

Elevated gene expression of S100A12 is correlated with the predominant clinical inflammatory factors in patients with bacterial pneumonia

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Abstract. Inflammation is the predominant characteristic of pneumonia. The present study aimed to identify a faster and more reliable novel inflammatory marker for the diagnosis of pneumonia. The expression of the S100A12 gene was analyzed by reverse transcription quantitative polymerase chain reaction in samples obtained from 46 patients with bacterial pneumonia and other infections, compared with samples from 20 healthy individuals, using the $2^{-\Delta\Delta C_t}$ method. The expression levels of S100A12 were increased in 12 patients with bacterial pneumonia. Compared with clinical inflammatory data, a positive correlation was observed between the expression of the S100A12 gene and levels of white blood cells, C-reactive protein (CRP), thrombocytocrit, neutrophils, erythrocyte sedimentation and soterocytes, and an inverse correlation was observed with the width of red blood cell volume distribution and platelet distribution, monocytes and hemoglobin, using Pearson's product-moment correlation method. The P-value of CRP and erythrocyte sedimentation were revealed to be statistically significant ($P < 0.05$). A sporadic distribution of

S100A12 was observed in a heatmap among the patients with different infections and bacterial pneumonia. Furthermore, the expression of S100A12 occurred in parallel to the number of clumps of inflamed tissue observed in chest computed tomography and X-ray. The value of gene expression of S100A12 (>1.0) determined using the $2^{-\Delta\Delta C_t}$ method was associated with more severe respiratory diseases in the patients compromised by bacterial pneumonia, sepsis and pancreatitis. These findings suggested that S100A12 is an effective marker for inflammatory diseases.

Introduction

The S100 gene family includes at least 19 members, which are located as a cluster on chromosome 1q21 (1-3). This gene family is predominantly expressed in marrow origin cells, including granulocytes, macrophages, monocytes and the endothelial cells involved in the inflammatory response, cell cycle and differentiation (4-7). S100A12 protein has a conserved domain of EF-hand calcium-binding proteins with low molecular weight, and is an important member of the S100 family (8-11). S100A12 is an acidic protein and functions as a receptor ligand for receptor for advanced glycation end products (RAGE) in the nuclear factor (NF)- κ B pathway (12-16). The S100A12 protein is derived predominantly from neutrophils involved in inflammation and is important for expressing vascular endothelial cell adhesion molecules, which activate the inflammatory cell chemoattractant effects (4,5).

Inflammation involves a series of highly coordinated events, which occur in vascular tissues as a response to detrimental stimuli, resulting in heat, swelling, redness and pain (17). It is a vital, acute protective response to injury, stimulating repair and preventing further disturbance to the affected tissues (18). Acute peripheral inflammatory pain is associated with immediate immune cell infiltration following tissue damage. Previous studies have demonstrated that S100A12 is functionally associated to inflammation (19), while

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S100A4 is involved in cell migration (20). The expression of S100A12 is increased in rheumatoid arthritis, systemic lupus erythematosus and several other inflammatory diseases (21,22) and is suggested an effective anti-inflammatory target protein due to its function in activating RAGE (12,13,23). S100A12 is an ideal marker due to its gene expression in human peripheral blood cells and is one of the most predictive factors for clinical diagnosis (24). S100A12 is expressed in the smooth muscle and inflammatory cells in ruptured coronary artery plaques in patients succumbing to cardiac mortality (25,26), suggesting the importance of S100A12 in vascular disease and atherothrombosis (25).

Previous studies have demonstrated that the S100A12 protein is an important inflammatory reactive protein and has been widely investigated in inflammatory bowel disease, rheumatic disease, cardiovascular disease, Kawasaki disease, neurodegenerative diseases, tumor samples and other diseases (27-31), however, no investigations have been performed in respiratory tract infections. The present study aimed to investigate the gene expression of S100A12 in patients infected with various pathogens and to analyze its correlation with current common clinical data from whole blood analysis to examine a faster, simpler and more reliable marker for the diagnosis of infectious and inflammatory diseases.

Materials and methods

Reagents. The following reagents and kits were purchased: TRIzol™ LS reagent (Thermo Fisher Scientific, Waltham, MA, USA), a QIAamp RNA Blood kit (Qiagen, Hilden, Germany), Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega Corporation, Madison, WI, USA) and a cDNA Synthesis kit (Fermentas, Ontario, Canada).

Sample collection. The present study was approved by the Ethics Committee of Beijing Friendship Hospital (Beijing, China) and written informed consent was provided by each patient.

Blood samples (5 ml) were collected into EDTA tubes from 46 inpatients (23 female and 23 male; median age, 50 years; range, 19-72 years) with major symptoms of fever and chest pain, who were admitted to the Beijing Friendship Hospital affiliated with Capital Medical University (Beijing, China) between November 2012 and January 2013. The blood samples of 20 healthy subjects were collected from volunteers (11 female and 9 male; median age, 45 years; range, 23-68 years). All the blood collection tubes were inverted five times for mixing immediately following collection and prior to storage at 22°C, 4°C or -20°C, as indicated in the experimental protocol.

Analysis of clinical blood samples. The blood samples from the patients and healthy volunteers were collected into EDTA tubes (Kangjian, Jiangsu, China) and routinely processed in the Beijing Friendship Hospital laboratory. A hematological analyzer (ABX Pentra DF 120; Horiba, Ltd., Kyoto, Japan) was used to accurately measure the cellular identifications based on electronic impedance variation as previously described (32). Expression of C-reactive protein (CRP) was measured using a colloidal gold immunoturbidimetric assay kit (Wuhan

J.H. Bio-Tech Co., Ltd., Wuhan, China) with the ABX Pentra DF 120 (Horiba, Ltd., Kyoto, Japan) and a PCT Biomerieux mini VIDAS immunoassay (BioMerieux, Durham, NC, USA).

RNA extraction from blood samples. The total RNA from whole blood samples (5 ml) collected into EDTA tubes, was isolated using an organic extraction method (TRIzol LS), according to the manufacturer's instructions and was further purified with a QIAamp™ spin column and on-column DNase digestion (Qiagen, Shanghai, China). Finally, 500 µl of RNase free water was used to elute total RNA from the column. Briefly, 1 ml RNase-free water was mixed with 6 ml TRIzol LS reagent and 1 ml of blood sample in a screw-top centrifuge tube, mixed thoroughly and centrifuged for 10 min at 8,000 x g at 4°C. Chloroform (1.6 ml; Best-Reagent Chemical Co., Chengdu, China) was subsequently added to each tube and the contents were mixed again. The tubes were incubated on ice for 5 min and centrifuged at 10,000 x g at 4°C for 10 min, and the upper aqueous phase was collected and transferred into clean tubes. Alcohol precipitates of the nucleic acids at a ratio of 1:1 were resuspended in 240 µl RNase-free water. The RNA samples (200 µl), 840 µl Buffer RLT (QIAamp RNA Blood kit) and 600 µl absolute ethanol were mixed and briefly centrifuged to remove excess liquid from the cap. The total sample (700 µl) was applied to a QIAamp spin column and centrifuged at 12,000 x g for 5 min at 4°C for 1 min. Any remaining sample was used to repeat the filter wash. The QIAamp spin column was washed with 600 µl RW1 wash buffer (Qiagen, Valencia, CA, USA) and centrifuged for 1 min at full speed, then washed twice more with 500 µl RPE wash buffer (Qiagen) following DNase (Qiagen) treatment and centrifugation for 1 and 3 min between washes, respectively. The RNA was then eluted twice using 40 µl RNase free water.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). For RT, the total RNA (0.2-1.0 µg) was primed with random hexamers using AMV reverse transcriptase (Promega Corporation), according to the manufacturer's instructions. The RNA integrity was evaluated by electrophoresis using a 1.2% agarose gel (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). No degradation of the total RNA was exhibited by the 28S and 18S bands. First-strand cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis kit (Fermentas, Pittsburgh, PA, USA), with the Oligo dT-Adaptor Primer (Qiagen). The reaction mixture containing 4 µl MgCl₂ (Dalian All World I/E Co., Ltd., Dalian, China), 3 µl AMV buffer (Qiagen), 2 µl dNTP (Qiagen), 0.5 µl recombinant RNasin (Qiagen), 2 µl AMV retro (Qiagen), 0.5 µl Oligo (dT)15, 2 µl total RNA and 6 µl water was incubated at 37°C for 1 h. The qPCR controls consisted of separate reactions, in which either the template or the reverse transcriptase was eliminated. No product was observed in the negative controls following 40 cycles of amplification at 94°C for 30 sec, 60°C for 45 sec and 72°C for 1 min. The GAPDH copy number was determined from 2 µl of each eluate using the GAPDH RT-qPCR assay from PE Applied Biosystems (Foster City, CA, USA). qPCR amplification was performed using gene-specific qPCR primers. The primer sequences were designed according to the mRNA sequences in the gene bank (National Institutes of Health, Bethesda, MA, USA) and synthesized by Invitrogen

Table I. Primers used for reverse transcription quantitative polymerase chain reaction.

Gene	Primer (5'-3')	Length (bp)	Amplified fragment (bp)
S100A12 ^a			
Forward	CACATTCCTGTGCATTGAGG	20	147
Reverse	TGCAAGCTCCTTTGTAAGCA	20	
GAPDH ^b			
Forward	GAGTCAACGGATTTGGTCGT	20	238
Reverse	GACAAGCTTCCCCTTCTCAG	20	
18s rRNA ^c			
Forward	GTAACCCGTTGAACCCATT	20	151
Reverse	CCATCCAATCGGTAGTAGCG	20	

Based on the template of nucleic acid accession nos. ^aXM_005541788, ^bNG_007992 and ^cM11188.1.

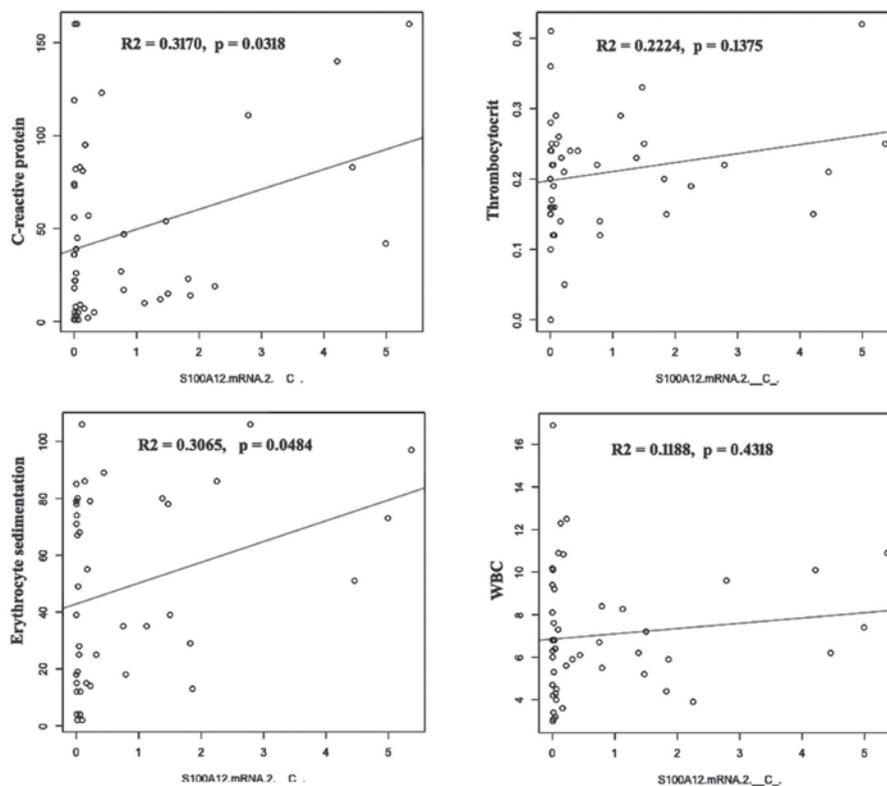


Figure 1. Scatter plots indicate positive correlations between S100A12 and certain major clinical inflammatory factors, including CRP, erythrocyte sedimentation, thrombocytocrit and WBC. Regression lines were constructed to summarize the association between the expression of S100A12 and these variables. CRP, C-reactive protein; WBC, white blood cells.

Life Technologies (Shanghai, China). The sequences of the primers and their expected product sizes are summarized in Table I [accession numbers from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>)]. The correct qPCR fragment was confirmed using a commercial sequencing service company (BGI, Beijing, China).

Control samples from normal healthy subjects and housekeeping gene. The normal subject cDNA was triply amplified in parallel with GAPDH and S100A12, respectively. The expression levels of S100A12 in the 46 patients with bacterial pneumonia were determined following subtraction of the expression levels of the

housekeeping gene, with the value for the healthy individuals designated as 1, using the $2^{-\Delta\Delta C_t}$ method (33).

Calculations of the gene expression of S100A12 using the $2^{-\Delta\Delta C_t}$ method. The qPCR determinations for each gene in each of the samples were performed in quadruplicate. Any replicates unsuitable, according to Source Precision Medicine quality-control standards for range and skew (33), were eliminated. The relative mRNA expression was determined in the healthy sample PCR products using the $2^{-\Delta\Delta C_t}$ method. The cycle threshold (C_t), is the cycle at which the qPCR product crosses the detection threshold, usually at mid-log stage of

Table II. Representatives of inflamed lung computered tomography in parallel to clinical data of 46 patients.

Patient ID	S100A12 ($\mu\text{g/ml}$)	Erythrocyte sedimentation (male: 0-15 mm/h; female: 0-20 mm/h)	White blood cells ($\times 10^9/l$)	Thrombocytocrit (0.18-0.22%)	C-reactive protein (0-8mg/l)
A3496397	0.0039	79	6.8	0.36	56
B3590123	0.0057583	78	6.3	0.28	73
C3571267	0.1617	15	3.6	0.14	7
D3614871	0.4409	89	6.1	0.24	123
E1333480	1.4721	78	5.2	0.33	54
F3612302	4.2113	Not available	10.1	0.15	140

A-F Patient samples from computed tomography images shown in Fig. 4. Data were collected prior to chemotherapy.

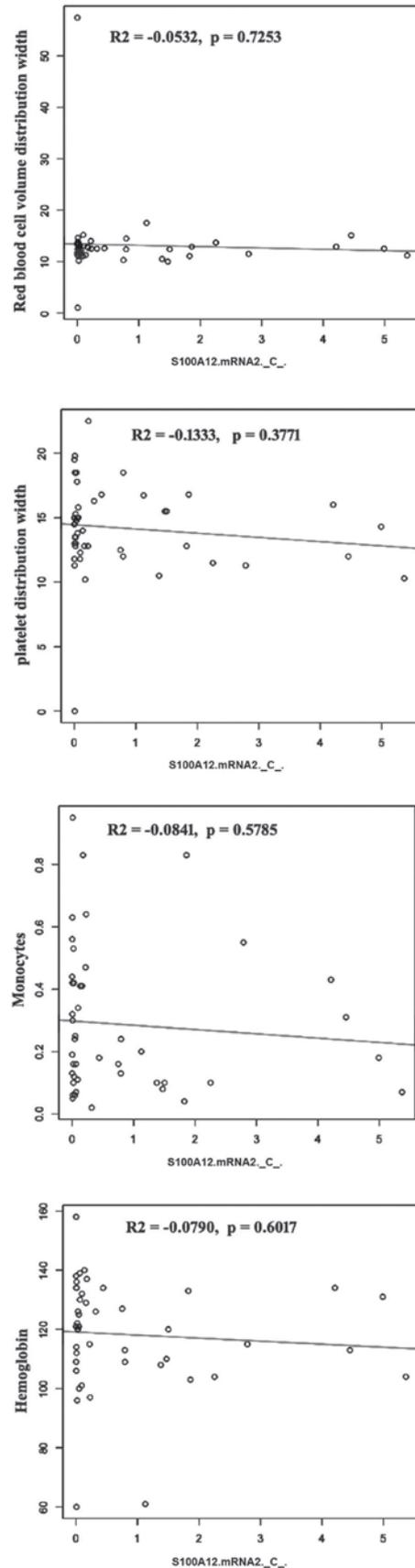


Figure 2. Scatter plots demonstrate an inverse correlation between S100A12 and certain major clinical inflammatory factors, including RDW, PDW, monocytes and hemoglobin. Regression lines were constructed in order to summarize the association between these variables and S100A12 expression. RDW, red blood cell volume distribution width; PDW, platelet distribution width.

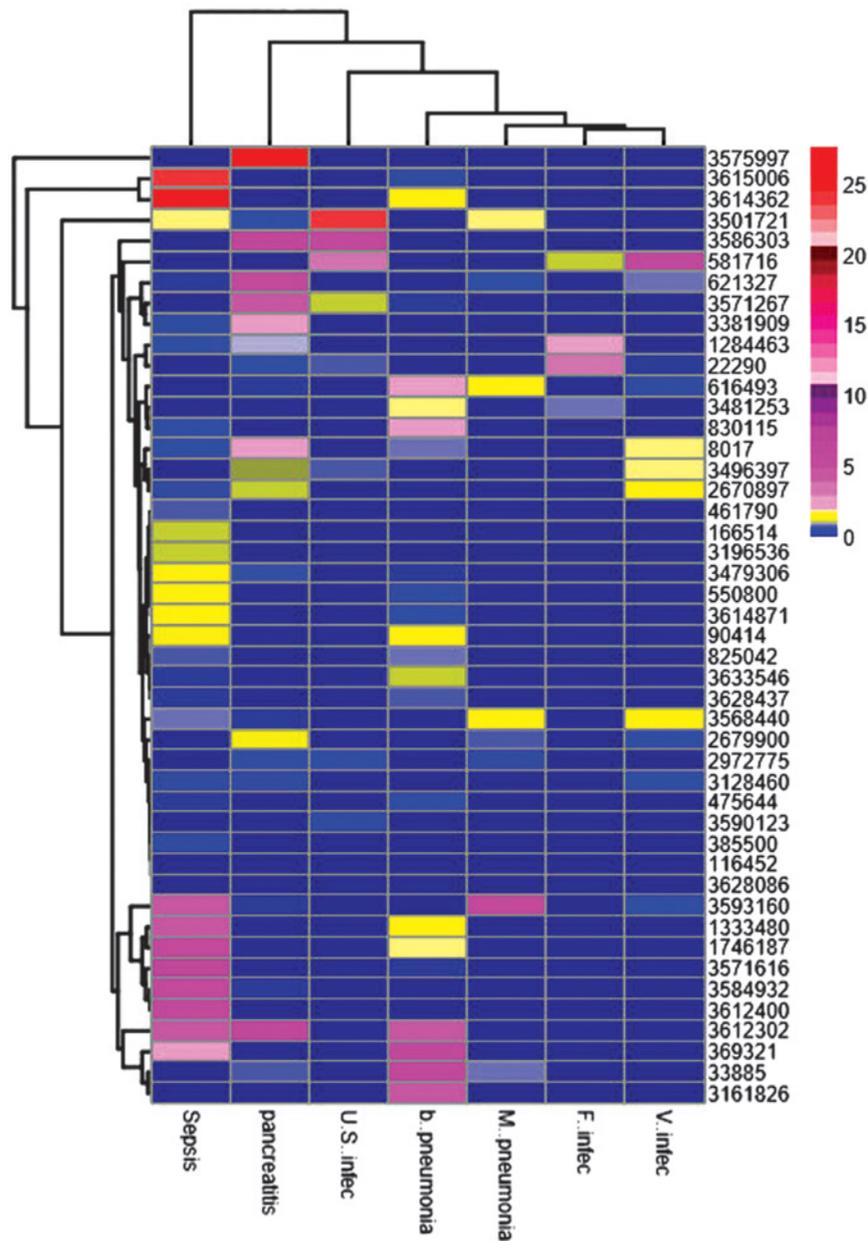


Figure 3. Heatmap representation of the gene expression results. The differential gene expression of S100A12 in 46 patients with V. infec, F. infec, M. pneumonia, b. pneumonia, U.S. infec, pancreatitis and sepsis. The patient IDs are listed on the y-axis and the respective diseases are listed on the x-axis. High gene expression values are indicated in red and low gene-expression values are indicated in green. V. infec, virus infected; F. infec, fungal infected; M., mycoplasma; b., bacterial; U.S. infec, urinary system infection.

qPCR amplification. Each qPCR reaction was multiplexed with specific primer sets for two genes in each well, the gene of interest and an endogenous control (18S rRNA); rRNA was selected as it is highly resistant to nuclease degradation (34). The difference between the mRNA C_t value and the 18S RNA C_t value was associated as $\Delta C_t = C_{t(\text{mRNA gene})} - C_{t(18S \text{ rRNA})}$. The relative mRNA abundance ($\Delta\Delta C_t$) represents the difference between the ΔC_t values for a pair of conditions. The relative mRNA expression, assuming 100% qPCR efficiency, is exponential and is defined by the formula: $[\text{mRNA}] = 2^{-\Delta\Delta C_t}$. The standard error for $\Delta\Delta C_t$ was calculated using the method of Livak and Schmittgen (33).

Heatmap presentation of the gene expression for different infections. To present the gene expression as a heatmap

using the relative expression ($2^{-\Delta\Delta C_t}$) values, a moderated two-sample t-test was performed using the Limma package, version 1.6.1 (35) to identify differential gene expression between the infectious groups.

Correlation of S100A12 with clinical data by Pearson's product-moment correlation (PPMC). PPMC was calculated using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) to determine the association between the gene expression of S100A12 and the selected clinical data from the 46 patients.

Pre-operative computed tomography (CT) scans. Prior to clinical diagnosis, spiral chest scanning pre-operative CTs were performed on all patients using a GE 9800 scanner (General Electric, Milwaukee, WI, USA).

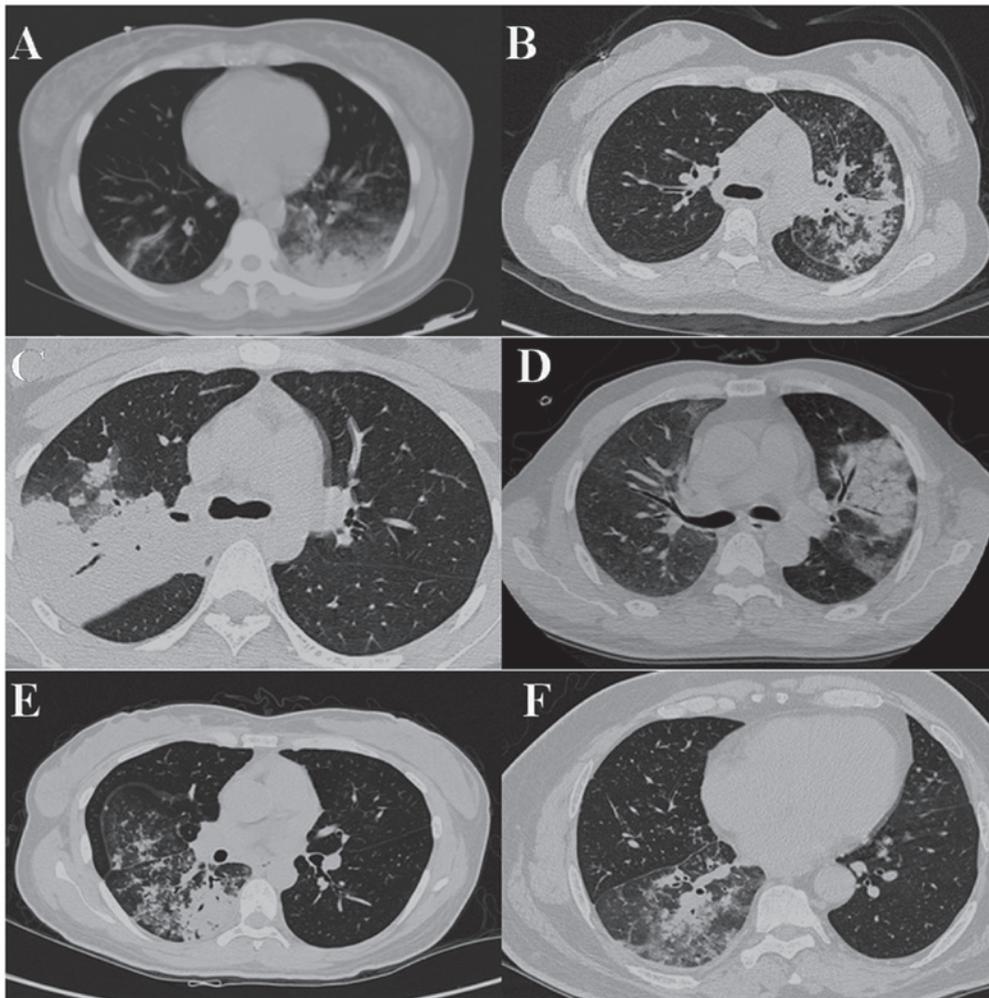


Figure 4. High-resolution computed tomography of patients with chest lung inflammation. (A) Patient 3496397: Lungs had scattered ground-glass opacities and small nodular opacities, left lower lobe (right side of the image) revealed bronchial wall thickening and dilation. (B) Patient 3590123: Left lung (right side of image) demonstrated multiple small nodules, ground-glass opacities and pulmonary consolidation was observed locally. (C) Patient 3571267: right upper lobe demonstrated consolidation and ground-glass density consolidation, which was identified as BOOP. (D) Patient 3614871: Left lung revealed multiple patchy opacities and BOOP. (E) Patient 1333480: Right lung demonstrated multiple patchy, blotchy consolidation, ground-glass opacity and small acinar nodules. (F) Patient 3612302: Right lower lobe had multiple nodules and ground-glass density lesions. BOOP, bronchiolitis obliterans organizing pneumonia.

Statistical analysis. The difference between the means were analyzed by one-way analysis of variance and the least significant difference was used for multiple comparisons. All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Positive correlation between S100A12 and the predominant clinical inflammatory factors. To determine the correlation between the expression of the S100A12 gene with other clinical factors and inflammatory status, Pearson's metrics was used to measure the similarities between S100A12 and individual clinical data. A positive correlation was observed with white blood cells (WBCs), CRP, thrombocytocrit and erythrocyte sedimentation, and the majority of samples were within the range of 0.01-0.1 $\mu\text{g/ml}$. Correlations between the replicate samples varied between 0.97 and 0.99, demonstrating high reproducibility (Fig. 1).

Inverse correlation. An inverse correlation was observed between the gene expression of S100A12 and red blood cell volume distribution width, platelet distribution width, monocytes and hemoglobin in PPMC analyses (Fig. 2), suggesting that these factors were reduced by S100A12, even in the absence of a direct indication of downregulation. These factors are not directly associated with inflammation, lesion or hyperplasia, although they are normally regarded as inflammatory factors (36).

2^{-ΔΔC_t} value of the gene expression of S100A12 is sporadically distributed in different diseases.

The patients in the present study had dilated airways to retain large bronchial smooth breath, however persistent inflammation was present throughout the lungs from different infections. A heatmap was used to summarize the qPCR data and the effect of the gene expression of S100A12 in different infections. Following the triple amplification of healthy patient cDNA with GAPDH and S100A12, respectively, the relative S100A12 value was obtained following subtraction from the housekeeping gene,

systemically. The gene expression levels of S100A12 were increased in 12 of the patients with bacterial pneumonia (>26%). The $2^{-\Delta\Delta Ct}$ of the 46 patients with bacterial pneumonia and other diseases are distributed in the heatmap shown in Fig. 3.

CT scan of the chest in parallel to inflammatory tissues. Cross-sectional CT images at a 'lung window' setting (-600H level, 1 600H width) were captured to demonstrate the inflammatory status of the lungs (Fig. 4). During CT scanning, a thin X-ray beam rotating around the chest was applied to support the CT analysis (data not shown). In the overlay of the CT scan, inflammation was distinguished, however, the inflammatory degree was not defined. Combined with the clinical features, the images in Fig. 4A and B were determined as exhibiting possible inflammation and in Fig. 4C-F were determined as exhibiting inflammation. Thus, the CT observations were consistent with the gene expression levels of S100A12 (Table II).

Discussion

Several factors have been used for the diagnosis of inflammation, including C-reactive protein, erythrocytes, sedimentation rate, thrombocytocrit and WBCs. CRP is an inflammatory marker, which has been used for several decades, particularly as they are non-specific to patient ethnicity or geographical origin (37-39). Their positive correlation with S100A12 is significantly supported by infection types, while the highest expression of S100A12 is observed in sepsis and, due to inflammation being important in bronchiectasis, corticosteroid therapy is theoretically beneficial (36).

Since S100A12 is involved directly and indirectly in several aspects, which trigger inflammatory-associated factors in NF- κ B cell signaling (40), it is a small molecule and it is easy to perform qPCR from whole blood samples, S100A12 is regarded that the collective gene expression of S100A12 for all types of leukocytes and blood plasma, reflects the patient's individual status. The present study demonstrated that the expression of S100A12 correlated with CRP, WBCs, thrombocytocrit, erythrocyte sedimentation and the major inflammatory clinical data, and was also in parallel to the CT scanning. Combined with the heatmap data, it was concluded that a value of gene expression of S100A12 in the $2^{-\Delta\Delta Ct}$ method >1.0 was associated with more severe respiratory diseases in patients compromised with bacterial pneumonia, sepsis and pancreatitis. However, further confirmation is required using a larger population sample.

The RT-qPCR results of the 66 samples (46 patients and 20 controls), the clinical laboratory data and the CT images revealed that S100A12 was positively correlated with the major clinical inflammatory factors and paralleled with CT observation. Therefore, it was concluded that S100A12 is a potential clinical inflammatory marker, particularly for patients with severe pathogen infections.

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