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Original Article

Expression profiles and association between OATP4A1 and PGE_2 in adenomyosis tissue

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Abstract: Objective: To measure the expression of OATP4A1 and PGE_2 in adenomyosis tissues, and to explore their roles in the incidence and development of adenomyosis-induced dysmenorrhea. Methods: OATP4A1 mRNA and protein in the Aadenomyosis tissues with/without dysmenorrhea and normal uterus were detected. The expression of PGE_2 was measured. Results: OATP4A1 protein was mainly expressed on the cell membrane and in the cytoplasm of endometrial glandular cells. The IRS of OATP4A1 in the two adenomyosis groups were 3.030±1.903, and 5.200±1.789, significantly lower than 9.110±3.457 in the control group (both $P<0.05$). The expression levels of OATP4A1 protein in the endometrial-myometrial interface (EMI) of adenomyosis women were 15.217±6.106 and 20.085±3.633, considerably lower than 38.873±7.899 in the control group (both $P<0.05$). The expression levels of OATP4A1 mRNA in the adenomyosis groups were 0.593±0.281 and 0.805±0.440, significantly lower than 1.910±0.499 in the control group (both $P<0.05$). The expression levels of PGE_2 in the three groups were 62.329±6.505, 45.099±3.192, 39.446±3.807 (pg/ml), respectively. OATP4A1 protein was negatively correlated with PGE_2 expression in three groups ($r = -0.598$, $P = 0.019$; $r = -0.967$, $P = 0.002$; $r = -0.663$, $P = 0.007$). Conclusion: OATP4A1 is negatively correlated with PGE_2, suggesting that low expression of OATP4A1 probably causes the topical accumulation of PGE_2 which is proportional to the degree of pain.

Keywords: Adenomyosis, dysmenorrhea, OATP4A1, PGE_2

Introduction

Adenomyosis is a common chronic benign disease in women of childbearing age. The diagnosis of adenomyosis can merely rely on pathological examination [1, 2]. The incidence of adenomyosis among ordinary women ranges from 5% to 70%, which significantly varies due to the differences in race, region or investigation methods [3]. The incidence of adenomyosis has been demonstrated to be approximately 20%-30% in hysterectomy specimens [4].

Adenomyosis-induced dysmenorrhea is caused by multiple factors including inflammation, nervous system, hormone and immunity. Inflammatory mediators, nerve mediators, nerve fibers, hormones, and other aspects have been regarded as possible inducing factors. Previous studies have demonstrated that the expression levels of a large quantity of inflammatory mediators and dysmenorrhea-inducing cytokines are dysregulated in adenomyosis tissues, which may directly cause nociceptive stimulation, activate downstream molecules, and trigger classical pain signaling pathways. The role of prostaglandin E_2 (PGE_2) in the occurrence of dysmenorrhea associated with adenomyosis has been recognized. The expression levels of prostaglandin (PGs) are significantly up-regulated in the ectopic endometrium of adenomyosis patients, and the eutopic endometrium, peritoneal fluid, and menstrual blood of the endometriosis patients. PGs have been proven to be associated with the incidence of dysmenorrhea [5]. Peng *et al*. [6] have demonstrated that the PG level in the adenomyosis lesions is significantly higher compared with that in normal uterus and the expression level of PGE_2 is positively correlated with the severity of dysmenorrhea. Currently, multiple PGs transporters have been identified, such as multidrug resistance-

associated protein 4 (MRP4), organic cation transporter 1 (OCT1), organic anion transporter 1-4 (OAT1-4), OATP-1 (LST-1), OATP2B1, OATP-2A1 (PGT) and OATP4A1 (OATP-E) [7]. Whether these PGs transporters are involved with the abnormal expression of PGs in the adenomyosis lesions remains elusive.

Organic anion transporter polypeptides 4A1 (OATP4A1) is a member of OATP family, which acts as the main transport pump of the influx of substances on the cell membrane to absorb and transport various endogenous complexes across the membrane. OATP4A1 has been proven to be involved in transporting estrogen and its various precursors and metabolites, PGE_2 and thyroid hormone [7]. At present, the expression of OATP4A1 at the mRNA and protein levels has been rarely investigated. Abnormal expression of OATP4A1 has been detected in breast cancer [8-11], bone tumor [12], inflammatory bowel disease [13], colorectal tumor [14] and primary liver cancer [15].

In endometriosis patients, slight duplication is detected in the 20q13.33 region of the chromosome (where OATP4A1 is located). RT-PCR confirms that the duplication of the copy number of OATP4A1 gene may lead to abnormal expression of OATP OATP4A1 [16]. Although adenomyosis and endometriosis are two different diseases, they share similarities in the mechanism, occurrence, and development of respective illness. Whether OATP4A1 is abnormally expressed in adenomyosis tissues and whether OATP4A1 is correlated with PGE_2 in the mechanism of adenomyosis-induced dysmenorrhea needs to be investigated.

In this study, real-time PCR, western blot, immunohistochemistry, and alternative methods were used to detect the expression of OATP4A1 at the mRNA and protein levels. ELISA was performed to detect the expression level of PGE_2 and statistically compared among adenomyosis patients with/without dysmenorrhea and healthy counterparts. The potential association between OATP4A1 and PGE_2 and their roles in the incidence of adenomyosis-induced dysmenorrhea were investigated, aiming to unravel the mechanism underlying adenomyosis-induced dysmenorrhea from the perspective of substance transport and provide evidence for medical therapy of adenomyosis.

Materials and methods

Baseline data

Paraffin-embedded tissue samples: Thirty-five adenomyosis patients undergoing hysterectomy in Department of Pathology, Xiangya Hospital of Central South University from February 2013 to July 2014, aged 31-50 years with a median age of 40.5 years and (42.7±4.5) years on average, were enrolled and divided into dysmenorrhea (n = 30) and non-dysmenorrhea groups (n = 5). Normal uterus collected from 25 women was used as normal controls, aged 32-52 years with a median age of 42 years, (43.0±5.3) years on average.

Fresh tissue samples: Endometrial-myometrial interface (EMI) tissues were collected from 21 adenomyosis patients undergoing hysterectomy in the Department of Obstetrics and Gynecology, Xiangya Hospital of Central South University from July to November 2014, aged 34-48 years with a median age of 42.0 years and mean 41.67±3.83 years. Among them, 15 patients were assigned into dysmenorrhea and 6 into the non-dysmenorrhea groups. Normal EMI tissues were obtained from 14 women, aged 39-49 years with a median age of 44 years, (43.70±2.83) years on average. The fresh tissues were stored in the liquid nitrogen at -80°C and prepared for total protein and RNA extraction and ELISA analysis.

Inclusion criteria: In accordance with the diagnostic criteria for adenomyosis [17]; Clinical symptoms: progressive aggravation of secondary dysmenorrhea; Menstrual changes including excessive menstrual volume and prolonged menstrual period; accompanied by infertility, unexplained vaginal bleeding during the middle menstrual period, and decreased sexual desire. Gynecologic examination signs: the uterus is spherical and homogeneous or has local nodules and bulges; the uterine position can be tilted backwards with hard texture, andtenderness becomes more evident during the menstrual period. B-mode ultrasound: the uterus is enlarged in a spherical shape to different degrees or no changes in the shape; lesions are primarily located in the posterior wall, manifested as thickening of the posterior wall of the uterus, shifting or unclear endometrial lines; uneven myometrial echo; the spot echo was

coarsely enhanced at the lesions, with honeycomb-, palisade-shaped and patchy changes between the areas with no or low echo; there was no pseudocapsule and no obvious boundary between the lesion and normal muscle layers [18]. Laboratory examination: the serum level of tumor marker CA125 was increased by a slight to moderate degree. The diagnosis can be confirmed by the comprehensive consideration of clinical symptoms, signs, imaging, and laboratory examination.

Exclusion criteria: Those in special physiological periods, such as pregnancy, lactation, and menopause; those with cognitive impairment, intellectual impairment, mental illness; those complicated by primary diseases of endometriosis or other important organs or systems, such as heart, brain, liver, kidney, blood, or gynecologic malignancies.

RT-PCR

Approximately 30-50 mg of corresponding tissues stored at -80C were supplemented with 1 ml of Trizol for total RNA extraction, the obtained RNA solution was diluted to 0.5 μg/μl for subsequent cRNA synthesis. Nuclease-free water was utilized to dilute each primer and cDNA stock solution was diluted to an appropriate concentration, PCR reaction mixture was supplemented with 4 μl of the cDNA stock solution diluted by 5 times. β-actin: forward primer: CTACGTCGCCCTGGACTTCGAGC; reverse primer: GATGGAGCCGCCGATCCACACGG; OATP4A1: forward primer: CGTCACCTACCTGGATGAGAA; OATP4A1 reverse primer: AGATATTCAGCAGGGCACCTC. RT-PCR results were expressed as $2^{-\Delta\Delta Ct}$.

Western blot

A portion of 0.25 tissues were lysed with 300 μl of RIRA for 30 min on the ice, centrifuged at 12000 *rpm* at 4°C for 15 min and stored at -80°C for subsequent BCA assay. PVDF membrane was soaked in methanol and then soaked with filter paper in membrane transfer buffer. After membrane transfer, the membrane was taken out and washed once in 1 × TBST for 5 min. The efficiency of protein transfer was tested by Ponceau S staining the membrane, washed with 5% skimmed milk powder prepared with 1 × TBST. After the membrane was immersed, it was shaken and sealed at room temperature for 1 h. The primary antibody was diluted (OATP4A1 = 1:250, β-actin = 1:4000), the membrane was incubated with the primary antibody overnight at 4°C. Then, the HRP-labeled second antibody was diluted at a dilution ratio of 1:3000, the diluted second antibody was incubated with the membrane for 60 min, washed in 1 × TBST for 3 times, 15 min for each wash and ECL visualization.

ELISA

A portion of 100 mg tissues were washed with cold PBS and prepared for tissue homogenate, centrifuged at 5000 rpm for 5 min at 4°C, and the supernatant was collected for analysis. Except the blank control, 50 μl of enzyme conjugate and 50 μl of antibody were added into each well, fully mixed, and incubated at 37°C for 1 h, then supplemented with 50 μl of visualizer A solution and visualizer B solution, thoroughly mixed, and kept in dark at 37°C for 15 min. We added 50 μl of stop solution. The optical density (OD) value was measured at A wavelength of 450 nm. The experiment was conducted in triple replicates and the mean value was calculated as the expression level of PGE_2.

Immunohistochemical staining

4-μm sections were sliced, dried at 60°C for 3 h, 70°C for 5 min and 37°C for 10 min for dewaxing, placed in a gradient concentration of ethanol (100%-95%-85%-70%, 5 min for each gradient), mixed with PBS three times, 5 min for each wash, cooled at room temperature after boiling with sodium citrate, washed with PBS 3 times, added to 50 μl of BSA sealing solution, incubated with the primary antibody (1:100 dilution) at 4°C overnight, washed with PBS 3 times, incubated with secondary antibody at 37°C for 30 min, stained with DAB, 60 s hematoxylin for nuclear staining, 3 s $LiCO_3$ for counterstaining, dehydrated in a gradient concentration of ethanol (70%-85%-95%-100%, 40 s for each gradient), neutral balsam mounting, dried, and observed under a fluorescent microscope (× 200). The staining intensity and the percentage of positive cells were evaluated.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by SPSS 19.0 statistical software (SPSS Inc.,

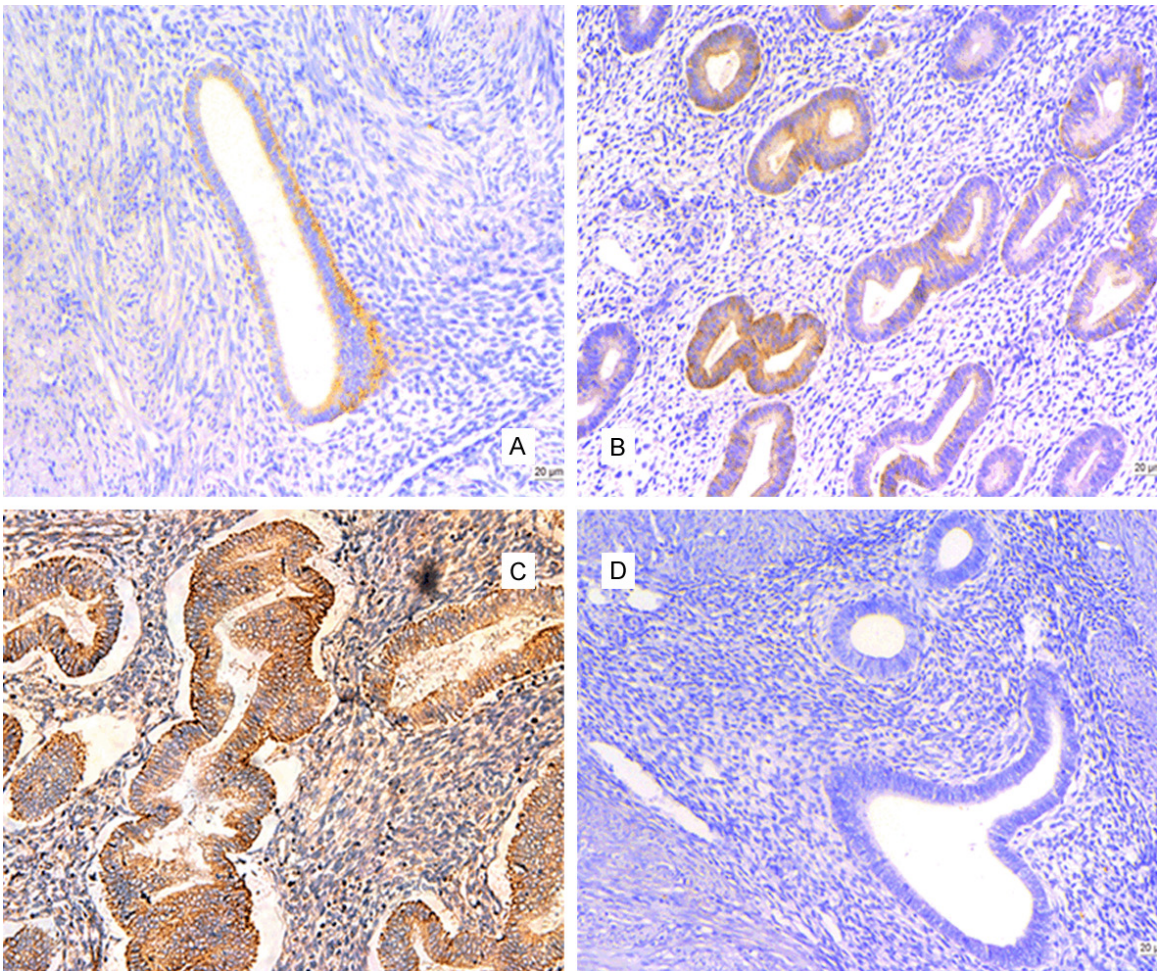

Figure 1. Expression profile of OATP4A1 protein in the uterus (× 200). A. Dysmenorrhea adenomyosis group. B. Non-dysmenorrhea adenomyosis group. C. Control group. D. EMI region in negative controls.

Chicago, IL, U.S.). One-way ANOVA was adopted to compare the differences among three groups. Pearson correlation analysis was utilized to analyze the correlation between the expression levels of OATP4A1 and PGE_2. A *P* value of less than 0.05 was considered significant.

Results

Expression profile of OATP4A1 protein in uterus

Immunohistochemical staining demonstrated that the OATP4A1 protein was mainly expressed on the cell membrane and in the cytoplasm of endometrial glandular cells rather than in the nucleus. In addition, it was slightly expressed in the cytoplasm of certain lumen epithelial cells. OATP4A1 protein was not expressed in endometrial stromal cells or uterine smooth muscle cells (**Figure 1**).

Semi-quantitative expression profile of OATP4A1 protein in uterus

Semi-quantitative immunohistochemical analysis demonstrated that the IRS of the OATP4A protein in the dysmenorrhea adenomyosis group and non-dysmenorrhea adenomyosis group was 3.030±1.903, 5.200±1.789, significantly lower than 9.110±3.457 in the control group (both $P<0.05$). No statistical significance was observed between the two adenomyosis groups ($P>0.05$).

Quantitative expression profile of OATP4A1 protein in uterus

Western blot showed that the expression levels of OATP4A1 protein in the dysmenorrhea adenomyosis group and non-dysmenorrhea adenomyosis group were 15.217±6.106 and 20.085±3.633, significantly lower compared

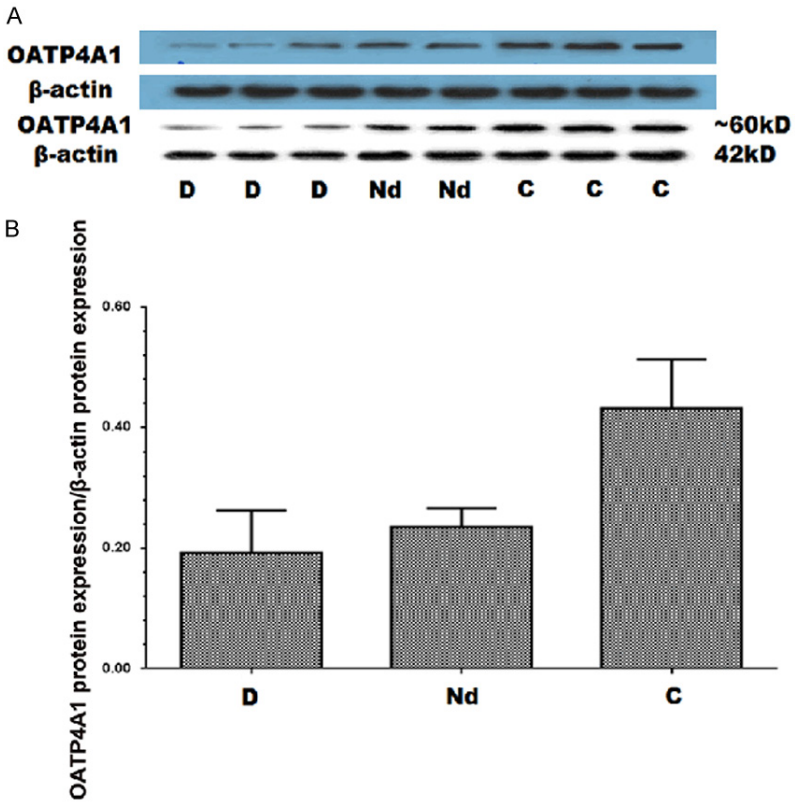

Figure 2. A. Quantitative expression profile of OATP4A1 protein in uterus. Abbreviation: D: denotes dysmenorrhea adenomyosis group, Nd: represents non-dysmenorrhea adenomyosis group, C: control group. B. Quantitative expression profile of OATP4A1 protein in uterus after the internal reference protein was normalized.

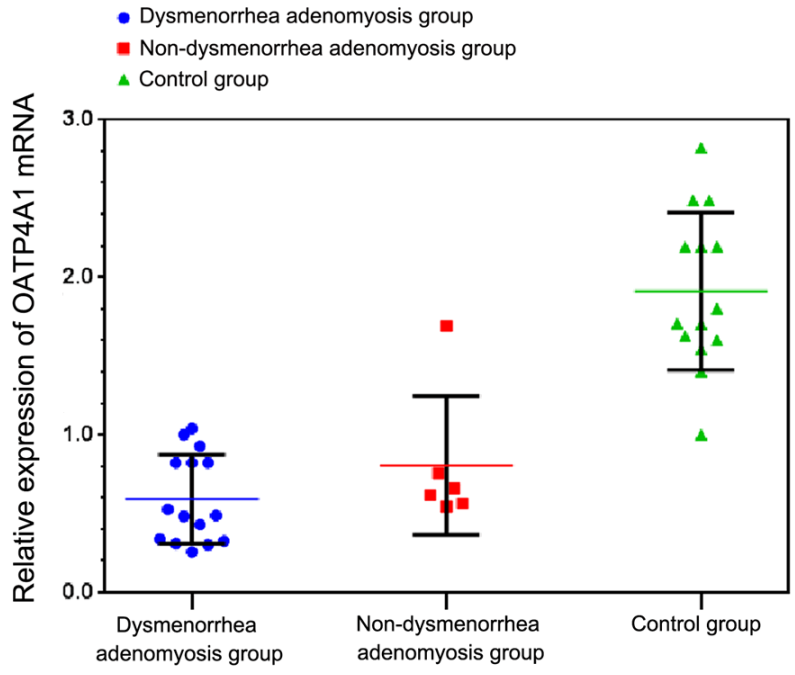

Figure 3. Expression profile of OATP4A1 mRNA in uterus.

with 38.873±7.899 in the control group (both $P<0.05$). No statistical significance was observed between the two adenomyosis groups ($P>0.05$), as illustrated in **Figure 2A**, **2B**.

Expression profile of OATP4A1 mRNA in uterus

RT-PCR demonstrated that the expression levels of OATP4A1 mRNA in the dysmenorrhea adenomyosis group and non-dysmenorrhea adenomyosis group were calculated as 0.593±0.281 and 0.805±0.440, significantly lower compared with 1.910±0.499 in the control group (both $P<0.05$), whereas the results did not significantly differ between the dysmenorrhea adenomyosis group and non-dysmenorrhea adenomyosis group ($P>0.05$), as illustrated in **Figure 3**.

Expression profile of PGE_2 in uterus

ELISA revealed that the expression level of PGE_2 in the dysmenorrhea adenomyosis group was the highest, up to (62.329±6.505) pg/ml, significantly higher compared to (45.099±3.192) pg/ml in the non-dysmenorrhea adenomyosis group, followed by (39.446±3.807) pg/ml in the control group (all $P<0.05$).

Correlation analysis between OATP4A1 protein and PGE_2

Pearson correlation analysis demonstrated that the expression level of OATP4A1 protein was significantly negatively correlated with that of PGE_2 ($r = -0.598$, $P = 0.019$) in the dysmenor-

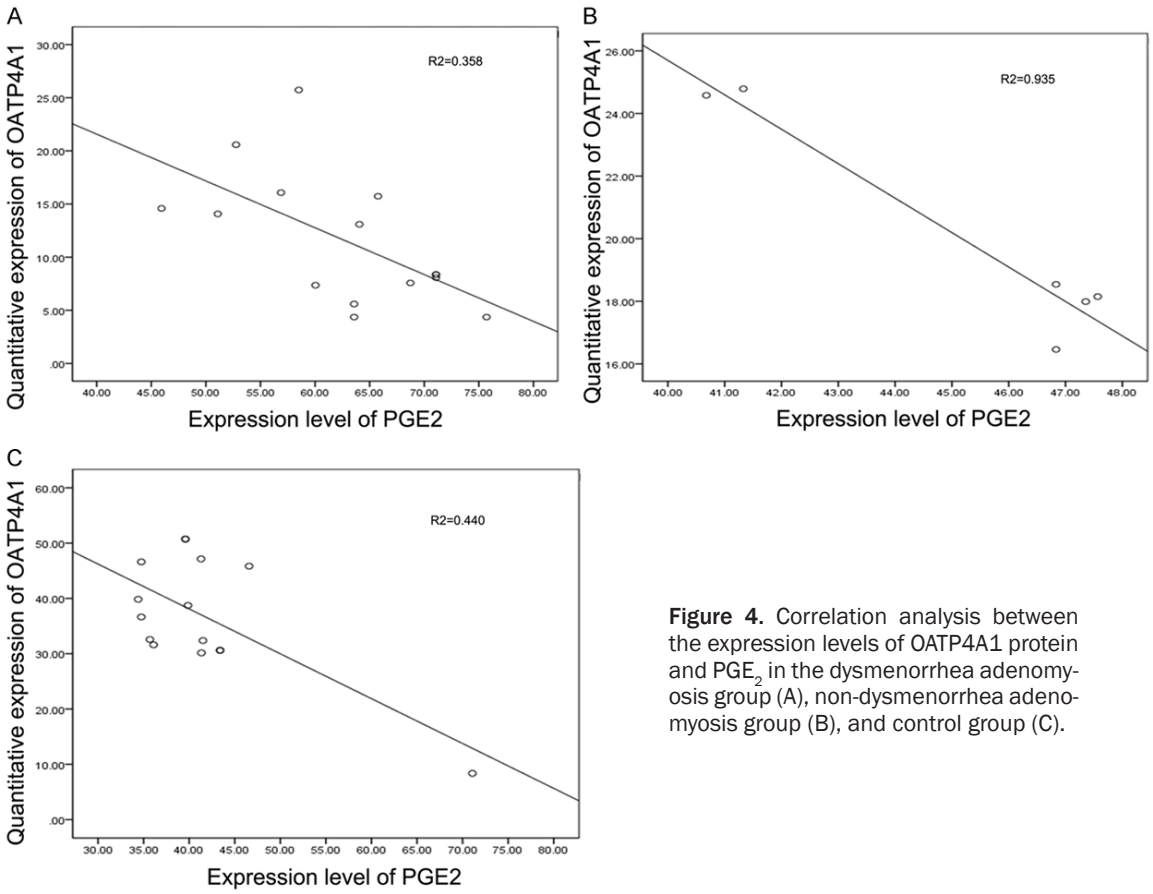

Figure 4. Correlation analysis between the expression levels of OATP4A1 protein and PGE_2 in the dysmenorrhea adenomyosis group (A), non-dysmenorrhea adenomyosis group (B), and control group (C).

rhea adenomyosis group. In the non-dysmenorrhea adenomyosis group, a negative association was noted between them (r = -0.967, P = 0.002). In the control group, the expression of OATP4A1 protein was also negatively associated with that of PGE_2 (r = -0.663, P = 0.007), as demonstrated in **Figure 4**.

Discussion

OATP4A1 belongs to the family of organic anion transport polypeptides ($OATP_S$), which is a transmembrane protein consisting of 722 amino acids with 12 transmembrane regions and multiple phosphorylation and glycosylation sites [8]. $OATP_S$ is a member of the solute transport carrier superfamily (SLC_S), including 11 transport polypeptides from six families (OAT P1-OATP6). Some members, especially the OATP3 and OATP4 families, are widely distributed in human tissues, whereas alternative members have certain tissue specificity, e.g. OATP 2 only exists in liver tissues. As the main non-sodium ion-dependent inward pump on the cell membrane, $OATP_S$ plays a major role in the uptake and transport of various endogenous metabolites across the membrane [19]. The family members are highly expressed in certain secretory functional tissues, such as small intestine, liver, kidney, and biological barrier tissues, such as brain, placenta, ovary and testis [20], indicating that $OATP_S$ participates in the absorption, distribution and excretion of regulatory substances as an outward pump by coordinating with intracellular metabolic enzymes and extra-membrane outward pumps [21].

OTAP4A1 gene is widely expressed in human tissues and tumor cell lines, especially in the fetal tissues, the mRNA expression is high in the placenta and heart [10]. OATP4A1 is not detected only in human blood cells, and it is the only transporter among the $OATP_S$ family detected in the skeletal muscle [22], prompting that OATP4A1 probably participates in cell proliferation and differentiation by transporting the essential elements or substances required for cell proliferation and growth.

The expression level of OATP4A1 in malignant tumors is higher than that in benign tissues. OATP4A1 transcription level in the endometrium of PCOS patients is lower than that in normal endometrium [23], which is consistent with the results of the present study. In this investigation, the OATP 4A1 expression level in the EMI region of normal uterus was the highest, whereas it was still lower than that of housekeeping gene β-actin. The expression level of OATP4A1 was significantly decreased in the adenomyosis groups, whereas no significant difference was noted between the dysmenorrhea and non-dysmenorrhea adenomyosis groups.

At present, the relationship between OATP4A1 and endometriosis has been seldom studied. Yang *et al.* [17] confirmed that the chromosome 20q13.33 region (where SLCO 4A1 is located) is slightly duplicated after detecting the ectopic endometrium in 11 patients with ovarian endometriosis. RT-PCR confirmed that the copy number of SCLO4A1 gene is duplicated, which may lead to overexpression of OATP4A1. However, in this study, the expression of OATP4A1 in adenomyosis patients was down-regulated compared to normal counterparts. It can be concluded that adenomyosis is a benign disease with common features with endometriosis and possesses malignant invasion and proliferation behavior, but these two diseases significantly differ in other aspects. In this study, the OATP4A1 protein was expressed in the cell membrane and cytoplasm of endometrial glandular cells and in the cytoplasm of some lumen epithelium of the EMI region of normal uterus and adenomyosis tissues. No expression was found in endometrial stromal cells or uterine smooth muscle cells. Therefore, it can be inferred that the down-regulation of OATP4A1 expression level in the EMI region of adenomyosis may lead to an imbalance of local substance transport in the EMI region. A large quantity of OATP4A1 transporting basal substances, such as estrogen, its metabolites and prostaglandins, accumulate outside the endometrial glandular cells. However, many endometrial stromal cells and smooth muscle cells around the EMI region of adenomyosis uterus may also lack OATP4A1 and similar inward transport pump proteins, resulting in the accumulation of estrogen products, PGE_2 and other substances in the topical lesions, which seems to explain that adenomyosis always expresses high levels of estrogen and PGE_2 in the local foci, whereas the peripheral hormone levels are not significantly changed.

PGE_2 is mainly synthesized by endometrial cells in human uterus, but it mainly acts on uterine smooth muscle cells. PGE_2 participates in and affects the occurrence of dysmenorrhea in adenomyosis patients. Significant increases in prostaglandins can be detected in the focus of adenomyosis patients, ectopic endometrium of endometriosis patients, eutopic endometrium, peritoneal fluid and menstrual blood, which is correlated with the occurrence of dysmenorrhea [5, 24-26]. PGE_2 can inhibit the spontaneous activity of uterine smooth muscle and relax the uterine smooth muscle. PGE_2, as a well-known inflammatory mediator, plays a well-known role in inflammatory pain. PGE_2 can promote local vasodilation, increase capillary permeability, and show inflammatory manifestations of redness, swelling, heat and pain. PGE_2 itself is not only a pain-causing factor, it can directly stimulate peripheral nociceptors to cause pain, but also can participate in other signaling pathways to induce pain. PGE_2 and other types of PGs may cause uterine contraction polarity disorder. In addition, PGS produced in tissue fluid cannot be rapidly metabolized due to the proliferation of tissues surrounding the adenomyosis lesions, resulting in sustained dysmenorrhea.

The results of this study demonstrate that there is a negative correlation between OATP4A1 protein and PGE_2 expression in adenomyosis and normal uterus. It can be inferred that the down-regulating of OATP4A1 expression in the EMI region of uterus is related to the topical accumulation of PGE_2 and the expression of OATP4A1 in adenomyosis uterus is down-regulated, suggesting that both two events may play a role in the incidence of adenomyosis and even the mechanism of dysmenorrhea. However, OATP4A1 is only one of the proteins involved in transporting PGE_2. In addition, HPRC, MRP4, OCT1, OAT1-4, OATP-1 (LST-1), OATP2B1, OATP2A1 (PGT), OATP 4A1 (OATP-E) [6] are also the transporters of PGE_2, especially OATP2A1 (PGT) and OATP4A1 belong to the same family and share certain structural and functional similarities, and thereby are involved in transporting PGE_2. It can be speculated that since OATPs

is an inward transport pump on the cell membrane, the ability to absorb PGE_2 into the endometrial glandular epithelial cells is decreased after the expression level is down-regulated in the EMI region of adenomyosis uterus tissues. A large quantity of PGE_2 is accumulated outside the cellular wall, possibly entering into interstitial cells and surrounding uterine smooth muscle cells through other transport channels, thereby causing dysmenorrhea.

In this investigation, the expression of OATP4A1 at the mRNA and protein levels was detected in the EMI region of both normal and adenomyosis uterus, whereas the expression of OATP4A1 was down-regulated in the EMI region of adenomyosis uterus, suggesting that OATP4A1 probably participates in the incidence of adenomyosis-induced dysmenorrhea by affecting the efficiency of substance transportation. The up-regulated expression of PGE_2 in adenomyosis uterus was positively correlated with the severity of dysmenorrhea, confirming that PGE_2 is a key factor involved in the occurrence of adenomyosis-induced dysmenorrhea. The abnormal expression of PGE_2 in the EMI region of adenomyosis uterus may disrupt the balance of substance transport and metabolism in local tissues, thus promoting the formation of abnormal hormone levels and inflammatory factors and causing pain symptoms. The conservative treatment of adenomyosis dysmenorrhea yields limited clinical efficacy, probably because it fails to consider the changes in the steady state of material transport in the local environment of adenomyosis lesions. Consequently, the role of alternative factors and transporters of OATPs family in the incidence of adenomyosis remains to be elucidated.

Conclusion

The expression of OATP4A1 is down-regulated in adenomyosis tissues, suggesting that OATP4A1 may be involved in the occurrence of adenomyosis-induced dysmenorrhea by influencing the substance transportation. The up-regulated expression of PGE_2 in adenomyosis tissues is positively correlated with the severity of dysmenorrhea, suggesting that PGE_2 is associated with the occurrence of dysmenorrhea in adenomyosis patients. The expression of OATP4A1 protein is negatively correlated with PGE_2 in both the adenomyosis and normal uterus, suggesting that the low expression of OATP 4A1 probably causes the topical accumulation of PGE_2 in adenomyosis tissues and participates in promoting the occurrence and progression of adenomyosis-induced dysmenorrhea.

Acknowledgements

This study is approved by the relevant Ethics Committee and obtained a signed informed consent from all patients.

Disclosure of conflict of interest

None.

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