



Original Research Article

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The study on the expression of sulfotransferases2A1 in uterine Leiomyoma and its clinical significance

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Abstract

Aims: The objective of this study was to examine the expression of Sulfotransferases2A1 in Uterine Leiomyoma and to determine its Clinical Significance using Western Blot method.

Methods: A hospital based cross sectional study was conducted in Department of Obstetrics and Gynecology, Shengjing Hospital, China Medical University from December 2013 to June 2014. Patients included were of age 36 - 45 years, in proliferative phase of menstrual cycle, with no history of diabetes, hyperthyroidism, ovarian cancer, other comorbidities or hormonal therapy for last 3 months. Tissue samples were taken from fibroid tissue and extra capsular myometrium in 30 uterine leiomyomatous patients and from normal myometrium in 10 uterine prolapse cases. Western Blot assay was used to detect SULT2A1 expression in uterine leiomyoma tissue, uterine leiomyoma adjacent tissue and normal myometrial tissue.

Results: The expression of SULT2A1 was seen in 12 (40%) specimens of fibroid tissue, 17 (56.6%) specimens of extra capsular myometrium and 6 (60%) specimens of normal myometrium. Significant difference was seen between specimens of fibroid tissue and extra capsular myometrium (p-value <0.05), fibroid tissue and normal myometrium (p-value <0.05) but not between extra capsular myometrium and normal myometrium (p-value >0.05).

Conclusion: This study gives evidence of SULT2A1 expression in uterine fibroid, fibroid extra capsular tissue and normal myometrium. The higher level of SULT2A1 protein expression in the normal myometrium compared to extra capsular tissue or uterine fibroid tissue, points to its possible role in the tumor genesis. This provides a promising theoretical basis for advent of non-surgical treatment of uterine fibroid through specific protein expression targeting.

Keywords: Uterine fibroid; SULT2A1; Western Blot

Introduction

At present, uterine leiomyoma stands as a major health problem worldwide. It is the most common uterine tumor in women of reproductive age group. Approximately 70-80% of women of reproductive age are afflicted with this disease [1, 2]. More than 60% of the laparotomies done for pelvic diseases are attributed to uterine leiomyoma (ULMs) [2]. Besides, it accounts for various morbidities like abdominal pain, dysmenorrhea, menorrhagia, infertility [1, 3, 4]. In addition, it is the commonest indication for hysterectomy in most of the countries and necessitates treatment cost hiking over billions annually [1, 3, 5].

Despite the high prevalence rate and health disturbances, exact pathogenesis still remains to be elucidated. A number of literatures describe the risk factors associated to fibroid and its tumor genesis. Early menarche, nulliparity, increasing age, obesity, diet including red meat and ham, and certain race and geography favor its occurrence. However, menopause, smoking and exercise prevent its growth. The role of OCP and HRT still remain inconclusive [6]. It is evident that ULMs are highly dependent on ovarian steroids. Recent studies have shown increased expression of estrogen receptor (ER), aberrant micro RNA, HSD17B1 rs605059 gene polymorphism associated with ULMs [7, 8, 9, 10, 11]. Literature reports steroid sulfotransferase (SULT) as major enzyme catalyzing sulfation of steroids, drugs and xenobiotic. SULT2A1 is the major isoform that catalyzes sulfation of various steroids including DHEA to DHEAS, the precursors of both androgen and estrogen [12, 13,14]. Abnormal SULT1A1 and SULT1E1 gene expression have been indicated in leiomyoma and endometrial Cancer [15]. Also, estrogen sulfotransferase (EST) is known to be related to progression and metastasis of estrogen dependent breast cancer [16]. Although the enzymatic activity of SULT2A1 has been studied in some detail, little is known about its expression in any tissue. Therefore, this study is an attempt to explore SULT2A1 expression in uterine leiomyoma and to determine its clinical significance.

Materials and Methods

A cross sectional study was conducted in the Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University from December 2013 to June 2014.

Specimens were collected from 30 uterine leiomyoma patients after total or subtotal hysterectomy and 10 uterine prolapse patients (not with uterine fibroids, adenomyosis or endometrial hyperplasia) after total hysterectomy. The inclusion criteria were a) patient's age 36 - 45 years, b) proliferative phase of menstrual cycle, c) not under any hormonal therapy for last 3 months and d) with no history of diabetes, hyperthyroidism, ovarian cancer and other comorbidities. In uterine leiomyoma specimen, tissue sample was taken each 1cm deep from the fibroid tissue and extra capsular myometrium (adjacent to fibroid capsule). In uterine prolapse specimen, tissue sample was taken from normal myometrium. Western Blot assay was used to detect SULT2A1 expression in uterine leiomyoma tissue, uterine leiomyoma adjacent tissue and normal myometrial tissue.

Statistical analysis was done using SPSS 17.0 software. ANOVA comparative analysis and Student-Newman-Keuls (SNK) pair wise comparison were done to test the difference of expression of SULT2A1 between the different tissues. P-value <0.05 indicated that the analysis was statistically significant and P <0.01 indicating that it was very significant. The study was approved by the hospital ethical committee. Informed written consent was collected from the patients.

Experimental reagents and instruments used

1. SULT2A1 antibody of rabbit anti-human polyclonal from Beijing Biosynthesis Biotechnology Co., Ltd.
2. Secondary antibody of donkey anti-human polyclonal from British Abcam.
3. BCA protein assay kit from Beijing Cell Chip Biotechnology Co., Ltd.
4. PVDF membrane (0.45um) from America Millipore.

5. SDS-PAGE protein sample buffer (5x) from Shanghai Beyotime Biotechnology Co., Ltd.
6. Western Blot kit from Shanghai Beyotime Biotechnology Co., Ltd.
7. Western Blot with a protein marker from Lithuania Fermentas.
8. Constant temperature table: ZHWY-211C from Shanghai Yiheng technology.
9. Gel Doc 1000 gel imaging systems from the United States BIO-RAD company.
10. Electric heater water bath Model HH-SY11-Ni produced from Beijing Changfeng Instruments Corporation.

with an electric homogenizer, then centrifuged in a micro centrifuge (4 °C, 12000rpm,15min).The tubes were gently removed from the centrifuge. The protein containing supernatant was aspirated and put in fresh tube, separately and saved at (-80 °C).

(4) Protein quantization

(a) Working solution was prepared according to the standard solution and number of samples. BCA reagent A: reagent B=50:1 formulation, an appropriate amount of BCA working solution was made. BCA working solution was stable at room temperature within 24 hours.

(b) Dilution of standard solution with PBS buffer was done to give final concentration of 0.5mg/ml and standard curve was prepared for the fluid.

(c) 15 µl PBS buffer and 5 µl sample were added to the sample bottle and repeated three times.

(d) 200 µl BCA working solution was added to each well at 37 °C and left for 30 minutes.

(e) It was then cooled down to room temperature. Using Elisa A562, the protein concentration was determined according to the standard curve.

(5) After boiling the protein samples and the sample buffer mix (100 °C for 5 min), these were instantly then placed on ice and cooled to room temperature.

(B) Electrophoresis, Transfer of proteins and staining (Western Blotting)

(1) Preparation of PAGE Gel

The proteins were separated, according to their molecular weight, on polyacrylamide gel using standard technique SDS-polyacrylamide agarose gel electrophoresis (SDS-PAGE).

Experimental Method

Western Blot –detection of protein expression

(A) Sample preparation

(1) Selection of sample: The specimens were removed from freezer (-80 °C) and checked one by one for any damage. Then 0.1g sample was taken from each specimen, washed twice with cold PBS and put into fresh EP tube.

(2) Configuration of Cell lysate: RIPA Lysis buffer (750ml) was added to each EP tube and mixed well. PMSF was also added making final concentration 1mM. Then cell culture was centrifuged in a microcentrifuge (4 °C, 12000rpm, 15min).The tubes were gently removed from the centrifuge. The supernatant was aspirated and put in fresh tube, separately and saved at (-80 °C).

(3) Configuration of tissue lysate: Tissues were dissected in ultrasonic cell disruptor and placed in EP tubes. These were then immersed in liquid nitrogen, lysis buffer was added and homogenized

Table 1: Showing the Gel formulation

Gel	H2O (ml)	30% Acrylamid ((ml)	1.5M Tris (ml) (pH8.8)	10% SDS (ml)	10% APS (ml)	TEMED (ml)
10% Separating gel 5ml	1.3	1.7	1.9	0.05	0.05	0.002
5% Stacking gel 2ml	1.4	0.33	0.25 (pH6.8)	0.02	0.02	0.002

- (2) The samples were mixed with 4µl of pre-stained protein marker on the Marker track.
- (3) Electrophoresis was done by setting the voltage at 80V and adjusting the voltage to 120V until marker bands were seen separated into the separating gel. Electrophoresis was continued for about two hours.
- (4) After electrophoresis, the gel was removed and placed in transfer buffer. PVDF membrane and filter were cut to appropriate size depending on the gel size. PVDF membrane was pretreated by immersing it in methanol for 1min. The membrane was then put with filter paper into the transfer buffer.
- (5) The gel and membrane were sandwiched between filter paper and cotton pads (three layers of filter paper - gel - PVDF membrane - triple filter paper) and finally covered with cotton pad. Ensuring that there were no air bubbles between gel and membrane, all were finally clamped in electric transfer folder.
- (6) The clip was put into the transfer slot, to make the clip black-face to face slot black-face and clip white face to face slot red face. A piece of ice was placed at the side of the slot to cool when the transfer produced heat.
- (7) Blocking: 5% skimmed milk was configured with TBS-T as blocking buffer. The PVDF membrane was rinsed once with TBS-T for 5min. The film was incubated for 2 hours at 4⁰ C under agitation.
- (8) Primary antibody incubation: Post dilution, the antibody was incubated overnight at 4⁰ C with membrane in a hybridization bag, sealed air tight after removing air bubbles.
- (9) Secondary antibody incubation: The membrane was then rinsed in TBS-T thrice, 10 min per wash, to remove residual primary antibody. The appropriate secondary antibody

was selected and diluted in blocking buffer. It was incubated with membrane, at room temperature for 2 hours in a hybridization bag, sealed air tight after removing air bubbles.

(10) Development: After secondary antibody incubation, it was rinsed with TBS-T thrice, 10 min per wash. Then ECL + detection kit was used and film was developed in a darkroom. Lastly, photographs were taken to save the picture.

Results

In specimens from 30 uterine leiomyoma patients, the expression of SULT2A1 was seen in 12 (40%) specimens of fibroid tissue and 17 (56.6%) specimens of fibroid extracapsular myometrium. Whereas, in 10 uterine prolapse patients, the expression of SULT2A1 was seen in 6 (60%) specimens. (Table 2)

Significant difference was seen in expression of SULT2A1 between specimens of uterine fibroid tissue and fibroids extra capsular myometrium (p-value <0.05), uterine fibroid tissue and normal myometrium (p-value <0.05). However, there was no significant difference in expression of SULT2A1 between fibroids extra capsular myometrium and normal myometrium (p-value >0.05).

ANOVA comparative analysis of SULT2A1 protein content in the three types of tissues showed significant variance (P<0.001)

Student-Newman-Keuls (SNK) pair wise comparisons showed difference between each groups.

Table 2: SULT2A1 protein relative content ($\bar{x} \pm s$) analysis results

Specimen	Frequency	Percent	SULT2A1 (Mean±SD)
Uterine fibroids	12	40.00%	0.207±0.058
Extra capsular tissue	17	56.67%	0.332±0.108
Normal myometrium	6	60.00%	0.532±0.108

Figure 1: Pattern of expression of SULT2A1 in uterine leiomyoma, myoma extra capsular myometrium and normal myometrium tissue

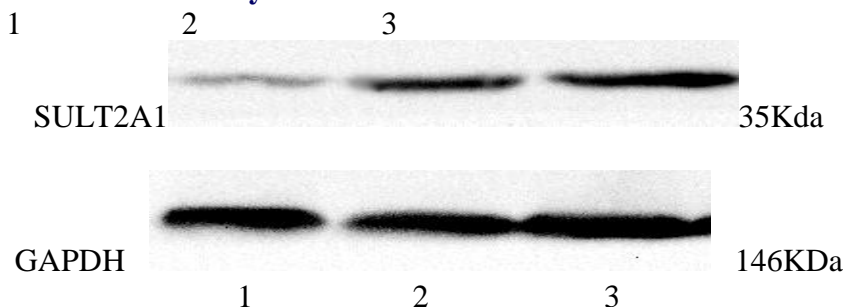


Figure 1: Western Blot method to detect the expression of SULT2A1.

- 1: SULT2A1 expression in uterine fibroids tissue
- 2: SULT2A1 expression in the extra capsular tissue of uterine fibroids
- 3: SULT2A1 expression in normal myometrium tissue

Discussion

Recent studies found that uterine fibroids, endometrial cancer and other gynecological tumors, abnormal expression of SULT1E1 and SULT1A1, and found SULT1E1 and SULT1A1 polymorphism associated with gynecologic cancer susceptibility.

SULT1A1 catalyze the sulfate conjugation of phenolic compounds including steroid hormones, catecholamines and phenolic drugs, and also participate in the bio activation of procarcinogens.

SULT1E1 strongest affinity of estrogen in all the SULTs [8], and it's the most closely relationship with physiological metabolism of estrogen [9]. As an important detoxification enzymes, catalytic estrogens (estrone and 17 - estradiol) and its metabolites (such as catechol estrogens) sulfate metabolism. It can form the inactive form of estrogen also is estrogen sulfate compound, estrogen sulfate.

Prof Wang Ming use chemiluminescent method for determination of content of E2 and progesterone and testosterone in uterine leiomyoma patients and healthy women's serum and tissue homogenates, and immunohistochemical staining SULT1E1 protein expression. Result show that [11,12]:In the uterine fibroid tissue,E2 were significantly higher

than in leiomyoma extra capsular tissue ,In the uterine fibroid tissue,SULT1E1 protein expression levels were significantly lower than in leiomyoma extra capsular tissue .Therefore infer the occurrence of uterine myoma was related to the decrease of SULT1E1 enzyme activity. Its mechanism may be:SULT1E1 catalyzed endogenous estrogen sulfating reaction, formation of estrogen inactive form - sulfating estrogen, this compound cannot be combined with estrogen receptor and lost activity, and it can reduce circulation and target tissue estrogen exposure levels.SULT1E1 lower expression in uterine fibroids organizations can lead to elevated levels of endogenous estrogen, estrogen has raised PR content effect, PR was positively related to the intensity of ER protein expression, higher content of ER alpha indirectly, promote the growth of uterine fibroids.

Hydroxysteroid sulfotransferase SULT2A1 catalyzes the sulfation of hydroxysteroids. It plays an important role in the detoxification of hydroxyl-containing xenobiotics and in the regulation of the biological activities of hydroxysteroids. ERR is an orphan member of the nuclear receptor superfamily that is closely related to estrogen receptor alpha (ER). Here we report that the mRNA expression of human SULT2A1 was suppressed by ERR in myometrium cells. To investigate the mechanisms of this regulation, the effects of ERR on human

SULT2A1 promoter transcription in myoma cells were investigated. Reporter luciferase assay results showed that ERR significantly represses human SULT2A1 promoter transcription in myoma cells. Deletion analysis indicated that human SULT2A1 promoter region between positions -188 and -130 is necessary for its repression by ERR in myoma cells. The 5' DNA -188 to -130 region of human SULT2A1 contains IR2 and DR4 hormone response elements and two putative ERR response elements (ERREs) (ERRE188: GCAAGCTCA and ERRE155: ATAAGTTCA). Our results suggest that ERR may play an important role in promoting the leiomyoma and in regulating endogenous hydroxysteroid activities via the regulation of SULT2A1. Sulfuric acid transferase enzyme members, SULT2A1 uterine fibroids, may play an important role in the development.

Human SULT2A1 has been mapped to chromosome 19q13.3 (Luu-The et al. 1995, Otterness et al. 1995).

SULT2A1 is located on chromosome 19:48373723bp-48389654bp, molecular weight: 35kDa. SULT2A1 in the liver, colon, kidney, testicle, lymph nodes, cerebral cortex and adrenal tissue were expressed, especially in colorectal cancer, liver cancer, pancreatic cancer, stomach cancer, bladder cancer has a high expression [17]. There are studies that RORs can activate the SULT2A1 promoter, and the expression of SULT2A1 is positively correlated with the expression of RORs, which indicates that it can regulate the expression of SULT2A1. It is also reported that vitamin D receptor (VDR) can stimulate the expression of endogenous SULT2A1. Estrogen receptor associated receptor ERR can down regulate the expression of SULT2A1.

In summary, SULT gene family associated with uterine fibroids, endometrial cancer and other gynecological tumor's development, the mechanism may be estrogen SULT abnormal, made topical estrogen synthesis and metabolism, increased target organ estrogen exposure. SULT2A1, as a member of the SULT2 family, whose main catalytic DHEA, pregnelone and dehydroepiandrosterone ketone, hydroxy

steroid sulfation, like SULT1E1, SULT1A1, SULT1A3, SULT2A1 also catalyze the metabolism of estrogen. SULT2A1 relationship between estrogen metabolism and suggest it occurs in uterine fibroids, may play an important role in development.

SULT2A1 protein expression in uterine leiomyoma and clinical significance.

SULT2A1 protein in uterine fibroids, fibroids extracapsular muscle tissue and normal myometrium were expressed. SULT2A1 protein relative content in uterine fibroids was 0.207 ± 0.058 , SULT2A1 protein relative content in fibroids extracapsular tissue was 0.332 ± 0.108 , SULT2A1 protein relative content in normal myometrium tissue was 0.532 ± 0.108 . SULT2A1 protein relative content comparative analysis of variance F value is 28.379, $P < 0.001$ in three different tissues, there is a significant difference. Student-Newman-Keuls (SNK) pairwise comparisons showed that, between any two groups are not the same numbers.

Conclusion

The existing high prevalence rate of leiomyoma, associated morbidities and the financial burden of hysterectomies necessitate that alternative therapies are developed. This target can be attained only with better understanding of leiomyoma at cellular level.

This study provides evidence of SULT2A1 protein expression in uterine fibroid, fibroid extra capsular tissue and normal myometrium. The expression level of SULT2A1 protein in the normal myometrium was significantly higher than in leiomyoma extra capsular tissue or uterine fibroid tissue, inferring that this protein may have a role in the pathogenesis and development of uterine fibroid. Its expression pattern shows that the mechanism may be inhibiting factor in the incidence of uterine fibroids. This finding provides promising theoretical basis for advent of non-surgical treatment of uterine fibroid through specific protein expression targeting.

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