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Comparison of estrogen receptor-α, progesterone receptor and calponin expression in gonadotrophin-releasing hormone agonist-sensitive and -resistant uterine fibroids

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Objective
This study was aimed to compare immunohistochemical expression of estrogen receptor (ER)-α, progesterone receptor (PR), and calponin in gonadotrophin-releasing hormone agonist (GnRH-a)-sensitive and -resistant uterine fibroids.

Methods
We collected data retrospectively. The sensitive group consisted of women who had reduction in uterine volume greater than 40% following GnRH-a treatment. Uterine volume was either reduced by less than 10%, or was increased in the resistant group. A tissue microarray was constructed using formalin-fixed, paraffin-embedded tissues, 31 and 26 patients for the sensitive and resistant groups, respectively. Tissue sections were immunostained with antibodies against ER-α, PR, and calponin. The intensity and area of the immunohistochemical reactions were evaluated using a semi-quantitative scoring system. The Mann-Whitney *U*-test, Fisher's exact test, and Spearman's rank correlation test were used for analysis of data.

Results
PR ($P=0.04$) and calponin ($P=0.03$) showed a significantly higher staining intensity in the resistant group than in the sensitive group. Both groups showed comparable expression of ER-α ($P=0.23$). In correlation analysis between changes in uterine volume after GnRH-a therapy and clinicopathological factors, the immunohistochemical intensity of PR ($P=0.04$) and calponin ($P=0.03$) was significantly correlated with changes in uterine volume.

Conclusion
This study shows that GnRH-a resistance of uterine fibroids is not related to ER-α content, but the expression of PR and calponin is related with GnRH-a resistance.

Keywords: Calponin; Estrogen receptor; Gonadotropin-releasing hormone agonist; Leiomyoma; Progesterone receptor

Introduction

Uterine fibroids are ovarian steroid-dependent tumors. The mechanism of the growth of uterine fibroids has yet to be fully ascertained. The growth and maintenance of uterine fibroids are mainly stimulated by estrogen and are affected by cyclic hormonal changes. In addition, the existence of estrogen receptors (ERs) and progesterone receptors (PRs) have been identified in fibroid tissue [1-3].

For this reason, gonadotrophin-releasing hormone agonists

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(GnRH-a) have been used in the treatment of uterine fibroids since the 1980s. Their main mechanism is to suppress the pituitary-ovarian axis to establish hypoestrogenism [4,5]. Several studies have reported that GnRH-a therapy reduces the size of uterine fibroids by ≤70% within a short time [6,7]. Calponin, an actin-binding protein in smooth muscle, was recently reported as a sensitive marker of smooth muscle differentiation in human neoplasms. This protein is used as a marker for myoepithelial and smooth muscle cell tumors [8].

Despite administration of GnRH-a therapy, sufficient size reduction cannot be obtained for some uterine fibroids, indicative of GnRH-a-resistant fibroids. The mechanism for this resistance is unknown.

Previous reports have described a correlation between uterine fibroids and ER, PR, and calponin. This study was aimed to compare the immunohistochemical expression of ER-α, PR, and calponin between GnRH-a-sensitive and -resistant uterine fibroids.

Materials and methods

1. Fibroid tissue specimens

Fifty-seven patients included in this study were from a subset of 159 patients who had undergone a total hysterectomy after GnRH-a (goserelin or leuprolide acetate) therapy because of uterine fibroids, between January 2000 and March 2009 at Kyungpook National University Hospital (KNUH).

We examined formalin fixed, paraffin-embedded tissue samples obtained from the 57 women. Exact measurement of the change in volume for each uterine fibroid proved difficult owing to variation in the number, size, and location of the fibroids. Therefore, measurement of the change in volume for each uterine fibroid was replaced with the change in total uterine volume before and after GnRH-a treatment.

Study participants were selected from patients who had ≥300 cm^3 uterine volume prior to GnRH-a therapy. In addition, participants had <10% difference between uterine volume measured by vaginal ultrasonography 1 day before the operation and postoperative pathological uterine volume. Patient chart reviews were performed retrospectively for patient characteristics and clinicopathological parameters.

The patients were divided into two groups based on changes in uterine volume after GnRH-a therapy. Uterine volume was measured before and after using a GnRH-a by vaginal ultrasonography. The volume was calculated using the prolate ellipsoid method and the formula V=0.5233 (D1×D2×D3), where D1, D2, and D3 are the longitudinal, transverse, and cross sectional diameters of the uterus, respectively.

Patients in the sensitive group had a ≥40% reduction in uterine volume, following GnRH-a therapy. The uterine volume of patients in the resistant group was either reduced by 10%, or was increased, following GnRH-a therapy. Postoperative uterine volume was calculated using our preparatory experiment for uterine density. Mean uterine density (mean ± standard deviation), which contained uterine fibroids, was 0.96 ± 0.15 g/cm^3. The KNUH does not require approval from the institutional review board for retrospective chart reviews. Therefore, this analysis was exempt from the approval process.

2. Construction of microarray

A microarray instrument (Tissue Microarray Set TMA01, MediKorea, Seoul, Korea) was used. Conventional hematoxylin and eosin (H&E) slides were reviewed and representative areas without necrosis or hemorrhage selected. Paraffin cores, 2 mm in diameter, were taken from the tissue blocks at sites corresponding to the selected areas on the H&E slides and placed in empty blocks in the kit. The cores were punched at 1 mm intervals, and arranged six by 10. Each block contained thyroid and endometrial tissue as negative and positive controls, respectively. A grid system with each core having a coordinate reference (X axis, Y axis) was used to allow for cross-referencing between the core location and parent case. Once the microarrays were complete, all blocks were incubated at 60°C for 30 minutes. The slides were dewaxed and rehydrated prior to antigen retrieval.

3. Immunohistochemical staining and interpretation

Immunohistochemical staining was performed using an automatic stainer (BenchMark, Ventana Medical Systems Inc., Tucson, AZ, USA). Tissue sections 4 μm in thickness were mounted on positively charged slides. Antibodies against calponin (EP798Y, 1:100; Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA), ER-α (SP1, 1:200; NeoMarkers Inc., Fremont, CA, USA), and PR (SP2, 1:400, NeoMarkers Inc.) were used as the primary antibodies. An iView DAB Detection kit (Ventana Medical Systems Inc.) was used as the secondary antibody, and hematoxylin (Ventana Medical Systems Inc.) was used for counter staining. Calponin was interpreted

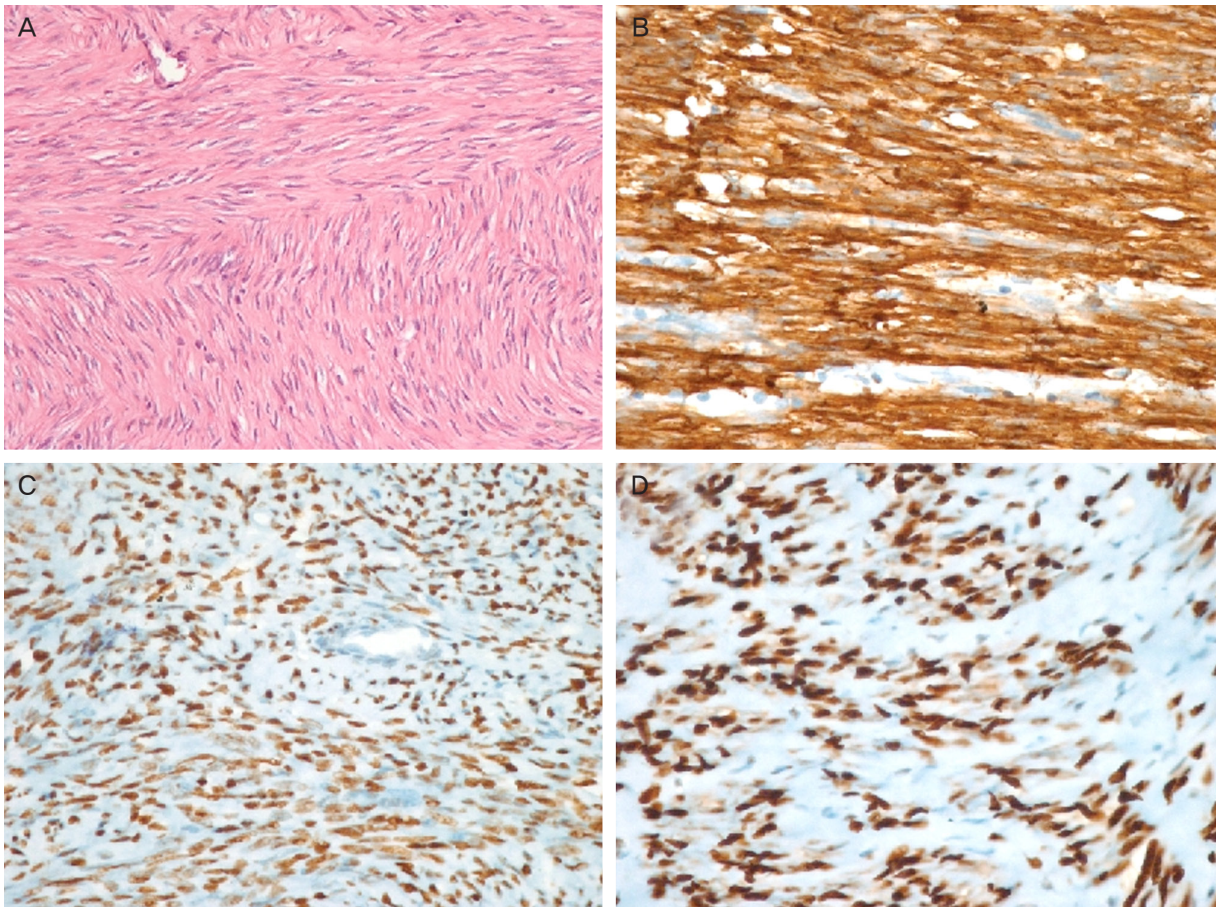

Fig. 1. (A) Uterine fibroid composed of interwoven bundles of densely packed smooth muscle (hematoxylin and eosin [H&E], ×200). (B) Expression of calponin in the cytoplasm can be seen in the majority of smooth muscle cells (immunohistochemistry [IHC], ×400). (C) Expression of estrogen receptor-α within the nucleus can be seen most smooth muscle cells (brown spots; IHC, ×400). (D) Expression of progesterone receptor within the nucleus can be seen most smooth muscle cells (brown spots; IHC, ×400).

as positive when tumor cells showed cytoplasmic staining. ER-α and PR were interpreted as positive when tumor cells showed nuclear staining. Immunohistochemical results were scored using a semi-quantitative scoring system [9]. This system assesses the percentage of positive cells (none, 0; <1%, 1; 1%–10%, 2; 11%–33%, 3; 34%–67%, 4; and >67%, 5) and the intensity of staining (none, 0; weak, 1; intermediate, 2; and strong, 3). The intensity and percentage scores are added to give a final score of 0 to 8. The slides were reviewed by two pathologists (Fig. 1).

4. Statistical analysis

Statistical analyses were performed using the Mann-Whitney *U*-test and Fisher's exact test when comparing differences between two groups. Spearman's rank correlation test was used to assess correlations between the expression of ER-α, PR, and calponin and other clinicopathological characteristics. A $P<0.05$ was considered significant.

Results

All data are expressed as the mean±standard deviation. The mean age at the time of surgery was 45.9±4.2 years for the sensitive group and 45.9±3.7 years for the resistant group ($P=0.10$). Body mass index was 24.7±3.2 and 25.9±3.0 kg/m^2 ($P=0.18$) in the sensitive and resistant groups, respectively. The obstetric history was not different between the groups. GnRH-a drugs used by the two groups were goserelin and leuprolide acetate. Within the sensitive group, 16 patients were treated with goserelin and 15 patients with leuprolide acetate. In the resistant group, goserelin was used by 11 pa-

tients and leuprolide acetate by 15 ($P=0.67$). The number of injections of GnRH-a administered to the study participants was between three and six (3.8±0.8 in the sensitive group and 3.9±0.9 in the resistant group [$P=0.78$]).

The interval between administration of the final GnRH-a injection and hysterectomy was 2.0±1.7 months in the sensitive group and 2.0±1.1months in the resistant group, respectively ($P=0.83$). Prior to injection with GnRH-a, uterine volume was 559.5±217.0 cm^3 in the sensitive group and 493.7±188.0 cm^3 in the resistant group ($P=0.07$) .

After injection, the uterine volume was significantly lower (262.1±113.7 cm^3) in the sensitive group than in the resistant group (494.3±143.8 cm^3) ($P<0.01$). With regard to changes in uterine volume before and after administration of GnRH-a, a decrease of 46.7±7.5% was observed in the sensitive group and an increase of 106.6±29.5% was observed in the resistant group. In the current study, the mean uterine volume measured by ultrasonography for 159 patients after GnRH-a injection was decreased to 73.1±26.0% compared with uterine volume prior to its administration. Of the 159 cases analyzed, a decrease in uterine volume was observed for 138 (86.7%) cases, no change was observed in four (2.6%), and 17 (10.7%) showed an increase in uterine volume despite GnRH-a treatment. Across both groups, all samples had >70% cells positive for the three antibodies tested (percentage score, 5), but there were different immunohistochemical staining intensities. The intensity of ER-α was not different between the sensitive and resistant groups (1.4±0.6 vs. 1.6±0.7, $P=0.23$). However, the intensity of PR (1.8±0.8 vs. 2.6±0.6, $P=0.04$) and calponin (2.6±0.7 vs. 3.0±0.0 $P=0.03$) was significantly lower in the sensitive group than in the resistant group.

Table 1. Patients' characteristics and immunohisctochemical intensity of calponin, ER-α, and PR

	Sensitive group (n= 31)	Resistant group (n= 26)	*P*-value
Age (yr)	45.9±4.2	45.9±3.7	0.10[a]
Body mass index (kg/m^2)	24.7±3.2	25.9±3.0	0.18[a]
Obstetric history			
Gravidity	3.7±1.5	3.8±2.1	0.70[a]
Parity	2.1±0.7	2.0±0.8	0.78[a]
Abortion	1.5±1.8	1.6±2.1	0.94[a]
GnRH agonist type			0.67[b]
Goserelin	16	11	
Leuprolide acetate	15	15	
No. of injection	3.8±0.8	3.9±0.9	0.78[a]
Uterine volume before GnRH-a (cm^3)	559.5±217.0	493.7±188.0	0.07[a]
Uterine volume after GnRH-a (cm^3)	262.1±113.7	494.3±143.8	<0.01[a]
Volume change after GnRH-a (%)	46.7±7.5	106.6±29.5	<0.01[a]
Immunohisctochemical intensity			
ER-α	1.4±0.6	1.6±0.7	0.23[a]
PR	1.8±0.8	2.6±0.6	0.04[a]
Calponin	2.6±0.7	3.0±0.0	0.03[a]
Hysterectomy			0.45[b]
Vaginal	18 (58)	10 (38)	
Laparoscopy	8 (26)	7 (27)	
Laparotomy	5 (16)	9 (35)	
Interval between GnRH-a injection and hysterectomy	2.0±1.7	2.0±1.1	0.83[a]

Data are expressed as the mean±standard deviation or n (%).
GnRH, gonadotrophin-releasing hormone; ER, estrogen receptor; PR, progesterone receptor.
[a]Mann-Whitney *U*-test; [b]Fisher's exact test.

In the sensitive group, vaginal hysterectomy was performed in 18 (58%) patients, laparoscopic-assisted hysterectomy in eight (26%) patients, and laparotomic hysterectomy in five (16%) patients. In the resistant group, vaginal hysterectomy was performed in 10 (38%) patients, laparoscopic-assisted hysterectomy in seven (27%) patients, and laparotomic hysterectomy in nine (35%) patients. No significant difference was observed for the type of surgery between the groups ($P=0.45$) (Table 1).

We examined correlations between changes in uterine volume and clinicopathological factors (age, body mass index, doses of GnRH-a administered, calponin, ER-α and PR expression, and GnRH-a type). Only the immunohistochemical staining intensity of PR ($P=0.04$) and calponin ($P=0.03$) showed a significant correlation with a change in uterine volume. All other clinicopathological factors failed to show any significant correlations.

Discussion

According to systematic reviews of the efficacy of preoperative GnRH-a injections in women with uterine fibroids, a larger uterus results in a greater reduction in volume. However, no trial stratifying the effects of GnRH-a on fibroid volumes has been reported [10].

Determination of the cutoff value for the allocation of cases to one of two groups (sensitive or resistant) was based on the following parameters. If the reduction in uterine volume was <10%, this reduction was within the error of measurement between preoperative and postoperative uterine volumes. Therefore, we considered this reduction to be indicative of GnRH-a resistance. We consider that hysterectomy by the vaginal approach is feasible and safe when the uterine volume is under 350 cm^3 [11]. Based on these experiences, the GnRH-a-sensitive group was determined as having a greater than 40% reduction in uterine volume.

There are no reports regarding unchanged or increased uterine volumes in response to GnRH-a treatment. Likewise, there are no data on GnRH-a-resistant fibroids. Nakayama et al. [12] suggested that GnRH-a resistance was due to the presence of mast cells and insulin-like growth factor-1 in uterine fibroids. Horiuchi et al. [13] reported the effects of heparin secretion from mast cells located in uterine smooth muscle in myometrium and fibroids. They showed an inhibitory effect of heparin on the proliferation of myometrial and leiomyoma smooth muscle cells. Cirkel et al. [14] analyzed ER and PR expression in enucleated uterine fibroids after luteinizing hormone-releasing agonist therapy by immunohistochemistry. They reported that the extent of fibroid regression showed a close association with ER content in uterine fibroids; however, it was independent of PR.

In our study, the immunohistochemical intensity of ER-α was slightly higher in the resistant group than in the sensitive group, but this was not significant. Therefore, the ER-α content in fibroids was not strongly associated with GnRH-a resistance.

In the study conducted by Cirkel et al. [14], only the largest enucleated fibroids were selected for determination of volume change. In contrast, the current study investigated the uterus, which was enlarged mainly with fibroids. Cirkel et al. reported that under physiological conditions fibroid ER concentrations exceeded those of the myometrium. This fact can offset the correlation between ER-α and uterine volume changes observed in the current study, because the measurement of volume change included measurements for both fibroids and myometrium.

In the current study, all 57 tissue samples were immunostained with ER-α and PR. However, in Cirkel et al. [14]'s study, six cases were positive for PR and one was negative for ER. A considerable discrepancy in immunostaining results exists between the two studies.

Although not statistically significant, a positive relationship was observed between ER-α expression and a change in uterine volume in the current study. Considering the results of the current study and those of previous studies, we cannot exclude a correlation between these two parameters.

Many studies have indicated that progesterone is an important factor for uterine fibroid growth. Reinsch et al. [15] reported that the effect of antiprogestogen RU 486 on reducing uterine volume and blood flow is equal to that of leuprolide acetate. Harrison-Woolrych and Robinson [16] reported a rapid increase in fibroid size in menopausal women who had taken high doses of progesterone, and subsequent decreases in fibroid size when the medications were stopped. In our study, correlation analysis showed relationship between a change in uterine volume following GnRH-a therapy and immunohistochemical staining intensity of PR. Significant differences were also observed between the two groups. Previous studies have reported rapid decrease in PR content following

GnRH-a therapy. In the current study, uterine volume was marginally decreased in the group with a limited reduction in immunohistochemical staining intensity of PR, there is likely to be a relationship between GnRH-a resistance and PR.

In our study, the expression of calponin was 100% for all cases analyzed. Twenty-six of 31 cases in the sensitive group showed strong immunohistochemical staining intensities. But, all samples in the resistant group showed strong immunohistochemical staining intensities. Statistical significance was observed in the two groups.

Regarding correlations between the immunohistochemical staining intensities for calponin and a change in uterine volume, there was a significant relationship. In the sensitive group, the immunohistochemical intensity score for calponin was 1 for five patients, and 3 for the remainder. The intensity score was 3 for all patients in the resistant group.

Calponin is a sensitive marker of smooth muscle differentiation in human neoplasm and shows expression of 89.3% and 100% in canine and human fibroid, respectively [17]. The higher expression of calponin in the resistant group might be explained as a result of active smooth muscle differentiation or proliferation which might surpass the inhibitory effects of GnRH-a. However, Horiuchi et al. [13] reported that heparin inhibits proliferation of myometrial and leiomyomal smooth muscle cells through induction of alpha-smooth muscle actin, calponin h1, and p27. The results of the current study are not in agreement with these findings. According to Horiuchi et al. [13]'s study, calponin expression have increased in the sensitive group, and decreased in the resistant group. The mechanism of how calponin affects uterine fibroids should be investigated in the future. There are limitations to this study. The current study was a retrospective study. In addition, measurement of a change in volume for each uterine fibroid was replaced with the measurement of a change in total uterine volume before and after GnRH-a treatment. These problems are critical limitations in this study.

In conclusion, this study shows that GnRH-a resistance of uterine fibroids is not related to ER-α content. But, the expression of PR and calponin is related with GnRH-a resistance.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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