

MMP-1/TIMP-1 expressions in rectal submucosa of females with obstructed defecation syndrome associated with internal rectal prolapse

Long-Lei Cao^{1*}, Jie Yu^{1*}, Zhang-Ling Yang¹, Xu Qiao², Hui Ye¹, Chang-Lei Xi¹, Qi-Chang Zhou¹, Cheng-Cheng Hu¹, Chao-Jun Zhao³ and Zhi-Lin Gong¹

¹Department of Anal-colorectal Surgery, ²Digestive Endoscopy Center, Jingzhou Central Hospital, the Second Clinical Medical College, Yangtze University, Jingzhou and ³Song Zi Liu Jia Chang Town Hospital of Hubei Province, China

* These authors contributed equally to this study and share first authorship

Summary. Objective. To explore the MMP-1/TIMP-1 expressions rectal submucosa of females with obstructed defecation syndrome (ODS) associated with internal rectal prolapse (IRP). Methods. Fifty-six female patients with ODS associated with IRP were enrolled as Case group, and 43 female hemorrhoids of stages III-IV without constipation and IRP were enrolled as Control group. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry were performed to test the expressions of MMP-1/TIMP-1 in the rectal submucosa. Western blotting was used to examine protein expressions of MMP-1/TIMP-1 and pro-inflammatory cytokines (IL-6 and TNF- α) in the rectal submucosa. EVG staining was conducted to detect collagen and elastic fibers in rectal submucosa. Results. The increased expression of MMP-1 was negatively linked to the decreased TIMP-1 level in the rectal submucosa of patients with ODS associated with IRP. Besides, the expressions of IL-6 and TNF- α were increased in the Case group as compared with the Control group. Additionally, ODS severity and the pro-inflammatory cytokines was positively linked to MMP-1, but negatively related to TIMP-1 in Case group. EVG staining showed that the area ratios of collagen and elastic fibers were lower in Case group than Control group. Through Pearson's correlation analysis, the area

ratios of collagen and elastic fibers were positively associated with MMP-1 expression, but negatively correlated with TIMP-1 expression in rectal submucosa of patients with ODS associated with IRP. Conclusion. Elevated MMP-1 and reduced TIMP-1 were found in ODS associated with IRP, which was related to the ODS severity, inflammation and contents of collagen and elastic fibers.

Key words: Internal rectal prolapse, Obstructed defecation syndrome, MMP-1, TIMP-1

Introduction

Internal rectal prolapse (IRP) is well-known as a full-thickness intussusception of the rectum during defecation, which was first proposed by Tuttle et al. in 1903 (Ganio and Giani, 2008). As for obstructed defecation syndrome (ODS), it has been recognized as a common clinical problem frequently suffered by middle-aged females, who have an impaired ability to satisfactorily evacuate the rectum (Hasan and Hasan, 2012), and coincidentally, IRP was one of the major risk factors for ODS (Wijffels et al., 2011), such as rectal tenesmus, desire to defecate or defecation difficulties, seriously affecting the patients' health and quality of life (Mcnevin, 2016). The etiology of IRP has been suggested by several lines of previous evidences to have a relation with sliding hernia, intussusception, descending perineum syndrome, and relaxed pelvic floor syndrome (Wedell et al., 1980; Hawkins et al., 2016).

Offprint requests to: Zhi-Lin Gong, Department of Anal-colorectal Surgery, Jingzhou Central Hospital, the Second Clinical Medical College, Yangtze University, Renmin Road 1, 434020, Jingzhou, China. e-mail: gong_zhilin@sina.com
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Among them, the relaxed pelvic floor has been widely recognized, in which it is believed that the lack of fixed tissue around the rectum, like lateral ligament relaxation, would result in prolapse (Rickert and Kienle, 2015).

Matrix metalloproteinases (MMPs), a group of zinc endopeptidases, are mainly synthesized and secreted by connective tissue cells, epithelial cells and macrophages (Fireman et al., 2002; Vishnuvardhan et al., 2013). Functionally, MMPs could not only degenerate effectively components of extracellular matrix (ECM), but also regulate cell adhesion, and directly or indirectly participate in normal physiological processes, such as embryonic development, tissue remodeling and wound repair (Lee and Yang, 2013). On the other hand, TIMPs (tissue inhibitor of metalloproteinases), are widely distributed in connective tissues, which form a compound via binding to the corresponding active MMP in 1:1 non-covalent irreversible bonding, thus inhibiting the degeneration of MMP on corresponding substrates (Gueders et al., 2006; Fenol et al., 2014). MMP-1 is an interstitial collagenase degenerating collagen fibers at the highest expression in MMPs family, whereas TIMP-1 acts as the tissue inhibitor of MMP-1 to suppress its biological activity (Lambert et al., 2009; Hong et al., 2015). Notably, the abnormal expressions of TIMP-1 and MMP-1 were associated with prolapse of pelvic floor organs in recent studies, for example, Hu et al. reported that low TIMP-1 expression and high MMP-1 expression in vaginal wall exerted vital roles in the pathogenesis of vaginal prolapse (Hu et al., 2017). As far as we know, pelvic floor organs also include the vagina, uterus, bladder, urethra and rectum (Lee et al., 2014). But it is still unknown whether TIMP-1 and MMP-1 are abnormally expressed in rectal submucosa, thereby affecting IRP progression.

As a consequence, this study enrolled 56 female patients with ODS associated with IRP and 43 female hemorrhoids of stages III-IV without constipation and IRP. We conducted quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry to determine expressions of MMP-1 and TIMP-1, used Western blotting to test protein expressions of MMP-1/TIMP-1 and pro-inflammatory cytokines (IL-6 and TNF- α), and performed EVG staining to detect collagen and elastic fibers in rectal submucosa; thereby investigating the expressions of MMP-1 and TIMP-1 in ODS associated with IRP and analyzing the possible pathogenesis.

Materials and methods

Ethics statement

In this study, all related clinical experiments got the permission of the Ethics Committee of our hospital and strictly followed the Declaration of Helsinki (World Medical, 2013). Besides, all subjects volunteered to participate in this clinical trial and all specimens were obtained through patients' informed consents.

Participants

A total of 56 female patients aged 54.63 ± 11.55 (36-76 years) with ODS associated with IRP underwent stapled transanal rectal resection (STARR) at our hospital from December 2015 to December 2017 were recruited as the Case group. The recognized constipation scoring system (CSS; range: 0-30 at increments of 1; no symptoms=0) (Agachan et al., 1996), Longo's ODS score (range: 0-40 at increments of 1; no symptoms=0) (Zhang et al., 2010), and symptom severity score (SSS; range: 0-36 at increments of 1; no symptoms=0) (Schwandner et al., 2010) were used to quantify the severity of ODS, and the CSS, ODS, and SSS scores were 17.59 ± 5.55 , 18.71 ± 4.72 and 14.66 ± 2.97 , respectively. All patients finished the related examination, including digital rectal examination, defecography, colonoscopy, anorectal manometry, and other tests before surgery to exclude other related diseases. Inclusive criteria: patients had at least 3 or more ODS specific indicators, that is, incomplete defecation, painful defecation, defecation difficulty, change of defecation posture, and assistance in defecation; ineffective conservative treatment, including drinking over 1500 mL water per day, high fiber diet, regular use of laxatives and continuous biofeedback treatment for 6 months; defecography presented internal rectal mucosal intussusception (>10 mm). Meanwhile, 43 female hemorrhoids of stages III-IV aged 56.05 ± 13.47 (34-77 years) without constipation and IRP were enrolled as the Control group. No significant differences were found in age between the Case group and the Control group ($P > 0.05$). Exclusive criteria: patients had malignant tumor or other diseases; abnormal or injured liver and kidney function; or in lactation and pregnancy; or severe mental illness who were difficult to cooperate with the investigation and physical examination.

Specimen collection

During the operation, the rectal submucosa tissues were taken and washed by 0.9% NaCl solution. One third of the specimens were fixed in 10% formalin solution, dehydrated, embedded with paraffin, and cut into serial sections of 4 μ m for immunohistochemistry and Elastica van Gieson (EVG) staining. Another section of the specimens were loaded into the EP tube containing RNA Later solution and saved at -80°C in the refrigerator for qRT-PCR and Western blotting. The last section was stored at -80°C in the refrigerator immediately for spare.

qRT-PCR

The Trizol reagent was used to extract RNA, whose OD 260/280 ratio was 1.8 to 2.0, proving the purity of RNA was good (without DNA and protein pollution). The reverse transcription kit (Invitrogen, Carlsbad, CA,

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USA) was applied to generate cDNA. The qRT-PCR was performed using LightCycler® 480 Real-Time PCR system (Roche Applied Science, Penzberg, Germany). The total reaction system of 20 µl, consisting of 1 µl cDNA, 10 µl SYBR premix buffer solution, 0.5 µl Forward primer, 0.5 µl Reverse primer, and sterile water (till 20 µl). Based on the annealing temperature of each gene, the three-step method was used to complete the amplification with 40 cycles and GAPDH as internal reference. Table 1 presents the primer sequences of GAPDH, MMP-1 and TIMP-1. The relative quantitative formula $2^{-\Delta\Delta Ct}$: $\Delta\Delta Ct = Ct_{(\text{target gene})} - Ct_{(\text{internal reference gene})}$ was adopted in this experiment.

Western blotting

The Bradford method was used to determine the total concentration of the supernatant. Next, the protein was isolated by polyacrylamide gel electrophoresis, and transferred to poly (vinylidene fluoride) (PVDF) membrane (Bio-Rad, USA) using a semi-dry blotter (Bio-Rad Laboratories, Hercules, CA, USA). After sealing with skim milk powder at room temperature, PVDF membrane was washed with poly (butylene succinate-co-butylene terephthalate) (PBST) buffer solution and then added with anti-MMP1 antibody (ab137332, 1/1000), anti-TIMP1 antibody (ab109125, 1/1000), anti-IL-6 antibody (ab6672, 1/500) and anti-TNF- α -antibody (ab6671, 1/1000) (all purchased from Abcam, Cambridge, MA, USA) for 1 h hybridization at room temperature. With PBST washing for 3 min \times 5 times, PVDF membrane was incubated with horseradish peroxidase (HRP) labeled IgG (Abcam, Cambridge, MA, USA) for 1 h. Washed with PBST for 3 min \times 5 times, HRP substrate (Bio-Rad Inc., Hercules, California, USA) was used to develop the target protein with β -actin as loading control. The relative content of target protein was measured by the ratio of gray value of the target protein/ β -actin.

Immunohistochemistry

After dewaxing and rehydration, endogenous peroxidase blocking, and microwave antigen retrieval blocking of non-specific protein binding, the sections were incubated with anti-MMP1 antibody (ab137332, 1/500) and anti-TIMP1 antibody (ab109125, 1/8000) (both purchased from Abcam, Cambridge, MA, USA) at 4°C in the refrigerator for 16-20 h. After washing 3 times with PBS, 20 min of incubation at room temperature was conducted through addition of 50µl secondary antibody. Next, sections were washed 3 times with PBS, and the color was developed with DAB (diaminobenzidine) under the microscope. The sections were rinsed with flowing water, and then counterstained with hematoxylin (blue), dehydrated with graded ethanol, dried and sealed with neutral gum.

EVG staining

The paraffin sections were dewaxed and stained with Verhoeff Van Gieson's stain at room temperature. Washed by running water, sections were differentiated by 2% ferric chloride for 10-20s, and fibers were black under the microscope. Then the sections were treated with 5% sodium thiosulfate for 1 min and counterstained

Table 1. Primer sequences of GAPDH, MMP-1 and TIMP-1 used in this study.

Gene	Target fragment	Primer sequences
GAPDH	78kb	Forward: 5'-ACAGTCAGCCGCATCTTCT-3' Reverse: 5'-ACTCCGACCTTCACCTTCC-3'
MMP-1	168kb	Forward: 5'-CAAACCTGTGACCCAGTT-3' Reverse: 5'-TACCAGTTGGAAGGGATGGC-3'
TIMP-1	108kb	Forward: 5'-GGGGCTTACCAA GACCTAC-3' Reverse: 5'-GGAAGCCCTTTTCAGAGCCT-3'

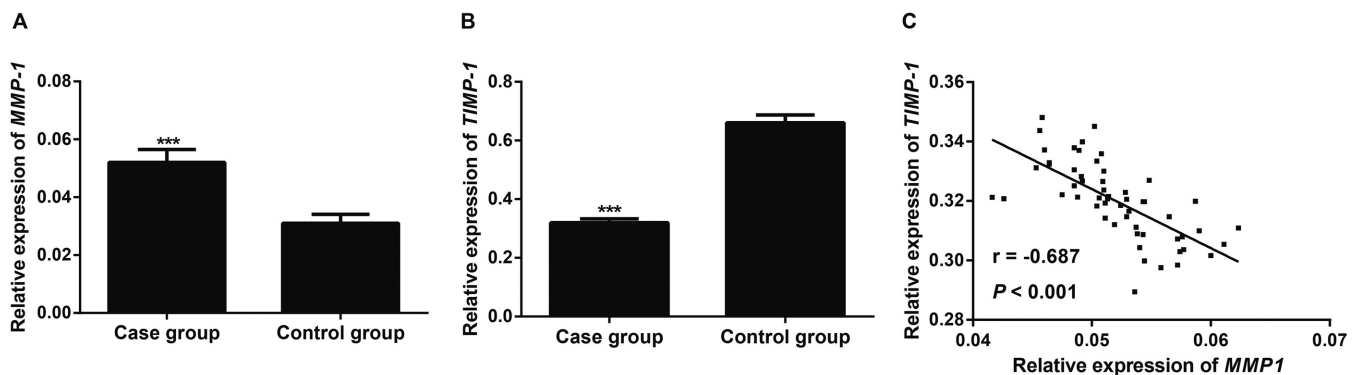


Fig. 1. The mRNA levels of MMP-1 and TIMP-1 in rectal submucosa detected by qRT-PCR. **A-B.** The relative mRNA expressions of MMP-1 (**A**) and TIMP-1 (**B**) in rectal submucosa of Case group and Control group detected by qRT-PCR, *** $P < 0.001$, compared with Control group. **C.** The correlation analysis between mRNA levels of MMP-1 and TIMP-1 in patients with ODS associated with IRP.

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with Verhoeff Van Gieson's stain for 3-5 min, washed with absolute ethyl alcohol, dehydrated by gradient alcohol, and finally sealed in neutral gum. Under the microscope, the structure and distribution of elastic and collagen fibers in rectal submucosa were observed. Then

three target fields in each section were selected and photographed under the microscope (× 200). The image-processing software (Image ProPlus 6.0, Media Cybernetics, USA) was used to analyze the expression of collagen and elastic fibers, and the area ratio of two

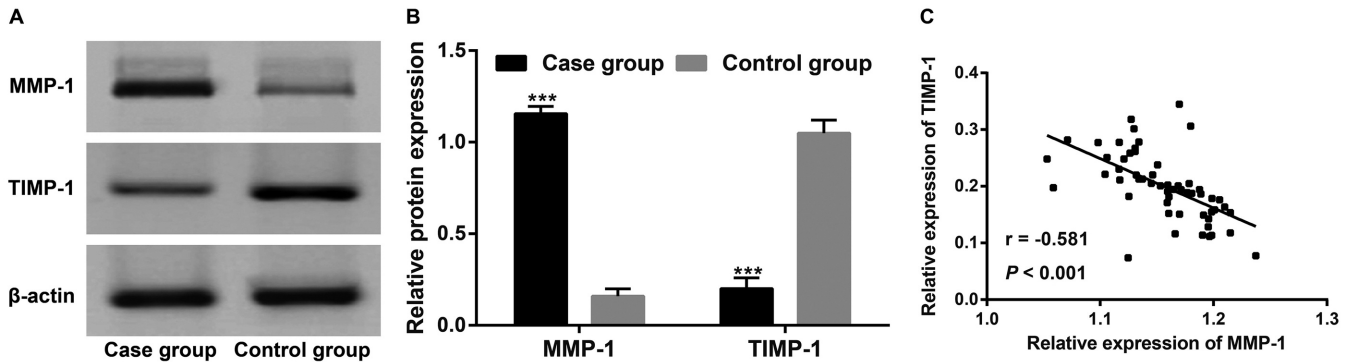


Fig. 2. The protein expressions of MMP-1 and TIMP-1 in rectal submucosa detected by Western blotting. **A-B.** The protein expressions of MMP-1 and TIMP-1 in rectal submucosa of Case group and Control group detected by Western blotting, *** $P < 0.001$, compared with Control group. **C.** The correlation analysis between MMP-1 and TIMP-1 protein expressions in patients with ODS associated with IRP.

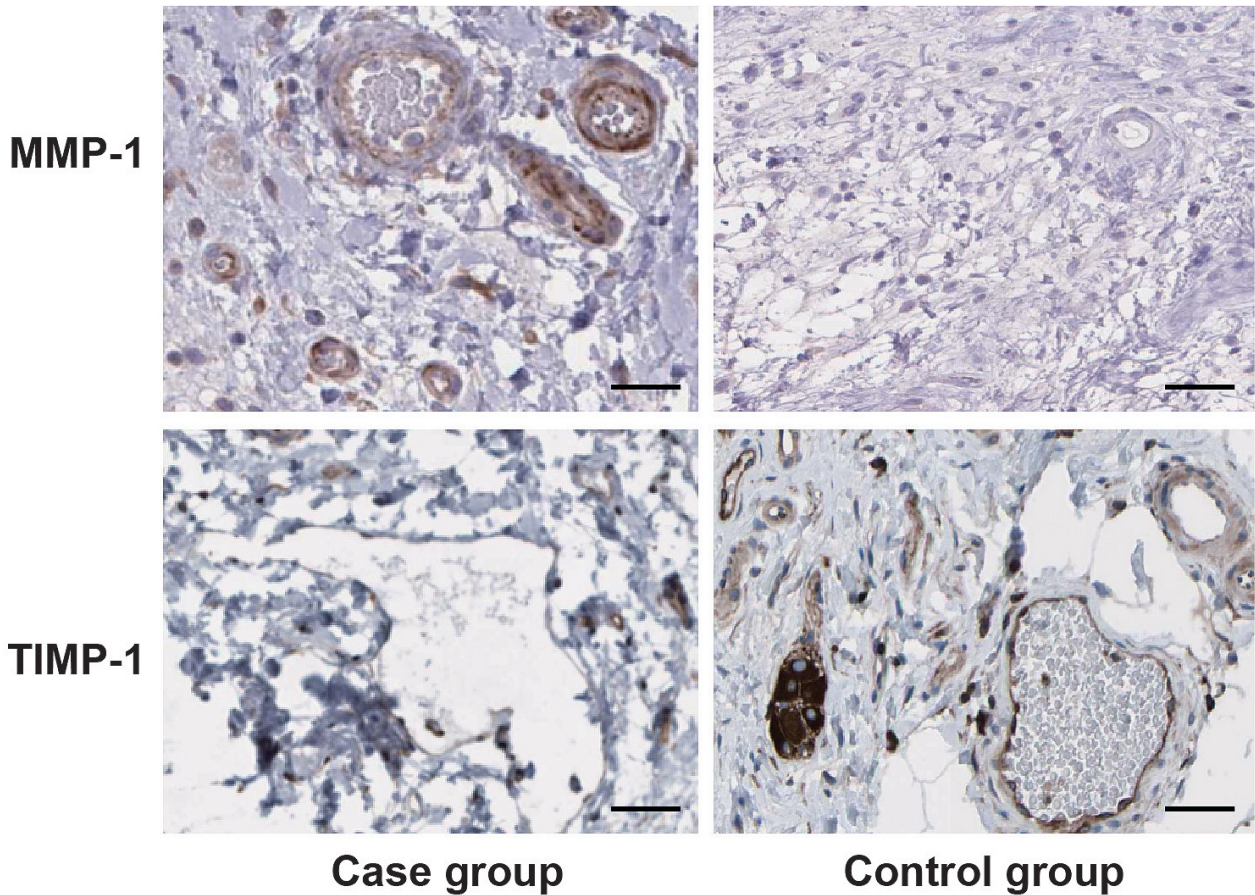


Fig. 3. The protein expressions of MMP-1 and TIMP-1 in rectal submucosa detected by immunohistochemistry. Scale bars: 200 μm .

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fibers was calculated.

Statistical analysis

All data were processed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as mean \pm standard deviation (SD), and the comparison between two groups was analyzed by student's t test. As represented as frequency, comparison of enumeration data was analyzed by χ^2 test. The correlation analysis was carried out by Pearson's correlation test. The value of $P < 0.05$ was regarded as a statistical difference.

Results

The mRNA levels of MMP-1 and TIMP-1 in case and control groups

In Fig. 1A,B, Case group showed an evident increase in *MMP-1* expression and a great decrease in *TIMP-1* expression, as compared with Control group ($P < 0.001$). In addition, there was a negative correlation between the mRNA levels of *MMP-1* and *TIMP-1* in Case group, as presented in Fig. 1C ($P < 0.001$).

Protein expressions of MMP-1 and TIMP-1 in Case and Control groups

Western blotting in Fig. 2A,B showed that the

protein expressions of MMP-1 and TIMP-1 were 1.15 ± 0.04 and 0.20 ± 0.06 in Case group, and were 0.16 ± 0.04 and 1.05 ± 0.07 in Control group, respectively. In contrast to Control group, Case group presented a significant increase in MMP-1 protein expression and a great decrease in TIMP-1 protein expression (both $P < 0.05$). Besides, MMP-1 protein expression was negatively associated with TIMP-1 protein expression in female patients of ODS associated with IRP ($P < 0.05$, Fig. 2C). Immunohistochemistry analysis of MMP-1 and TIMP-1 in rectal submucosa in Fig. 3 demonstrated that the female patients of ODS associated with IRP presented a higher staining degree of MMP-1 and a lower staining degree of TIMP-1, as compared with Control group, which presented the similar expression pattern as the Western blotting.

Correlation analysis between protein expressions of MMP-1/TIMP-1 and ODS severity in female patients of ODS associated with IRP

Pearson's correlation analysis demonstrated (Fig. 4) a positive correlation of MMP-1 protein expression with CSS, ODS and SSS scores (r values were 0.609, 0.640 and 0.570, respectively, all $P < 0.001$), but a negative association of TIMP-1 protein expression with CSS, ODS and SSS scores (r values were -0.471, -0.533 and -0.495, respectively, all $P < 0.001$) in rectal submucosa of patients with ODS associated with IRP.

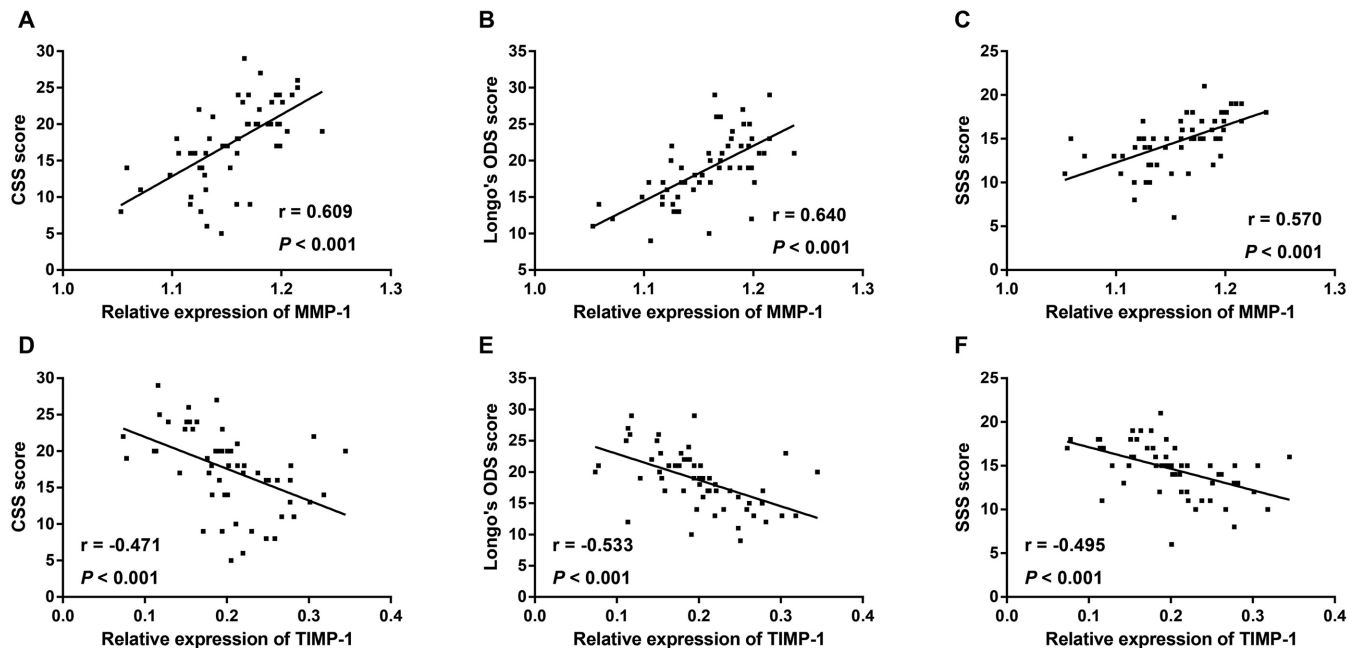


Fig. 4. The correlation analysis between the protein expressions of MMP-1/TIMP-1 and ODS severity in female patients of ODS associated with IRP. **A-C.** The correlation analysis of MMP-1 protein expression with CSS (A), ODS (B), and SSS (C) scores in female patients of ODS associated with IRP. **D-F.** The correlation analysis of TIMP-1 protein expression with CSS (D), ODS (E), and SSS (F) scores in female patients of ODS associated with IRP.

Comparison of inflammatory factors in rectal submucosa of two groups

When compared to Control group, the patients in Case group showed increased pro-inflammatory cytokines (IL-6 and TNF- α) (both $P < 0.05$, Fig. 5A-B). Moreover, the pro-inflammatory cytokines (IL-6 and TNF- α) were positively associated with MMP-1 protein expression but negatively correlated with TIMP-1 protein expression (all $P < 0.05$, Fig. 5C-F).

Comparison of collagen and elastic fibers in rectal submucosa of two groups

As shown in Fig. 6A, EVG staining revealed that the collagen fibers in rectal mucosa had a thin structure and were distributed sparsely, while elastic fibers was broken in fragments and disorder in Case group, as compared with Control group. In Fig. 6B-C, the area ratio of collagen and elastic fibers in rectal submucosa of Case group was significantly lower than that in Control group

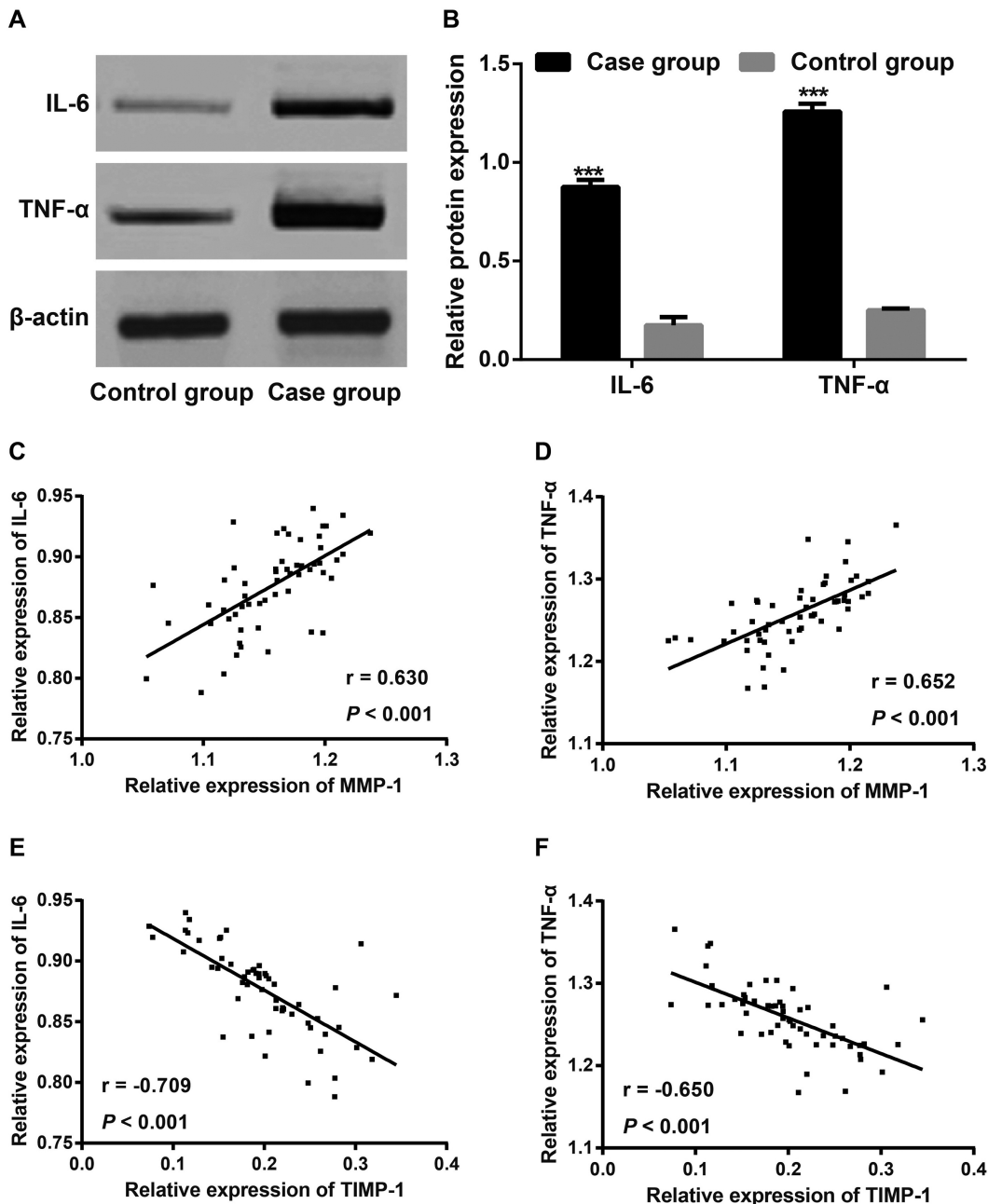


Fig. 5. The protein expressions of pro-inflammatory cytokines (IL-6 and TNF- α) in rectal submucosal. **A-B.** The protein expressions of IL-6 and TNF- α in rectal submucosa of Case group and Control group detected by Western blotting, *** $P < 0.001$, compared with Control group. **C-D.** The correlation analysis of MMP-1 protein expression with IL-6 (C) and TNF- α (D) and MMP-1 in female patients of ODS associated with IRP. **E-F.** The correlation analysis of TIMP-1 protein expression with IL-6 (E) and TNF- α (F) and MMP-1 in female patients of ODS associated with IRP.

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(both $P < 0.05$). Pearson's correlation analysis found that the protein expression of MMP-1 in rectal submucosa of female patients with ODS associated with IRP was positively related to the area ratios of collagen ($r = 0.576$) and elastic fibers ($r = 0.586$) (both $P < 0.001$, Fig. 6D-E). Besides, the protein expression of TIMP-1 shared a negative relation with the area ratios of collagen ($r = -0.442$) and elastic fibers ($r = -0.659$) ($P < 0.001$, Fig. 6F-G).

Discussion

In modern medicine, although the etiology of IRP is not entirely clear so far, it is generally believed that the long-term high intra-abdominal pressure of the body, together with hypoplasia and atrophy of the levator muscles of the anus and pelvic floor fascia, leads to pelvic floor weakness, which cannot support the rectum in the normal position, and weakens the fixation of perirectal tissue on the rectum, eventually resulting in IRP (Sun et al., 1989; Peters et al., 2001).

MMPs and TIMPs are well-documented to participate in various physiological and pathological proteolytic processes, since they have been suggested as the vital regulators during the degradation of extra cellular matrix (ECM) (Liu et al., 2017). In recent years, the MMPs and these inhibitor TIMPs were frequently reported to play important roles in pelvic organ prolapse. For instance, MMP-2 and MMP-9 were observed to be highly expressed in women with vaginal prolapse (Budatha et al., 2011). Moreover, Liang et al. found an

enhancement in MMP-2 mRNA and a reduction in TIMP-2 mRNA in uterosacral ligament, which was linked to uterine prolapse in females without urinary incontinence (Gabriel et al., 2006). It is worth mentioning that rectum, also belonging to pelvic organs, may prolapse resulting from the lack of surrounding fixed tissues, such as lateral ligamentous laxity, relatively free mesentery, and laxity of pelvic floor and muscles around the anal canal, thereby resulting in ODS, which further leads to a vicious circle of straining with deterioration of prolapse (Scaglia et al., 1993; Gouvas et al., 2015). In this study, we performed qRT-PCR, Immunohistochemistry and Western blotting to detect the rectal submucosa expressions of MMP-1/TIMP-1 of patients with ODS associated with IRP, and found an increase in MMP-1 and a decrease in TIMP-1. Similarly, uterosacral ligaments of women with pelvic organ prolapse (POP) presented a dramatic increase in MMP-1 (Strinic et al., 2009; Vulic et al., 2011). Meanwhile, as Dviri et al. reported, MMP-1 was highly expressed when compared with controls in vaginal wall biopsies of vaginal prolapse (Dviri et al., 2011). Moreover, TIMP-1 was found to be lower in POP patients than non-POP patients (Wang et al., 2014), indicating that abnormal expressions of MMP-1/TIMP-1 exert effects on the occurrence and development of pelvic floor prolapse, including IRP, possibly because inflammation diffuses from mucosa to other layers of the intestinal wall, and invades the supportive tissues around the rectum, consequently resulting in laxity of supportive tissues and prolapse (Rahman et al., 2016). In this experiment, we

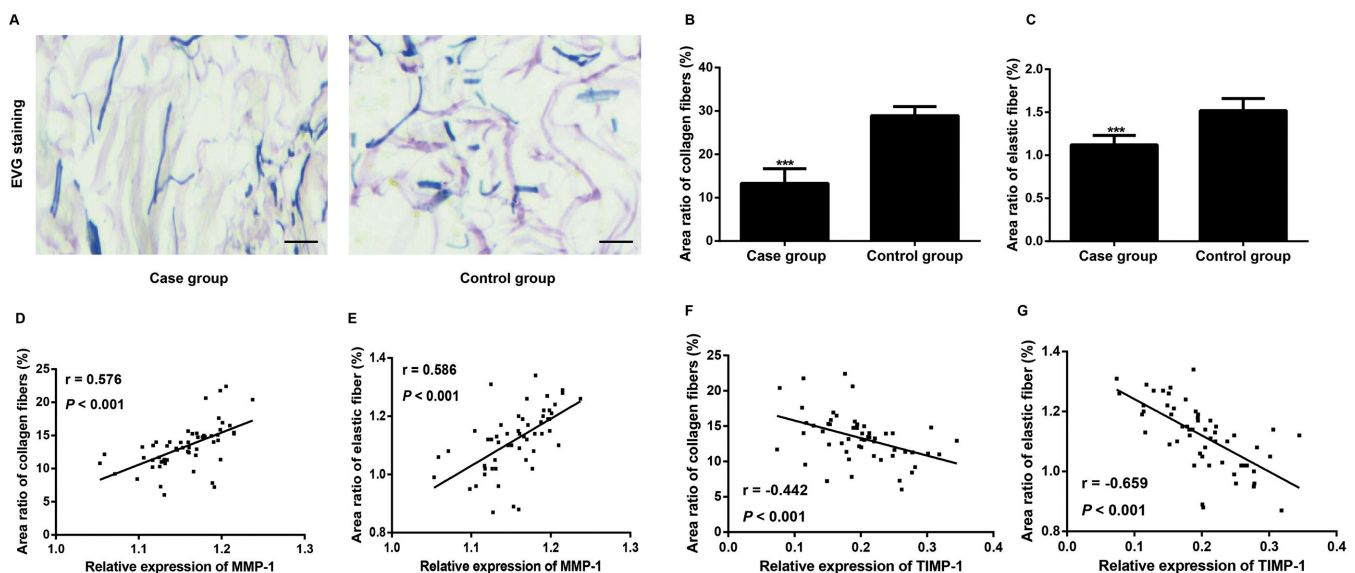


Fig. 6. Collagen and elastic fibers in rectal submucosa detected by EVG staining. **A-C.** The collagen fibers (**A, B**) and elastic fibers (**A, C**) in Control group and Case group detected by EVG staining. **D-E.** The correlation analysis of MMP-1 protein expression with area ratios of collagen fibers (**D**) and elastic fibers (**E**). **F-G.** The correlation analysis of TIMP-1 protein expression with area ratio of collagen fibers (**F**) and elastic fibers (**G**). Scale bars: 100 μ m.

found the patients in Case group showed increased pro-inflammatory cytokines (IL-6 and TNF- α), which were positively associated with MMP-1 protein expression but negatively correlated with TIMP-1 protein expression. As reported, the balance between MMP-1 and TIMP-1 was important in modulation of the degradation of collagen proteins during inflammation (Arihiro et al., 2001). In addition, our study also applied the Pearson's correlation analysis to analyze the relationship between MMP-1/TIMP-1 expressions and ODS severity (according to the CSS, Longo's ODS, and SSS scores), and our findings showed that the female patients with more severe ODS exhibited higher MMP-1 and lower TIMP-1 in rectal submucosa may cause more serious constipation, along with the increasing abdominal pressure, as well as the decline of pelvic floor and more serious prolapse. However, the mechanism still needs further investigation.

Currently, the reduced synthesis of collagen and elastic fibers of pelvic floor supporting structure was found to cause the movement of organs in female pelvic floor, including bladder, uterus, vaginal stump and rectum, down along the normal position (Soderberg et al., 2009). Besides, our finding revealed that the area ratio of collagen and elastic fibers were evidently lower in rectal submucosa of female patients with ODS associated with IRP than the female hemorrhoids without constipation and IRP. A previous study has shown that the decrease of collagen and elastic fibers may be associated with the activity change of collagen metabolic enzymes such as MMPs and TIMPs, further leading to the decrease of collagen content (Kalinin et al., 2016). Furthermore, there was a study which found that collagen participates in the rectal prolapse (Joshi et al., 2015). Additionally, oxidative stress may indirectly regulate MMP-1 and TIMP-1 in hUSLFs, so as to facilitate collagen metabolic disorder in a severity dependent way, thereby participating in the pathophysiology of POP (Liu et al., 2016). Besides, Pearson's correlation analysis in this study demonstrated that MMP-1 protein expression shared a positive correlation with the area ratios of collagen and elastic fibers, while TIMP-1 was in a negative correlation with them. Meanwhile, Altaş et al. also found a vital role of MMP-1 in turnover of collagen fibers in the intercellular matrix (Altas et al., 2010), and the responsibility of MMP-1 up-regulation in the lysis of dermal collagen and elastic fibers during skin aging (Wu et al., 2012). What's more, the overexpressed TIMP1 protein was one of main features for mice lungs with fibrosis (Ovet and Oztay, 2014). In the study by Taguchi's team, the elastase-treated animals presented a reduction in collagen and elastic fiber deposition and an enhancement in TIMP-1 expression after sakuranetin treatment (Taguchi et al., 2015). All related evidence indicated that MMP-1/TIMP-1 was correlated with the synthesis of collagen and elastic fibers to affect collagen content, giving rise to the occurrence of ODS associated with IRP.

Taken together, the enhanced MMP-1 shared a

negative correlation with the reduced TIMP-1 in rectal mucosa of patients with ODS associated with IRP. In addition, the collagen and elastic fibers were decreased in rectal submucosa of patients with ODS associated with IRP, but the pro-inflammatory cytokines (IL-6 and TNF- α) were increased. Besides, the expressions of MMP-1/TIMP-1 were correlated with ODS severity, inflammation and contents of collagen and elastic fibers.

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