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ORIGINAL ARTICLE

Can an Immunohistochemistry Method Differentiate Intestinal Tuberculosis from Crohn's Disease in Biopsy Specimens?

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Abstract
Background It is sometimes difficult to diagnose whether a patient has intestinal tuberculosis or Crohn's disease because both have similar clinical, pathologic, and endoscopic features. However, their therapies are completely different and a mistake in diagnosis can result with deterioration. Many laboratory methods for the diagnosis of tuberculosis require considerable time to receive a diagnostic result. We wanted to evaluate whether an immunohistochemical tuberculosis staining method can be helpful for faster differentiation of biopsy materials.
Methods We used formalin-fixed paraffin-embedded histologically diagnosed small intestine ($n = 1$), colon ($n = 7$), skin ($n = 8$), lung ($n = 5$), lymph node ($n = 24$) tuberculosis and Crohn's disease ($n = 28$) biopsy materials only with granulomas. Demographic characteristics like age and gender were also obtained. Pathology specimens were stained immunohistochemically with an antibody to VP-M660, targeting the 38-kDa antigen of *Mycobacterium tuberculosis*.
Results In the *M. tuberculosis* group, 33/45 of patients have positive immunohistochemistry (IHC) staining (73% sensitivity, 93% specifity), whereas only two of 28 patients have positive staining in the Crohn's group ($p < 0.001$). The positive staining with IHC was detected as 85.7, 75, 75, and 60% in colon, lymph node, skin, and lung granulomas, respectively, in *M. tuberculosis* patients.
Conclusions Immunohistochemical staining of biopsy specimens with anti-VP-M660 seems to be a simple and fast technique with 73% sensitivity and 93% specificity for establishing an earlier differentiation of *M. tuberculosis* from Crohn's disease.

Keywords Immunohistochemistry · *Mycobacterium tuberculosis* · Crohn's disease · Differential diagnosis

Introduction

Abdominal tuberculosis (AT) commonly presents in the developing world. The global incidence of AT has also increased over the past decade as a consequence of immigration and increased world travel opportunity as well as increasing prevalence of HIV infection [1–7]. In general, AT can be classified according to its involvement as intestinal, mesenteric lymph nodes, or peritoneal. A frequently involved area of the gastrointestinal tract is the ileocecal region and associated peritoneal tuberculosis with/without ascites are often present. Endoscopically, gross appearance of intestinal tuberculosis can be divided into four categories: (1) ulcerative lesions characterized by multiple superficial ulcers, especially transverse ulcers to colon lumen axis (2) hypertrophic lesions characterized by scarring, fibrosis, and mass lesions, (3) ulcero-hypertrophic lesions combining features of the two entities mentioned above in 1 and 2, and (4) fibrotic strictures [8]. Patients with gastrointestinal tuberculosis may also present with intestinal and perianal fistulas [3].

Histologically, gastrointestinal tuberculosis is characterized by granuloma formation, which is not specific to

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this disease, however, the presence of caseified granulomas strengthens the probability of tuberculosis as the diagnosis. Unfortunately, caseation is infrequently seen in gastrointestinal mucosal diseases (although more commonly found in affected mesenteric lymph nodes).

Less than one-third of patients demonstrate acid-fast staining of the organism using Ziehl-Neelsen's (ZN) staining of intestinal tissue samples. In the absence of a positive ZN staining, a positive culture of intestinal biopsies require for the definitive diagnosis. Furthermore, tissue culture for *M. tuberculosis* may take several weeks, resulting in delays in confirmation of the diagnosis as well as in the initiation of appropriate treatment.

Intestinal tuberculosis mimics many other abdominal diseases. The main differential diagnosis of intestinal tuberculosis is Crohn's disease, a chronic inflammatory bowel disease of unknown etiology. Intestinal tuberculosis and Crohn's disease have similar clinical, endoscopic, and pathologic findings.

There is no single gold standard for the diagnosis of Crohn's disease and diagnosis is made by clinical evaluation including detailed history, physical examination, and combination of endoscopic findings, histology, radiologic findings, and laboratory investigations. For tuberculosis, neither clinical signs, laboratory, radiological and endoscopic methods, nor bacteriological and histopathological findings provide a gold standard by themselves in the diagnosis of abdominal TB. However, an algorithm of these diagnostic methods leads to considerably higher precision in the diagnosis of this insidious disease, which primarily necessitate a clinical awareness of this serious health problem.

The treatments of these disorders (Crohn's and tuberculosis) are quite different. Corticosteroids, immunosuppressive, and anti-tumor necrosis factor (anti-TNF) agents are employed in Crohn's therapy, whereas they are contraindicated in tuberculosis. Additionally, anti-TNF agents also can induce reactivation of tuberculosis. It is very difficult to differentiate these two disorders in the absence of a chest X-ray suggesting pulmonary tuberculosis, a positive ZN stain of a gut biopsy, caseification necrosis in the granulomas, or a positive tissue culture for tuberculosis.

The diagnosis of intestinal tuberculosis is often difficult because of its diverse clinical manifestations and low positivity of ZN staining in tissue granulomas [9, 10]. Various nucleic acid amplification assays have been developed that have shown excellent sensitivity and specificity in respiratory specimens. However, usage of these tests in clinical settings for non-respiratory specimens still requires validation and is still out of reach in routine diagnosis in developing countries [11]. Additionally, there are samples both acid-fast bacilli and culture-negative. Therefore, diagnosis is usually made on the basis of classical histological changes of chronic granulomatous inflammation, suggestive of tuberculosis. These histological features can be found in various diseases other than tuberculosis and in immunocompromised tuberculosis patients leading to considerable difficulty and delay in diagnosis [12]. Differentiation of intestinal tuberculosis and Crohn's disease is important, since treatment differs considerably [13, 14].

The present study was undertaken to assess the usefulness of immunohistochemical staining with species-specific monoclonal antibody to the 38-kDa antigen of *M. tuberculosis* complex to facilitate differentiation of tuberculosis from Crohn's disease in archival formalin-fixed paraffin-embedded tissue sections.

Patients and Methods

The study was performed on various site granulomas [skin ($n = 8$), small intestine ($n = 1$), colon ($n = 7$), lung ($n = 5$), lymph nodes ($n = 24$)], clinically and histologically diagnosed as tuberculosis ($n = 45$), and Crohn's disease ($n = 28$) biopsy specimens, which were obtained from archives of the Department of Pathology, Haydarpaşa Numune Education and Research Hospital, Istanbul, Turkey, between January 1, 2004, and December 31, 2008. Only specimens that contained granuloma were selected among Crohn's and tuberculosis groups and all used stored tissue samples were paraffin-fixed. Because of the limited number of intestinal tuberculosis specimens with granuloma, we added lymph node, lung, and skin tuberculosis specimens to study for control cases. Therefore, among 150 Crohn's and 200 tuberculosis specimens, 28 Crohn's and 45 tuberculosis specimens were appropriate for study. Only one sample was used from each patient and pathology specimens were stained immunohistochemically with an antibody to VP-M660, targeting the 38-kDa antigen of *M. tuberculosis*.

Inclusion criteria for the tuberculosis group ($n = 45$) were as follows: either one or more than one test positivity for specific tests of tuberculosis; culture [Löwenstein-Jensen ($n = 23$), Bactec ($n = 32$)], TBC PCR ($n = 20$) and EZN ($n = 12$) as well as appropriateness of clinical (loss of weight, fever, cough, sputum, abdominal pain, diarrhea, constipation), laboratory (high sedimentation, PPD positivity), pathology (granulomas with caseification necrosis), and colonoscopic appearances as described before and response to anti-tuberculosis therapy. Because of the limited number of intestinal biopsy specimens in the pathology unit, we used the tuberculosis specimens of different part of body like skin, lymph node, and lung as positive control. It is also thought that the results of study can be used for differential diagnosis of extraintestinal granulomatous

Table 1 Diagnostic categories and immunohistochemical (IHC) staining rates of the granulomas tested

Diagnosis, site, and immunohistochemical staining rate	Lymph nodes	Small intestine	Colon	Lung	Skin
Tuberculosis (Tbc)	24	1	7	5	8
Crohn's disease (CD)	0	14	14	0	0
Immunohistochemical staining rate					
Tbc	18/24 (75%)	0/1 (0%)	6/7 (85.7%)	3/5 (60%)	6/8 (75%)
CD	Not available	2/14 (14.2%)	0/14 (0%)	Not available	Not available

diseases. Inclusion criteria for the Crohn's group were: known Crohn's disease patients that had been selected with small intestinal or bowel involvement with appropriate clinical (pain, diarrhea, constipation, fever), laboratory (increased CRP and sedimentation level) and colonoscopic (cobble-stoning, ulcers, exudation, fistula, fissure) findings.

The diagnostic categories included in the study for *M. tuberculosis* and Crohn's disease are shown in Table 1. After the diagnosis of tuberculosis, a full course of anti-tuberculosis therapy with four drugs (rifampicin, isoniazid, ethambutol, pyrazinamide) was employed in all patients.

We also obtained the demographic characteristics of patients such as age and gender.

The histological diagnosis of extrapulmonary tuberculosis of various sites was based on the classical caseous granulomas observed on the histopathological examination of hematoxylin and eosin-stained formalin-fixed paraffin-embedded tissue sections. All the sections were subjected to immunohistochemical staining. The researcher that performed the laboratory analyses was blind to the original diagnoses.

Immunohistochemical Staining (IHC)

Immunohistochemical staining was performed by using the IgG1 type mouse monoclonal antibody to *M. tuberculosis* (Vector Laboratories, Inc., Burlingame, CA, USA) and standardized in our pathology department laboratory. The staining was carried out with *M. tuberculosis* species-specific (MTSS) mouse monoclonal antibody raised to 38-kDa antigen of the *M. tuberculosis* complex.

The study kit was just for the usage in formalin-fixed paraffin blocks, not for fresh tissues. Tissue sections 4 μm thick were cut from paraffin blocks on lam that was covered with poly-L-lysine. These sections were kept in an incubator overnight at a temperature of 37°C for physical deparaffinization and then deparaffinized in fresh xylene (10 min, three times) and then rehydrated with 96% ethanol (10 min, three times) for chemical deparaffinization. Alcohol was removed by distilled deionized water (5 min). Sections were kept in citrate buffer (pH 6) and antigen retrieval was followed by microwave heating at 800 W (10 min). Sections were brought to room temperature and washed in Tris buffer (pH 6) three times (5 min each). The endogenous peroxidase activity of the tissue was blocked by incubating the tissue in 3% hydrogen peroxidase for 20 min. Again, the sections were washed in Tris buffer three times (5 min each). Sections were treated with biotinylated goat serum (20 min) to block the non-specific binding sites, followed by washing with Tris buffer. Excess buffer was drained. Sections were then covered with primary antibody diluted in Tris buffer solution (primary antibody: Tris buffer solution = 1:40) and incubated for 24 h in a moist chamber at 4–7°C. The next day, the sections were brought to room temperature and rinsed in Tris buffer twice (5 min each). Link antibody was added and sections were incubated for 20 min and this was again followed by Tris washing. Streptavidin peroxidase conjugate was then added to the sections for 20 min. Finally, the chromogen was diluted in the substrate buffer.

Evaluation of Immunoreactivity

Immunohistochemical expression of *M. tuberculosis* antibody was evaluated under light microscopy for distribution of stain in the cytoplasm of epithelioid histiocytes and multinucleated giant cells. Tuberculosis lymphadenitis sections were used for external control. Sections of external control and cases taken on to the every slide. There was no internal control. Granular staining was observed as in the cytoplasm of epithelioid histiocytes and multinucleated giant cells. Staining rate (the distribution of stain) was graded as semiquantitative in the positively stained cells and put into the following categories: 0: (0–4%), 1: (5–25%), 2: (25–50%), 3: (50–100%). Intensity of staining: 0: negative; 1+ :mild, 2+ :moderate; 3+ :strong. The immunoreactive score was calculated by multiplying the ratio of positive stained cells by the scores obtained from staining intensity.

Ethics and Statistical Analysis

All samples and clinical details were collected after approval by the Institutional Ethics Committee (Date and number of approval; 26.03.2008/08–12).

All statistical analyses were made by SPSS 15.0 for Windows (SPSS, Inc, Chicago, IL, USA). Mann–Whitney *U* test was used for numeric variables for comparing two groups, ANOVA was used for continuous variables and the results were presented as mean ± standard deviation (mean ± SD). Chi-square test (χ^2) was used for categorical variables and p value < 0.05 was accepted as significant.

Results

Overall, 73 patients' formalin-fixed paraffin-embedded pathology specimens were retrieved from the pathology department (45 tuberculosis and 28 Crohn's disease patients). Demographic features of the patients are shown in Table 2.

Histology

Both necrotic and non-necrotic granulomas with epithelioid cells and multinucleated giant cells characteristic of tuberculosis were observed in tuberculosis patients. Necrotic granulomas were found in 75.5% of the tuberculosis patients whereas 24.5% had both necrotic and non-necrotic granulomas.

IHC

IHC with anti-VP-M660 was positive in 18 (75%), six (85.7%), six (75%), three (60%) and 0 (0%) cases from lymph nodes, colon, skin, lung, and small intestine, respectively, in the tuberculosis group and two (14.2%) in the Crohn's disease group. The IHC staining rates of the various site granulomas of the two groups are shown in Table 1. The sensitivity and specificity of the anti-VP-M660 was found to be 73 and 93%, respectively, in order to differentiate *M. tuberculosis* from Crohn's disease.

VP-M660 antigen was detected as granular cytoplasmic staining in the Crohn's patients and in the granuloma cells of the tuberculosis cases (Fig. 1). The surrounding normal tissues in the tuberculosis lymph nodes did not show any staining. Antigen was mainly detected in the epithelioid cells and giant cells within granulomas. Necrotic centers were negative in the majority of patients. The intensity and distribution of staining was different among patients. In some, most of the cells in the granulomas were strongly positive, while in others, only a few cells showed staining.

In the tuberculosis group, IHC staining was positive in 33 out of 45 (73.3%) patients, whereas only in two out of 28 (7%) patients was stained with IHC in the Crohn's disease group. When we compared the two groups for the IHC staining, the tuberculosis group significantly showed more staining than the Crohn's group ($p < 0.001$).

Table 2 Demographic features of patients

	Crohn's disease	Tuberculosis	Total	*p* value
Age (mean ± SD)	39 ± 17	51 ± 14	47 ± 16	0.001*
Female/male	10/18	28/17	38/35	0.03**

* Patients in the tuberculosis group were significantly older than in the Crohn's disease group

** The tuberculosis group had significantly more female patients than the Crohn's disease group

Discussion

There have been some reports on IHC staining about establishing the diagnosis of *M. tuberculosis* [15–24] but most of them had used polyclonal antibodies, resulting in false-positive reactions due to antigenic cross reaction with other bacteria and fungi and only completed in tuberculosis patients with or without healthy controls. There is only one study in the English-language literature evaluating immunohistochemical staining with species-specific monoclonal antibody to 38-kDa antigen of *M. tuberculosis* complex in archival formalin-fixed paraffin-embedded tissue sections of extra-pulmonary tuberculosis [17]. We have used IHC staining with specific monoclonal antibody to 38-kDa antigen of *M. tuberculosis* complex to establish the differential diagnosis of pulmonary and extrapulmonary tuberculosis and Crohn's disease. In a study by Marchetti et al., four nested PCR assays for the detection of *M. tuberculosis* from formalin-fixed, paraffin-embedded tissues have been studied and 80–87% sensitivity rates have been reported [25]. Another study by Sekar et al. to evaluate the role of PCR in the laboratory diagnosis of different forms of extrapulmonary tuberculosis in comparison to conventional bacteriological techniques found 18, 22, and 63% sensitivity for smear, culture, and PCR, respectively [26]. Kivihya-Ndugga et al. reported 93% sensitivity for diagnosis of tuberculosis with the Amplicor PCR method [27].

We found that IHC staining showed positive staining with *M. tuberculosis* antigens in histological section of 33 out of 45 patients (73.3%) of pulmonary and extrapulmonary tuberculosis and 2 out of 28 (7.1%) cases of Crohn's disease, whereas ZN stain for acid-fast bacilli was negative in both of the two *M. tuberculosis* patients. Goel et al. reported 100% specificity and sensitivity with the same monoclonal antigen that we used, but our study did not produce similar results. Our results are similar to reported rates of IHC studies with polyclonal and monoclonal antibodies for diagnosis of *M. tuberculosis*, which changed between 68 and 100% [17].

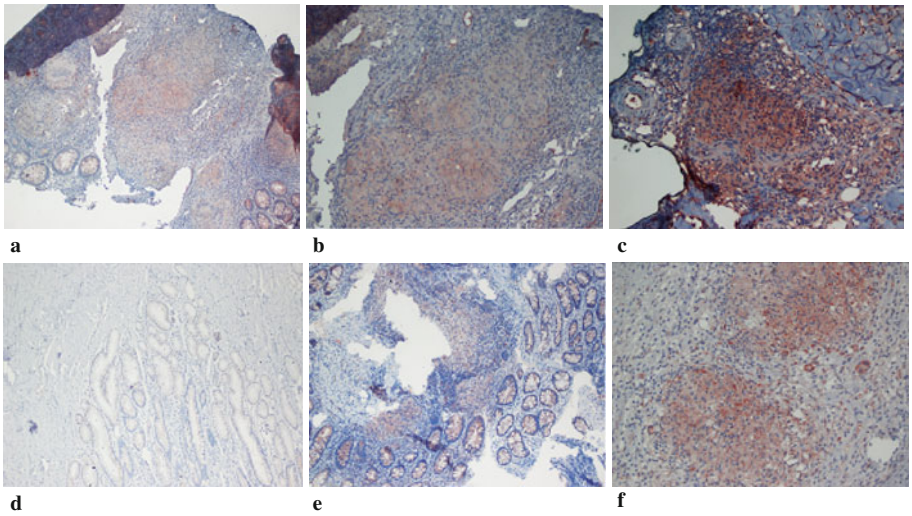

Fig. 1 Immunohistochemical staining patterns in *M. tuberculosis* and Crohn's disease granulomas with antibody to 38-kDa antigen (**a–f**). **a** +2 immunostaining in the cytoplasm of epithelioid histiocytes which make up granulomas that localized in the lamina propria and submucosa in the endoscopic colon biopsy (H&E 10 × 10). **b** +2 positive staining (H&E 20 × 10). **c** +3 immunostaining in the cytoplasm of epithelioid histiocytes, generate granulomas in the subcutaneous fatty tissue of cutaneous tuberculosis case (H&E 20 × 10). **d** No immunostaining with MT in the colon mucosa, submucosa, and muscularis propria layer, which showed morphological features consistent with Crohn's disease (H&E 10 × 10). **e** Granulomas generating histiocytes showing +1 immunostaining in the lamina propria layer of colon mucosa biopsy (H&E 4 × 10). **f** +2 cytoplasmic immunostaining in granulomas and multinucleated giant cells in tuberculosis lymphadenitis (H&E 20 × 10)

Although it is difficult to explain the positive staining in two out of 28 Crohn's patients, one can argue that in fact these two patients had latent tuberculosis, which is unlikely, though possible, in a country of endemic tuberculosis, but they were diagnosed with Crohn's disease. One of these patients was 37 years old and had Crohn's disease for 11 years and also received various treatments including corticosteroids, azathioprine, and anti-TNF and had no history of tuberculosis. The other patient was 32 years old and had enterocutaneous fistulas and classic histopathological findings in the small intestine resection material. Another explanation is that foreign-body granulomas are well known for giving non-specific staining by immunohistochemistry and the positive results in these two patients might be due to residual irrelevant antibodies in the absorbed anti-VP-M660.

Mycobacterial culture is generally used for the validation of any new diagnostic test. It also takes a long time, which is crucial for both tuberculosis and Crohn's disease. 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 tuberculosis, as is the case in Crohn's disease, it creates a diagnostic dilemma. The positive IHC staining with species-specific antibodies in these cases will rule out the differential diagnosis of Crohn's disease, sarcoidosis, or other non-specific tuberculoid granulomas.

In conclusion, the immunohistochemical diagnosis of *M. tuberculosis* complex antigen with antibody to 38-kDa antigen may be an efficient diagnostic adjunct to clinical, laboratory, and histopathological examinations for the diagnosis of tissue granuloma of the tuberculosis, especially in patients who need fast, certain differentiation. Immunohistochemical staining of biopsy specimens with anti-VP-M660 seems to be a simple, fast, and easy-to-perform technique with 73% sensitivity and 93% specificity for establishing an earlier differentiation of *M. tuberculosis* from Crohn's disease.

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