

# Increased serum calpain activity is associated with HMGB1 levels in systemic sclerosis

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## Research article

**Keywords:** calpain, systemic sclerosis, interstitial lung disease, calpain, HMGB1, microarray analysis

**Posted Date:** March 10th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-16478/v1>

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**Version of Record:** A version of this preprint was published at Arthritis Research & Therapy on May 11th, 2020. See the published version at <https://doi.org/10.1186/s13075-020-02195-y>.

# Abstract

**Background** Systemic sclerosis (SSc) or scleroderma is an intractable autoimmune disorder that affects multiple organs. The objectives were to investigate clinical correlations of serum calpain activity and high mobility group box 1 (HMGB1) levels with immunological and clinical traits.

**Methods** A total of 31 patients with SSc, 20 age- and gender-matched healthy control subjects (HC) and 10 patients with other connective tissue diseases (CTD) were recruited in the study. We measured serum calpain activity and HMGB1 levels and analyzed the datasets (GSE40839, GSE48149, GSE76808, GSE81292 and GSE33463) from gene expression omnibus (GEO) database to explore potential mechanism by which calpain exerts its function through bioinformatics methods.

**Results** Serum calpain activity was significantly increased in patients with SSc compared with those in HC and in patients with CTD and was correlated with serum HMGB1 levels, modified Rodnan skin score, erythrocyte sedimentation rate, mean platelet volume and plateletcrit. Notably, serum calpain activity and HMGB1 levels in SSc patients with interstitial lung disease (ILD) were significantly higher than that in SSc patients without ILD. Serum calpain activity and HMGB1 levels could be the independent risk factors for SSc-ILD and novel biomarkers in patients with SSc.

**Conclusion** This is the first study that reports increased serum calpain activity and the correlation between calpain and HMGB1 in patients with SSc or SSc-ILD. The serum calpain activity and HMGB1 levels may serve as measures of ILD in patients with SSc. Also, calpain and HMGB1 could be potentially therapeutic targets for patients with SSc or SSc-ILD in the future.

## 1. Background

Systemic sclerosis (SSc) or scleroderma is an intractable autoimmune disorder characterized by vasculopathy and fibrosis of the skin and visceral organs including heart, kidney and lung, which has high mortality and reduced survival than other autoimmune diseases [1]. Although SSc often involves multiple organs, lung diseases such as interstitial lung disease (ILD) and pulmonary arterial hypertension (PAH), are considered the main causes of mortality[2]. To date, the pathogenesis and mechanism of SSc have been poorly understood. Progress in the development of effective therapies for SSc has been slow [3]. Thus, besides novel biomarkers reflecting the progression of disease there is also need to identify the potential targets for accurate therapy in SSc or SSc-ILD.

Calpain is a family of calcium-dependent, non-lysosomal neutral cysteine endopeptidases [4]. Humans can express 15 calpain genes including CAPN1 to CAPN16 with the exception of CAPN4, which can be categorized as conventional and unconventional subunits [5]. Conventional subunits comprised of CAPN1 and CAPN2 are the most studied members of calpain family, and both are ubiquitously expressed in all tissues, which function as the catalytic subunits of calpain-1 and calpain-2, respectively[6, 7]. Calpain small subunit 1 (CAPNS1), also known as CAPN4, is the common regulatory subunit of calpain-1 and calpains-2, and plays a important role in maintaining the activity and stability of calpain [8]. Notably

calpain activities disorder contribute to the pathogenesis of many diseases such as neurodegenerative diseases, brain ischemia, cardiovascular disorders and cancers[5]. Several studies have reported that calpains may involve in the pathogenesis of Alzheimer's disease (AD) [9–12]. It has been previously reported that elevated calpain activity and microparticle levels in cerebrospinal fluid of AD patients and could become a potential prognostic and diagnostic biomarker for AD[13]. A recent study has demonstrated that inhibition of calpain preserves memory and reduces accumulation of A $\beta$  and of p25 in mouse model of AD[14]. Moreover, the function of calpain in cardiovascular diseases have been extensively investigated [15–19]. Although there is accumulating evidence that targeted inhibition of calpain serves as a potential therapeutic strategy for calpain related diseases in animal models and clinical trials, few studies have investigated the roles of calpains in autoimmune diseases[5, 20]. In particular, the roles of calpains in the pathogenesis of SSc have never been investigated.

High Mobility Group Box-1 (HMGB1), an endogenous damage-associated molecular pattern (DAMP) is secreted when macrophages and monocytes are activated and is passively released as a consequence of cell damage or necrosis[21]. HMGB1 is also a nuclear nonhistone chromatin-binding protein, which participates in the regulation of transcription and stabilization of nucleosome structure[22]. Several studies have reported that serum HMGB1 level is increased and is associated with platelet-derived microparticles indicating that platelets are a source of DAMP HMGB1 in patients with SSc[23–26]. Moreover, active calpain is also associated with platelet-derived microparticles and are known as a source of active calpain in the blood[27]. Thus, calpain activity in the serum and the levels of HMGB1 seem to be directly correlative. So far, study concerning the correlation between calpain activity and HMGB1 level in the serum from SSc or SSc-ILD patients are still missing.

In this study, we attempted to investigate clinical correlations of serum calpain activity and HMGB1 levels with immunological and clinical traits in 31 Chinese patients with SSc, 20 healthy control subjects (HC) and 10 patients with other connective tissue diseases (CTD). Furthermore, integrative microarray datasets of lung samples and peripheral blood mononuclear cells (PBMCs) samples from patients with SSc or SSc-ILD and HC were utilized to explore underlying mechanism by which calpain exerts its function in the pathogenesis of SSc through bioinformatic analysis. Finally, we found that SSc patients showed significantly elevated serum calpain activity and higher HMGB1 levels compared with HC and patients with other CTD. In addition, serum calpain activity was significantly positively correlated with HMGB1 levels in patients with SSc. Our findings may point to serum calpain activity and HMGB1 levels could be promising treatment targets for SSc or SSc-ILD, and provide powerful evidences for better understanding the pathogenesis of SSc.

## 2. Methods

### 2.1 *Patients and controls*

A total of 31 patients with a positive diagnosis as SSc according to ACR/EULAR 2013 classification criteria was recruited at Zhongshan Hospital (Fudan University, Shanghai, China) [28]. 10 patients with

other CTD were recruited as disease control subjects. 20 people with no history of pulmonary, autoimmune, cardiovascular, or other diseases were recruited as HC. Detailed characteristics of SSc patients, HC and patients with other CTD were shown (Table 1). The study was approved by the Zhongshan Hospital Research Ethics Committee. Written informed consent was acquired from all subjects.

## **2.2 Blood sampling**

Blood samples were collected in serum tubes with a gel separation plug (BD Biosciences, USA). All samples were gently mixed, and the serum tubes were placed at room temperature for coagulation for 30 minutes. Then, all samples were then centrifuged at 3000g for 20 minutes at 4°C and the top volumes of the serum were collected in 1.5 mL centrifuge tube (Axygen, USA). All samples were frozen within 30 minutes and preserved at -80°C before tests. Further detection was done within one month.

## **2.3 Calpain activity measurement**

Calpain activity kit (Raybiotech, USA) was utilized to measure calpain activities in serum or plasma. Eighty-five µL of serum was diluted in 10 µL of 10X calpain reaction buffer and 5 µL of calpain substrate Ac-LLY-AFC with or without 100 µM Calpeptin (Abmole, USA). Free AFC was quantified using fluorometer (excitation λ400 nm, emission λ505 nm) after incubating at 37°C for 1 hour in the dark. The difference of calpain activity was determined by comparing the relative fluorescent unit (RFU) of samples with and without calpeptin. The calpain activity was expressed as RFU per µL serum of each sample.

## **2.4 Measurement of HMGB-1 concentrations in serum**

The measurement of the serum HMGB-1 level was performed by enzyme linked immunosorbent assays (IBL-International, Hamburg, Germany) according to the manufacturer's instructions. The detection limit of this assay was 0.313 ng/ml. Each sample was tested in duplicate.

## **2.5 Data information**

Microarray datasets and high-throughput sequencing datasets from NCBI Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) were thoroughly searched for available datasets involving SSc. Included datasets should meet the following criteria: (a) datasets with SSc or SSc-ILD lung tissue, skin or blood samples; (b) datasets with platform information; (c) datasets with healthy people as control. According to these criteria, six microarray datasets (GSE40839, GSE48149, GSE76808, GSE81292

and GSE33463) were obtained from GEO database. Details of each microarray study, including samples descriptions and platform information, were shown in Table S1.

## **2.6 Data processing**

For datasets of lung tissue samples (GSE40839, GSE48149, GSE76808 and GSE81292), 50 SSc-ILD patients and 28 HC were included for further analysis. For datasets of PBMCs samples (GSE33463), 69 SSc-ILD patients and 41 HC were included. First, the raw data of each dataset was preprocessed by the R packages *affy* (under the R environment, version 3.6.1) and *annotate* methods to make normalized expression profiles with official gene names. Since datasets of lung samples were from different studies and based on different platforms, all lung samples of five datasets (GSE40839, GSE48149, GSE76808 and GSE81292) were integrated by batch normalization using *sva* package in R software to reduce batch effects and heterogeneity among different samples so as to significantly improve sample size (50 SSc-ILD vs 28 HC). Next, the differential expression analysis ( $\text{Log}_2\text{FC} > |1|$ ,  $P\text{-value} < 0.05$ ) of calpain related genes was performed by comparing SSc or SSc-ILD samples to HC samples using *limma* package. The boxplot was also utilized to visualize the expression of calpain related genes.

## **2.7 Bioinformatic analysis**

To explore the function of calpain related genes in SSc patients, we removed HC lung samples ( $n=20$ ) and clustered SSc-ILD patients ( $n=50$ ) into two clusters based on the expression status of calpain related genes using “ConsensusClusterPlus” package in R[29]. Then, the differential expression analysis was performed using the *limma* package in two clusters. The cutoff value was  $\text{log}_2\text{FC} > |1|$ ,  $P\text{-value} < 0.05$ . Next, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis of differently expressed genes (DEGs) in two clusters were performed using *GOplot* package in R. To build the protein-protein interaction (PPI) network, we imported the genes into STRING database (<http://string-db.org>) and visualized these genes by Cytoscape.

## **2.8 Statistical analysis**

We utilized the Student *t*-test, the Mann-Whitney *U* test, or the chi-squared test, as appropriate, to test comparisons of each groups for statistical significance, and used univariate logistic regression (ULR) and multivariate logistic regression (MLR) to determine risk factors by SPSS 22.0. We also utilized receiver operating characteristic curve (ROC) to calculate the area under the curve (AUC) by SPSS 22.0 and utilized Spearman's rank correlation to evaluate the correlation between two continuous parameters by GraphPad Prism 6.0. A *P* value of  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1 *Laboratory and clinical characteristics of the patients and controls*

SSc patients and HC with matched age and gender showed no significant differences (Table 1). Moreover, we found that there were no significant differences between laboratory and clinical parameters in SSc patients with and without ILD (Table 1).

### 3.2 *Overexpression of calpain related genes in SSc or SSc-ILD patients*

To explore the relationship between calpains and SSc, we compared the expression levels of calpain related genes between SSc or SSc-ILD patients and HC in five microarray datasets from lung biopsy samples and one from PBMCs samples. For the comparison of integrated microarray datasets of lung samples, the expression levels of *CAPN1*, *CAPNS1*, *CAPN6* and *CAPN7* were significantly higher in lung samples from SSc-ILD patients than that from HC (Fig. 1a, 50 SSc-ILD versus 28 HC). For the comparison of PBMCs samples, the expression levels of *CAPN1*, *CAPN5*, *CAPN11* and *CAPN12* were significantly higher in PBMCs samples from SSc patients than that from HC (Fig. 1b, 69 SSc versus 41 HC). Notably the differential expression of *CAPN1* was overlapped in these microarray datasets from lung samples and PBMCs samples, indicating that significant overexpression of *CAPN1* in patients with SSc (Fig. 1c and 1d,  $p=0.034$  and  $p=0.042$ , respectively). Therefore, calpain activity was measured in serum from SSc and SSc-ILD patients to further verify overexpression of calpains.

### 3.3 *Serum calpain activity and HMGB1 level were increased in SSc patients*

SSc patients showed significantly increased calpain activity in serum compared with HC ( $p<0.05$ ) and patients with other CTD ( $p<0.05$ ; Fig. 2a). Serum calpain activity in patients with diffuse cutaneous systemic sclerosis (dSSc) was significantly higher than those in patients with limited cutaneous systemic sclerosis (lSSc) ( $p<0.05$ ; Fig. 2b). Serum calpain activity in patients with SSc-ILD was significantly higher than those in SSc patients without ILD ( $p<0.05$ ; Fig. 2c). Furthermore, SSc patients showed significantly elevated HMGB1 level in serum compared with HC ( $p<0.001$ ) and patients with other CTD ( $p<0.001$ ; Fig. 2d). Serum HMGB1 level in patients with dSSc were significantly elevated relative to patients with lSSc ( $p<0.05$ ; Fig. 2e). Serum HMGB1 level in patients with SSc-ILD were significantly elevated in comparison to SSc patients without ILD ( $p<0.05$ ; Fig. 2f). To further assess the relationship between serum calpain activity and HMGB1 in the pathogenesis of SSc, we evaluated whether serum calpain activity are correlated with serum HMGB1 level and found that serum calpain activity was correlated positively with HMGB1 level ( $r=0.3891$ ,  $p<0.05$ ; Fig. 2g).

### ***3.4 Clinical and laboratory correlation***

Next, we evaluated clinical and laboratory correlation of serum calpain activity and HMGB1 level in SSc patients (Table 2). The cutoff value was set at 0.437 RFU/ $\mu$ L for calpain activity and 13.37 ng/mL for HMGB1 concentration, which were calculated as the mean+2 SD for HC. A total of 10 patients were defined as the calpain high group, while the remaining 21 patients were defined as the calpain low group. For HMGB1 level, a total of 12 patients were defined as the HMGB1 high group, while the remaining 19 patients were defined as the HMGB1 low group. SSc patients with serum-elevated calpain activity had significantly higher frequency of negative anti-Scl-70 antibody ( $p<0.05$ ), higher modified Rodnan skin score (mRSS) ( $p<0.05$ ), higher plateletcrit (PCT) ( $p<0.05$ ), higher platelet large cell ratio (P-LCR) ( $p<0.05$ ) and decreased erythrocyte sedimentation rate (ESR) ( $p<0.05$ ). Furthermore, serum calpain activity was correlated positively with mRSS ( $r=0.4990$ ,  $p<0.05$ ), mean platelet volume (MPV) ( $r=0.3682$ ,  $p<0.05$ ) and PCT ( $r=0.3988$ ,  $p<0.05$ ) and was correlated inversely with ESR ( $r=-0.4186$ ,  $p<0.05$ ; Fig. 3). However, serum HMGB1 level showed no significant correlations with any clinical and laboratory parameters. Therefore, these results suggested elevated serum calpain activity was associated with the skin thickness, ESR, anti-Scl-70 antibody and platelet abnormalities in patients with SSc.

### ***3.5 Diagnostic value of serum calpain activity and HMGB1 levels***

Next, we applied ULR and MLR and found that mRSS, serum calpain activity and HMGB1 levels could become independent risk factors for SSc-ILD in SSc patients (Table 3). However, the results of MLR analyses indicated that serum calpain activity and HMGB1 levels were not significantly correlated with the diagnosis of SSc-ILD (Table 3). Next, to explore the diagnostic capabilities of serum calpain activity and HMGB1 levels for patients with SSc or SSc-ILD, we performed ROC that the AUC was 0.750 ( $p<0.05$ ) for serum calpain activity, 0.746 ( $p<0.05$ ) for HMGB1 levels, 0.824 ( $p<0.001$ ) for the combination of them (Fig. 4a). Then, we confirmed the potential utility of serum calpain activity and HMGB1 levels as novel diagnostic biomarkers of SSc-ILD. The AUC for serum calpain activity, HMGB1 levels, and combination of them when distinguishing SSc-ILD patients from SSc patients were 0.781 ( $p<0.05$ ), 0.767 ( $p<0.05$ ), 0.833 ( $p<0.05$ ) (Fig. 4b). In summary, above results indicated a high diagnostic accuracy of serum calpain activity and HMGB1 levels as a new biomarker for SSc or SSc-ILD and they could be the independent risk factors for SSc-ILD.

### ***3.6 Underlying mechanisms in SSc or SSc-ILD***

To further explore the biological role of calpain related genes and underlying mechanisms in SSc or SSc-ILD, we removed 28 lung samples from HC and clustered 50 lung samples from SSc-ILD into two groups using the ConsensusClusterPlus package. On the basis of the expression similarity of calpain related genes,  $k = 2$  seemed to be an adequate selection with clustering stability increasing from  $k = 2$  to 10 (Fig.

S1a, b and c). Next, we used the differential expression analysis of cluster1 relative to cluster2 to confirm differentially expressed genes associated with calpain expression. 72 up-regulated and 65 down-regulated genes were identified as to be significantly associated with calpain related genes expression between cluster1 and cluster2 (Fig. S1d). These abnormal genes were also shown as an expression heatmap (Fig. S1e). Then, we identified these aberrant genes through PPI network (Fig. S1f). GO enrichment analysis revealed that these genes were mainly distributed in reproductive structure development, reproductive system development, cornification, renal system development and urogenital system development (Fig. 5a). Moreover, to understand the biological mechanism as associated with SSc or SSc-ILD, we performed KEGG analysis of these abnormal genes. These genes were mainly distributed in genes encoding for transcriptional mis-regulation in cancer, toll-like receptor signaling pathway, pancreatic secretion and complement and coagulation cascades, suggesting that the levels of these pathways were correlated with SSc or SSc-ILD advances (Fig. 5b)

## 4. Discussion

The present study is the first to reveal that calpain activity was increased in serum samples from SSc patients in comparison to HC and patients with other CTD. We also demonstrated that calpain activity in serum of patients with SSc was positively correlated with HMGB1 levels, mRSS, MPV, and PCT, but correlated inversely with ESR. Moreover, elevation of serum calpain activity was accompanied by the presence of increased mRSS, PCT, P-LCR, negative anti-Scl-70 antibody and decreased ESR, indicating that serum calpain activity was associated with platelet dysfunction and skin thickness. Furthermore, serum-elevated calpain activity and HMGB1 levels were also the independent risk factors for SSc-ILD. Finally, we explored the potential mechanisms and provided new insights into the pathogenesis of SSc or SSc-ILD. Collectively, our research suggested that serum calpain activity and HMGB1 levels were a new potential serological biomarker for diagnosis of SSc-ILD. In addition, calpain and HMGB1 could be therapeutic targets for accurate therapy of SSc or SSc-ILD in the future.

Calpain has been widely studied to play a critical role in vascular remodeling and collagen synthesis [30, 31]. Peng and colleagues reported that calpain induces myocardial hypertrophy and fibrosis [8]. Several studies have reported that inhibition of calpain attenuates bleomycin-induced pulmonary fibrosis in mice [32–35]. Furthermore, recent study has demonstrated that calpain activation by renin-angiotensin system induce collagen-I synthesis and pleural fibrosis [36]. However, studies involving the calpain activation and skin fibrosis or SSc related pulmonary fibrosis have never been reported. It is worth mentioning that our study is the first clinical research which provides insightful viewpoints in the relationship between calpain activation and SSc or SSc-ILD and have demonstrated that increased serum calpain activity is associated with skin thickness and could predict the development of ILD in SSc patients. Moreover, several studies have demonstrated that microparticles are higher in the blood of patients with SSc compared with HC and that most of them are derived from platelets, indicating that platelet could be a critical actor in the activation of innate immunity [37, 38]. Manfredi and colleagues have revealed that platelets-derived microparticles express DAMP HMGB1 and lead to initiate fibrosis and endothelial damage when injected into mice, indicating that platelets-associated HMGB1 may be a



potential indicator of SSc [25]. Consistent with previous findings, our results also showed that serum calpain activity was correlated with serum HMGB1 levels and platelet-related parameters (MPV, PCT and P-LCR), which suggest that activated platelet-derived calpain and HMGB1 may be responsible for the vascular remodeling and endothelial damage in patients with SSc.

HMGB1 has been widely reported as a central role in the pathogenesis of many CTD, such as SLE, polymyositis or dermatomyositis with ILD, and RA[39–43]. Our results clearly demonstrate that serum HMGB1 levels from SSc patients are significantly higher compared with that from HC. Consistent finding has also been reported in a cohort of 70 patients with SSc and 25 HC, which indicates that increased HMGB-1 levels have more frequent involvement of multiple organs and abnormalities of immunological parameters and correlate positively with mRSS[23]. Several mechanisms have been reported for HMGB1 to contribute to the of SSc. A recent study reported that activated platelets-derived microparticles expressed DAMP HMGB1 and induced neutrophil activation in SSc patients, contributing to neutrophil extracellular trap production and autophagy [25]. Moreover, DAMP HMGB-1 induces pro-inflammatory effects partly through interaction with toll-like receptor 4 (TLR4), resulting in activation of nuclear factor-kappa B (NF-κB) pathway and several inflammatory genes [44–47]. Notably, according to experimental findings of myocardial infarction mouse model with cardiomyocyte-specific deletion of Capn4, Capn4 knock-out correlated with restoration of IκB protein and inhibition of NF-κB activation, resulting in inhibition of proinflammatory cytokine expression and inflammatory cell infiltration in the Capn4 knock-out heart after myocardial infarction[48]. Consistent with previous results, our finding suggests that toll-like receptor signaling pathway could be the potential mechanism which calpain and HMGB1 commonly functions through bioinformatics analysis of integrative microarray datasets.

A limitation of this study is that our limited samples from largely middle-aged female patients cannot represent the general population. Therefore, our findings may not generalize total SSc or SSc-ILD patients. Further studies with larger sample size and less heterogeneity are needed for the generalization of our findings. In spite of the limitation, our study firstly identified the serum calpain activity was elevated in patients with SSc and highlight the potential mechanisms for further research.

## 5. Conclusions

In conclusion, this is the first study that reports increased calpain activity in serum of patients with SSc or SSc-ILD. We also highlight the correlations between serum calpain activity and HMGB1 levels. Serum calpain activity could potentially serve as a novel biomarker in patients with SSc-ILD and therapeutic target for SSc or SSc-ILD. In the future, there remains a strong need for well-designed and prospective clinical studies with more homogenized populations and larger samples size to study the underlying function of calpain and HMGB1 in the development of SSc.

## List Of Abbreviations

SSc: Systemic sclerosis; ILD: interstitial lung disease; PAH: pulmonary arterial hypertension; CAPNS1: Calpain small subunit 1; AD: Alzheimer's disease; HMGB1: High Mobility Group Box-1; DAMP: damage-associated molecular pattern; HC: healthy control subjects; CTD: connective tissue diseases; PBMCs: peripheral blood mononuclear cells; GEO: Gene Expression Omnibus; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; DEGs: differently expressed genes; PPI: protein-protein interaction; ULR: univariate logistic regression; MLR: multivariate logistic regression; ROC: receiver operating characteristic curve; AUC: area under the curve; dSSc: diffuse cutaneous systemic sclerosis; lSSc: limited cutaneous systemic sclerosis; mRSS: modified Rodnan skin score; PCT: higher plateletcrit; P-LCR: platelet large cell ratio; ESR: erythrocyte sedimentation rate; MPV: mean platelet volume.

## **Declarations**

### **Ethics approval and consent to participate**

This study was carried out in accordance with the recommendations of Zhongshan Hospital Research Ethics Committee. The protocol was approved by the Ethical Committee of Zhongshan Hospital with the approval number-B2019-192R. The written informed consent was obtained from all patients.

### **Consent for publication**

Informed consent was obtained from all participants for publication.

### **Availability of data and materials**

Publicly available datasets were analyzed in this study. Datasets (GSE40839, GSE48149, GSE76808, GSE81292, GSE33463) were downloaded from GEO (<http://www.ncbi.nlm.nih.gov/geo/>).

### **Competing interests**

The authors declare that they have no competing interests

### **Funding**

This study was supported by the grants awarded to Dr Qiang Wang from the National Natural Science Foundation of China (81641087), the Key Biomedical Program of Science and Technology Commission of Shanghai Municipal (18401931700), the Research Fund of Shanghai Municipal Commission of Health

and Family Planning (201640071,2016ZJP001), and the Research and Development Fund of Zhongshan Hospital Affiliated to Fudan University (2016ZSFZ46).

**Authors' contributions**

Zheng JN, Li Y, and Wang Q designed the study. Shao WQ collected blood samples. Zheng JN, Li Yang and Yan YM did the statistical analyses and prepared figures. Zheng JN, Li Y, Yan YM, Yu Y, Shao WQ and Wang Q reviewed the results and wrote the manuscript. All authors have made an intellectual contribution to the manuscript. All authors read and approved the final manuscript.

**Acknowledgments:**

Special thanks to Dr Zhen Yao for continued support and company.

**Supplementary Materials**

**Table S1. Details of SSc associated microarray datasets from GEO database**

**Figure S1—Genome-wide genes associated with calpain related genes expression**

- (a) Consensus matrix heat map for the chosen optimal cluster number (k = 2).
- (b) Consensus clustering cumulative distribution function (CDF) for k = 2 to 9.
- (c) Relative change in area under CDF curve for k = 2 to 9.
- (d) Volcano plot of differential genes between cluster1 and cluster2.
- (e) Heat map of the two clusters. \* P < 0.05, \*\* P < 0.01
- (f) Protein–protein interactions network of differentially expressed genes between two clusters.

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## Tables

**Table 1. Laboratory and clinical characteristics of the patients and control subjects included in the study**

Characteristics	All SSc (n=31)	Non-ILD-SSc (n=10)	SSc-ILD (n=21)	Other CTD (n=10)	HC (n=20)
Age	51±13		53±12		
(mean± SD)		49±14		47±11	40±11
Female n (%)	27 (87)	10 (91)	17 (85)	8 (80)	17 (85)
Course of disease (mean± SD)	8±7		9±8		NA
lcSSc/dcSSc %	55/45	45/55	60/40		
PAH n (%)	3 (10)	0 (0)	3 (15)		
Raynaud's phenomenon n (%)	28 (90)		19 (45)		
Anti-Scl-70 antibody n (%)	13 (42)	9 (82) 4 (36)	9 (45)		
ANA n (%)	30 (97)	10 (91)	20 (100)		
Anti-RNP n (%)	8 (26)	4 (36)	4 (20)		
Anti-centromere n (%)	8 (26)		6 (30)		
mRSS (mean± SD)	12±10	2 (18)	10±7		
n=25		21±14			
Immunosuppressive therapy ever n (%)	11 (35)	5 (45)	6 (25)		
Cyclophosphamide n (%)	6 (19)		3 (15)		
Methotrexate n (%)	2 (6)	3 (27) 0 (0)	2 (5)		
Mycophenolate mofetil n (%)	3 (10)		1 (5)		
FVC % predicted	74.5±14.2	2 (18)	75.6±16.2		
n=17					
(mean± SD)		78±8.5			
DL <sub>CO</sub> % predicted	74.2±22.3		76.2±27.8		
n=8					
(mean± SD)		70.9±3.5			
FEV1/FVC%	85.5±11.6		88.4±11.1		
n=17					
(mean± SD)		76.2±7.5			
ESR, mm/h (mean± SD)	17.10±11		16.76±9.13		
PLT, 10 <sup>9</sup> /L (mean ± SD)	206±67	17.80±14.36	204.62±61.60		
MPV, fL (mean ± SD)	11.95±1.49	209.40±79.98 11.23±1.95	10.81±1.24		



PDW, fL (mean ± SD)	0.22±0.07	0.23±0.08	0.22±0.07	
PCT, % (mean ± SD)	31.17±11.13	34.69±14.07	29.49±9.36	
P-LCR, % (mean ± SD)	13.47±3.30		13.16±2.63	
RA n (%)		14.12±4.50 NA		1 (10)
ANCA-associated vasculitis n (%)				1 (10)
SLE n (%)				2 (20)
pSS n (%)				1 (10)
Dermatomyositis n (%)				2 (20)
Polymyositis n (%)				3 (30)
				NA

**Abbreviation:** SSc: systemic sclerosis; ILD: interstitial lung disease; PAH: pulmonary arterial hypertension; CTD: connective tissue diseases; HC: healthy control subjects; SD: standard deviation; dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis; ANA: antinuclear antibodies; Anti-RNP: anti ribonucleoprotein antibody; mRSS: modified Rodnan skin score; FVC: forced vital capacity; DL<sub>CO</sub>: diffusing capacity of the lung for carbon monoxide; FEV1: forced the first second of expiratory volume; ESR: erythrocyte sedimentation rate; PLT: platelet count; PDW: platelet distribution width; PCT: plateletcrit; MPV: mean platelet volume; P-LCR: platelet large cell ratio; RA: rheumatoid arthritis; ANCA: anti-neutrophil cytoplasmic antibodies; SLE: systemic lupus erythematosus; pSS: Sjogren syndrome; NA: not available.

**Table 2. Clinical and laboratory characteristics of SSc patients according to serum calpain activity and HMGB1 levels**

Characteristics	Calpain <sup>high</sup> n=10	Calpain <sup>low</sup> n=21	P value	HMGB-1 <sup>high</sup> n=12	HMGB-1 <sup>low</sup> n=19	P value
Age	48±13	53±13				
(mean± SD)			0.311	49±14	53±12	0.358
Female n (%)	8 (80)	19 (90)	0.577	11 (92)	16 (84)	0.958
Course of disease	10±11	7±6				
(mean± SD)			0.299	7±2	8±2	0.946
dSSc/lSSc	5/5	12/9	0.709	8 (67)	6 (32)	0.075
PAH n (%)	2 (20)	3 (14)	0.690	4 (33)	1 (5)	0.060
SSc-ILD n (%)	8 (80)	16 (76)	0.813	11 (92)	13 (68)	0.201
Raynaud's phenomenon n (%)	9 (90)	18 (86)				
Anti-Scl-70 antibody	1 (10)	12 (57)	0.734	11 (92)	16 (84)	0.958
n (%)			0.020*	5 (42)	8 (42)	0.981
ANA n (%)	10 (100)	20 (95)	0.483	12 (100)	18 (95)	0.999
Anti-RNP n (%)	4 (40)	4 (19)	0.381	4 (33)	4 (21)	0.676
Anti-centromere n (%)	3 (30)	5 (24)				
mRSS (mean± SD)	19±13	9±6	0.715	5 (42)	3 (16)	0.206
n=26			0.012*	9±5	15±12	0.098
FVC % predicted	64.85±35.29	54.27±35.40		52.63±33.50	63.80±36.46	0.472
n=17			0.545			
(mean± SD)						
DL <sub>CO</sub> % predicted	81.74±32.99	66.57±11.25		61.35±20.29	80.79±27.21	0.387
n=8			0.488			
(mean± SD)						
FEV1/FVC%	88.42±15.84	85.53±8.49		83.58±9.22	88.97±12.79	0.344
n=17			0.638			
(mean± SD)						
ESR, mm/h	11.10±5.04	19.95±11.77	0.031*	13.92±8.45	19.11±11.90	0.167
(mean± SD)						
Platelets count, 10 <sup>9</sup> /L (mean ± SD)	201±71	209±66	0.772	194±83	214±56	0.442
MPV, fL (mean ± SD)	11.59±1.66	10.64±1.33	0.096	11.25±1.82	10.75±1.25	0.373
PDW, fL (mean ± SD)	0.22±0.07	0.22±0.07	0.867	0.21±0.08	0.23±0.06	0.480
PCT, % (mean	37.74±11.69	28.04±9.62	0.021*	30.98±12.95	31.29±10.20	0.944

$\pm$ SD)						
P-LCR, %	15.17 $\pm$ 3.94	12.66 $\pm$ 2.68	0.045*	14.08 $\pm$ 4.01	13.08 $\pm$ 2.81	0.465
(mean $\pm$ SD)						

**Abbreviation:** SSc: systemic sclerosis; ILD: interstitial lung disease; PAH: pulmonary arterial hypertension; HC: healthy control subjects; SD: standard deviation; dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis; ANA: antinuclear antibodies; Anti-RNP: anti ribonucleoprotein antibody; mRSS: modified Rodnan skin score; FVC: forced vital capacity; DL<sub>CO</sub>: diffusing capacity of the lung for carbon monoxide; FEV1: forced the first second of expiratory volume; ESR: erythrocyte sedimentation rate; PLT: platelet count; PDW: platelet distribution width; PCT: plateletcrit; MPV: mean platelet volume; P-LCR: platelet large cell ratio; RA: rheumatoid arthritis; ANCA: anti-neutrophil cytoplasmic antibodies; SLE: systemic lupus erythematosus; pSS: Sjogren syndrome; NA: not available. \**P* < 0.05.

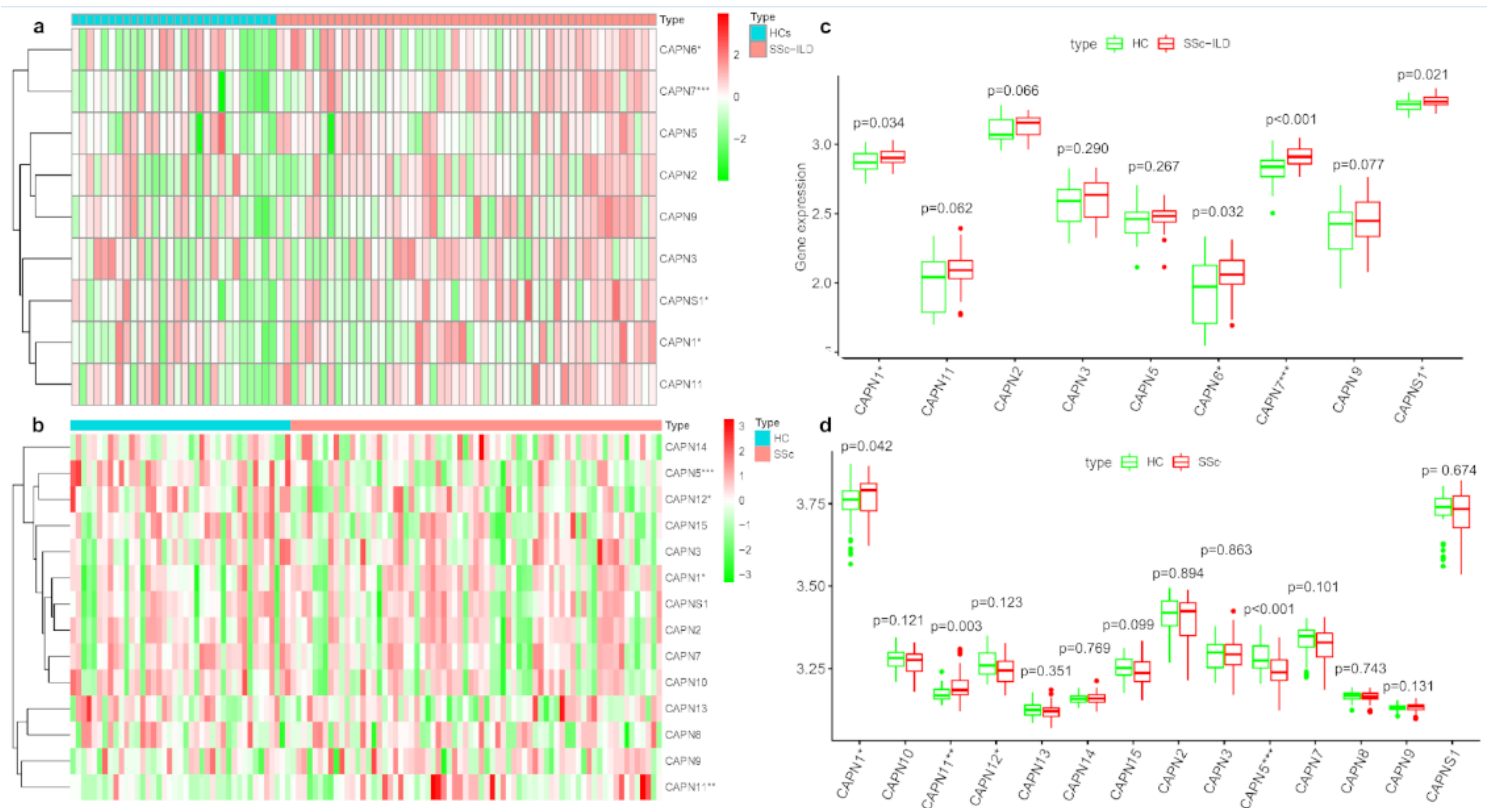
**Table 3. Univariate and multivariate logistic regression of clinical and laboratory parameters to ILD in patients with SSc**

Hub genes/clinical traits	Univariate logistic regression			Multivariate logistic regression		
	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value
Gender	1.578	0.218, 7.618	0.929			
Age	0.943	0.878, 1.013	0.108			
Course of disease	0.991	0.898, 1.093	0.857			
Raynaud's phenomenon	0.421	0.050, 3.532	0.425			
Anti-Scl-70 antibody (+)	2.121	0.428, 10.515	0.357			
ANA (+)	0.970	0.982, 1.0010	0.834			
Anti-RNP (+)	0.353	0.066, 1.874	0.221			
Anti-centromere (+)	4.500	0.471, 42.970	0.191			
mRSS	0.876	0.770, 0.997	0.044*	0.917	0.807, 1.042	0.184
FVC (%predict)	1.003	0.973, 1.033	0.865			
DL <sub>CO</sub> (%predict)	1.071	0.934, 1.228	0.325			
FEV1/FVC%	1.087	0.944, 1.253	0.245			
ESR, mm/h	0.991	0.925, 1.062	0.800			
PLT, 10 <sup>9</sup> /L	0.999	0.987, 1.010	0.850			
MPV, fL	0.829	0.503, 1.365	0.461			
PDW, fL	0.282	0.000, 21.236	0.825			
PCT, %	0.959	0.895, 1.028	0.235			
P-LCR, %	0.916	0.730, 1.149	0.449			
Calpain activity	1.057	1.002, 1.115	0.041*	1.063	0.968, 1.166	0.202
HMGB1	1.190	1.006, 1.408	0.043*	1.414	0.953, 2.096	0.085

**Abbreviations:** CI, confidence interval; OR, odds ratio; SSc: systemic sclerosis; ILD: interstitial lung disease; ANA: antinuclear antibodies; Anti-RNP: anti ribonucleoprotein antibody; mRSS: modified Rodnan skin score; FVC: forced vital capacity; DL<sub>CO</sub>: diffusing capacity of the lung for carbon monoxide; FEV1: forced the first second of expiratory volume; ESR: erythrocyte sedimentation rate; PLT: platelet count; PDW: platelet distribution width; PCT: plateletcrit; MPV: mean platelet volume; P-LCR: platelet large cell ratio; RA: rheumatoid arthritis; ANCA: anti-neutrophil cytoplasmic antibodies; NA: not available.

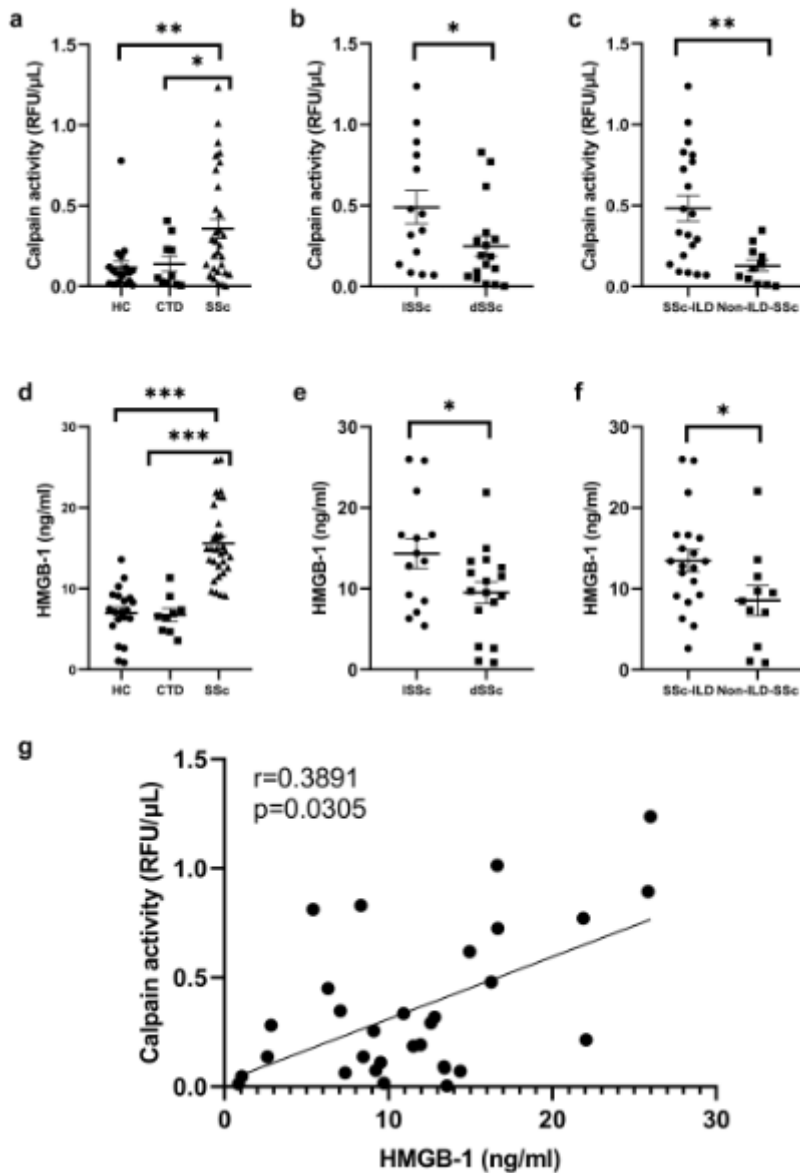
\**P* < 0.05.

## Figures



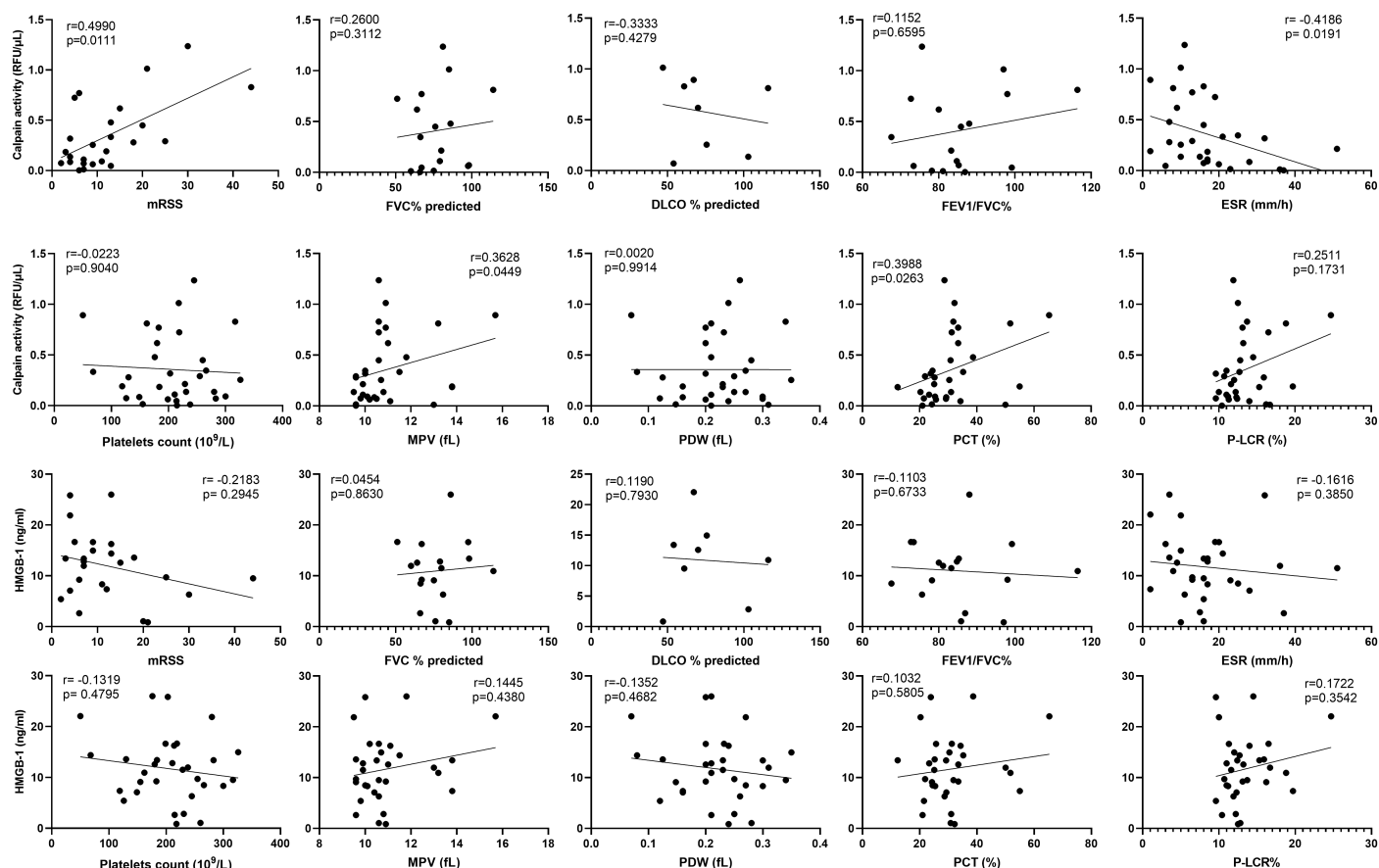
**Figure 1**

Expression of calpain related genes in patients with SSc or SSc-ILD (a) Differentially expressed heatmap of calpain associated genes in lung samples of patients with SSc-ILD (GSE40839, GSE48149, GSE76808, GSE81292). (b) Differentially expressed heatmap of calpain associated genes in PBMCs samples of patients with SSc (GSE33463). (c) Differentially expressed boxplot of calpain associated genes in lung samples of patients with SSc-ILD (50 SSc-ILD vs 28 HC). (d) Differentially expressed boxplot of calpain associated genes in PBMCs samples of patients with SSc (69 SSc vs 41 HC). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  Abbreviations: SSc: systemic sclerosis; ILD: interstitial lung disease; HC: healthy control subjects;



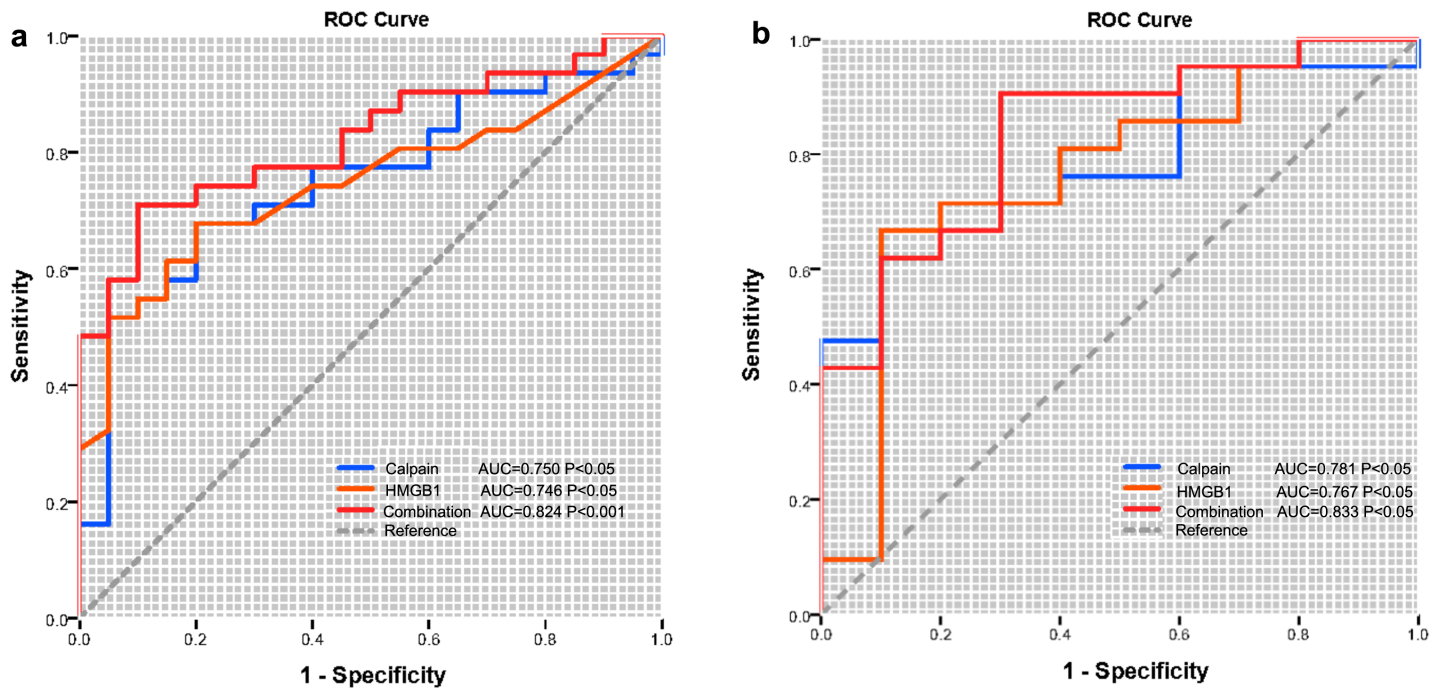
**Figure 2**

Increased serum calpain activity and HMGB1 levels in SSc patients (a) Significantly higher serum calpain activity and HMGB1 levels (d) were found in SSc patients than in HC and in patients with other CTD. (b) Patients with dSSc showed significantly higher serum calpain activity and HMGB1 levels (e) than patients with ISSc. (c) SSc patients with ILD showed significantly higher serum calpain activity and HMGB1 levels (f) than SSc patients without ILD. (g) Correlations between serum calpain activity and HMGB1 levels from patients with SSc. The short bar indicates mean $\pm$ 2SD in each group. \*\*\* < ,0.001, \*\* <0.01, \* <0.05 Abbreviations: SSc: systemic sclerosis; ILD: interstitial lung disease; CTD: connective tissue diseases; HC: healthy control subjects; SD: standard deviation; dSSc: diffuse cutaneous systemic sclerosis; ISSc: limited cutaneous systemic sclerosis;



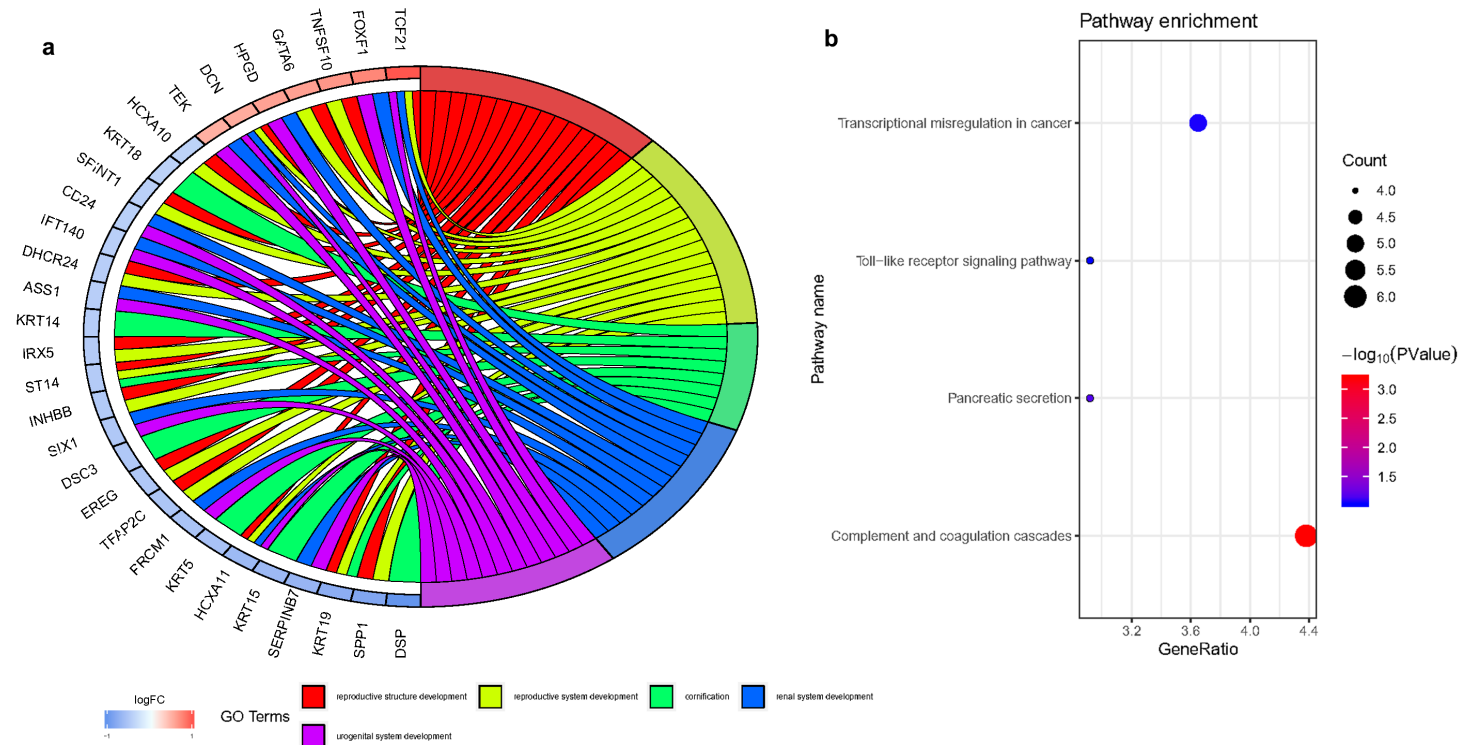
**Figure 3**

The correlation of serum calpain activity and HMGB1 levels with clinical parameters in patients with SSc. Abbreviations: mRSS: modified Rodnan skin score; FVC: forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; FEV1: forced the first second of expiratory volume; ESR: erythrocyte sedimentation rate; PLT: platelet count; PDW: platelet distribution width; PCT: plateletcrit; MPV: mean platelet volume; P-LCR: platelet large cell ratio;



**Figure 4**

Receiver operating characteristic curve (ROC) Serum calpain activity and HMGB1 levels for the diagnosis of patients with SSc (a) or SSc-ILD (b)



**Figure 5**

KEGG pathway and GO enrichment analysis (a) The top 5 GO enrichment pathways of differentially expressed genes between cluster1 and cluster2. (b) The size of each circle means the amounts of genes.



The different color of each circle means P value. GeneRatio means the number of genes in differentially expressed genes that belong to this pathway divided by the number of genes in the background gene cluster that belong to this pathway. Abbreviations: GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes;

## Supplementary Files

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