

Original Article

High expression of calcium channel subtypes in uterine fibroid of patients

Xiaoping Ke^{1,2}, Zhongping Cheng², Xiaoyan Qu², Hong Dai², Wenchao Zhang², Zi-Jiang Chen¹

¹Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University & Shandong Provincial Key Laboratory of Reproductive Medicine, 324 Jingwu Road, Jinan 250021, China; ²Department of Obstetrics and Gynecology, Yangpu District Central Hospital, 450 Tengyue Road, Shanghai 200090, China

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Abstract: Aim: To investigate the expression of calcium channel protein in uterine fibroids, and to explore the relationship between calcium signaling pathway and the pathogenesis of uterine fibroids. Methods: Uterine fibroid tissues (UFC) and adjacent healthy uterine smooth muscle tissues (SMC) were collected from 30 cases of uterine fibroids. Real-time quantitative PCR and western blot were used to detect cell membrane calcium channel protein subtypes: TRPC1, TRPC3, TRPC4, TRPC6, TRPM6 and TRPM7. The effects of genes exhibiting most-notable differences on cell proliferation were examined using gene interference techniques. Results: We found that calcium channel protein subtypes expressed differently in fibroids and the surrounding smooth muscles. The mRNA and protein expressions of TRPC1 and TRPM7 were higher in uterine fibroid tissues than in smooth muscle ($P < 0.05$), while no obvious difference was found in terms of other subtypes (TRPC3, TRPC4, TRPC6 and TRPM6). In cultured uterine leiomyoma cells, modifying the expressions of TRPC1 and TRPM7 significantly affected the proliferation rate of uterine fibroids. Conclusion: Calcium channel subtypes TRPC1 and TRPM7 exhibit different expression patterns in uterine fibroids and surrounding smooth muscles, suggesting that calcium signaling pathway regulated by these calcium channel proteins may be associated with the incidence of uterine fibroids.

Keywords: Uterine fibroids, TRPC, TRPM, calcium signaling

Introduction

Uterine fibroid is the most common benign tumor of female reproductive organs with a clinical incidence of 20%-40%, and a detection rate of 77% for pathology [1-3]. The common symptoms of uterine fibroid include abnormal uterine bleeding, infertility, and pelvic mass which may cause a serious threat to women's health and quality of life.

Previous studies indicate that uterine fibroid is associated with estrogen, androgen, and abnormal cell proliferation. However, the exact pathogenesis still remains unclear so far. In 1980-90, some researchers monitored intrauterine pressure through pressure sensors placed in the uterine cavity of patients with uterine fibroids, and found that patients showed abnormal uterine contraction. More recently, studies on the physiology of non-pregnant uterus contraction suggested that deep uterine myometrium, which is also subendocardial muscle,

exhibited "creep-like contraction" [4-6]. It was also observed that there was a reduced motor function but enhanced proliferation in leiomyoma cells [7]. All these studies have shown that abnormal smooth muscle contraction could play an important role in the pathogenesis of uterine fibroids whereas the molecular mechanism underlying was still not clear and worthy of further exploration.

Calcium ions are important upstream signaling molecules to initiate cell contraction. The calcium channel proteins are molecular switches in signal transduction and they not only regulate cell contraction but also are involved in cell cycle regulation which is closely associated with a variety of tumors and oncogenic pathways.

Studies on the relationship between abnormal contraction and abnormal uterine fibroids have been focused on the role of the transient receptor potential (TRP) in the pathogenesis of uter-

Table 1. Primers used in Real Time PCR

Gene Name	Forward primer	Reverse primer
TRPC1	GGCCAGCCCTTGAAAGAATAG	TTCTGCCACCACTGTAGGATG
TRPC3	ACTCCTTCAGCCACTCAC	ATCCAGCACACCCACTAC
TRPC4	TCTGCCTACTCCCTTCAATG	CGCCTATGCTGTGTTCTTAC
TRPC6	TTGCCATTGGACTGCCCTTC	AGGCTGCGTGTGCTACAAAC
TRPM6	GGAGACCATGCTGGGATAGA	GATGGGTGTGCTCTCCATCT
TRPM7	CCTCTTCTGGTGCCTTATTC	GGCTGAGATGGTGTACTAAC
GAPDH	GACCGCCTAAATGTCTACAC	GCCTTCTCCATGGTGGTGAA

ine fibroids. TRP proteins are localized in the cell membrane and belong to cation channel protein family. There are at least 28 homologous proteins discovered in mammalian cells and they are in six subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP and TRPML. The main function of TRPC is to regulate the influx of $\text{Ca}^{2+}/\text{Na}^{+}$ depending on PKC pathway activation induced by the G-protein-coupled receptors (GPCR) [8]. Studies have shown multiple TRPC proteins are expressed in normal muscle and smooth muscle tumors, in which TRPC and TRPM subfamily proteins are most closely associated with smooth muscle physiology and pathology [9, 10]. TRPC1, 3, 4, 6 and TRPM6, 7 are the main subtypes of TRPC family and TRPM family. All of them regulate Ca^{2+} influx, but expressed at different levels in different tissues.

In this study, we measured the expression levels of TRPC, TRPM in uterine leiomyoma and normal smooth muscles using real-time quantitative PCR and western blot.

We found that the proteins were expressed in different levels in the different tissues, and interference techniques were used to identify the relationship between calcium signaling pathways and uterine fibroids.

Materials and methods

Clinical samples

Uterine fibroids and adjacent uterine smooth muscles were collected from 30 patients with uterine fibroids who were surgically treated in Yangpu District Central Hospital and underwent laparoscopic surgery from May, 2011 to December 2011. They are 30-54 years old (mean age, 42.3 ± 4.6 years). Uterine fibroids were classified as single or multiples (ranging from 1 to 4 fibroids) and the largest tumor

diameter was greater than 5 cm. All of them are intramural fibroids. Patients with adenomyosis or receiving hormone therapy in the previous 6-months were excluded from the study. Uterine fibroid tissue and normal muscle tissue were confirmed by H&E staining. The potentially malignant was excluded by measuring the serum levels of AFP,

CEA, CA125, CA153 and CA199. The intramural fibroid tissues were removed from patients receiving laparoscopic surgery and the control samples were uterine smooth muscle tissue collected from tumor adjacent tissues with a distance greater than 0.5 cm.

Real time PCR

Total RNA from a 100 mg sample of fibroid or smooth muscle tissue was extracted with Trizol (Sigma-Aldrich, US) and reverse transcribed into DNA (MBI, Lithuania). Real-time PCR was performed using a quantitative polymerase chain reaction kit (TaKaRa, JAPAN). The total reaction volume is 10 μl , and the reaction consisted of an initial denaturation step (5 min at 95°C) followed by 40 cycles of denaturation (15 s at 95°C), annealing (35 s at 60°C), and extension (30 s at 72°C). GAPDH was used as internal control and the data were analyzed by ABI Prism 7500 SDS software. Primers used in the real time PCR are listed in **Table 1**.

Western blot

Uterine fibroids and smooth muscle tissues, 0.5 cm \times 0.5 cm in size, were stored in -80°C . The tissue (100 mg) was homogenized on ice and total protein was extracted. Total protein (40 μg per well) was prepared for electrophoresis and then transferred to a nitrocellulose membrane (Whatman, USA). Membrane was blocked in 5% skim milk at room temperature for 30 min and incubated with the following antibodies: TRPC group TRPC1 1:1000; TRPC3 1:500; TRPC4 1:1000, TRPC6 1:500; TRPM6 1:1000; TRPM7 1:500 (Abcam, USA); GAPDH 1:2000. Goat anti-mouse antibody (1:2000) was used as secondary antibody. The experiment was repeated three times.

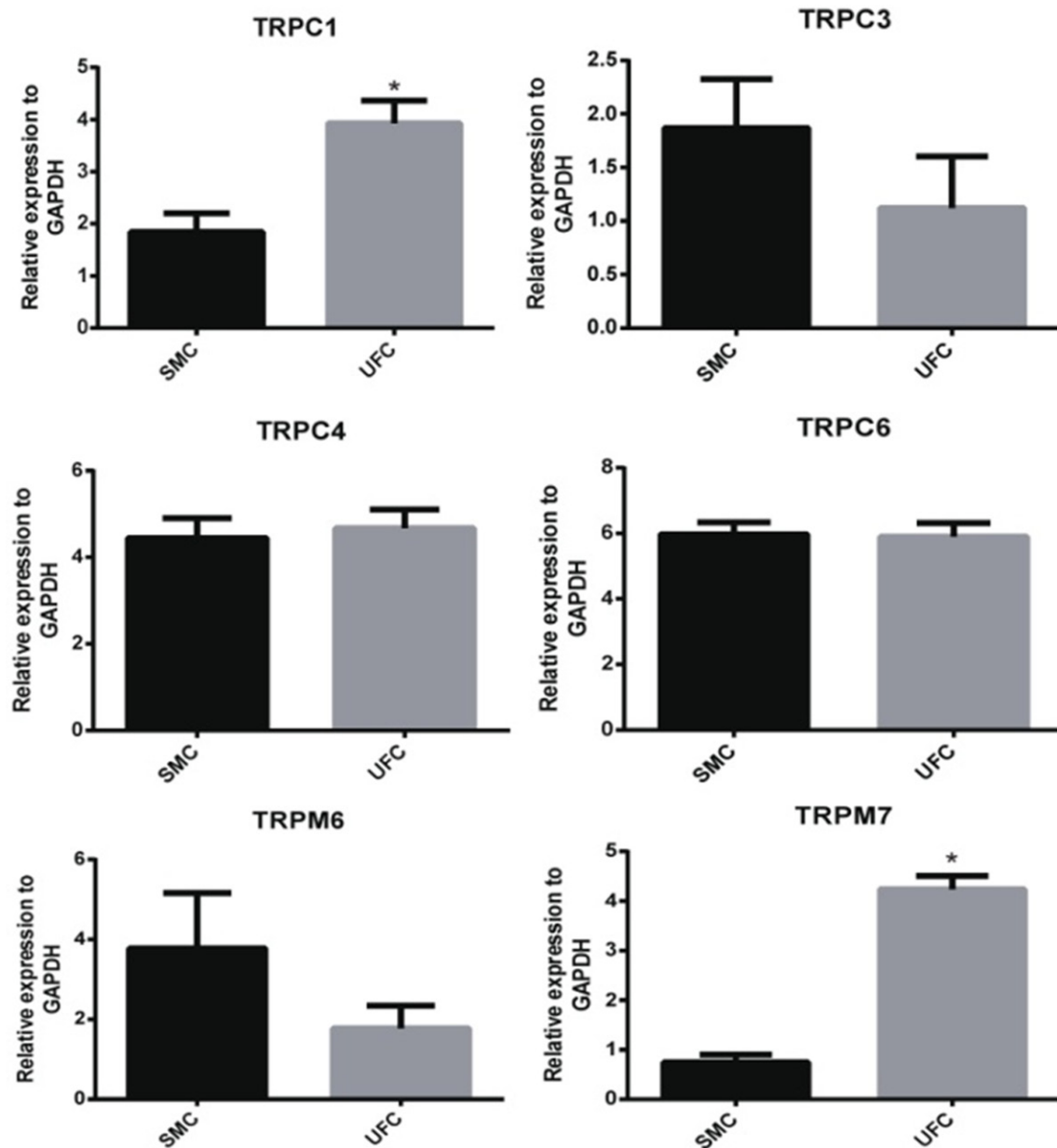


Figure 1. mRNA expression of calcium channel subtypes determined by real time RT-PCR. TRPC1 and TRPM7 were significantly increased in uterine fibroid cells (UFC) as compared to smooth muscle cells (SMC), while TRPC2, TRPC4, TRPC6 and TRPM6 did not have significant difference between UFC and SMC; *P < 0.05 as compared to SMC.

Primary cell culture

Fresh uterine fibroid was isolated and cut into pieces in HBSS buffer containing penicillin and streptomycin. After adding 0.4% collagenase (containing DNase I 20 µg/ml), 0.05% trypsin 2 ml, the sample was shaken at 37°C for three hours for digestion. Following the digestion, cells were washed and seeded in culture flasks containing 10% FBS and 100 U/ml of penicillin

and 100 µg/ml of streptavidin in DMEM-F12 medium. The adherent cells were fibroblasts within 20 minutes, and no adherent cells were transferred to new flasks, after 3 cycles of 20 min adherent, the non-adherent cells were smooth muscle cells and attached overnight.

RNA interference

The GIPZ lentiviral vector with TRPC1 and TRPM7 shRNA was purchased from Open bio-

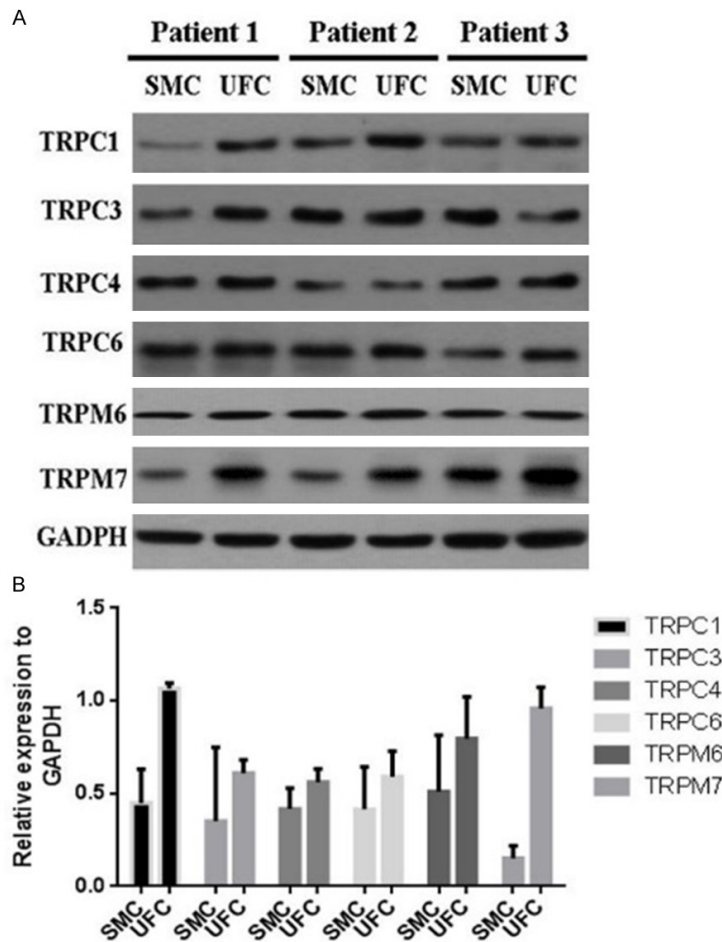


Figure 2. Protein expressions of calcium channel subtypes determined by Western blotting. Similar patterns of change were observed for each protein as those observed for the mRNA levels. A. Western blot analysis; B. Quantitative of bands by Image J; TRPC1 and TRPM7 were significantly increased in uterine fibroid cells (UFC) as compared to smooth muscle cells (SMC), while TRPC2, TRPC4, TRPC6 and TRPM6 did not have significant difference between UFC and SMC; * $P < 0.05$ as compared to SMC.

system, and the mature antisense sequence for TRPC1 is TAGTCTATTCTTTCAAGGG, the mature antisense sequence for TRPM7 is ACA-GCTAGAAAATCTAGCA. Cells were transiently infected with lentiviral shRNA for 72 h for cell proliferation assay.

MTT assay

Uterine fibroid cells were seeded in 96-well plates with 5000 cells per well. Cells were divided into control group and experimental groups and treated for different intervals and then 20 μ L of a final concentration of 0.5 mg/mL of MTT was added to each well for 4 h. After

removal of the culture supernatant from each well, 150 μ L of DMSO was added and the cultures were shaken for 10 min. A wavelength of 490 nm was used to detect the absorbance of each well using a microplate reader, and the growth rate was calculated as the relative growth to control group.

Data analysis

ANOVA was utilized to compare the differences in the mean among the groups when the data approximately represented a normal distribution. P -value ≤ 0.05 (two-sided test) was considered as statistically different.

Results

The expression of calcium channel protein subtypes in uterine fibroids

The mRNA expressions of calcium channel subtypes were compared in 30 pairs of uterine fibroids and surrounding smooth muscles and determined by quantitative PCR. As shown in **Figure 1**, there was no significant difference in terms of most of the calcium channel isoforms, such as TRPC3, TRPC4,

TRPC6, and TRPM6 between these two tissues. However, TRPC1 and TRPM7 had a higher expression in leiomyoma tissues than in smooth muscle tissue.

In addition, western blot was used to assess the protein expression levels of calcium channel subtypes. As shown in **Figure 2A** and **2B**, the expression levels of TRPC1 and TRPM7 were higher in the fibroid tissue than in surrounding smooth muscles which is consistent with their mRNA expression results, whereas the expressions of other isoforms including TRPC3, TRPC4, TRPC6, TRPM6 were not significantly different between the 2 groups.

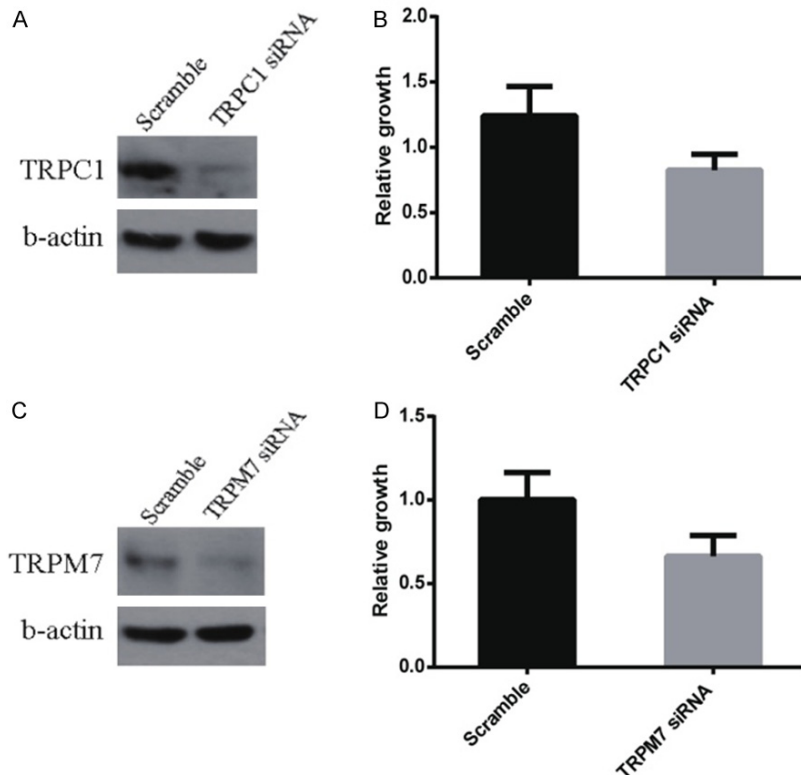


Figure 3. Knock down of TRPC1 and TRPM7 expression in UFC. A. Verification of the knock down efficiency of TRPC1; B. Cell proliferation rate was decreased as a result of TRPC1 knock down in UFC; C. Verification of the knock down efficiency of TRPM7; D. Cell proliferation rate was decreased as a result of TRPM7 knock down in UFC; *P < 0.05 as compared to control group.

Knockdown the expression of TRPC1 inhibits cell proliferation

Since TRPC1 expression increased in uterine fibroids, we further investigated its impact on the proliferation of uterine fibroids. As shown in **Figure 3A**, siRNA virus significantly lowered TRPC1 expression. Cell proliferation was measured by MTT method, and **Figure 3B** showed that knockdown of TRPC1 significantly inhibited uterine leiomyoma cell proliferation.

Knockdown TRPM7 expression inhibits cell proliferation

Since TRPM7 protein increased in uterine fibroids, we used RNA interference technique to knockdown the TRPM7 expression and examined its effect on uterine leiomyoma cell proliferation. As shown in **Figure 3C**, TRPM7 expression was markedly inhibited. Using MTT method, we observed knockdown of TRPM7

resulted in significant proliferation inhibition of uterine leiomyoma cells (**Figure 3D**).

Discussion

It is known that uterine smooth muscle excitation-contraction coupling is regulated by two signaling pathways [11] in which calcium signaling pathway plays a vital role in cellular response. In smooth muscle cell contraction, the elevation of cytosolic Ca^{2+} is induced through two pathways. The first one is triggered by the activation of transmembrane proteins to induce the influx of calcium. There are two types of transmembrane calcium channel proteins: calcium transient receptor potential channel (TRPC), and calcium channel delay (long-lasting potential channel, LPC). The activation of G protein-coupled receptor (GPCR) is

regulated by the activation of calcium channel proteins. The second pathway is the release of calcium from the intracellular calcium stores in the endoplasmic reticulum (ER). GPCRs activate PLC and hydrolyze PIP2 in the plasma membrane and generate DAG and IP3 which binds to IP3 receptors (IP3R) on the ER, causing release of calcium.

Recent studies found that TRP proteins regulating intracellular Ca^{2+} homeostasis are also involved in tumor genesis and development [12]. Abnormal TRPC expressions are exhibited in various human tumor tissues [12, 13]. Many studies show that TRPC proteins are associated with the proliferation of breast cancer [14], prostate cancer [15], glioma cell proliferation [16] and glioma cell migration [17]. It has been shown that the upregulation of TRPC1 expression enhanced intracellular Ca^{2+} concentration, promoted cell proliferation and inhibited apop-

tos. Meanwhile, cell migration is a critical step of tumor metastasis and invasion. In this process, TRPC1 plays a very important role by increasing the Ca^{2+} gradient and affecting cell migration [18], which causes nasopharyngeal cancer lymph node metastasis. TRPM7 (Transient receptor potential cation channel, subfamily M, member 7) is a recently discovered dual function protein acting as an ion channel and protein kinase. It induces the permeability of a number of divalent and monovalent including their Ca^{2+} , Mg^{2+} , K^+ , Na^+ as a non-selective cation channel, including a number of divalent and monovalent cations permeability, as a protein kinase which induces substrate phosphorylation. In this study, we found that the expression mRNA and protein of TRPC1 and TRPM7 in uterine fibroids were higher than in surrounding smooth muscles, indicating that these two calcium channel subtypes may be closely related to the incidence of uterine fibroids.

It is reported that TRPC1 and TRPM7 calcium channel proteins are associated with abnormal uterine fibroid contraction, but whether they are associated with the proliferation of uterine fibroids is still not clear. In this study, we found that fibroid cell proliferation decreased after the knockdown of the expression of TRPC1 or TRPM7 in vitro. Our results suggest that the expression of TRPC1 and TRPM7 protein in uterine fibroids is not only involved in abnormal contraction but also participates in uterine leiomyoma cell proliferation, reflecting that calcium channel proteins have multiple functions. These results also support our hypothesis that abnormal uterine contraction is associated with the occurrence of uterine fibroids, in which TRPC1 and TRPM7 protein may play an important role.

In conclusion, this study demonstrated that calcium channel subtypes TRPC1 and TRPM7 were highly expressed in uterine fibroid and involved in both cell contraction and cell proliferation, suggesting that TRPC1 and TRPM7 mediated calcium signaling may participate in the development of uterine fibroid.

Address correspondence to: Dr. Zi-Jiang Chen, Center for Reproductive Medicine, Provincial Hospital Affiliated to Shandong University & Shandong Provincial Key Laboratory of Reproductive Medicine, 324 Jingwu Road, Jinan 250021, China. E-mail:

chenzjiang@hotmail.com; Dr. Zhongping Cheng, Department of Obstetrics and Gynecology, Yangpu District Central Hospital, 450 Tengyue Road, Shanghai 200090, China. E-mail: zpcheng_63@126.com

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