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Original Article

Effect of PDIA3 gene silence on colonic mast cells and visceral sensitivity of rats with irritable bowel syndrome

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Abstract: This study aimed to elucidate the role of PDIA3 in visceral hypersensitivity of rats with irritable bowel syndrome (IBS). Fourty eight SD rats were randomly divided into four groups (n=12): control group, IBS-empty virus group (IBS-1), IBS-PDIA3 silence group (IBS-2), and IBS-the control group (IBS-3). Visceral hypersensitivity models were established by using acetic acid enema combined with restraint stress, and assayed by abdominal withdrawal reflexes (AWR). Mast cells (MCs) in ileocecal mucosa were counted with toluidine blue staining. Degranulation of MCs was observed under electron microscopy. Serum and mucosal levels of IL-4 and IL-9 were measured with ELISA and QT-PCR. Intestinal tryptase and PAR-2 expression was examined with ELISA and Western blot. The Results showed that PDIA3 plays an important role in the formation of visceral hypersensitivity by increasing systemic and colon mucosal expressions of IL-4 and IL-9, activating mast cells and upregulating PAR-2 expression of target organs.

Keywords: PDIA3, visceral hypersensitivity, mast cells, PAR-2, IBS

Introduction

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder. So far, its pathogenesis is still unclear, and there is rare effective method for clinical treatment. It has been reported that colon mast cells (MCs) infiltration and subsequent mediator release are important factors causing IBS symptoms, which are also closely associated with visceral hypersensitivity in IBS patients [1]. The activated MCs release a series of bioactive substances, in which tryptase selectively activates protease-activated receptor-2 (PAR-2). PAR-2 is a kind of nociceptors. When PAR-2 which is located in the colonel spinal dorsal root and neuronal membrane of afferent sensory fibers was activated, prolonged neuronal hyper excitability will take place [2].

In previous literature, it is revealed that protein disulfide isomerase A3 (PDIA3) was specifically expressed in colon mucosa in IBS rats. Similar to the finding in IBS rats, PDIA3 expression in colon mucosa of IBS patients was significantly increased, suggesting that PDIA3 most likely involved the IBS pathogenesis [3]. PDIA3 was also found to play an important role in endogenous antigen presentation [3, 4]. Therefore, this study was designed to explore the effects of PDIA3 on MCs activation and visceral sensitivity in rats.

Materials and methods

Animals

Forty-eight healthy SD rats (body weight 250± 10 g) were purchased from the Animal Center of Third Military Medical University. All rats arrived without deformity, trauma, or skin infections. The rats were raised in cages with pellets feeding, food and water ad libitum, which were purchased from the Animal Center of Third Military Medical University, and 12 h light. Animal experiments were performed after 1 week adaptive feeding. Animal experiments have approved by the Yangzhou University ethics committee.

Reagents and instruments

The PDIA3 interference lentiviruses (Westernbiotechnology Co., Ltd., Chongqing) and glacial

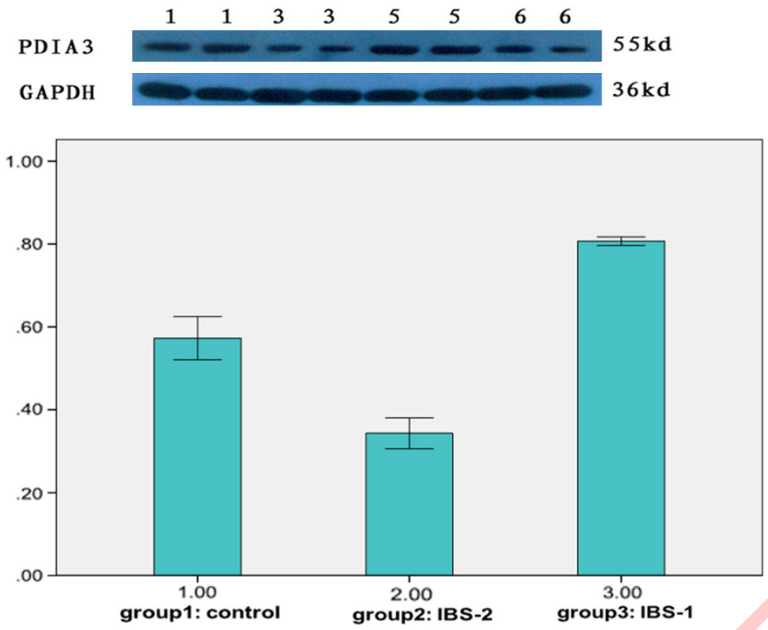

Figure 1. Protein expression of PDIA3. Group 1: control; Group 5: IBS-empty virus (IBS-1); Group 3, 6: IBS-PDIA3 gene silence (IBS-2).

acetic acid were used. The 8F catheter was used as an internal colorectal balloon dilatation catheter (Kankan Medical Devices Co., Ltd., Zhejiang).

Animal experiment

Grouping: Forty-eight rats were raised in room with normal food and water ad libitum for one week. These rats were then divided into blank control group (Control, n=12) and visceral hypersensitivity group (Model, n=36). The latter were subdivided into empty virus group (IBS-1, n=12), PDIA3 gene silence group (IBS-2, n=12), and the model control group (IBS-3, n=12). The rats were 3/cage only raised in room at 22-24°C, humidity <60%, and noise <50 db with normal food and water ad libitum. Animal experiments have approved by the Yangzhou University ethics committee.

Visceral hypersensitivity model: The rats in IBS-1 and IBS-2 group were injected empty virus and PDIA3 silence virus into tail vein, respectively. The injection was performed daily for 3 days, 150 μl each injection, and the virus titer was $5*10^8$ TU. Three days later, acetic acid enema combined with restraint stress was performed to establish visceral hypersensitivity model [5, 6].

Assessment of visceral hypersensitivity model: The visceral sensitivity was assessed with the international common score criteria of rat abdominal withdrawal reflex (AWR) [7].

Sample collection: On the second day following acetic acid enema, 2 rats each group were randomly selected and anesthetized with 3% pentobarbital (40 mg/kg). Colon tissues (1 cm length) were collected 6 cm above anus to evaluate acute intestinal mucous damage induced by acetic acid. On the 10th day after the successful model, the rest rats were anesthetized and decapitated. Peripheral blood (about 5 mL) was collected and centrifuged at 3000 rpm for 10 min. The serum was saved at -80°C until use. The ileocecal tissues and the 1-cm colon tissues 6 cm above anus were removed, longitudinally cut, cleaned with saline, and divided into two parts, which were soaked in 10% neutral formalin and liquid nitrogen until use.

Measurements

The colon tissues collected on the 10th day were used for Western blot to detect PDIA3 expression. The colon tissues near anus were performed HE staining. The serum levels of cytokines IL-4, IL-9 and total Ig-E were measured by using ELISA. The ileocecal tissues were performed toluidine blue staining to examine MCs (cell number was counted in 5 successive high-powered microscopy fields in each random sample and the averages were calculated). MCs degranulation was examined under electron microscopy. The mRNA levels of mucosal IL-4 and IL-9 were measured by QT-PCR. PAR-2 expression was measured by Western blot.

Statistical analysis

The results were analyzed using SPSS17.0 statistical software. The quantitative data were presented as mean ± SD. Independent samples T test was performed to compare two sam-

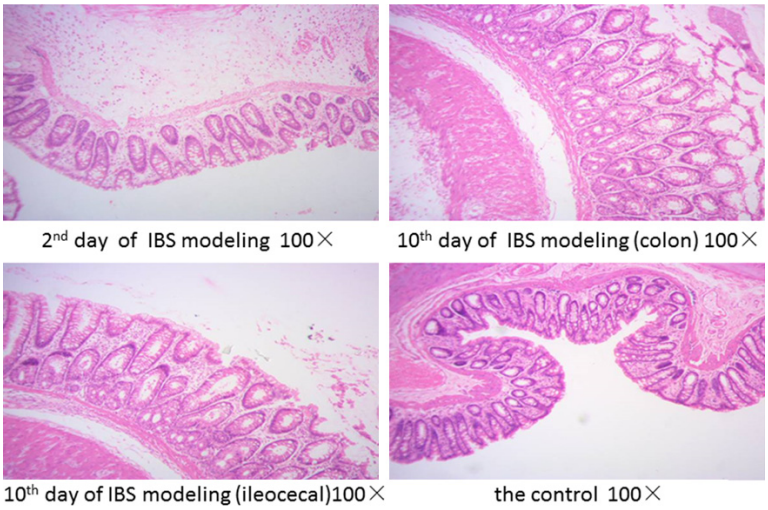

Figure 2. Inflammatory response in colon and ileocecal tissues.

Table 1. Pressure difference of balloon dilation in colorectal tissues when AWR was 3

Groups	Mean ± SD (mmHg)
Control	77.21±7.35**
IBS-1	50.25±5.62*
IBS-2	79.14±8.06**
IBS-3	41.67±5.62*

*P<0.05 vs control group. **P<0.05 vs IBS-3 group.

ple means. P<0.05 was considered statistically significant.

Results

General information

The rats treated with PDIA3 gene interference virus or empty virus exhibited good general condition and normal behavior. The conditions of diet and feces were consistent with those in blank control group. In the first 1-2 days after modeling, all rats exhibited restless, watery stool, perianal contamination with fecal residues, increased drinking, and decreased food consumption. On the 4-5th day, rats in the 3 modeling subgroups evacuated soft feces with less water. Starting at 7th day when restraint stress was exerted, all rats in visceral hypersensitivity model mainly evacuated soft stools, occasionally particle-like feces. After acetic acid enema combined restraint stress was completed, rats in all modeling subgroups appeared listlessness, shrugging hairs, reduced activity, slow response, thin and soft stool, perianal fecal residue, significantly reduced water and food consumption.

PDIA3 gene silence

Empty virus or virus with PDIA3 gene interference was injected into rat tail vein and the expression of PDIA3 in ileocecal tissues was detected by Western blot, as shown in **Figure 1**. The results showed that protein expression of PDIA3 decreased in PDIA3 gene interference group, as compared to control group and empty virus group (P< 0.05). The gray values were 0.34±0.04, 0.81±0.34, 0.57±0.04, respectively. This demonstrates PDIA3 gene silence model is successful. Hence, these rats can be used for further experiments.

HE staining of colon and ileocecal tissues in rats after 0.4% acetic acid enema

On the second day following acetic acid enema, thickened colon wall with congestion and hemorrhage was observed under naked eyes in model group. Under optical microscope, mucosal and submucosal layers presented edema and flaky bleeding spots in acetic acid stimulated model group as compared to blank control group, as shown in **Figure 2**. On the 10th day and after colorectal distension (CRD) test, the collected colon mucosa in both groups did not appear edema, congestion or hemorrhage. Under optical microscope, as compared to blank control group, colon tissue in model group was observed more intact mucosal structure and crypt, epithelium, and all layers of tissue. Pathological changes, including ulcer, were not seen. These results indicate that acetic acid-induced acute inflammatory mucosal damage has recovered.

IBS model assessment

In **Table 1**, the assessment results showed the visceral sensitivity in IBS-1 and IBS-3 groups was significantly increased, as compared to blank control group (P<0.05). As compared to IBS-1 and IBS-3 groups, the visceral sensitivity in IBS-2 group was significantly decreased

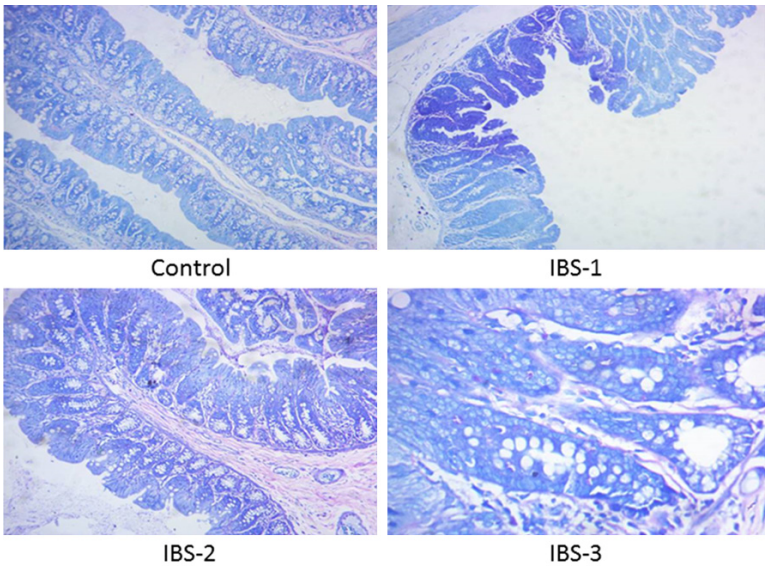

Figure 3. Toluidine blue staining of ileocecus.

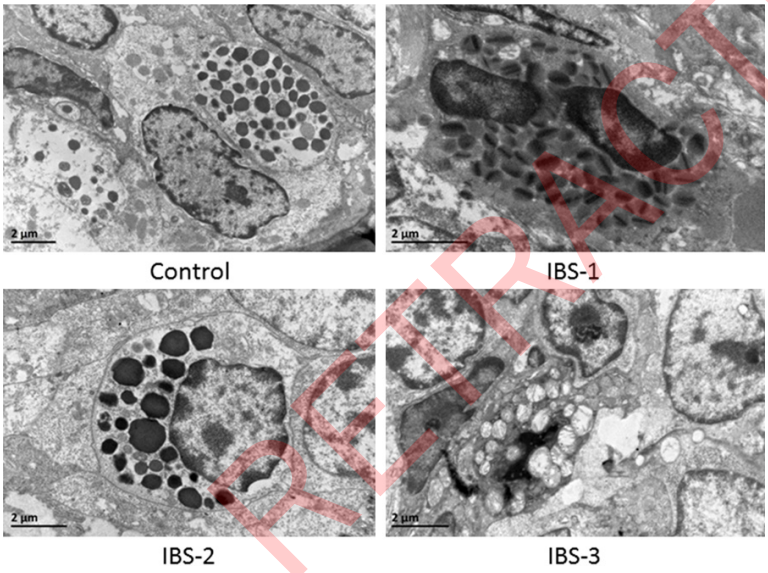

Figure 4. Degranulation of MCs in ileocecus.

(*P*<0.05). The visceral sensitivity in IBS-2 group did not show significant difference with that in blank control group.

Toluidine blue staining

MCs in toluidine blue staining exhibited purple cytoplasm and blue nuclei under optical microscope, shown in **Figure 3**. These cells scattered in the submucosa and lamina propria (cell number was counted in 5 successive high-powered microscopy fields in each random sample and the averages were calculated). The number of MCs in ileocecus in IBS-3 group was significantly increased as compared to blank control group (2.17±0.72 *vs* 0.58±0.51, *P*<0.05). The number of MCs in ileocecus in IBS-2 group was significantly decreased as compared to IBS-3 group (0.67±0.65 *vs* 2.17±0.72, *P*<0.05), while no significant difference with that in blank control group.

Activation and degranulation of MCs

Under electron microscope, undegranulated MCs showed intact membrane, uniform cytoplasm. The degranulated MCs appeared membrane rupture with particulate emission, irregular cells and vacuolization, as shown in **Figure 4**. Our results showed that MCs in IBS-3 group exhibited obvious degranulation, shrunk membrane, irregular cells and intracellular vacuoles. In IBS-1 group, shrunk membrane, irregular cells and suspicious vacuolization were observed. No apparent degranulation was seen in IBS-2 and blank control groups.

Serum expression of total IgE, IL-4, IL-9

Serum levels of IL-4, IL-9 and total IgE in IBS-3 group were significantly increased as compared to blank control group (*P*<0.05), as shown in **Table 2**. Serum levels of those three factors in IBS-2 group were significantly decreased as compared to IBS-3 group (*P*<0.05). No significant difference of these factors was seen between IBS-2 and blank control groups.

Expression of IL-4 and IL-9 in colon mucosa

In the results shown in **Figure 5**, the mRNA expression of IL-4 and -9 in ileocecal tissues in IBS-3 group were significantly increased, comparing to blank control group (*P*<0.05). These

Table 2. Serum levels of IL-4, IL-9, total IgE and colon tryptase expression

	Control	IBS-1	IBS-2	IBS-3
IgE (μg/L)	0.10±0.00**	0.20±0.00*	0.10±0.00**	0.28±0.05*
IL-4 (μg/L)	0.04±0.01**	0.14±0.01*	0.05±0.01**	0.15±0.02*
IL-9 (μg/L)	0.08±0.01**	0.10±0.01*	0.08±0.01**	0.21±0.01*

*P<0.05 *vs* control. **P<0.05 vs IBS-3 group.

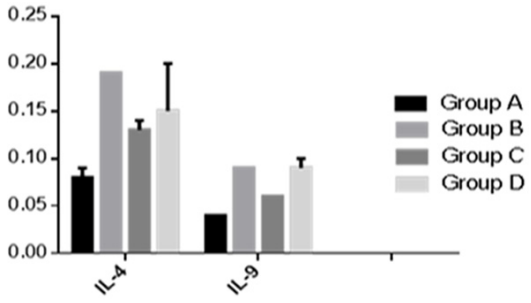

Figure 5. mRNA expression of IL-4 and 9 in colon mucosa. Group A: Control; Group B: IBS-GFP; Group C: IBS-2; Group D: IBS-3.

Table 3. Tryptase levels in colon tissues

Groups	n	Mean ± SD
Control	10	0.08±0.00
IBS-1	10	0.17±0.01*
IBS-2	10	0.11±0.01**
IBS-3	10	0.23±0.02*

*P<0.05 *vs* control. **P<0.05 vs IBS-3 group.

expressions were significantly decreased by PDIA3 gene silence (P<0.05 IBS-2 group *vs* IBS-3 group). There is no significant difference between IBS-2 and blank control groups.

Intestinal proteases tryptase and PAR-2 expression in colon tissue

In **Table 3**, the expression of tryptase in ileocecal tissues in IBS-3 group were significantly increased comparing to blank control group (P<0.05). This expression was significantly decreased by PDIA3 gene silence (P<0.05 IBS-2 *vs* IBS-3 group). No significant difference was seen between IBS-2 and blank control groups.

In **Figure 6**, Protein expression of PAR-2 in ileocecal tissues was remarkably upregulated in IBS-3 group, as compared to blank control group (Gray value 0.62±0.00 *vs* 0.41±0.00, P<0.05). Comparing to IBS-3 group, PAR-2 expression in IBS-2 group was significant decreased (0.20±0.02 *vs* 0.62±0.00, P<0.05). The difference of PAR-2 expression between IBS-2 group and blank control group was not significant.

Discussion

Recent researches have shown that mucosal immune dysfunction plays an important role in the pathogenesis of IBS. Abnormal immune response exists in colon mucosa in IBS patients [8, 9]. In the colonic mucosal immune function, mucosal antigen-presenting cells (antigen presenting cells, APC) play a crucial role [4, 10]. The related studies showed that PDIA3 plays an important role in the process of antigen presentation [11].

PDIA3 (also named ERp57), is one member of protein disulfide isomerase family. PDIA3 catalyzes disulfide redox isomerization reaction of protein or polypeptide. Recent studies revealed that PDIA3 is important for endogenous antigen presentation. Antoniou *et al* reported that in MHC molecules antigen-presentation, PDIA3 molecules directly entered and bound to the peptide binding groove of MHC-I molecules to make the groove more suitable for the binding of antigen peptides [12]. Santos *et al* further proved that PDIA3 formed couples with MHC-I and tapasin to help MHC-I bind to antigenic peptides in the process of antigen presentation [13]. Stepensky *et al* found that PDIA3 enhanced the binding stability of MHC-I and antigenic peptide [14].

The important role of MCs in IBS has been demonstrated. MCs, once activation, release histamine, serotonin, plasmin and other media, which subsequently activate sensory neurons including in the gastrointestinal tract, leading to hyperalgesia or allodynia. Plasmin can activate PAR-2, which is located in neuronal membrane of spinal dorsal root of colon, and result in lasting high excitability of nociceptors [11]. Domestic and international studies have shown that the number of MCs was increased in the mucosa of large intestine and small intestine [15]. The proportion of activated degranulated MCs was also increased [16]. Degranulated MCs densely appear around efferent neurons in IBS patients, suggesting that MCs may be involved in sensory and motor abnormalities in IBS [16, 17]. Clinical and animal investigations showed that the application of MCs stabilizer

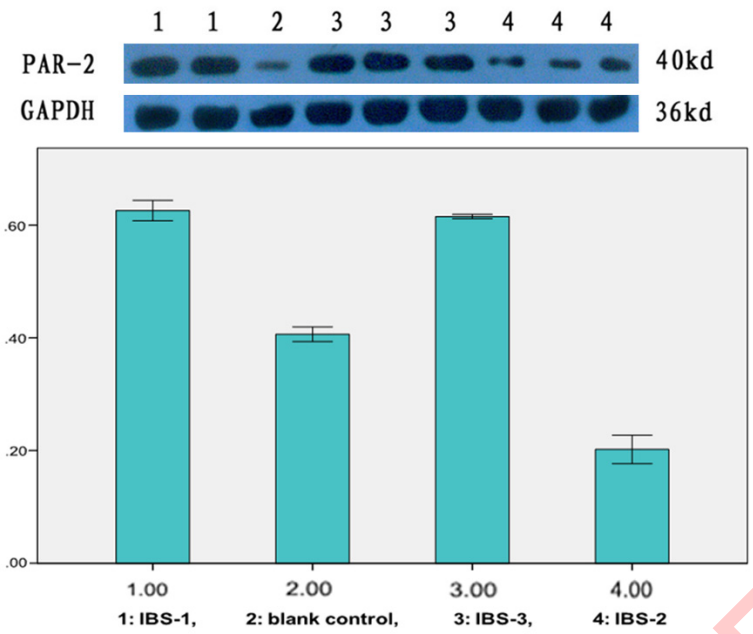

Figure 6. PAR-2 expression in ileocecal tissues.

ketotifen alleviated visceral hypersensitivity and IBS symptoms [18].

MCs are activated by a variety of pathways, among which, the most classic one is the recognition and bridging between IgE and its receptor FcεRI, which subsequently lead to degranulation [19]. IL-4 is a specific IgE promoting cytokine. The upregulation of IL-4 induced antigen-specific IgE production, the latter then lead to MCs activation [20]. IL-9 can increase FceRIα expression in MCs surface, making MCs more easily to be activated [21].

When the colonic mucosa immune shows abnormalities, the antigen presenting of colon would be enhanced, and the secretion of IL-4 and IL-9 would be enhanced. Related studies revealed that the expression of intestinal IL-4 was upregulated during acute stage in post-infective IBS. IL-4 expression returned to normal level or even much lower after successful IBS modeling [22]. Previous studies showed that IL-9 is a Th2 cytokine. However, IL-9 is also secreted by various T cells, including Th9 cells, Th17 cells and Treg cells [23]. MCs are well-known major IL-9-secreted cells. MCs promotes IgE cross-linking at cell surface through IL-9 autocrine manner, initiates downstream signaling, which causes MCs degranulation and rapid release of histamine, IL-1β and other media. This forms a positive feedback to promote IL-9 secretion. Finally, MCs growth and proliferation in immune response are enhanced [24].

Therefore, in the early stage of IBS, the antigen presenting of colon is enhanced. The quantity of IL-4 was larger than that in normal state, activating MCs and causing a series of subsequent immune response. Similar to IL-4, IL-9 also makes MCs in more easily activated state. Moreover, IL-9, after MCs activation, promoted MCs to secrete more IL-9 by positive feedback, thereby facilitating MCs to take effect in immune response. McKernan *et al* compared the expression of plasma cytokines in 30 IBS patients. They found IL-6 and IL-8 were upregulated, while IL-4 had no significant change as compared to control groups [25]. Our study showed that serum and mucosal levels of IL-4 and IL-9 were elevated in IBS rats with visceral hypersensitivity.

Experimental data have shown that increased MCs number and cells activation, increased secretion of IL-4 and IL-9 in serum and colon mucosa, as well as upregulated expression of nociceptors PAR-2 in IBS rats. This indicates that in the process of visceral hypersensitivity, T lymphocytes secret more IL-4 and IL-9, which results in MCs activation and subsequently promoting the formation of visceral hypersensitivity. PDIA3 gene silence reduced the secretion of IL-4 and IL-9, reduced the number and degranulation of intestinal MCs, inhibited the mucosal expression of PAR-2, and blocked the formation of visceral hypersensitivity. Therefore, PDIA3 plays an important role in the development of visceral hypersensitivity in IBS.

According to all data in the present study, we speculated that increased number or abnormal hyperfunction of T cells may exist in IBS rats with visceral hypersensitivity. This results in abnormal production of IL-4 and IL-9 different from their physiological amount. IL-4 and IL-9 act on MCs to increase their number, make them in a state of high sensitivity, even degran-

ulation, and to release biological media. These biological media binds to PAR-2 nociceptors to cause IBS visceral hypersensitivity. PDIA3 gene silence may decrease cytokines secretion from T lymphocytes in systemic and local stress response, and then reduce MCs activation and visceral sensitivity. Therefore, we believe PDIA3 would mediate MCs activation by influencing the secretion of cytokines from T lymphocytes, and play an important role in the formation of visceral hypersensitivity.

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Disclosure of conflict of interest

None.

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