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Differential expression of estrogen receptor α and β isoforms in multiple and solitary leiomyomas

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ABSTRACT

Uterine leiomyomas are benign myometrial neoplasms that function as one of the common indications for hysterectomy. Clinical and biological evidences indicate that uterine leiomyomas are estrogen-dependent. Estrogen stimulates cell proliferation through binding to the estrogen receptor (ER), of which both subtypes α and β are present in leiomyomas. Clinically, leiomyomas may be singular or multiple, where the first one is rarely recurring if removed and the latter associated to a relatively young age or genetic predisposition. These markedly different clinical phenotypes indicate that there may different mechanism causing a similar smooth muscle response. To investigate the relative expression of ERα and ERβ in multiple and solitary uterine leiomyomas, we collected samples from 35 Chinese women (multiple leiomyomas n = 20, solitary leiomyoma n = 15) undergoing surgery to remove uterine leiomyomas. ELISA assay was performed to detect estrogen(E_2) concentration. Quantitative real-time PCR analysis was performed to detect ERα and ERβ mRNA expression. Western blot and immunohistochemical analysis were performed to detect ERα and ERβ protein expression. We found that ERα mRNA and protein levels of in multiple leiomyomas were significantly lower than those of solitary leiomyomas, whereas ERβ mRNA and protein levels in multiple leiomyomas were significantly higher than those in solitary leiomyomas, irrespectively of the menstrual cycle stage. In both multiple and solitary leiomyomas, ERα expression was higher than that of ERβ. E_2 concentration in multiple and solitary leiomyomas correlated with that of ERα expression. ERα was present in nuclus and cytoplasma while estrogen receptor β localized only in nuclei in both multiple and solitary leiomyomas. Our findings suggest that the difference of ERα and ERβ expression between multiple and solitary leiomyomas may be responsible for the course of the disease subtypes.

1. Introduction

Uterine leiomyomas (also called fibroids or myomas) are benign myometrial neoplasms composed of unorganized smooth-muscle cells and enriched extracellular matrix [1,2]. They are present in the 30–40% of premenopausal women, making leiomyomas one of the common indications for hysterectomy [3,4] and a major public health problem, with a consequent heavy financial burden [5,6].

The exact etiology and pathophysiology of leiomyomas are still unknown, but clinical and biological evidence indicates that the development of uterine leiomyoma is estrogen-dependent [5,7]. Estrogens are hormones regulating cell proliferation in different organs, including uterus and breast [8]. Estrogens stimulate cell proliferation through binding to the estrogen receptor (ER), whose subtypes ERα and ERβ are both present in leiomyomas [9,10].

Till now, previous studies treated leiomyomas as a single disease. Clinically, however, leiomyomas may be both singular and multiple. The first one rarely recurs once removed, while the other

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is common at young age or may be associated with genetic predisposition [11,12]. These markedly different clinical phenotypes suggest that there may different mechanisms causing a similar smooth muscle response.

We hypothesized that this phenotypic variation might be associated with differences in the expression of ERα and ERβ in multiple and solitary leiomyomas. Therefore, we investigated a possible correlation between ER subtype expression and leiomyoma subtypes.

2. Materials and methods

2.1. Patients and sample collection

The study was conducted on 35 Chinese women diagnosed with uterine leiomyomas by transvaginal ultrasound(TVS) (multiple leiomyomas n = 20, solitary leiomyoma n = 15) and undergoing myomectomy or hysterectomy at the First Affiliated Hospital of Chongqing Medical University, Chongqing, People's Republic of China, from August 2013 to February 2015. The diagnosis of leiomyoma was confirmed by histologic analysis, and adenomyosis or malignancy were excluded in all cases. Leiomyoma samples were obtained from the leiomyoma tissues just beneath the capsule of the tumor. The samples of multiple leiomyomas were collected from the largest leiomyoma of each patient. Patients did not receive any medication or hormonal therapy for the 3 months before surgery. Use of human tissue specimens was approved by the Ethical Committee of Chongqing Medical University with written informed consent from each patient. Samples of multiple and solitary leiomyomas were divided into several pieces and either frozen in liquid nitrogen after removal and stored at −80 °C for ELISA assay and mRNA and protein isolation or fixed in 4% paraformaldehyde for immunohistochemical analysis. All samples were examined histologically by an experienced pathologist.

2.2. Patients' clinical history and index studies

Patients were required to fill in a questionnaire asking them about age when diagnosed with leiomyomas, age of menarche, number of pregnancies, deliveries and the time from last labor. Body mass index (BMI) was calculated according to body height and weight. The phase of the cycle was obtained according to the date of the last menstrual period and endometrial histology. Leiomyomas were localized using the TVS report. Uterine volume was calculated preoperatively by TVS using the prolate ellipsoid formula [13]. Women further reported their clinical symptoms including sensation of mass, menorrhagia, dysmenorrhea due to leiomyomas.

2.3. ELISA assay

Estrogen concentration in leiomyoma tissues was determined using a commercial ELISA kit (USCN Life Science Inc, China). In brief, enzyme-linked immunosorbent assay plates were coated with human estradiol monoconol antibody in 100 μl 0.05 M carbonated bicarbonate (PH 9.6) by overnight incubation at 4 °C. After washing five times with 0.05% Tween-20 in PBS (PBST), the plates were blocked with 5% skim milk in PBST at 37 °C for 1 h and washed again as above. The first monoclonal antibody was added to each well and incubated at 37 °C for 1 h. The plates were washed again before adding horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Zhongshan Goldenbridge Biotechnology Co., Ltd) to each well. The plates wee incubated at 37 °C for 1 h. The plates were washed again with PBST as above, and 100 μl 3,3′,5,5′-Tetramethylbenzidin (TMB) substrate was added to each well. The reaction was stopped using 50 μl 0.5 N H_2SO_4 after incubation at room temperature for 30 min. The optical density (OD) value at 450 nm of each well was immediately read using a microtiter plate reader (Labsystems Multiskan MS).

2.4. RNA extraction, reverse transcription, and quantitative real-time PCR analysis

Total RNA was extracted from approximately 50–100 mg of uterine leiomyoma tissues using TRLZOL reagent (Invitrogen) following manufacturer's instructions. RNA concentration was determined by spectrophotometric measurement the ratio of A260/A280. Primer sequences of ERα(NCBI NO.:NM 0001253.3), ERβ(NCBI NO.:NM 001040275.1) and TBP (synthesized by Sangon Biotech Col., Ltd, Shanghai China) are as follows: ERα sense: GGTCAGTGCCTTGTTGGATG, antisense: TGCCAGGTTGGTCAGTAAGC; ERβ sense: ACTGGGATTGTGTGGTCAGC, antisense: AGAGGATAGGCATCGGCATT; TBP sense: CCACAGCTCTTCCACTCACA, antisense: CTGCGGTACAATCCCAGAAC. Quantitative real-time PCR (qRT-PCR) was carried out by BIO-Rad CFX 96 using SYBR Green (TaKaRa Code: RR820A). 100 ng cDNA template was added to 12.5 μl of 2× SYBR Premix Ex Taq and 0.5 μl of each specific primer, and water to a final volume of 25 μl we used a 40 amplification cycles with following parameters: denaturation 95 °C, 30 s; annealing 95 °C, 10 s; extension 56 °C, 30 s. The fluorescence signal was plotted at the end of the extension phase of each cycle. Melting curve analysis was performed by the instrument automatically. All values of the target genes were normalized to the expression of the reference gene TBP. Relative expression was calculated according to the equation $2^{-\Delta\Delta ct}$ and analyzed by Mann–Whitney U test.

2.5. Western blot analysis

Approximate 100 mg uterine leiomyoma tissues were homogenized in 200 μl lysis buffer (Beyotime, China) and 2 μl chemiosttin phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF). Total protein was extracted using the total protein Tripure reagent kit (Beyotime, China) according to the manufacturer's instructions. Protein concentration was determined by Bradford assay. Proteins samples (about 45 μg each sample) were run on a 10% SDS-PAGE gels and then transferred onto PVDF membranes using a Bio-Rad electroblot apparatus (Bio-Rad, Beijing, china). The membrane was incubated in blocking PBS containing 0.05% Tween-20 (PBST) and 5% nonfat milk for 1 h at room temperature and then was subsequently incubated with polyclonal rabbit anti-ERα antibody (1:2000, Gene Tex, GTX100634), monoclonal mouse anti-ERβ antibody(1:3000, Gene Tex, GTX70174) or β-actin (1:1000, Zhongshan Goldenbridge Biotechnology Co., Ltd TA-09) at 4 °C for 12 h, washed for three times with PBST (5 min each time), followed by incubated with secondary antibody (goat anti-rabbit IgG for ERα and goat anti-mouse IgG for ERβ) conjugated with horseradish peroxidase (Zhongshan Goldenbridge Biotechnology Co., Ltd) for 1.5 h at room temperature, and then washed three times with PBST(5 min each time). Protein bands were visualized using diaminobenzidine tetrahydrochloride (DAB). Densitometry analysis of ERα and ERβ expression was performed using Quantity One Software. β-actin protein was used as the internal control.

2.6. Immunohistochemical analysis

Immunohistochemical analysis was carried out on paraffin sections (5 μm) according to the SP-9001 Reagent Kit manual (Zhongshan Goldenbridge Biotechnology Co., Ltd). Paraffin sections were deparaffinized with xylene and then hydrated by descending concentrations of ethanol. Sections were incubated in PBS for