Original article

Decreased expression of human heat shock protein 70 in the endometria and pathological lesions of women with adenomyosis and uterine myoma after GnRH agonist therapy

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Short Title: HSP70 expression in reproductive diseases

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Condensation

A variable amount of tissue stress reaction occurred in adenomyosis and uterine myoma that can be effectively suppressed after treatment with GnRH agonist.

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Abstract

Objective : A prominent stress reaction in the pelvis of women with endometriosis and the role of human heat shock protein 70 (HSP70) in inflammation and the growth of endometriosis has been recently demonstrated. We report here expression of HSP70 in tissues derived from GnRH agonist (GnRHa)-untreated and -treated women with adenomyosis and uterine myoma.

Study Design: This is a case-controlled biological study. Biopsy specimens were collected from pathological lesions and eutopic endometria/autologous myometria of 30 women with adenomyosis, 35 women with uterine myoma and 15 control women during laparoscopy, laparotomy and hysteroscopy. Fourteen women with adenomyosis and 20 women with uterine myoma were treated with GnRHa for a variable period of

3-6 months before surgery. The immunoexpressions of HSP70 and CD68-positive M ϕ in endometria, lesions/myometria were examined by immunohistochemistry. The immunoreactivity of HSP70 in tissues was analyzed by quantitative-histogram (Q-H) scores.

Results: Comparing to control women, HSP70 immunoexpression was significantly higher in endometria/myometria and pathological lesions of women with adenomyosis and myoma. A significant positive correlation between Q-H scores of HSP70 and CD68-positive M ϕ numbers was found in the endometria derived from women with adenomyosis (r=0.388). Treatment with GnRHa significantly decreased Q-H scores of HSP70 in pathological lesions and endometria/myometria of women with adenomyosis and uterine myoma comparing to similar tissues derived from GnRHa-untreated women.

Conclusion: A variable amount of tissue stress reaction occurred in endometria and pathological lesions of women adenomyosis and uterine myoma that can be effectively suppressed after GnRHa treatment.

Keywords: adenomyosis, uterine myoma, HSP70, inflammation, GnRHa

Introduction

Human heat-shock proteins (HSPs) function as molecular chaperons in general and are reported to be produced by macrophages (M ϕ), vascular endothelial cells, smooth muscle cells, endometrial cells and other dendritic cells [1,2]. Molecular chaperons are critical for the folding and regulation of a wide array of cellular proteins (3). Heat shock proteins are the most representative group of chaperons. As a marker of tissue stress reaction, endogenous HSPs including HSP70 may be released under a wide variety of stressful stimuli such as heat shock, viral or bacterial infections, internal physical stress, chemical stress and pelvic inflammation [4,5]. Endometrial tissue breakdown during menstruation and neurogenic or painful stimuli may also elicit a stressful reaction to the tissues.

Most of the published reports on HSPs described their expression in either endometrium or in endometriosis. A prominent stress reaction in the pelvis of women with endometriosis and the role of HSP70 in Toll-like receptor (TLR) 4-mediated inflammation and growth of endometriosis has been recently reported from our laboratory [6]. Persistent and changing inflammatory reaction in pelvic environment and

growth/invasion of cells may induce variable stress reaction in tissues derived from women with endometriosis. An additive effect between HSP70 and inflammation has been observed in a recent study [7]. Despite enough information on endometriosis, the detailed analysis of HSP70 immunoreactivity in adenomyosis or in uterine myoma is limited.

The hyperplastic and/or hypertrophic changes of smooth muscle cells in response to the invaginating glands/stroma into the myometrium of patients with adenomyosis may exert stressful insult to the adenomytic lesions or to the surrounding myometrium. A similar pattern of tissue stress reaction may occur in the endometrium or myometrium in response to the growing myoma nodules. A differential inflammatory reaction in submucosal, intramural and subserosal myomas has been reported [8]. However, the tissue stress reaction and its association with tissue inflammatory reaction in women with adenomyosis and uterine myoma are yet to be investigated.

In addition to pituitary suppression, we recently demonstrated that GnRHa has multiple biological functions in peripheral tissues of women with different reproductive diseases [9]. Since, GnRH receptors were expressed by both eutopic endometria and

pathological lesions of women with endometriosis, adenomyosis, and uterine myoma [9], we believe that multiple biological functions could be the direct tissue effect of GnRHa or indirect effect of GnRHa-mediated estrogen suppression. In fact, GnRHa was able to significantly suppress tissue inflammatory reaction, angiogenesis, cell proliferation and induced apoptosis in women with endometriosis, adenomyosis and uterine myoma [9]. In a separate study, suppression of HSP70 expression in response to GnRHa and in women with endometriosis has been recently reported [10]. However, the role of GnRHa in the tissue expression of HSP70 in women with adenomyosis and uterine myoma is unknown.

Therefore, in the first part of our study, we aim to examine the tissue expression of human HSP70 in pathological lesions and endometria derived from GnRHa-untreated women with adenomyosis and uterine myomas. We speculated that infiltrating immune cells and micro-vessels within tissues might also suffer a variable amount of stress reaction. Therefore, secondly, we examined the co-localization of HSP70 and CD68 (M ϕ marker) and von Willebrand factor (VWF, vessel marker) in the serial section of tissues derived from these women. Finally, we compared the pattern

of changes in the immunoreaction of HSP70 in pathological lesions and autologous endometria/myometria between GnRHa-treated and -untreated women with adenomyosis and uterine myoma.

Materials and Methods

Subjects. This is a case-controlled biological research using prospectively collected biopsy samples of women with and without adenomyosis/uterine myoma and their retrospective evaluation. The subjects in this study were women of reproductive age (age range 23-51yrs). From July 2009 to April 2012, biopsy specimens were collected from a total of 30 women with adenomyosis and 35 women with uterine myomas who underwent laparoscopy, laparotomy or hysteroscopy. All these fertile women were admitted to our hospital with the complaint of pelvic pain, dysmenorrhea, abnormal genital bleeding, hypermenorrhoea or anemia. The control group consisted of 15 fertile women between 23 and 37 years of age without any evidence of peritoneal or ovarian endometriosis and operated on for dermoid cysts by laparoscopy. We selected patients with dermoid cysts as control group with the assumption that tissues of these patients may suffer less stress reaction (but not zero) than in tissues derived from other

reproductive diseases as demonstrated recently [6,11]. The diagnosis of adenomyosis and uterine fibroids in all these women was done by clinical manifestations, by ultrasonograghapy or magnetic resonance image before operation and subsequently confirmed by surgery and histopathology. The distribution of women who had GnRHa treatment for a period of 3-6 months is as follows: adenomyosis (n=14) and uterine myoma (n=20). Groups of women without GnRHa treatment in this study did not receive oral contraceptives or any other hormonal medication within 12 months prior to surgery. The phases of the menstrual cycle in women without hormonal therapy was determined by histological dating of eutopic endometrial samples taken simultaneously with pathological lesions derived from these women.

Collection of biopsy specimens**.** Tissue samples from endometria of control women were collected at the time of laparoscopy. No hysterectomy was performed for control women. The visual absence of adenomyosis/uterine myoma in control women was confirmed by image. Biopsy specimens of adenomyotic lesions and autologous myometria from either anterior or posterior wall and corresponding endometria were obtained from 26 women with adenomyosis who underwent hysterectomy. Collection of

all samples of myometria in women with adenomyosis refers to myometria away from an adenomyotic lesion. We collected samples of only pathological lesions and endometria from four GnRHa-untreated women with adenomyosis who had conservative surgery with the purpose of future pregnancy. Biopsies were taken from nodules, endometria, and myometria of women with uterine myoma during hysteroscopic resection (n=5), laparoscopic myomectomy (n=10) or after hysterectomy (n=20). We collected samples of autologous myometrium from 20 women with myoma who underwent hysterectomy, 8 in GnRHa-untreated group and 12 in GnRHa-treated group. A total of three to four biopsy specimens from different anatomical locations (anterior wall, posterior wall, fundus) of the eutopic endometrium were also studied for women with adenomyosis and myoma who underwent hysterectomy. All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study. The details of physical collection of tissue biopsies were reported elsewhere [12-15].

All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval of the Nagasaki University Institutional

Review Board. A written informed consent was obtained from all women.

Antibodies used. We performed immunohistochemical analysis to investigate the immunoreaction of HSP70, CD68 and VWF using respective antibodies against target antigen as follows: (1) HSP70 (marker of stress reactive protein, 1:500), rabbit polyclonal, Dako, Denmark; (2) CD68 [marker of macrophage (M ϕ), 1:50], KP1, mouse monoclonal, Dako, Denmark; (3) von Willebrand factor (VWF, micro-vessel marker, 1:25), F8/86, M0616, mouse monoclonal, Dako, Denmark; (4) non-immune mouse immunoglobulin (Ig) G1 antibody (1:50, Dako) was used as a negative control.

Immunohistochemistry. Details of immunohistochemical procedures are described elsewhere [13,16]. Briefly, 5 μm thick paraffin embedded tissues were deparaffinized in xylene and rehydrated in phosphate buffered saline (PBS). After immersion in 0.3% H_2O_2/ methanol to block endogenous peroxidase activity, sections were pre-incubated with 10% normal goat serum to prevent non-specific binding and then incubated overnight at room temperature with respective antibody. The slides were subsequently incubated with biotinylated second antibody for 10 min, followed by incubation with avidin-peroxidase for 10 min and visualized with diaminobenzidine.

Finally, the tissue sections were counterstained with Mayers hematoxylin, dehydrated with serial alcohols, cleared in xylene and mounted.

All immunoreactive cells and tissues were examined in five different fields of one section (x200 or x400) by light microscopy. M ϕ numbers as immunoreactive to CD68 and total micro-vessel numbers as immunoreactive to VWF were counted. Total micro-vessel density of those areas that contained the highest number of capillaries and venules was analyzed. The intensity of HSP70-stained cells and numbers of CD68-stained M ϕ per field in each biopsy specimen were examined and confirmed by a second observer (MK).

Quantification of immunostained cells by Q-H score. The immunoreactive intensity of HSP70-stained gland cells, stromal cells and smooth muscle cells was quantified by a modified method of quantitative histogram scoring (Q-H score) as described previously [16-18]. The Q-H score was calculated using the following equation: Q-H score = $\Sigma pi (I + 1)$, where I = 1, 2 or 3 and pi is the percentage of stained cells for each intensity. The staining intensity was graded as 0 = no staining, 1 = weak, 2 = moderate, and 3 = strong. We calculated the mean Q-H scores of five different fields

of one section by light microscopy at moderate magnification (×200). The authors were blinded to the study groups during quantification.

As a main outcome measures, we investigated the pattern of changes in the tissue of expression of HSP70 in respective biopsy samples derived from GnRHa-treated and -untreated women with adenomyosis and uterine myoma. We also evaluated a panel of confounding variables such as, age, parity, phases of the menstrual cycle, severity of symptoms, presence of adenomyotic lesion/myoma nodule, and GnRHa treatment that might be involved as a potential source of tissue stress reaction and in the tissue expression of HSP70.

***Statistical Analysis*.** The clinical characteristics of the subjects were evaluated by one-way analysis of variance. All results are expressed as either mean ± SD or median and inter-quartile range (IQR). The differences between study group and control group, GnRHa-treated and -untreated group were compared using Mann-Whitney U-test or Student's t test. For comparison among groups, the Kruskal-Wallis test was used. Pearson's correlation coefficient was used to evaluate the relationship between two groups. Multiple analysis of covariance (ANCOVA) was performed to evaluate possible

confounding variables associated with tissue expression of HSP70. A p value <0.05 was considered statistically significant.

Results

The clinical profiles of control women, GnRHa-treated and -untreated women with adenomyosis and uterine myoma are shown in Table 1. The women with adenomyosis and uterine myoma were significantly older than control women ($p<0.01$ for each). No age difference was observed between women with adenomyosis and uterine myoma or between GnRHa-treated and -untreated women (Table 1). We did not find any influence of age difference in the tissue expression of HSP70. There was no difference in the number of women presenting with pain, anemia, reproductive desire or body mass index among study groups (data not shown). The distribution in the phases of menstrual cycle in GnRHa-untreated women and type of uterine myomas are shown in Table 1.

Immunoexpression of HSP70 in adenomyosis and uterine myoma. A moderate to strong imunoexpression of HSP70 was found in the control endometrium, adenomyotic lesions, myoma nodules and corresponding endometria or myometria

(Figure 1, A). HSP70 expression was found in both gland cells/stromal cells and smooth muscle cells derived from endometria, adenomyotic lesions/myoma nodules and myometria of women with adenomyosis and uterine myoma. Although we could not collect biopsy samples during the menstrual phases among these three groups of women, we found an apparent but insignificant increase in the HSP70 immunoexpression in the endometria collected in the secretory phase than in the proliferative phase of the menstrual cycle (data not shown). There was no obvious difference in HSP70 expression in different anatomical locations of endometria (data not shown). There was no difference in the immunoexpression of HSP70 between eutopic and ectopic endometria derived from women with adenomyosis.

Q-H scores of HSP70-immunoreactivity in adenomyosis and uterine myoma.

Since there was no significant difference in HSP70 expression between gland cells and stromal cells, we represented combined data of Q-H scores of gland/stromal cells in the eutopic endometria or pathological lesions derived from control women and women with adenomyosis/uterine myoma (Figure 1, B). We found that Q-H scores of HSP70 immunoreaction were significantly higher in the eutopic endometria/adenomyotic

lesions, eutopic endometria/myoma nodules and autologous myometria derived from women with adenomyosis and uterine myoma when compared with that of control endometria ($p<0.05$ for each of endometria, lesions and myometria) (Figure 1, B). There was no obvious difference in the Q-H scores of HSP70 immunoreaction between adenomyotic lesions and myoma nodules or between surrounding myometria derived from women with adenomyosis and uterine myoma (Figure 1, B).

Co-localization of HSP70 with CD68-positive Mφ and VWF-positive micro-vessels. To identify co-localization of HSP70, CD68-stained Mφ, and VWF-stained micro-vessels, we extended our experiment using serial sections of each specimen derived from eutopic endometria and myometria of women with adenomyosis and uterine myoma. We found that HSP70 immunostaining was co-localized with VWF-stained micro-vessels and CD68-stained infiltrating Mφ in the parallel section of biopsy specimens derived from the eutopic endometria (Figure 2, A, B, C) and autologous myometria (Figure 2, D, E, F) derived from women with adenomyosis. We also observed a similar pattern of co-localization in the immunostaining of HSP70, VWF and CD68 in the endometria and myometria derived from control women and

women with uterine myoma (data not shown).

***Correlation between Q-H scores of HSP70 and CD68-positive** Mφ.* We examined the possible association between tissue stress reaction and the amount of infiltrated Mφ in tissues. We found a significant positive correlation between Q-H scores of HSP70-immunoreactive cells and CD68-positive Mϕ numbers which was observed in the endometria derived from women with adenomyosis (r=0.388, p=0.0382) (Figure 3, B) but not in the endometria of control women (r=0.134) or in women with uterine myoma (r=0.082) (Figure 3, A, C). A similar pattern of positive correlation was observed between Q-H scores of HSP70 and VWF-positive micro-vessels or between Q-H scores of HSP70 and CD68-positive Mϕ in the endometria/myometria, derived from women with adenomyosis but not in control women or women with uterine myoma (data not shown).

Immunoexpression of HSP70 in tissues derived from GnRHa-treated and -untreated women with adenomyosis and uterine myoma. We found a moderate to strong immunoexpression of HSP70 in the endometria, myometria and adenomyotic lesions/myoma nodules derived from GnRHa-untreated women (Figure 4, A and B,

upper columns) comparing to similar samples derived from GnRHa-treated women (Figure 4, A and B, lower columns).

Q-H scores of HSP70-immunoreactivity in tissues derived from GnRHa-treated and -untreated women with adenomyosis and uterine myoma. The Q-H scores of HSP70 immunoreactivity were found to be significantly decreased in the endometria, pathological lesions (adenomyotic lesions and myoma nodules) and autologous myometria derived from GnRHa-treated women (hatched bar) with adenomyosis and uterine myoma (Figure 4, C, D) when compared with similar tissues derived from GnRHa-untreated women (white bar) (Figure 4, C and D).

The changes in the immunoreactivity of HSP70 expression in endometria, pathological lesions and myometria derived from GnRHa-treated and -untreated women with adenomyosis and uterine myoma are summarized in Table 2. Multiple ANCOVA with possible confounding variables as shown in Table 3 revealed that presence of adenomyotic lesions and myoma nodules were significantly and independently associated with the increased tissue expression of HSP70. Age was not an independent risk factor in increasing tissue expression of HSP70 in these two groups of women. This

difference in the tissue expression of HSP70 disappeared after GnRHa treatment (Table 3).

Discussion

We demonstrated a variable degree of tissue stress reaction in the pathological lesions and eutopic endometria or autologous myometria derived from women with adenomyosis and uterine myoma. This was demonstrated by the over-expression of HSP70 in pathological lesions and eutopic endometria or myometria derived from these two groups of women. Similar to our previous in vitro study in women with endometriosis [6,7], here we further confirmed a significant association between stress reaction and inflammation and between stress reaction and angiogenesis in intact tissues derived from women adenomyosis. However, this association was not observed in control women and in women with uterine myoma. Our co-localization study confirmed that infiltrating M ϕ and micro-vessels in tissues derived from these women also suffer from a variable amount of stress reaction.

Besides inflammation, endometriosis, adenomyosis and uterine myoma may induce a variable degree of physical stress (cell to cell contact, cell proliferation, cell

differentiation or tissue invasion) or chemical stress (receptor-ligand interaction) in pelvic environment or within endometrium or myometrium. This constant inflammatory, physical or chemical insult at the tissue level may be involved in the higher expression of HSP70 in the eutopic endometrium as well as in different pathological lesions derived from women with adenomyosis and uterine myoma. Our current findings coincide with the previously published reports [1,6,19]. We previous reported decreased levels of cell proliferation, inflammation and angiogenesis in tissues derived from control women than in women with endometriosis [14,16]. A decreasing order of tissue inflammation, angiogenesis, and prostaglandin production was observed in submucosal myoma, intramural myoma, and in subserosal myoma [8]. Here, we did not separately analyze the pattern of HSP70 expression in each of these three types of uterine myoma due to small sample size. This could explain the reason why we did not find any positive association between HSP70 expression and CD68-positive Mϕ or between HSP70 and VWF-positive micro-vessels in tissues derived from women with uterine myoma. Further studies using individual types of uterine myoma may clarify our findings.

Several reports have shown that heat shock proteins vary in accordance with the menstrual cycle. Tabibzadeh et al., [20] reported that constitutive form of HSP70 in human endometrium increased progressively during the late proliferative and early secretory phases, and diminished in the mid- to late secretory phases. Koshiyama et al., [21] found a markedly increased expression of HSP70 in human endometrium during the secretory phase. In our recent study in women with endometriosis [10], we found the highest expression of HSP70 in the menstrual phase, intermediate expression in the secretory phase and lower expression in the proliferative phase. In our present study, we did not find any significant difference in the tissue expression of HSP70 between the secretory phase and the proliferative phase of any of the control or study groups. This could be due to the difference in sample number or in our Q-H scoring system.

Increased HSP70 protein expression in the endometria and pathological lesions of women with adenomyosis and uterine myoma carries some biological significance: (1) As a molecular chaperon, HSP70 inhibits apoptosis of host cells by preventing recruitment of caspases to the apoptosome complex [22]. Therefore, increased expression of HSP70 may contribute to increased survival of

endometrioid/smooth muscle cells thereby facilitating the growth of adenomoytic lesions or myoma lesions. While adenomyosis originates from direct invagination of gland cells of basalis endometrium into myometrium, myoma lesions originate from monoclonal expansion of a single smooth muscle cell in myometrium. Both of these lesions are associated with hyperplastic/hypertrophic change of surrounding smooth muscle cells. As a reactive tissue insult, the resulting hyperplastic or hypertrophic change of the surrounding myometrium could be an additional trigger to the over-expression of HSP70. (2) Persistent endogenous stimulation in endometria/myometria or in the pelvis with inflammation or HSP70 may change the cell membrane permeability causing efflux of different cytokines out of immune cells or may cause shifting of resting cells (S0) to proliferative phenotype (S2) in cell cycle. This may also result in increasing cytokine levels by M ϕ or increase in cell proliferation as we reported recently [7].

An interesting finding in our current study is that treatment with GnRHa was able to significantly suppress tissue expression of HSP70 in the pathological lesions, endometria and autologous myometria when compared with similar tissues derived

from GnRHa-untreated women with adenomyosis and uterine myoma. ANCOVA analysis further indicated that while presence of adenomyotic lesions and myoma nodules independently predicted over-expression of HSP70, this tissue stress insult was not observed after GnRHa treatment. Aside from central action and multiple biological functions of GnRHa in peripheral tissues [9], our results provided an additional effect of GnRHa on the suppression of tissue stress reaction in these reproductive diseases. GnRHa may decrease tissue inflammation secondary to decreased levels of tissue stress reaction either directly or indirectly by causing estrogen suppression. In fact, HSP70/estrogen-induced inflammatory and pro-inflammatory response has been recently demonstrated [6,7,23]. This may further explain the therapeutic benefit of GnRHa in alleviating pain manifestation or in improving reproductive outcome in women with adenomyosis and uterine myoma.

There are some limitations in our current findings: (1) we did not analyze the pattern of HSP70 expression separately in focal/diffuse adenomyosis or in different types of uterine myoma. (2) Due to lack of samples during the menstrual phase, we could not examine menstrual phase-dependent changes in HSP70 expression, (3) we did

not measure tissue concentrations of soluble HSP70 and its link with tissue expression of HSP70 or with other inflammatory mediators. Further research is warranted to address these issues.

Finally we conclude that besides endometriosis, women with adenomyosis and uterine myoma may also suffer from different *in vivo* tissue stress reaction. This stress insult was not confined to pathological lesions and corresponding endometria/myometria, but also to infiltrated immune cells and micro-vessels. Besides multiple biological functions in peripheral tissues, GnRHa treatment may have an additional beneficial effect on the suppression of tissue stress reaction. We propose that besides hormonal factors and inflammation, an in-depth investigation on the role of a variety of stress-reactive proteins including HSP70 in different reproductive diseases is necessary to support our current findings.

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Figure legends

Figure 1. (A) Immunohistochemical staining of human heat shock protein 70 (HSP70) in the control endometrium (**a**), and endometrium, adenomyotic lesion, and autologous myometrium of woman with adenomyosis (**c, e, g**, repsecitvely) and endometrium, myoma nodule, and autologous myometrium of woman with uterine myoma (**d, f, h**, respectively). HSP70 expression was observed in gland cells, stromal cells and in smooth muscle cells. Negative controls (non-immune mouse IgG stain) are shown against each endometria of control women (**b**), and women with adenomyosis (**i**) and uterine myoma (**j**).

(B) Shows combined data of quantitative-histogram (Q-H) scores of HSP70 immunoreactive gland/stromal cells derived from eutopic endometria/adenomyotic lesions (white box/hatched box) and in smooth muscle cells of nodule/myometria (hatched box) derived from women with adenomyosis and uterine myoma. Q-H scores of HSP70 immunoexpression in eutopic endometria/lesions/nodule/myometria derived from women with adenomyosis and uterine myoma were significantly higher when compared with eutopic endometria of control women (*$p<0.05$ for each). Boxes represent the distance (interquartile range) between the first (25%) and third (75%)

quartiles, and horizontal lines in the boxes represent median values. Magnification of each slide (x200).

Figure 2. Co-localization of human heat shock protein 70 (HSP70)-immunostained cells (**A, D**), von Willebrand factor (VWF)-stained micro-vessels (**B, E**), and CD68-stained macrophages (**C, F**) at the same locations (arrows/arrow heads) in the serial sections of eutopic endometria (**A, B, C**, upper panel) and myometria (**D, E, F**, lower panel) derived from woman with adenomyosis (x400).

Figure 3. The correlation between quantitative-histogram (Q-H) scores of human heat shock protein 70 (HSP70)-immunostained cells and CD68-immunoreactive mean macrophage (M ϕ) numbers per field in the eutopic endometria derived from control women (**A**), in the eutopic endometria of women with adenomyosis (**B**), and in the eutopic endometria of women with uterine myoma (**C**). A significant positive correlation was found between Q-H scores of HSP70 expression and tissue infiltration of M ϕ in women with adenomyosis (r=0.388, p=0.0382, **B**) but not in control women (**A**) or in women with uterine myoma (**C**) .

Figure 4. The immunohistochemical staining of human heat shock protein 70

(HSP70) in the endometria, pathological lesions, and autologous myometria derived from GnRHa-untreated [GnRHa (-), upper columns] and GnRHa-treated [GnRHa (+), lower columns] women with adenomyosis (**A**) and uterine myoma (**B**).

The quantitative-histogram (Q-H) scores of HSP70 immunoreactive cells in endometria, pathological lesions and autologous myometria derived from GnRHa-treated (hatched box) and -untreated (white box) women with adenomyosis (**C**) and uterine myoma (**D**). We found significantly lower Q-H scores of HSP70 expression in respective samples derived from GnRHa-treated women than in GnRHa-untreated women with adenomyosis and uterine myoma. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. Magnification of each slide (**A**, **B)** (x200).

Table 1. Clinical profiles of women with adenomyosis, uterine myoma, and control group.

	GnRHa (-)	GnRHa (+)
Adenomyosis (n=30)	16	14
age in yrs (mean ± SD)	41.6 ± 4.2	42.8 ± 2.5
menstrual cycle: P/S/M/A	4/12/0/0	0/0/0/14
duration of therapy (month)		3-6
hysterectomy done (n=26)	12	14
Uterine myoma (n=35)	15	20
age in yrs (mean ± SD)	40.6 ± 5.9	37.2 ± 5.3
size in cm (mean ± SD)	4.8 ± 2.3	5.8 ± 3.3
menstrual cycle: P/S/M/A	5/10/0/0	0/0/0/20
duration of therapy (month)		3-6
type of myoma: SMM/IMM/SSM	4/9/2	4/12/4
hysterectomy done (n=20)	8	12
Control group (n=15)	15	
age in yrs (mean ± SD)	28.6 ± 4.1	
menstrual cycle: P/S/M/A	4/11/0/0	

The results are expressed as mean ± SD. GnRHa (-), without GnRH agonist therapy; GnRHa (+), with GnRH agonist therapy; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; SMM, submucosal myoma, IMM, intramural myoma, SSM, subserosal myoma.

Table 2. Differences in Q-H scores of HSP70 expression in pathological lesions, endometria and myometria that were collected from GnRH agonist (GnRHa)-treated and -non-treated women with adenomyosis and uterine myma.

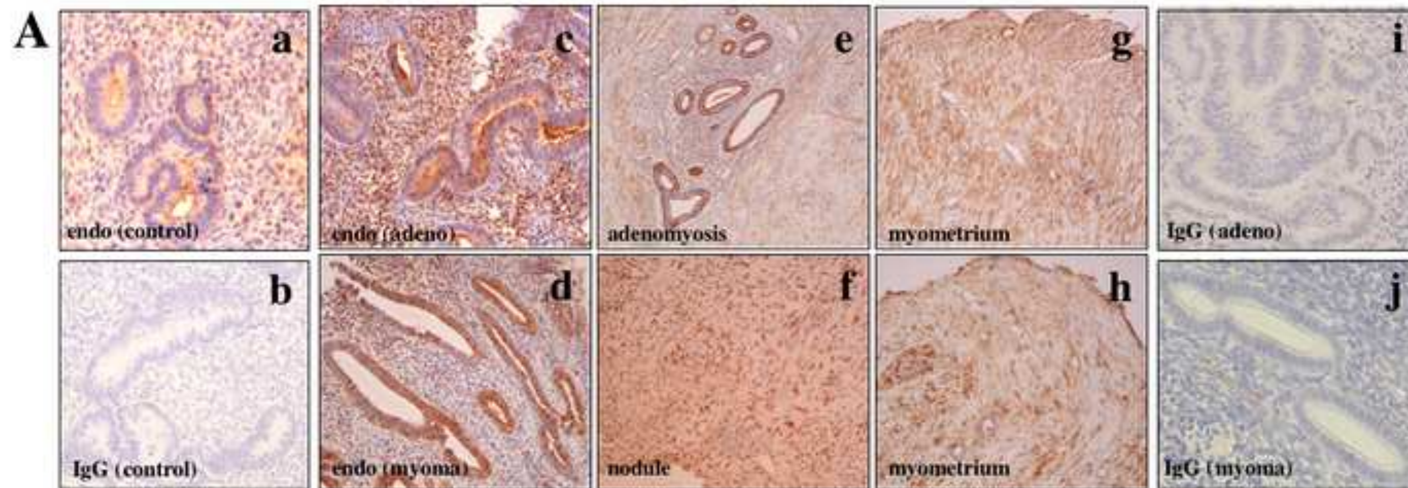
	GnRHa (-)	GnRHa (+)	P value
Adenomyosis (n=30)	16	14	
endometrium	69.1 ± 7.1	38.6 ± 5.5	0.0059
adenomyotic lesion	62.3 ± 6.1	30.1 ± 2.4	0.0364
myometrium	55.6 ± 5.7	26.6 ± 2.5	0.0031
Uterine myoma (n=35)	15	20	
endometrium	63.5 ± 7.2	31.4 ± 4.2	0.0052
myoma nodule	54.8 ± 5.1	25.9 ± 2.7	0.0246
myometrium	64.1 ± 7.9	36.3 ± 3.2	0.0075

The results are expressed as mean ± SEM. GnRHa (-), without GnRH agonist treatment; GnRHa (+), with GnRH agonist treatment; HSP70, human heat shock protein 70; Q-H scores, quantitative-histogram scores.

Table 3. Multivariate analysis of covariance to evaluate the contributors to tissue expression of heat shock protein 70.

Confounding variables	F value	P value
Age (yrs)	1.52	0.47
Parity	0.49	0.92
Menstrual phase	2.26	0.39
Severity of symptoms	4.12	0.09
Presence of adenomyotic lesion	6.11	0.02
Presence of myoma nodule	5.45	0.03
GnRH agonist treatment	2.61	0.52

Figure 1



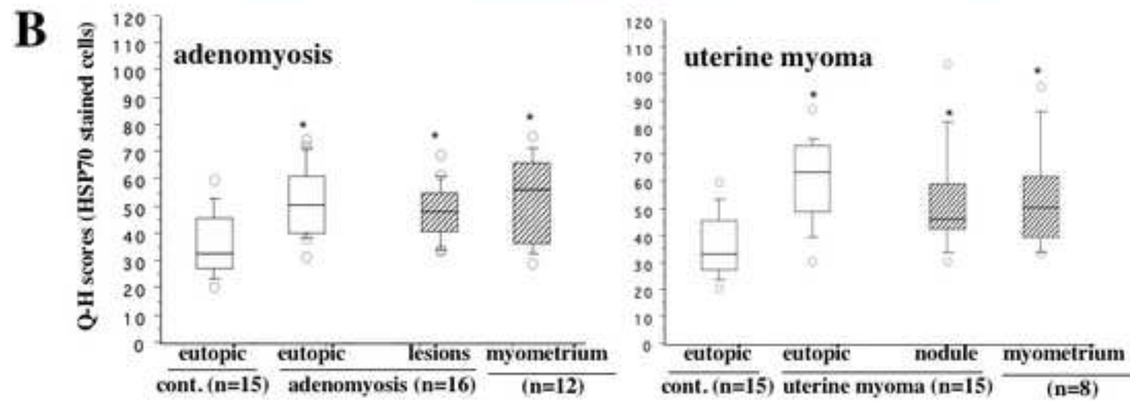


Figure 2

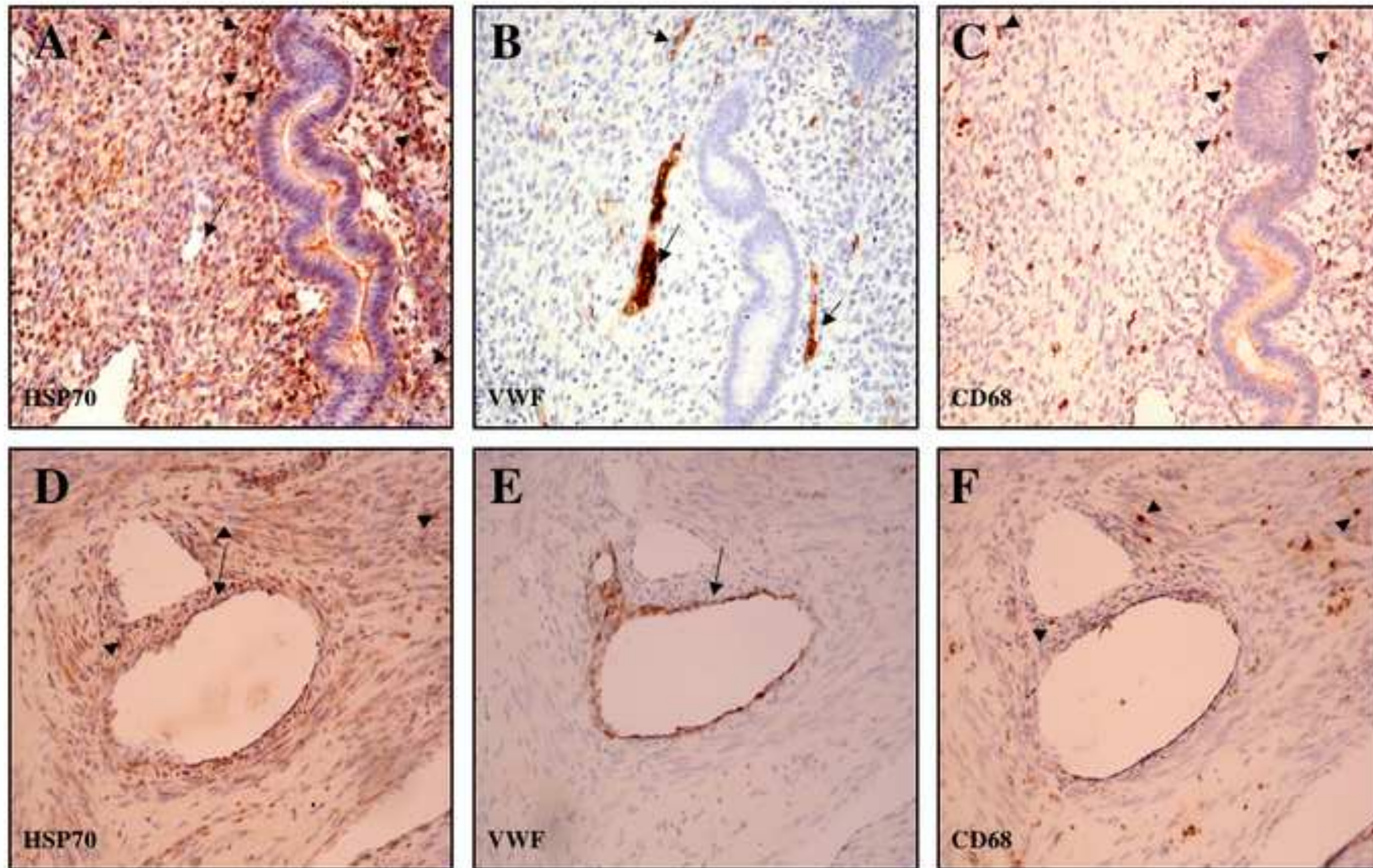


Figure 3

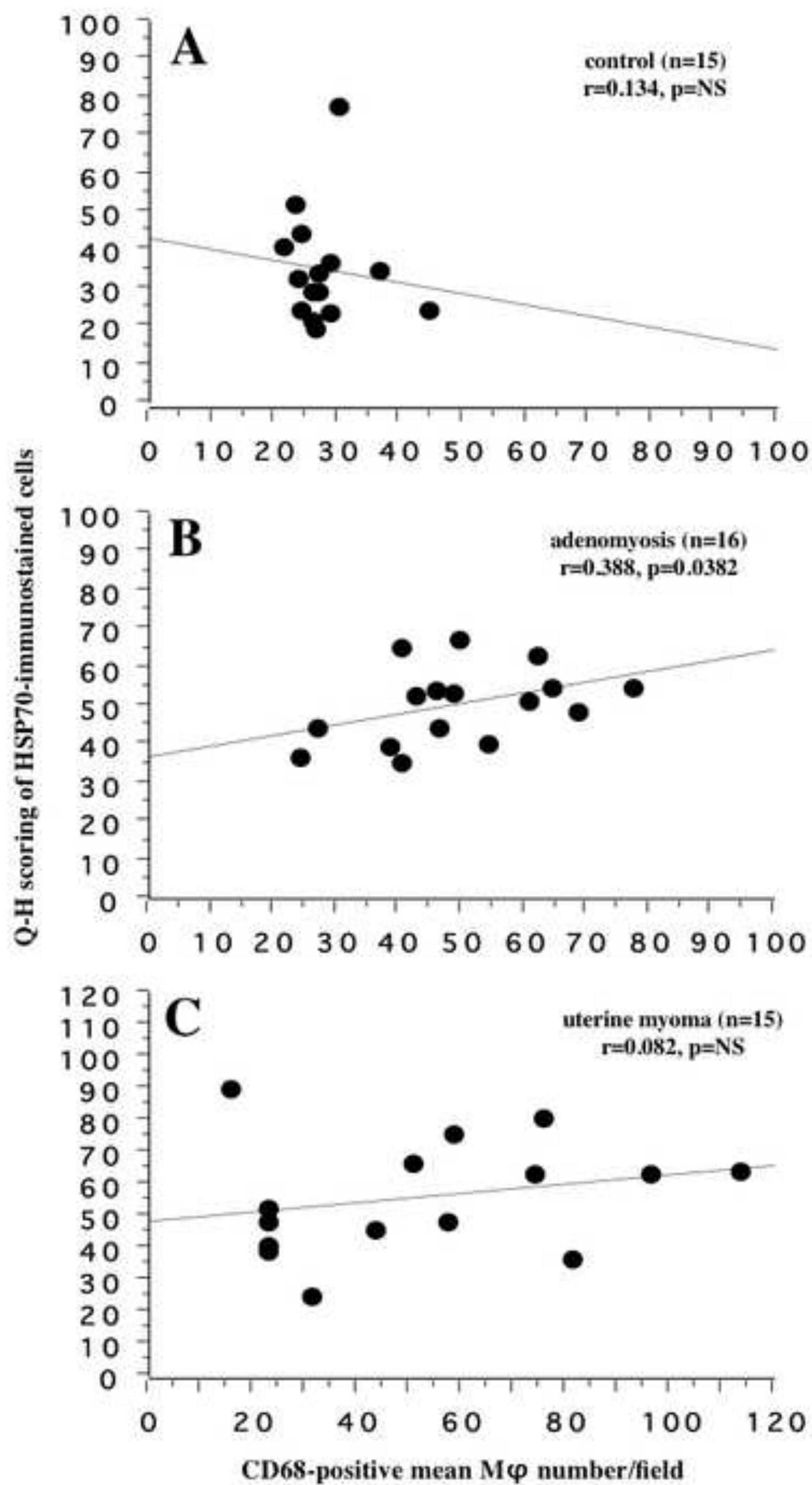


Figure 4