



Vitamin D receptor and cyclooxygenase-2 expression in uterine leiomyoma tissues and their correlation

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Background: To explore vitamin D receptor (VDR) and cyclooxygenase-2 (COX-2) expression in uterine leiomyomas and normal myometrium tissues, and further analyze their correlation in the etiopathogenesis of uterine leiomyomas.

Methods: We collected uterine leiomyoma tissues and their paired adjacent normal myometrium tissues from 20 cases of uterine leiomyomas and examined the expression of VDR and COX-2 in the tissues by real-time polymerase chain reaction and Western blotting, respectively. We further analyzed the correlation between VDR and COX-2 as well as their expressions with correlation to the size of uterine leiomyomas.

Results: The expression of VDR mRNA in 15 of 20 uterine leiomyoma tissues decreased compared with the healthy uterine smooth muscle tissues ($P < 0.05$). The expression of COX-2 mRNA in 13 of 20 uterine leiomyoma tissues was higher than in the healthy uterine smooth muscle tissues ($P < 0.05$). The Western blotting results also showed that VDR expression decreased and COX-2 expression increased in uterine leiomyoma tissues compared with the expression in healthy uterine smooth muscle tissues. There were 11 cases with VDR expression decreased and COX-2 expression increased in uterine leiomyoma tissues. And there was a negative relevance between VDR and COX-2 expression in the 11 cases ($r = -0.628$, $P < 0.05$ for transcriptional level; $r = -0.612$, $P < 0.05$ for protein level). However, there was not significant correlation between VDR and COX-2 expression in total specimen ($r = -0.209$, $P > 0.05$ for transcriptional level; $r = -0.165$, $P > 0.05$ for protein level). There was not significant correlation between VDR expression and uterine leiomyoma size, as well as between COX-2 expression and uterine leiomyoma size ($r = -0.008$, $P > 0.05$; $r = 0.150$, $P > 0.05$, respectively).

Conclusions: VDR expression decreased and COX-2 expression increased in uterine leiomyomas compared with the healthy uterine smooth muscles. Decreased VDR expression and high COX-2 expression may play a role in the etiopathogenesis of uterine leiomyomas. There may be a negative relevance between VDR and COX-2 in leiomyomas.

Keywords: Uterine leiomyoma; vitamin D receptor (VDR); cyclooxygenase-2 (COX-2)

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Introduction

Uterine leiomyomas are the most common gynecological benign tumors in females, clinically affecting more than 25% of reproductive age females and causing significant morbidity (1,2). Although some leiomyomas don't cause any symptoms, symptomatic leiomyomas are usually associated with specific symptoms such as anemia, abnormal uterine bleeding, abdominal distention, pelvic pain and frequent micturition. Uterine leiomyomas are the most common diseases for hysterectomy in the United States and they cost at least 6 billion dollars annually (3). Despite the fact that uterine leiomyomas are very common and cause significant medical and economic burdens, the etiology is still partially understood. The occurrence and development of uterine fibroids may be relevant to certain risk factors such as estrogen, progesterone, growth factors and ethnicity, but the exact causes are not yet well known (4,5). Recently, some studies indicated that lower serum vitamin D levels are inversely relevant to leiomyoma burden in different ethnic groups and vitamin D inadequacy may be associated with the occurrence of uterine leiomyomas (6,7). Inflammation can be considered as the 7th hallmark of cancer (8). The causality between the inflammation and tumor proliferation may be explained that the injury caused by the infection causes cellular proliferation and the extracellular matrix growth due to pro-inflammatory and growth factors. Furthermore, it decreases cellular apoptosis and abnormal tissue repair. And chronic inflammation may be a risk for the development of uterine leiomyomas (9). Cyclooxygenase-2 (COX-2) plays a key role in inflammatory processes and in control of cell growth, and it has also been associated with tumorigenesis. Vitamin D can inhibit the inflammatory process by vitamin D receptor (VDR). So far, there are few studies about VDR and COX-2 concomitant expression in uterine leiomyomas. In this study, we found that VDR expression decreased and COX-2 expression increased in uterine leiomyomas, and there was a certain correlation between VDR and COX-2, which may play a role in the etiopathogenesis of uterine leiomyomas.

Methods

Patients and tissues

The study was approved by Institutional Review Board of China-Japan Friendship Hospital (No. 2016-GZR-16), and it was agreed by each patient. Written informed consent was obtained from every patient before the study

began. Specimens of benign leiomyomas and adjacent matched normal myometrium tissues were obtained from 20 premenopausal women undergoing hysterectomy or myomectomy. Normal myometrium tissues were harvested at least 1cm far from the leiomyomas. The age of patients ranged from 31 to 53 years old. None of them had received any hormone, vitamin D or vitamin D analog therapy for at least 3 months before the surgery. The size of uterine leiomyomas was determined by transvaginal ultrasound or scale during the surgery. Volume of all leiomyoma lesions were determined according to the formula ($a \times b \times c \times 0.523$) (6), where a is length, b is width, and c is height. Tissue specimens were taken from the nucleus of intramural leiomyomas and adjacent normal myometrium tissues. Tissue specimens were taken into ice-cold box and immediately sent to the laboratory.

Reagents and antibodies

Trizol reagent was ordered from Invitrogen. RT-PCR kit was purchased from Takara. VDR rabbit monoclonal antibody (anti-VDR), COX-2 rabbit monoclonal antibody (anti-COX-2) and GAPDH rabbit monoclonal antibody was ordered from Cell Signaling Technology. Horseradish peroxidase-labeled goat anti-rabbit IgG, RIPA buffer and SDS-PAGE sample loading buffer was purchased from Beyotime Biotechnology.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from the tissues using Trizol reagent according to manufacturer's protocol. The concentration of total RNA was examined using a NanoDrop 2000 system (Gene Company Limited, China). Five micrograms of total RNA were reverse transcribed in a 40- μ L volume using an quantitative RT-PCR kit. After incubation at 42 °C for 60 min, the reverse transcription reaction was finished by heating at 75 °C for 5 min. The synthesized cDNA was amplified by quantitative real-time PCR (qPCR). qPCR was conducted using Stratagene Mx3000P (Agilent Technologies, USA) according to the manufacturer's instructions. Primer sequences of genes were as follows: VDR primer, forward: 5'-GTGGACATCGG CATGATGAAG-3', reverse: 5'-GGTCGTAGGTCTTATGGTGGG-3'; COX-2 primer, forward: 5'-TCAAGTCCCTGAGCATCTACGGTT-3', reverse: 5'-CTGTTG TGTTCGCCGACCCAGATT-3'; GAPDH primer, forward: 5'-GGACCTGACCTGC CGTCTAG-3',

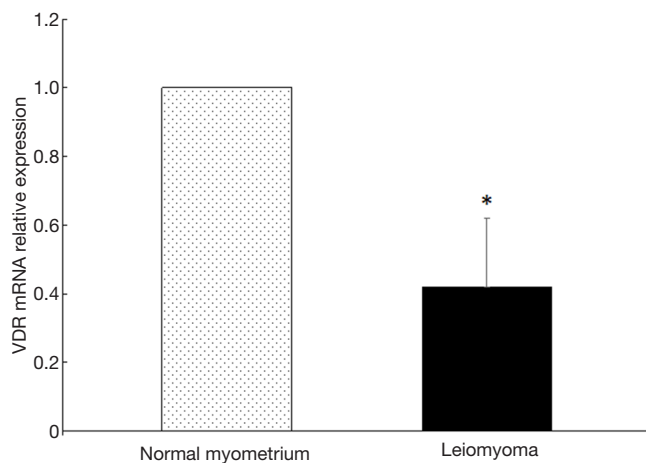


Figure 1 VDR mRNA relative expression in uterine leiomyoma tissues and normal myometrium tissues. *, $P < 0.05$, compared with normal myometrium tissues. VDR, vitamin D receptor.

reverse: 5'-TAGCCCAGGATGCCCTTGAG-3'. qPCR was performed with the following cycling conditions: 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. GAPDH expression was used as internal control to standardize the VDR and COX-2 results. The experiment was repeated 3 times with two replicates for each specimen.

Western blotting analysis

Total protein was extracted from the tissues followed the manufacturer's instructions. And protein concentrations were examined using Bio-Rad protein assay reagents according to the manufacturer's instructions. Samples were resolved by 10% SDS-PAGE. Protein samples were transferred onto polyvinylidene fluoride membranes and the membranes were immersed with specific primary antibodies respectively (anti-VDR, 1:1,000; anti-COX-2, 1:1,000). Specific protein bands were observed after exposure to enhanced chemiluminescence reagent and by E-Gel Imager. The band intensity of each protein was evaluated by image analysis software and normalized against corresponding GAPDH. The experiment was repeated three times for each specimen.

Statistical analysis

Data were expressed as mean \pm standard deviation. Differences in the expression levels of VDR and COX-2 in uterine leiomyoma tissues and healthy uterine smooth muscle tissues were performed by paired *t*-test using SPSS

20.0 software. The correlation between VDR expression and COX-2 expression was performed by Pearson correlation test. P value < 0.05 was considered statistically significant at a 95% confidence level.

Results

VDR expression in uterine leiomyoma tissues

The expression of VDR mRNA in uterine leiomyoma tissues and their paired adjacent healthy myometrium tissues were determined by qPCR. The expression of VDR mRNA in 15 of 20 uterine leiomyoma tissues decreased compared with the healthy myometrium tissues. Relative expression of VDR mRNA in 20 uterine leiomyoma tissues were significantly lower than that in healthy myometrium tissues ($P < 0.05$) (Figure 1). The other hand, VDR proteins were also determined by Western blotting. The result showed the same trend of VDR expression as mRNA level. The protein level of VDR in leiomyoma tissues were significantly lower than that in normal myometrium tissues (Figure 2).

COX-2 expression in uterine leiomyomas tissues

The expression of COX-2 mRNA in uterine leiomyoma tissues and their paired adjacent healthy uterine smooth muscle tissues were determined by qPCR. The expression of COX-2 mRNA in 13 of 20 uterine leiomyoma tissues increased compared with the healthy uterine smooth muscle tissues. Relative expression of COX-2 mRNA in 20 uterine leiomyoma tissues were significantly higher than that in healthy uterine smooth muscle tissues ($P < 0.05$) (Figure 3). The Western blotting result also showed COX-2 protein expressions in leiomyoma tissues were significantly higher than that in healthy uterine smooth muscle tissues (Figure 4).

The correlation between VDR expression and COX-2 expression

We supposed that there was some correlation between VDR expression and COX-2 expression. Among the 20 cases, there was not significant correlation between VDR and COX-2 expression ($r = -0.209$, $P > 0.05$ for transcriptional level; $r = -0.165$, $P > 0.05$ for protein level). However, among the 11 cases with VDR expression decreased and COX-2 expression increased in uterine leiomyoma tissues, there was negative correlation between VDR and COX-2 expression ($r = -0.628$, $P < 0.05$ for transcriptional level; $r = -0.612$, $P < 0.05$

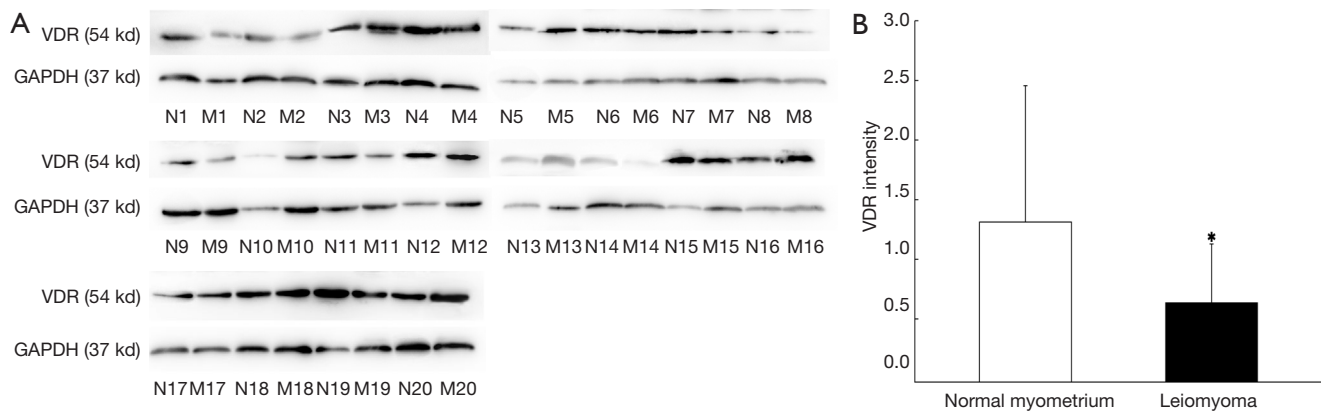


Figure 2 VDR protein expression in uterine leiomyoma tissues and normal myometrium tissues. (A) VDR protein in the tissues were determined by Western blotting (N, normal myometrium; M, leiomyoma); (B) bar graph showing the levels of VDR protein in the tissues. *, $P < 0.05$, compared with normal myometrium tissues. VDR, vitamin D receptor.

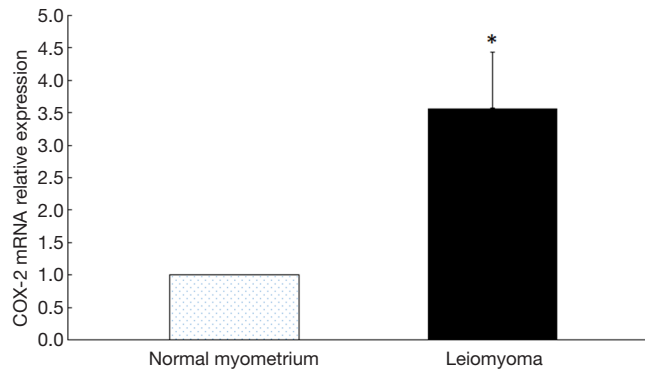


Figure 3 COX-2 mRNA relative expression in uterine leiomyoma tissues and normal myometrium tissues. *, $P < 0.05$, compared with normal myometrium tissues. COX-2, cyclooxygenase-2.

for protein level).

The correlation between VDR expression, COX-2 expression and uterine leiomyoma size

The diameter of the leiomyomas was from 3.5 to 9.2 cm, and the volume ranged from 16.03 to 205.99 cm^3 . There was not significant correlation between VDR expression and uterine leiomyoma size, as well as between COX-2 expression and uterine leiomyoma size ($r = -0.008$, $P > 0.05$; $r = 0.150$, $P > 0.05$, respectively).

Discussion

Vitamin D mainly consists of two highly lipophilic

substances: cholecalciferol (vitamin D₃) and ergocalciferol (vitamin D₂), which is barely active. It needs further activation steps to become the biologically active 1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. VDR is the natural ligand of 1,25(OH)₂D₃, which is a transcriptional factor of the nuclear receptor superfamily (10). Vitamin D plays a physiological role by liganded VDR binding to vitamin D response elements in the promoter and regulatory region of vitamin D target genes (11). The main function of serum 1,25(OH)₂D₃ is regulation of bone mineralization and calcium/phosphate homeostasis (12). Autocrine and paracrine actions of the locally synthesized 1,25(OH)₂D₃ include improving of the innate immune system, lowering autoimmune disease, reducing risk of heart attack, and exerting antitumorigenic activities (13). What's more, many studies demonstrate that 1,25(OH)₂D₃ is able to delay tumor formation and prevent spread of metastases by altering cellular energy metabolism, prohibiting proliferation, inducing differentiation, activating apoptosis and inhibiting angiogenesis of the tumor (14,15). However, human clinical data are much less conclusive in showing cancer preventive effects of vitamin D (16). Due to the influence on tumors, it interests the researchers to investigate the role of vitamin D in the pathogenesis of uterine leiomyomas. Some studies demonstrated that the serum vitamin D of the persons with uterine leiomyomas were significantly lower than that of the persons without leiomyomas and vitamin D may play a role in the occurrence and development of uterine leiomyomas (17,18). Additionally, *in vitro* and animal studies indicated that vitamin D could inhibit uterine leiomyoma cells growth and leiomyomas shrunk by vitamin D treatment

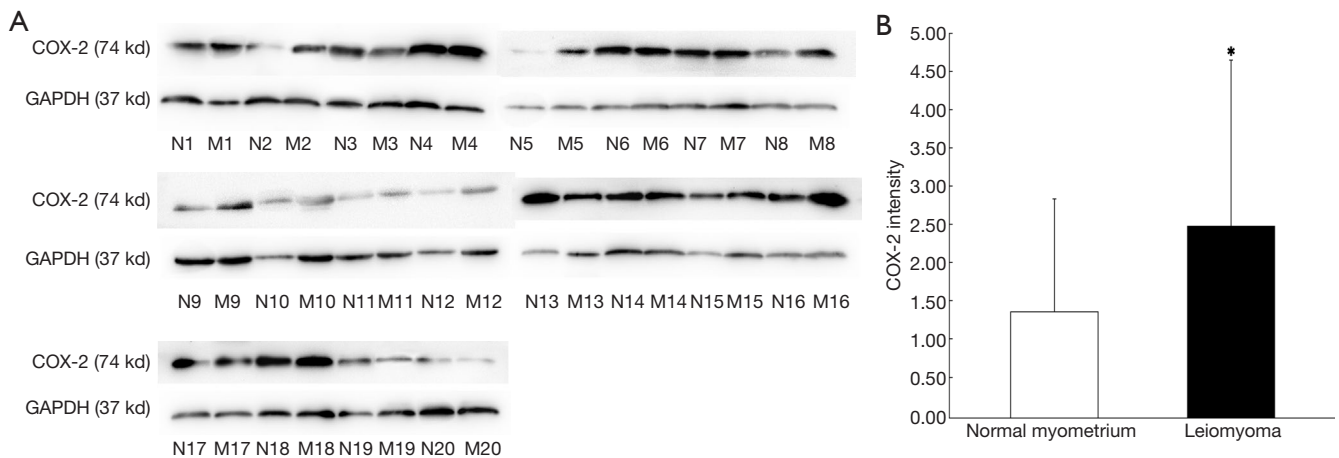


Figure 4 COX-2 protein expression in uterine leiomyoma tissues and normal myometrium tissues. (A) COX-2 protein in the tissues were determined by Western blotting (N, normal myometrium; M, leiomyoma); (B) bar graph showing the levels of COX-2 protein in the tissues. *, $P < 0.05$, compared with normal myometrium tissues. COX-2, cyclooxygenase-2.

(19,20). So far, the mechanism of vitamin D impacting on uterine leiomyomas is unknown. Vitamin D performs physiological role by VDR, but VDR expression in uterine leiomyomas are not verified. Only a few researches found that most uterine fibroid tissues expressed low levels of VDR compared to adjacent normal myometrium, and there was inverse correlation between higher levels of estrogen receptor, progesterone receptor and lower levels of VDR (19,21). We also found that the expression of VDR in uterine leiomyomas was significantly lower compared to adjacent normal myometrium. Although one study demonstrated that serum levels of vitamin D₃ are inversely related to leiomyoma sizes (6), the correlation between VDR and leiomyoma sizes is unknown. In this study the result indicated that there was not significant correlation between VDR and leiomyoma sizes.

Prostaglandin signaling plays an important role in inflammation-associated tumor formation (22). Prostaglandin E₂ is a lipid mediator that is synthesized by COX-2 in all cell types. COX-2 is inducible by inflammatory stimuli, including cytokines, growth factors, and tumor promoters, and is upregulated in a variety of malignancies and favors the growth of malignant cells by stimulating proliferation and angiogenesis (23-25). The expression of COX-2 and its role in uterine leiomyomas is not conclusive. There are a few reports that COX-2 expression in uterine leiomyomas increased significantly compared to normal myometrium tissues (26,27), while other study indicated that COX-2 expression in smooth muscle tumors is not a prominent mark (28). In addition,

celecoxib could inhibit the leiomyoma cell growth by blocking the COX-2 inflammatory pathway, suggesting that COX-2 plays an important role in the pathogenesis of uterine fibroids (27,29). In this study we found that the expression of COX-2 in 13 of 20 uterine leiomyoma tissues was higher than in the healthy uterine smooth muscle tissues. It was in accordance with the previous studies.

Some researches demonstrated that there was a negative correlation between VDR and COX-2 in cancerous tissues (30,31). And *in vitro* study showed that 1,25(OH)₂D₃ significantly inhibited inflammatory responses, such as IL-8 expression and prostaglandin activity (32). In mice, calcitriol inhibited COX-2 expression and reduced subsequent prostaglandin synthesis in macrophages (33). However, to date there is no study examined the correlation of VDR with COX-2 in human uterine leiomyomas. We postulated that there was an inverse relevance between VDR and COX-2 in uterine leiomyomas. And vitamin D may play a role in leiomyomas by the COX-2 signaling pathway. Unexpectedly, we found that there was not significant correlation between VDR and COX-2 expression in leiomyomas among the total specimens. However, among the 11 cases with VDR expression decreased and COX-2 expression increased in uterine leiomyoma tissues, there was an inverse relevance between VDR and COX-2 expression. As far as we were concerned, the small quantity of the specimens led to the discrepancy. The inverse correlation of VDR with COX-2 may be confirmed by another big population study in future.

In conclusion, in this study we found that VDR

expression decreased and COX-2 expression increased in uterine leiomyomas compared with normal myometrium tissues. Decreased VDR expression and high COX-2 expression may play a role in the pathogenesis of uterine leiomyomas. There may be an inverse correlation between VDR and COX-2 in leiomyomas.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2018.01.19>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of China-Japan Friendship Hospital (No. 2016-GZR-16). Written informed consent was obtained from every patient before the study began.

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