Brief Report

Association between erythema nodosum/erythema induratum of Bazin and *Mycobacterium tuberculosis* infection in Koreans

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Abstract

Background: Erythema nodosum and erythema induratum of Bazin are similar inflammatory diseases of the lower extremities. These are clinically distinguishable entities, though overlap can occur. Both diseases are reported to be related to *Mycobacterium tuberculosis* infection, but it is very difficult to identify *Mycobacterium tuberculosis* in skin lesions.

Aim: This study aimed to develop a new nested polymerase chain reaction targeting the IS6110 insertion sequence of *M. tuberculosis* to improve the *M. tuberculosis* detection rate in skin lesions of erythema nodosum or erythema induratum of Bazin.

Methods: From May 2016 to Jan 2018, 14 patients with clinically suspicious erythema nodosum or erythema induratum were enrolled in the study. Two cases were classified as erythema nodosum and 12 as erythema induratum. Individual patients were subjected to a 4-mm punch biopsy, and their venous whole blood was sampled immediately after diagnosis.

Results: Eight patients were tested for *M. tuberculosis* using QuantiFERON, of which seven (87.5%) were positive. IS6110-nested polymerase chain reaction on all 14 patients identified 11 (78.6%) positive cases. Four of the eight (50%) individuals tested with QuantiFERON were also positive in the IS6110 nested polymerase chain reaction. The difference between the outcomes of the QuantiFERON and the IS6110-nested polymerase chain reaction tests was not statistically significant. There was also no significant agreement between the results of both assays. Sequencing the IS6110-nested polymerase chain reaction products showed a 97%–100% nucleotide sequence identity with the H37Rv genome.

Conclusion: It is important to test for tuberculosis in patients with multiple tender subcutaneous nodules on their lower extremities in high-burden tuberculosis countries like Korea.

Limitations: We need to register more suspicious patients to verify the association between erythema nodosum/erythema induratum of Bazin and *M. tuberculosis*. Furthermore, it is necessary to improve the more sensitive polymerase chain reaction technique to identify *M. tuberculosis* directly in cutaneous lesions.

Key words: Erythema induratum of Bazin, erythema nodosum, *Mycobacterium tuberculosis*

Introduction

Erythema nodosum is an acute, self-limited reaction pattern consisting of several nonulcerative, tender, erythematous nodules or plaques minimally elevated 1–10 cm and located predominantly on the extensors of the lower extremities. Erythema nodosum is the archetype of septal panniculitis. Fat necrosis is usually minimal and vasculitis unexpected, but Miescher's radial granuloma can be observed. The terms erythema induratum of Bazin and nodular vasculitis are sometimes used interchangeably, although others

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reserve the diagnosis of erythema induratum for cases associated with tuberculosis and nodular vasculitis for the remainder. Clinically, erythema induratum usually occurs in young or middle-aged women as firm violaceous nodules and plaques with a predilection for the posterior of the legs (calves). It is a lobular or mixed lobular-septal panniculitis with vasculitis. Overt lipophagic and ischemic fat necrosis, and usually eosinophilic necrosis (coagulative, caseous and fibrinous), are hallmarks of all stages of erythema induratum. It may be triggered by tuberculosis and nodular vasculitis may be idiopathic but has been associated with Crohn's disease, ulcerative colitis, rheumatoid arthritis and hepatitis B virus infection.[1] Both erythema nodosum and erythema induratum can be improved by systemic corticosteroid administration and if a simple small and superficial biopsy is performed, it may be more likely to be nondiagnostic or misinterpreted.

Vaccination of infants with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is mandatory in Korea and many Asian countries. It is difficult to use the tuberculin skin test to distinguish between the immune responses from fresh *Mycobacterium tuberculosis* infection and BCG in the vaccinated individuals.[2] Interferon-γ release assay has been approved as a new standard for the diagnosis of *M. tuberculosis* infection in recent years, as it is not affected by previous BCG vaccination or exposure to nontuberculous mycobacteria. But a positive release assay does not necessarily mean that the patient has an active tuberculosis infection, and therefore, further tests are needed for confirmation (e.g., chest X-ray, sputum cultures, or polymerase chain reaction.[3]

Korea has the highest prevalence, incidence and mortality rate of tuberculosis per 100,000 people among the 34 Organization for economic cooperation and development-affiliated countries in 2015 according to the World Health Organization.[4] In one study, 22 patients in Korea, who were diagnosed with erythema induratum of Bazin clinicopathologically, demonstrated a positive response to the interferon-γ release assay and a good response to anti-tuberculosis treatment.[5] However, *M. tuberculosis* is rarely found directly in erythema nodosum and erythema induratum skin lesions in this region. In this study, we performed nested polymerase chain reaction, targeting the IS6110 insertion sequence of *M. tuberculosis*, to demonstrate the presence of *M. tuberculosis* in the cutaneous lesions of patients with suspected cases of erythema nodosum or erythema induratum and interferon-γ release assay to prove the importance of *M. tuberculosis* infection in the development of both diseases in Korea.

Methods

Patients and samples

From May 2016 to January 2018, patients who had multiple tender erythematous to violaceous subcutaneous nodules on their lower extremities were recruited in the Kangdong Sacred Heart Hospital, Seoul, Korea. The purpose of this study is to help diagnose and treat erythema nodosum/erythema induratum by detecting *M. tuberculosis* using our new PCR method. The ambiguous cases were excluded, and only in a clear case, the patients were classified by considering clinical features and histologic findings. Written informed consent was obtained from the involved patients, and the experimental protocol was approved by the Institutional Review Board of the Kangdong Sacred Heart Hospital in Seoul, Korea (no. 2018-04-012). Individual patients were subjected to a 4-mm punch biopsy, and their venous whole blood was sampled immediately after diagnosis. An incisional biopsy is the ideal biopsy method for diagnosis, but we did a 4-mm punch biopsy (7-mm depth) because of time, cost, and patient preference in the Korean medical environment. Patients were subjected to a complete physical examination and history taking for any type of tuberculosis.

Designing primers for a nested polymerase chain reaction amplification

The *IS6110* gene is a mobile genetic element usually present in multiple copies within the genomes of *M. tuberculosis* and has been used extensively as a genotypic marker in epidemiological studies.[6] Known primers targeting a 123-base pair DNA fragment of the *IS6110* gene were used.[7] To improve sensitivity using nested polymerase chain reaction, a new set of primers amplifying a 247-base pair DNA fragment surrounding this 123 base pair sequence were designed using the Primer3 web-based program (http://primer3.ut.ee).

Nested polymerase chain reaction amplification of the *IS6110* gene in tissue DNA

The first round of the nested polymerase chain reaction used the primers IS6110-OUT-F1 and IS6110-OUT-R1 in a final volume of 20 mL, containing 5 mL of sample DNA, 1 mL of each 10 pM primers, 3 mL of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA) and 10 mL of polymerase chain reaction master mix (BioFACT™ H-star *Taq* polymerase chain reaction Master Mix; Biofact, Daejeon, Korea). The product of the first round polymerase chain reaction was purified with a QIAquick polymerase chain reaction purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions to remove any polymerase chain reaction inhibitors. The second round of polymerase chain reaction was then performed with primers IS1 and IS2 using 8 mL of the purified first-round polymerase chain reaction product, 1 mL of each 10 pM primers and 10 mL of polymerase chain reaction master mix. Amplification was carried out with an automated thermal cycler (LifePro Thermal Cycler, Bioer, Hangzhou, China) following the program in Table 1. About 4 mL of the final product was electrophoresed in a 1.7% agarose gel in 1× Tris-acetate–ethylenediaminetetraacetic acid buffer at 120 V for 30 min with a 100-base pair molecular marker running in parallel. The gel was stained with a fluorescent dye (EcoDye™ Nucleic Acid Staining Solution; Biofact)

and photographed under ultraviolet light illumination. Genomic DNA from *M. tuberculosis* strain H37Rv (ATCC® 25618™) was used as a positive control. Negative controls included genomic DNA from nontuberculous mycobacteria, which are known as cutaneous pathogens, and from patients with other skin diseases. The nontuberculous mycobacteria consisted of *Mycobacterium abscessus* (KCTC® 19621™), *Mycobacterium chelonae* subspecies *chelonae* (KCTC® 9505™), *Mycobacterium fortuitum* subspecies *fortuitum* (KCTC® 9510™), *Mycobacterium haemophilum* (ATCC® 29548™), *Mycobacterium intracellulare* (KCTC® 9514™), *Mycobacterium kansasii* (KCTC® 9515™), *Mycobacterium szulgai* (KCTC® 9520™), *Mycobacterium massiliense* (KCTC® 19086™), *Mycobacterium ulcerans* (ATCC® 19423™) and *Mycobacterium scrofulaceum* (ATCC® 19981™). Other skin diseases included a case of granuloma annulare, discoid lupus erythematosus, nummular dermatitis and urticaria.

Sequencing

A mixture of 1 mL of cleaned polymerase chain reaction product, 4 mL of terminator ready reaction (Applied Biosystems, Foster City, CA, USA), 1 mL of primer and 4 mL of sterile water was amplified in a thermocycler for 30 cycles of 96°C for 10 ds, 50°C for 5 ds and 60°C for 4 min. Both strands of the polymerase chain reaction products were mixed with 15 mL of loading buffer, highly deionized formamide, and then sequenced with an ABI PRISM 3730XL DNA analyzer (Applied Biosystems) according to the manufacturer's instructions. The resulting DNA sequences were analyzed by a BLASTn search against the online nonredundant nucleotide collection (nr/nt) database of NCBI.

Results

A total of 14 patients who were clinically suspected to have erythema nodosum or erythema induratum were recruited. None of our patients has had any type of tuberculosis. Based on a pathology assessment of hematoxylin and eosin-stained slides from 4-mm punch biopsy specimens, two cases were classified as erythema nodosum and 12 as erythema induratum of Bazin [Table 2]. Eight of the patients were tested for *M. tuberculosis* with QuantiFERON, seven of whom were positive (87.5%, 95% confidence interval, 46.7%–99.3%) [Table 3]. IS6110-nested polymerase chain reaction on all 14 patients identified 11 positive cases (78.6%, 95% confidence interval, 48.8%–94.3%) [Table 3]. Four of the eight individuals tested with QuantiFERON also tested positive in the IS6110-nested polymerase chain reaction (50%, 95% confidence interval, 17.5%–82.6%). The difference between the outcomes of the QuantiFERON and the IS6110-nested polymerase chain reaction was not statistically significant (P = 0.408). Three patients who tested positive by QuantiFERON had negative IS6110-nested polymerase chain reaction results. One patient with indeterminate QuantiFERON result tested positive by IS6110-nested polymerase chain reaction. There was no significant agreement between the results of the assays (kappa value = -−0.231, P = 0.408).

Sequencing the IS6110-nested polymerase chain reaction products showed 97%–100% nucleotide sequence identity with the H37Rv genome (GenBank accession no. NC_000962.3) [Figure 1]. The DNA sequences obtained in this study were deposited in GenBank (accession nos. MH883881 to MH883894).

Discussion

M. tuberculosis infection usually induces Th1 responses, which secrete interferon-γ from T cells and can be detected by interferon-γ release assay. There are two interferon-γ release assay assays currently approved by the United States Food and Drug Administration, the QuantiFERON-tuberculosis Gold In-Tube test and the T-SPOT.tuberculosis test. US tuberculosis screening guidelines indicate that they can be used in place of the tuberculin skin test for latent tuberculosis screening. Furthermore, interferon-γ release assay tests may be more predictive in patients with prior BCG immunization because of their lack of cross-reactivity with antigens.[3] Due to the lack of a gold standard, the performance of QuantiFERON in detecting latent tuberculosis is hard to evaluate. In evaluating active tuberculosis, a meta-analysis concluded that the pooled sensitivity for 22 QuantiFERON studies was 76% (95% confidence interval, 72%–80%), and the pooled specificity for 16 QuantiFERON studies was 98% (95% confidence interval, 96%–99%).[8] The results of interferon-γ release assay tests may be positive, negative or indeterminate. Indeterminate tests

Table 1: Primers and PCR programs used in this study

Target gene and primer name	Round of nested PCR	PCR program for each primer
IS6110		
IS6110-OUT-F1	First	95°C for 10 min; 50 cycles of 95°C for 1 min, 58.7°C for 1 min, and 72°C for 1 min; a final extension at 72°C for 10 min
IS6110-OUT-R1		
IS1	Second	95°C for 10 min; 50 cycles of 95°C for 1 min, 63.8°C for 1 min, and 72°C for 1 min; a final extension at 72°C for 10 min
IS2		
β-Globin		
GH20-F	NA	95°C for 10 min; 40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; a final extension at 72°C for 10 min
PC04-R		

PCR: polymerase chain reaction; NA: not applicable

Table 2: Demographic and pathological classification of the study population

Characteristics	Male	Female
Sex	None	*n*=14
Age (years), mean±SD	None	55.2±11.19
Pathological classification		
EN	0	2
EIB	0	12

SD: standard deviation; EN: erythema nodosum; EIB: erythema induratum of Bazin

Table 3: Outcomes of QuantiFERON and IS6110 nested PCR amplification according to pathological classification

Pathological classification	QuantiFERON	IS6110 nested PCR
EN	NA	1 (1/1, 100%)
EIB	7 (7/8, 87.5%)	10 (10/13, 76.9%)
Total	7 (7/8, 87.5%)	11 (11/14, 78.6%)

PCR: polymerase chain reaction; EN: erythema nodosum; EIB: erythema induratum of Bazin; NA: not applicable

can occur in cases of anergic response, immunosuppression, high initial levels of interferon-γ, active infection, extremes of age, or improper handling of the samples.[3] One study reported that there was an agreement between QuantiFERON and real-time polymerase chain reaction assays for detecting tuberculosis infection. While QuantiFERON detects the immune response to *M. tuberculosis*-specific antigens in individuals with latent tuberculosis infections, the real-time polymerase chain reaction assay detects the bacterium-specific genomic sequences which are necessarily present in tuberculosis infections. Given that *M. tuberculosis*-specific T-cell immunity can control bacterial replication and infection, it may be reasonable to expect QuantiFERON responses and negative polymerase chain reaction results and vice versa in immunocompromised individuals.[2] In this study, there was one case that showed indeterminate QuantiFERON results but was positive for the IS6110-nested polymerase chain reaction assay. Three cases were positive by QuantiFERON but negative by the polymerase chain reaction test. The lack of agreement between these two assays (kappa value = -0.231) may not be surprising.

The IS6110 belongs to the IS3 family, and most members of the *M. tuberculosis* complex, including *M. tuberculosis*, contain multiple IS6110 copies although *M. bovis* generally contains only a single copy. As with all other members of the IS3 family, the IS6110 sequence contains two partially overlapping reading frames: *orfA* and *orfB*. By way of translational frameshifting, an OrfAB protein, which acts as a transposase, may also be produced.[6] The presence of IS6110 in the erythema nodosum and erythema induratum lesions remains controversial. While Baselga *et al*.[9] reported the presence of IS6110 in 77% of the formalin-fixed, paraffin-embedded tissues of 65 patients with erythema induratum, others failed to detect it in erythema nodosum and erythema

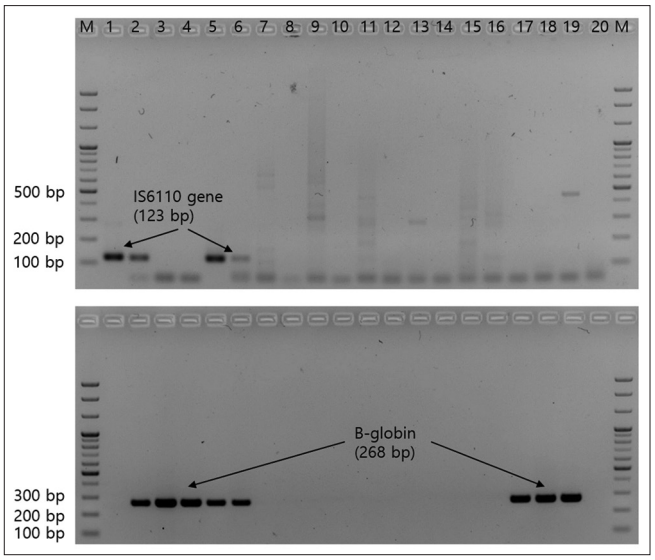

Figure 1: Results of IS6110-nested polymerase chain reaction of patient's samples and positive and negative controls. Lanes: M: molecular marker, 1: H37Rv (positive control), 2: QuantiFERON(+) and nested polymerase chain reaction (+), 3–4: QuantiFERON(+) but nested polymerase chain reaction (−), 5–6: indeterminate by QuantiFERON but positive by nested polymerase chain reaction, 7–16: nontuberculous mycobacteria species, 17: granuloma annulare, 18: discoid lupus erythematosus, 19: nummular dermatitis, 20: all polymerase chain reaction reagents (master mix, primers, nuclease-free water)

induratum tissues.[10] Chen *et al*. reported that they detected IS6110 in 30.4% and 38.9% of fresh erythema nodosum and nodular vasculitis tissues, respectively.[2] We detected IS6110 in 78.6% of the formalin-fixed, paraffin-embedded tissues from patients with patients erythema nodosum and erythema induratum which is similar to the result of Baselga *et al*.'s research (77%). The relatively high sensitivity in this study, despite using formalin-fixed, paraffin-embedded tissues, maybe due to three factors. First, we used a nonphenolic, nonenzymatic, rapid salting-out method of DNA isolation. The kit-based silica column method is more widely used because it is user-friendly and rapid, but the absorbance ratios and DNA yield are lower than those obtained by the salting-out method.[11] Second, nested polymerase chain reaction using new outer primers which amplify a 247-base pair fragment of the *IS6110* gene in addition to the known primers was designed to improve sensitivity. Finally, the product of the first round polymerase chain reaction was further purified to remove any inhibitors, such as organic solvents, residual salts, ethylenediaminetetraacetic acid, ionic detergents or hemoglobin.

All QuantiFERON-positive cases or IS6110-nested polymerase chain reaction-positive cases (total 14 cases) were treated for 6 months of anti-tuberculosis treatment (four-drug regimen [isoniazid, rifampin, pyrazinamide and ethambutol]). Although the dose varies according to the weight, it was administered based on the general weight (60 kg) as follows: isoniazid 300 mg, rifampicin 600 mg, ethambutol 900 mg, and pyrazinamide 1500 mg. About a year later, there were two cases with new

lesions around previous lesions (simple recurrence rate: 14.2%). In our additional studies, interferon-γ release assay positivity rates in Korean erythema nodosum/erythema induratum patients were significantly higher, with 50% in erythema nodosum and 75% in erythema induratum.

Limitations

Our study has some limitations. The main limitation is that we need to register more suspicious patients to verify the association between erythema nodosum/erythema induratum and *M. tuberculosis*. Also, we performed a skin punch biopsy instead of our ideal incisional biopsy and did not perform the interferon-γ release assay on all patients, because of time, cost and patient preference.

Conclusion

We need to consider and evaluate the presence of tuberculosis in patients with multiple tender subcutaneous nodules on their lower extremities in high-burden countries like Korea and attempt anti-tuberculosis treatment in patients with suspected tuberculosis. Given the long-term, multi-drug regimen of tuberculosis treatment, we recommend that conventional treatment be done first, followed by tuberculosis treatment, in patients with multiple relapse or no improvement. For patients with frequent relapses, if IGRA test or PCR is positive, tuberculosis treatment is recommended if the patient agrees, even considering the prospect of tuberculosis prevention. It is also necessary to introduce the more sensitive polymerase chain reaction technique to identify *M. tuberculosis* directly in cutaneous lesions.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Conflicts of interest

There are no conflicts of interest.

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