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Expression of Cannabinoid Receptors in Myometrium and its Correlation With Dysmenorrhea in Adenomyosis

Xue Shen, MD[1], Hua Duan, MD, PhD[1], Sha Wang, MD, PhD[1], Wei Hong, MD[2], Yu-Yan Wang, MD[1], and Si-Li Lin, MD[1]

Abstract

The myometrium, especially the junctional zone (JZ), is now well documented to have a role in the pathogenesis of adenomyosis. Cannabinoid receptors have been shown to participate in the establishment of endometriosis and its pain perception. However, its relation to adenomyosis has not been identified yet. The aim of this study was to investigate the expression of cannabinoid receptor type I (CB1) and type II (CB2) in myometrium of uteri with and without adenomyosis and determine the correlation between their levels and clinical parameters of adenomyosis. We collected tissue samples of JZ and the outer myometrium from 45 premenopausal women with adenomyosis and 34 women without adenomyosis. CB1 and CB2 messenger RNA (mRNA) and protein expression levels were evaluated by the use of Western blotting and real-time quantitative polymerase chain reaction from all samples. Clinical information on the severity of dysmenorrhea and other data were collected. We found both CB1 and CB2 mRNA and protein levels in women with adenomyosis were significantly higher than those of controls, and CB1 expression levels in JZ were positively correlated with the severity of dysmenorrhea. These data suggest that cannabinoid receptor CB1 may be involved in the pathogenesis of dysmenorrhea in adenomyosis and may be a potential therapeutic target.

Keywords

adenomyosis, endometriosis, dysmenorrhea, myometrium, cannabinoid receptor

Introduction

Adenomyosis is a common chronic gynecological disorder characterized by symptoms of dysmenorrhea, abnormal uterine bleeding, and infertility.[1] It refers to the presence of endometrial islands and stroma within the myometrium, while endometriosis is defined as the endometrial tissue outside the uterus. Owing to the lack of standard diagnostic criteria, the precise incidence of adenomyosis is unknown; correspondingly, its prevalence has been reported to vary widely from 5% to 70%.[2] Although people have been paying great attention to adenomyosis, it remains an enigmatic disease without an adequate understanding of either its etiology or its physiopathology. Therefore, continued research based on its pathogenesis is needed urgently at present.[3]

As for the pathogenesis of adenomyosis, much of our understanding derives from studies on the eutopic and ectopic endometrium of uteri with adenomyosis, while many basic questions on it still remain unknown. In the recent years, with advances on magnetic resonance imaging as a noninvasive diagnostic technique for adenomyosis, the myometrium, especially the junctional zone (JZ; also known as the endometrial-myometrial interface or subendometrial myometrium) is now well documented to have a role in the pathogenesis of adenomyosis.[4]

The JZ, which is used to describe a low-signal-intensity myometrial zonal anatomy between the high-signal-intensity endometrium and the medium-signal-intensity outer myometrium (OM) on magnetic resonance imaging, was first described by Hricak et al in 1983.[5] Over the years, several studies have reported that there are structural and functional differences between JZ and the OM of uteri, and abnormal JZ might contribute to the development of adenomyosis.[6-9] Our previous work has also confirmed that the myocytes from JZ and OM are ultrastructurally different,[10] and estrogen may affect the myocytes of JZ and uterine JZ contraction in adenomyosis.[11,12] Furthermore, some studies report that uterine

[1] Department of Minimally Invasive Gynecologic Center, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing, People's Republic of China
[2] Department of Obstetrics and Gynecology, Beijing Luhe Hospital, Capital Medical University, Beijing, People's Republic of China

Corresponding Author:
Hua Duan, Department of Minimally Invasive Gynecologic Center, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, No. 17 Qi Helou Road, Dong Cheng District, Beijing 100006, People's Republic of China.
Email: duanhuasci@163.com

contractility is associated with dysmenorrhea,[13] which is the primary reason for patients' final choice for hysterectomy, and uterine contractility originates exclusively from the JZ.[6,14] However, the molecular mechanisms involved in that process remain largely unknown.

Herbal cannabis has been used as a pain reliever for thousands of years. In the early 1990s, endocannabinoids were discovered in the study of the mechanisms of $\triangle^9$-tetrahydrocannabinol (THC), the main psychoactive component in cannabis.[15] Since then, considerable research has confirmed that endocannabinoids via cannabinoid receptors can trigger several signal pathways, producing a variety of physiological and pathological effects, such as anti-inflammation, antifibrosis, antitumor, pain control, and reproductive regulation.[16-22] Cannabinoid receptors, which are essential for endocannabinoids to produce their biological effects, were proved to be associated with the establishment of endometriosis and its pain perception.[23,24] Particularly, accumulating evidence suggests that the endocannabinoid system (ECS) may play a role in the pathophysiology of endometriosis, and it has emerged as a potential target to treat endometriosis.[23-28] However, such data for adenomyosis are sparse.

Given the close relationship between adenomyosis and endometriosis, we hypothesize that the alternations in the ECS may also exist in the pathogenesis of adenomyosis, which may be associated with dysmenorrhea. Based on our previous research on the differences between JZ and OM of uteri,[11,12,29] we designed this study to evaluate the cyclic expression of the 2 main receptors of endocannabinoids, cannabinoid receptor type I (CB1) and type II (CB2), in JZ and OM of uteri in adenomyosis and to compare their levels with myometrium of the disease-free women. We also aim to determine the relationship between their levels and clinical parameters of adenomyosis.

Materials and Methods

Collection of Tissues and Clinical Parameters

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki and approved by the Ethical Committee of Clinical Research of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, China (reference No. 2016-KY-012). Written informed consent was obtained from all participants before surgery. Uterine samples with adenomyosis were collected from 45 women undergoing hysterectomy from July 2016 to January 2018 in Beijing Obstetrics and Gynecology Hospital. We initially recruited 76 patients with suspected adenomyosis based on symptoms of dysmenorrhea, menorrhagia, or both, combined with the transvaginal ultrasound report. Among them, 50 were confirmed by pathological examination of the hysterectomy specimen after surgery, 26 were excluded because of a combination of uterine fibroids or endometrial pathology, and other 5 were excluded because their ultrasound reports couldn't be found in medical charts when we analyzed the data. Thirty-four women undergoing hysterectomy for early-stage cervical cancer or ovarian cancer during the same time period were included as the control group.

All women were premenopausal and had regular menses (lengths ranged from 21 to 35 days). Exclusion criteria included fibroids, endometrial polyps, endometrial cancer, and use of hormones or intrauterine devices within 3 months before surgery. None of them had any visible endometriosis or pelvic inflammation during the hysterectomy. The day of the menstrual cycle was determined by histologic criteria.

Samples of JZ and OM were obtained as soon as the uterus was removed during the surgery. Based on the thickness of JZ and OM reported in normal reproductive women,[30-33] we took samples of JZ from 2 mm beneath the endometrium and OM from 2 mm beneath the uterine serosa. All samples were excised from the anterior fundal wall of each uterus and divided into 2 parts: one for RNA extraction and the other for protein. All of them were snap frozen in liquid nitrogen and then stored at −80°C refrigerator. After their diagnosis was confirmed by postoperative pathological examination, we took them out and conducted the following experiments.

For all patients, age at surgery, gravidity, parity, Pictorial Blood Loss Assessment Chart Scores (PBAS; calculated as described elsewhere[34]), and the severity of dysmenorrhea measured by a 10-cm Visual Analogue Scale (VAS; estimated as reported elsewhere[35]) score were collected by interviewing patients before surgery. The uterus size, calculated as uterine volume $= D_1 \times D_2 \times D_3 \times 0.52$, as others reported,[36] was collected by reviewing medical records retrospectively.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from tissues according to the manufacturer's instructions for RNAiso Plus (Takara Bio Inc, Shiga, Japan). The quality and quantity of RNA were assessed using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific Inc, Massachusetts).

Total RNA of 1 μg in a total volume of 20 μL was converted to complementary DNA using the PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara). A 2-step quantitative polymerase chain reaction (PCR) was performed according to the protocol of the SYBR Premix Ex Taq II (Tli RNaseH Plus; RR820A; Takara) on an AB 7500 Real-Time PCR System (Applied Biosystems, Grand Island, New York). The cycling conditions were composed of 1 cycle of 95°C for 30 seconds to denature, 40 cycles of 5 seconds at 95°C, and 34 seconds at 60°C. The relative expressions of CB1 and CB2 were, respectively, calculated using the comparative C_T method as reported,[37] where $\triangle C_T = C_T$ (gene of target) $- C_T$ (internal control), and the $2^{-\triangle CT}$ data were presented for statistical tests. The following primers specific to CB1 were used for quantitative real-time PCR (qRT-PCR): sense primer, 5′-CCT AGA TGG CCT TGC AGA TAC C-3′ and antisense primer, 5′-GAA TGT CAT TTG AGC CCA CGT A-3′. The primers used for CB2 were: forward, 5′-CAG GTC AAG AAG GCC TTT GC-3′ and reverse, 5′-GCA TAG ATG ACA GGG TTG ACC AT-3′. The primers for internal control β-actin were as follows: forward, 5′-TGC CGA CAG GAT GCA GAA G-3′ and reverse, 5′-CTC AGG AGG AGC AAT GAT CTT GA-3′.

Western Blotting

Total protein from each sample was extracted with RIPA buffer (R0010; Solarbio Life Sciences, China) containing 1 mmol/L phenylmethylsulfonyl fluoride. A protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, Missouri) was added to inhibit degrading of proteins in the extracts. Protein concentration was established with an Enhanced BCA Protein Assay Kit (P0010; Beyotime Institute of Biotechnology, China). Protein Samples (30 μg) were loaded on 12% sodium dodecyl sulfate-polyacrylamide gels (Solarbio Life Sciences), electrophoretically separated, and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts), which had been wetted in 100% methanol for 15 seconds previously. Nonfat dry milk 5% in Tris-buffered Saline Tween 20 (TBST; containing 0.1% Tween 20) was used to block the membranes for 2 hours to reduce nonspecific bindings. Next, the membranes were incubated with a CB1 rabbit polyclonal antibody (C2866; Sigma-Aldrich), a CB2 rabbit polyclonal antibody (ab3561; Abcam, United Kingdom), or an a-tubulin rabbit polyclonal antibody (Abclonal, China) in proper dilutions overnight at 4°C with gentle agitation. After that, the membranes were washed with TBST 3 times and incubated with HRP goat anti-rabbit IgG (H+L; Abclonal) for 1 hour with gentle agitation at room temperature. Signals were detected by ChemiDocTM XRS+ with Image Lab software (Bio-Rad, Hercules, California) and quantified as the ratio of a target protein to a-tubulin.

Statistical Analysis

All normally distributed data were represented as mean ± standard deviation (SD). The difference in gene and protein expression between women with and without adenomyosis was analyzed using generalized estimating equation (GEE) modeling considering the 2 locations (JZ and OM) within a patient and the variables age, parity, gravidity, and menstrual phase. For each group, unpaired Student *t* test or nonparametric test was used to compare the results between proliferative phase and secretory phase, and paired *t* test or nonparametric test was used to compare the results between JZ and OM. Pearson correlation coefficient was used to examine the correlation between VAS and PBAS, VAS and uterus volume, or PBAS and uterus volume. To identify possible effects of age, gravidity, parity, uterus size, PBAS, CB1 or CB2 mRNA / protein levels on VAS score, a multivariate linear regression model for original data was used. All statistical analyses were performed using GraphPad Prism for Mac, version 6.0 (GraphPad Software Inc., California) and SPSS 23.0 for Mac (SPSS, Inc., Chicago, Illinois). $P < .05$ was regarded as statistically significant.

Results

Patient Characteristics

A total of 79 patients were included in our study. The characteristics of them are listed in Table 1. There was no significant difference in age, gravidity, parity, or menstrual phase between the 2 groups. However, women with adenomyosis had significantly higher VAS, PBAS, and uterus volume than the control group (Table 1). In patients with adenomyosis, no relationship was found between VAS and PBAS (Pearson correlation, $r = .001$, $P = .997$), VAS and uterus volume (Pearson correlation, $r = .148$, $P = .333$), or PBAS and uterus volume (Pearson correlation, $r = .02$, $P = .897$).

Table 1. Characteristics of the Patients in Both Groups.[a]

Variables	Adenomyosis, n = 45	Controls, n = 34	*P* Value
Age, years	46.0 ± 3.8 (37-53)	44.2 ± 5.0 (32-53)	.078
Menstrual phase			
Proliferative	23 (51.1%)	22 (64.7%)	.227
Secretory	22 (48.9%)	12 (35.3%)	
Gravidity			
0	2 (4.4%)	0 (0.0%)	.473
1	10 (22.2%)	4 (11.8%)	
2	12 (26.7%)	14 (41.2%)	
≧3	21 (46.7%)	16 (47.1%)	
Parity			
0	5 (11.1%)	1 (2.9%)	.233
1	38 (84.4%)	31 (91.2%)	
≧2	2 (4.4%)	2 (5.9%)	
PBAS	170 (85-510)	105 (42-210)	<.001
VAS	6 (0-10)	0 (0-8)	<.001
Uterus volume, in mm^3	255.5 (54.6-799.6)	83.9 (44.2-344.3)	<.001

Abbreviations: PBAS, Pictorial Blood Loss Assessment Chart Scores; VAS, Visual Analogue Scale.
[a]Data are presented as mean ± standard deviation (range), or number (percentage), or median (range).

CB1 and CB2 mRNA Expression in Adenomyosis and Control Groups

The GEE modeling accounting for repeated measures (JZ and OM) and age, parity, menstrual phase, and the interactions of these factors demonstrated that CB1 and CB2 mRNA in adenomyosis and control patients were both significantly different, which were higher in adenomyosis (for CB1 mRNA, $P < .001$, odds ratio [OR] = 0.919, 95% confidence interval [CI]: 0.907-0.932; for CB2 mRNA, $P < .001$, OR = 0.955, 95% CI: 0.944-0.966). Neither CB1 nor CB2 mRNA expression was significantly different between the 2 menstrual phases. CB1 mRNA levels were significantly higher in JZ than in OM in the adenomyosis group but not in the control group (Figure 1A). CB2 mRNA levels showed no differences between JZ and OM in both adenomyosis and control group (Figure 1B).

CB1 and CB2 Protein Expression in Adenomyosis and Control Groups

To further confirm the above mentioned results, we also used Western blots to examine CB1 and CB2 protein expression levels in each group. We obtained similar results in that both CB1 and CB2 protein expression levels were significantly higher in adenomyosis with GEE modeling accounting for

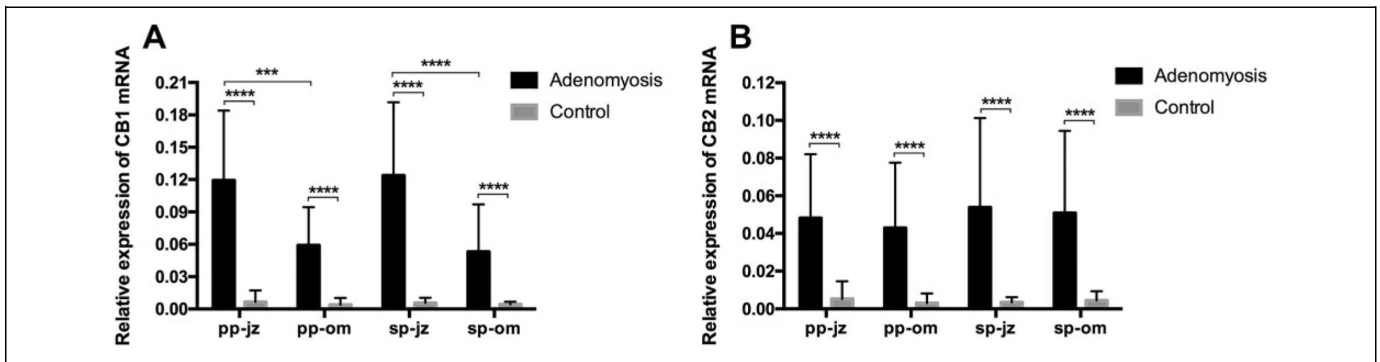

Figure 1. Relative expressions of cannabinoid receptor type I (CB1; A) and type II (CB2; B) messenger RNA (mRNA) in myometrium of uteri with or without adenomyosis.

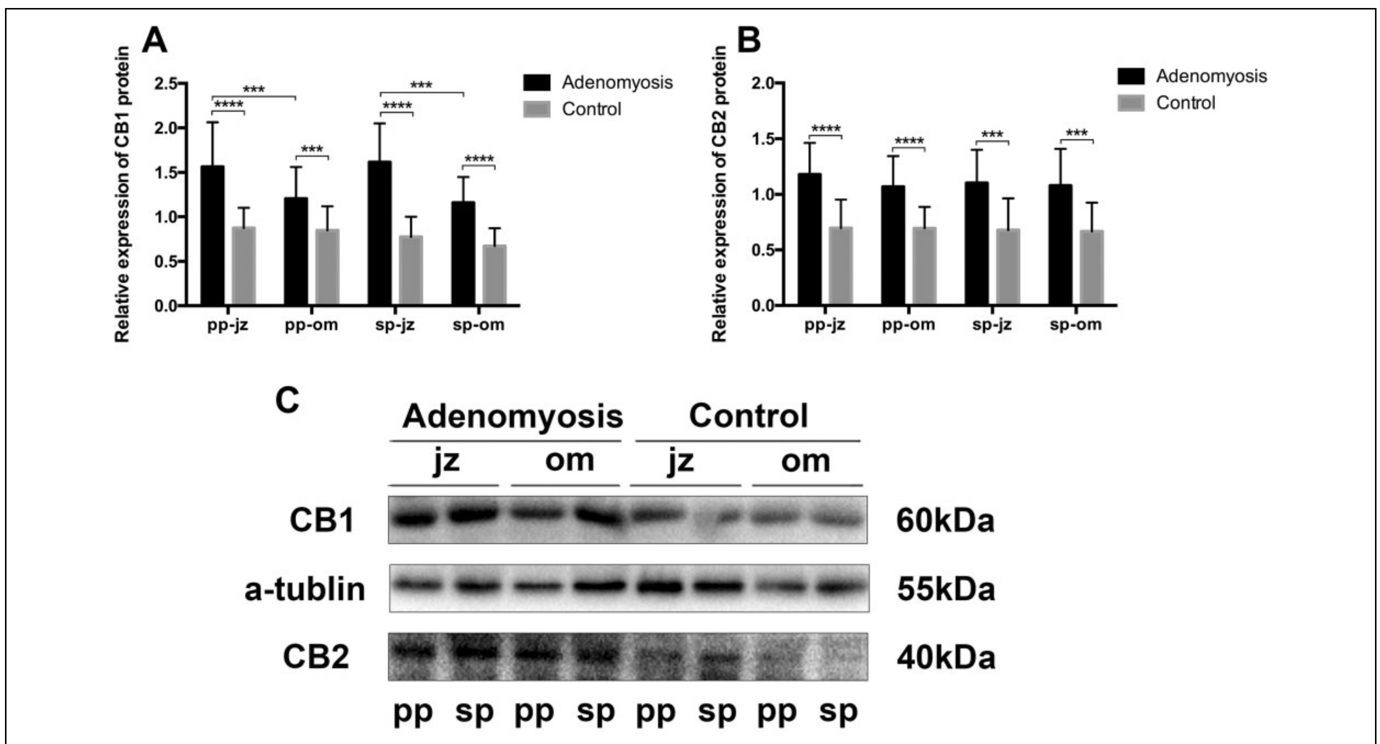

Figure 2. Relative expressions of cannabinoid receptor type I (CB1; A) and type II (CB2; B) protein in myometrium of uteri with or without adenomyosis. Representative Western blotting of CB1, CB2, and a-tublin (C).

repeated measures (JZ and OM) and age, parity, menstrual phase, and the interactions of these factors (for CB1 protein, $P < .001$, OR = 0.569, 95% CI: 0.506-0.641; for CB2 protein, $P < .001$, OR = 0.655, 95% CI: 0.6-0.714). For CB1 protein expression, it was higher in JZ than in OM in adenomyosis, but it did not vary with menstrual phase (Figure 2A and C). For CB2 protein expression, no menstrual cycle variation or anatomical variation was observed in each group (Figure 2B and C).

CB1 mRNA and Protein Expressions in JZ and its Correlation With Severity of Dysmenorrhea

We found that CB1 expression levels in JZ was positively correlated with the severity of dysmenorrhea (for CB1 mRNA expression levels, $r = .677$, $P < .001$ for both groups, and $r = .353$, $P = .017$ for adenomyosis group; for CB1 protein expression levels, $r = .708$, $P < .001$ for both groups, and $r = .54$, $P < .001$ for adenomyosis group). For patients with adenomyosis, a multivariate linear regression comprising age, gravidity, parity, uterus size, PBAS, and CB1 or CB2 mRNA levels as covariates revealed that CB1 mRNA levels in JZ were the only covariate associated with the VAS score ($R^2 = .125$, $P = .017$; Figure 3A). For both groups, a multivariate linear regression comprising age, gravidity, parity, uterus size, PBAS, and CB1 or CB2 mRNA levels as covariates revealed that CB1 mRNA levels in JZ and uterus volume were associated with the VAS score ($R^2 = .536$, $P < .001$). Similar results were obtained for the association of CB1 protein levels in JZ with the VAS

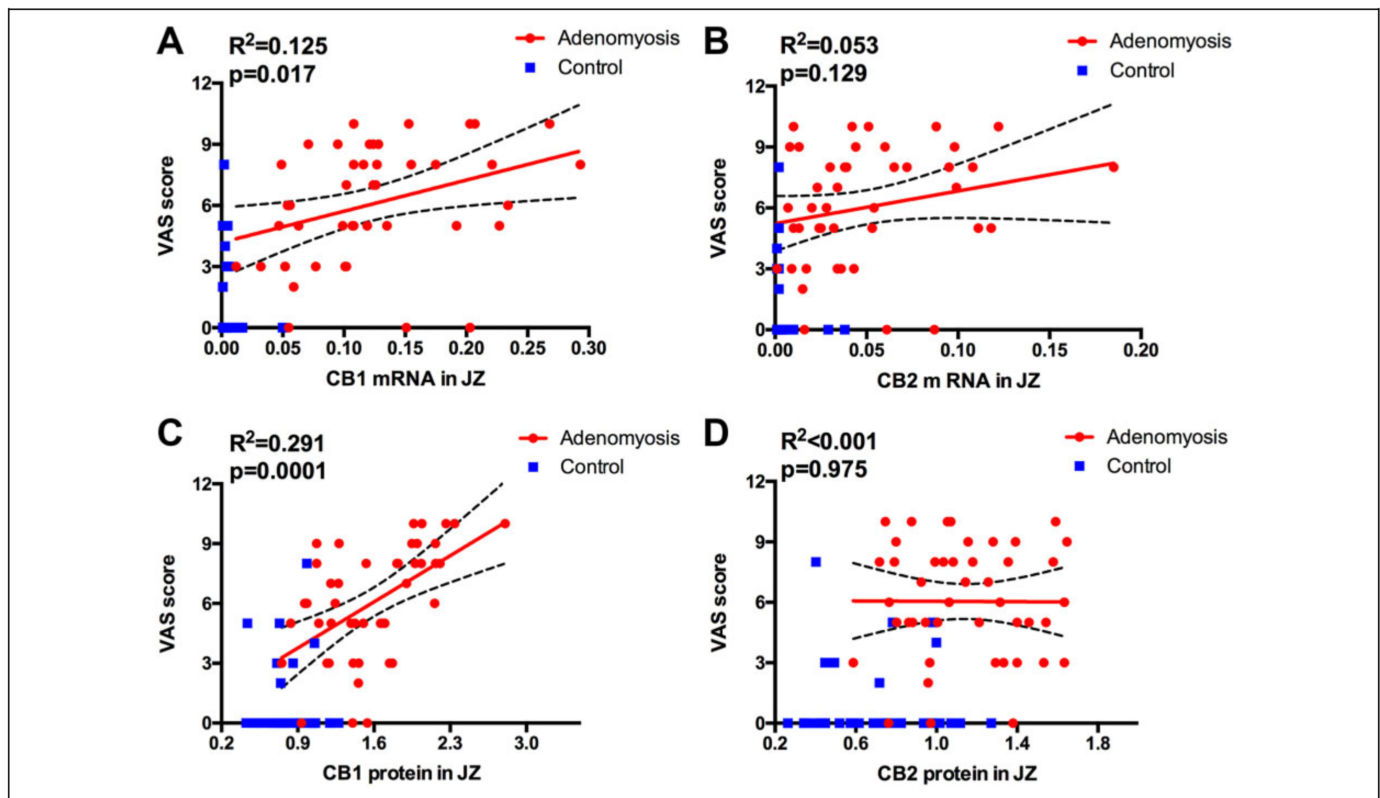

Figure 3. Correlation of cannabinoid receptor type I (CB1) messenger RNA (mRNA)/protein expression (A/C) or cannabinoid receptor type II (CB2) mRNA/protein expression (B/D) in junctional zone (JZ) and Visual Analogue Scale (VAS) score. The red and blue dots represent data from patients with and without adenomyosis. The red line indicates a linear regression fit of the data.

score ($R^2 = .291$, $P < .001$ for adenomyosis group and $R^2 = .54$, $P < .001$ when CB1 protein levels in JZ and uterus volume were both predictors for both groups; Figure 3C).

Discussion

In the present study, we found that CB1 and CB2 mRNA and protein expression levels in women with adenomyosis were significantly higher than the control group, both in JZ and in OM, and in spite of the menstrual cycle differences. CB1 expression levels in JZ were positively correlated with the severity of dysmenorrhea. These findings suggest that ECS may be involved in the pathogenesis of symptomatology of adenomyosis, and CB1 may be a potential therapeutic target for adenomyosis.

Endocannabinoid system is comprised of cannabinoid receptors, endogenous cannabinoids, and their metabolic enzymes that regulate their biosynthesis, transport, and degradation.[38] CB1 and CB2 receptors, which are both 7-transmembrane G protein-coupled receptors (GPCRs), are the main receptors that endogenous cannabinoids depend on to exert their bioactivities.[38-40] Since CB1 receptor was discovered in the mouse uterus in 1995, considerable research has reckoned that uterus may be a target for cannabinoid ligand-receptor signaling.[25,27,41-44] But as far as we know, almost all the existing data are about the expression of components of ECS in the endometrium, plasma,[27,42,43] or mouse myometrium,[44] and little data have been published about their levels in human myometrium except pregnant myometrium.[45] Our findings provide evidence of the expression of CB1 and CB2 in human myometrium and further confirm the previous hypothesis that uterus may be a target for cannabinoid ligand receptor signaling.

Although adenomyosis and endometriosis have been generally regarded as 2 different diseases, their histological characteristics are essentially the same and they do have something in common in clinical manifestations and pathogenesis.[46-49] As for the role of cannabinoid receptors in endometriosis, there are some conflicting results. Resuehr et al[43] and Sanchez et al[27] reported a decreased CB1 expression in endometriosis, immunohistochemical staining of human endometrial tissues, and PCR of mRNA extracted from endometrial stromal cells were used respectively. Leconte and colleagues concluded that CB1 and CB2 were equally expressed in endometrial cells obtained from women with and without endometriosis.[25]

In our study, we observed that myometrium of uteri with adenomyosis expressed significantly higher levels of cannabinoid receptors CB1 and CB2 than the controls. This seems to be consistent with the results about their effect of promoting the development of endometriosis.[23,50,51] Sanchez and coworkers showed that the cannabinoid receptor CB1was able to facilitate the development of endometriosis in a mouse model,[23] and

high expression of CB1 was detected on somata and fibers of both the sensory and the sympathetic neurons that govern the abnormal growth of ectopic lesions in a rat model of endometriosis.[24] Also, it has been shown that activation of CB1 can enhance endometrial cell invasiveness[50] and induce migration of the human endometrial HEC-1B cells.[52] It is also important to note that there are inconsistent reports that cannabinoid agonists exert antiproliferative effects and inhibition of fibrosis in deep infiltrating endometriosis,[25] and they mediate proapoptotic effects in Ishikawa and ovarian endometriosis cyst wall stromal cell lines.[53] The biphasic effects demonstrated in the current studies about cannabinoids in endometriosis is consistent with reports of cannabinoids in tumors, which may be explained by the evidence that its effect changes with its concentration as some scholars reviewed.[20]

However, little is currently known regarding whether ECS has the same impact on adenomyosis. There has been only 1 report about the expression of cannabinoid receptors in adenomyosis which found that CB1 and CB2 receptor immune labeling in the endometrium of adenomyosis was lower compared to the control group.[53] This is consistent with the results of Resuehr et al[43] and Sanchez et al[27] but seems conflicting with ours. The difference may attribute to methodological differences such as different tissues, different technologies, or to unsuitable controls which did not exclude myoma.

Since ECS expression in the human uterus was initially reported in 2010,[42] several studies have demonstrated that sex steroids have an impact on ECS.[43,54-58] Through a cross-sectional and longitudinal study, Talatini et al[59] found plasma anandamide (AEA; one of the main endocannabinoids) reached the peak level at ovulation, and it was positively correlated with estradiol levels. Resuehr et al[43] and Sanchez et al[27] found that CB1 mRNA and protein expression reached peak level in the secretory phase of normal endometrium and the lowest level in the ectopic endometrium of endometriosis; thus, Resuehr et al[43] thought CB1 expression was progesterone dependent. Sanchez et al[27] also found levels of AEA and 2-arachidonylglycerol (2-AG; another endocannabinoid) in the plasma of patients with endometriosis were upregulated during the secretory phase, whereas the AEA and 2-AG levels in the control group did not change significantly across the menstrual cycle. These results suggest that the expression of components of ECS may be regulated in part by sex hormones. However, in this study, we failed to find any cyclic menstrual variation in the expression of CB1 or CB2 in the JZ and OM of both the groups. This may be due to a difference in response between endometrium and myometrium. After all, myometrium does not show obvious cyclic responses to fluctuating hormone levels as the endometrium, which presents continuous dynamic changes.

Although the contractility originated from JZ is known to be involved in dysmenorrhea of adenomyosis,[13,14,60] the mechanisms are unclear. In our study, we observed that CB1 expression in JZ in adenomyosis is significantly higher than OM, while there was no difference for CB2 expression in different anatomical zones. These results showed that CB1 may play a more critical role than CB2 in adenomyosis and further established that abnormal JZ is involved in the pathogenesis of adenomyosis. In this study, we also observed that there was a positive relationship between CB1 levels and the severity of pain which was measured as the VAS score. This is consistent with the reports that for endometriosis-associated pain, CB1 is able to promote sprouted innervation in endometrial ectopic growth by mitogen-activated protein kinase activation.[51] Besides that, cannabinoids are reported to relax human pregnant myometrium via the CB1 receptor,[45] and CB1 receptor activation shows selective inhibition of myometrial spontaneous contractility in mouse myometrium.[44] All these data demonstrate that CB1 may be a potential therapeutic target for disease associated with abnormal uterus contractility, such as preterm birth, impaired transport of embryos resulting in infertility, and dysmenorrhea.

As far as we know, our study is the first to systematically investigate the expression of cannabinoid receptors CB1 and CB2 in human myometrium with adenomyosis and determine their association with the severity of dysmenorrhea. However, there are still some limitations in our study. First, we used VAS score to assess the severity of dysmenorrhea, which may be inaccurate due to recall bias and its subjectivity. Second, we didn't detect the serous expression of endocannabinoids, which might affect levels of CB1 and CB2. Third, this was just a preliminary observational study, and we didn't provide a functional experiment to demonstrate the role of CB1 in the pathogenesis of adenomyosis. Fourth, we took all samples from the anterior fundal wall in order to minimize variability, while since adenomyosis is commonly located in the posterior uterine wall, sampling with both sides (anterior and posterior) might be better. In addition, given the moderate sample size, some differences could be masked. Therefore, more functional studies with a larger sample size will further clarify this issue.

In summary, we found that cannabinoid receptors CB1 and CB2 gene and protein expression were significantly increased in myometrium with adenomyosis compared to normal myometrium. Furthermore, we found that the myometrial CB1 expression in JZ was associated with the severity of dysmenorrhea in women with adenomyosis. Based on our results, we conclude that aberrant expression of CB1 in the myometrium might be involved in the pathogenesis of adenomyosis. However, further functional experiments are still needed in elucidating the role of cannabinoids in adenomyosis.

Authors' Note

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Xue Shen conceived, drafted, revised the article, carried out the entire experiment, and performed statistical analysis; Hua Duan participated in study design and revised it; Sha Wang participated in conceiving the article and patient recruitment; Wei Hong, Yu-Yan Wang, and Si-Li Lin participated in sample collection and acquisition of data. All authors approved the final version of the article.

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Declaration of Conflicting Interests

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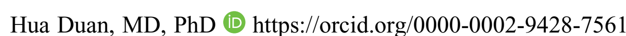
Hua Duan, MD, PhD https://orcid.org/0000-0002-9428-7561

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