TRANSFORMING GROWTH FACTOR-β SIGNALING IS UPREGULATED IN SPORADIC INCLUSION BODY MYOSITIS

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ABSTRACT: *Introduction:* In this study we aimed to determine whether transforming growth factor-β (TGF-β) signaling is dysregulated in sporadic inclusion body myositis (sIBM) muscle samples. *Methods:* We examined TGF-β signaling markers in muscle samples from 24 sIBM patients and compared them with those from 10 dermatomyositis (DM) patients using immunohistochemistry and Western blot analyses. *Results:* Compared with the DM muscle fibers, the sIBM muscle fibers exhibited greater TGF-β, TGF-β receptor type I (TβRI), and TGF-β receptor type II (TβRII) immunoreactivity in the cytoplasm, as well as greater phosphorylated Smad2 (pSmad2) immunoreactivity in the myonuclei. The signal intensities of TGF-β, TβRI, and TβRII immunoreactivity correlated significantly with muscle fiber cross-sectional areas. Western blot analyses indicated higher expression levels of TGF-β, TβRI, TβRII, and pSmad2 in the sIBM muscle samples than in the DM muscle samples. *Conclusions:* These data indicate that upregulation of TGF-β signaling may be an important molecular event in sIBM.

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Although polymyositis (PM), dermatomyositis (DM), necrotizing myopathy, sporadic inclusion body myositis (sIBM), and non-specific myositis have been classified as major inflammatory myopathies, these entities exhibit clinically distinct characteristics.[1,2] sIBM appears to be the most common age-associated muscle disease in individuals >50 years old, and it accounts for 30% of all inflammatory myopathies.[3,4] In Japan, the number of sIBM patients is approximately 1,250[5] and continues to increase as the size of the elderly population increases.[6] Several mechanisms for sIBM have been proposed, such as β-amyloid (Aβ) accumulation, immune system abnormalities, viral infection, and genetic susceptibilities.[3,4] The identification of Aβ accumulation has raised the possibility that sIBM may be a primary degenerative muscle disorder,[7,8] but there are other opinions.[9] It is not known which, if any, of these mechanisms play a central role in the etiopathogenesis of sIBM.

Muscle biopsies of individuals with sIBM characteristically reveal endomysial inflammation, small groups of atrophic fibers, and muscle fibers with rimmed vacuoles lined with granular material.[10,11] These pathological features indicate both degenerative and inflammatory processes. The degenerative process results in atrophy of muscle fibers, vacuolar degeneration, and accumulation of multiple proteins in vacuolated or non-vacuolated muscle fibers.[7,12] The inflammatory process is characterized by invasion of muscle fibers expressing major histocompatibility complex (MHC)-I by CD8-positive lymphocytes.[13]

Transforming growth factor-β (TGF-β) plays several important roles in developmental, physiological, and pathological processes.[14,15] TGF-β binds to its transmembrane receptor, a serine/threonine kinase complex that contains both TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) subunits.[14] Ligand interaction with a homodimer of TβRII recruits and activates TβRI, which in turn phosphorylates the receptor-regulated Smad proteins (Smad2 and 3).[15] Then, phosphorylated Smad2/3 (pSmad2/3) translocates to the nucleus, where these phosphoproteins regulate transcription of their target genes.[15] Dysregulation of TGF-β signaling critically contributes to the pathogenesis of a range of neurodegenerative diseases, including Alzheimer disease (AD)[16,17] and motor neuron diseases.[18,19]

The TGF-β signaling cascade, including TβRI and TβRII, has not been studied in detail in sIBM

Additional Supporting Information may be found in the online version of this article.

Abbreviations: Aβ, β-amyloid; AD, Alzheimer disease; BMP1, bone morphogenetic protein 1; CK, creatine kinase; DAB, diaminobenzidine; DM, dermatomyositis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; MHC-I, histocompatibility complex I; MRC, Medical Research Council; NADH, nicotinamide adenine dinucleotide hydrogen; PBS, phosphate-buffered saline; pSmad2/3, phosphorylated Smad2/3; pSmad1/5/8, phosphorylated Smad1/5/8; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; sIBM, sporadic inclusion body myositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II

Key words: degenerative disease; dermatomyositis; inflammatory myopathy; phosphorylated Smad2; sporadic inclusion body myositis; transforming growth factor-β; signaling

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muscle samples. In this study, we examined whether TGF-β signaling is dysregulated in sIBM muscle samples compared with DM muscle samples.

METHODS

We examined consecutive muscle biopsy samples from 24 sIBM patients (17 men and 7 women) who were referred to our institution for a diagnostic muscle biopsy between 2011 and 2014. According to the European Neuromuscular Centre IBM research diagnostic criteria,[20] 10 patients were diagnosed with clinically and histologically defined inclusion body myositis (IBM), and 14 patients were diagnosed with clinically defined IBM. These patients were diagnosed by clinical examination and muscle biopsy. No patient was aware of any family history of sIBM.

Muscle samples taken from 10 DM patients (4 men and 6 women) were tested as disease controls. According to the criteria of Dalakas and Hohlfeld,[21] 5 patients were diagnosed with definite DM, and 5 patients were diagnosed with probable DM. No skin rash was present in the 5 probable DM patients. We diagnosed these patients according to other clinical signs or muscle biopsy findings.

This study was approved by the medical ethics committee of Nagoya University Graduate School of Medicine, and informed consent was obtained from all patients who participated.

Muscle biopsies were performed as previously described.[22–24] Muscle biopsies were taken from a clinically involved muscle, which in most cases was the biceps muscle of the arm or the vastus lateralis of the quadriceps muscle. After excision, the biopsy specimens were immediately frozen in isopentane, chilled on dry ice, and stored at –80°C until further use. Serial transverse 10-μm-thick sections were cut with a freezing microtome at –25°C. For histological examination, the sections were stained with hematoxylin and eosin, modified Gomori trichrome, nicotinamide adenine dinucleotide hydrogen (NADH)–tetrazolium reductase, routine adenosine triphosphatase, Congo red, succinate dehydrogenase, and cytochrome *c* oxidase.

Immunohistochemistry was performed as previously described.[22–24] Serial transverse 10-μm-thick sections of the muscle samples were air dried and fixed in ice-cold acetone for 3 minutes. The samples were incubated with normal horse serum or normal goat serum for 60 minutes, followed by incubation with primary antibodies. After washing with phosphate-buffered saline (PBS), the sections were incubated with biotinylated horse anti-mouse immunoglobulin G (IgG) or biotinylated rabbit anti-goat IgG (Vector Laboratories) for 60 minutes. Next, the sections were washed extensively, exposed to avidin–biotin complex (Vector Laboratories) for 60 minutes, and then covered with diaminobenzidine (DAB) for 10 minutes.

The following primary antibodies were used for immunohistochemistry: anti–HLA-ABC (555551, Becton-Dickinson, 1:6,000); anti-CD8 (M0707, Dako, 1:150); anti-ubiquitin (Z0458, Dako, 1:1,000); anti–pan-specific TGF-β (AB-100-NA, R&D Systems, 1:1,000); anti-TβRI (RB-10455, Thermo Scientific, 1:1,000); anti-TβRII (RB-10345, Thermo Scientific, 1:1,000); anti-pSmad2 (AB3849, Millipore, 1:1,000); anti–bone morphogenetic protein 1 (BMP1; ab118520, Abcam, 1:1,000); and anti-pSmad1/5/8 (9511, Cell Signaling Technology, 1:1,000).

Muscle biopsy specimens from 3 patients without clinical or histological evidence of myopathy served as normal controls.

For quantitative analysis of immunohistochemistry, randomly chosen sections containing at least 500 individual muscle fibers were used to determine the signal intensities of TGF-β, TβRI, and TβRII immunoreactivity in the immunostained muscle fibers. We did not include the immunoreactivity of fat or connective tissue for the quantitative study. The signal intensities and muscle fiber cross-sectional areas were analyzed using ImageJ software (NIH, Bethesda, Maryland).

Western blot analyses were performed as described in previous studies.[18,25] Approximately 50 mg of muscle sample was homogenized in Cel-Lytic lysis buffer (Sigma-Aldrich) containing a phosphatase inhibitor mixture (Sigma-Aldrich) and a protease inhibitor mixture (Thermo Scientific). The homogenates were centrifuged at 2,500*g* for 15 minutes at 4°C. The supernatant fractions were separated on 5%–20% sodium dodecylsulfate–polyacylamide gel electrophoresis (SDS-PAGE) gels and then transferred to Hybond-P membranes (GE Healthcare) using 25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS, and 10% methanol as transfer buffer. Primary and secondary antibodies were diluted with Can Get Signal, a signal enhancer solution (NKB-101, Toyobo). The Western blot bands were digitized (LAS-3000 Imaging System, Fujifilm), and the signal intensities of independent blots were quantified with Image Gauge software, version 4.22 (Fujifilm). Random homogeneous areas around the Western blot bands were selected to serve as background signals. Primary antibody binding was probed using horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) at a dilution of 1:1,000, and the bands were detected using an ECL Plus kit (GE Healthcare). The membranes were reprobed with antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to control for protein loading.

The following primary antibodies were used for Western blot analyses: anti–pan-specific TGF-β

(AB-100-NA, R&D Systems, 1:1,000); anti-TβRI (RB-10455, Thermo Scientific, 1:1,000); anti-TβRII (RB-10345, Thermo Scientific, 1:1,000); anti-pSmad2 (AB3849, Millipore, 1:1,000); anti-BMP1 (ab118520, Abcam, 1:1,000); anti-pSmad1/5/8 (9511, Cell Signaling Technology, 1:1,000); and GAPDH (MAB374, Millipore, 1:5,000).

Quantitative data are presented as mean ± standard deviation. Statistical comparisons were performed using the Student *t*-test or the Welch *t*-test, as appropriate. Differences were considered significant at $P < 0.05$. A regression analysis was used to assess correlations between TGF-β signaling markers and clinicopathological features of the sIBM patients.

RESULTS

The clinicopathological features of the 24 sIBM patients are listed in Table S1 (refer to Supplementary Material, available online). The mean age at onset was 66.4 ± 7.3 (range 51–84) years, and the mean age at biopsy was 69.8 ± 6.8 (range 55–86) years. The mean disease duration before biopsy was 39.9 ± 24.4 (range 10–105) months. The mean Medical Research Council (MRC) grade of the biopsy site was 3.1 ± 1.0 (range 1–5). The mean serum creatine kinase (CK) level was 515 ± 260 U/L (range 127–1,075 U/L). The mean percentage of fibers with rimmed vacuoles was 4.9 ± 3.5% (range 0.9%–15.0%). The clinical and pathological features of the 10 DM patients are also listed in Table S1. The mean age at onset was 57.6 ± 15.7 (range 52–84) years, and the mean age at biopsy was 58.2 ± 15.9 (range 32–71) years. The mean disease duration before biopsy was 6.8 ± 6.8 (range 1–24) months. The major clinical features of the sIBM and DM groups were equivalent statistically, except that the disease duration was longer in the patients with sIBM (see Table 1).

The immunohistochemical findings of the sIBM and DM muscle fibers are shown in Figure 1. TGF-β was not expressed on the collagen fibers or perimysium of the sIBM or DM muscle specimens.

Table 1. Comparison of sIBM and DM patients

Characteristics	sIBM (*n* = 24)	DM (*n* = 10)	*P*-value
Age at onset (years) (mean ± SD)	66.4 ± 7.3	57.6 ± 15.7	0.14
Age at diagnosis (years) (mean ± SD)	69.8 ± 6.8	58.2 ± 15.9	0.06
Gender (% men)	70.8	40.0	0.10
Duration (months) (mean ± SD)	39.9 ± 24.4	6.8 ± 6.8	<0.005
CK (U/L) (mean ± SD)	515 ± 260	3219 ± 3594	0.05

sIBM, sporadic inclusion body myositis; DM, dermatomyositis; CK, creatine kinase.

The percentage of myonuclei in the sIBM muscle fibers that had pSmad2 immunoreactivity was 15.1 ± 12.1% (range 4.3%–46.7%). In contrast, pSmad2 immunoreactivity in the DM myonucluei was weak and unsuitable for quantification. Immunoreactivities of TGF-β, TβRI, and TβRII were observed in the cytoplasm of the sIBM muscle fibers with and without rimmed vacuoles. Compared with the DM and normal muscle fibers, the sIBM muscle fibers exhibited greater TGF-β, TβRI, and TβRII immunoreactivity in the cytoplasm (Fig. 2), as well as greater pSmad2 immunoreactivity in the myonuclei.

The correlations between signal intensities and muscle fiber cross-sectional areas in the sIBM muscle fibers are shown in Figure 3. In the cytoplasm of the sIBM muscle fibers, the signal intensities of TGF-β, TβRI, and TβRII immunoreactivity correlated strongly with muscle fiber cross-sectional areas. The immunoreactivities of BMP1 and phosphorylated Smad1/5/8 (pSmad1/5/8), the downstream signaling molecules regulated by BMP,[14] were similar between the sIBM and DM muscle fibers.

The results of the Western blot analyses are shown in Figure 4. A significant increase in TGF-β, TβRI, TβRII, and pSmad2 expression was found in the sIBM muscle samples compared with the DM muscle samples. In contrast, the sIBM and DM muscle samples did not show significant differences with regard to expression of BMP-1 and pSmad1/5/8.

The correlations between the expression of TGF-β signaling markers and disease duration before biopsy in the sIBM and DM patients are shown in Figure 5. The expression levels of pSmad2 and disease duration before biopsy were strongly correlated in the sIBM patients. In contrast, no correlation was observed in the DM patients. According to the regression analysis, there was no correlation between expression of other TGF-β signaling markers (TGF-β, TβRI, TβRII, or pSmad2) and clinicopathological features (age at onset, age at diagnosis, MRC grade of the biopsy site, serum CK levels, or percentage of fibers with rimmed vacuoles) in the sIBM patients. The results were similar in the DM patients (data not shown).

DISCUSSION

Although sIBM is classified as a major inflammatory myopathy, some studies have suggested that non-inflammatory mechanisms also participate in its pathogenesis.[7] Whereas the immunotherapies for sIBM have only a transient or no beneficial effect,[26] degenerative histopathological features exist and clearly distinguish sIBM from other forms of myositis.[1,4]

Various studies have shown that TGF-β signaling participates in the pathogenesis of

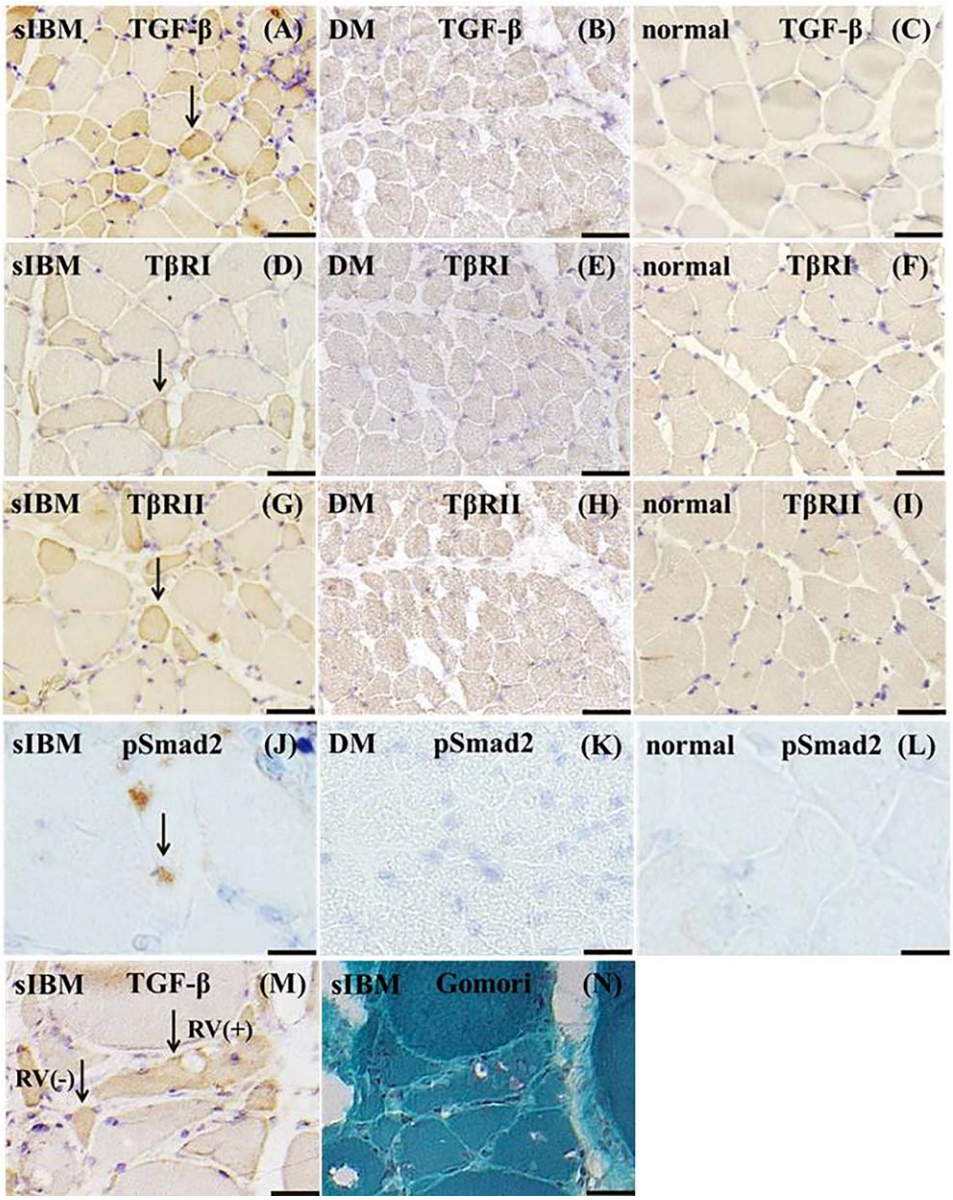

FIGURE 1. Immunohistochemical stains of muscle biopsies from an sIBM patient **(A, D, G, J)**, another sIBM patient **(M, N)**, a DM patient **(B, E, H, K)**, and a normal patient **(C, F, I, L)**. Stains include: TGF-β **(A–C, M)**; TβRI **(D–F)**; TβRI **(G, H, I)**; and pSmad2 **(J–L)**. Modified Gomori trichrome stain **(N). (M)** and **(N)** are consecutive sections. Immunoreactivity is indicated by black arrows **(A, D, G, J, M). (A–I)** Scale bars = 50 μm; **(J–N)** scale bars = 25 μm. sIBM, sporadic inclusion body myositis; DM, dermatomyositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II; pSmad2, phosphorylated Smad2; RV, rimmed vacuoles.

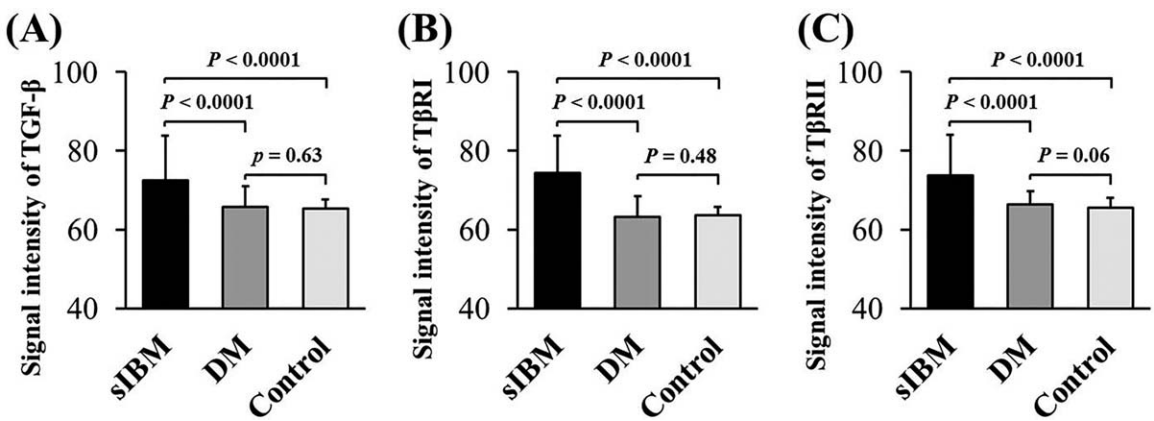

FIGURE 2. Signal intensities in sIBM, DM, and normal muscle fibers. Compared with the DM and normal muscle fibers, the sIBM muscle fibers exhibited greater TGF-β (DM, $P < 0.0001$; normal, $P < 0.0001$), TβRI (DM, $P < 0.0001$; normal, $P < 0.0001$), and TβRII (DM, $P < 0.0001$; normal, $P < 0.0001$) immunoreactivity. **(A)** TGF-β. **(B)** TβRI. **(C)** TβRII. sIBM, sporadic inclusion body myositis; DM, dermatomyositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II.

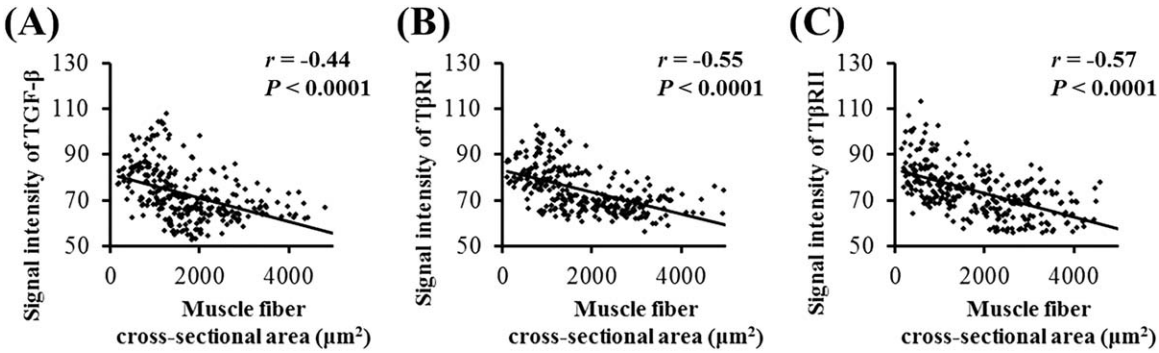

FIGURE 3. Correlations between signal intensities and muscle fiber cross-sectional areas. In the cytoplasm of the sIBM muscle fibers, the signal intensities of TGF-β, TβRI, and TβRII immunoreactivity correlated strongly with muscle fiber cross-sectional areas (TGF-β, $r = -0.44$, $P < 0.0001$; TβRI, $r = -0.55$, $P < 0.0001$; TβRII, $r = -0.57$, $P < 0.0001$). **(A)** TGF-β. **(B)** TβRI. **(C)** TβRII. sIBM, sporadic inclusion body myositis; DM, dermatomyositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II.

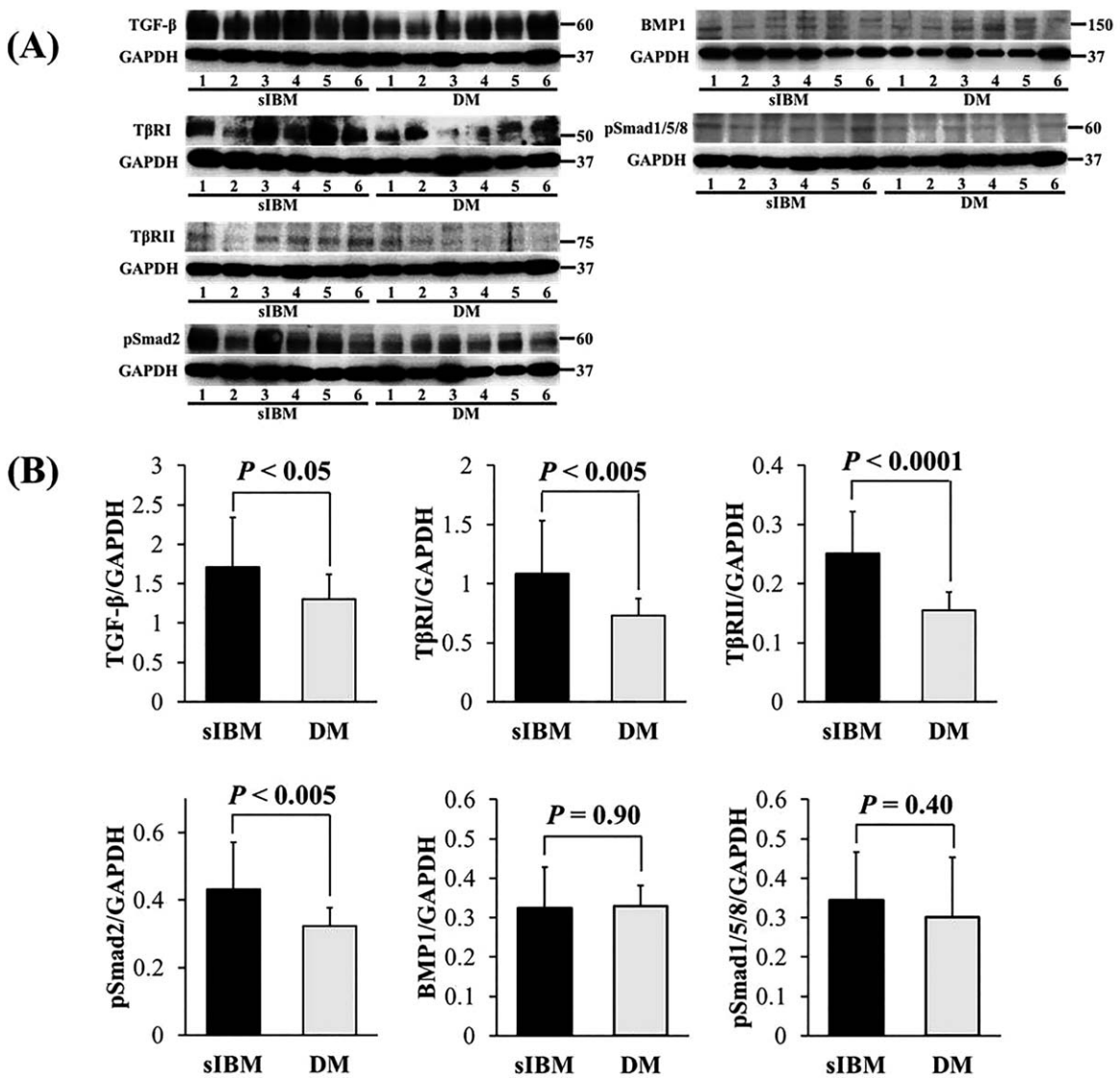

FIGURE 4. Western blot analyses of TGF-β signaling markers, including TGF-β, TβRI, TβRII, pSmad2, BMP1, and pSmad1/5/8. A significant increase in TGF-β, TβRI, TβRII, and pSmad2 expression was found in the sIBM muscle samples (TGF-β/GAPDH, 1.71 ± 0.64; TβRI/GAPDH, 1.09 ± 0.45; TβRII/GAPDH, 0.25 ± 0.07; pSmad2/GAPDH, 0.43 ± 0.14) compared with the DM muscle samples (TGF-β/GAPDH, 1.30 ± 0.32, $P < 0.05$; TβRI/GAPDH, 0.73 ± 0.14, $P < 0.005$; TβRII/GAPDH, 0.16 ± 0.03, $P < 0.0001$; pSmad2/GAPDH, 0.32 ± 0.05, $P < 0.005$). In contrast, the sIBM and DM muscle samples did not exhibit significant differences regarding the expression of BMP1 [BMP1/GAPDH, 0.32 ± 0.10 (sIBM) vs. 0.33 ± 0.05 (DM); $P = 0.90$] and pSmad1/5/8 [pSmad1/5/8/GAPDH, 0.34 ± 0.12 (sIBM) vs. 0.30 ± 0.15 (DM); $P = 0.40$]. **(A)** Representative autoradiograms. **(B)** Densitometric quantification of the Western blotting results. sIBM, sporadic inclusion body myositis; DM, dermatomyositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II; pSmad2, phosphorylated Smad2; BMP1, bone morphogenetic protein 1; pSmad1/5/8, phosphorylated Smad1/5/8; GAPDH; glyceraldehyde 3-phosphate dehydrogenase.

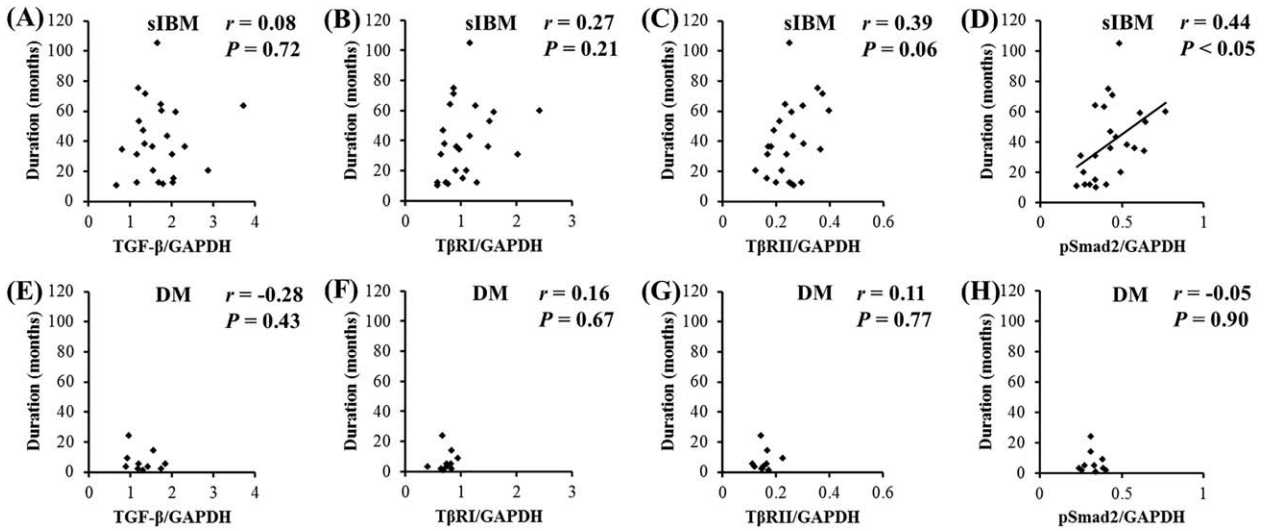

FIGURE 5. Correlations between the expression of TGF-β signaling markers and disease duration before biopsy of the sIBM (**A–D)** and DM **(E–H)** patients. The expression levels of pSmad2 and disease duration before biopsy correlated strongly in the sIBM patients ($r = 0.44$, $P < 0.05$). **(A, E)** TGF-β. **(B, F)** TβRI. **(C, G)** TβRII. **(D, H)** pSmad2. sIBM, sporadic inclusion body myositis; DM, dermatomyositis; TGF-β, transforming growth factor-β; TβRI, TGF-β receptor type I; TβRII, TGF-β receptor type II; pSmad2, phosphorylated Smad2.

neurodegenerative diseases.[18,19,27] TGF-β has been found to exacerbate vascular pathology in AD[28] and aggravate neurodegeneration in amyotrophic lateral sclerosis (ALS).[29] We hypothesize that the dysregulation of TGF-β signaling represents the cellular response initiated to cope with myodegeneration in sIBM.

We observed via immunohistochemistry and Western blot analyses that the sIBM muscle samples exhibited increased expression levels of TGF-β, TβRI, TβRII, and pSmad2 compared with DM muscle samples. Compared with the DM muscle fibers, the sIBM muscle fibers exhibited greater TGF-β, TβRI, and TβRII immunoreactivity in the cytoplasm and greater pSmad2 immunoreactivity in the myonuclei, which suggests that dysregulation of TGF-β signaling appears to underlie the degenerative process in sIBM.

These results show that TGF-β activation was associated with muscle atrophy in sIBM. Dysregulation of TGF-β signaling has been implicated in various pathological conditions that affect skeletal muscle,[30] and several members of the TGF-β family have been shown to play major roles in regulating muscle growth and atrophy.[31] Abnormal TGF-β signaling has also been linked to several forms of muscular dystrophy.[32–34] Because the degenerative process results in muscle fiber atrophy, an increase in TGF-β signaling may be related to muscle atrophy in sIBM.

In addition, TGF-β is a cytokine with well-documented roles in promoting inflammation and fibrosis.[35,36] After injury to skeletal muscles, TGF-β expression increases.[37] Inhibiting TGF-β promotes early recovery of muscle function.[38] The biological effects of TGF-β may promote the inflammatory process and subsequent suppression of muscle regeneration in sIBM. These insights suggest that successful manipulation of the myoprotective and myotoxic properties of TGF-β may contribute to development of therapies for sIBM.

We also showed that the expression levels of pSmad2 and disease duration before biopsy were strongly correlated in the sIBM patients. There is a possibility that the longer the disease duration, the more pSmad2 is expressed in the sIBM patients. Not only are the levels of Smads in the nucleus important, but the length of time Smads reside in the nucleus is also significant.[39] Further study is needed to determine whether these findings are relevant.

The molecular basis of the trigger for onset of sIBM is unknown, and the exact downstream cellular mechanisms of TGF-β signaling in sIBM have remained elusive. Recently, Amato *et al.*, found that muscle pSmad2/3 expression was higher in patients with sIBM than in those with other muscle diseases. They also showed that sIBM patients treated with BYM338, which prevents the binding of ligands to activin type receptor II, improved their 6-minute walking distance, which peaked at 16 weeks, compared with patients treated with a placebo.[40] Because we found that the expression levels of TGF-β signaling markers were higher in the sIBM patients than in the DM patients, we expect positive results in the trial of BYM338 (NCT01925209).

Our findings indicate that TGF-β signaling may play a role in the pathogenesis of myodegeneration in sIBM. Upregulation of TGF-β signaling in

sIBM patients may produce an unfavorable environment for muscle fibers. Greater attention should be paid to the active role of TGF-β signaling in sIBM, and additional studies are needed to clarify the causes of the alterations in TGF-β signaling in sIBM.

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