THE PRESENCE OF MYCOBACTERIAL ANTIGENS IN SARCOIDOSIS ASSOCIATED GRANULOMAS

Masoud Shamaei1, Mihan Pourabdollah2, Hamed Mousaei1, Mehdi Mirsaeidi1, Mohamad Reza Masjedi2

1 Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; 2 Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; 3 Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, NE2 4AX, UK; 4 Division of Pulmonary and Critical Care, Department of Medicine, University of Miami, FL, US; 5 Tobacco Prevention and Control Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

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 IS6110. 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 IS6110 DNA sequence that is the most common target used for MTB diagnosis is not sensitive method to detect MTB in sarcoidal granulomas. However, tissue IHC for anti-PPD antibody shows higher performance to detect MTB in sarcoid granulomas reveals a mycobacterial signature in sarcoidosis tissue with negative IS6110 assay. This finding supports Mycobacterium tuberculosis may have an etiologic role in sarcoidosis. (Sarcoidosis Vas Diffuse Lung Dis 2017; 34: 236-241)

Keywords: 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
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\textsuperscript{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 \textit{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\textit{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 etiologies 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 (IS6110) 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 IS6110 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,
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 IS6110 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 IS6110 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.

| Variable          | Tuberculosis | Sarcoidosis | Control |
|-------------------|--------------|-------------|---------|
| 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. 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 coarse cytoplasmic positivity of epithelioid macrophage as non-homogeneous staining suggest fragmented bacilli seen in or around necrotizing granuloma of TB section (B)

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, IS6110, rpoB and 16S rDNA were used for detection of MTB DNA by PCR targeting. The analysis for IS6110 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, IS6110 is regarded as the reason for varying results in different studies. IS6110 has normally 1-25 repeats in DNA of MTB Complex. For instance, M. bovis BCG possesses a single copy of IS6110 (27) whereas the number of repeats is higher in M. tuberculosis and there are some reports of M. tuberculosis without any copy for IS6110 (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 IS6110 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 IS6110 in 5 (3.8%), 115(89%) and 7 (5.4%) subjects, respectively. They also reported two isolates with no copy of IS6110 (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 IS6110 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.

References

1. Fidler HM, Rook GA, Johnson NM, McFadden J. Mycobacterium tuberculosis DNA in tissue affected by sarcoidosis. Brmj 1993; 306(6877): 546-9.
2. Chen ES, Moller DR. Etiologies of sarcoidosis. Clinical reviews in allergy & immunology 2015; 49(1): 6-18.
3. Hunninghake G, Costabel U, Ando M, Baughman R, Cordier J, Du Bois R, et al. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. Sarcoidosis, vasculitis, and diffuse lung diseases: official journal of WASOG/WORLD Association of Sarcoidosis and Other Granulomatous Disorders 1999; 16(2): 149.
4. Drake WP, Pei Z, Pride DT, Collins RD, Cover TL, Blaser MJ. Molecular analysis of sarcoidosis tissues for mycobacterium species DNA. Emerging infectious diseases 2002; 8(11): 1334-41.
5. Gupta D, Agarwal R, Aggarwal AN, Jindal SK. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. European Respiratory Journal 2007; 30(3): 508-16.
6. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. New England Journal of Medicine 1990; 323(23): 1573-80.
7. Gupta D, Agarwal R, Aggarwal AN, Jindal SK. Sarcoidosis and tuberculosis: the same disease with different manifestations or similar manifestations of different disorders. Current opinion in pulmonary medicine 2012; 18(5): 506-16.
8. Bloom CI, Graham CM, Berry MP, Rozakeas F, Redford PS, Wang Y, et al. Transcriptional blood signatures distinguish pulmonary tuberculosis, pulmonary sarcoidosis, pneumonias and lung cancers. PloS one 2013; 8(8): e70630.
9. Comert S, Fidan A, Dogan C, Tahtasakal S, Caglayan B. Sarcoidosis: Is Clinical And Radiological Diagnosis Sufficient? Am J Respir Crit Care Med. 2014;189:A4085.
10. Agrawal R, Kee AR, Ang L, Hang YT, Gupta V, Kon OM, et al. Tuberculosis or Sarcoidosis: Opposite ends of the same disease spectrum? Tuberculosis 2016. 
11. Popper HH, Klemen H, Hoefler G, Winter E. Presence of mycobacterial DNA in sarcoidosis. Human pathology 1997; 28(7): 796-800.
12. Li N, Bajoghi A, Kubba A, Bhawan J. Identification of mycobacterial DNA in cutaneous lesions of sarcoidosis. Journal of cutaneous pathology 1999; 26(6): 271-8.
13. Brett G. Epidemiological trends in tuberculosis and sarcoidosis in a district of London between 1958 and 1963. Tubercle 1965; 46(4): 412-6.
14. Goel MM, Budhwar P. Immunohistochemical localization of Mycobacterium Tuberculosis complex antigen with antibody to 38 kDa antigen versus Ziehl Neelsen staining in tissue granulomas of extra-pulmonary tuberculosis. Indian Journal of Tuberculosis 2007; 54(1): 24.
15. Karimi S, Shamaei M, Pourabdollah M, Sadr M, Karbasi M, Kiani A, et al. Histopathological findings in immunohistochemical staining of the granulomatous tissue reaction associated with tuberculosis. Tuberculosis research and treatment 2014; 2014.
16. Kutzner H, Argényi ZB, Requena L, Rütten A, Hügel H. A new application of BCG antibody for rapid screening of various tissue microorganisms. Journal of the American Academy of Dermatology 1998; 38(1): 56-60.
17. Dugassa H, Demisie T. Review on different diagnostic methods for detection of Mycobacterium avium subsp. Paratuberculosis infections. World Journal of Medical Sciences 2014; 11(3): 273-88.
18. Goel MM, Budhwar P, Jain A. Immunocytochemistry versus nucleic acid amplification in fine needle aspirates and tissues of extrapulmonary tuberculosis. Journal of Cytology 2012; 29(3): 157.
19. Allen SS, Evans W, Carlisle J, Hajizadeh R, Nadaf M, Shepherd BE, et al. Superoxide dismutase A antigens derived from molecular analysis of sarcoidosis granulomas elicit systemic Th-1 immune responses. Respiratory research 2008; 9(1): 1-12.
20. Song W, Strack RL, Svensen N, Jaffrey SR. Plug-and-Play Fluorophores Extend the Spectral Properties of Spinach. Journal of the American Chemical Society 2014; 136(4): 1198-201. doi: 10.1021/ja410819s. PubMed PMID: PMC3929357.
21. Celada LJ, Hawkins C, Drake WP. The etiologic role of infectious antigens in sarcoidosis pathogenesis. Clinics in chest medicine 2015; 36(4): 561-8.
22. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gottsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. PloS one 2008; 3(8): e3056.
23. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proceedings of the National Academy of Sciences of the United States of America 2006; 103(3): 732-7.
24. Mootha VK, Agarwal R, Aggarwal AN, Gupta D, Ahmed J, Verma I, et al. The Sarcoid-Tuberculosis link: evidence from a high TB prevalence country. Journal of Infection 2010; 60(6): 501-3.
25. Richter E, Greinert U, Kirsten D, Rösch-Gerdes S, Schlüter C, Duchrow M, et al. Assessment of mycobacterial DNA in cells and tissues of mycobacterial and sarcoid lesions. American journal of respiratory and critical care medicine 1996; 153(1): 375-80.
26. Bocart D, Lecossier D, De Lassence A, Valeyle D, Battesti J-P, Hance AJ. A search for mycobacterial DNA in granulomatous tissues from patients with sarcoidosis using the polymerase chain reaction. American Journal of Respiratory and Critical Care Medicine. 1992; 153(1): 1142-8.
27. van Soolingen D, De Haas P, Hermans P, Groenew P, Van Embden J. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. Journal of clinical microbiology 1993; 31(8): 1987-95.
28. Thoe S, Tay L, Sng E. Evaluation of Amplificor-and IS6110-PCR for direct detection of Mycobacterium tuberculosis complex in Singapore. Tropical Medicine & International Health 1997; 2(11): 1095-101.
29. Farnia P, Mohammad F, Masjedi MR, Varnere AT, Zarifi AZ, Tabatabae J, et al. Evaluation of tuberculosis transmission in Tehran: using RFLP and spoligotyping methods. Journal of Infection. 2004; 49(2): 94-101.
30. Amand ALS, Frank DN, De Groote MA, Pace NR. Use of specific rRNA oligonucleotide probes for microscopic detection of Mycobacterium avium complex organisms in tissue. Journal of clinical microbiology 2005; 43(4): 1505-14.