Tuberculosis and Respiratory Diseases 결핵 및 호흡기질환, Vol. 53, No. 2, Aug, 2002

□ 원 저 □

Detection of *Mycobacterium Tuberculosis* in Bronchial Specimens Using a Polymerase Chain Reaction in Patients with Bronchial Anthracofibrosis

Joo Ock Na, M.D.*, Chae-Man Lim, M.D., Sang Do Lee, M.D., Younsuck Koh, M.D., Woo Sung Kim, M.D., Dong Soon Kim, M.D., Won Dong Kim, M.D., Tae Sun Shim, M.D.

Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

=국문초록=

기관지 탄분 섬유화증 환자의 기관지내시경 검체에서 PCR을 이용한 결핵균의 검출

울산대학교 의과대학 서울아산병원 내과학교실

나주옥, 임채만, 이상도, 고윤석, 김우성, 김동순, 김원동, 심태선

연구배경 : 저자들은 기관지 내시경 검사상 관찰된 탄분 섬유화증 (Anthracofibrosis. 이하 AF)과 결핵과의 연관성을 기관지 내시경 검체 결핵 PCR을 이용하여 알아보고자 하였고, 또한 결핵 PCR이 AF에 동반된 활동성 폐결핵의 신속한 진단에 도움이 되는지를 알아보고자 하였다.

방 법 : 기관지 내시경 검사상 기관지 협착이나 폐쇄를 동반한 기관지 점막의 탄분 침착을 갖는 25명의 여성과 5명의 남자를 포함한 총 30명의 환자를 대상으로 하였다. 기관지 세척액과 AF부위 조직을 대상으로 *Mycobacterium tuberculosis*의 IS6110분절을 대상으로 하는 PCR을 시행하였고, 또한 환자의 세균학적 검사 결과, 임상상, 및 임상경과를 분석하고 비교하였다.

결 과 : 임상분석상 18명(60%)이 결핵과 연관되어 있었고, 이 중 9명은 과거에 결핵을 앓은 병력이 있었으며 나머지 9명은 세균학적 검사상 활동성 결핵으로 판명되었다. 객담과 기관지 세척액에서 항산균 도말은 4명에서 양성 소견을 보였고, 항산균 배양양성은 9명 (30%)이었으며 AF의 조직검사 상 결핵을 보인 경우는 모두 5명이었다.

Address for correspondence :
Tae Sun Shim, M.D.
Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-dong, Songpa-gu, Seoul 138-600, Korea
Tel : 82-2-3010-3892 Fax : 82-2-3010-6968 E-mail : shimts@amc.seoul.kr
*Present address: Department of Internal Medicine, Soonchunhyang University Chonan Hospital 23-20, Bongmyung-Dong Choan, Korea.

기관지 세척액과 AF조직에서의 TB-PCR은 각각 11명(37%), 5명(17%)에서 양성 소견을 보였다. PCR방법은 항산균 도말보다 폐결핵 진단에 민감하였으며 (민감도 89 % *vs* 22 %, respectively, $p < 0.05$) AF조직에서 결핵으로 진단된 5명의 환자 모두 AF조직 및 기관지 세척액에서 TB-PCR 양성소견을 보였다. TB-PCR 양성이면서 조직학적 또는 세균학적으로 결핵의 증거를 보이지 않았던 3명의 환자 중 2명은 임상적으로 활동성 결핵이 의심되었고 나머지 한명은 과거에 결핵을 앓은 적이 있었던 환자이었다.

결 론 : 세균학적 및 조직학적 결핵 진단법과 비교하여 TB-PCR 방법이 추가로 AF와 결핵과의 연관성을 밝혀내지는 못하였으나, 기관지 세척액 TB-PCR은 AF와 동반된 활동성 폐결핵의 신속한 진단에는 도움이 될 것으로 기대된다. 조직학적 검사상 결핵의 증거가 없이 AF소견만 보인 모든 조직에서 결핵 PCR음성을 보인 점은 AF 병변 자체가 결핵의 감염 부위이거나 활동성 결핵 자체이기 보다는 결핵에 의한 이차적인 변화일 가능성이 높을 것으로 생각된다. **(Tuberculosis and Respiratory Diseases 2002, 53:161-172)**

Key words : Anthracofibrosis, Tuberculosis, Polymerase chain reaction

Introduction

Anthracofibrosis (AF) is defined as a finding which shows dark anthracotic pigmentation associated with bronchial narrowing or obliteration on bronchoscopic examination[1]. Dark anthracotic pigmentation in the bronchial mucosa has been regarded as a bronchoscopic finding of pneumoconiosis or evidence of heavy exposure to atmospheric soot in the past[2]. However, most patients with bronchial AF have had no exposure to mining or industry and no history of smoking. Recently, some reports showed that bronchial AF was associated with tuberculosis (TB) in some patients[1,3,4]. However, in others patients no cause could be found for AF, and there was no evidence of TB by histological and bacteriological methods. At this point, we do not know whether these non-TB-associated AF lesions are really unrelated to TB status, or that an association with TB was not apparent due to a failure of current techniques in detecting *M. tuberculosis*.

Bronchoscopy is essential for the diagnosis of bronchial AF. When anthracofibrotic lesions are found during bronchoscopic procedure, bronchial specimens such as bronchial wash fluid, bronchoalveolar lavage fluid, or mucosal tissue are obtained for the correct diagnosis of pulmonary and bronchial lesions. These bronchial specimens have been used successfully in the diagnosis of pulmonary TB in AFB smear-negative patients[5]. In recent years, polymerase chain reaction (PCR) and other amplification-based techniques have been widely used in the diagnosis of TB[6-9]. Some researchers have used bronchoscopic specimens for the amplification of *M. tuberculosis* DNA in the diagnosis of TB, and have shown promising results[10,11]. In the context of these results, we hypothesize that most AF lesions may be associated with TB, and that we can detect this association by PCR technology. To evaluate this hypothesis, nested-PCR was performed to detect *M. tuberculosis* on

bronchial specimens. Clinically this study will also give us information on whether PCR on bronchial specimens could be useful in the rapid diagnosis of active pulmonary TB in patients with bronchial AF.

Material and method

1. Bronchoscopic procedures

Among a total of 1,597 patients who underwent bronchoscopy at the Asan Medical Center from September 1999 to July 2000, 54 patients (3.4%) showed bronchial anthracofibrotic lesions. Excluding twenty-four patients in whom biopsy specimens could not be obtained or PCR was omitted, thirty patients were enrolled in this study. Diagnosis of AF was made solely on the basis of bronchoscopic findings. Patients showing only anthracotic pigmentation on bronchial mucosa without bronchial stenosis were excluded[1]. In all patients, sputum was obtained for bacteriological and cytological study. After examination of the bronchial trees, 5-10 ml of normal saline was instilled into the orifice of the bronchial segment in which the abnormalities were located, and then aspirated back. Bronchial biopsies were performed at the site of anthracofibrotic lesions. After taking two to five tissues, we verified that the biopsies were performed at the correct site by confirming that the tissue contained the dark pigmentation grossly. Mostly, significant bleeding from biopsy sites was the factor limiting the number of biopsy tissues. Biopsy specimens were evenly separated for PCR and histologic examination.

2. DNA extraction

Bronchial tissues and bronchial wash fluids were digested in lysis buffer (10mM Tris, pH 8.5; 10mM EDTA, pH 8.0;0.5% SDS; 100mM NaCl) with proteinase K (500ug/ml, GiBCO BRL) at 60 ℃ overnight. Genomic DNA was extracted by traditional method using phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. Pellet was washed with 70% cold EtOH followed by air-drying and resuspended in dextrose water. Extracted DNAs were stored at -20℃ until use.

3. Polymerase chain reaction

IS6110 segment was amplified by nested-PCR using the commercial primer set N-5820 (297 base pair) and N-5821 (285 base pair) (Bioneer, Seoul, Korea) (Table 1).

PCR was done separately using each primer set. N-5820 primer set was designed to amplify 130 to 426th base sequence, and N-5821 was designed for the sequence 590 to 874th. The PCR results were reported as positive if one of the two PCR analyses were positive. PCR was performed as follows:Sample DNA was denatured at 94℃ for 5 min and amplified by 35 cycles at 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, and followed by a final extension for 10 min at 72℃. The first PCR was carried out in a 25 ul reaction mixture containing 0.8 μg of genomic DNA, 10 pmol of primers, 0.2 mM of dNTP and 1 unit of Taq® polymerase (Takara, Otsu, Japan). The second PCR was performed using 1.5 μl of the first PCR product in a 25

Table 1. The sequences of primers for targeting IS6110 segment

Primer set	Name	Primer sequence
N-5820	KBN1	5′-GGATGGTCGCAGAGATCCGC-3′
	KBN2	5′-CGATGCCCTCACGGTTCAGG-3′
	KBN3	5′-GGTCAGCACGATTCGGAGTG-3′
	KBN4	5′-CGGGGCCCTCGCGGTGGCCC-3′
N-5821	KBN5	5′-CTCAAGGAGCACATCAGC-3′
	KBN6	5′-TAGTGCATTGTCATAGGAGCTTCCGACC-3′
	KBN7	5′-CTACGGTGTTTACGGTGCCC-3′
	KBN8	5′-TAGGCGTCGGTGACAAAGGC-3′

μl reaction mixture containing the same as the first PCR. The final PCR product was analyzed on a 2.5% agarose gel and visualized by UV illumination after ethidium bromide staining. Size of the final PCR product was 297 bp with the primer set N-5820 and 285 bp with N-5821. Genomic DNA from the sample identified to be infected with *M. tuberculosis* was used for the positive control and a normal genomic DNA for the negative control.

4. Data analysis

The patients' clinical histories were reviewed, including previous medical history, occupation, life style, smoking status, current symptoms, and the response to the anti-tuberculosis treatment. Plain chest radiograph and chest CT were also reviewed. The results of sputum and bronchial wash fluid Ziehl-Neelson staining for acid-fast bacilli (AFB), culture, and histologic findings of bronchial tissue were analyzed and compared with the results of PCR on bronchial wash fluid or mucosal tissue. The diagnosis of TB was confirmed only when cultures of sputum or bronchial aspirates were positive for *M. tuberculosis*, or if lung or bronchial biopsy specimens showed chronic granulomatous inflammation with caseation necrosis. Clinical TB was defined as radiological findings compatible with TB that improved with antituberculosis drug treatment, but lacked bacteriological or histological confirmation of TB. In cases of clinical TB, TB was considered to be confirmed if histologic findings showed chronic granulomatous inflammation even without caseation necrosis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of PCR were calculated using bacteriological or histological diagnoses as the ultimate diagnostic standard for TB. In the case of patients with false-positive PCR results, the clinical history and radiographic results were investigated.

5. Statistical analysis

Statistical analysis was performed using SPSS

for Windows v10.0 (SPSS Inc., Chicago, IL, USA). The McNemar test was used to compare of the sensitivity of direct AFB smear and PCR. Data are expressed as mean±SD, and statistical significance was defined as $p<0.05$.

Results

1. Clinical characteristics

The patients were composed of 25 women and 5 men, ranging in age from 53 to 88 (median, 71 years). Only one patient was a current smoker, whereas 20 were non-smokers and 9 were ex-smokers. No patients had an occupational history of exposure to mining or industry. Twelve patients had lived in urban areas, and the rest resided in rural areas. Nine patients had a past history of pulmonary tuberculosis, of whom seven were treated with antituberculous medication, and the remaining two had old tuberculous lesions on chest radiographs.

Fifteen patients had a cough, and 13 patients complained of variable amounts of sputum. Other presenting clinical symptoms were 11 dyspnea, 8 hemoptysis, 4 chest discomfort and 3 fever. Eleven patients were asymptomatic and were referred to the Asan Medical Center based on abnormal findings in routine chest radiograph.

The most common radiographic finding of the plain chest radiographs at presentation was segmental or subsegmental atelectasis (18/30) especially involving the right middle lobe (13/30). Other principal findings of plain chest radiographs were central mass-like lesions or consolidation (10/30) and normal except

Table 2. Chest CT findings in 24 patients with AF

CT findings	Number (%)
Lobar or segmental collapse	15 (62)
Lymph node enlargement	14 (58)
with calcification	6 (25)
without calcification	8 (33)
Mass shadow	6 (25)
Bronchial narrowing	16 (67)
Consolidation	8 (33)
Nodulo-streaky density	8 (33)
Bronchiectasis	3 (12)
Pleural effusion	3 (12)
Normal	1 (4)

fibrostreaky density at both upper lobes suggesting inactive pulmonary tuberculosis (1/30). In one patient, the chest radiographs were unremarkable. Chest CT scans were performed in 24 patients. Chest CT finding of all patients(23/24) was observed collapse or mass lesion or only bronchial narrowing except one patient who showed normal chest CT finding. The most common CT finding was atelectasis distal to the narrowed bronchus (15/24) **(Table 2)**. The possibility of lung cancer could not be excluded by CT findings in patients with lung masses or atelectasis because of dense fibrotic lesions around the involved bronchus. Therefore, in most cases bronchoscopic examination was indicated including one patient who showed normal chest radiograph and chest CT and developed hemoptysis.

The principal bronchoscopic findings were anthracotic pigmentation on bronchial mucosa and bronchial luminal narrowing by fibrotic

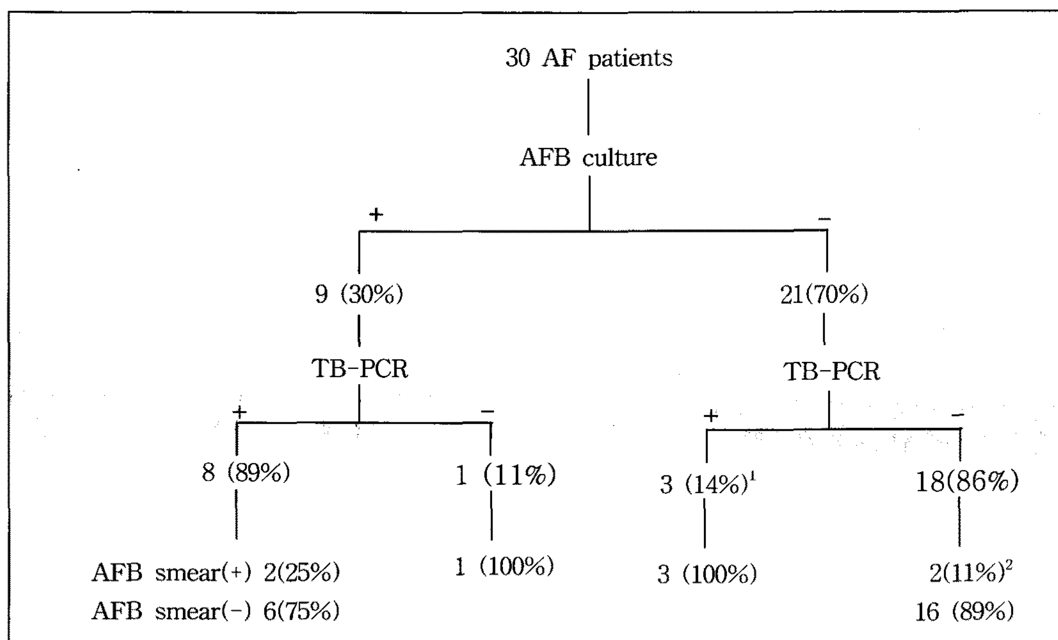

Fig. 1. Summary of AFB smear, culture, and PCR results in 30 AF patients. 1: Among these three patients, two showed improvement of their symptoms and chest radiographs after anti-TB medication. 2: Two patients who had negative AFB culture but positive smear result also had negative TB-PCR results, and showed improvements in clinical symptoms and chest radiographic findings after administering antituberculous medication.

change. Fifteen patients showed bilateral anthracofibrosis, thirteen patients with right sided lesions and two patients with left sided lesions. The right middle lobe bronchus was the most commonly involved site (23/30) followed by the right upper lobe (21/30), and the left upper lobe (13/30). In most cases, affected bronchial mucosa had excessive bleeding-tendency during bronchoscopic biopsy.

The main histologic finding was mucosal or submucosal fibrosis with black pigmentation. Chronic granulomatous inflammation suggesting active tuberculosis was found in 5 patients, among whom three were also positive for AFB staining. These five patients all had positive bronchial wash fluid AFB culture results.

The diagnosis of active tuberculosis was confirmed in nine patients (30%) by AFB cultures of sputum or bronchial wash fluid (Fig. 1). All nine patients who had past history of tuberculosis showed negative AFB smear and culture. Positive AFB smear of sputum and bronchial aspirate were observed in four patients among whom only two had positive AFB culture results (22% sensitivity of AFB smear). The other two patients who had negative AFB culture but positive smear result also had negative TB-PCR results, and showed improvements in clinical symptoms and chest radiographic findings after administering antituber-

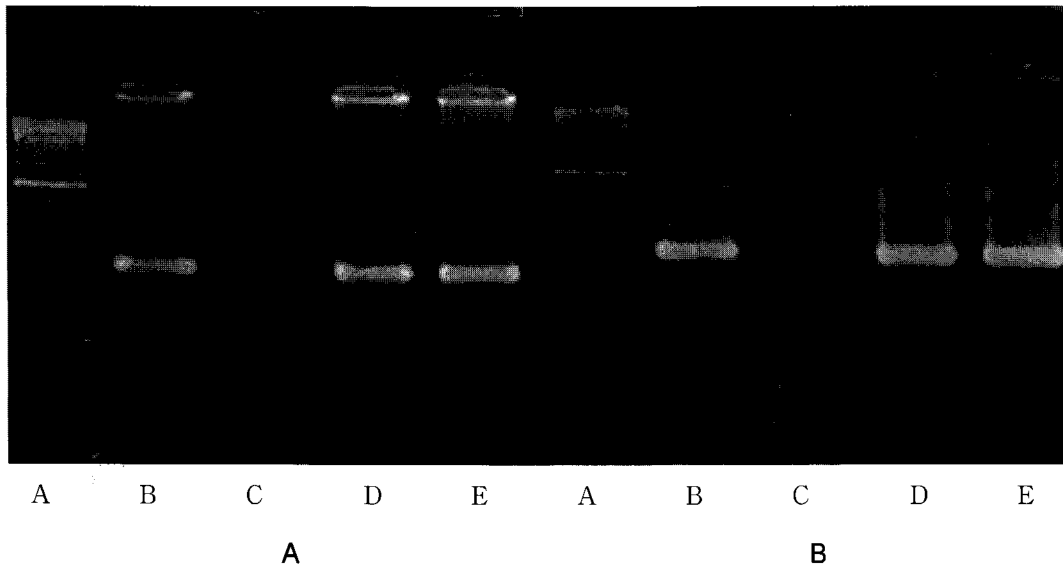

Fig. 2. PCR results using primer set N-5821 (*left*, A) and N-5820 (*right*, B) in bronchial wash fluid of one representative patient with AF and confirmed TB. PCR analysis showed positive results in both primer sets. A: DNA marker, B: Positive control, C: Negative control, D and E: Duplicate of bronchial wash sample.

culous medication.

2. PCR analysis

Positive TB-PCR on bronchial wash fluid was found in 11 patients (37%) **(Fig. 1, Fig. 2)**. Six patients were positive using both sets of primers, whereas two were positive only in the N-5820 set, and the remaining three were positive only in the N-5821 set. The eight patients who had positive a PCR result in both primer sets or only in N-5820 primer set were confirmed as having active TB. One confirmed TB patient showed negative TB-PCR result. Hence, the sensitivity of TB-PCR on bronchial wash fluid was 89% (8/9) and the specificity was 86% (18/21). Of the three patients who had positive TB-PCR but negative AFB culture, two patients showed improvement in their symptoms and chest radiographic findings after anti-TB medication suggesting that they might have active tuberculosis clinically. The other one patient who had positive TB-PCR but negative AFB culture had a past history of TB and showed no evidence of active TB clinically. Positive TB-PCR results of AF tissue were found in 5 patients. However, all of these patients also had positive histologic finding for TB and also positive TB-PCR results of bronchial wash fluid, so, tissue TB-PCR did not provided any additional benefits in the diagnosis of TB.

3. Clinical outcomes

Patients who showed hemoptysis or AFB culture

positive or smear positive or progressive collapse during follow up period took anti-TB medication by clinical judgment. Fifteen patients including 9 confirmed TB patients took anti-TB medication. Excluding two patients who were lost to follow up and 2 patients who showed no radiographic improvement and 1 patient who showed radiographic aggravation, ten out of fifteen patients (including two patients who had positive TB-PCR but negative culture results) showed radiographic improvement after medication. One patients who received anti-TB medication for 4 months without no bacteriologic evidence of TB showed progressive aggravation of mass like lesion of right middle lobe and she was recommended further evaluation but, she refused evaluation and were lost to follow up. One other patient who had positive TB-PCR but negative culture result was not treated with anti-TB medication and there was no interval change on the chest radiographs after one year. Among fifteen patients who did not take anti-TB medication, 5 patients showed spontaneous radiographic improvement one year after initial bronchoscopy and the other 2 patients showed no interval change. Nine patient who did not take anti-TB medication were lost to follow up **(Table 3).**

Discussion

Although anthracofibrosis was reported to be associated with tuberculosis in some patients[1,3,4], clinicians face the dilemma of whether AF is associated with active tuberculosis or not, which questions the need to prescribe antituberculous medications. This study showed that PCR for *M. tuberculosis* on bronchial wash fluid could be helpful in the rapid diagnosis of tuberculosis associated with AF. By using bronchial wash fluid instead of mucosal tissue, we can reduce the risk of bleeding complication by obtaining a minimal amount of bronchial tissue for histologic examination.

Because of the slow growth rate of *M. tuberculosis*, isolation, identification, and drug susceptibility testing of this organism using traditional methods takes several weeks or longer. TB-PCR methods can potentially reduce the diagnostic time from weeks to days[12]. However, the current sensitivity of PCR testing to diagnose TB is disappointing. Even FDA-approved amplification techniques are relatively insensitive in smear-negative pulmonary TB samples[13]. Hence, we used another commercial nested-PCR kit targeting IS6110 repetitive segment of *M. tuberculosis* complex to increase sensitivity. IS6110 segment is 1,361 bp long and contains 28-bp imperfect inverted repeats at its extremities with three mismatches and 3-bp direct repeats that probably results from repetition of the target sequence. Among the various mycobacterial species examined, IS6110 was detected only in species belonging to the *M. tuberculosis* complex and had been used as a target for the identification of *M. tuberculosis*[14]. However, even though we used two primer sets in the IS6110 segment in order to increase the sensitivity, we could not demonstrate more distinctive findings as compared with traditional methods. Actually, the N-5821 primer set did not yield any more benefits in increasing the

sensitivity of PCR in this study. TB-PCR showed positive results in only 3 out of 21 TB-unproven patients. One had no clinical evidence of TB, and the other two patients showed improvement radiologically and clinically after treatment with anti-TB medication, suggesting that they indeed had clinical TB. However, we were not convinced that they really had active TB because we sometimes observed spontaneous improvement in chest radiographs of patients with bronchial AF without any treatment. This finding was also observed in this study where six patients showed spontaneous radiographic improvement even without treatment. Collectively, we could not show an increased association between bronchial AF and TB using PCR techniques compared with previous reports.

However, in spite of these findings, the results in this study have some important implications. The most critical clinical decision in case of AF is to exclude the possibility of lung cancer. If lung cancer is excluded, the question is whether anti-TB medication needs to be prescribed when other causes of AF are ruled out. The sputum or bronchial aspirate AFB smear was positive only in 4 study patients, and culturing, although it gave more positive results, took a long time. Because TB-PCR on bronchial aspirates showed more sensitive results than the AFB smears for the diagnosis of TB (22% vs 89%, respectively, $p<0.05$), it can be used for a more rapid diagnosis and to decide whether anti-TB medication should be prescribed.

We used both bronchial wash fluid and bronchial tissue for the PCR analysis for *M.tuberculosis*. If the anthracofibrotic site is the origin of TB lesions, we can easily suspect that TB-PCR on AF tissue will produce more sensitive results compared with bronchial wash fluid. However, in our study, PCR on bronchial aspirates were more sensitive than on bronchial tissue. This may have resulted from low sensitivity of the test due to the small amount of tissue obtained. Anthracofibrotic tissue usually tends to bleed, so multiple biopsies could not be taken at once to be used for histologic examination. Regardless of this fact, the findings suggest important clinical and pathophysiological implications. Pathophysiologically, the results suggest that an AF lesion itself is not an active or original site of infection, but a secondary change of TB. At this point, even though we only know that there is some association between AF and TB, the cause-effect relationship between them is unknown. TB may develop at the distal portion of the AF bronchial segment because of decreased defense mechanisms due to AF, or endobronchial TB itself may be a cause of bronchial AF. Some findings suggest that endobronchial TB is one of the most likely causes of bronchial AF. Endobron-chial TB usually develops in young women and bronchial AF lesions are usually found in older women. The endobronchial TB lesion may leave bronchial defects which make the bronchi more susceptible to anthracofibrotic mucosal change in old age. These mucosal defects may be more vulnerable to the recurrence of tuberculosis, and recurrence of TB is sometimes shown at the AF lesion or the distal lung parenchyma. The aforementioned scenarios assume that the AF

lesion is not an active or original site of infection, but just a secondary change of TB or other diseases. Clinically, we can minimize the amount of tissue biopsied for histologic examination in patients with AF, but not for TB-PCR analysis. Bleeding risk in AF patients could be reduced using TB-PCR analysis because bronchial wash fluid, rather than tissue, is sufficient for testing.

As mentioned above, the PCR method we used in this study is not FDA approved. In-house PCR sensitivity and specificity usually vary greatly between institutions, and may also depend on the expertise of the laboratory workers[15,16]. Although there is no worldwide standardization, we have used this PCR protocol in our hospital for several years, and it usually yielded a higher sensitivity. Using two primer sets in clinical practice, one PCR result was not always concordant with the other PCR result. IS6110 segment is present in high copy numbers in *M. tuberculosis*. Therefore, even though there are deletions or mutations in some IS6110 segments, other segments make it possible to be detected in PCR analysis targeting IS6110 segment. We don't know yet why the two primers show a discrepancy in PCR results. Regardless of the difference between primers, the addition of one more primer set N-5821 in this study did not give any more sensitivity over the single primer set N-5820.

In conclusion, even though TB-PCR did not reveal an increased association between bronchial AF and TB compared with traditional methods, PCR on bronchial wash fluid seemed to be useful for the rapid diagnosis of pulmonary TB in patients with bronchial AF. TB-PCR on AF bronchial tissue itself did not yield additional benefits in the diagnosis of TB over bronchial wash fluid, suggesting that an AF lesion itself may not be an active or original site of infection, but a secondary change of TB.

Summary

Background : To Investigate the association between bronchial anthracofibrosis (AF) and tuberculosis (TB), and the clinical utility of a polymerase chain reaction (PCR) on bronchial specimens for rapid diagno-sis of active pulmonary TB in patients with bronchial AF.

Method : Thirty patients (25 women and 5 men ranging in age from 53 to 88), who were diagnosed with bronchial AF by a bronchoscopic exami-nation, were enrolled in this study. PCR targeting the IS6110 segment of *Mycobacterium tuberculosis* was performed on the bronchial wash fluid and anthracofibrotic bronchial tissue. The PCR results were compared with the bacteriological, histological, and clinical findings.

Results : Eighteen of the 30 patients (60%) were associated with TB, nine of whom were confirmed as having active TB. The remaining 9 had a past history of TB. The sputum or bronchial aspirate AFB smear, culture, and histological findings were positive in 4 (13%), 9 (30%), and 5 (17%) patients, respectively. PCR of the AF tissue and bronchial wash fluid was positive in 5 (17%) and 11 (37%) of the 30 patients, respectively. PCR was more sensitive than the AFB smears for diagnosing pulmonary

TB (22 % *vs* 89 %, respectively, p<0.05). All 5 patients with positive AF tissue PCR results also had both histological findings and positive bronchial wash fluid PCR results. Of the 3 patients with positive PCR but negative bacteriological or histological results, 2 of these patients appeared to have active tuberculosis on a clinical basis.

Conclusion : Although TB-PCR did not reveal an increased association between bronchial AF and TB compared with traditional methods, PCR on the bronchial wash fluid appears to be useful for the rapid diagnosis of pulmonary TB in patients with bronchial AF. TB-PCR on AF bronchial tissue itself did not yield additional benefits for diagnosing TB, which suggests that an AF lesion itself may not be an active or original site of the infection, but a secondary change of TB.

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