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RESEARCH ARTICLE

# Roles of TGF-β/Smad signaling pathway in pathogenesis and development of gluteal muscle contracture

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**Abstract**

*Purpose of the study*: Gluteal muscle contracture (GMC) is a chronic fibrotic disease of gluteal muscles which is characterized by excessive deposition of collagen in the extracellular matrix. Transforming growth factor (TGF)-βs have been shown to play an important role in the progression of GMC. However, the underlying mechanisms are not entirely clear. We sought to explore the expression of TGF-β/Smad pathway proteins and their downstream targets in gluteal muscle contracture disease.

*Materials and methods*: The expression levels of collagens type I/III, TGF-β1, Smad2/3/4/7 and PAI-1 (plasminogen activator inhibitor type 1) in gluteal muscle contraction (GMC) patients were measured using immunohistochemistry, reverse transcription and polymerase chain reaction (RT-PCR) and western blot assays.

*Results*: The expressions of collagens type I/III and TGF-β1 were significantly increased in the contraction band compared with unaffected muscle. In addition, R-Smad phosphorylation and Smad4 protein expression in the contraction band were also elevated, while the expression of Smad7 was significantly decreased in the fibrotic muscle of the GMC patients compared to the unaffected adjacent muscle. The protein and mRNA levels of PAI-1 were also remarkably increased in the contraction band compared with adjacent muscle. Immunohistochemical analysis also demonstrated that the expression levels of TGF-β1 and PAI-1 were higher in contraction band than those in the adjacent muscle.

*Conclusion*: Our data confirm the stimulating effects of the TGF-β/Smad pathway in gluteal muscle contracture disease and reveal the internal changes of TGF-β/Smad pathway proteins and their corresponding targets in gluteal muscle contracture patients.

**Keywords**

Gluteal muscle contracture, PAI-1, Smad2/3, Smad7, TGF-β/Smad pathway

**History**

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## Introduction

Gluteal muscle contracture (GMC) is now recognized as a common clinical syndrome with multiple etiologies, characterized by fibrosis and contracture of the gluteal muscles and their tendons (1). The primary symptom of GMC is the difficulty in adducting the hip or squatting, which ultimately progresses to serious functional disorder of the hip (2). It is thought that repeated intramuscular injection into the gluteal region during childhood is a major cause for GMC (3). GMC is more widely reported in China than in the US and Europe, which is likely attributable to frequent use of benzyl alcohol as a diluent for intramuscular injections of penicillin in some regions of China (4). Previous studies have demonstrated that the extracellular matrix (ECM) deposition, which consists mainly of type I collagen, is increased during the development of fibrosis in GMC patients (5). Other studies have also confirmed that collagen type I and III are major components of the contraction band and are markedly elevated compared to the unaffected adjacent muscle (6).

It is known that GMC is a complicated pathological process involving various cytokines and multiple cell signaling pathways. Transforming growth factor (TGF)-βs are members of a superfamily of polypeptides that play an important role in the pathogenesis of many fibrotic diseases (7,8). One subtype, TGF-β1, is critical for tissue fibrosis due to its role as a potent fibrogenic cytokine involved in every step of the process by stimulating fibroblasts (9). TGF- β1 promotes excess ECM generation through the TGF- β/Smad signaling pathway, which leads to increases in the synthesis of matrix proteins and decreases in the production of matrix-degrading proteases (10). It is reported that the increased expression of TGF-β1 is associated with deposition of

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collagen types I and III in the fibrotic muscle of GMC patients. In addition, other forms of (TGF)-βs, namely TGF-β2 and-β3, are known to contribute to the process of fibrotic diseases (6).

TGF-β proteins transmit their signal from the activated transmembrane receptor to the nucleus mainly through the Smad family proteins (11,12). It was found that Smad2 and Smad3 were highly phosphorylated in some tissue fibrosis, and inhibition of Smad2 and Smad3 by RNA interference resulted in a significant decrease in collagen expression (13,14). In contrast, the expression of Smad6 and Smad7 was reduced in diseased tissue compared with normal tissue (15). Consistent with these results, the fibrogenic roles of each of the three isoforms of TGF-β have been documented during GMC procession, although the detailed underlying mechanism remains unknown. This study sought to detect the changes of TGF-β1/Smad downstream signaling molecules which elaborate the effects of TGF-β1Ssmad pathway in the GMC pathological process.

## Methods

### Tissue specimens and antibodies

In this study, 28 patients (12 males and 16 females between the ages of 14 and 35 years) who had GMC release with radiofrequency energy under arthroscopic guidance were selected between May 2010 and January 2011 from the Department of Sports Medicine and Rehabilitation, Peking University Shenzhen Hospital. All of these selected patients had failed to improve with stretching exercises. In contrast to the traditional operation method, we applied radiofrequency coblation to cut the high tensor fascia and the gluteus medius (Figure 1). Tissues obtained from these 28 GMC patients (28 contraction bands and 28 adjacent muscles) during surgical treatment were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. The size of biopsies we collected was approximately 1 × 1 cm. Informed consent was obtained from all of the patients. This study was approved by the Ethical Board of Peking University Shenzhen Hospital, and followed the Declaration of Helsinki Guidelines.

Mouse anti-human monoclonal antibodies against collagen types I and III were purchased from Abcam (CAT: ab6308 and ab6310, Cambridge, UK), and mouse anti-human monoclonal antibodies against TGF-β1 (CAT: sc-130348) and mouse anti-Smad7 antibody (CAT: sc-365846) and rabbit/mouse anti-β-actin antibody (CAT: sc-7210 and sc-8432) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human phospho-Smad2 antibody (CAT: BS4172) and rabbit anti human Smad2 antibody (CAT: BS2993) were purchased from Bioworld Technology (St. Louis, MO). Rabbit anti-human phospho-Smad3 antibody (CAT: 9520), rabbit anti-human Smad3 antibody (CAT: 9513), rabbit anti-Smad4 (CAT: 9515) and rabbit anti-PAI-1 antibody (CAT: 11907) were purchased from Cell Signaling Technology (Boston, MA).

### Collagen assay

Tissue samples were accurately weighed and washed by PBS for three times. The tissues were cut into 1 mm cubes and incubated in 10 mL acetic acid (0.5 mol/L) overnight. Then the swelled tissue was homogenized on ice with normal saline for 5 min using the homogenizer (Allegra 64R, Beckman Coulter). The samples were stirred and digested by pepsin at 4 °C for 6 h [enzyme: tissue (v/v) = 1:100]. The pH was adjusted to 7.4 after enzymolysis and the extraction was centrifuged at 12 400 × $g$ (RCF = $1.118 \times 10^{-5} \times N^2 \times R$, N: rpm, R: 7.5 cm) for 45 min. The supernatant was then collected and salted out with 0.7 mol/L NaCl at 4 °C overnight, then centrifuged at 6000 × $g$ for 45 min at 4 °C. The powder was weighed after lyophilization for 2 h. The samples were then dissolved in normal saline for other experiments.

### Western blot analysis

Tissue samples were homogenized using a modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl and 1 mM EDTA) supplemented with protease and phosphatase inhibitors (1 mM phenylmethyl sulfonyl fluoride, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 1 mg/ml aprotinin, 1 mg/ml pepstatin, 0.5 mg/ml leupeptin, 1 mM NaF, 1 mM $Na4P_2O_4$ and 2 mM $Na_3VO_4$). The extract was centrifuged at 16 800 × $g$ (RCF = $1.118 \times 10^{-5} \times N^2 \times R$, N: rpm, R: 7.5 cm) for 15 min at 4 °C to remove cell debris. The supernatant was harvested and the protein levels were quantified using the BCA protein assay (Rockford, MA), followed by boiling for 5 min with sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 12% β-mercaptoethanol, 20% glycerol and 0.01%

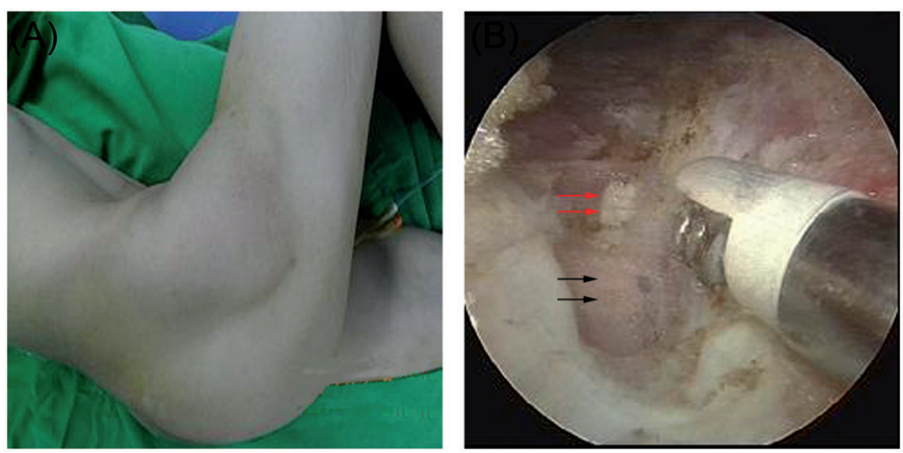


Figure 1. The morphology changes in gluteal muscle contracture patients. (A) The greater trochanter was blocked by the abnormal gluteus maximus when GMC patient's hip was flexed and extended. (B) Abnormal thickening of the gluteal fascia and the related atrophic muscle. The red arrows indicate the thicking gluteal fascia (contraction band); the black arrows indicate the related atrophic muscle (adjacent muscle).

bromophenol blue) at the equivalent protein level. The samples were subjected to SDS-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 10% fat-free skim milk in Tris Buffer Saline containing 0.1% Tween 20, then incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 2 h at room temperature after a series of TBST washes. The immunoreactivity proteins were visualized by ECL (Amersham Pharmacia Biotech, USA) and autoradiography. Densitometry analysis was carried out with Quantity One software (Bio-Rad, Hercules, CA).

### Reverse transcription and polymerase chain reaction (RT-PCR) and real-time reverse transcription-polymerase chain reaction

The expression of various genes from GMC patient tissues was analyzed by RT-PCR and real-time PCR. Total mRNA of samples was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol, and then converted to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). cDNA was subjected to PCR with primers for collagen type I (forward, 5′-GTCGAGGGCCAAGACGAAG-3′ and reverse, 5′-CAGATCACGTCATCGCACAAC-3′), collagen type III (forward, 5′-TGGTCCCCAAGGTGTCAAAG-3′ and reverse, 5′-GGGGGTCCTGGGTTACCATTA-3′), TGF-β1 (forward, 5′-GGCCAGATCCTGTCCAAGC-3′ and reverse, 5′-GTGG GTTTCCACCATTAGCAC-3′), PAI-1 (forward, 5′-CGGAGC ACGGTCAAGCAAGTG-3′ and reverse, 5′-GTTGAGGGCA GAGAGAGGCGC-3′), and β-actin (forward, 5′-CTCCAT CCTGGCCTCGCTGT-3′ and reverse, 5′-GCTGTCACC TTCACCGTTCC-3′). All target sequences were separately amplified for 30–31 cycles of the following protocol: 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C. The reaction products were separated by agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed with 290 nm ultraviolet illumination. The density of each band was measured by Quantity One software (Bio-Rad, Hercules, CA).

Real-time PCR was then performed on each sample using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μL fast on the 7900HT Real-time PCR system (Applied Biosystems) as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. β-actin was used as the reference gene. The relative levels of gene expression were represented as $\triangle Ct = Ct_{gene} - Ct_{reference}$, and the fold change of gene expression was calculated by the $2^{-\triangle\triangle Ct}$ Method. Experiments were repeated in triplicate.

### Immunohistochemistry

Tissue samples were excised and embedded in paraffin blocks, then sectioned onto slides. Slides were dried overnight at 37 °C, dewaxed in xylenes, rehydrated through graded alcohol, and soaked in 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. For antigen retrieval, slides were boiled in tris (hydroxymethyl) aminomethane-EDTA buffer (pH 8.0) in a pressure cooker for 10 min. The sections were incubated with 10% goat serum at room temperature for 20 min to block non-specific protein binding. Subsequently, the blocked sections were incubated with antibody against TGF-β1 (1:500) or antibody against PAI-1 (1:400) for 60 min at 37 °C in a moist chamber. After rinsing five times with 0.01 mol/L phosphate-buffered saline (PBS, pH = 7.4) for 10 min, the slides were incubated with secondary antibody at a concentration of 1:100 for 30 min at 37 °C. Slides were then stained with DAB (3,3-diaminobenzidine) after washing in PBS again to visualize immunoreactive protein. The staining intensities of TGF-β1 and PAI-1 in the contraction band and unaffected adjacent muscle were graded semi-quantitatively using the following scale: (−) = no staining; (+) = weak staining, less than 30% of the highest protein expression; (++) = moderate staining, between 30% and 60% of the highest protein expression; and (+++) = strong staining, more than 60% of the highest protein expression. The degree of staining intensities was expressed as the mean of ten different fields in each slide. The results were scored by two independent pathologists who were blinded to the identities of the slides.

### Statistical analyses

Statistical analysis was performed using the SPSS Software, Version 17.0 (SPSS Inc., Chicago, IL). All values were expressed as mean ± SD. Statistics between groups were compared using the Student's *t*-test and the Mann–Whitney rank sum test, which was used for the degree of staining intensities. $p < 0.05$ were considered as statistically significant difference.

## Results

### Expression of collagen types I and III is upregulated in the contraction band

To determine if fibrosis within GMC contraction bands is associated with increased collagen production, we first aimed to examine the expression of collagen types I and III in these tissues. Expression of these proteins was upregulated significantly in contraction bands compared to unaffected adjacent muscle at both mRNA and protein levels as examined by Western blotting and RT-PCR, respectively (Figure 2A). Semi-quantitative densitometry analysis indicated that the protein expression of collagen types I and III was increased 4.2- and 5.6-fold, respectively, and the mRNA levels of collagen types I and III were increased 3.8- and 4.5-fold, respectively, in the contraction band tissue compared with those in unaffected muscle of the GMC patients (Figure 2B, mean of 28 cases, $p < 0.01$). Our real-time PCR results also confirmed that collagen types I and III mRNA expression was up-regulated in contraction band compared with that in unaffected adjacent muscle (Figure 2C). These results confirm that GMC tissues have increased collagen synthesis, which is characteristic of pathological fibrosis.

### Expression of TGF-β1 is especially upregulated in contraction bands

Considering the key role of TGF-β1 in fibrosis, we next wanted to determine if this protein was also induced in GMC

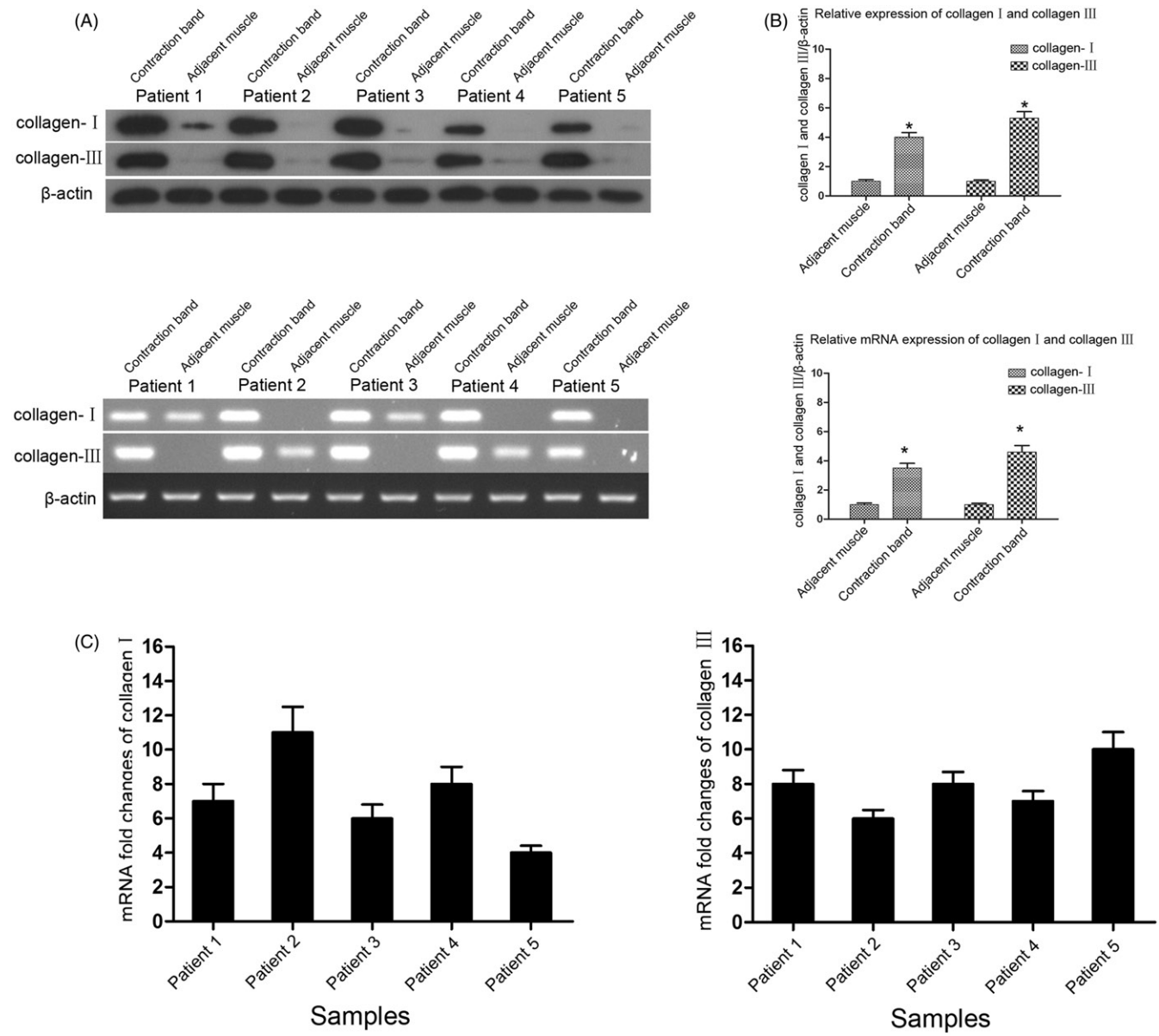


Figure 2. Protein expression of collagen types I and III examined by western blotting assay and mRNA level of collagen types I and III by RT-PCR assay in gluteal muscle contracture patients. (A) Top: Mouse anti-human monoclonal antibodies against collagen types I and III were used to detect the collagen protein in the contraction band and adjacent muscle in five GMC patients by SDS-PAGE gel electrophoresis. Bottom: mRNA levels of collagen types I and III in the contraction band and adjacent muscle in five GMC patients. (B) Densitometric analysis of protein expression and mRNA levels of collagen types I and III in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. $*p<0.05$ compared with the adjacent muscle. (C) Up-regulated expression of collagen types I and III mRNA was examined by real-time PCR in five GMC patients, when compared with adjacent muscle tissues. Expression levels were normalized to β-actin ($n=3$).

contraction bands. As shown in Figure 3(A), protein expression of TGF-β1 measured by western blot and mRNA expression of TGF-β1 measured by RT-PCR were both significantly upregulated in the contraction bands compared with those in the unaffected adjacent muscle (five representative cases). Semi-quantitative densitometry analysis demonstrated that the protein expression and mRNA level of TGF-β1 were increased 6.8- and 8.3-fold, respectively, in GMC contraction bands compared with the unaffected adjacent muscle (Figure 3B, mean of 28 cases, $p<0.01$). The real-time PCR results also demonstrated that mRNA level of TGF-β1 was elevated in GMC contraction bands compared with the unaffected adjacent muscle (Figure 3D). Immunohistochemical analysis was also carried out to measure the expression of TGF-β1 in the contraction band and adjacent muscle (Figure 3C). Quantification of the staining confirms that TGF-β1 overexpression was largely restricted to the contraction band, but not unaffected adjacent muscle (Table 1).

### R-Smad phosphorylation induced by TGF-β1 is upregulated in GMC contraction bands

Phosphorylation of R-Smads is a key event in the signaling pathway induced by TGF-β1. Western blotting results indicate that more phosphorylated Smad2 and Smad3 were present in GMC contraction bands compared to adjacent unaffected muscle, as shown in Figure 4(A). Semi-quantitative densitometry analysis of the blots confirmed that Smad2

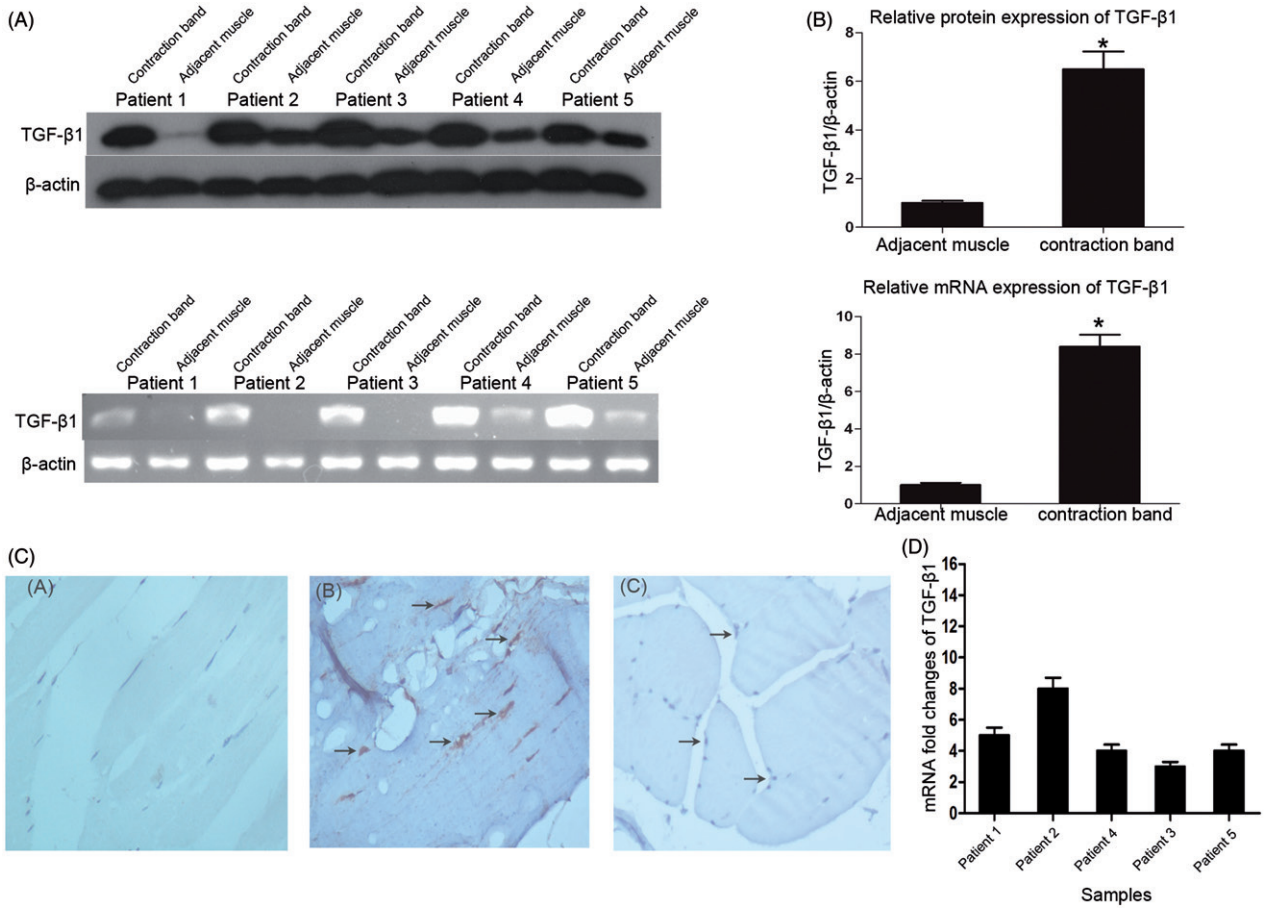


Figure 3. The protein expression of TGF-β1 examined by western blotting and mRNA level of TGF-β1 measured by RT-PCR and immunohistochemistry assays. (A) Top: Mouse anti-human monoclonal antibodies against TGF-β1 were used to measure the protein of TGF-β1 expression in the contraction band and adjacent muscle in five GMC patients by SDS-PAGE gel electrophoresis. Bottom: mRNA levels of TGF-β1 were measured by Reverse transcription and polymerase chain reaction assay. (B) Densitometric analysis of protein expression and mRNA levels of TGF-β1 in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. (C) Immunohistochemistry analysis for TGF-β1 in the contraction band and adjacent muscle. ((A)) normal muscle tissue without antibody (control); ((B)) the signals around nuclei show high TGF-β1 expression in contraction band; ((C)) the stain of TGF-β1 show low expression in adjacent muscle. *$p < 0.05$ compared with the adjacent muscle. The arrows indicate the cell nucleus staining with TGF-β1. (D) Up-regulated expression of TGF-β1 was examined by real-time PCR in contraction band of five GMC patients compared with adjacent muscle tissues. Expression levels were normalized to β-actin ($n = 3$).

Table 1. Immunohistochemical analysis of TGF-β1 in the contraction band and adjacent muscle.

| | TGF-β1 | | | | |
|---|---|---|---|---|---|
| | (−) | (+) | (++) | (+++) | *p* |
| Adjacent muscle | 12 | 6 | 4 | 2 | |
| Contraction band | 1 | 4 | 11 | 12 | 0.05* |

Semi-quantitative scale: (−) = no staining; (+) = weak staining; (++) = moderate staining; and (+++) = strong staining.
*$p < 0.05$ compare with the adjacent muscle.

and Smad3 phosphorylation in the contraction band was increased 5.3- and 9.4-fold, respectively, compared with that in adjacent muscle in the same patients (Figure 4B, mean of 28 cases, $p < 0.01$).

## Expression of Smad4 is increased, while Smad7 is decreased, in GMC contraction bands

Following phosphorylation, R-Smads typically bind to the common mediator Smad and Smad4. Consistent with increased R-Smad phosphorylation, expression of Smad4 was also markedly increased in the contraction band compared with adjacent muscle of GMC patients analyzed by western blot, while the expression of the inhibitor Smad and Smad7, was significantly decreased. Semi-quantitative densitometry analysis confirmed that Smad4 protein expression in the contraction band was increased 6.8-fold while Smad7 expression was decreased 9.2-fold, compared to that in adjacent muscle in the same patients (Figure 4D, mean of 28 cases, $p < 0.01$).

## Expression of plasminogen activator inhibitor type 1 (PAI-1) in contraction band induced by TGF-β1

As shown in the Figure 5(A), the protein and mRNA levels of PAI-1 in the contraction band were both dramatically increased in GMC contraction bands compared to unaffected adjacent muscle (five representative cases are shown). Semi-quantitative densitometry analysis showed that the protein expression and mRNA level of PAI-1 were increased 4.4- and 9.6-fold, respectively (Figure 5B, mean of 28 cases, $p < 0.01$), in the contraction bands compared with adjacent muscle in

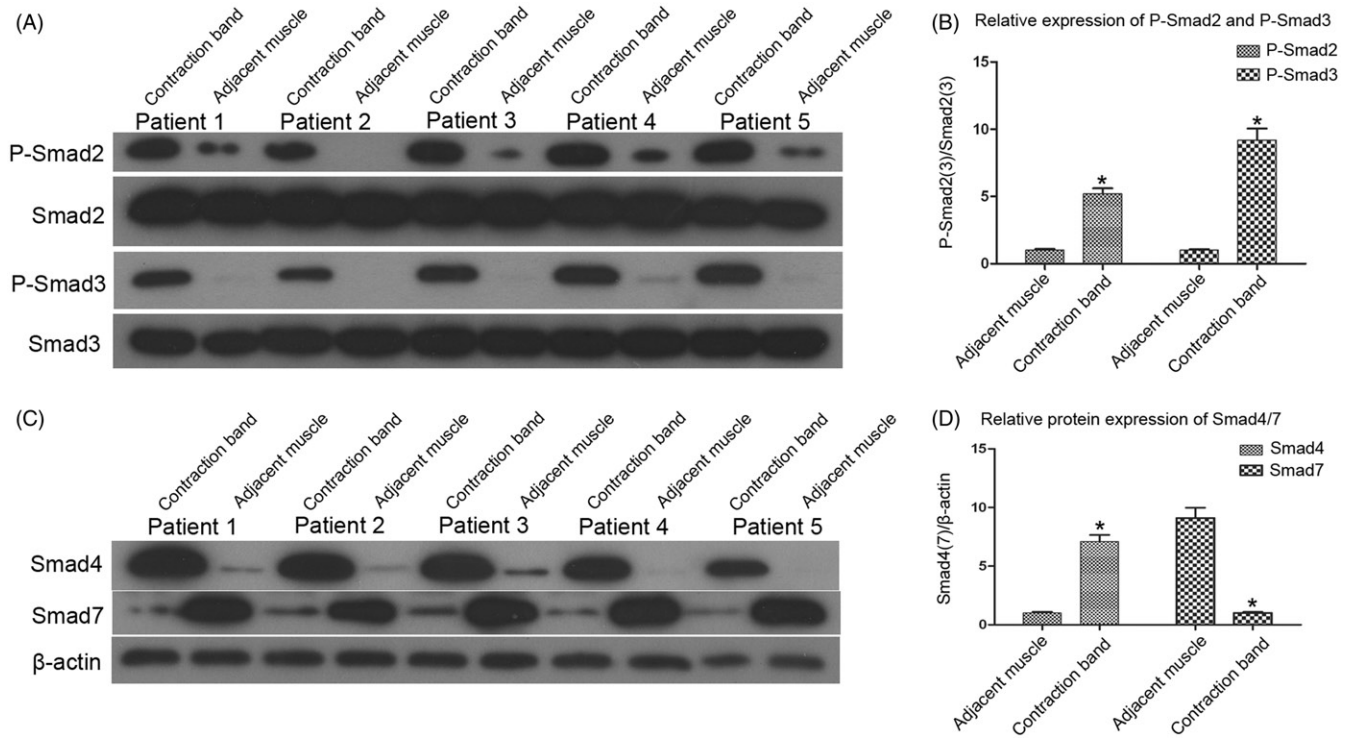


Figure 4. R-Smad phosphorylation and Smad4 and Smad7 expression detected by western blotting assay in the contraction band and adjacent muscle. (A) Rabbit anti-human phospho-Smad2 antibody, rabbit antihuman Smad2 antibody, rabbit anti-human phospho-Smad3 antibody, rabbit anti-human Smad3 antibody were used to detect the phosphorylation status of R-Smad in the contraction band and adjacent muscle by SDS-PAGE gel electrophoresis. (B) Densitometric analysis of R-Smad phosphorylation in the contraction band and adjacent muscle. The results are shown as the relative phosphorylation in the contraction band compared with that in adjacent muscle of 28 GMC patients. (C) Rabbit anti-Smad4, anti-Smad7 and rabbit anti-β-actin antibody were used to detect the expression of Smad4 and Smad7 in the contraction band and adjacent muscle by SDS-PAGE gel electrophoresis. (D) Densitometric analysis of Samd4 and Smad7 expression in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. $*p < 0.05$ compared with the adjacent muscle.

GMC patients. The real-time PCR results also certified that mRNA level of PAI-1 was enhanced in the contraction bands compared with the unaffected adjacent muscle (Figure 5D). Immunohistochemistry (Figure 5C) indicated that PAI-1 was weakly present in the adjacent muscle compared to the contraction band. Quantification confirmed that high expression of PAI-1 (+++) was mainly detected in the contraction band of GMC patients (Table 2).

## Discussion

GMC is a clinical fibrotic disease of the gluteal muscle (16). In recent years, extensive work has focused on the clinical treatment and rehabilitation of affected patients, such as the precise diagnosis criteria, better operation methods and effective means of rehabilitation (17–20). Despite clinical progress, the mechanism of GMC still remains unclear. Researchers speculated that the trigger factors for GMC, and the resulting muscle stiffness and joint dysfunction, include the repeated injections of benzyl alcohol into the buttocks (21). Some retrospective studies have found that post-operative patients have a tendency to develop fibrosis in other organs, such as hypertrophy scars in the skin or keloid in the incision site (22,23). These secondary fibrotic lesions have a strong impact on the treatment and prognosis of GMC patients. Therefore, understanding the mechanism of GMC is essential to improve clinical treatment and post-operative rehabilitation. In this study, for the first time, we demonstrated that the expression of collagen types I and III was significantly increased in contraction bands compared to that in adjacent muscle, suggesting that an imbalance of extracellular matrix (ECM) proteins might be the driving force in the development of GMC disease (6).

TGF-β1 is well known as the crucial fibrogenic cytokine promoting ECM production and tissue fibrosis, which is well-established in GMC progression (17). Chenguang Zhao et al. recently discovered that Sphingosine-1-phosphate (S1P) significantly contributes to the process of gluteal muscle scarring in GMC patients, because it stimulates the expression of TGF-β1 and collagens in the contraction band (24)]. Our study has also shown that the protein and mRNA levels of TGF-β1 are remarkably increased in the contraction band compared with adjacent muscle. Immunohistochemistry also confirmed that high expression of TGF-β1 is mainly present in the contraction band. These data suggest that up-regulation of TGF-β1 in the contraction band significantly contributes to muscle fibrosis in GMC patients. It is implied that TGF-β1 production is higher than required under long-term fibrotic stimulation, especially in some scar diathesis. This is supported by evidence showing increased TGF-β3 in GMC patients, which could explain why TGF-β3 repression is not decreased in GMC patients by a negative feedback loop under long term stimulation of fibrotic factors (25,26). The negative feedback loop also could be seen in other tissue fibrosis such as keloid fibroblasts (27).

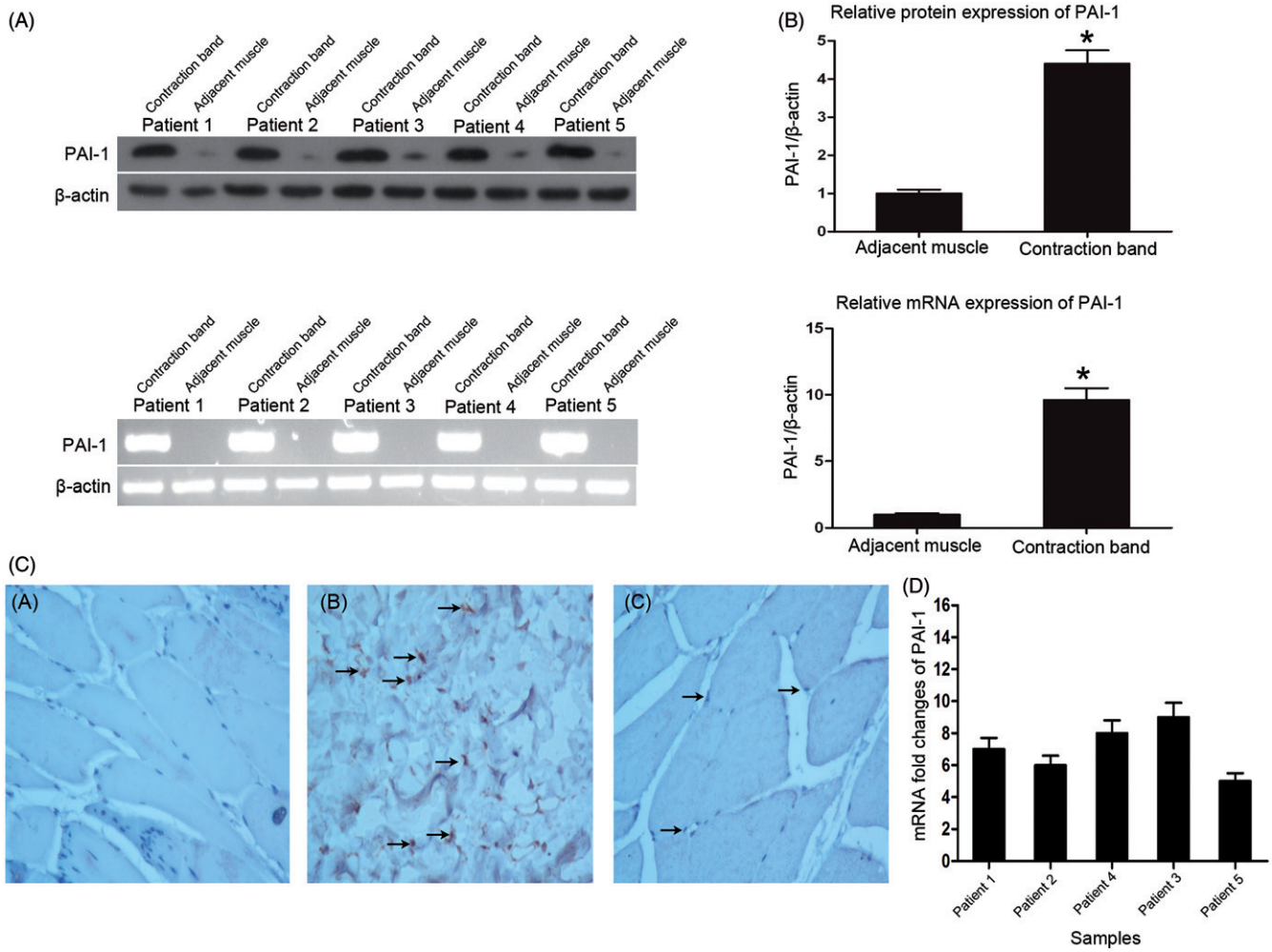


Figure 5. The protein expression of PAI-1 induced by TGF-β/Smad signaling pathway was detected by western blotting assay and mRNA level of PAI-1 was measured by RT-PCR and immunohistochemistry assays. (A) Top: Rabbit anti-PAI-1 antibody was used to measure the expression of PAI-1 in the contraction band and adjacent muscle in five of the GMC patients by SDS-PAGE gel electrophoresis. Bottom: mRNA levels of PAI-1 were measured by reverse transcription and polymerase chain reaction assay. (B) Densitometric analysis of protein expression and mRNA levels of PAI-1 in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. (C) Immunohistochemistry for PAI-1 in the contraction band and adjacent muscle. ((A)) normal muscle tissue without antibody (control); ((B)) the signals of PAI-1 around the nuclei show a high expression in the contraction band; ((C)) the stain of PAI-1 shows a low expression in adjacent muscle. *$p < 0.05$ compared with the adjacent muscle. The arrows indicate the cell nucleus staining with PAI-1. (D) Up-regulated expression of PAI-1 was examined by real-time PCR in 5 GMC patients, when compared with adjacent muscle tissues. Expression levels were normalized to β-actin ($n = 3$).

Table 2. Immunohistochemical analysis of PAI-1 in the contraction band and adjacent muscle.

| | PAI-1 | | | | |
|---|---|---|---|---|---|
| | (−) | (+) | (++) | (+++) | *p* |
| Adjacent muscle | 13 | 8 | 6 | 1 | |
| Contraction band | 3 | 4 | 8 | 13 | 0.05* |

Semi-quantitative scale: (−) = no staining; (+) = weak staining; (++) = moderate staining; and (+++) = strong staining.
*$p < 0.05$ compare with the adjacent muscle.

Smad family proteins are located downstream of TGF-β in the TGF-β/Smad signaling pathway and are responsible for transmitting the signal from cell surface receptors to the nucleus, leading to activation or inhibition of their target genes (28–32). R-Smads are receptor-regulated Smads. R-Smads are directly phosphorylated through their intracellular kinase domain on their C-terminus, leading to R-Smad activation (33). Many reports have shown that TGF-β receptors (TbR) I and II have serine/threonine protein kinase activity that can promote R-Smad phosphorylation (34). Phosphorylated R-Smad (Smad2 and Smad3) combine with Smad4 to form a complex in the nucleus, resulting in enhanced expression of intracellular and extracellular fibrogenic proteins and inhibition of ECM degrading-proteins (35). Numerous studies have demonstrated that Smad2 and Smad3 are key factors in tissue fibrosis, suggesting that down-regulation of the phosphorylation of Smad2/3 could inhibit the pathological process (36). We have shown that the protein expression of R-Smad is significantly elevated in the contraction band identified with other tissue fibrosis process. R-SMAD proteins bind to the SMAD4 protein and form a protein complex, which then moves to the cell nucleus. In the nucleus, the SMAD protein complex binds to specific areas of

DNA, where it controls the activity of particular genes and regulates cell growth and division (proliferation). Other studies discovered that some molecules like MicroRNA or Small heat shock protein resist fibrosis through modulating Smad4 expression (37,38). Smad7 can restrain R-Smad phosphorylation by competitively inhibiting the TbR I receptor due to its similarity in structure with R-Smads (39,40). It has been established that a decrease in the expression of Smad7 is a feature of systemic sclerosis in skin fibroblasts (41). In our study, we found that R-Smad phosphorylation and protein expression of Smad4 were significantly increased in the contraction band compared with adjacent muscle of GMC patients, which corresponded with significantly suppression of Smad7 in contraction bands. These data suggest that the increased R-Smad phosphorylation and Smad4 expression could generate more complexes in the nucleus, resulting in superfluous fibrotic protein expression. In addition, decreased expression of Smad7 could exacerbate progression of fibrosis. Taking into account other findings on GMC, we propose that the formation of GMC is due, at least in part, to an increase in TGF-β/Smad signaling.

Plasminogen activator inhibitor type 1 (PAI-1) is one of the furthest downstream target genes of TGF-β/Smad signaling (42). It is the primary physiological inhibitor of the urokinase-plasminogen activator (uPA) and tissue-plasminogen activator (tPA) systems (43). The fibrinolytic system, which includes uPA and tPA systems, is the key to regulating the activity of matrix metalloproteinases (MMPs) and ECM degradation factors (42,44). Previous studies have demonstrated that overexpression of PAI-1 can induce fibrosis in many organs, especially in liver, where it can lead to hepatocellular carcinoma (45). Suppressing the expression of PAI-1 could postpone the progress of liver fibrosis (46). Our study has shown that the protein and mRNA levels of PAI-1 are both significantly increased in the contraction band, compared with the adjacent muscle of GMC patients. Immunohistochemistry confirmed that the expression of PAI-1 is higher in the contraction band than in adjacent muscle. These results suggest that the abnormally high expression of PAI-1 might play an important role in GMC disease development.

In this study, we have demonstrated that the expression of collagen types I and III, TGF-β1, and Smad4, as well as R-Smad phosphorylation, were significantly increased in contraction bands of GMC patients compared to adjacent unaffected muscle, whereas the expression of Smad7 was significantly decreased. In addition, the protein and mRNA levels of plasminogen activator inhibitor type 1 (PAI-1) in the contraction band were elevated in contraction bands compared to adjacent muscle. These results provide a clue for further research into the mechanism and treatment of GMC disease.

## Conclusion

In summary, this study not only confirmed that GMC is a fibrotic disease of gluteal muscle characterized by accumulation of collagen types I and III, but also verified the importance of the TGF-β/Smad pathway and its downstream effectors in GMC. Our findings are beneficial to comprehending the development and progression of GMC and could provide a new avenue for treatment of this debilitating disease.

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## Declaration of interest

The authors declare that they have no competing interests.

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