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BRIEF ARTICLE

Diagnosis of intestinal tuberculosis using a monoclonal antibody to *Mycobacterium tuberculosis*

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Abstract

AIM: To investigate the utility of immunohistochemical (IHC) staining with an antibody to *Mycobacterium tuberculosis* (*M. tuberculosis*) for the diagnosis of intestinal tuberculosis (TB).

METHODS: We retrospectively identified 10 patients (4 males and 6 females; mean age = 65.1 ± 13.6 years) with intestinal TB. Clinical characteristics, including age, gender, underlying disease, and symptoms were obtained. Chest radiograph and laboratory tests, including sputum Ziehl-Neelsen (ZN) staining, *M. tuberculosis* culture, and sputum polymerase chain reaction (PCR) for tubercle bacilli DNA, as well as Tuberculin skin test (TST) and QuantiFERON-TB gold test (QFT), were examined. Colonoscopic records recorded on the basis of Sato's classification were also reviewed, in addition to data from intestinal biopsies examined for histopathological findings, including hematoxylin and eosin staining, and ZN staining, as well as *M. tuberculosis* culture, and PCR for tubercle bacilli DNA. For the present study, archived formalin-fixed paraffin-embedded (FFPE) intestinal tissue samples were immunohistochemically stained using a commercially available species-specific monoclonal antibody to the 38-kDa antigen of the *M. tuberculosis* complex. These sections were also stained with the pan-macrophage marker CD68 antibody.

RESULTS: From the clinical data, we found that no patients were immunocompromised, and that the main symptoms were diarrhea and weight loss. Three patients displayed active pulmonary TB, six patients (60%) had a positive TST, and 4 patients (40%) had a positive QFT. Colonoscopic findings revealed that all patients had type 1 findings (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), all of which were located in the right hemicolon and/or terminal ileum. Seven patients (70%) had concomitant healed lesions in the ileocecal area. No acid-fast bacilli were detected with ZN staining of the intestinal tissue samples, and both *M. tuberculosis* culture and PCR for tubercle bacilli DNA were negative in all samples. The histopathological data revealed that tuberculous granulomas were present in 4 cases (40%). IHC staining in archived FFPE samples with anti-*M. tuberculosis* monoclonal antibody revealed positive findings in 4 patients (40%); the same patients in which granulomas were detected by hematoxylin and eosin staining. *M. tuberculosis* antigens were found to be mostly intracellular, granular in pattern, and primarily located in the $CD68^+$ macrophages of the granulomas.

CONCLUSION: IHC staining with a monoclonal antibody to *M. tuberculosis* may be an efficient and simple diagnostic tool in addition to classic examination methods for the diagnosis of intestinal TB.

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Key words: Colonoscopy; Intestinal tuberculosis; Immunohistochemistry; Monoclonal antibody; *Mycobacterium tuberculosis*

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INTRODUCTION

Tuberculosis (TB), a chronic granulomatous infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), is still a significant cause of morbidity and mortality worldwide. Due to the increasing prevalence of human immunodeficiency virus (HIV) infection and immunosuppressive therapy for various diseases, the global incidence of TB has increased[1].

The gastrointestinal tract is a common site of extrapulmonary TB. The ileocecal region is frequently involved in most patients diagnosed with intestinal TB, although the diagnosis of intestinal TB is often difficult because of its diverse clinical manifestations and very low positivity using current diagnostic tests including Ziehl-Neelsen (ZN) staining and *M. tuberculosis* culture from intestinal tissue samples[2]. More recently, detection of tubercle bacilli DNA by polymerase chain reaction (PCR) has been developed as a diagnostic tool with excellent sensitivity and specificity in respiratory specimens. However, diagnosis by PCR in clinical settings still requires validation[3]. Therefore, diagnosis is generally made on the basis of the classical histopathological demonstration of a caseating epithelioid cell granuloma, which is suggestive of TB. However, it may be difficult to differentiate intestinal TB from Crohn's disease based on this technique due to the fact that intestinal TB and CD have similar clinical, colonoscopic, and pathological findings. Although it is well known that caseating granulomas are a feature of TB, and non-caseating granulomas are that of CD, the prevalence of caseation is low in clinical settings for intestinal tuberculous granulomas[4,5].

The present study was conducted to investigate the utility of immunohistochemical (IHC) staining with a species-specific monoclonal antibody to the 38-kDa antigen of the *M. tuberculosis* complex to diagnose intestinal TB in archived formalin-fixed paraffin-embedded (FFPE) intestinal tissue sections of suspected intestinal TB patients.

MATERIALS AND METHODS

Patients

We retrospectively identified 10 patients (4 males and 6 females; mean age, 65.1 ± 13.6 years) with intestinal TB between 1996 and 2011. All cases were obtained from the archives of the Department of Infectious, Respiratory, and Digestive Medicine at the University of the Ryukyus Hospital, Okinawa, Japan. The diagnosis of intestinal TB was made by at least one of the following criteria: (1) a positive culture of *M. tuberculosis* from the intestinal tissue; (2) histopathological demonstration of acid-fast bacilli (AFB) in the intestinal tissue; (3) histopathological demonstration of a caseating epithelioid cell granuloma in the intestinal tissue; (4) detection of tubercle bacilli DNA by PCR from the intestinal tissue; and (5) typical endoscopic features together with a favorable response to a trial of antituberculous therapy. These patients were all treated with a full course of antituberculosis therapy (rifampicin, isoniazid, ethambutol, pyrazinamide) following diagnosis. The clinical and colonoscopic records of these patients were obtained, as well as archived FFPE intestinal tissue sections. This study was approved by the Ethics Committee of our institute.

Colonoscopy and histopathology

Colonoscopy was performed with standard colonoscopes (Olympus, Tokyo, Japan). All patients diagnosed with intestinal TB were examined from the rectum to terminal ileum after lavage bowel preparation with a polyethylene glycol electrolyte solution. Colonoscopic findings were recorded on the basis of Sato's classification[6]. Open ulcers or erosions were classified into 4 types: type 1 (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), type 2 (round or irregular-shaped isolated small ulcers arranged circumferentially without nodules), type 3 (multiple erosions restricted to the colon), and type 4 (small aphthous ulcers or erosions restricted to the ileum). Healed lesions in the ileocecal area were also recorded, including the patulous ileocecal valve (PV), pseudodiverticular deformity (PD), and atrophic mucosal area (AMA) with multiple ulcer scars[6]. During colonoscopy, biopsy specimens were obtained in a routine fashion using standard forceps. The specimens were prepared for ZN staining, tuberculous culture, PCR for tubercle bacilli DNA, and hematoxylin and eosin (HE) staining.

Table 1 Clinical, laboratory and bacteriologic findings in patients with intestinal tuberculosis

Case	Age/gender	Underlying disease	Symptoms	Chest radiograph	Sputum ZN stain	Sputum culture	Sputum PCR	TST	QFT
1	38/F	Epilepsy	Fever	Active TB	+	-	+	+	ND
2	66/F	Lumber neuralgia	Diarrhea	Normal	-	-	-	+	ND
3	81/F	Cecal cancer	Diarrhea, weight loss	Active TB	+	-	-	ND	ND
4	72/M	Post-herpes neuralgia	Abdominal pain	Normal	-	-	-	+	+
5	43/M	Diabetes mellitus	Diarrhea	Normal	-	-	-	-	ND
6	76/M	Gout	Diarrhea	Normal	-	-	-	+	+
7	58/M	Ulcerative colitis	Diarrhea	Normal	-	-	-	-	-
8	74/F	Gastric ulcer	Weight-loss	Active TB	+	-	-	+	+
9	74/F	Colonic polyp	None	Normal	-	-	-	ND	+
10	70/F	Rectal cancer	Diarrhea	Inactive TB	-	-	-	+	ND

TB: Tuberculosis; ZN: Ziehl-Neelsen; PCR: Polymerase chain reaction; TST: Tuberculin skin test; ND: Not determined; QFT: QuantiFERON-TB gold test; M: Male; F: Female.

IHC staining

IHC staining was performed using the IgG1 type mouse monoclonal antibody against the 38-kDa antigen of the *M. tuberculosis* complex (Vector Laboratories, Burlingame, CA, United States). 5 μm thick sections were prepared from formalin-fixed, paraffin-embedded tissue. IHC was carried out using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, United States) as described elsewhere[7-11]. Briefly, after deparaffinization and rehydration, the sections were exposed to antigen retrieval (Target retrieval solution pH 6.0, DakoCytomation, CA, United States) in high temperature water (98 ℃) for 30 min, and then cooled for 20 min at room temperature. Endogenous peroxidase activity was inhibited by incubating the sections with hydrogen peroxide for 20 min. To prevent non-specific binding, these sections were incubated in normal mouse serum for 20 min. Primary antibody (anti-*M. tuberculosis* mouse monoclonal antibody in 1:80 dilutions) was applied to the sections overnight. This step was followed by washing and 40-min incubation with a biotinylated secondary antibody. These sections were then subjected to an avidin biotin-peroxidase complex for 40 min. Visualization was performed using ImmPACT DAB (Vector Laboratories, Burlingame, CA, United States), which was applied for 10 s. Sections were counter-stained with hematoxylin. A negative control in which the primary antibody was substituted with antibody diluent was used. IHC expression of the *M. tuberculosis* antigen was evaluated under light microscopy for the distribution of stain in the cytoplasm of epithelioid histiocytes and multinucleated giant cells. Additional IHC staining was performed to evaluate the relationship between *M. tuberculosis* antigen and macrophage distribution in the colonic specimens. These paraffin sections were stained with anti-*M. tuberculosis* rabbit antibody (Abcam, Cambridge, MA) at a 1:200 dilution, and the pan-macrophage marker CD68 antibody (Leica Microsystem, Buckinghamshire, United Kingdom) at a 1:80 dilution using the streptavidin-biotin peroxidase method as described previously.

RESULTS

Clinical features

Clinical features of the 10 patients are summarized in Table 1. No patients were immunocompromised, including immunosuppressive medication use and HIV infection. Primary symptoms were diarrhea and weight loss. Although no patients had respiratory symptoms, chest radiograph and sputum ZN staining in 3 patients revealed active pulmonary TB. Six patients (60%) had a positive Tuberculin skin test (TST) and four patients (40%) had a positive QuantiFERON-TB gold test (QFT), of which three patients had both a positive TST and QFT test.

Colonoscopic, bacteriological, and histopathological findings

Colonoscopic, bacteriological, and histopathological findings of the 10 patients are summarized in Table 2. All patients had type 1 findings (linear ulcers in a circumferential arrangement or linear ulcers arranged circumferentially with mucosa showing multiple nodules), all of which were located in the right hemicolon and/or terminal ileum (Figure 1A). Seven patients (70%) had concomitant healed lesions, including PV, PD, or AMA, in the ileocecal area (Figure 1B). No AFB was detected with ZN staining of the intestinal tissue samples. Tuberculous culture and PCR for tubercle bacilli DNA were negative in all intestinal tissue samples.

In the histopathological studies, tuberculous granulomas were identified in 4 cases (40%) (Figure 2A). IHC staining with anti-*M. tuberculosis* monoclonal antibody was positive in these same 4 patients (40%). The mycobacterial antigens were mainly detected as granular cytoplasmic staining in the epithelioid cells and giant cells within granulomas (Figure 2B). All samples in which granulomas were detected by HE staining were positive for TB by IHC staining. As for the relationship between *M. tuberculosis* antigens and macrophage distribution in the colonic consecutive specimens, the mycobacterial

Table 2 Colonoscopic, bacteriologic and histopathological findings in patients with intestinal tuberculosis

Case	Type	Colonoscopic or macroscopic findings			Histopathological findings		
		Location	PV, PD, AMA	AFB in ZN staining	Culture/PCR	Granuloma	TB by IHC staining
1	1	TI, C, A	PV, AMA	-	-	-	-
2	1	TI, C, A	PV, AMA	-	-	+	+
3	1	TI	-	-	-	+	+
4	1	C, A	PV, PD, AMA	-	-	-	-
5	1	TI, C, A, T, D, S	PV, PD, AMA	-	-	-	-
6	1	TI	-	-	-	-	-
7	1	TI, C, A, T, D, S	PV, PD, AMA	-	-	-	-
8	1	TI, C	PV, AMA	-	-	+	+
9	1	TI, C, A, S	PD, AMA	-	-	-	-
10	1	TI	-	-	-	+	+

TI: Terminal ileum; C: Cecum; A: Ascending colon; T: Transverse colon; D: Descending colon; S: Sigmoid colon; PV: Patulous ileocecal valve; PD: Pseudodiverticular deformity; AMA: Atrophic mucosal area; AFB: Acid-fast bacilli; ZN: Ziehl-Neelsen; PCR: Polymerase chain reaction; IHC: Immunohistochemical; TB: Tuberculosis.

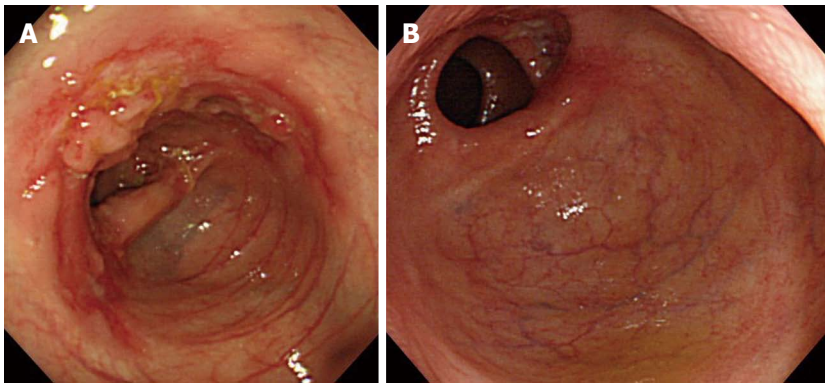

Figure 1 Typical colonoscopic views of intestinal tuberculosis. A: Colonoscopy shows a circumferential ulcer with edematous flared nodules in the ascending colon (patient 1); B: Colonoscopy shows a whitish mucosal area with an absence of the normal vascular pattern of healed ulcer scars in the ascending colon. Note the concomitant active ulcers in the proximal colon (patient 2).

antigens were seen predominantly as coarse granular immunopositive material in $CD68^+$ macrophage cytoplasm (Figure 2C, D).

Clinical course and outcome

All patients were suspected to have intestinal TB on the basis of clinicopathologic findings. The regimen for TB was combined chemotherapy containing isoniazid, rifampicin, and ethambutol for six months and pyrazinamide for two months. All patients responded to antituberculous therapy and follow-up colonoscopies showed improvement of the colonic lesions.

DISCUSSION

This study has highlighted several important considerations. Most patients in this study had abdominal symptoms, and although no patients had respiratory symptoms, 4 (40%) had concomitant active or inactive pulmonary TB, consistent with prior reports in which pulmonary TB was apparent in less than 25% of patients with intestinal TB[2,12]. Based on these findings, we should be aware of the possibility of tuberculous involvement in multiple organs despite apparent symptoms.

Although the TST has long been used as a reliable diagnostic examination, the recently developed QFT has been increasingly applied. There is controversy as to whether or not the QFT is effective for the diagnosis of extrapulmonary TB. Kim *et al*[13] reported that in a prospective study of 128 patients, QFT was a limited but useful aid in combination with the TST in the diagnosis of intestinal TB. In agreement with their findings, the QFT and TST had a good agreement in our study.

In this study, the vast majority of cases were colonoscopically diagnosed with TB. Similar to previous studies[6,14], all patients in our study had a type 1 appearance among colonoscopic findings. Although this type has been established as a reliable colonoscopic feature, recent studies have emphasized that healed lesions in the ileocecal area, including PV, PD, and AMA with multiple ulcer scars, can coexist with active tuberculous inflammation. Sato *et al*[6] reported that 91% of patients in their study have AMA lesions, and so concluded that the AMA was the most frequently recognized endoscopic manifestation of intestinal TB. Our data, in which 60% of patients had these lesions, correlates strongly with their findings.

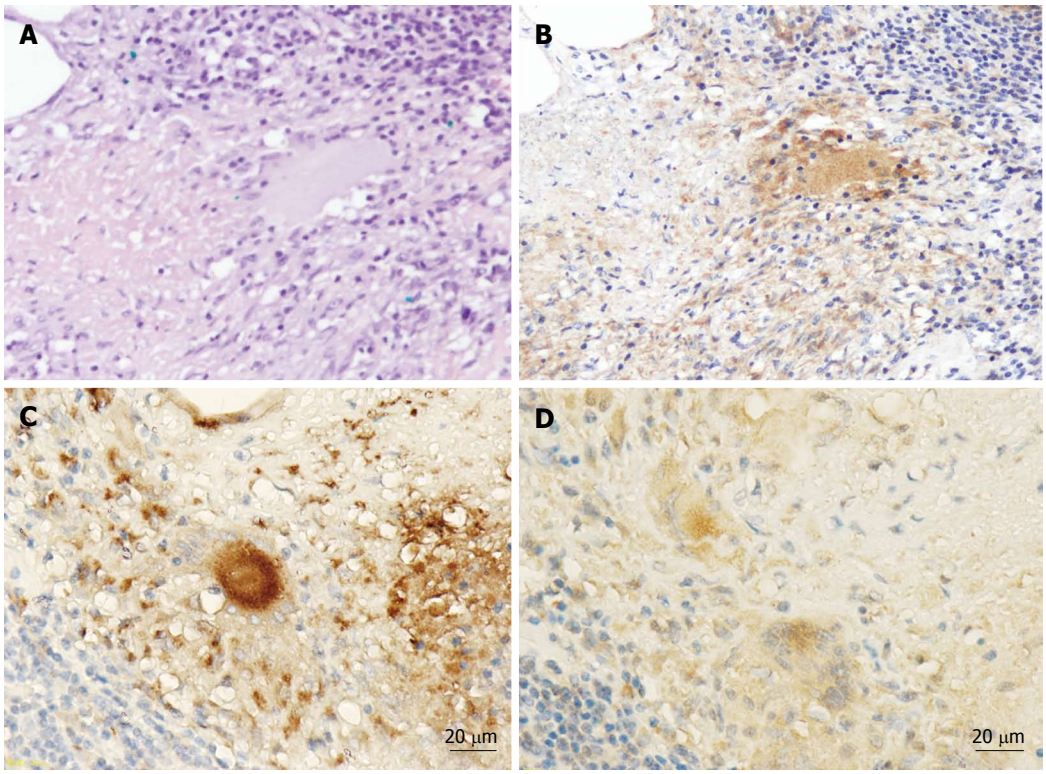

Figure 2 Histopathological views of tuberculous granuloma and localization of the mycobacterial antigen in the colonic lesion (patient 8). A: A granuloma, surrounded by inflammatory lymphocytes, is present in the lamina propria (hematoxylin and eosin, × 200); B: Immunohistochemical staining view of the colonic specimen (× 200). Note that the mycobacterial antigens (brownish granular matter) are present in the cytoplasm of epithelioid histiocytes and multinucleated giant cells in the granuloma; C: Cells stained with the pan-macrophage marker CD68 antibody are present in the granuloma (× 400); D: The mycobacterial antigens (brownish granular matter) are present in the cytoplasm of the macrophages in the granuloma (× 400).

Numerous studies have compared the diagnostic methods of intestinal TB[15]. A study by Sekar *et al*[16] evaluated the role of PCR in the laboratory diagnosis of different forms of extrapulmonary TB in comparison with conventional bacteriologic techniques. They found an 18%, 22%, and 63% sensitivity for smear, culture, and PCR, respectively. A recent study clearly demonstrated that using real-time PCR technology with fluorescence resonance energy transfer probes has much higher sensitivity for the detection of tubercle bacilli DNA in tissue biopsy samples and FFPE surgically resected tissues of the gastrointestinal tract than traditional PCR[17]. Our negative AFB and PCR tests may be due to a small biopsy sample size.

There have been several studies on IHC staining for the diagnosis of TB in which the most used polyclonal antibodies[18,19] resulted in false-positive reactions. There are only two studies that have evaluated IHC staining with a species-specific monoclonal antibody to 38-kDa antigen of *M. tuberculosis* complex in archival FFPE tissue sections of intestinal TB. Goel *et al*[7] demonstrated that 2/2 samples tested had positive IHC staining (100%), whereas Ince *et al*[8] demonstrated IHC positivity in 6/8 (75%) intestinal tuberculous granulomas. Our results revealed 4 positive samples using IHC staining out of 4 tuberculous granulomas, whereas ZN staining for AFB was negative in all of these sections. Our results demonstrating that granulomas detected with HE staining are all positive with IHC have confirmed these previous findings. It is important to note that this was a retrospective study on previously diagnosed TB; hence we cannot determine the false positivity rate of IHC staining for intestinal TB.

Granular cytoplasmic staining of *M. tuberculosis* in IHC staining is considered to be due to fragments or debris of the bacilli[7]. Low positivity of AFB could be due to the fact that only the intact bacilli take up the stain. The fact that fragments or debris of *M. tuberculosis* can be detected easily using IHC staining may be a great advantage. To the best of our knowledge, this is the first report that demonstrates that *M. tuberculosis* antigens are located as coarse granular immunopositive material in the cytoplasm of $CD68^+$ macrophages within human intestinal TB. This finding may help to uncover the unknown relationship between *M. tuberculosis* and macrophages[20].

Distinguishing intestinal TB from CD is still challenging[21-25], and the treatments of TB and CD are quite different. Corticosteroids, immunosuppressive and anti-tumor necrosis factor (TNF) agents are widely applied in the treatment of CD, whereas they may be harmful in TB. For example, anti-TNF agents can induce reactivation of TB; therefore differentiating TB from CD is extremely important. Histopathological features en-

countered frequently in intestinal TB include granulomas that are confluent, large (> 200 μm), and multiple in number (> 5 per section)[26]. The classical histological picture of tuberculous granulomatous inflammation is not a diagnostic problem in tissue samples; however, when sections show non-caseous epithelioid granulomas mimicking TB as is the case in CD, it creates a diagnostic dilemma. In the present study, we did not conduct any experiments using intestinal samples of CD; however Ince *et al*[8] concluded that positive IHC staining with species-specific antibodies to TB can rule out the diagnosis of CD with high sensitivity and specificity. We plan to conduct a large-scale study to confirm these findings in the future.

In conclusion, IHC staining using a monoclonal antibody to *M. tuberculosis* antigen, which is a novel translational implication, can potentially be an efficient and simple diagnostic tool to compliment classic clinicopathological examinations for the diagnosis of intestinal TB.

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COMMENTS

Background

The diagnosis of intestinal tuberculosis (TB) is often difficult because of its diverse clinical manifestations and very low positivity using current diagnostics, including acid-fast bacilli in Ziehl-Neelsen staining and culture of *Mycobacterium tuberculosis* (*M. tuberculosis*) from intestinal tissue samples.

Research frontiers

Detection of *M. tuberculosis* DNA by polymerase chain reaction has been established as a diagnostic tool with excellent sensitivity and specificity in pulmonary TB. However, the diagnosis of intestinal TB still requires validation.

Innovations and breakthroughs

In this study, the authors have shown that immunohistochemical (IHC) staining using a monoclonal antibody to *M. tuberculosis* antigen in archived formalin-fixed paraffin-embedded intestinal tissue samples, which is a novel translational implication, can be an efficient and simple diagnostic tool to compliment classic clinicopathological examinations for the diagnosis of intestinal TB.

Applications

Although further studies are needed, IHC staining may be beneficial to differentiate TB from Crohn's disease (CD) with similar microscopic features, including the presence of granulomas.

Terminology

Monoclonal antibodies are monospecific antibodies which have monovalent affinity. IHC staining is widely applied to investigate the distribution of antigens in biological tissues by using specific antibodies.

Peer review

In this manuscript, the authors have clearly illustrated the potential advantage for the diagnosis of intestinal tuberculous granuloma. The importance of the research and its significance show that this topic is an important issue. The differentiation between TB and CD is very important in gastroenterology.

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