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Full Length Research Paper

Decreased ADAM9 expression in patients with pulmonary sarcoidosis

Jingyu Liu[1,2] and Zhenhua Li[1]*

[1]China Medical University, ShenYang city, Liao Ning province, 110001, China.
[2]Liaoning Medical University, Jinzhou 121001, China.

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The pathogenesis of sarcoidosis is still poorly understood. Genome-wide gene expression profiling can provide novel genetic data involved in the pathogenesis of disease. In this study, using normal bronchoalveolar lavage (BAL) and sarcoidosis BAL as models for cDNA microarray analysis, we detected an elevation of ADAM9 (a disintegrin and metalloprotease) expression on pulmonary sarcoidosis progression. This result was further confirmed by real-time quantitative RT-PCR and immunohistochemistry. It was found that ADAM9 was significantly down-regulated in pulmonary sarcoidosis in comparison to the normal tissue on mRNA level. On protein level, ADAM9 was also highly decreased in pulmonary sarcoidosis patients. The present study provides novel candidate molecules and suggests a potential local role for ADAM9 as mediators of lung damage or remodeling and as markers of disease activity.

Key words: Pulmonary sarcoidosis, ADAM9, cDNA microarray, quantitative RT-PCR, immunohistochemistry.

INTRODUCTION

Sarcoidosis is a systemic granulomatous disease of unknown cause, which primarily affects the lung and lymphatic system of the body (Gil et al., 2010; Morimoto et al., 2008; Morgenthau and Padilla, 2009). It causes small lumps, or granulomas, which generally heal and disappear on their own. However, for those granulomas that do not heal, the tissue can remain inflamed and become scarred, or fibrotic. Pulmonary sarcoidosis can develop into pulmonary fibrosis, which distorts the structure of the lungs and can interfere with breathing. Bronchiectasis, a lung disease in which pockets form in the air tubes of the lung and become sites for infection, can also occur (Gil et al., 2010; Bourbonnais and Samavati, 2008; Tokuyasu et al., 2010). So far, the cause and mechanisms behind pulmonary sarcoidosis remain unclear. In the past years, several genes have been identified as related to the development of pulmonary sarcoidosis, such as IFN-γ, TNF-α, IL-2, IL-12, IL-16, IL-18, IL-27, MIP-1, MIP-3, MCP-1, RANTES, and IP-10. However, considering the complexity of the genome, it is most likely that most of the molecular changes causing pulmonary sarcoidosis still need to be elucidated. Moreover, there is still a need for prognostic markers in this devastating cancer disease.

cDNA microarray technology is a burgeoning molecular biology technology over recent years，which can provide access to enormous genetic data sets with opportunities to discover novel disease mechanisms. cDNA microarray technique has unexampled advantages in disease study with the characteristics of high flux and high efficiency. This approach has been applied to diseases of unknown cause to create new hypotheses relating to disease pathogenesis (Calvano et al., 2005; Ergun et al., 2007) and is shown to have prognostic (Korkola et al., 2007) and diagnostic applications (Takahashi et al., 2005; Aldred et al., 2004). Using the global genomic approach, we sought to identify novel gene transcripts engaging in common biological processes operating at the tissue level in pulmonary sarcoidosis, therefore, cDNA microarray were utilized in the present study to detect corresponding gene transcripts on pulmonary sarcoidosis. It was found that AMAD9 of expression was significantly decreased in the lungs of patients with sarcoidosis.

ADAM9, one of the first ADAM proteins to be identified and characterized, a membrane-anchored metallo-

*Corresponding author. E-mail: lizhenhua104@yahoo.cn. Tel: +86-024-8328-2002. Fax: +86-024-8328-2002.

proteinase with an N-terminal prodomain followed by a metalloprotease domain, a disintegrin domain and cysteine-rich region, an epidermal growth factor (EGF) repeat, a transmembrane domain, and a cytoplasmic tail with potential SH3 ligand domains (Weskamp et al., 1996). ADAM9 is catalytically active in both biochemical and cell-based assays and can cleave several membrane proteins (Peduto et al., 2005; Roghani et al., 1999; Horiuchi et al, 2007). In addition, ADAM9 is thought to participate in cell-cell interactions by binding to integrins (Nath et al., 2000). In the current study, we evaluated the ADAM9 expression on protein and transcript level to clarify a diagnostic or prognostic value of ADAM9 in pulmonary sarcoidosis.

MATERIALS AND METHODS

Study population and tissue

Gene expression analysis was performed on lung tissues from patients who met the operational diagnosis of sarcoidosis. Eight patients with pulmonary sarcoidosis (3 men and 5 women) aged 33–54 were recruited from the Department of Respiratory Diseases, the First Affiliated Hospital of China Medical University, from April 2006 to December 2007. The diagnosis of all patients were confirmed to be sarcoidosis on the basis of clinical, imaging, and histopathological findings (Spagnolo et al., 2003). Eight persons (4 men and 4 women) aged 35–52 years who are volunteers as controls. Healthy control individuals were all nonsmokers with no history of lung disease and were confirmed to be normal on the basis of the results of chest radiography and bronchfiberoscopy. For the bronchoalveolar lavage (BAL) studies, subjects were recruited at the time of diagnostic bronchoscopy and were not using systemic medications for sarcoidosis. BAL was performed by instilling three 50-ml aliquots of warmed saline in a segmental bronchus. Processing of BAL specimens was done as previously reported (Schwettmann et al., 2001). For immunostaining, we used 8 paraffin-embedded pulmonary sarcoidosis samples harvested over the years by the Institution of Respiratory Diseases in China Medical University and 7 normal lung tissues resected during operations as control. Informed written consent was obtained from all subjects, and the study was approved by the Ethics Committee of China Medical University, China.

Isolation and validation of high-quality RNA for gene chip analysis and PCR

Total RNA was isolated from frozen lung tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol and as described in the online supplement. In short, the tissue was ground with mortar and pestle cooled by liquid nitrogen of the ground tissue, 100 mg was incubated with 1 mlTRIzol for 5 min at room temperature (RT). Cell debris was removed by centrifugation (12,000×g at 4°C for 10min) and 0.4 ml chloroform was added. After vortexing the mix was incubated for 5 min at RT. The phases were separated by centrifugation (12,000×g at 4°C for 15 min) and the aqueous phase was transferred to a new tube. 0.6× volume of isopropyl alcohol and a 0.1× volume of 3 M sodium acetate were added to this aqueous phase and incubated for 10 min at 4°C. The precipitated RNA was pelleted by centrifugation (12,000×g at 4°C for 15 min) and after the removal of the supernatant the RNA was washed twice with 70% ethanol. After drying, the RNA was resuspended in 30 µl DEPC-treated water.

The quality and quantity of the RNA was verified by the presence of two discrete electropherogram peaks corresponding to the 28S and 18S rRNA at a ratio approaching 2:1.

cDNA microarray

Isolation and validation of high-quality mRNA from the pulmonary sarcoidosis and the normal were reversely transcribed to cDNAs, then, cDNAs were labeled with the directly incorporated fluorescently dUTP (cy-5 or cy-3) to prepare the hybridization probes. The mixed probes were hybridized to the cDNA microarray. After high-stringent washing, the cDNA microarray was scanned using the fluorescent signals and showed differences between two tissues by means of statistical analysis program.

Real-time quantitative RT-PCR

In order to detect mRNA expression level of ADAM9, quantitative RT-PCR was performed as described in the online supplement. Briefly, using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's directions. Real-time quantitative PCR experiments were conducted with an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA) and SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer sequences were as follows: ADAM9 forward, 5′-TGCCCCCAAAGATTGTTTCAT-3′; reverse, 5′-CACCCTGTTGCTG TAGCCAAA-3′. GAPDH (Applied Biosystems) served as the internal control. Each sample was normalized on the basis of its GAPDH content. The thermal cycling conditions were as follows: 2 min at 95˚C, followed by 40 cycles of 95°C for 15 s and 59°C for 1 min.

Immunohistochemical analysis

Immunohistochemical was performed using the Streptavidin-Peroxidase system (Ultrasensitive™ SP; MaiXin, Fuzhou, China). Frozen lung tissue samples obtained from patients with sarcoidosis and control subjects were processed and cut at 4µm for slide preparation. The sections were deparaffinized in xylen and rehydrated with graded alcohols. For heat-induced epitope retrieval, the sections were immersed in 0.01 M citrate buffer solution (pH 6.0) for 10 min. Then, they were cooled for 1 h at room temperature and washed in water and phosphate-buffered saline (PBS). Next, 0.3% hydrogen peroxide was applied to block endogenous peroxidase activity, and the sections were incubated with normal goat serum to reduce nonspecific binding. They were then incubated overnight at 4˚C with primary rabbit polyclonal anti-human ADAM9 antibody (1:50; Minneapolis, MN). Biotinylated goat anti-rabbit serum IgG was used as a secondary antibody. After the sections were washed 3 times in PBS, they were incubated with streptavidin-biotin conjugated with horse-radish peroxidase and visualized by chromogenic detection of the conjugated peroxidase with diaminobenzidine as the substrate. The slides were counterstained with hematoxylin. For the negative control, primary antibodies were replaced with PBS.

Statistics analysis

To calculate the statistical differences between the control and sarcoidosis, the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. Student's *t* test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p values less than 0.05 were considered statistically significant.

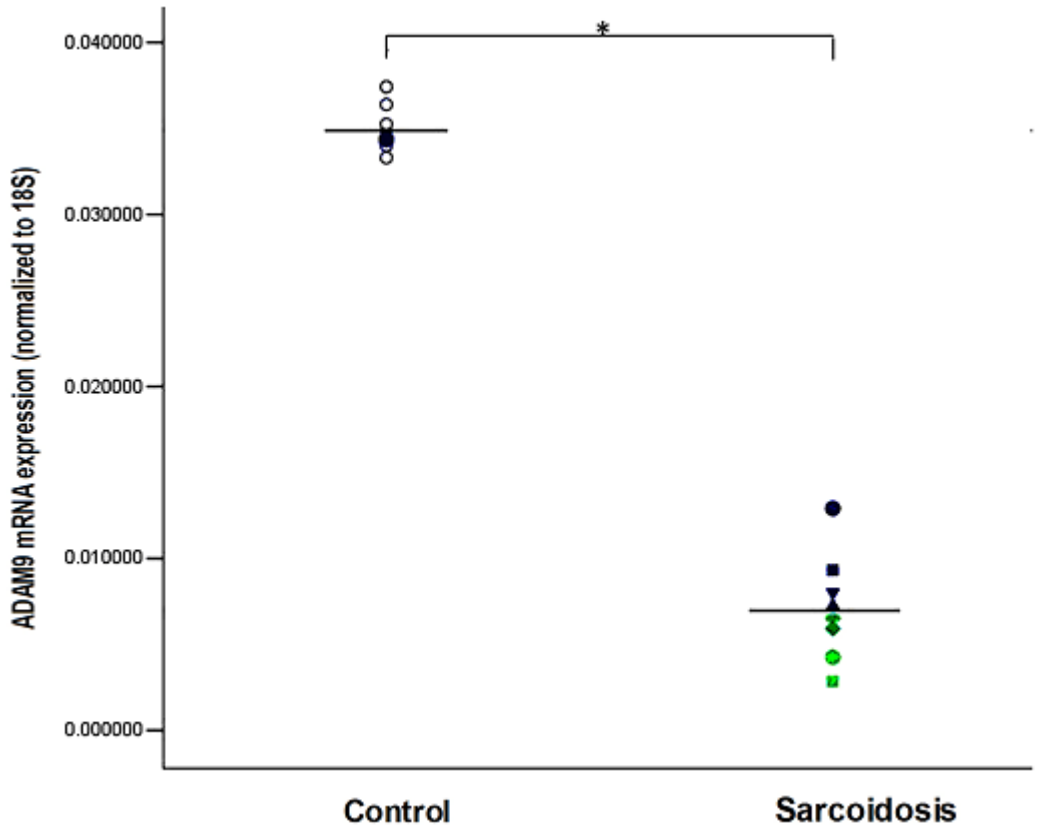

Figure 1. Real-time quantitative RT-PCR analysis of ADAM9 mRNA expression in pulmonary sarcoidosis. The symbol ○ represents the control group; the solid figures such as ■, ●, and ▲ represent the data of the 8 cases in the pulmonary sarcoidosis group. The presented data are the relative values after the standardization of a relative to 18S of each sample. The horizontal line represents the mean value in each group. The * symbol indicates a statistically significant ($P < 0.001$) difference between the 2 groups.

RUSULT

ADAM9 of differentially expressed in lung tissue from patients with sarcoidosis and the control group by cDNA microarray

We were interested in identifying functionally related genes that distinguished the patients with sarcoidosis from the control subjects. It was observed that the permutation P values were < 0.05 for the patients with sarcoidosis versus the control subjects. There were 305 differentially expressed transcripts genes in the patients with sarcoidosis compared with the control subjects, 116 genes were upregulated while 189 were downregulated in the pulmonary sarcoidosis. The gene list is available for review on the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query). One of these genes was identified as ADAM9, a member of ADAM family, which was significantly down-regulated in pulmonary sarcoidosis in comparison to the normal tissue by cDNA microarray.

Real-time quantitative RT-PCR analysis of ADAM9 expression

In order to detect the mRNA expression of ADAM9 in patients with sarcoidosis, Real-time quantitative RT-PCR was conducted. As shown in Figure 1, the expression of ADAM9 was significantly lower in BAL with sarcoidosis than the normal BAL on mRNA level ($P < 0.01$). This result further confirmed that ADM9 expression down-regulated in pulmonary sarcoidosis in comparison to the normal BAL.

Immunohistochemical analysis of ADAM9 expression

The immunostainings were performed to detect protein expression of ADAM9 using a multiheaded microscope. ADAM9 was expressed in lung tissue with sarcoidosis and nomal tissue (Figure 2). Brown-yellow ADAM9 staining was observed in the cell membrane and cytoplasm of both endothelial cells of the alveolar wall and macrophages of the alveolar space of normal lung tissue

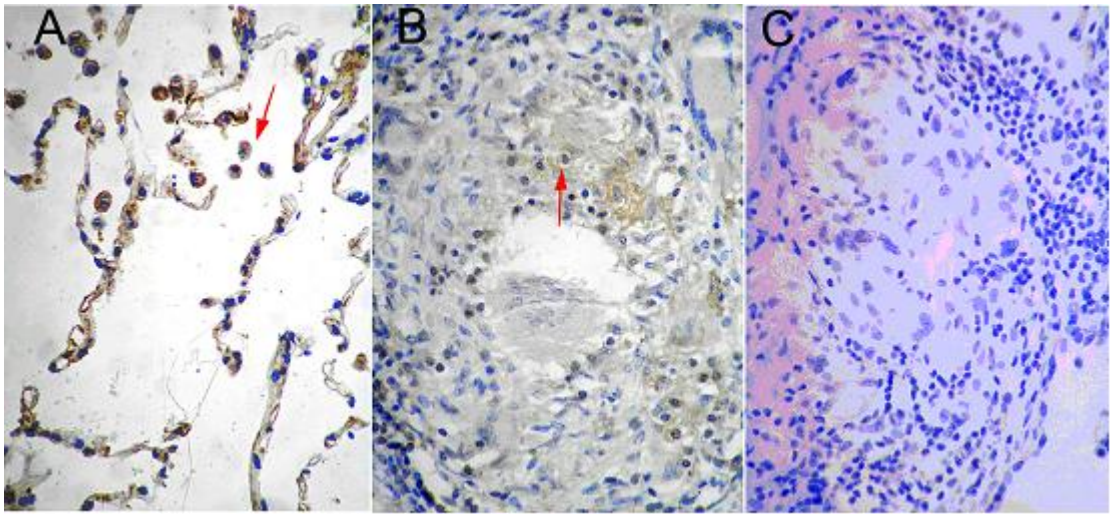

Figure 2. Immunohistochemical analyses of ADAM9 protein expression in pulmonary sarcoidosis (200×). Immunohistochemical staining of frozen lung samples for ADAM9 from representative control subjects and patients with sarcoidosis. A: Normal lung tissue showed strong staining. B: ADAM9 staining was depleted in granulomas and adjacent lung tissue. C: negative control, where the primary antibody is replaced by PBS, did not show non-specific staining.

(Figure 1A), but ADAM9 staining was scarce in granulomas or adjacent lung tissues (Figure 1B). The negative control did not show non-specific staining (Figure 1C). These results indicated that ADAM9 expression was decreased in sarcoidosis tissues, which was compatible with mRNA expression of ADAM9 in BAL with sarcoidosis.

DISCUSSION

In this study, cDNA microarray analysis was used to determine functionally related genes that may be expressed at different levels Between BAL with sarcoidosis and controls. Using pooled samples, 116 genes were markedly upregulated while 189 genes were down-regulated in the sarcoidosis. ADAM9, selected for further study, was downregulated in the sarcoidosis by bioinformatics analysis. Real-time PCR verified that there were decreased in the mRNA encoding ADAM9, Furthermore, immunoreactive ADAM9 was more intensely stained in nomal lung tissue compared with sarcoidosis lung tissue. The current study provides preliminary data on the involvement of previously unidentified ADAM protein in the pathogenesis of sarcoidosis.

ADAM9 have been described in various diseases on mRNA and/or protein level in and have often been associated with adverse prognostic parameters or shorter patient survival (McGrath et al., 2005; Grutzmann et al., 2003; Hirao et al., 2006; Yamada et al., 2007). Our results are in line with this notion and demonstrate a prognostic value of ADAM9 for sarcoidosis. Significantly reduced expression of mRNA for ADAM9 was observed in lens epithelial cells from patients with anterior polar cataracts (Lim et al., 2002), which was consistent with our results. However, ADAM9 overexpression has been described in a wealth of solid tumours including lung cancer (McGrath et al., 2005; Grutzmann et al., 2003; Hirao et al., 2006; Yamada et al, 2007), which seems not to be compatible with our results and might be that ADAM9 used different biologic function in different kinds of diseases.

ADAM9, a member of the ADAM family, is involved in various biological processes (Moss et al., 2001), and previous research have showed that ADAM9 could regulate the expression of laminin, vitronectin, collagen types I and IV; regulate cell-cell and cell-matrix interactions; and maintain the normal morphology and functions of cells through interactions between its disintegrin domain and the α1β1, α3β1, α6β1 and αvβ1 protein in the β1 integrin family (Spagnolo et al., 2003). The result of immunohistochemistry revealed high levels of expression of ADAM9 in the cytoplasm of both endothelial cells of the alveolar septum and macrophages of the alveolar space of normal lung tissues, which indicate that ADAM9 may play similar roles in cell-cell and cell-extracellular matrix interactions in lung tissues. However, the expression of ADAM9 significantly decreased in both the granulomas and BALF of pulmonary sarcoidosis tissues compared with nomal lung tissue. It may therefore be speculated that the ability of regulating cell-cell and cell-extracellular matrix interactions was decreased, with the expression of ADAM9 decrease, and the balance between cells and extracellular matrix was disrupted, which then consequently creates a possibility of granuloma formation. Moreover, the mechanisms that cause granuloma

formation might be that ADAM9 decomposition fibronectin and gelatin was reduced due to ADAM9 expression decrease, as a result it cannot induce proteolysis of the extracellular matrix, which lead to cell accumulation and granuloma formation (Schwettmann et al., 2001). If this proved true, ADAM9 might play a role in granuloma progression, and might be used not only for prognostic and diagnostic purposes but also for novel therapeutic approaches. However, the detail functions of ADAM9 and its effects in pulmonary sarcoidosis were largely unknown; elucidation of these questions would depend on further studies and investigation.

In conclusion, we have demonstrated that ADAM9 was significantly down-regulated in pulmonary sarcoidosis in comparison to the normal tissue. The current study provides a new approach for studying the mechanism underlying the pathogenesis of pulmonary sarcoidosis. This suggests that decreased ADAM9 expression may be a useful diagnostic marker and could also become a potential target in the treatment of sarcoidosis.

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