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Genome-wide DNA methylation analysis in dermal fibroblasts from patients with diffuse and limited systemic sclerosis reveals common and subset-specific DNA methylation aberrancies

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Abstract

Background—The aetiology of systemic sclerosis (SSc) is not clear, but there is an emerging evidence of gene-specific epigenetic dysregulation in the pathogenesis of SSc.

Methods—We performed a genome-wide DNA methylation study in dermal fibroblasts in six diffuse cutaneous SSc (dSSc) patients, six limited cutaneous SSc (lSSc) patients compared with 12 age-matched, sex-matched and ethnicity-matched healthy controls. Cytosine methylation was quantified in more than 485 000 methylation sites across the genome. Differentially methylated CpG sites between patients and controls with a fold difference ≥ 1.2 were identified. Quantitative real-time RT-PCR was performed to assess correlation between DNA methylation changes and gene expression levels.

Results—We identified 2710 and 1021 differentially methylated CpG sites in dSSc and lSSc, respectively. Of the differentially methylated sites, 61% in dSSc and 90% in lSSc were

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Contributors

All authors listed contributed and fulfil authorship criteria as follows: substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing Interest

None.

Ethics approval

IRB at the University of Michigan.

Provenance and peer review

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hypomethylated. There were only 203 CpG sites differentially methylated in both dSSc and lSSc, representing 118 hypomethylated and 6 hypermethylated genes. Common hypomethylated genes include *ITGA9*, encoding an α integrin. Other relevant genes such as *ADAM12*, *COL23A1*, *COL4A2* and *MYO1E*, and transcription factors genes *RUNX1*, *RUNX2* and *RUNX3* were also hypomethylated in both dSSc and lSSc. Pathway analysis of differentially methylated genes in both dSSc and lSSc revealed enrichment of genes involved in extracellular matrix–receptor interaction and focal adhesion. We demonstrate significant correlation between DNA methylation status and gene expression in the majority of genes evaluated.

Conclusions—Our data highlight common and subset-specific aberrancies in dSSc and lSSc fibroblasts at the epigenomic levels and identify novel candidate genes in SSc.

INTRODUCTION

Scleroderma (systemic sclerosis (SSc)) is a complex multisystem autoimmune disease characterised by vascular damage, autoimmunity and activation of fibroblasts leading to excessive deposition of collagen in the skin and internal organs.¹ Depending on the extent of skin involvement, SSc is categorised as limited cutaneous (lSSc) if skin thickening is confined to the extremities distal to the elbows and knees with or without facial involvement, whereas SSc is categorised as diffuse cutaneous (dSSc) if skin thickening involves areas proximal to the elbows and knees, including the trunk.² The aetiology of SSc remains unclear despite vigorous research efforts. However, there is growing evidence of gene-specific epigenetic alterations in SSc.^{3–5} Supported by the observation of striking geographic clustering of SSc,^{6,7} these epigenetic alterations might indicate a possible role for environmental–epigenetic factors in the pathogenesis of SSc.

Tissue fibroblasts are considered the core synthetic cells of the extracellular matrix (ECM) components, including collagen.⁸ Fibroblasts also play a role in activation of the immune system via production of numerous cytokines and chemokines and upregulation of adhesion and costimulatory molecules. It is well established that SSc fibroblasts differ from normal fibroblasts in several ways that include, but not limited to, persistently activated phenotype characterised by excessive production of collagen,⁹ increased proliferation and decreased apoptosis in vitro.¹⁰ Therefore, studying SSc fibroblasts is of paramount importance in identifying ‘fibroblasts-mediated’ biological pathways, which might serve the relentless search for disease-modifying therapy for this often-fatal disease.

DNA methylation is considered the core epigenetic mechanism that regulates gene expression by altering transcriptional accessibility of regulatory regions within gene sequences.¹¹ In this study, we performed a genome-wide DNA methylation study in dermal fibroblasts from patients with dSSc and lSSc compared with fibroblasts from control subjects. Our aim was to gain a fundamental understanding of the pathogenesis of SSc through identifying the differentially methylated genes in SSc and characterising biological pathways enriched by these genes. We identified aberrant methylation in key common genes and pathways between dSSc and lSSc, as well as subset-specific genes and pathways that are pertinent to the pathogenesis of SSc. Moreover, we demonstrate good correlation between

gene-specific DNA methylation status and gene expression in the majority of genes that we evaluated in this study.

METHODS

Systemic sclerosis patients and controls

We studied 6 patients with dSSc, 6 patients with lSSc and 12 healthy controls. Patients and controls were matched for age (± 5 years), sex and ethnicity. Demographic features of the study participants are shown in table 1. All patients had skin thickening of the fingers extending proximal to the metacarpophalangeal joints and fulfilled classification criteria of SSc.^{12–14} The clinical features of patients with SSc included in this study are shown in table 2. The institutional review board at the University of Michigan approved this study. All study participants signed a written informed consent prior to participation.

Fibroblasts isolation

Two 4 mm Punch skin biopsies were obtained under strict aseptic technique from the dorsum of the forearm, distal to the elbow, from all subjects involved in this study. Skin specimens were incubated briefly in RPMI media with an Antibiotic-Antimycotic (Life technologies, Carlsbad, California, USA), then treated with a mix of digestion enzymes that include 2.4 units/mL dispase (Sigma-Aldrich, Carlsbad, California, USA), 650 units/mL type II collagenase (Life Technologies) and 10 000 Dornase units/mL DNase (Millipore, Temecula, California, USA), and incubated for 2 h at 37°C. After digestion, cells were resuspended with complete growth media (EGM-2MV, New Bullet, Lonza) and plated on a gelatin-coated plate. Cells were harvested after 7–10 days. All fibroblasts used in the DNA methylation study were isolated from primary cell culture (without subculturing or cell passage, passage 0) by negative selection after depleting the endothelial cells from cultured cells using CD31 Microbead kit (Miltenyi Biotec, Cambridge, Massachusetts, USA), as described by the manufacturer's protocol. Isolation of genomic DNA was performed using the DNeasy Kit (Qiagen, Valencia, California, USA) as described in the manufacturer's protocol.

Gene expression analysis

Total RNA was extracted from fibroblasts for gene expression analysis by using RNeasy kits (Qiagen) as previously described.¹⁵ cDNA was prepared using Verso cDNA synthesis kits (Thermo Scientific). Quantitative PCR was performed using SYBR Green PCR master mix (Applied Biosystems). All samples were run in duplicate using Applied Biosystems Real-Time PCR System and analysed using 7500 Applied Biosystems software. Gene quantification cycle values were normalised to β -actin expression using a standard curve methodology to obtain relative cell equivalents. We evaluated expression levels of eight genes that were hypomethylated in both diffuse and limited SSc (*ADAM12*, *COL23A1*, *COL4A2*, *ITGA9*, *MYO1E*, *PAX9*, *RUNX2* and *RUNX3*) in 24 samples that were mostly independent samples (n=8 dSSc-, 8 lSSc-, and 8 control fibroblasts). Primers for RT-PCR are available upon request.

DNA methylation studies

Genome-wide DNA methylation in fibroblasts from SSc patients and controls included in this study was assessed using the Illumina Infinium HumanMethylation450 BeadChip array, which allows for the interrogation of over 485 000 methylation sites within the entire genome. This array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene across the promoter region, 5' untranslated region (5'-UTR), first exon, gene body and 3'-UTR. It also covers 96% of CpG islands. Non-CpG-methylated sites recently identified in human stem cells are also covered as well as microRNA promoter regions.

DNA methylation analysis was performed using the GenomeStudio methylation analysis package (Illumina) as previously described.¹⁶¹⁷ The average level of DNA methylation (β) on each CpG site was compared between patients and controls. To identify differentially methylated CpG sites between cases and controls, we used three data filtering criteria: (i) CpG site with an average difference in DNA methylation level of at least 1.2-fold; (ii) differential methylation score of ≥ 2.2 ($p < 0.01$) after adjusting for multiple testing using a false discovery rate (FDR) of 5%; and (iii) exclusion of CpG sites assessed by probes with a genetic variant located within 10 bp of the 3' end of the probe. The p value for differential methylation between patients and controls was calculated by the following equation:

$$P = z \left(\frac{(|\beta_{\text{patient}} - \beta_{\text{control}}|)}{\left(\left(\frac{s_{\text{control}}^2}{N_{\text{control}}} \right) + \left(\frac{s_{\text{patient}}^2}{N_{\text{patient}}} \right) \right)} \right)$$

where 's' represents the SD estimate and 'z' represents the two-sided tail probability of the standard normal distribution. The SD estimate 's' is a function of β and was calculated by the following equation:

$$s = A\beta^2 + B\beta + C, \text{ where } A = -0.1511, B = 0.1444, \text{ and } C = 0.01646$$

where A, B and C values were derived by Illumina from repeatedly measuring loci with known methylation fractions ranging from 0 to 1 and fitting a parabola to the SD as a function of β . A differential methylation scores for each probe was calculated as

$$\text{Diff Score} = 10 * \text{sgn}(\beta_{\text{patient}} - \beta_{\text{control}}) * -\log_{10}(p),$$

$$\text{where } \text{sgn}(i) = \begin{cases} i > 0; +1 \\ i = 0; 0 \\ i < 0; -1 \end{cases}$$

To systematically highlight the most over-represented biological terms, we performed gene ontology (GO), network and pathway analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) V.6.7.¹⁸ In pathway and GO analysis, we used Expression Analysis Systematic Explorer (EASE) Score threshold of < 0.1 for the detection of gene enrichment analysis (EASE score represents a modified Fisher's exact p

value, which is considered a measure to examine the significance of gene-term enrichment), in addition to fold enrichment of 1.5 and FDR for correction of multiple testing <10%.¹⁹

RESULTS

We evaluated DNA methylation changes in dermal fibroblasts from two independent sets consisting of dSSc and lSSc patients and age-matched, sex-matched and ethnicity-matched controls. Of the 485 000 CpG sites that were evaluated at a genome-wide level, we identified a total of 3528 differentially methylated CpG sites in fibroblasts from patients with SSc compared with healthy controls. A total of 2710 CpG sites were identified in dSSc fibroblasts, the majorities were hypomethylated (1653 CpG sites) compared with healthy matched controls, representing 818 hypomethylated and 513 hypermethylated gene regions. On the other hand, we identified 1021 differentially methylated CpG sites in lSSc fibroblasts; the majorities are hypomethylated (916 CpG sites), representing 496 hypomethylated and 56 hypermethylated gene regions. Surprisingly, there were only 203 (~6%) common differentially methylated CpG sites between diffuse and limited SSc, representing 118 hypomethylated and 6 hypermethylated genes (table 3, see online supplementary table S1 and figure S1). Further, there were 57 hypomethylated and 7 hypermethylated additional common genes, with unique differentially methylated CpG sites, in both dSSc and lSSc (see online supplementary table S2). This finding highlights an interesting diversity of DNA methylation profile in dermal fibroblasts, at the genome-wide level, between diffuse and limited SSc subsets.

None of the patients included in this study were taking any medications that are known to affect DNA methylation. One patient in the lSSc group was taking mycophenolate mofetil and another patient was on a small dose of prednisone (table 1). The mean DNA methylation levels in the differentially methylated CpG sites were not different between these two patients and the rest of the patients included in the lSSc group who are not on any medications. This was further confirmed using hierarchical clustering analysis (not shown). Therefore, it is unlikely that medication exposure is affecting the DNA methylation differences observed between patients and controls in this study.

Common differentially methylated genes in both dSSc and lSSc

As demonstrated above, around 6% of all differentially methylated CpG sites in fibroblasts were shared between dSSc and lSSc subsets; the vast majority of these were hypomethylated in reference to control fibroblasts. Hypomethylated common genes between diffuse and limited SSc include *ITGA9*, encoding for integrin α 9, which is an integral membrane glycoprotein that mediates cell–cell and cell–matrix adhesion. We identified one hypomethylated CpG site in the body of *ITGA9*. Other relevant differentially methylated genes in both diffuse and limited SSc include *ADAM12*, which encodes for ADAM metalloproteinase domain 12, and has extracellular metalloprotease and cell-binding functions. We identified three hypomethylated CpG sites in the body of *ADAM12* in dSSc, two of which were also hypomethylated in lSSc.

Other genes hypomethylated in both dSSc and lSSc include *COL4A2* and *COL23A1*, which encode for collagen type IV α 2 and collagen type XXIII α 1, respectively. Of note, we

identified other genes that encode for collagens, which were hypomethylated in SSc subsets; specifically, *COL8A1*, *COL16A1* and *COL29A1* were hypomethylated in dSSc fibroblasts, whereas *COL1A1*, *COL6A3* and *COL12A1* were hypomethylated in lSSc fibroblasts. Moreover, *PAX9* was hypomethylated in both dSSc and lSSc (four and two CpG sites, respectively). This gene encodes the pro- $\alpha 2$ chain of type I collagen, which is found in most connective tissues. Among genes encoding for ECM glycoproteins, we identified hypomethylation of *TNXB* in diffuse and limited SSc.

Among the transcription factors, *RUNX1*, *RUNX2* and *RUNX3* were all hypomethylated in fibroblasts from both dSSc and lSSc patients. Table 4 lists some of the top-rank relevant differentially methylated common genes between dSSc and lSSc.

Ontologies and pathways enriched by differentially methylated CpG sites common between dSSc and lSSc

We used DAVID software¹⁸ to facilitate the systematic identification and grouping of differentially methylated genetic loci into biological networks. Canonical pathway analysis identified ECM–receptor interaction ($p=2.92E-03$) and focal cell adhesion ($p=8.86E-03$) as the most significant pathways unifying the differentially methylated genes in fibroblasts from patients with dSSc and lSSc (table 5). GO analysis of hypomethylated common genes between dSSc and lSSc demonstrated enrichment of genes involved in negative regulation of MAP kinase activity ($p=2.14E-04$), cell projection morphogenesis ($p=9.72E-04$), axonogenesis ($p=1.11E-03$), cell part morphogenesis ($p=1.29E-03$), cell adhesion ($p=1.89E-03$), cell motility ($p=3.97E-03$) and positive regulation of transcription, DNA-dependent ($p=6.17E-03$), among other ontologies (table 5).

Genes, gene ontologies and pathways enriched by differentially methylated genes in dSSc

We identified differential methylation of several genes that are likely related to the pathogenesis of SSc in fibroblasts isolated from patients with dSSc. We identified two hypomethylated CpG sites in *CDH11*, which encode for cadherin-11, both CpG sites are located in the 5'UTR, with an average differential score of -51.8 , and average fold change of 1.30 compared with healthy controls. Additionally, *PDGFC*, which encode for platelet-derived growth factor isoform C, was hypomethylated in diffuse SSc fibroblasts. PDGFC is a profibrotic factor that is overexpressed in SSc²⁰ and that contributes to fibroblast activation and transformation to myofibroblast in SSc.

The Wnt/ β -catenin signalling pathway has been established as a critical pathway in activation of fibroblasts in wound healing and maintenance of fibrogenesis in SSc.^{21–23} In this study, we demonstrate hypomethylation of *CTNNA2* and *CTNNB1* in dSSc.

We demonstrate hypermethylation of *SOX2OT*, which encode for a long non-protein-coding RNA. *SOX2OT* was represented on the microarray by 91 CpG sites across the gene. We identified 53 hypermethylated CpG sites in fibroblasts from patients with dSSc compared with control fibroblasts, around 1/3 of these CpG sites were located in proximity to transcription start site. Of particular interest, none of these CpG sites in *SOX2OT* were differentially methylated in lSSc fibroblasts.

Online Supplementary table S3 provides a list of all differentially methylated CpG sites in diffuse SSc fibroblasts compared with control fibroblasts.

GO analysis of all hypomethylated genes in dSSc demonstrated enrichment of genes involved in cell adhesion ($p=1.51E-09$), cell morphogenesis involved in transformation ($p=3.11E-06$), neuron projection development ($p=7.71E-06$), skeletal system development ($p=6.01E-05$), blood vessel morphogenesis ($p=2.92E-03$) and immune system development ($p=5.40E-03$). Whereas GO ontologies of hypermethylated genes in dSSc include genes involved in embryonic morphogenesis ($p=1.93E-04$), negative regulation of mitotic cell cycle ($p=2.26E-04$), actin cytoskeleton organisation ($p=9.90E-04$), negative regulation of transcription ($p=1.26E-03$), regulation of cell migration ($p=1.76E-03$) and actin filament-based process ($p=1.87E-03$), among others. Pathway analysis of the differentially methylated genes in dSSc demonstrated gene enrichment of pathways involved in focal adhesion ($p=2.84E-06$), vascular smooth muscle contraction ($p=1.34E-04$), adherens junction ($p=1.60E-03$), ECM–receptor interaction ($p=3.57E-03$) and gap junction ($p=5.91E-03$) (see online supplementary table S4).

Genes, gene ontologies and pathways enriched by differentially methylated genes in ISSc

The Wnt/ β -catenin pathway was represented by hypomethylation of *CTNNA3* and *CTNND2* in ISSc. In addition, *COL1A1*, *COL6A3* and *COL12A1* were hypomethylated in ISSc fibroblasts as mentioned above (see online supplementary table S5 which provides all differentially methylated CpG sites in ISSc fibroblasts).

GO analysis of hypomethylated genes in ISSc demonstrated enrichment of genes involved in embryonic morphogenesis ($p=9.20E-05$), skeletal system development ($p=1.55E-04$), cell adhesion ($p=1.61E-04$), positive regulation of transcription ($p=1.66E-04$), actin filament-based process ($p=1.09E-03$) and regulation of cell-substrate adhesion ($p=5.60E-03$), among other ontologies. Pathway analysis demonstrated enrichment of genes involved in ECM–receptor interaction ($p=1.75E-04$), and focal adhesion ($p=8.85E-04$) (see online supplementary table S6).

Correlation between methylation status of representative genes and gene expression

To assess the functional effect of gene methylation status on gene expression, we performed RT-PCR analysis on a sample of genes that were hypomethylated in both dSSc and ISSc (*ADAM12*, *COL23A1*, *COL4A2*, *ITGA9*, *MYO1E*, *PAX9*, *RUNX3* and *RUNX2*). We found significant overexpression of the majority of these genes in SSc fibroblasts compared with control fibroblasts (figure 1).

DISCUSSION

In this study, we performed an unbiased genome-wide DNA methylation study in fibroblasts from patients with dSSc and ISSc, and identified several differentially methylated genes and involved pathways compared with healthy matched controls. The most striking finding in this study is the distinct and characteristic methylation pattern displayed in fibroblasts from dSSc and ISSc subsets, as reflected by the number of the differentially methylated CpG sites and corresponding genes in dSSc and ISSc (ratio 2.6:1, respectively). Nevertheless, we also

demonstrate an interesting similarity as highlighted by the observation that around 6% (203 out of 3528) of the differentially methylated CpG sites were indeed common CpG sites between diffuse and limited SSc.

The second important finding in this study is the demonstration of several hypomethylated and overexpressed genes that are integral to the pathogenesis of SSc (figure 1). Of particular interest, most of the hypomethylated CpG sites in these genes were detected in gene bodies, but are nonetheless associated with significant expression changes.

We demonstrate hypomethylation and mRNA overexpression of *ITGA9* in both dSSc and lSSc fibroblasts. Upregulation of integrins was demonstrated previously in both SSc fibroblasts^{24–26} as well as in lung fibroblasts in patients with fibrotic lung disease.²⁷ Overexpression of integrins contributes to myofibroblast differentiation²⁸ and activation of TGF- β .²⁹ There is evidence of an extensive bidirectional crosstalk between integrins and TGF- β signalling in fibrosis, with TGF- β inducing integrin expression and integrins modulating TGF- β activation and signalling.³⁰ Our results indicate a possible role of *ITGA9* hypomethylation in its overexpression in SSc, which may contribute to TGF- β upregulation.

It has been increasingly apparent that *ADAM12* overexpression contributes to the process of fibrosis through enhancing TGF- β signalling pathway.^{31–34} Knockdown of *ADAM12* in fibroblasts has been shown to be sufficient to limit collagen accumulation in vitro.³⁵ We demonstrate hypomethylation of *ADAM12* in both dSSc and lSSc fibroblasts compared with control fibroblasts, and we confirmed *ADAM12* overexpression, especially in dSSc fibroblasts.

Tissue fibrosis in SSc is orchestrated by fibroblasts via production of excessive amount of ECM components, including collagens. Among the common differentially methylated genes in dSSc and lSSc, we identified hypomethylation of several genes that encode for collagens and demonstrate overexpression of two collagen genes (*COL23A1*, *COL4A2*) in dSSc and lSSc fibroblasts compared with control fibroblasts. Moreover, *TNXB* was hypomethylated in dSSc and lSSc fibroblasts. *TNXB* encodes a member of the tenascin family of ECM glycoproteins, which are involved in matrix maturation.³⁶

RUNX1 and RUNX2 are transcription factors that induce expression of SOX5 and SOX6, which leads to the induction of type II collagen expression via the direct regulation of promoter activity, and therefore, collagen synthesis.^{37,38} RUNX3, another member of the Runx family, is also likely to contribute to collagen synthesis in association with RUNX2.³⁹ We identified hypomethylation of *RUNX1*, *RUNX2* and *RUNX3* in dSSc and lSSc. Furthermore, we evaluated mRNA expression of *RUNX2* and *RUNX3* and demonstrate overexpression of *RUNX3* in SSc fibroblasts, but no statistically significant difference in the expression of *RUNX2* in either dSSc or lSSc compared with controls (figure 1). The observation that the hypomethylated genes that we evaluated are generally overexpressed argues for a possible role of DNA methylation defects in overexpression of these genes and suggests a possible role of DNA methylation in the pathogenesis of SSc. However, although hypomethylated DNA in gene regulatory regions represents an epigenetic mark for accessibility of the transcriptional machinery to the chromatin, DNA hypomethylation

should not be viewed as the sine qua none for genomic transcription as other factors, like transcription factors, are needed for active transcription.

There has been tremendous interest to understand the factors that lead to recapitulation of Wnt/ β -catenin signalling pathway in SSc,^{2240–43} which is generally required for embryonic development, and cell fate determination. We identified aberrant methylation of genes representative of the Wnt/ β -catenin pathway in SSc. Specifically, we identified hypomethylation *CTNNA2*, *CTNNA1* in dSSc fibroblasts, and *CTNNA3*, and *CTNND2* in ISSc fibroblasts compared with control fibroblasts. Our observation supports a role of Wnt/ β -catenin in SSc pathogenesis and provides evidence for epigenetic alterations in this pathway in SSc fibroblast.

In diffuse SSc fibroblasts, we identified hypomethylation of *CDH11*, which encode for cadherin-11. Cadherins are a group of transmembrane glycoproteins that mediate cell-to-cell adhesion. It has been established that *CDH11* has a role in the process of fibrosis,⁴⁴ wound healing, lung fibrosis⁴⁵⁴⁶ and SSc.⁴⁷⁴⁸ Indeed, *CDH11*-deficient mice had decreased fibrotic endpoints in bleomycin-induced fibrosis.⁴⁵ We postulate that hypomethylation of *CDH11* contributes to its overexpression, which facilitates the differentiation of resident tissue fibroblasts into myofibroblasts in SSc.

Multiple studies in a variety of cell types have demonstrated altered DNA methylation patterns in a number of autoimmune and fibrotic diseases, providing important new directions towards understanding the pathogenesis of immune-mediated diseases.^{1749–55} Epigenetic studies in fibroblasts in autoimmunity included recent genome-wide DNA methylation profiling of synovial fibroblasts in rheumatoid arthritis.⁵⁰⁵⁵ De la Rica and colleagues⁵⁵ reported differential methylation of 2571 CpG sites, associated with 1240 different genes in rheumatoid arthritis synovial fibroblasts (RASf). Of interest, 330 of these genes were also differentially methylated in at least one subset of SSc fibroblasts in our study. Out of the 330 common differentially methylated genes, 125 were hypomethylated and 79 were hypermethylated in both RASf and SSc fibroblasts, and the rest showed mixed methylation pattern (see online supplementary table S7). Moreover, we identified 30 hypomethylated and 4 hypermethylated common genes between RASf and both subsets of SSc (ISSc and dSSc) (see online supplementary table S7). Canonical pathway analysis identified ‘tight junction’ (hsa04530, $p=2.46E-03$, FDR=2.8%) as the most significant pathway representing common differentially methylated genes in RASf and SSc fibroblasts (n=330).

In summary, we identified genome-wide DNA methylation changes for the first time in SSc fibroblasts. Our study demonstrates a striking diversity of DNA methylation profile, as demonstrated by more intense aberrant DNA methylation in dSSc compared with ISSc fibroblasts, which provides a new perspective to the heterogeneity of SSc. We evaluated the effect of aberrant DNA methylation on gene expression in a set of genes that were hypomethylated in both dSSc and ISSc, and established good correlation between DNA methylation pattern and gene expression. These data indicate that abnormal DNA methylation exists in genes involved in integral pathways involved in fibrogenesis, such as the TGF- β and Wnt/ β -catenin signaling pathways. Our findings emphasise the potential role

of DNA methylation changes in the pathogenesis of SSc, and the potential for epigenome-wide studies to identify novel aspects in complex diseases. Replication studies and studies looking at DNA methylation aberrancies associated with specific features of SSc are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Correlation of gene expression and methylation status. To evaluate the functional significance of methylation status on gene expression, we performed RT-qPCR for eight selected genes that were hypomethylated in both diffuse and limited SSc fibroblasts. Overall most of the hypomethylated genes were overexpressed in SSc fibroblasts. The columns in the graph show the average individual gene expression level in limited and diffuse SSc in reference to control fibroblasts, error bars represent the SD. t test was used to compare the mean of individual gene expression levels in each SSc subset, as well as all SSc samples. *p value 0.05.

Table 1

Demographic characteristics of the study participants

SSc patients						Controls			
ID	Type	Age (years)	Sex	Ethnicity	Immunosuppressive medications	ID	Age (years)	Sex	Ethnicity
P1	dSSc	60	Male	EA	None	C1	65	Male	EA
P2	dSSc	25	Female	EA	None	C2	23	Female	EA
P3	dSSc	71	Male	EA	None	C3	67	Male	EA
P4	dSSc	30	Female	AA	None	C4	29	Female	AA
P5	dSSc	45	Female	EA	None	C5	44	Female	EA
P6	dSSc	65	Female	EA	None	C6	67	Female	EA
P7	lSSc	63	Female	EA	None	C7	64	Female	EA
P8	lSSc	38	Female	EA	None	C8	38	Female	EA
P9	lSSc	61	Male	EA	None	C9	61	Male	EA
P10	lSSc	62	Female	EA	Mycophenolate mofetil	C10	62	Female	EA
P11	lSSc	61	Female	EA	Prednisone (5 mg daily)	C11	58	Female	EA
P12	lSSc	74	Female	EA	None	C12	70	Female	EA

Mean age is 49.3 years for patients with dSSc, 49.1 years for their controls, and 59.8 years for lSSc, and 58.8 years for their controls, p Value=0.99 and 0.88, respectively. AA, African-American; dSSc, diffuse cutaneous systemic sclerosis; EA, European-American; lSSc, limited cutaneous systemic sclerosis.

Table 2

Clinical and laboratory characteristics of SSc patients and healthy controls included in this study

Clinical characteristic	dSSc (n=6)	lSSc (n=6)	Controls (n=12)
Age (mean±SD)	49.3±19.0	59.8±11.7	54.0±16.2
Sex (M/F)	2/4	1/5	3/9
Ethnicity (AA/EA)	1/5	0/6	1/11
Disease duration (years)	3.9±6.0	7.6±8.7	NA
MRSS (mean±SD)	20.6±10.7	7.0±4.8	NA
Organ involvement			
Interstitial lung disease	4	2	NA
Raynaud's phenomena	6	6	NA
Gastrointestinal involvement	2	3	NA
Scleroderma renal crisis	1	0	NA
Pulmonary hypertension	2	2	NA
Serological characteristics			
Anti-topoisomerase I (Scl-70)	4	0	NA
Anti-centromere	0	1	NA
Anti-RNA polymerase III	1	0	NA
Anti-RNP	0	0	NA

AA, African-American; EA, European-American; MRSS, modified Rodnan skin score; NA, not applicable.

Table 3

Summary of the differentially methylated CpG sites and genes in diffuse and limited systemic sclerosis fibroblasts compared with healthy control fibroblasts

	Increased methylation	Decreased methylation	Total
dSSc			
CpG sites	1057	1653	2710
Fold change	1.20–21.40	1.20–11.58	
(Average)	1.59	1.52	
Differential score	22.0 to 338.5	–22.0 to –336.2	
(Average)	41.2	–46.8	
Genes	513	818	1269*
lSSc			
CpG sites	105	916	1021
Fold change	1.20–21.20	1.22–6.53	
(Average)	2.35	1.52	
Differential score	22.0 to 337.0	–22.0 to –183.9	
(Average)	81.7	–36.5	
Genes	56	496	548*
Common between dSSc and lSSc			
CpG sites	15	188	203
Fold change	1.20–13.11	1.21–4.56	
(Average)	2.37	1.62	
Differential score	22.4 to 338.4	–22.1 to –268.6	
(Average)	74.2	–44.2	
Genes	6	118	121*

* This total represents total differentially methylated genes, some with both hypomethylated and hypermethylated CpG sites.

dSSc, diffuse cutaneous systemic sclerosis; lSSc, limited cutaneous systemic sclerosis.

dSSc					ISSc						
#	Gene	# CpG	Location	Meth status	Diff score: mean (range)	Fold change: mean (range)	# CpG	Location	Meth status	Diff score: mean (range)	Fold change: mean (range)
1	<i>NCLN</i>	1	Body	HypOMeth	−268.64	1.53	1	Body	HypOMeth	−83.55	1.32
2	<i>SORCS2</i>	2	Body	HypOMeth	−91.76 (−27.17 to −156.34)	1.96 (1.21–2.72)	2	Body	HypOMeth	−23.96 (−23.52 to −24.40)	1.42 (1.40–1.43)
3	<i>CAMK1D</i>	2	Body	HypOMeth	−101.84 (−76.65 to −127.04)	2.06 (1.83–2.28)	2	Body	HypOMeth	−46.53 (−37.07 to −55.99)	1.57 (1.42–1.71)
4	<i>KRT12</i>	1	Body	HypOMeth	−116.64	1.93	1	Body	HypOMeth	−28.68	1.40
5	<i>IMMP2L</i>	2	Body	HypOMeth	−81.69 (−53.22 to −110.16)	2.13 (1.28–2.98)	2	Body	HypOMeth	−47.62 (−42.01 to −53.23)	1.57 (1.32–1.82)
6	<i>CTDSPL</i>	1	Body	HypOMeth	−108.00	2.25	1	Body	HypOMeth	−26.56	1.50
7	<i>NRGN</i>	1	Body	HypOMeth	−106.00	1.58	1	Body	HypOMeth	−52.06	1.28
8	<i>RAPGEF4</i>	2	Body	HypOMeth	−83.27 (−64.02 to −102.53)	2.05 (1.51–2.59)	1	Body	HypOMeth	−78.63	2.02
9	<i>LFNG</i>	2	TSS1500; Body	HypOMeth	−77.32 (−56.16 to −98.47)	1.94 (1.38–2.50)	2	TSS1500; Body	HypOMeth	−37.24 (−32.55 to −41.92)	1.70 (1.58–1.83)
10	<i>IFT88</i>	1	Body	HypOMeth	−37.37	1.41	1	Body	HypOMeth	−93.06	1.78
11	<i>ODZ4</i>	2	5'UTR; Body	HypOMeth	−57.31 (−26.94 to −87.68)	1.58 (1.30–1.86)	2	5'UTR; Body	HypOMeth	−45.07 (−34.86 to −55.28)	1.49 (1.30–1.67)
12	<i>WIPF1</i>	2	5'UTR; TSS1500	HypOMeth	−53.95 (−27.41 to −80.49)	1.36 (1.32–1.39)	1	5'UTR; TSS1500	HypOMeth	−39.91	1.23
13	<i>RCBTB2</i>	1	Body	HypOMeth	−76.25	1.90	1	Body	HypOMeth	−83.20	1.89
14	<i>COL4A2*</i>	1	Body	HypOMeth	−50.34	1.35	1	Body	HypOMeth	−28.64	1.25
15	<i>ESRRG</i>	1	5'UTR	HypOMeth	−47.74	2.03	1	5'UTR	HypOMeth	−87.36	2.46
16	<i>NRPI</i>	3	Body; 3'UTR	HypOMeth	−62.34 (−43.35 to −81.46)	1.49 (1.37–1.73)	1	Body	HypOMeth	−83.24	2.21
17	<i>ITGA9*</i>	1	Body	HypOMeth	−41.40	1.35	1	Body	HypOMeth	37.29	1.80

dSSc							ISSc				
#	Gene	# CpG	Location	Meth status	Diff score: mean (range)	Fold change: mean (range)	# CpG	Location	Meth status	Diff score: mean (range)	Fold change: mean (range)
18	<i>COL23A1</i> *	1	Body	HypOMeth	-40.35	1.25	1	Body	HypOMeth	-136.16	1.25
19	<i>CACNA1C</i>	1	Body	HypOMeth	-32.87	1.67	1	Body	HypOMeth	-27.13	1.55
20	<i>PAX9</i> *	4	5'UTR; TSS1500; body	HypOMeth	-31.95 (-27.35 to -39.98)	1.48 (1.34-1.77)	2	5'UTR	HypOMeth	-29.97 (-26.44 to -33.50)	1.35 (1.24-1.49)
21	<i>NID2</i>	2	TSS1500; Body	HypOMeth	-32.01 (-22.60 to -41.43)	2.10 (1.36-2.85)	2	TSS1500	HypOMeth	-46.09 (-23.02 to -69.16)	1.58 (1.46-1.71)
22	<i>RUNX2</i> *	2	5'UTR; 1st Exon; body	HypOMeth	-36.41 (-29.99 to -42.83)	1.30 (1.25-1.35)	1	5'UTR; 1st Exon	HypOMeth	-46.20	1.27
23	<i>ADAM12</i> *	3	Body	HypOMeth	-38.31 (-23.04 to -47.87)	1.38 (1.22-1.75)	2	Body	HypOMeth	-42.41 (-32.80 to -52.01)	1.58 (1.39-1.77)
24	<i>MYO1E</i> *	3	Body	HypOMeth	-59.18 (-53.72 to -63.75)	1.85 (1.49-2.17)	2	Body	HypOMeth	-26.38 (-23.51 to -29.25)	1.37 (1.21-1.58)
25	<i>RUNX3</i> *	6	5'UTR; TSS200; TSS1500; 1st Exon	HypOMeth	-62.37 (-31.45 to -95.09)	1.27 (1.21-1.48)	1	TSS1500	HypOMeth	-55.14	1.32
26	<i>MIR154</i>	1	TSS1500	HypOMeth	-63.76	1.90	1	TSS1500	HypOMeth	-24.18	1.51
27	<i>RUNX1</i>	3	5'UTR; TSS1500; 1st Exon; Body	HypOMeth	-71.94 (-38.88 to -92.95)	1.79 (1.46-2.09)	2	Body	HypOMeth	-36.88 (-28.59 to -45.16)	1.58 (1.53-1.64)
28	<i>MYOM2</i>	2	Body	HypOMeth	-81.74 (-79.46 to -84.01)	3.41 (2.27-4.56)	2	Body	HypOMeth	-36.21 (-29.06 to -43.36)	1.70 (1.58-1.83)
29	<i>WDSUB1</i>	1	5'UTR; 1st Exon	HypERMeth	26.07	9.80	1	5'UTR; 1st Exon	HypERMeth	47.97	13.11
30	<i>OSR2</i>	5	Body	HypERMeth	58.17 (39.73 to 91.80)	1.38 (1.27-1.48)	1	Body	HypERMeth	47.88	1.27

* RT-qPCR performed for these genes.

dSSc, diffuse cutaneous systemic sclerosis; HypERMeth, hypermethylated; HypOMeth, hypomethylated; ISSc, limited cutaneous systemic sclerosis; Meth status, methylation status; TSS1500, within 1500 bps from transcription start site; 5'UTR, 5' untranslated region.

Table 5

Canonical pathways and gene ontologies enriched by differentially methylated genes with common differentially methylated CpG sites between dSSc and ISSc

Category	ID	Term	p Value	Fold enrichment	FDR
(A) Canonical pathways					
KEGG_PATHWAY	hsa04512	ECM–receptor interaction	2.92E-03	5.95	0.03
KEGG_PATHWAY	hsa04510	Focal adhesion	8.86E-03	3.32	0.09
(B) Gene ontologies of hypomethylated common genes between dSSc and ISSc *					
GOTERM_BP_FAT	GO:0043407	Negative regulation of MAP kinase activity	2.14E-04	16.63	0.00
GOTERM_BP_FAT	GO:0048858	Cell projection morphogenesis	9.72E-04	4.40	0.02
GOTERM_BP_FAT	GO:0007409	Axonogenesis	1.11E-03	4.96	0.02
GOTERM_BP_FAT	GO:0032990	Cell part morphogenesis	1.29E-03	4.21	0.02
GOTERM_BP_FAT	GO:0007155	Cell adhesion	1.89E-03	2.57	0.03
GOTERM_BP_FAT	GO:0003012	Muscle system process	2.71E-03	4.99	0.04
GOTERM_BP_FAT	GO:0030030	Cell projection organisation	3.47E-03	3.25	0.05
GOTERM_BP_FAT	GO:0030029	Actin filament-based process	3.88E-03	3.97	0.06
GOTERM_BP_FAT	GO:0051674	Localisation of cell	3.97E-03	3.51	0.06
GOTERM_BP_FAT	GO:0048870	Cell motility	3.97E-03	3.51	0.06
GOTERM_BP_FAT	GO:0000904	Cell morphogenesis involved in differentiation	4.15E-03	3.93	0.06
GOTERM_BP_FAT	GO:0010604	Positive regulation of macromolecule metabolic process	4.64E-03	2.24	0.07
GOTERM_BP_FAT	GO:0042325	Regulation of phosphorylation	5.26E-03	2.83	0.08
GOTERM_BP_FAT	GO:0006469	Negative regulation of protein kinase activity	5.85E-03	6.88	0.09
GOTERM_BP_FAT	GO:0045893	Positive regulation of transcription, DNA-dependent	6.17E-03	2.76	0.09
GOTERM_BP_FAT	GO:0043405	Regulation of MAP kinase activity	6.31E-03	5.09	0.09
GOTERM_BP_FAT	GO:0051254	Positive regulation of RNA metabolic process	6.54E-03	2.74	0.10
GOTERM_BP_FAT	GO:0033673	Negative regulation of kinase activity	6.59E-03	6.65	0.10

* Number of hypermethylated common genes between dSSc and ISSc is inadequate to evaluate hypermethylated ontologies.

ECM, extracellular matrix; FDR, false discovery rate; GOTERM_BP_FAT, Gene Ontology term biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes.