSc* limited cutaneous systemic sclerosis

[a]*IQR* interquartile range

assessed using a NanoDrop spectrophotometer at 260 and 280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA).

Gene expression by real-time quantitative PCR

Primers (ITGB2, SELL and β2M as a housekeeping gene) were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). The Basic Local Alignment Search Tool on the US National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to determine accuracy and specificity of primers. Quantitative analysis of the genes expression in PBMCs was investigated by ABI StepOnePlus real-time PCR System and SYBR Green master mix. Each mixture of reaction (20 µl total volume) comprised of 10 µl of master mix, 2 µl of cDNA, 0.8 µl of forward primer, 0.8 µl of reverse primer, and 6.4 µl of H_2O. The quantitative real-time PCR was fulfilled under these conditions: 10 min at 95 °C, then 40 cycles for 15 s at 95 °C, and 60 s at 60 °C. To analyze relative gene expression, a common approach, the comparative C_T method was used as previously described by Schmittgen and Livak [26]. Relative mRNA expression level of target genes in samples was calculated and normalized to the corresponding β2M expression level as a housekeeping gene. After that, relative expression of each gene in the samples was calculated using this equation: relative mRNA expression $= (2^{-\Delta Ct}) \times 10^3$.

DNA extraction

DNA of 80 samples were extracted by phenol–chloroform method [27]. Yield and purity of extracted DNAs were determined using a NanoDrop spectrophotometer at 260 and 280 nm (NanoDrop ND-2000C Spectrophotometer, Thermo Fisher Scientific, USA).

DNA treatment via bisulfite conversion

The extracted DNA samples were used for bisulfite treatment according to a manual described by Kate Patterson et al. [28]. This method changes unmethylated C bases to U bases and has no effect on methylated C bases and was previously implemented at our lab [29–31].

Nested PCR, gel electrophoresis and sequencing

Our real-time quantitative PCR results led us to investigate methylation status of *ITGB2* promoter region, due to its significantly different transcript profile between patients and controls.

The amplification target DNA was 251 bp on chromosome 21q22.3, located in a CpG island with 576 bp length, from promoter to exon 1. There was 22 CpG sites in the target DNA (Fig. 1) but 16 CpG sites were detectable in our sequencing and took the analysis criteria.

Because the amplification of bisulfite treated DNA was difficult, nested PCR was designed for the amplification of treated DNA. To amplify the sequence containing CpG islands in promoter of ITGB2 (21q22.3), in the first stage, an outer forward primer (F1 sequence: AAGGGTGATGTC GTTAGGGTT) and an outer reverse primer (R1 sequence: CGCGCACCGACTAAACAAC) with 919 bp product length was designed. After that, this PCR product was used as templet for second run. At this run, an inner forward primer (F2 sequence: GGATAGTGAGGTTGGGAGTTTAT) and an inner reverse primer (R2 sequence: TCCAAAACACCTAAC TAAAACCTAC) with 251 bps product size was designed. Both pairs of primers were designed by Methprimer website (http://www.urogene.org/cgibin/methprimer/methprimer.cgi).

Each tube for PCR comprised of 10 µl of PCR master mix (including PCR Taq polymerase and buffer), 0.75 µl of forward primer, 0.75 µl of reverse primer, 4 µl of bisulfite-treated DNA, and H_2O to a total volume of 25 µl. It is

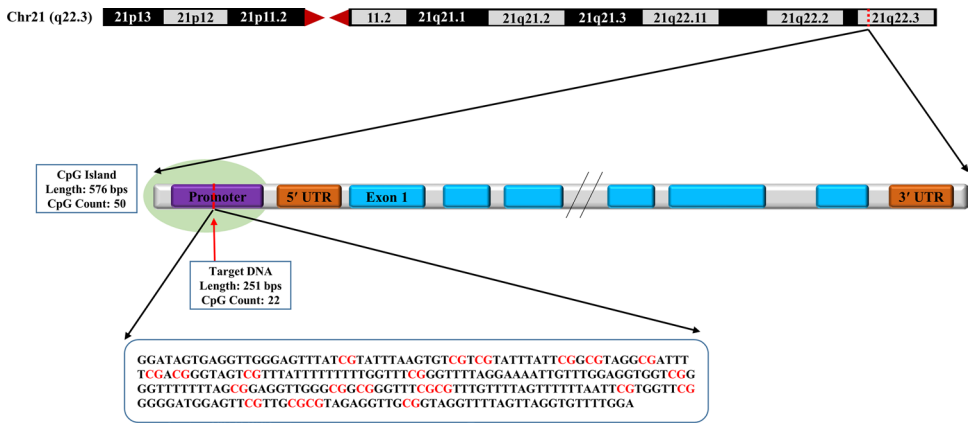

Fig. 1 Schematic picture of the target CpG sites of selected CpG island in the promoter of *ITGB2* gene. The amplification target DNA was 251 bps on chromosome 21q22.3, which was covered within a CpG island with 576 bps length from promoter to exon 1. Twenty-two CpG sites were found in the chosen region of *ITGB2* promoter. Sixteen CpG sites methylation status were surveyed

important to note that at second run 1.5 µl of each forward and reverse primers were used. PCR was fulfilled under these conditions: 35 cycles for DNA denaturation at 94 °C for 30 s, primer annealing at an optimized temperature of 58.4 °C (outer primer) and 61.5 °C (inner primer) for 45 s, and an extension stage at 72 °C for 45 s. Finally, the extension at 72 °C for 10 min was fulfilled.

Gel electrophoresis of final PCR product was done to validate amplification (Fig. 2) and, then, sequenced via difficult Sanger sequencing method (Macrogen, Seoul, Korea). The results of DNA sequencing were analyzed using CodonCode Aligner version 2 software.

Statistical analysis

Analysis of data was performed using SPSS software version 22 (SPSS, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to normality evaluation of Scale variables. Group comparisons of non-parametric variables were performed via the Mann–Whitney *U* test. To determine the relationship between parametric and nonparametric scale variables, Pearson's and Spearman's correlations were applied, respectively. The GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA; http://www.graphpad.com) was used for plotting the graphs. The study results were represented as Median and Interquartile Range (IQR), with statistical significance set at 0.05.

Results

mRNA expression level of ITGB2 and SELL in PBMCs

It was observed that mRNA expression level of *ITGB2* gene was significantly upregulated in PBMCs of SSc patients compared to that of healthy controls (fold change = 1.44; $P = 0.047$; Fig. 3A). *ITGB2* mRNA expression was nearly increased significantly twofold in PBMCs from dSSc patients compared to healthy controls (fold change = 1.81; $P = 0.038$; Fig. 3B). However, nearly similar level of *ITGB2* mRNA was found in lSSc patients compared to healthy controls; but the expression level difference was insignificant (fold change = 1.07; $P = 0.195$; Fig. 3C). On the other side, expression of *SELL* in SSc patients was a little lower than in healthy subjects; but this difference was not significant (fold change = 0.86; $P = 0.901$; Fig. 3D). As well, *SELL* mRNA expression in PBMCs of dSSc patients was nearly identical to that of healthy controls, and the difference of expression was not significant (fold change = 0.97; $P = 0.372$; Fig. 3E). Despite insignificantly, lSSc patients expressed lower *SELL* mRNA level compared to healthy controls (fold change = 0.74; $P = 0.480$; Fig. 3F).

Methylation status of *ITGB2* promoter in PBMCs

The methylation status of *ITGB2* promoter CpG sites in SSc and control groups has been shown in Table 2. Sixteen CpG sites in *ITGB2* promoter region were surveyed. CpG 12, 13 and 14 were hypomethylated significantly in patients compared to the control groups ($P = 0.046$, 0.018 and 0.043, respectively). In other words, the methylation status of the other 13 CpG sites were not significantly different. CpG12 showed decreased methylation level in patients compared with controls and its methylation level was associated significantly with the decreased risk of SSc ($P = 0.046$, OR 0.33, 95% CI 0.11–0.98). Moreover, methylation level of CpG13 in patients compared with controls was decreased and methylation of this CpG site demonstrated association with lower SSc risk ($P = 0.018$, OR 0.14, 95% CI 0.03–0.71). As well, a negative association was observed between decreased methylation level of CpG14 and SSc risk ($P = 0.043$, OR 0.36, 95% CI 0.14–0.97).

Taking all the CpG sites together, analysis of methylation status of CpG sites of *ITGB2* promoter region revealed that there was no significant difference between PBMCs from patients and control groups ($P = 0.46$, Fig. 4A). Furthermore, comparison of *ITGB2* promoter methylation level between dSSc PBMCs and healthy subjects showed no significant difference ($P = 0.24$, Fig. 4B). As well, there was no statistically significant difference of *ITGB2* promoter methylation status between PBMCs from lSSc and healthy subjects ($P = 0.36$, Fig. 4C).

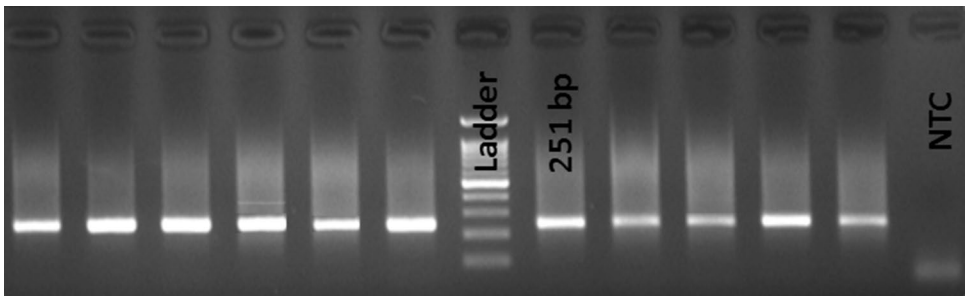

Fig. 2 Gel electrophoresis image of amplified bisulfite treated target DNA with 251-bps of samples from PBMCs of SSc patients and healthy controls

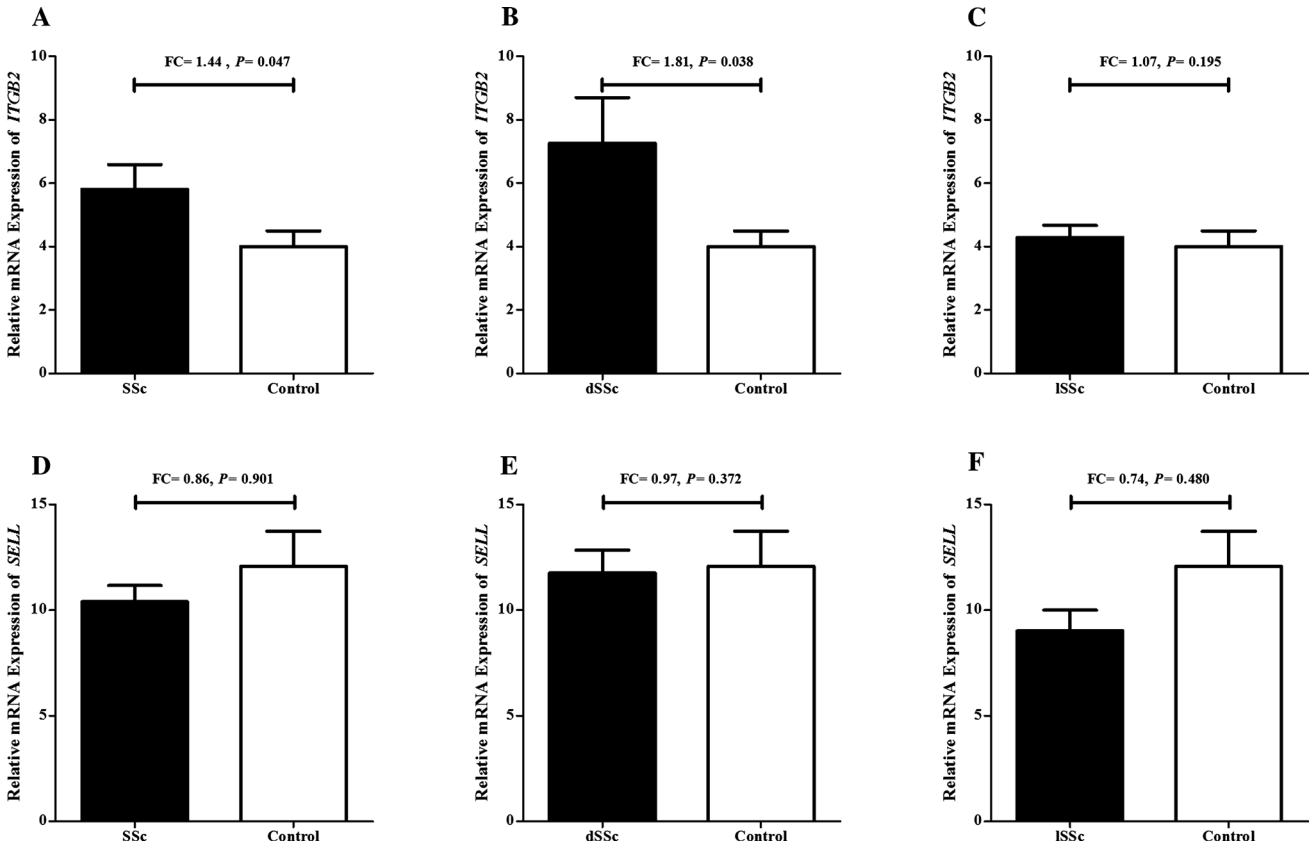

Fig. 3 Relative mRNA expression level of *ITGB2* and *SELL* in PBMCs of SSc patients vs. healthy control (**A, D**), dSSc patients vs. healthy control (**B, E**) and lSSc patients vs. healthy control (**C, F**). The Kolmogorov–Smirnov test revealed non-normal distribution of data and, therefore, the Mann–Whitney *U* test applied to calculating represented *P* values (*SSc* systemic sclerosis, *dSSc* diffuse SSc, *lSSc* limited SSc, *FC* fold change)

Correlation of *ITGB2* expression with its promoter methylation

In SSc patients, methylation percentage of *ITGB2* gene promoter in PBMCs did not display correlation with mRNA expression level of this gene ($P = 0.76$, $\rho = 0.044$; Supplementary Fig. 1A). Moreover, the methylation level of *ITGB2* promoter in PBMCs did not correlate significantly with mRNA expression level of the gene in dSSc patients ($P = 0.253$, $\rho = 0.237$; Supplementary Fig. 1B). Although methylation level of *ITGB2* gene promoter correlated negatively with mRNA expression of this gene in PBMCs of lSSc patients, the correlation was not significant ($P = 0.47$, $\rho = -0.153$; Supplementary Fig. 1C).

ITGB2 mRNA expression level in PBMCs from SSc patients in both methylated and unmethylated CpG sites is shown in Table 3. In some CpG sites, including CpG7, 9, 10, 11, 14, 15, and 16 expression level of *ITGB2* in methylated state was lower than in unmethylated state. Although, in other CpG sites, including CpG2, 5, 8, 12, and 13 *ITGB2* mRNA level was overexpressed in methylated state compared with unmethylated state. It was also observed that the expression level of *ITGB2* was significantly increased in hypermethylated status of CpG12 and 13 compared with unmethylated status (Table 3).

Correlation of *ITGB2* expression and methylation with Rodnan score of SSc patients

The Rodnan score was used to determining severity of skin involvement in SSc patients. There was no significant correlation between *ITGB2* expression level in PBMCs and Rodnan Score of SSc, dSSc, and lSSc patients, respectively (Supplementary Fig. 2A, C and E). Moreover, the methylation status of *ITGB2* promoter in PBMCs did not demonstrate statistically significant correlation with Rodnan score in SSc, dSSc, and, lSSc patients, respectively (Supplementary Fig. 2B, D and F).

Discussion

Nowadays, there is no doubt that epigenetic dysregulations play a paramount role in the etiopathogenesis of autoimmune disorders. Epigenetic modulations alter gene expression

Table 2 Methylation status of each 16 CpG sites in SSc and control groups

CpG sites	Frequency		OR (95% CI)	*P* value
	SSc patients ($n=50$)	Healthy controls ($n=30$)		
CpG1				
Methylated	1 (2%)	2 (6.7%)	0.29 (0.02–3.29)	0.315
Unmethylated	49 (98%)	28 (93.3%)		
CpG2				
Methylated	10 (20%)	0 (0%)	15.81 (0.89–280.53)	0.059
Unmethylated	40 (80%)	30 (100%)		
CpG3				
Methylated	1 (2%)	0 (0%)	1.85 (0.07–46.84)	0.709
Unmethylated	49 (98%)	30 (100%)		
CpG4				
Methylated	0 (0%)	0 (0%)	0.60 (0.01–31.23)	0.802
Unmethylated	50 (100%)	30 (100%)		
CpG5				
Methylated	16 (0%)	8 (6.6%)	1.29 (0.47–3.53)	0.614
Unmethylated	34 (100%)	22 (94.4%)		
CpG6				
Methylated	0 (0%)	0 (0%)	0.60 (0.01–31.23)	0.802
Unmethylated	50 (100%)	30 (100%)		
CpG7				
Methylated	7 (14%)	3 (10%)	1.46 (0.35–6.16)	0.602
Unmethylated	43 (86%)	27 (90%)		
CpG8				
Methylated	5 (10%)	2 (6.7%)	1.56 (0.28–8.57)	0.612
Unmethylated	45 (90%)	28 (93.3%)		
CpG9				
Methylated	3 (6%)	2 (6.7%)	0.89 (0.14–5.68)	0.905
Unmethylated	47 (94%)	28 (93.3%)		
CpG10				
Methylated	8 (16%)	6 (20%)	0.76 (0.24–2.46)	0.649
Unmethylated	42 (84%)	24 (80%)		
CpG11				
Methylated	10 (20%)	0 (0%)	15.81 (0.89–280.53)	0.059
Unmethylated	40 (80%)	30 (100%)		
CpG12				
Methylated	7 (14%)	10 (33.3%)	0.33 (0.11–0.98)	**0.046**
Unmethylated	43 (86%)	20 (66.7%)		
CpG13				
Methylated	2 (4%)	7 (23.3%)	0.14 (0.03–0.71)	**0.018**
Unmethylated	48 (96%)	23 (76.7%)		
CpG14				
Methylated	25 (50%)	22 (73.3%)	0.36 (0.14–0.97)	**0.043**
Unmethylated	25 (50%)	8 (26.7%)		
CpG15				
Methylated	4 (8%)	1 (3.3%)	2.52 (0.27–23.69)	0.418
Unmethylated	46 (92%)	29 (96.7%)		
CpG16				
Methylated	8 (16%)	4 (13.3%)	1.24 (0.34–4.52)	0.747
Unmethylated	42 (84%)	26 (86.7%)		

Statistically significant values ($P<0.05$) measured by Fisher's exact test are in bold

SSc systemic sclerosis, *OR* odds ratio, *CI* confidence interval

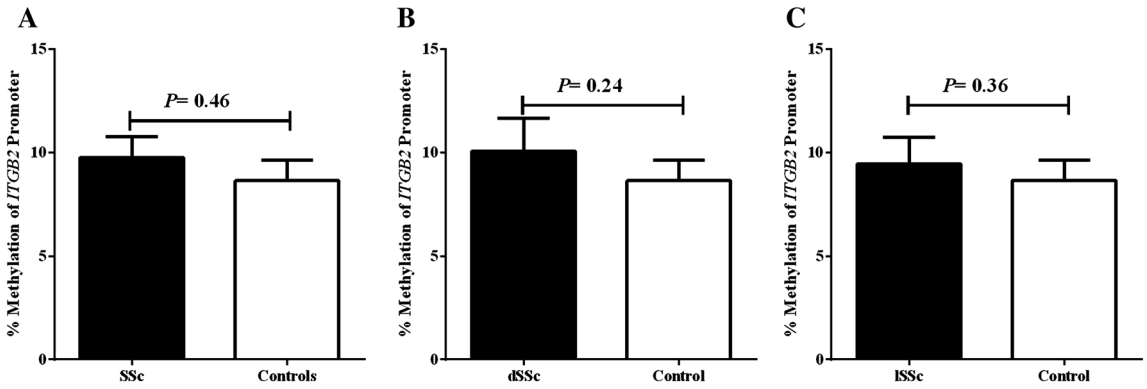

Fig. 4 Bar graphs illustrating the promoter methylation of *ITGB2* gene in **A** SSc patients and healthy controls, **B** dSSc patients and healthy controls, and **C** lSSc patients and heathy controls

Table 3 Comparison of the *ITGB2* mRNA expression level between SSc patients with and without methylated CpG sites

CpG site	Methylated Median (IQR)[a]	Unmethylated Median (IQR)[a]	Fold change; methylated vs. unmethylated	*P* value
CpG2	3.57 (1.54)	4.42 (2.67)	1.12	0.27
CpG5	4.24 (2.89)	4.30 (2.31)	1.24	0.57
CpG7	4.30 (2.89)	4.19 (2.24)	0.98	0.90
CpG8	4.39 (10.06)	4.14 (2.44)	1.26	0.98
CpG9	3.61 (0.12)	4.36 (2.6)	0.64	0.58
CpG10	4.63 (2.06)	3.98 (2.68)	0.98	0.65
CpG11	3.52 (2.42)	4.42 (2.67)	0.81	0.29
CpG12	5.47 (14.05)	3.90 (2.30)	1.90	**0.01**
CpG13	14.19 (7.01)	3.98 (2.31)	2.60	**0.04**
CpG14	4.43 (2.61)	4.30 (1.98)	0.99	0.44
CpG15	5.33 (2.22)	3.96 (2.30)	0.95	0.13
CpG16	4.04 (3.29)	4.30 (2.35)	0.92	0.81

CpG1 and CpG3 were not considered due to only one methylated patient

CpG4 and CpG6 were non-methylated in SSc cases

Statistically significant values ($P < 0.05$) measured by Mann–Whitney *U* test are in bold

[a]*IQR* interquartile range

without alteration in the gene sequence [32]. Epigenetic researches have concentrated preliminarily on the role of 5-methylcytosine (5-mC) in CpG islands in regulation of gene transcription in autoimmune disorders [33–37]. In this investigation, the possible implication of epigenetic dysmodulation in promoter of *ITGIB2* gene was evaluated in PBMCs of SSc patients compared with that of healthy subjects. The results of the experiments supported somewhat the role of CpG DNA methylation dysregulation in pathogenesis of SSc disease.

SSc pathogenesis includes an intricate interaction between vascular disorder, inflammation and autoimmunity, and severe tissue fibrosis [38]. Overexpression of cellular adhesion molecules, for example integrin beta 2 and Selectin L, provides the direct cellular binding to vascular endothelial cells and fibroblasts, which facilitates the infiltration of inflammatory cells to the sites of involvement. This interplay is believed to lead to vascular endothelial cells and fibroblasts activation, which manifests as vascular dysfunction and tissue fibrosis in SSc patients, respectively [39]. Moreover, DNA hypomethylation of dermal fibroblasts from dSSc and lSSc patients is related to the disease pathogenesis [40].

In the present study, it was shown that *ITGB2* mRNA level in patients with SSc was significantly increased compared to healthy individuals. This observation is in agreement with the study of Tan et al. [41], who reported several differently expressed genes in SSc PBC such as *ITGB2* and *SELL*. In contrast, we did not find significant difference in *SELL* mRNA level between patients and controls. In dSSc patients, *ITGB2* mRNA expression was upregulated significantly compared to healthy controls. But lSSc patients did not express *ITGB2* mRNA differently compared to healthy subjects.

Considerably, CpG islands are placed upstream of the transcriptional start site in promoter of gene [42]. DNA methyltransferase (DNMT) enzymes transfer methyl (CH3) group to the C5 position on a cytosine molecule to form the 5-methylcytosine (5mC). In this reaction, *S*-adenosylmethionine (SAM) plays as the methyl (CH3) group donor. Evidence shows that increased methylation level of CpGs in the promoter of genes leads to the suppression of gene transcription [18].

Furthermore, it has been found that DNA methyltransferase inhibitors are able to reverse epigenetic alterations and have protective effect against pulmonary fibrosis in mice [43]. Accordingly, epigenetic alterations appear to be reversible, suggesting they could emerge as new therapeutic options as anti-fibrotic therapy [44]. On the other

side, given to important contribution of integrin β2 family to inflammation [23], it seems that targeting *ITGB2* gene with elements modulating the methylation status might be promising in controlling the unwanted immune response and, therefore, disease treatment.

We observed that methylation status of *ITGB2* promoter had no significant difference between the patients and healthy individuals. Our study demonstrated that three CpGs 12, 13 and 14 were significantly hypomethylated in patients group. The hypomethylation of these three CpG sites was associated significantly with decreased risk of SSc. If this finding approve by other investigations, the methylation evaluation of these three CpG sites in *ITGB2* promoter can be beneficial measurement to predict the risk of SSc.

Methylation of DNA can repress gene expression in two ways: directly by preventing the binding of transcription factors and indirectly by employing the histone deacetylases (HDACs) via DNMT proteins and binding of proteins to methylated CpG [45]. Histone modifications, on the other hand, have been postulated to regulate gene expression [46]. It has also demonstrated that methylation and histone modifications are dependent events [47]. As well, DNMTs upregulation has been shown as a probable mechanism of gene hypermethylation [48]. In addition, Methyl-CpG-binding proteins (MECPs) were proposed as another mechanism of transcription inhibition through hypermethylation and following inhibition of transcription factors from binding to gene promoters [49]. Many studies have shown that CpGs methylation in promoter region of gene is only one of many possible regulatory mechanisms [50–52]. Other than these two, microRNAs might also potentially regulate the expression of *ITGB2* gene.

In our study, no statistically significant correlation was seen between methylation status of *ITGB2* promoter and mRNA expression of the gene in SSc patients compared to healthy group. Methylation events, as an important epigenetic mechanism, have wide effects during the evolution of immune system. By repression the immune system-related genes, epigenetic dysmodulation can deeply break self-tolerance and lead to autoimmunity disorder. This epigenetic dysmodulation has especially been observed in the genes involved in thymus formation, T cells development, and differentiation and effector function of these cells [53].However, no significant correlation was observed between *ITGB2* mRNA expression level as well as the methylation status of *ITGB2* promoter with Rodnan score of SSc patients in the present survey. Regarding to the overexpression of *ITGB2* was not correlated with *ITGB2* promoter hypomethylation in SSc patients, our study offers that upregulation of *ITGB2* expression in PBMCs from SSc patients probably is due to another regulatory mechanism other than the alteration of methylation.

In consideration of all, this study was performed to evaluate the methylation status of *ITGB2* gene and its possible effect on the mRNA expression of this gene in PBMCs from SSc patients. Here, we realized that methylation of *ITGB2* promoter did not impress the mRNA expression regulation of *ITGB2* in PBMCs from SSc patients. The mRNA expression of *ITGB2* was upregulated in PBMCs from SSc patients, which may participate in contribution of immune cells to migrate to the involved tissues. However, this contribution might not be mediated through CpG DNA methylation. Alternately, the methylation and expression levels of *ITGB2* in PBMCs from SSc patients did not correlate with disease severity with respect to Rodnan score. Epigenetic dysmodulation plays a role in SSc pathogenesis, requiring further evaluations to shed deep light on this puzzle.

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Compliance with ethical standards

Ethical approval All participants in the study were treated in agreement with the ethical standards of the Ethics Committee at Tehran University of Medical Sciences and with the revised Helsinki Declaration in 2000.

Informed consent Informed consent was obtained from all individual participants included in the study.

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