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Sputum endothelin-1 level is associated with active pulmonary tuberculosis and effectiveness of anti-tuberculosis chemotherapy

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Abstract. Pulmonary tuberculosis (TB) is a major global health problem. Endothelin (ET)-1 is an important pro-inflammatory factor in the airways, which acts as a chemoattractant and an upregulator of other inflammatory mediators. In the present study, the association of the sputum ET-1 level with active pulmonary TB and the effectiveness of anti-TB chemotherapy was explored for the first time. A total of 56 newly diagnosed patients with active pulmonary TB, 56 age- and gender-matched TB-free controls, and 43 subjects with latent TB were recruited to the study. Patients in the active TB group received standard anti-TB chemotherapy. Sputum samples were collected from all study subjects at baseline (day 0) and on days 1, 2, 4, 6, 10 and 14 of treatment for the active TB group and the ET-1 level was determined by enzyme-linked immunosorbent assay. The sputum ET-1 level in the active TB group was significantly higher than those in the latent TB and the non-TB groups at baseline. Following adjustment for confounders such as age, gender, severity of clinical presentation, plasma ET-1 level and comorbidities that might affect the sputum ET-1 level, multivariate logistic regression analysis revealed that sputum ET-1 level was an independent indicator for active pulmonary TB. In the active TB group during anti-TB chemotherapy, decrements in the sputum ET-1 level were in significant correlation with decrements in the number of colony-forming units and increments in the time to positivity in a Mycobacteria Growth Indicator Tube assay. In conclusion, this study indicates that an elevated sputum ET-1 level is an independent indicator of active pulmonary TB and suggests that decrements in the sputum ET-1 level could reflect the effectiveness of anti-TB chemotherapy.

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Introduction

Although various treatments for pulmonary tuberculosis (TB) are available, TB remains a major health problem worldwide, with an estimated 8.6 million new cases and 1.3 million mortalities in 2012 (1). The World Health Organization estimates that one-third of the global population has latent *Mycobacterium tuberculosis* (*M. tuberculosis*) infection, among which 5-10% are likely to develop active TB during their lifetime (1). A rapid and accurate diagnosis test for active TB would enable the early treatment of this disease and reduce transmission, thereby facilitating TB control (2). However, the diagnostic tests currently available have significant deficiencies, such as a lack of sensitivity and specificity (3). In addition, a marker that precisely reflects the effectiveness of antimicrobial therapy would be useful in assessing the response to anti-TB treatments.

Endothelin (ET)-1 is a potent vasoconstrictor that exerts various effects in the respiratory tract (4), including the stimulation of mucus secretion, airway edema, smooth muscle mitogenesis and bronchial hyperresponsiveness (5). In addition, it is considered to have important pro-inflammatory effects in the airways, where it acts as a chemoattractant and also upregulates other inflammatory mediators such as interleukin (IL)-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (4). ET-1 is produced by human airway epithelial and endothelial cells and macrophages (4). Sputum levels of ET-1 have been reported to increase in patients with chronic obstructive pulmonary disease (COPD) during exacerbation (5). In addition, sputum ET-1 levels are also elevated in patients with cystic fibrosis and COPD compared with the levels in normal subjects (6).

Our pilot study suggested that elevated sputum ET-1 levels might indicate active disease in patients with pulmonary *M. tuberculosis* infection. In the present study, the association of the sputum ET-1 level with active pulmonary TB and the effectiveness of anti-TB chemotherapy were explored.

Materials and methods

Subjects. From December 2012 to December 2013, 56 newly diagnosed patients with active pulmonary TB, 56 age- and gender-matched non-TB controls, and 43 subjects with latent TB were recruited at the Second Xiangya Hospital of Central South University (Changsha, China). Diagnosis of active pulmonary TB was based on clinical symptoms, chest radiography, microscopy for acid fast bacilli and sputum *M. tuberculosis* culture. The inclusion criteria for patients with active TB were as follows: i) Had not received any anti-TB treatment prior to entering the study; ii) Culture or molecular confirmation of infection with drug-susceptible *M. tuberculosis*; and iii) human immunodeficiency virus (HIV) negative. The symptoms of pulmonary TB include fever, productive cough, night sweats, weight loss, chest pain and malaise. According to the severity of clinical presentation, patients with active TB were divided in three groups (mild, moderate and severe). Subjects with latent TB were those who had contact with a person with confirmed active TB and had a positive tuberculin skin test; none of them showed clinical symptoms or chest X-ray signs suggesting active TB. The non-TB controls were those who had not contacted with any person with confirmed active TB and had a negative tuberculin skin test. All subjects in this study were HIV negative. Baseline characteristics of all subjects are shown in Table I. The study was approved by the Ethics Committee of the Second Xiangya Hospital. Written informed consent was obtained from all subjects.

Treatment. All patients with active pulmonary TB received standard anti-TB chemotherapy with a weight-adjusted fixed-dose of 55 mg/kg Rifafour e-275 (Sanofi-Aventis, Beijing, China) consisting of isoniazid, rifampin, pyrazinamide, and ethambutol. The treatment was administered on an inpatient basis.

Sputum sampling. For patients with active TB, first morning sputum samples were collected at baseline (day 0) and on days 1, 2, 4, 6, 10, and 14 during Rifafour e-275 treatment. For subjects with latent TB and non-TB controls, induced sputum samples were collected within 72 h after enrollment, as previously described (7). The samples were centrifuged at 2,000 x g for 10 min and the supernatant was collected. The ET-1 level in the supernatant was quantified with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (DET100; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For patients with active TB, an aliquot of the sputum sample was subject to log colony-forming unit (CFU) determination on 7H11 agar with Selectatab (polymyxin B, ticarcillin, amphotericin B and trimethoprim; Mast Group, Ltd., Bootle, Merseyside, UK) added. Log CFU determinations were performed on samples collected on days 0, 1, 2, 4, 6, 10 and 14. A second aliquot was decontaminated with 1% NaOH-N-acetyl-L-cysteine, diluted with phosphate-buffered saline (PBS) and centrifuged at 4˚C and 3,000 x g for 15 min.

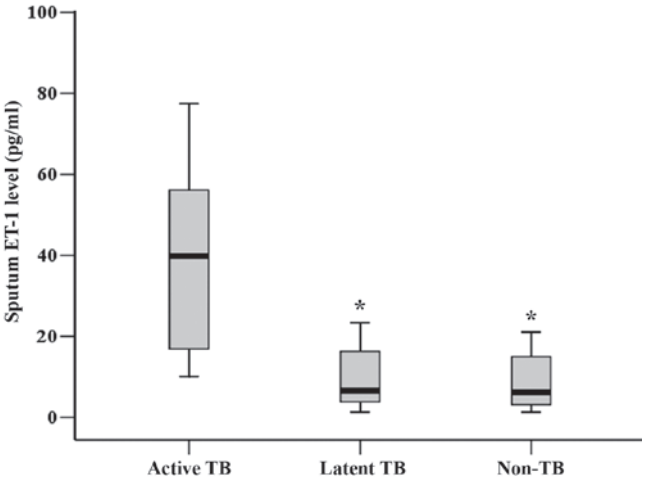

Figure 1. Sputum endothelin (ET)-1 levels in patients with active pulmonary tuberculosis (TB), subjects with latent TB, and non-TB controls. Baseline sputum ET-1 levels in the three groups are represented as boxplots. *P<0.05 vs. the active TB group.

The supernatant was discarded and the pellet resuspended in 1.5 ml PBS. Then, 500 ml of this suspension was used to inoculate a Mycobacteria Growth Indicator Tube (MGIT; BD Biosciences, Sparks Glencoe, MD, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC), and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin (PANTA). MGITs were incubated at 37˚C in a BACTEC MGIT 960 instrument (BD Biosciences) until they were flagged positive, or for a maximum of 42 days if no growth was detected. Time to positivity (TTP) in MGIT culture was recorded. Contamination was excluded by placing one drop of positive liquid culture on a blood agar plate (NHLS, Cape Town, South Africa) and by incubating for 48 h at 37˚C without visible growth.

Statistical analysis. Statistical analyses were performed using SPSS for Windows, version 13.0 (SPSS, Inc., Chicago, IL, USA). ET-1 levels were expressed as the median with interquartile range. Other continuous variables were expressed as mean ± standard deviation. Comparisons of sputum ET-1 levels among subject groups were performed with nonparametric Kruskal-Wallis H tests followed by pairwise comparisons using Nemenyi tests. Categorical variables were compared with Chi-square tests. Correlation analyses between the changes in sputum ET-1 level and the changes in log CFU or TTP results were examined using Spearman's rank tests. Multivariate logistic regression was performed to assess the odds ratio (OR) and its 95% confidence interval (CI). A two-tailed P<0.05 was considered statistically significant.

Results

Elevated sputum ET-1 level is associated with active pulmonary TB. As shown in Table I, there were no significant differences in age, gender and the prevalence of the co-morbidities hypertension, coronary artery disease, chronic bronchitis and COPD among the subject groups at baseline. The active TB group had a significantly higher sputum ET-1 level, but not plasma ET-1 level than the latent TB and the non-TB groups at baseline (Table I).

Table I. Baseline characteristics of study subjects.

Characteristic	Active TB (n=56)	Latent TB (n=43)	Non-TB (n=56)	P-value
Age (years)[a]	59.7±16.9	55.2±18.5	57.1±16.4	0.83
Age group (years)				
15-29	6 (10.7)	6 (14.0)	6 (13.3)	0.95
30-44	7 (12.5)	6 (14.0)	7 (13.3)	
45-59	12 (21.4)	12 (27.9)	12 (21.4)	
≥60	31 (55.4)	19 (44.1)	31 (55.4)	
Age range (years)	18-72	17-69	18-72	
Male gender	39 (69.6)	27 (62.8)	39 (69.6)	0.72
M. tuberculosis culture positivity	41 (73.2)	0 (0)	0 (0)	1.00
Clinical presentation				
Mild	5 (8.9)	-	-	-
Moderate	40 (71.4)	-	-	
Severe	11 (19.6)	-	-	
Sputum ET-1 level (pg/ml)	39.7 (17.9-56.4)	6.3 (3.1-16.7)[a]	5.7 (2.6-15.2)[a]	<0.01
Plasma ET-1 level (pg/ml)	1.5 (1.3-1.8)	1.3 (1.0-1.8)	1.2 (1.0-1.7)	0.08
Co-morbidities				
Hypertension	27 (48.2)	19 (44.2)	24 (42.9)	0.84
CAD	23 (41.1)	16 (37.2)	21 (37.5)	0.90
Chronic bronchitis	17 (30.4)	11 (25.6)	10 (17.9)	0.30
COPD	10 (17.9)	5 (11.6)	5 (8.9)	0.36

Age is presented as the mean ± standard deviation. Sputum and plasma ET-1 levels are presented as the median (interquartile range). Comparisons of sputum and plasma ET-1 levels among subject groups were performed with nonparametric Kruskal-Wallis H tests followed by pairwise comparisons using Nemenyi tests. All categorical variables are expressed as n (%) and comparisons were performed with Chi-square tests. CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; TB, tuberculosis; *M.tuberculosis*, *Mycobacterium tuberculosis*. [a]P<0.05 vs. active TB.

Table II. Logistic regression analysis of factors significantly associated with sputum *M. tuberculosis* culture positivity.

Factor	Point estimate	Standard error	Wald Chi-square	P-value	Odds ratio	95% CI for odds ratio
Severity of clinical presentation	1.01	3.68	9.61	<0.01	2.74	1.04-7.22
Sputum ET-1 level (pg/ml)	1.87	0.49	4.17	0.04	6.50	1.32-32.02

Multivariate logistic regression analysis was performed with sputum culture results (*M. tuberculosis* negative=0, *M. tuberculosis* positive=1) as the dependent variable. Age, gender (female=0, male=1), severity of clinical presentation (mild=1, moderate=2, severe=3), sputum ET-1 level, plasma ET-1 level, and co-morbidities (hypertension and/or coronary artery disease, no=0, yes=1; chronic bronchitis and/or COPD, no=0, yes=1) were used as independent variables. Severity of clinical presentation and sputum ET-1 level entered the logistic regression model. *M. tuberculosis*, *Mycobacterium tuberculosis*; ET, endothelin; CI, confidence interval.

As shown in Fig. 1, the sputum ET-1 level in the active TB group was significantly higher than those in the latent TB and the non-TB groups at baseline (P<0.01).

In order to identify the factors that significantly affected the sputum *M tuberculosis* culture positivity, multivariate logistic regression analysis was performed using sputum culture results (*M. tuberculosis* negative=0, *M. tuberculosis* positive=1) as the dependent variable. Age, gender (female=0, male=1), severity of clinical presentation (mild=1, moderate=2, severe=3), sputum ET-1 level, plasma ET-1 level, and co-morbidities (hypertension and/or coronary artery disease, no=0, yes=1; chronic bronchitis and/or COPD, no=0, yes=1) were used as independent variables. As shown in Table II, the severity of clinical presentation and the sputum ET-1 level entered the logistic regression model. The results indicated that the severity of clinical presentation (OR=2.74, 95% CI=1.04-7.22, P<0.01) and the sputum ET-1 level (OR=6.50, 95% CI=1.32-32.02, P=0.04) were significantly associated with sputum *M. tuberculosis* culture positivity, which

Table III. Correlation between changes in sputum ET-1 level and changes in CFU/ml in patients receiving anti-tuberculosis chemotherapy.

Day	Correlation coefficient (r)	P-value
1	0.44	<0.01
2	0.51	<0.01
4	0.31	0.02
6	0.36	<0.01
10	0.42	<0.01
14	0.54	<0.01

Patients with active pulmonary TB were treated with a weight-adjusted fixed-dose of Rifafour e-275. Changes in the sputum ET-1 level (pg/ml) and CFU/ml from baseline (day 0) on days 1, 2, 4, 6, 10 and 14 were expressed as ΔSputum ET-1 and Δlog CFU/ml, respectively. Spearman rank tests were performed to analyze the correlation between ΔSputum ET-1 and Δlog CFU/ml at each time point. ET, endothelin; CFU, colony-forming unit.

Table IV. Correlation between changes in sputum ET-1 level and changes in TTP in patients receiving anti-tuberculosis chemotherapy.

Day	Correlation coefficient (r)	P-value
1	-0.52	<0.01
2	-0.42	<0.01
4	-0.43	<0.01
6	-0.46	<0.01
10	-0.49	<0.01
14	-0.56	<0.01

Patients with active pulmonary TB were treated with a weight-adjusted fixed-dose of Rifafour e-275. Changes in the sputum ET-1 level (pg/ml) and in TTP from baseline (day 0) on days 1, 2, 4, 6, 10 and 14 were expressed as ΔSputum ET-1 and ΔTTP, respectively. Spearman rank tests were performed to analyze the correlation between ΔSputum ET-1 and ΔTTP at each time point. ET, endothelin; TTP, time to positivity.

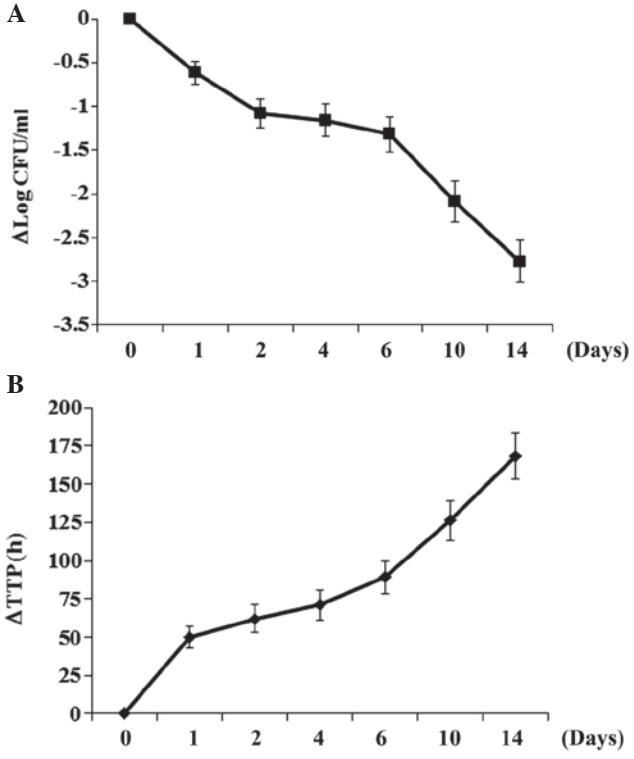

Figure 2. Changes in colony-forming unit (CFU) counts and time to positivity (TTP) in patients receiving anti-tuberculosis (TB) chemotherapy. Patients with active pulmonary TB were treated with a weight-adjusted fixed-dose of Rifafour e-275. Changes in CFU/ml and TTP (hours) from baseline (day 0) on days 1, 2, 4, 6, 10 and 14 were expressed as (A) Δlog CFU/ml and (B) ΔTTP and plotted against time.

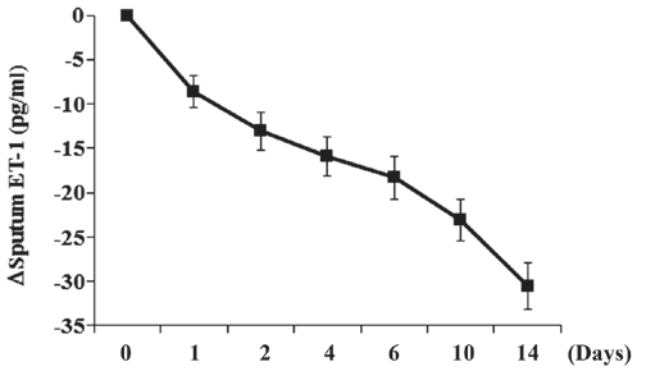

Figure 3. Changes in sputum endothelin (ET)-1 level in patients under anti-tuberculosis (TB) chemotherapy. Patients with active pulmonary TB were treated with a weight-adjusted fixed-dose of Rifafour e-275. Changes in the sputum ET-1 level (pg/ml) from baseline (day 0) on days 1, 2, 4, 6, 10 and 14 were expressed as ΔSputum ET-1 and plotted against the time.

suggests that these two factors are independent indicators of active pulmonary TB.

Change in sputum ET-1 level correlates with patient response to anti-TB chemotherapy. In order to determine the association between the level of ET-1 in the sputum and the patient response to anti-TB chemotherapy, patients in the active TB group were treated with a weight-adjusted fixed-dose of 55 mg/kg Rifafour e-275 and the sputum ET-1 level, the number of CFU/ml and TTP were measured at baseline (day 0) and on days 1, 2, 4, 6, 10 and 14. As shown in Fig. 2, the number of CFU and TTP decreased and increased, respectively, over the time of treatment. The sputum ET-1 level decreased over the time of treatment (Fig. 3), with a trend similar to that of the number of CFU. As shown in Table III, correlation analyses with Spearman rank tests revealed that decrements (from baseline) in sputum ET-1 level were in significant positive correlation with decrements (from baseline) in the number of CFU at each time point during the treatment, with the correlation coefficient ranging from 0.31 on day 4 to 0.54 on day 14 (all $P<0.05$). By contrast, decrements (from baseline) in the sputum ET-1 level were in significant negative correlation with increments (from baseline) in TTP at each time point during the treatment, with the correlation coefficient ranging from -0.42 on day 2 to -0.56 on day 14 (all $P<0.01$; Table IV).

Discussion

The present study, to the best of our knowledge, provides the first evidence that the sputum ET-1 level is significantly associated with active pulmonary TB and the effectiveness of anti-TB chemotherapy.

ET-1, produced by airway epithelial and endothelial cells and macrophages (8-10), functions as a pro-inflammatory factor in the airways, where it acts as a chemoattractant and upregulates other important inflammatory mediators such as IL-6 and GM-CSF (5,11). A systemic rise of ET-1 levels occurs in response to a variety of factors, including sepsis and ischemia (5,12). In the present study, it was observed that the sputum ET-1 level was significantly elevated in patients with active pulmonary TB compared with patients with latent TB and TB-free controls. Following adjustment for confounders such as age, gender, severity of clinical presentation, plasma ET-1 level and comorbidities that might affect the sputum ET-1 level, multivariate logistic regression analysis revealed that the sputum ET-1 level was an independent indicator for active pulmonary TB. Since the plasma ET-1 level was not significantly increased, it is likely that the elevation of sputum ET-1 levels in patients with active pulmonary TB was due to the pulmonary, not systemic, inflammatory responses to active *M. tuberculosis* infection.

The viability of bacilli and the susceptibility to anti-TB therapy is usually monitored by culture (13), which remains the gold standard in the diagnosis and follow-up of mycobacterial infections. However, it is a time-consuming process, since *M. tuberculosis* grows slowly and several weeks or months are required for its detection in clinical samples (14). Inflammation-related factors have been suggested as potential biomarkers for active TB (15,16). Travar *et al* (15) reported that the sputum level of interferon λ-2 was significantly higher in patients with active pulmonary TB than in patients with latent TB and healthy controls. Cai *et al* (16) reported that the expression level of complement C1q in the peripheral blood was able to discriminate patients with active TB from those with latent TB infection and healthy controls. The results of the present study show that the sputum ET-1 level was significantly higher in the patients with active pulmonary TB than in those with latent TB and the TB-free controls. Whether these factors are connected in active pulmonary TB and how remain to be explored in our future studies.

In this study, decrements in the sputum ET-1 level significantly correlated with decrements in CFU and increments in TTP during anti-TB chemotherapy. This corroborates the finding that the sputum ET-1 level is significantly associated with active pulmonary TB, and also suggests that decrements in the sputum ET-1 level could be a potential indicator of the effectiveness of anti-TB chemotherapy. Determination of the sputum ET-1 level by ELISA is fast (<5 h) and easy, which supports the feasibility of using the sputum ET-1 level as a biomarker for active pulmonary TB and the effectiveness of anti-TB chemotherapy. We plan to explore the clinical application value of the sputum ET-1 level for patients with active pulmonary TB in a future study with a large patient sample.

The present study has several limitations: i) Only HIV-negative subjects were enrolled to minimize the potential effects of immunodeficiency on the sputum ET-1 level, since ET-1 is profoundly involved in inflammatory responses in the airways. ii) Only newly diagnosed patients infected with drug-susceptible *M. tuberculosis* and without previous anti-TB treatment were enrolled in the active TB group to exclude possible confounding effects of drug-resistant *M. tuberculosis* on the effectiveness of anti-TB chemotherapy in this study (17). Nevertheless, the findings of this study provide a solid basis for future studies with a more extensive patient sample.

In conclusion, this study indicates that an elevated sputum ET-1 level is an independent indicator of active pulmonary TB and suggests that decrements in the sputum ET-1 level may reflect the effectiveness of anti-TB chemotherapy.

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