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Original Article

A possible complementary tool for diagnosing tuberculosis: a feasibility test of immunohistochemical markers

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Abstract: Differentiation of tuberculous granuloma (TG) from non-tuberculous granuloma (NG) is histopathologically difficult. We evaluated the usefulness of selected immunohistochemical markers to differentiate tuberculous granuloma (TG) and non-tuberculous granuloma (NG). We selected six biomarkers (FoxP3, TNF-beta, E-selectin [ESEL], indoleamine 2,3-dioxygenase [IDO], lactoferrin [LACT], and tartrate-resistant acid phosphatase [TRAP]) and immunohistochemically analyzed their expression in the presence of two types of granulomatous tissue samples, TG (n = 36) and NG (n = 31), using a microarray format. Three of those six biomarkers (LACT, IDO, and TNF-beta) were moderately accurate in discriminating TG from NG, individually and in combination, according to ROC analysis (AUC = 0.7-0.89, sensitivity = 55.6-77.8%, specificity = 71.0-100%). Our data indicate that selected immunohistochemical markers (LACT, IDO, and TNF-beta) can be used in ancillary tests to differentiate TG from NG in tissue samples. Further large-scale studies are required to validate our results.

Keywords: Tuberculosis, extrapulmonary tuberculosis (EPTB), biomarkers, immunohistochemistry, granuloma, lactoferrin (LACT), FoxP3, indoleamine 2,3-dioxygenase (IDO), TNF-beta, E-selectin (ESEL)

Introduction

Granulomatous inflammation comprises a spectrum of morphologically and biologically diverse lesions. Tuberculosis (TB) is a well-known cause of chronic granulomatous inflammation and is the most common etiology of granuloma, especially in developing countries. Therefore, from a pathological point of view, the first step in differentiating a granulomatous lesion in clinical practice is to microscopically determine the presence or absence of caseous necrosis within the lesion. However, because of the diverse granuloma etiologies and common overlapping histopathologic features, differentiation between tuberculosis granuloma (TG) and non-tuberculosis granuloma (NG) can be diagnostically difficult for pathologists, especially when caseous necrosis is not obvious.

Based on clinical manifestation, tuberculosis (TB) can be categorized as either pulmonary (PTB) or extrapulmonary (EPTB) [1]. EPTB involves organs other than the lungs (e.g., pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, or meninges) [1, 2]. A definitive diagnosis of TB can only be established by a culture positive for *Mycobacterium tuberculosis* [1]. Due to the low sensitivity (range 0-40%) of conventional smear microscopy, negative results do not confidently exclude a TB etiology [3]. Since about 10-50 % of EPTB patients have concomitant pulmonary involvement, screening for concomitant PTB is required for all suspected EPTB cases [1].

In a recent review, Mehta et al. summarized the various diagnostic tests currently in use [2]. To diagnose EPTB, smear microscopy has a variable sensitivity value (0-40%) [4, 5]. Depending upon the type of extrapulmonary tissue, *M. tuberculosis* culture identification is also variably sensitive (0-80%); additionally, culture has the drawback of requiring a relatively long turnaround time (generally 4-8 weeks) [6, 7]. Histopathologic diagnosis of EPTB from tissue sam-

Table 1. Distribution of anatomic sites according to granulomatous lesion type

Sites	TG	NG
Skin	0	16
LN	15	12
Pleura	8	0
Lung	5	2
Soft tissue	2	1
Kidney	2	0
Adrenal gland	1	0
Bone	1	0
Epididymis	1	0
Uterus	1	0
Total	*36*	*31*

TG = tuberculous granuloma; NG = non-tuberculous granuloma.

ples can be established when granulomatous inflammation and caseous necrosis are observed [8, 9]. However, histopathology cannot differentiate between EPTB and other infectious or non-infectious etiologies that can cause granulomatous lesions, such as leprosy, deep fungal infections, sarcoidosis, or systemic lupus erythematosus [10].

One of the most widely used diagnostic tests is polymerase chain reaction (PCR) because of its superior accuracy. According to one review, however, the diagnostic accuracy of PCR for EPTB cases ranges from 2.8-100% sensitivity and 67-100% specificity, depending on the gene targets, type of PCR, and type of EPTB [2].

Another diagnostic approach for suspected EPTB cases is immunohistochemistry (IHC) using a monoclonal antibody to tuberculosis, anti-MPT64, which specific to the *Mycobacterium tuberculosis* complex. Anti-MPT64 has been reported to have a sensitivity and specificity of 90% and 83%, respectively [11]. However, studies of immunohistochemical markers specifically related to the pathogenesis of granulomatous lesions are lacking, which is the motivation of this study.

Herein, we hypothesize that differences in immune reaction to TG and NG are underpinned by differential expression of immunohistochemical markers, which serve as an indication of chronic granulomatous inflammation. We evaluated the feasibility of six selected immunohistochemical markers [FoxP3, TNF-beta, E-selectin (ESEL), Indoleamine 2,3-dioxygenase (IDO), lactoferrin (LACT), and tartrate-resistant acid phosphatase (TR)] in ancillary testing to differentiate between tuberculous granuloma (TG) and non-tuberculous granuloma (NG).

Materials and methods

Case selections

We retrospectively searched the pathology database of St. Vincent's Hospital of the Catholic University for tissue-confirmed cases of two well-known types of non-tuberculosis granuloma (NG), sarcoidosis and foreign-body reaction (FBR), and cases of tuberculosis granuloma (TG); we limited our search to cases from 2009 to 2012. The Institutional Review Board of St. Vincent's Hospital approved this study (VC13SISI0188).

Among the identified cases, we selected 17 cases of FBR, 14 cases of sarcoidosis, and 36 cases of TB on the basis of paraffin block condition and amount of tissue sample available for additional sectioning and staining (**Table 1**). Among the TG tissue samples (n = 36), 15 were lymph-node samples, eight were pleura tissue, five were lung tissue, two were soft tissue, two were kidney tissue, one was an adrenal gland, one was bone, one was epididymis tissue, and one was uterine tissue. Among the NG samples (n = 31), the 17 FBR samples included one joint cavity and 16 samples of skin tissue, involving ruptured epidermal inclusion cysts. The 14 sarcoidosis samples included two pulmonary samples and 12 lymph node samples.

We included cases that met the following criteria for TB as either "confirmed" or "probable" (TG, n = 36; confirmed TB = TG1; probable TB = TG2). Cases in which the histological diagnosis was suggestive of TB and showed positivity in at least one of three diagnostic tests were designated as "TG1" ("confirmed" TB: n = 12): 1) special staining for acid fast bacilli (Ziehl-Neelsen stain), 2) culture, 3) PCR method. Cases in which the patient showed a clinical response to empirical anti-TB medication, even when all of the above tests were negative, were categorized as TG2 (n = 24). For comparison, sarcoidosis cases (n = 14) and FBR (n = 17) cases were selected and designated to the NG group.

Specimens were fixed in 10% formalin, processed using routine methods, and embedded

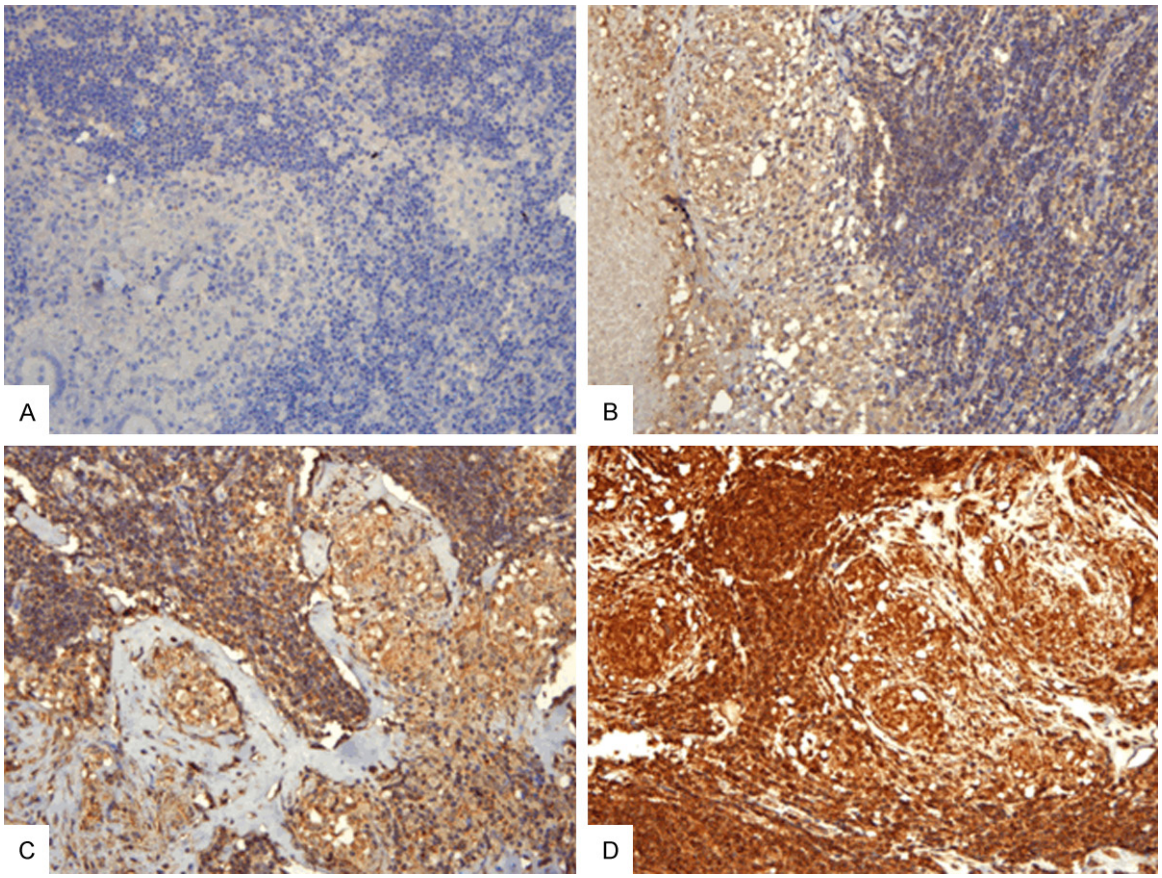

Figure 1. Representative photographs of immunohistochemistry stains of the six immunohistochemical markers (TNF-beta), scored according to staining intensity: 0 (A), 1 (B), 2 (C), and 3 (D) (original magnification, ×200).

in paraffin. All of the original hematoxylin and eosin (H&E)-stained and/or newly cut and H&E-stained sections were reviewed.

Tissue microarray (TMA) and immunohistochemical (IHC) stains

We created a tissue microarray (TMA) of all TG and NG cases (N = 67; TG1, n = 12; TG2, n = 24; NG-1 (sarcoidosis), n = 17; NG-2 (FBR), n = 14). The TMA hole-size was 5.0 mm for all samples.

Immunohistochemical staining (IHC) was performed for each case, using either the labeled streptavidin-biotin peroxidase method (LSAB kit; Dako, Glostrup, Denmark) or the polymer-based detection system (Bond Polymer Refine Detection; Vision BioSystems, Norwell, MA, USA). An antigen-retrieval technique was applied where needed for each antibody.

The antibodies used were FoxP3 (Monoclonal; 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), TNF-beta (monoclonal; 1:100; Santa Cruz Biotechnology), E-selectin (ESEL) (monoclonal, 1:50; Santa Cruz Biotechnology), IDO (monoclonal, 1:50; Santa Cruz Biotechnology), lactoferrin (monoclonal, 1:50; Santa Cruz Biotechnology), and TR (monoclonal, 1:100; Abnova, Taipei, Taiwan).

IHC evaluation

We first identified the granuloma area (GA) and the inflammatory cell area (IC) of each case on H&E slides and then separately assessed the immunoreactivity of the six markers in the two regions. We scored according to intensity of immunostaining: 0 = no signal, 1 = weak, 2 = moderate, or 3 = marked (**Figures 1-6**). The sum of the two scores for GA and IC was designated as the total score (TS = GA + IC). All stained slides were interpreted by two pathologists who were blinded to each other's scores (KJ Seo and CY Yoo). In cases where the score difference was ≥ 2, the slides were re-examined, and a consensus was reached by the observers (**Table 2**).

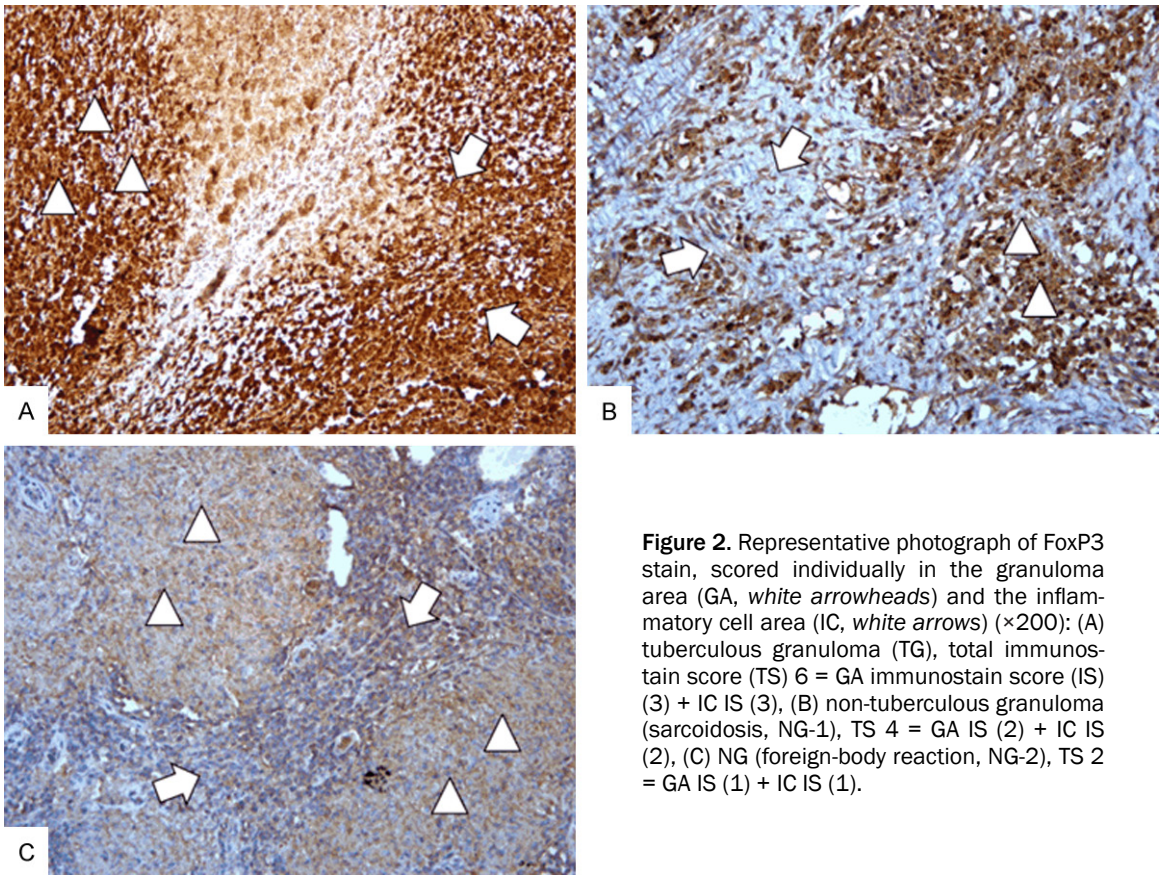

Figure 2. Representative photograph of FoxP3 stain, scored individually in the granuloma area (GA, *white arrowheads*) and the inflammatory cell area (IC, *white arrows*) (×200): (A) tuberculous granuloma (TG), total immunostain score (TS) 6 = GA immunostain score (IS) (3) + IC IS (3), (B) non-tuberculous granuloma (sarcoidosis, NG-1), TS 4 = GA IS (2) + IC IS (2), (C) NG (foreign-body reaction, NG-2), TS 2 = GA IS (1) + IC IS (1).

Statistics

We performed Mann-Whitney U tests to compare the mean IHC scores between the TG subgroups (TG1 vs. TG2). All reported *p*-values are two-sided. Statistical analyses were performed using MedCalc Statistics for Biomedical Research, version 12.4.0.0 (MedCalc Software, Ostend, Belgium). Using ROC analysis, we classified test accuracies in a five-category system suggested by Sets [12, 13]: 1) non-informative (AUC ≤ 0.5), 2) low accuracy (0.5 < AUC ≤ 0.7), 3) moderately accurate (0.7 < AUC ≤ 0.9), 4) highly accurate (0.9 < AUC < 1), and 5) perfect (AUC = 1).

Results

When comparing the diagnostic test results and IHC scores of the two TG subgroups, TG1 and TG2, we found no difference in mean IHC score (Mann-Whitney test, *P > 0.05*; **Table 3**).

When comparing the diagnostic accuracy of the six IHC markers (FoxP3, TNF-beta, ESEL, IDO, LACT, and TR) for differentiating between TG and NG, we found the following AUC values, in descending order: LACT (0.89), IDO (0.83), TNF-beta (0.70), ESEL (0.67), FoxP3 (0.63), and TR (0.60) (**Table 4**; **Figure 7A**). Among the six immunohistochemical markers, three (LACT, IDO, TNF-beta) were categorized as moderately accurate (0.7 < AUC ≤ 0.9) [12, 13].

When applied individually, four of the six analyzed markers (LACT, ESEL, TR, and IDO) had a sensitivity > 50%, ranging from 55.6% to 77.8%. With regard to specificity, four of the analyzed markers (LACT, IDO, FoxP3, and TNF-beta) had sufficient power to discriminate TG from NG when used alone, with specificities ranging from 71.0% to 100.0% (**Table 4**).

The combined predictor, which is the sum of the total score (TS) of the three markers (LAC + IDO + negative TNF-beta), slightly increased the overall accuracy into the moderate category (AUC = 0.9, sensitivity = 66.7%, and specificity = 96.8%; **Table 4**; **Figure 7B**).

Discussion

The pathogenesis of tuberculosis is fairly well established. Among people infected with *M.*

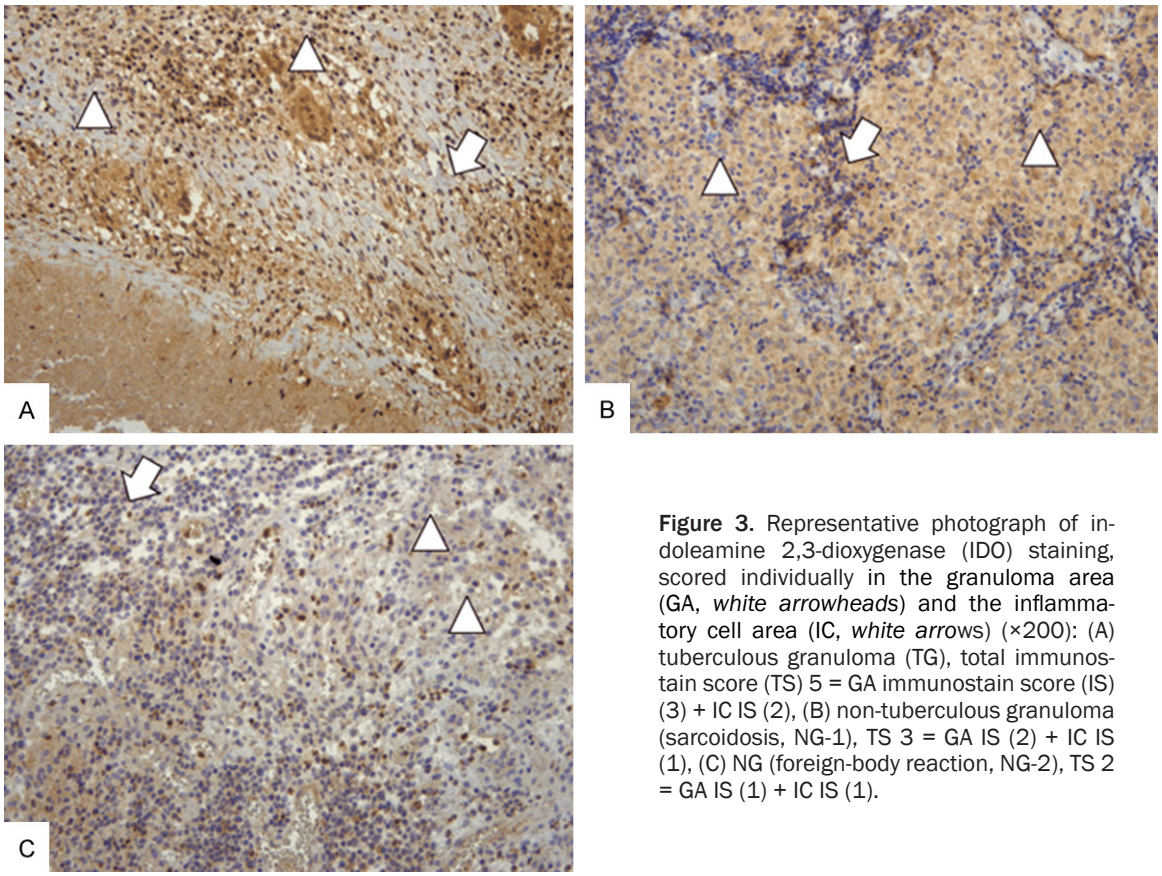

Figure 3. Representative photograph of indoleamine 2,3-dioxygenase (IDO) staining, scored individually in the granuloma area (GA, *white arrowheads*) and the inflammatory cell area (IC, *white arrows*) (×200): (A) tuberculous granuloma (TG), total immunostain score (TS) 5 = GA immunostain score (IS) (3) + IC IS (2), (B) non-tuberculous granuloma (sarcoidosis, NG-1), TS 3 = GA IS (2) + IC IS (1), (C) NG (foreign-body reaction, NG-2), TS 2 = GA IS (1) + IC IS (1).

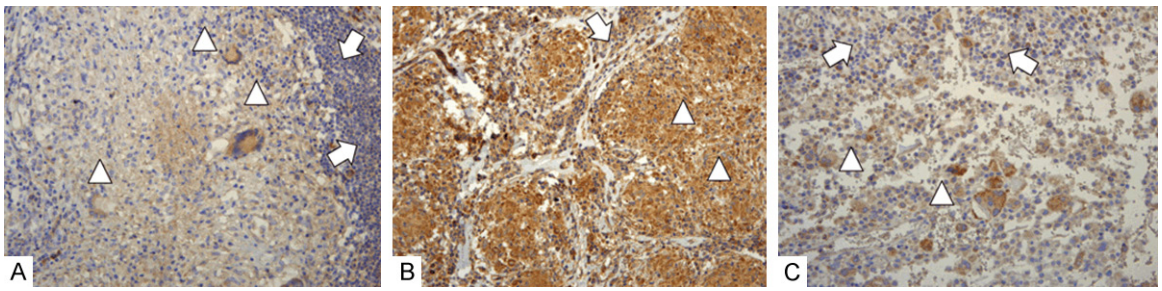

Figure 4. Representative photograph of E-selectin (ESEL) staining, scored individually in the granuloma area (GA, *white arrowheads*) and the inflammatory cell area (IC, *white arrows*) (×200): (A) tuberculous granuloma (TG), total immunostain score (TS) 2 = GA immunostain score (IS) (2) + IC IS (0), (B) non-tuberculous granuloma (sarcoidosis, NG-1), TS 5 = GA IS (3) + IC IS (2), (C) NG (foreign-body reaction, NG-2), TS 4 = GA IS (3) + IC IS (1).

tuberculosis, only about 10% are thought to have an active infection. In most cases, the host immune system can suppress growth and spread of *M. tuberculosis* so that the infection remains inactivate [14]. However, if the host's immune system weakens with age or becomes compromised, the infection can proceed to an active state.

Recurrent infections after treatment have the same pathogenetic pattern as first-time infections. In active tuberculosis, there are two well-understood key pathogenicity steps: 1) proliferation of CD4+ T-cells by IL-12 and 2) activation of phagocytes by IFN-gamma as a result of CD4+ T-cell proliferation, leading to engulfment of the *M. tuberculosis* organisms. Some other immune factors known to be involved in TB pathogenesis are CD4+ regulatory T-cells and CD8+ cytotoxic T-cells [14]. The role of B-cells in TB pathogenesis, however, is still not well understood. From an immunological perspec-

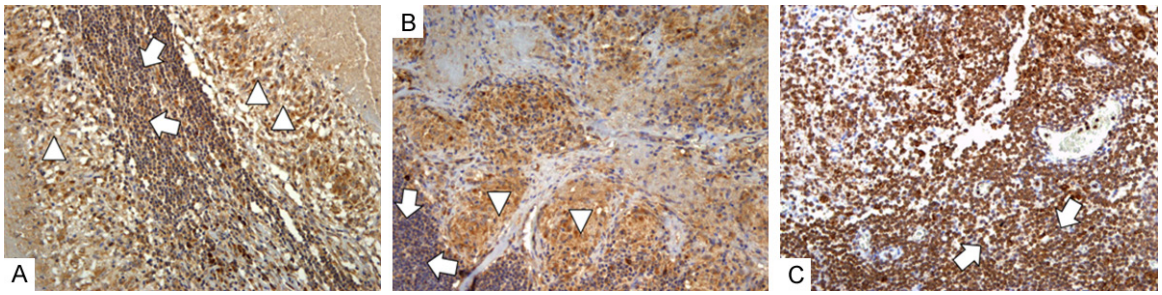

Figure 5. Representative photograph of TNF-beta staining, scored individually in the granuloma area (GA, *white arrowheads*) and the inflammatory cell area (IC, *white arrows*) (×200): (A) tuberculous granuloma (TG), total immunostain score (TS) 5 = GA immunostain score (IS) (3) + IC IS (2), (B) non-tuberculous granuloma (sarcoidosis, NG-1), TS 5 = GA IS (3) + IC IS (2), (C) NG (foreign-body reaction, NG-2), TS 5 = GA IS (2) + IC IS (3).

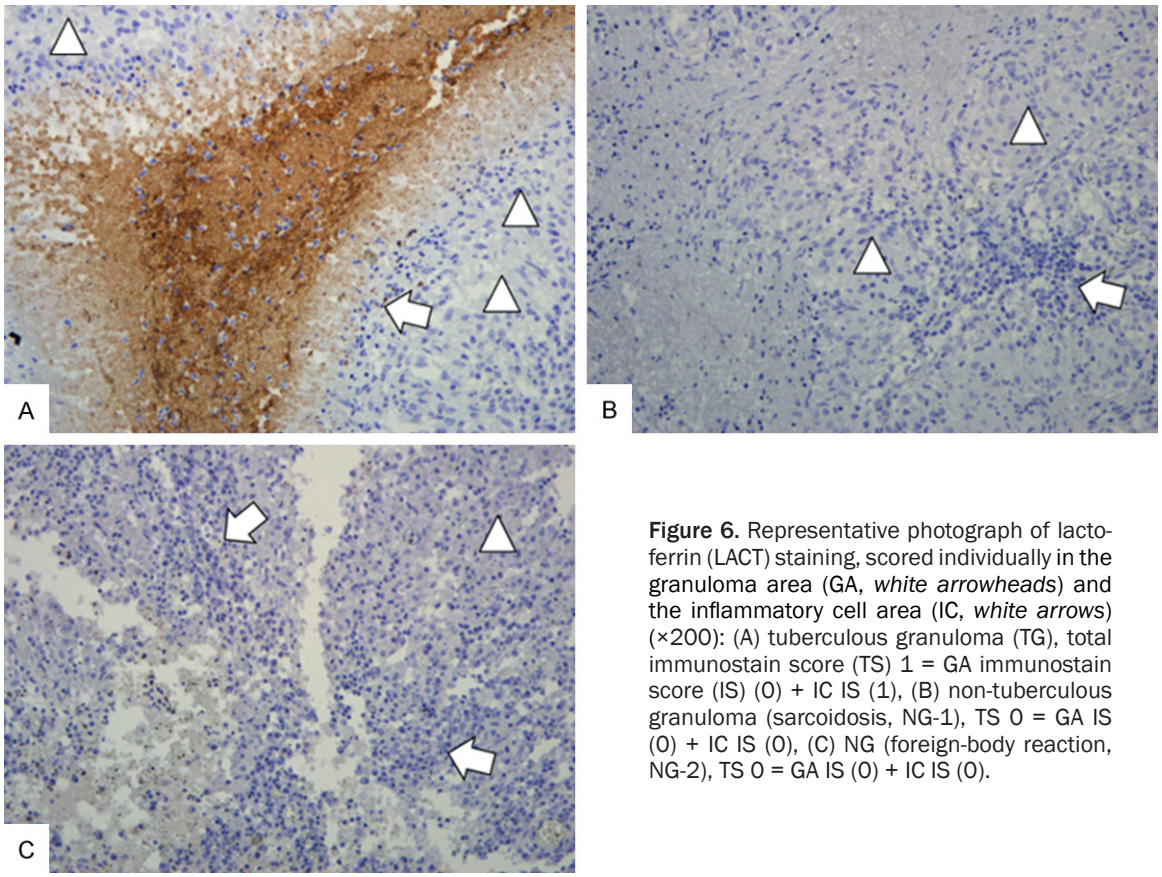

Figure 6. Representative photograph of lactoferrin (LACT) staining, scored individually in the granuloma area (GA, *white arrowheads*) and the inflammatory cell area (IC, *white arrows*) (×200): (A) tuberculous granuloma (TG), total immunostain score (TS) 1 = GA immunostain score (IS) (0) + IC IS (1), (B) non-tuberculous granuloma (sarcoidosis, NG-1), TS 0 = GA IS (0) + IC IS (0), (C) NG (foreign-body reaction, NG-2), TS 0 = GA IS (0) + IC IS (0).

tive, TB can be interpreted as a continuous immune reaction and response between the host and the pathogen [15, 16].

Clinically, there are two types of tuberculosis (TB): pulmonary TB (PTB) and extrapulmonary TB (EPTB) [1]. EPTB refers to TB that has spread to organs other than the lungs (e.g., pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, or meninges) [1, 2]. Diagnosing TB, especially EPTB, is challenging. The strictest standard for a definitive diagnosis of TB requires a tissue culture that is positive for *Mycobacterium tuberculosis* [1]. However, the low sensitivity (up to 40%) of conventional smear microscopy makes it impracticable to exclude a TB diagnosis solely based on the absence of TB on a conventional smear [3].

According to a recent review by Mehta et al., the various diagnostic tests currently used for EPTB diagnosis have several disadvantages [2]: 1) Smear microscopy sensitivity is low and highly variable (0-40%) [4, 5]. 2) Previous stud-

Table 2. Comparison of diagnostic test results and IHC scores of the two subgroups of tuberculous granuloma (TG, n = 36): confirmed TB (TG1, n = 12) and probable TB (TG2, n = 24)

Case No.	Subgroup	Sex	Age	Sites	Tests used for diagnosing extrapulmonary tuberculosis (EPTB)					*Total Score (TS) of IHC						
					Clinical symptoms of pulmonary TB	AFB test	Culture	PCR	†Resp.	FoxP3	IFN	TNF	ESEL	IDO	LACT	TR
Case 1	*confirmed*	F	43	pleura	*P*	*P*	N	N/A	Yes	2	2	2	2	2	2	2
Case 2	*confirmed*	M	26	lymph node	*P*	*P*	N	N/A	Yes	3	4	2	1	4	1	4
Case 3	*confirmed*	M	26	lung	*P*	*P*	N	N/A	Yes	6	6	4	3	3	0	5
Case 4	*confirmed*	F	29	lung	*P*	*P*	N	*P*	Yes	2	2	3	4	2	1	4
Case 5	*confirmed*	F	26	pleura	*P*	*P*	N	N/A	Yes	3	2	2	3	2	1	3
Case 6	*confirmed*	M	66	adrenal gland	*P*	*P*	N/A	N/A	Yes	4	6	2	2	2	1	5
Case 7	*confirmed*	M	35	lymph node	*P*	N	*P*	N/A	Yes	6	2	1	1	2	1	2
Case 8	*confirmed*	F	23	pleura	*P*	N	N	*P*	Yes	6	4	1	2	4	1	6
Case 9	*confirmed*	F	44	lymph node	No	*P*	N/A	N/A	Yes	6	5	4	2	3	0	5
Case 10	*confirmed*	F	29	lymph node	No	*P*	N	N	Yes	6	6	5	2	2	0	2
Case 11	*confirmed*	F	53	soft tissue	No	*P*	N	N/A	Yes	3	2	1	3	2	1	4
Case 12	*confirmed*	F	20	lymph node	No	N	*P*	N/A	Yes	2	2	1	1	2	1	2
Case 13	probable	F	51	uterus	*P*	N	N	N/A	Yes	2	2	1	2	2	1	2
Case 14	probable	M	19	lung	*P*	N	N/A	N/A	Yes	5	4	3	2	4	1	2
Case 15	probable	M	5	pleura	*P*	N	N	N	Yes	6	6	5	2	4	1	3
Case 16	probable	M	31	pleura	*P*	N	N	N	Yes	3	2	1	3	2	1	3
Case 17	probable	F	37	pleura	*P*	N	N	N/A	Yes	6	6	3	2	3	2	4
Case 18	probable	F	51	lymph node	*P*	N	N	N/A	Yes	4	4	2	3	4	1	4
Case 19	probable	M	31	kidney	*P*	N	N	N	Yes	5	4	2	2	4	1	2
Case 20	probable	F	63	lung	*P*	N	N/A	N/A	Yes	5	4	2	2	4	1	4
Case 21	probable	M	71	lung	*P*	N	N	N/A	Yes	3	3	2	1	3	1	2
Case 22	probable	M	54	pleura	*P*	N	N	N/A	Yes	5	6	4	2	3	0	3
Case 23	probable	M	18	pleura	*P*	N	N	N	Yes	6	3	3	2	3	2	6
Case 24	probable	M	35	soft tissue	*P*	N	N	N/A	Yes	3	3	1	2	3	1	6
Case 25	probable	M	70	kidney	*P*	N/A	N	N/A	Yes	6	5	4	3	4	0	4
Case 26	probable	F	36	lymph node	*P*	N/A	N/A	N/A	Yes	2	4	3	2	4	1	3
Case 27	probable	F	67	lymph node	No	N	N/A	N/A	Yes	2	6	4	3	2	1	3
Case 28	probable	F	63	bone	No	N	N	N/A	Yes	6	3	4	2	3	0	2
Case 29	probable	F	77	lymph node	No	N	N/A	N	Yes	3	4	1	3	3	1	5
Case 30	probable	M	35	epididymis	No	N	N	N	Yes	3	2	3	2	2	1	4
Case 31	probable	M	33	lymph node	No	N	N	N/A	Yes	3	4	2	2	4	2	5
Case 32	probable	F	55	lymph node	No	N	N	N/A	Yes	4	4	4	2	3	0	3
Case 33	probable	F	39	lymph node	No	N	N	N/A	Yes	3	2	2	1	2	0	5
Case 34	probable	M	29	lymph node	No	N/A	N	N/A	Yes	2	2	2	1	2	1	4
Case 35	probable	F	50	lymph node	No	N/A	N/A	N/A	Yes	3	2	2	3	2	2	2
Case 36	probable	M	21	lymph node	No	N/A	N/A	N/A	Yes	5	6	3	1	4	2	3

*TS of the six immunohistochemical markers (FoxP3, TNF, ESEL, IDO, Lac, TR) was the sum of semi-quantitative intensity scores (0, 1, 2, 3) in the inflammatory cell area (IC) and the granuloma area (GA) of the same lesion (TS = IC IHC score + GA score). †Resp. = clinical response to anti-TB medication; EPTB = extrapulmonary tuberculosis; AFB = acid-fast bacilli test; TNF = TNF-beta; ESEL = E-selectin; IDO = Indoleamine 2,3-dioxygenase-1; LACT = lactoferrin; TR = tartrate-resistant acid phosphatase; *P* = positive; N = negative; N/A = not available.

ies have shown that the sensitivity of *M. tuberculosis* culture-based diagnosis is also highly variable (0-80%) and can involve a relatively long turnaround time (generally 4-8 weeks) [6, 7].

Histopathologic examination plays an important role in EPTB diagnosis, wherein EPTB can be confirmed based on the presence of granulomatous inflammation and caseous necrosis [2, 8, 9]. However, histopathology is limited in its ability to differentiate between EPTB and other infectious or non-infectious etiologies that can cause granulomatous lesions, especially when caseous necrosis is not easily observed [2, 10].

One diagnostic test that has been widely incorporated into clinic settings to overcome these limitations is polymerase chain reaction (PCR).

Table 3. Distribution of IHC scores and conventional diagnostic test results (acid-fast bacilli stain, culture, PCR) of the two subgroups of tuberculous granuloma

Group	N	IHC total score (*TS)						Presence of pulmonary tuberculosis symptoms (%)	AFB† positivity (%)	Culture† positivity (%)	PCR† positivity (%)
		FoxP3	TNF	ESEL	IDO	LACT	TR				
TG	36	2-6	1-5	1-4	2-4	0-2	2-6	22/36	9/31 (29)	2/27 (7)	2/9 (22)
TG1	12	2-6	1-5	1-4	2-4	0-2	2-6	8/12 (67)	9/12 (75)	2/10 (20)	2/3 (67)
TG2	24	2-6	1-5	1-3	2-4	0-2	2-6	14/24 (58)	0/19 (0)	0/17 (0)	0/6 (0)
p-value‡		0.820	0.400	0.880	0.052	0.470	0.730				

*TS of the six immunohistochemical markers (FoxP3, TNF, ESEL, IDO, Lac, TR) was the sum of semi-quantitative intensity scores (0, 1, 2, 3) in the inflammatory cell area and granuloma area of the same lesion (TS = IHC score in IC + score in GR). †The acid-fast bacilli (AFB) test, culture, and PCR were not all performed in all cases. ‡Mann-Whitney test ($P < 0.05$). TNF = TNF-beta; ESEL = E-selectin; IDO = Indoleamine 2,3-dioxygenase-1; LACT = lactoferrin; TR = tartrate-resistant acid phosphatase.

Table 4. Predictive and cut-off values of six immunohistochemical markers, individually and in combination, to differentiate tuberculous granuloma and non-tuberculous granuloma (ROC curve analysis)

Comparisons	Marker	AUC	SE[a]	95% CI[b]	Sensitivity	Specificity	Cut-off
Tuberculous granuloma (TG) vs. Non-tuberculous granuloma (NG)	*LACT*	0.89	0.04	0.788 to 0.953	77.8	100.0	> 0
	IDO	0.83	0.04	0.721 to 0.912	58.3	90.3	> 2
	TNF	0.70	0.06	0.574 to 0.804	55.6	71.0	≤ 2
	ESEL	0.67	0.06	0.540 to 0.776	72.2	58.1	≤ 2
	FoxP3	0.63	0.07	0.508 to 0.749	41.7	83.9	> 4
	TR	0.60	0.07	0.470 to 0.715	72.2	48.4	> 2
	*Combination	0.90	0.04	0.798 to 0.958	66.7	96.8	> 0

Italicized predictors (Lac, IDO, and TNF-beta) and combined predictors (Combination*) were categorized as "moderately accurate" markers ($0.7 < AUC \leq 0.9$), according to Greiner et al. [12]. *Combination: Sum of the scores of the three markers (= sum of total scores [†TS]s of LAC + IDO + negative TNF-beta). †TS of the six immunohistochemical markers (FoxP3, TNF, ESEL, IDO, Lac, TR) was the sum of semi-quantitative intensity scores (0, 1, 2, 3) in the inflammatory cell area (IC) and the granuloma area (GA) of the same lesion (TS = IC IHC score + GA score). Cut-off: Score of immunohistochemical stain used as the cut-off value. LACT = lactoferrin; IDO = Indoleamine 2,3-dioxygenase-1; TNF = TNF-beta; ESEL = E-selectin; TR = tartrate-resistant acid phosphatase; AUC = area under the ROC curve; SE = standard error.

Although the diagnostic accuracy of PCR varies depending on the type of PCR, target gene, etc., it appears to have greater accuracy than other diagnostic tests [2].

Another new diagnostic approach for TB (especially EPTB) is immunohistochemistry using a monoclonal antibody to *Mycobacterium tuberculosis* (anti-MPT64). This test has shown sensitivity and specificity of 90% and 83%, respectively, which are sufficient to allow the test to serve as a valuable ancillary test [11].

However, studies on immunohistochemical markers, which are thought to be related to the pathogenesis of granulomatous inflammation, are lacking. Therefore, in this study, we analyzed whether there is a difference in expression of immunohistochemical markers between TG and NG. We also evaluated the feasibility of six immunohistochemical markers for use in an ancillary test to differentiate between tuberculous granuloma (TG) and non-tuberculous granuloma (NG).

There has recently been an increased interest in biological markers of TB, which can be useful not only for early detection, but also for determining the treatment response [17-21]. In latent and active TB cases, in addition to the proliferation of CD4+ T-cells, IFN-gamma, IL-2, TNF, and IL-17 numbers also increase in serum [21-24]. In a recent study, messenger RNA expression of IL-8, FOXP3, and IL-12 beta was used to differentiate between latent and active tuberculosis [25]. However, according to that study, the IL-8/FOXP3/IL-12 beta levels should be interpreted as combination results, and the time sequence of the infection should be considered (i.e., whether the patient was newly diagnosed or had experienced at least short-term treatment).

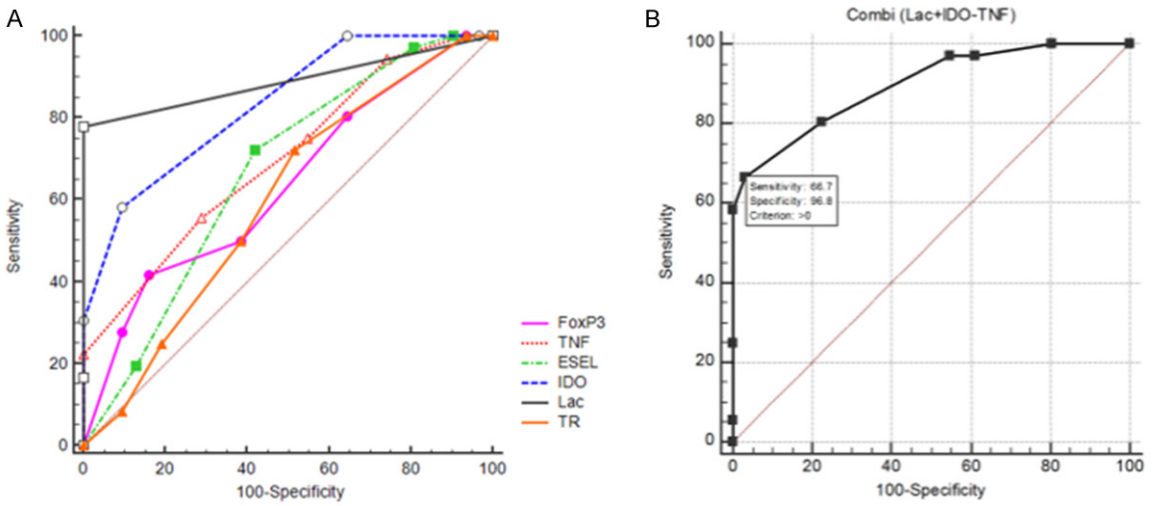

Figure 7. Receiver operating characteristic (ROC) curves of each of the six immunohistochemical markers, individually (A) and in combination (B): (A) ROC analysis of each of the six antibodies: The area under the curve (AUC) represents an optimal summary statistic for comparing the sensitivities and specificities of immunohistochemistry (IHC) for each of the four antibodies (see **Table 4**, column 3) for differentiating between tuberculous granuloma (TG) and non-tuberculous granuloma (NG). The six IHC markers are listed in descending AUC order: Lac (0.89), IDO (0.83), TNF (0.70), ESEL (0.67), FoxP3 (0.63), and TR (0.60). (B) The ROC curve of the combined predictor (designated as Combination* in **Table 2**), which was defined as the sum of the scores of three individual markers (LAC + IDO + negative TNF), shows increased accuracy compared to all the individual curves (AUC = 0.9, sensitivity = 66.7%, and specificity = 96.8%) and improves the test to a moderate level of accuracy. TNF = TNF-beta; ESEL = E-selectin; IDO = Indoleamine 2,3-dioxygenase; Lac = lactoferrin; TR = tartrate-resistant acid phosphatase.

A recent study by Suzuki et al. found that IDO activity was associated with pathogenesis of tuberculous pleurisy, which implicates IDO as a biomarker of active TB [26]. In another recent study, tartrate-resistant acid phosphatase (TR) was associated with multi-nucleated giant cells. The expression pattern on immunohistochemistry was shown to be useful as a marker to differentiate TB from other chronic granulomatous inflammations, especially sarcoidosis [27].

In addition to our experimental work, we also conducted a review of the current research on each of our selected biomarkers. Wu et al. have found that the expression of three genes, IL-8, FOXP3, and IL-12, was predictive of active TB versus latent Mycobacterial infection. Thus, measurement of Ag-specific expression of these three genes might offer a specific and noninvasive means to differentiate between latent *Mycobacterium tuberculosis* infection and active TB [25].

Aung et al. studied the expression of several cytokines, including transforming growth factor (TNF)-alpha, TNF-beta, interferon (IFN)-gamma, and interleukin (IL)-4, using immunohistochemical staining of lung tissue from tuberculosis patients [28]. They found that only TNF-beta expression was elevated in granulomatous pulmonary lesions, suggesting an important role of TGF-beta 1 in TB immunopathology. Interestingly, in our analyses, TNF-beta expression was lower in tuberculous necrosis cases than in non-tuberculous granuloma cases. We interpreted this result as reflecting different cytokine expression between TG and NG in the lesions (**Table 2**, the cut-off value was ≤ 2).

In various inflammatory conditions, including tuberculosis, ESEL is known to be elevated and, thus, involved in pathogenesis mechanisms [29]. Interestingly, we found that ESEL expression was lower in the tuberculous necrosis (TG) cases than in the non-tuberculous granuloma (NG) cases. We interpreted this as reflecting different cytokine expression between TG and NG in the lesions (**Table 2**, the cut-off value was ≤ 2).

Suzuki et al. demonstrated that pleural tissue from TBP had enhanced IDO expression in the epithelioid granuloma regions, according to

immunohistochemistry results [26]. In our data, IDO expression values were as follows: AUC = 0.83, sensitivity = 58.3%, specificity = 90.3%, with a cut-off value > 2 (**Table 4**; **Figure 7A**). These results suggest that IDO is a valuable tool in ancillary testing to discriminate TG from NG, with the limitation that it demonstrates relatively low sensitivity (58.3%).

LACT is a transporter molecule with a high affinity for iron and is known to modulate host defenses by competing with microbes for iron [17, 30]. In a study using a murine model, a direct effect of LACT on *M. tuberculosis* infection was observed, in which correction of an iron overload by LACT inverted increased susceptibility to TB [17, 31]. LACT functions as a key component of the mammalian host defense. In our data, LACT expression values were as follows: AUC = 0.89, sensitivity = 77.8%, specificity = 100%, with a cut-off value > 0 (**Table 4**; **Figure 7A**). However, considering the relatively weak expression (i.e., the range of TS in TG of 0-2 and interobserver discrepancies with IHC), the expression difference is not sufficient to consider using lactoferrin in an independent discriminatory test.

Conclusion

We demonstrated that selected immunohistochemical markers (LACT, IDO and TNF-beta) can be used in ancillary tests to differentiate between TG and NG with formalin-fixed paraffin-embedded tissue samples (AUC = 0.7-0.89, sensitivity = 55.6-77.8%, specificity = 71.0-100%). Further studies are required to validate our results.

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Disclosure of conflict of interest

None.

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