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From Cell Biology to Tissue Engineering

Increased annexin A2 and decreased β-catenin in adenomyosis contribute to adenomyosis-associated dysmenorrhea

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Summary. Objective. To investigate the expression of annexin A2 (ANXA2) and β-catenin in eutopic and ectopic endometrium, and their relationships with adenomyosis-associated dysmenorrhea.

Methods. From December 2013 to June 2014, ectopic endometrium (n=30) and eutopic endometrium (n=30) of adenomyosis were collected as experimental group, and endometrium (n=30) of uterine myoma as control group from the department of gynecology and obstetrics, the affiliated hospital of Inner Mongolia medical university. The expression of ANXA2 and β-catenin was detected by immunohistochemical S-P method, followed by the Pearson correlations for the correlation analysis of ANXA2 and β-catenin with adenomyosis-associated dysmenorrhea. Meanwhile, the levels of preoperative serum ANXA2 of patients with adenomyosis (n=42) and uterine myoma (n=42) were also measured by enzyme-linked immunosorbent assay (ELISA).

Results. Immunohistochemistry and ELISA identified a higher expression of ANXA2 in eutopic and ectopic endometrium of adenomysis tissues, whereas β-catenin protein was down-regulated. Furthermore, there was a significant positive correlation between ANXA2 expression and dysmenorrhea degree, while there was a negative linear correlation between β-catenin expression and dysmenorrhea degree in ectopic endometrium.

Conclusion. These results suggested that increased ANXA2 and less expressed β-catenin were correlated to adenomyosis-associated dysmenorrhea. It may provide a new idea of diagnosis and treatment to adenomyosis-associated dysmenorrhea.

Key words: Annexin A2, β-catenin, Adenomyosis, Dysmenorrhea

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Introduction

Adenomyosis is a benign gynecological disease characterized by the presence of aberrant growth and invasion of endometrial tissue embedded within the myometrium, leading to dysfunctional myometrial hyperperistalsis, increased intrauterine pressure and impairment of proper uterine function (Tamai et al., 2005; Wang et al., 2009). It has been reported that the prevalence varies from 10.0% to 66.0% at the time of hysterectomy according to different diagnostic criteria (Vercellini et al., 2006). The most frequent clinical symptoms of adenomyosis include metrorrhagia (10-12%), dysmenorrhea (15-30%), menorrhagia (40-50%) (Bergeron et al., 2006). As one of the most common causes of dysmenorrhea, adenomyosis seriously injures women suffering from moderate or severe dysmenorrhea both in body and mentality, severely affecting quality of life. However, the underlying factors that regulate adenomyosis occurrence and its relationship with adenomyosis-associated dysmenorrhea are still poorly understood. Therefore, the causes of adenomyosis and adenomyosis-related dysmenorrhea urgently need to be

investigated.

Annexin A2 (ANXA2), a calcium-binding cytoskeletal protein existing in many cell types, has a diverse range of cellular functions including angiogenesis, proliferation, apoptosis, cell growth regulation and calcium signaling (Onishi et al., 2015; Pianta et al., 2015; Zhou et al., 2015). The up-regulated expression of ANXA2 has been reported in liver cancer, prostate cancer and pancreas cancer (Inokuchi et al. 2009; Zhang et al., 2012; Deng et al., 2013). Additionally, evidence-based data unraveled an active role for ANXA2 in the pathogenesis of adenomyosis through conferring the ability of endometrial carcinomas to metastatic and proangiogenic capacity (Alonso-Alconada et al., 2015). However, the understanding of ANXA2 in dysmenorrhea is still poorly elucidated.

β-catenin is the core component of the canonical Wnt signalling and plays a role in cell-cell adhesion (Brembeck et al., 2006). As an important molecule in the etiology and pathology of adenomyosis, abnormal activation of β-catenin contributes to adenomyosis development through epithelial-mesenchymal transition (Oh et al., 2013). Nonetheless, there is limited and controversial evidence of β-catenin expression in adenomyosis, with some suggesting that expression of β-catenin is decreased in endometriotic lesions (Scotti et al., 2000), while others demonstrate a higher β-catenin expression (Ueda et al., 2002; Oh et al., 2013).

Published *in vitro* studies also investigated ANXA2 and β-catenin together. Sarkar et al. (2011) suggested that ANXA2 expression was required for the biological effects of progastrin, and mediates the stimulatory effect of progastrin on p65NF-κ, β-catenin, CD44 and DCAMKL+1 *in vivo* and *in vitro*. Wang et al. (2015) further revealed that downregulation of ANXA2 could inhibit hepatoma cell growth through down-regulation of β-catenin and cyclin D1, which were involved in cell cycle inhibition. Cui et al. (2016) also found a direct interaction of CD147 with ANXA2 and DOCK3-β-catenin-WAVE2 signaling in cancer migration regulation. However, there was no study showing the expression of ANXA2 and β-catenin in women with adenomyosis, and their relationships with dysmenorrhea. Thus, in this study, we attempted to investigate the expression of ANXA2 and β-catenin in the ectopic and corresponding eutopic endometrium samples from women with adenomyosis, and their relationships with dysmenorrhea.

Materials and methods

Clinical summary

Freshly resected matched ectopic and eutopic endometrial tissues of adenomyosis and endometrium of uterine myoma were collected from the department of gynecology and obstetrics, the affiliated hospital of Inner Mongolia medical university from December 2013 to June 2014. None of premenopausal women had intra uterine device (IUD), typical endometrial hyperplasia; none had any unincorporated endocrine, immune and metabolic disease, malignant tumors; none received any hormone and immune agents prior to surgery for 3 months. Tissue and serum samples were immediately frozen in liquid nitrogen and stored at -80°C. The approval of local ethics committee and informed consents were obtained prior to the study.

Immunohistochemistry for ANXA2 and β-catenin

Experimental group

Ectopic and eutopic endometrium of 30 donors with an average of 44 years old (32 years old to 49 years old) were documented by the pathology department. Control group: 30 endometrium tissues were collected from patients with uterine myoma (an average of 42 years ranging from 34 to 51 years). There were 15 cases of proliferative phase and secretory phase in 15 cases with a history of uterine cavity operation no more than 2 times (diagnostic curettage or abortion, etc.), respectively. Of these, there were 4 cases of mild dysmenorrhea, 19 cases of moderate dysmenorrhea, and 7 cases of severe dysmenorrhea.

4 μm tissue sections were set in an oven at 60°C for 20 min, and then xylene was used to dewax twice for 10min each time. Tissue sections were incubated with 100% alcohol for 10 minutes twice, 95%, 90% and 80% alcohol for 5 minutes to block endogenous peroxidase activity respectively. After washing, sections were incubated with a repairing solution for 9 min. 50 μl ANXA2 antibody (diluted into 1:100, provided by Bio-synthesis, Lewisville, USA) or β-catenin antibody (diluted into 1:150, provided by Bio-synthesis, Lewisville, USA) was diluted in blocking solution and incubated in a humidified chamber at 4°C stay over. After washing, 50 μl secondary antibody (biotin-labeled goat anti-mouse IgG) was applied for 10 minutes at room temperature followed by 3 washing steps, 3 min each, and then 50 μl streptavidin peroxidase solution was used. Sections were then washed, and color was developed using DAB substrate chromogenic system (Zymed Inc., South San Francisco, CA, USA). Sections were counterstained with Mayer hematoxylin for 3 min. Finally, sections were analyzed with a microscope (BH-2 OLYMPUS, Tokyo, Japan).

ELISA for ANXA2

Experimental group

Preoperative serum samples were obtained from 42 adenomyosis patients, with an average of 43.5 years (ranging from 31 to 38 years old).

Control group

Preoperative serum samples of 42 patients with

uterine myoma ranged from 35 to 50 years old, with an average of 42 years.

The level of serum ANXA2 was detected using a human ANXA2 ELISA kit (Uscn Life Science Inc., Wuhan, China) following the manufacturer's instructions. 50 µl of serum sample or standard was separately added into each well, and then 50 µl of detection reagent was applied and incubated for 30 min at 37°C. Subsequently 50 µl color development reagent A and 50 µl reagent B were added and incubated for 15 min at 37°C. Finally, 50 µl of stop solution was added to each well, and absorbance was read at 450 nm. During the procedure, washing the plate was according to the ELISA routine method.

Evaluation criteria

Dysmenorrhea degree: the examiners evaluated the degree of dysmenorrhea using a visual analog scale (Hawker et al., 2011) (VAS, ranged 0-10 cm, a score of "10" entailed the best outcome, while a score of "0" entailed the worst). Pain assessment criteria: no dysmenorrhea (-), mild pain (+, l-4 cm), moderate pain (+ +, 5-7 cm) and severe pain (+ + +, 8-l0 cm).

Staining and scoring

ANXA2 exhibited positive expression with yellow or brown particles in membrane and cytoplasm; β-catenin expression was positive when brown particles appeared in endometrial glandular epithelial cell membrane. To evaluate the immunostaining, a score corresponding to the product of both (a) staining (0 = negative; 1 = canary; 2 = yellow; 3 = brown) and (b) percentage of positive cells (0 = < 5% positive cells; 1 = 5%-25% positive cells; 2 = 26%-50% positive cells; 3 =

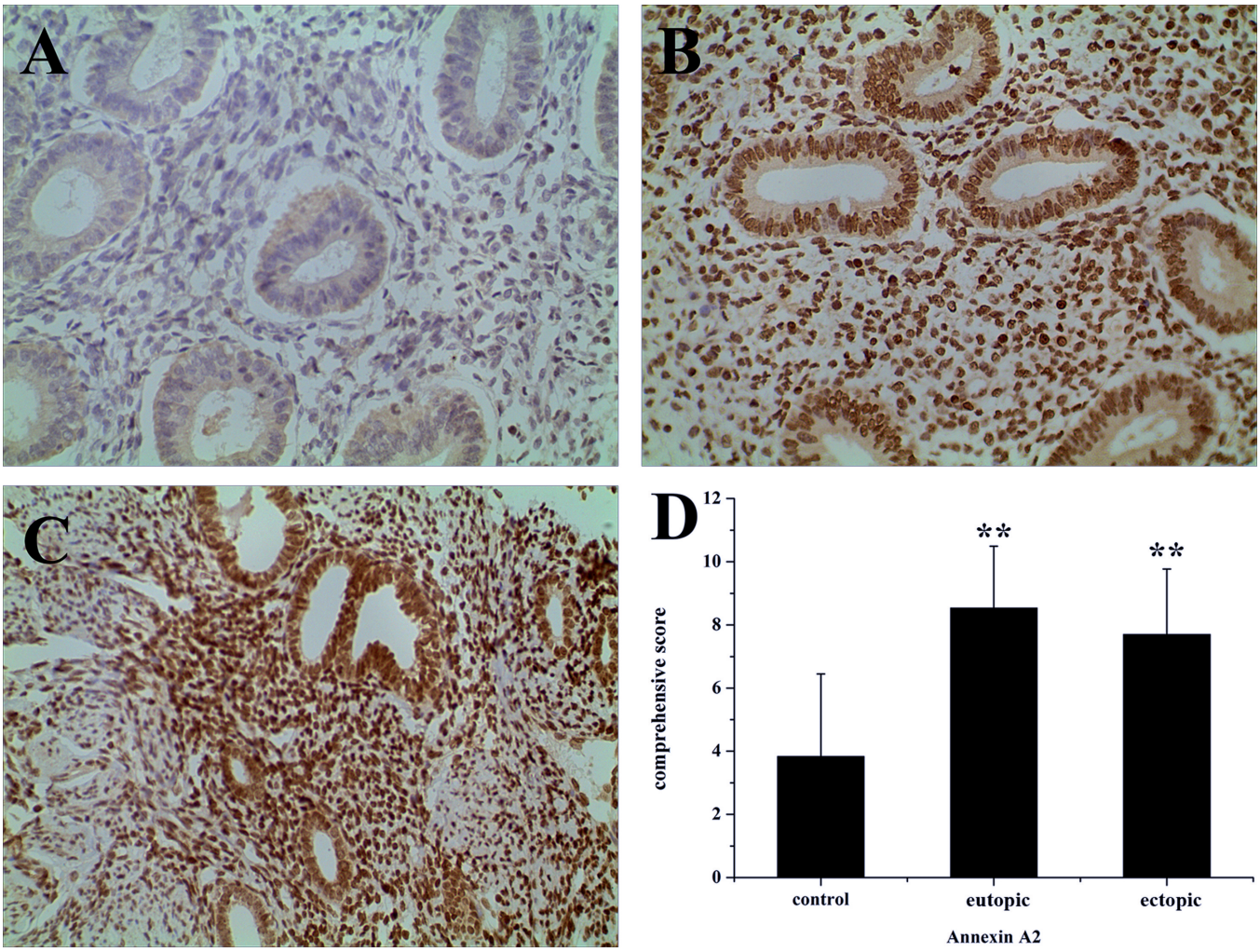

Fig. 1. The expression of ANXA2 in controls, eutopic endometrium and ectopic endometrium. **A.** ANXA2 was weakly positive expressed in normal endometrium tissues. **B.** ANXA2 was strongly positive expressed in eutopic endometrium. **C.** ANXA2 was positively expressed in ectopic endometrium. **D.** Semi-quantitatively for ANXA2 of three groups. *P<0.05, **P<0.01, compared with control group. x 400.

51-75% positive cells; 4 ≥ 75%) was established. The product of (a) * (b) was considered as comprehensive evaluation scores (0-2 = negative; 3-4 = weakly positive; 5-8 = positive; 9-12 = strongly positive). A score greater than 2 was the value of a positive immunohistochemical assay (Mattern et al., 1996; Zhao et al., 2005). The results of staining were evaluated by two independent pathologists without knowledge of the clinico-pathological features, and any difference in interpretation was resolved by consensus.

Statistical analysis

IBM SPSS statistical software, version 13 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The experimental data were shown as mean ± SD. One-way ANOVA was used with Tukey's multiple comparison tests for multiple groups. Bivariate Pearson correlation was used for the correlation analysis. The level of statistical significance was set at 0.05.

Results

ANXA2 was significantly increased in adenomyosis

According to the immunohistochemistry (Fig. 1A-C) and the comprehensive scoring criteria, ANXA2 showed strongly positive expression in eutopic endometrium and positive expression in ectopic endometrium. The result of semi-quantitative for ANXA2 showed that there was a significant difference between control group and adenomyosis groups (Fig. 1D). However, the expression of ANXA2 had no significant difference between eutopic endometrium group and ectopic endometrium group.

ANXA2 expression was positively correlated with dysmenorrhea degree in ectopic endometrium

As shown in Fig. 1, ANXA2 was significantly increased in eutopic endometrium and ectopic endometrium. Further, in order to investigate the correlation between ANXA2 expression and dysmenorrhea degree in eutopic endometrium and ectopic endometrium, Pearson correlations analysis was performed. The result revealed that there was a significant positive correlation between ANXA2 expression in ectopic endometrium and the degree of dysmenorrhea (r=0.577, P=0.001) (Fig. 2), while, ANXA2 expression in eutopic endometrium had no obviously linear dependence on dysmenorrhea degree (r=0.283, P=0.130).

β-catenin was significantly down-regulated in adenomyosis

β-catenin was also performed by immunohistochemistry, and the outcome suggested that β-catenin level was obviously reduced in adenomyosis tissues compared with that in normal endometrium tissues (Fig. 3A-C). Additionally, eutopic endometrium group exhibited a higher level of β-catenin than ectopic endometrium group (Fig. 3D).

β-catenin expression was negative correlated with dysmenorrhea degree in ectopic endometrium

Pearson correlations analysis revealed that there was a significant negative linear correlation between β-catenin expression and dysmenorrhea degree in ectopic endometrium (r=-0.469, P=0.009) (Fig. 4), however, no obviously linear dependence between dysmenorrhea degree and β-catenin expression in eutopic endometrium was observed (r=-0.113, P =0.550).

Annexin level was elevated in serum of patients with adenomyosis

The Annexin level in preoperative serum was further detected by ELISA (Fig. 5). Serum level of ANXA2 in adenomyosis group was significant higher than that in control group (P<0.01).

Discussion

Adenomyosis is a benign disorder that affects many women predominantly at the reproductive ages. Patients with adenomyosis can have a range of cardinal symptoms, such as menorrhagia and dysmenorrhea (Ferenczy, 1998), having a tremendous impact on women's well-being and health. Treatment of adenomyosis has been a challenge since invasive hysterectomy was the choice for severe and symptomatic adenomyosis. Hitherto, the mechanisms underlying adenomyosis-associated dysmenorrhea are poorly

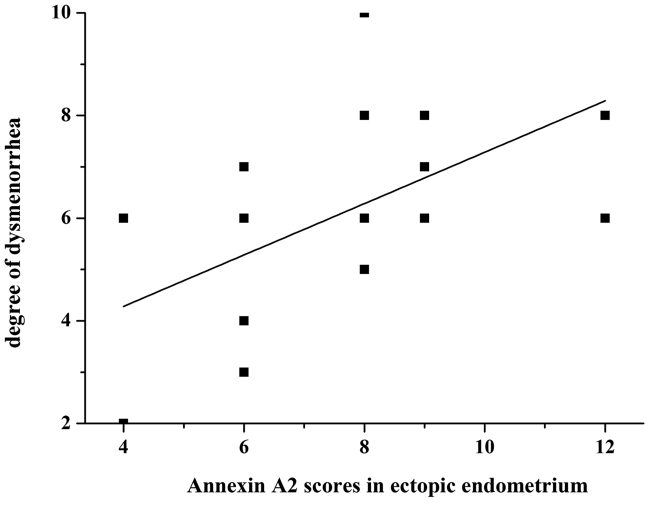

Fig. 2. ANXA2 expression was positively correlation with dysmenorrhea degree in ectopic endometrium.

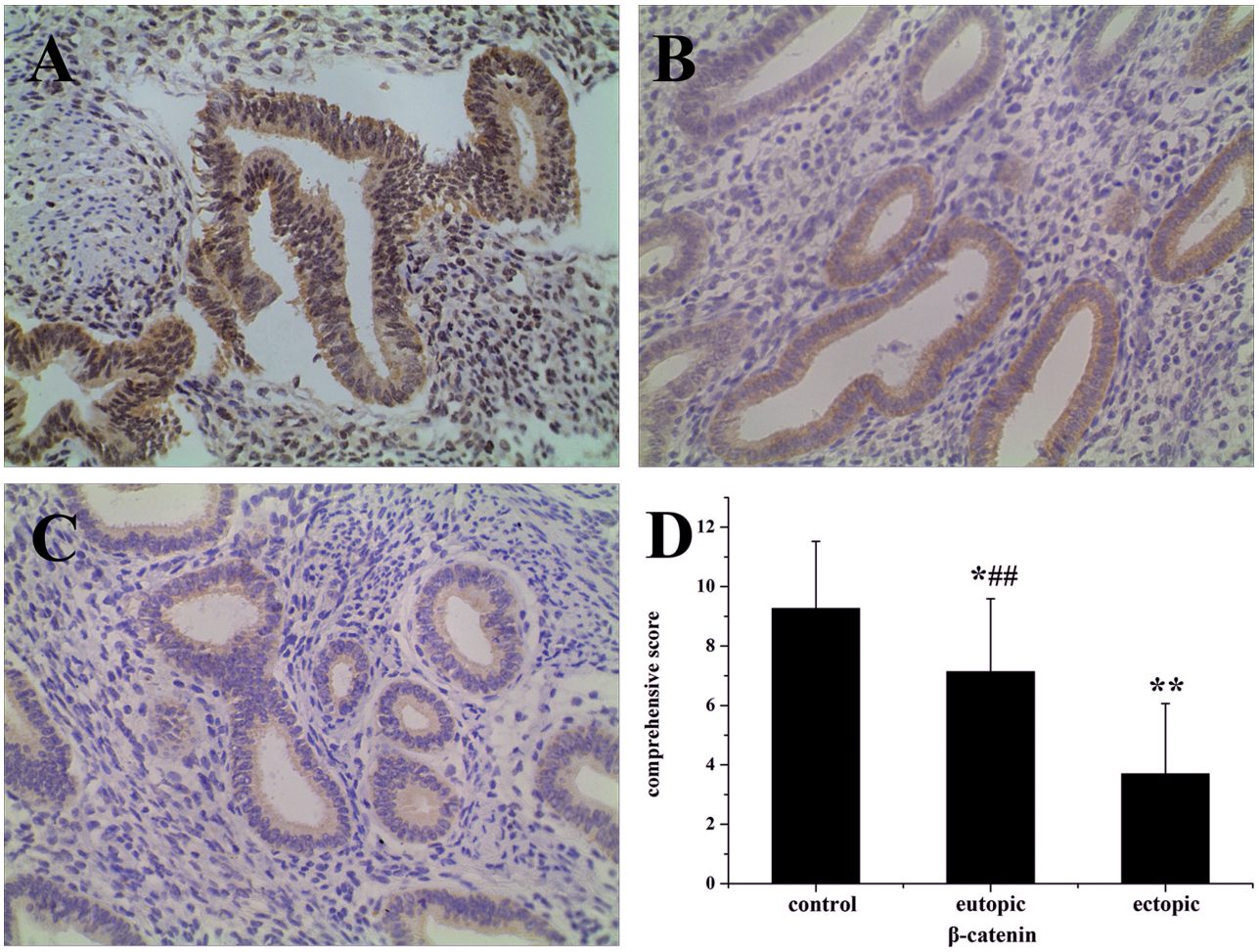

Fig. 3. The expression of β-catenin in controls, eutopic endometrium and ectopic endometrium. **A.** β-catenin was strongly positive expressed in normal endometrium tissues. **B.** β-catenin was positively expressed in eutopic endometrium. **C.** β-catenin was weakly positive expressed in ectopic endometrium. **D.** Semi-quantitatively for β-catenin of three groups. *P<0.05, **P<0.01, compared with control group; ##P<0.01, compared with ectopic endometrium group. x 400.

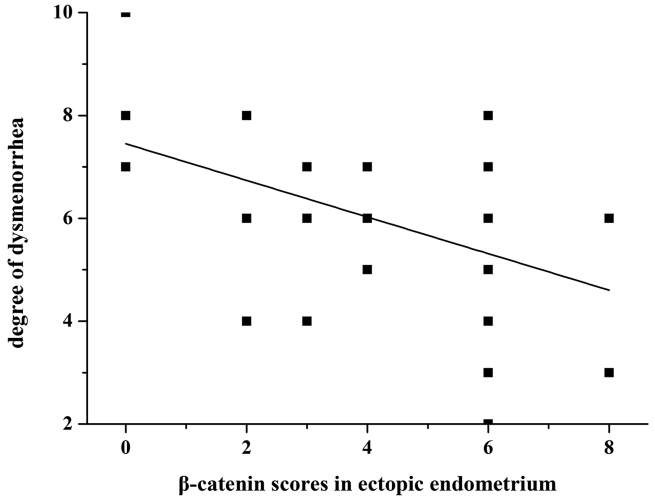

Fig. 4. β-catenin expression was negatively correlation with dysmenorrhea degree in ectopic endometrium.

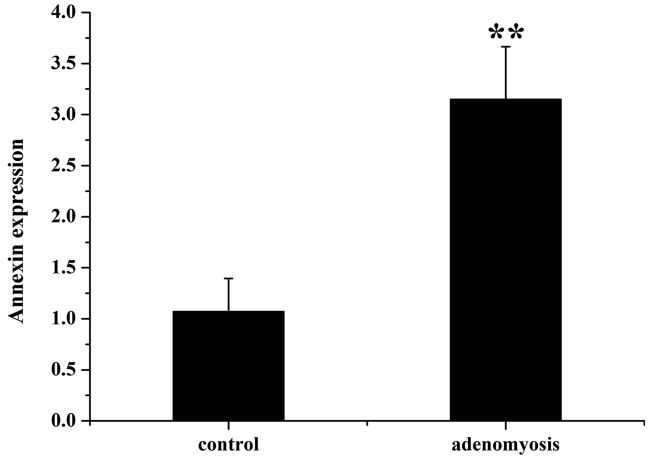

Fig. 5. The Annexin level in preoperative serum of control and adenomyosis groups. **P<0.01, compared with control group.

understood. Previous studies have demonstrated that both oxytocin receptor (OTR), increased immunoreactivity to nuclear factor κB and transient receptor potential vanilloid type 1 (TRPV1) might be positive correlated with dysmenorrhea and its severity in adenomyosis (Mechsner et al., 2010; Nie et al., 2010). These molecules are either directly or indirectly linked to inflammation and other pain mediators. Furthermore, emerging evidence suggests that the process of adenomyosis development is closely associated with ANXA2, as well as β-catenin. However, the role of ANXA2 and β-catenin, and their relationships with dysmenorrhea remain underexplored.

Herein, we investigated the expression of ANXA2 and β-catenin in the ectopic and corresponding eutopic endometrium. Further, the correlation between the expression of ANXA2 and β-catenin with adenomyosis-associated dysmenorrhea was assessed. In the current study, we implied a significant higher level expression of ANXA2 in ectopic and eutopic endometrium than normal endometrium, and its expression in ectopic lesion was proved to be positively correlated with severity of adenomyosis-associated dysmenorrhea. Meanwhile, decreased β-catenin and a negative correlation with adenomyosis-induced dysmenorrhea were markedly observed in patients with adenomyosis.

The above results coincided with the previous reports that elevated ANXA2 expression could be induced by higher estrogen concentration, and the expression of ANXA2 in ectopic lesion was tightly correlated with dysmenorrhea severity in women with adenomyosis (Zhou et al., 2012). In the present study, ELISA identified a higher expression of ANXA2 in preoperative serum compared to control group. The result suggested that overexpression of ANXA2 had a consistency both in tissues and in serum, confirming that ANXA2 might serve as a potential noninvasive target for the treatment of adenomyosis-induced dysmenorrhea. Interestingly, the expression of ANXA2 had no significant difference between eutopic endometrium and ectopic endometrium, indicating that the role of ANXA2 in the ectopic endometrium was consistent with the theory of 'eutopic endometrium determinism', that is the ectopic implantation of endometrial tissue depends on the differences in gene expression of the incumbent membrane (Tuerxun et al., 2014).

β-catenin has been reported to contribute to variety of diseases' progression, including prostate cancer, myeloid malignancies and ischemia-reperfusion injury (Al-bataineh et al., 2016; Bauman et al., 2016; Galán-Díez et al., 2016). Bodnar et al. (2014) demonstrated that intense expression of E-cadherin, β-catenin and Wnt-1 was found in ovarian cancer, indicating the Wnt/β-catenin pathway as a potential prognostic and predictive marker in patients with advanced ovarian cancer. Worth mentioning, contradictory results were obtained by Scotti et al. (2000), who found the expression of E-cadherin, together with β-catenin was reduced in endometriotic lesions, confirming the conclusion of other studies (Fujimoto et al., 1996; Shaco-Levy et al., 2008). In the present study, we also found that β-catenin was statistically decreased in adenomyosis compared with normal endometrium, especially in ectopic endometrium. A negative correlation with severity of adenomyosis-associated dysmenorrhea was also observed in ectopic endometrium. Therefore, a lack of β-catenin possibly plays an important role in the pathogenesis of adenomyosis, contributing to its invasive character.

In the present study, ANXA2 had no obvious correlation with β-catenin in eutopic and ectopic endometrium (data not shown). This was most likely because of their functions. On the one hand, ANXA2 promotes the occurrence of adenomyosis by the production of fibrinolytic enzyme and damage the basement membrane of endometrium. On the other hand, by changing the structure of the actin, ANXA2 induced transformation from endometrial epithelial to mesenchymal (EMT). As a result, the endometrial glandular cells were transformed into mesenchymal cells, and the adhesion of the cells was changed, so that the endometrial cells were easy to disperse, and then invaded and transferred to the myometrium. β-catenin is an important component of the Wnt signalling pathway and plays a role in cell-cell adhesion. It seems that ANXA2 initiated EMT, while β-catenin may be involved in the process of EMT, which can lead to adenomyosis by changing the cell adhesion. In fact, the real process is very complicated. The mechanism of these two proteins might involve a series of gene expression regulation in the chain reaction and various signal pathways. Therefore, the specific mechanism remains to be further studied.

This study also has some limitations. The expressions of ANXA2 and β-catenin in the ectopic and eutopic endometrium of women with adenomyosis was preliminarily studied without further confirmation, so the results still need to be tested by more experiments in the future. In addition, the underlying mechanism of ANXA2 and β-catenin on dysmenorrhea remain largely unrevealed, therefore more studies are still needed to enrich the mechanism of dysmenorrhea and its relationship with adenomyosis.

In conclusion, our study contributed to investigate the expression of ANXA2 and β-catenin in the ectopic and eutopic endometrium of women with adenomyosis, and their relationships with dysmenorrhea. These findings demonstrated important roles for ANXA2 and β-catenin in the adenomyosis and dysmenorrhea. Understanding ANXA2, β-catenin in the ectopic and eutopic endometrium of women with adenomyosis, and their relationship with adenomyosis-associated dysmenorrhea may pave the way for the development of efficient diagnosis targets and new therapeutic approaches in adenomysis-related dysmenorrhea.

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