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The presence of mycobacterial antigens in sarcoidosis associated granulomas

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Abstract. *Background:* Sarcoidosis is a multi-organ disorder with unknown etiology. The role of bacteria in pathogenesis of sarcoidosis is still controversial. This study analyses new aspects of *Mycobacterium Tuberculosis* (MTB) presence in sarcoidosis diseases. *Objectives:* To find MTB in paraffin embedded tissues of sarcoidosis patients, samples of 10 sarcoidosis, 12 confirmed pulmonary tuberculosis (PTB) and 5 controls associated with granulomatous tissues were analysed. *Methods:* The paraffin embedded tissue specimens of the selected patients from the pathology archive of a subspecialty pulmonary hospital in IRAN were evaluated by Real Time PCR for MTB DNA using IS*6110*. Immunohistochemistry (IHC) method using MTB purified protein derivative (PPD) antibody was used to detect mycobacterial antigens. *Results:* All sarcoidosis patients had negative MTB DNA results in Real time PCR analysis. This analysis resulted in 10 (83.3%) positive cases for TB patients. The IHC analysis for MTB anti-PPD antibody showed positive diffused cytoplasmic staining for all TB patients whereas this staining was positive for 3 sarcoidosis patients (30%). *Conclusion:* Amplification of the IS*6110* DNA sequence that is the most common target used for MTB diagnosis is not sensitive method to detect MTB in sarcoidosis granuloma. However, tissue IHC for anti-PPD antibody shows higher performance to detect MTB in sarcoidal granulomas reveals a mycobacterial signature in sarcoidosis tissue with negative IS*6110* assay. This finding supports *Mycobacterium tuberculosis* may have an etiologic role in sarcoidosis. *(Sarcoidosis Vasc Diffuse Lung Dis 2017; 34: 236-241)*

Key words: sarcoidosis, PCR analysis, immunohistochemistry, tuberculosis

Introduction

Sarcoidosis is a multisystem inflammatory disease with unknown etiology which mostly affects lungs. Although the etiology of the disease is still remained unexplained, there are certain evidences for antigen driven immunopathogenic process which ultimately presents as a granulomatous disease (1, 2). This granulomatous reaction can be triggered either by infection caused by bacteria and fungi or by non-infectious factors such as Beryllium (Be). Some microorganisms are proposed to play role in pathogenesis of sarcoidosis such as mycobacteria, *Propionibacterium acnes* and *Borrelia burgdorferi*, and viruses (*Human Herpes virus*, *Cytomegalovirus, Epstein-Barr virus* and *Coxsakie B*) (2, 3). The previous attempts

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to determine the pathogenic factor of sarcoidosis by histological staining and routine microbial culturing have mostly been unsuccessful (4). Polymerase chain reaction (PCR) has been widely used for determination of the associated pathogens enabling the analysis of DNA(5, 6). Because of clinical, radiological and pathologic similarities between tuberculosis (TB) and sarcoidosis, investigation of *Mycobacterium tuberculosis* (MTB) DNA in the sarcoidosis tissue samples has been generally considered (5, 7-9). In fact, sarcoidosis and tuberculosis are not differentiable with clinical symptoms and they are more difficult to distinguish in the case of negative microbiologic studies including Acid- Fast Bacilli (AFB) (10). Investigating the MTB DNA in sarcoidosis tissue samples has demonstrated wide range of results. It has been shown that MTB DNA have been detected about 30 to 50 percent of sarcoidosis cases in the US (11, 12). The odds of MTB DNA detection in sarcoidal granulomas depend on the epidemiology of TB in the studied region (10, 12, 13). Also the target of gene analysis could impact the results and hence using IS*6110*, rpoB, 16S rRNA may cause different results (4). So, genetic analysis alone may not be enough to investigate the role of tuberculosis in the pathogenesis of sarcoidosis.

Immunohistochemical evaluation of MTB antigens is recommended as efficient diagnostic adjunct to conventional staining in non-caseating epithelioid granulomas (14). We have recently used anti-BCG polyclonal antibody (pAbBCG) for histological diagnosis of TB in formalin-fixed paraffin-embedded (FFPE) tissue (15). Polyclonal anti-BCG antibody has been developed against sonicate of *M. bovis* strain BCG (Copenhagen stain) containing soluble and insoluble bacterial antigens and has been particularly useful in the detection of organisms surrounded by a dense overlying inflammatory cells (16).

Staining tissue with pAbBCG made two distinctive patterns, fine and coarse granules in the cytoplasm epithelioid histocytes. Cells were stained brown in uniform and scattered manner in the fine staining while in the second category, coarse granules were stained as fragmented bacilli (15). In comparison to PCR, immunohistochemistry (IHC) has an important advantage of discriminating preserved antigens in archived tissue, because antigen retrieval process can overcome some adverse effects of fixation in IHC (17). This issue has demonstrated that diagnostic value for Ziehl–Neelsen (ZN) and immunocytochemistry doesn't show significant difference between fresh and archived material, while sensitivity of nucleic acid amplification differed significantly in fresh biopsy (18). To the best of our knowledge, no study has previously compared the distribution of MTB antigens in pulmonary sarcoidosis granulomas with tuberculosis tissue specimens. Given advantage of IHC for antigen detection in FFPE tissue, we assessed the presence of MTB antigens utilizing polyclonal antibody in parallel with molecular genomic analysis of polymerase chain reaction targeting IS*6110* in sarcoidosis specimens.

Methods

Patient's selection

Lung and lymph node biopsy specimens of subjects who had a confirmed diagnosis of sarcoidosis or TB were selected from the archive of the Department of Pathology, Masih Daneshvari Hospital, a referral subspecialty hospital for pulmonary diseases in Tehran, Iran. The clinical records and laboratory data were reviewed and 33 confirmed pulmonary sarcoidosis and 27 subjects with culture positive pulmonary TB were screened. The following criteria were set for sarcoidosis in the study: (i) clinical symptoms were compatible with sarcoidosis, including chronic pulmonary disease along with erythema nodosum, hilar lymphadenopathy and arthritis (Löfgren syndrome) or indolent progressive pulmonary symptoms associated with radiology finding (i.e. hilar adenopathy, reticulonodular infiltration or pulmonary fibrosis), (ii) histopathologic data is compatible with sarcoidosis (noncaseating granulomas that is well circumscribed with surrounding tissue and different amounts of peripheral lymphatic infiltration). (iii) All microbial etiologis must be excluded. This should be confirmed by fungal, Haemotoxylin and Eosin (H&B, AFB and Auramine O staining and also routine tests for Bacteria, Fungi and culture for acid-fast Bacilli (19).

TB patients were enrolled to the study based on clinical symptoms compatible with tuberculosis, and AFB positive sputum smear and/or culture for TB, positive ZN analysis or positive molecular test for MTB DNA, along with positive response to empirical anti-TB drugs (20).

The medical records of sarcoidosis and TB subjects were reviewed by two pulmonologists. H&E stained slides and paraffin embedded tissue blocks were evaluated and only the cases with sufficient tissue volume for DNA extraction and immunohistochemical slide preparation were entered to the study. All samples were analysed by PCR amplification for MTB DNA. 12 TB and 10 sarcoidosis patients with suitable tissue specimens for histochemical analysis were subjected to TB antigen staining. Lung specimens of five subjects with granulomatous reaction and without sarcoidosis or TB were entered to the study as controls. The control individuals include fungal infections (aspergillosis, 3 cases), foreign body granulomas (one case) and one case of hydatid cyst in the lung tissue.

This study was conducted with the IRB approval of Masih Daneshvari Hospital ethic committee.

DNA Extraction

DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The tissue sample slides were prepared by 5 mm sections using microtome. Samples were dewaxed by xylene, followed by two washes with 100% ethanol (v/v) to remove xylene and rehydrate the tissue. After dissolving the paraffin, samples were combined with 200 µl of buffer ATL and 20 µl of proteinase K (17.8 mg/ml) and incubated overnight at 56°C. After digestion, samples were washed with 200 µl of buffer AL and incubated at 70°C for 10 min, followed by ethanol precipitation. The resulting solution was loaded onto a QIAamp spin column and washed according to the manufacturer's instructions. DNA was eluted using 100 µl of buffer AE.

Real Time PCR

Mycobacterium tuberculosis DNA analysis was performed using GeneProof MTB PCR Kit based on specific multi-copy insertion sequence (IS*6110*) amplification, followed by measuring the amplified products using PCR and fluorophore labelled probes according to the manufacturer's instructions. All PCRs were performed using StepOne™ Real-Time PCR Systems. The employed kit specifically detects strains of the MTB complex (*M. tuberculosis, M. bovis, M. africanum* and *M. microti*) in addition to vaccination strains (e.g. BCG). The presence of *M. tuberculosis* in the sample is expressed as FAM fluorophore fluorescence growth. The possible inhibition of the PCR reaction (ISIN version) and the DNA extraction quality (ISEX version) were controlled by including an Internal Standard (IS) in the reaction mix. Positive amplification of IS was detected in the HEX fluorophore fluorescence channel. The "hot start" technology utilized in the kit minimizes non-specific reactions and assures maximum sensitivity. The kit provides ready to use MasterMix containing uracil-DNA-glycosylase (UDG) which has the advantage of eliminating possible contamination of the PCR reaction by amplification products. MTB detection using the multi-copy insertion sequence IS*6110* is 16 times more sensitive compared to conventional single-copy gene detection methods.

Immunohistochemistry (IHC)

Ten sarcoidosis, 12 TB and 5 control samples were entered to the study for anti-*Mycobacterium tuberculosis* antibody, purified protein derivative (PPD) (ab905; Abcam, Cambridge, MA) staining. The antibody is reactive with *M. tuberculosis*, *M. parafortuitum*, *M. phlei* and *M. avium* and has no reaction with *Salmonella typhimurium*, *E. coli* K12, *Pseudomonas aeruginosa*, *Candida albicans*, *Neisseria meningitides* and *Streptococcus* (group B). Histochemical staining procedure was done as we described previously (15). To summarize, after antibody dilution (1:350), all slides were stained and reviewed. The granulomas were divided into fine and coarse granular staining pattern based on their staining characteristics. The fine granular pattern was defined as observing consistent and diffused (brown) cells, while the coarse granules occurred due to fragmented bacilli seen within or around granuloma (Figure 1). Given that the antibody does not show strong cross-reactivity with *M. bovis* antigens, vaccination with BCG would not interfere with the results.

Results

Out of 12 TB subjects with the mean (±SD) age of 40.1±16 years, 4 (25%) subjects were male. A total of 10 subjects with sarcoidosis were evaluated,

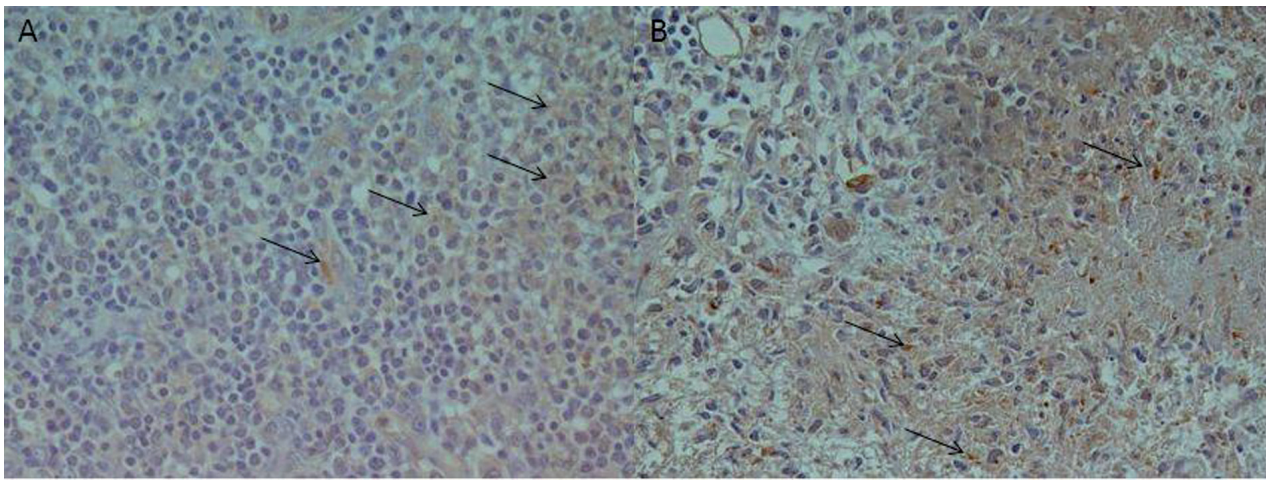

Fig. 1. Cytoplasmic positivity in plasma cell and lymphocyte, immunostaining of nongranulomatous reaction of TB case showing brown stained cytoplasm as fine and homogeneous staining suggest fine antigen dust of mycobacterial products (A) and course cytoplasmic positivity of epithelioid macrophage as non- homogeneous staining suggest fragmented bacilli seen in or around necrotizing granuloma of TB section (B)

2 (20%) individuals were male and the mean (±SD) age was 42.8±14.3 years.

TB tissue samples comprised of 4 pleural tissue (16.6%), 6 lymph node (50%) and two transbronchial lung biopsy (TBLB) specimens. Sarcoidosis tissue specimens contained 4 mediastinal lymph nodes (40%) and 6 cases of TBLB (60%).

The molecular analysis for MTB complex using IS*6110* amplification was positive in 10 (83%) samples in TB group while all specimens from sarcoidosis group were negative. The lung tissue specimens from control subjects had negative IS*6110* amplification results as well (Table 1).

In histochemical analysis, MTB antigen was found as granular cytoplasmic staining in all MTB patients where coarse granules staining were observed in 6 (50%) individuals. In sarcoidosis group, 3 (30%) specimens revealed positive results for granular cytoplasmic staining but no positive result for coarse granules was observed (Figure 2). The IHC staining was negative for all control specimens.

Table 1. Demographic, IS*6110* target amplification and immunohistochemical (IHC) staining with ab905 data in tuberculosis and sarcoidosis patients

Variable	Tuberculosis (n=12)	Sarcoidosis (n=10)	Control (n=5)
Sex (F/M)	8/4	8/2	2/3
Age (Mean±SD)	40.1±16	42.8±14.3	35.3±18.7
Positive Real-Time (IS6110)	10 (83.3%)	0	0
Positive IHC (ab905)	12 (100%)	3 (30%)	0

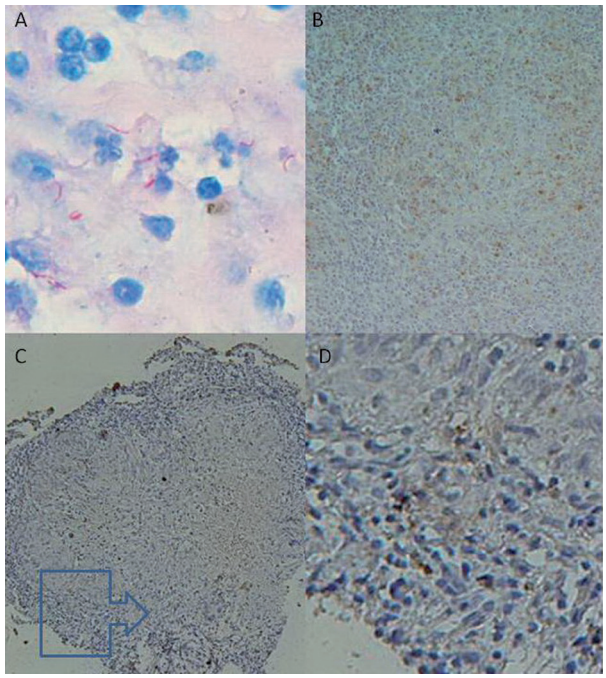

Fig. 2. Ziehl-Neelsen staining of a tuberculosis tissue sample (A), Fine and course positive brown immunostaining for MTB anti-PPD(ab905) in axillary lymph node section of TB patient showing mycobacterial products (B), fine cytoplasmic positivity in sarcoidosis mediastinal lymph node section showing diffuse brown cytoplasmic staining suggest mycobacterial antigen (C×10 & D×40)

Discussion

Limitation in diagnosis of microorganisms by tissue staining or culturing from pathologic tissue samples is still one of the main issues in addressing the possible role of infectious factors in pathogenesis of sarcoidosis (21). Although the specificity and sensitivity of molecular methods are improving, current culturing and staining methods are only able to discover less than 2% of microbial community from human specimens (22, 23).

In this study, the molecular method for detection of MTB DNA yielded negative results for all sarcoidosis patients. However, the result of immunohistochemical analysis for MTB complex was positive for 30% of sarcoidosis patients. This study shows for the first time that purified protein derivatives antigens of MTB are present in tissue cells even when MTB DNA could not be detected by most commonly used mycobacterial genomic method.

Gupta and colleagues have comprehensively studied the molecular and cellular aspects of TB and sarcoidosis. Their meta-analysis revealed the presence of MTB DNA in 30% of overall sarcoidosis patients (5) and 48% of new sarcoidosis patients (11, 24). Some other studies are consistent with our study which could not find MTB DNA in sarcoidosis tissue specimens (25, 26).

The first explanation for this variation could be the chance of DNA degradation during total DNA extraction which is more often in paraffin samples. It should be considered that using fresh specimens always provide more effective amplification in PCR analysis (4).

Moreover, the reactions which induce immune system and the granulomatous reaction with tendency to sarcoidosis can occur with lower number of pathogens and in the course of diagnosis and clinical symptom expression, this pathogenic load could be considerably lower. Therefore, there is a need for more sensitive diagnostic approaches wherein conventional molecular methods are impractical.

In the study conducted by Drake et al, MTB sequences, IS*6110*, rpoB and 16S rDNA were used for detection of MTB DNA by PCR targeting. The analysis for IS*6110* gene did not report any amplification while targeting rpoB and 16S rDNA were positive in 6 (24%) and 12 (48%) specimens respectively. Sequence analysis of 16S rDNA PCR product increased the number of positive amplifications to 60% of specimens (4).

In addition to the sensitivity of molecular methods, variable copy numbers of the target gene, IS*6110* is regarded as the reason for varying results in different studies. IS*6110* has normally 1-25 repeats in DNA of MTB Complex. For instance, *M. bovis* BCG possesses a single copy of IS*6110* (27) whereas the number of repeats is higher in *M. tuberculosis* and there are some reports of *M. tuberculosis* without any copy for IS*6110* (28, 29). So, the fewer copies of target gene presenting in *Mycobacterium* DNA means better quality of extracted DNA is required for detection.

The copy numbers of IS*6110* gene has been previously studies in Iran. Farnia *et al.* studied the TB transmission patterns using RFLP on 129 subjects and found the frequency of 1-3 copies, 6-15 copies and more than 16 copies of IS*6110* in 5 (3.8%), 115(89%) and 7 (5.4%) subjects, respectively. They also reported two isolates with no copy of IS*6110* (1.5%) (29).

In this study, based on immunohistochemical analysis, we have shown new evidence of mycobacterium presence in sarcoidosis tissues where the routine molecular analysis to find MTB DNA based on IS*6110* failed to demonstrate.

The findings of this study should be interpreted by caution. We did not evaluate the samples for presence of *Mycobacterium avium* complex (MAC) which may have cross reactivity with anti-PPD (ab905), while *Mycobacterium avium complex* has been isolated from sarcoidosis tissue previously (30).

The results of current study along with previous studies suggest the potential role of mycobacterial infection in the pathogenesis of sarcoidosis. Although the role of mycobacterium in initiating the reactions causing sarcoidosis is controversial, the antigenic induction by infectious and non-infectious factors is involved in the process. More studies are needed to show the efficacy of antigen detection assay in sarcoidosis and discovering the etiologic mechanism of mycobacterium in the pathogenesis of sarcoidosis.

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