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Expression of Exon 5 Deleted Estrogen Receptor Variant Messenger RNA in Human Uterine Myometrium and Leiomyoma

Kazuko Mito, Takaya Tamura*, Kenichi Hosokawa, Tokumasa Kondo, Takara Yamamoto and Hideo Honjo

Department of Obstetrics and Gynecology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-0841, Japan

To examine the relationship between uterine leiomyoma, an estrogen-dependent tumor and its estrogen receptor, the relative amounts of wild type estrogen receptor (WT) mRNA and exon 5 deleted estrogen receptor variant (D5-ER) mRNA to G3PDH mRNA were examined in human uterine myometrium and leiomyoma specimens obtained from 46 patients in 3 age groups (group A: 41–45 years old, group B: 46–50 years old, group C: 51–54 years old) using a quantitative reverse transcription polymerase chain reaction method (RT-PCR). D5-ER mRNA was co-expressed with WT mRNA in all myometrium and leiomyoma specimens. In myometrium, the relative amount of WT decreased with aging, but in leiomyoma, it was high in group B. The relative amount of D5-ER mRNA and the ratio of D5-ER mRNA to WT mRNA (D5/WT ratio) were significantly higher in group C in both myometrium and leiomyoma. The percentage of the patients whose D5/WT ratio was higher in leiomyoma than in myometrium (L/M ratio>1.0) increased with age. These findings suggest that D5-ER increases to supplement the decreasine in WT in uterine tissues toward menopause and that D5-ER plays a more active role in leiomyoma than in myometrium during the perimenopausal period. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Uterine leiomyoma is a common pelvic tumor occurring in one in four women during their reproductive years, and may cause dysmenorrhea, hypermenorrhea, and occasionally infertility. The tumor shrinks after menopause and is regarded to be estrogen-dependent. However, the growth of the tumor is not continuous during the reproductive age when the menstrual cycle is regular and circulating estradiol (E2) levels are normal. Moreover, some of them do not respond to gonadotropin-releasing hormone agonist (GnRH-a) therapy [1]. The genesis and the growth promoting factors of leiomyoma remain unclear including the role of estrogen and estrogen receptors (ER).

Many studies have been made on the estrogenic differences between uterine myometrium and leiomyoma. A higher tissue concentration of unconjugated E2 and aromatase activity [2] and lower E2 dehydrogenase activity in leiomyoma [3] and differences in steroid binding affinity [4] have been reported. Brandon *et al.* [5] and Sadan *et al.* [6] reported a higher concentration of ER in uterine leiomyoma than in myometrium at the mRNA and protein levels. Chrapusta *et al.* [7] showed the presence of more estrogen-binding ER protein in leiomyoma than in their parental myometrium. However, according to Vollenhoven *et al.* [8], the amount of cytosolic ER protein in leiomyoma was twice that in myometrium but that of ER mRNA was similar in both. They also reported that the ER binding capacity was independent of ER mRNA abundance both in myometrium and leiomyoma [8]. These lines of evidence suggest the presence of various kinds of ER with different functions in the uterine myometrium and leiomyoma at the mRNA and protein levels. In fact, in 1986, Kornyei *et al.* found two types of nuclear estrogen receptors by binding affinity analysis [9] and, recently, various kinds of estrogen receptors have

*Correspondence to T. Tamura. Tel. 251 5562; Fax: 212 1265.
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been identified both at the mRNA level and protein level [10, 11, 46].

Exon 5 deleted ER variant (D5-ER) mRNA was first identified by Fuqua *et al.* [12] in paradoxically ER-/progesterone receptor (PR) + breast cancer. It has ligand-independent *trans*-activating activity in a yeast-expression system, but its functions and roles *in vivo* have not been clarified yet. It is expressed widely in estrogen target organs and tissues [12–14] and has been speculated to be related with diseases in those tissues [15–19].

In this study, we measured the relative amounts of D5-ER mRNA and WT mRNA to G3PDH mRNA in myometrium and leiomyoma by the quantitative RT-PCR to examine the age-related changes in D5-ER in normal myometrium and leiomyoma.

MATERIALS AND METHODS

Materials

The tissue specimens were obtained with consent from 46 patients between 41 and 54 years old who had uterine leiomyoma and underwent a hysterectomy at our university hospital. The specimens were classified into three groups according to the patient's age (group A: 41–45 years old, group B: 46–50 years old, group C: 51–54 years old). All the patients in group A had a regular menstrual cycle, but 29 and 80% of the patients in group B and C, respectively, had irregular bleeding or prolonged menstrual period. None of the patients had received any hormonal therapy within 3 months before the operation. The external part of the largest leiomyoma and normal myometrium which was not adjacent to the leiomyoma were excised, cut into 100 mg pieces and then rapidly frozen in liquid N_2.

RNA extraction and RT/PCR

Frozen tumor samples were pulverized and total RNA was prepared using TRIzol Reagent (Life Technologies, NY), as recommended by the manufacturer. 1 μg of total RNA was denatured at 65°C for 3 min and reverse transcribed in the presence of 0.5 mM dNTP mixture, 10 mM dithiothreitol, 1 unit/μl RNase inhibitor, 2.5 μmol random primers, RT buffer (50 mM Tris–HCl; pH 8.3, 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$) and 10 units/μl avian myeloblastosis virus reverse transcriptase (Life Science, FL, U.S.A.) in a final volume of 20 μl for 10 min at 30°C and 60 min at 42°C followed by 5 min at 99°C.

Primer pairs used to coamplify both WT and D5-ER by PCR were the 4/6U primer [5′-GCTTCGATGATGGCTTACTG-3′(sense), located in exon 4] and the 4/6L primer [5′-T GCGGAACCGAGATGATGTA -3′ (antisense), located in exon 6] corresponding to the human ER cDNA sequence [20]. WT and D5-ER mRNA were detected as 292 base pairs (bp) and 153 bp PCR products, respectively. Human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as an internal standard was also amplified as 950 bp PCR products using the primer G3U primer (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, sense) and G3L primer (5′-CATGTGGGCCATGAGGTCCACCAC-3′, antisense) according to the published sequence [21].

PCR was performed with 1 μl of RT product, 2.5 mM dNTP mixture, PCR buffer [10 mM Tris–HCl; pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) autoclaved gelatin], 0.2 μM of each primer and 0.625 unit Taq polymerase (TaKaRa, Shiga, Japan) in a total volume of 25 μl. Each PCR consisted of 30 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) followed by 10 min at 72°C using a programmable thermal controller (The Minicycler; MJ Research, MA). Each PCR product was electrophoresed on a 1.8% agarose gel, stained with ethidium-bromide and visualized with a transilluminator (Dual-Intensity Transilluminator, UVP, Upland, CA) and photographed (T667, ISO3000; Polaroid, Hertfordshire, U.K.).

Cloning and sequence analysis for the preparation of standard templates

Following PCR and electrophoresis, the WT, D5-ER and G3PDH DNAs were collected from the bands on agarose gel using a GENECLEAN II Kit (BIO 101, CA) and subjected to direct sequence analysis using a Takara Taq Cycle Sequencing Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol and each objective gene was confirmed to have been amplified correctly. Each extracted DNA was also subjected to cloning using the Original TA Cloning Kit (Invitrogen, CA) according to the manufacturer's instructions. The insertions were characterized by restriction fragment length analysis with EcoRI and sequence analysis using a Sequencing PRO Kit (TOYOBO, Osaka, Japan), sequencing primers for SP6 and T7 promoter (Invitrogen, CA) which were in the plasmids and ^{35}S dATP (NEN, MA). The sequence ladder was visualized by autoradiography with a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan).

Quantitative analysis of mRNAs

The concentrations of each cloned plasmid DNA (WT, D5-ER and G3PDH) dissolved in distilled water were measured by spectrophotometry (GeneQuant RNA/DNA Calculator; Pharmacia Biotech, Cambridge, U.K.). Solutions of DNA at known concentrations (10^1–10^7 copies/μl) were prepared as standard templates for the quantitative PCR. These standard templates and RT products were amplified simultaneously using the same mastermix

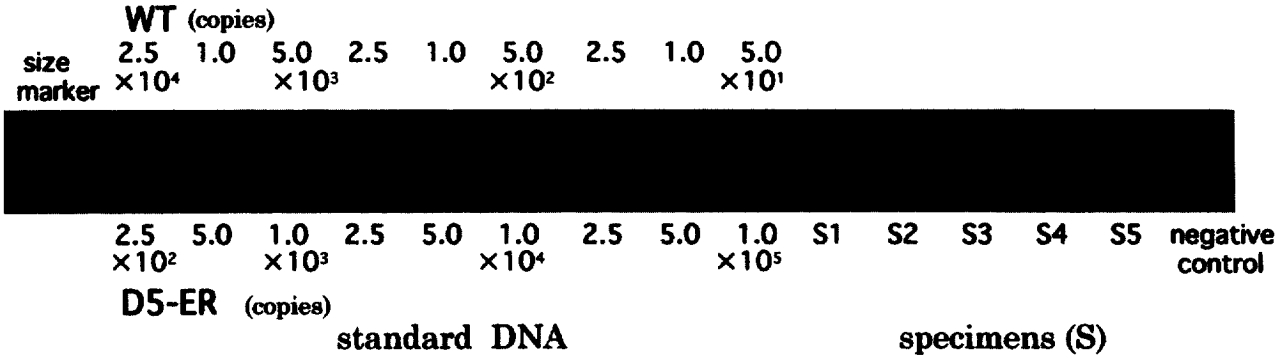

Fig. 1. Electrophoresis of small gradient standard template DNA mixture (WT and D5-ER). The species were amplified by PCR simultaneously (30 cycles). All species (S1–S5; 1 μl of cDNA solution) expressed both WT and D5-ER mRNAs and the intensities were within this standard range

solution and electrophoresed on agarose gel. The bands were photographed (T667, ISO3000; Polaroid, Hertfordshire, U.K.) and the integrated density was analyzed using an NIH image 1.52 (On-Line Software; NIH, MD). A standard sigmoid curve was drawn and the copy number in 1 μl of the RT product was roughly calculated. For exact quantitation, the RT product was amplified again with smaller gradient standard DNAs (Fig. 1). The amount of G3PDH (copies/1 μl of RT product) was measured similarly.

The relative amounts of WT and D5-ER mRNA versus G3PDH were calculated revising each WT and D5-ER (copies/1 μl of RT product) with corresponding G3PDH (copies/1 μl of RT product). Statistical analyses were conducted using an unpaired *T*-test between myometrium and leiomyoma in the same age groups (Figs 2, 3, 4 and 5) and a one-way ANOVA (post hoc test; Fisher's protected least significant difference) between the different age groups (Figs 3–5).

RESULTS

With 30 cycles of PCR amplification, two bands consistent with 292 bp (WT) and 153 bp (D5-ER) were detected in all specimens. The intensities of the bands varied with the specimen (Fig. 1) and in some specimen, it was stronger in the lower band than in the upper band.

By direct sequence analysis of PCR product, the 292 bp band was identical to human ER cDNA, while the 153 bp band lacked exon 5 completely and exon 4 was bound by exon 6 directly.

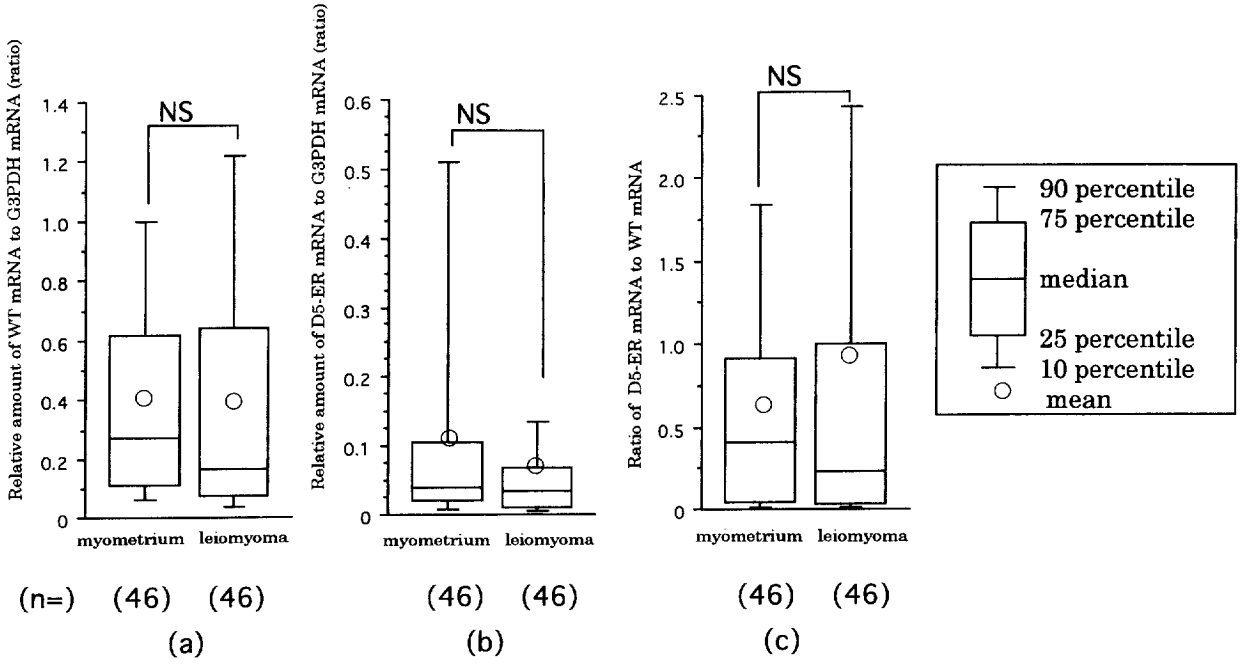

Fig. 2. Relative amounts of WT mRNA (a) and D5-ER (b) mRNA to G3PDH mRNA measured by a quantitative RT-PCR and D5/WT mRNA ratio (c). Data are expressed in percentile (median, 25–75 percentile of box, 10–90 percentile of bar) to show distribution. ○; mean, NS; not significant

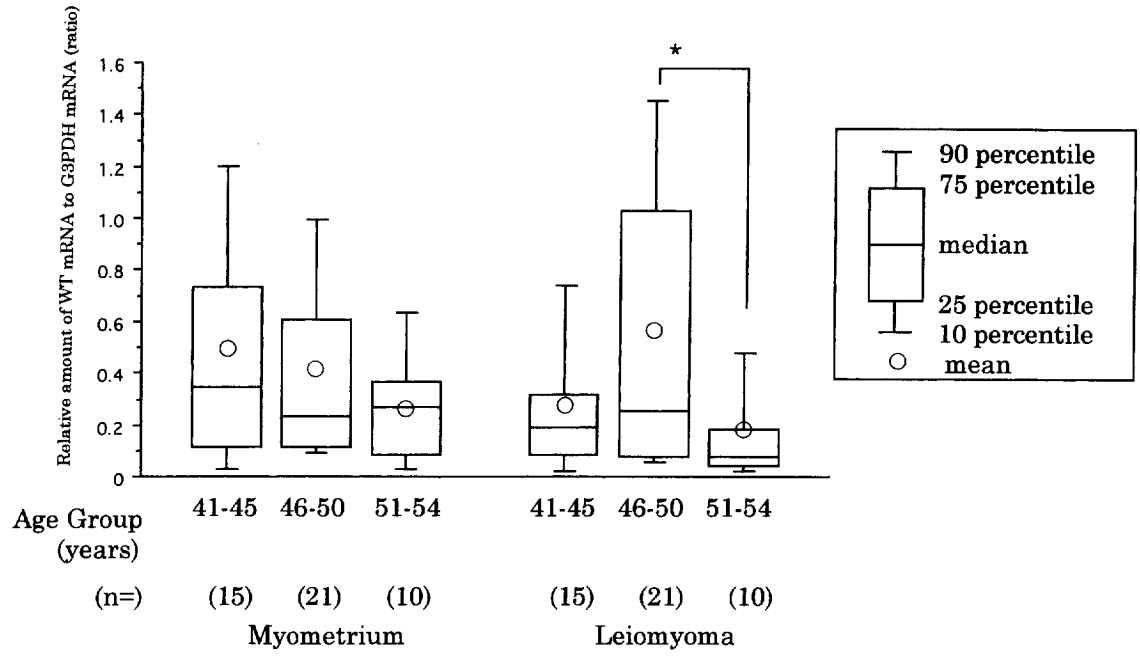

Fig. 3. Relative amount of WT mRNA to G3PDH mRNA measured by a quantitative RT-PCR. Group A: 41–45 years old ($n = 15$), group B: 46–50 years old ($n = 21$), group C: 51–54 years old ($n = 10$). *$p < 0.05$; one-way ANOVA (post hoc test: Fisher's PLSD)

The relative amounts of WT and D5-ER and the ratio of D5-ER mRNA to WT mRNA (D5/WT ratio) at any age are shown in Fig. 2(a), (b) and (c). The mean amount of D5-ER was 29% of that of WT in myometrium and 18% of that in leiomyoma [Fig. 2(a) and (b)]. The mean D5/WT ratio was 0.68 in myometrium and 0.88 in leiomyoma, and the median D5/WT ratio was 0.40 in myometrium and 0.23 in leiomyoma, respectively. About 20% of the samples had a ratio exceeding 1.0 both in myometrium and leiomyoma [Fig. 2(c)].

Figures 3 and 4 show the relative amounts of WT mRNA and D5-ER mRNA, respectively, and Fig. 5 shows the D5/WT ratio according to age group.

In myometrium, the expression of WT mRNA tended to decrease with age, although not significantly, but in leiomyoma, the relative amount of WT mRNA was highest in group B and lowest in group C

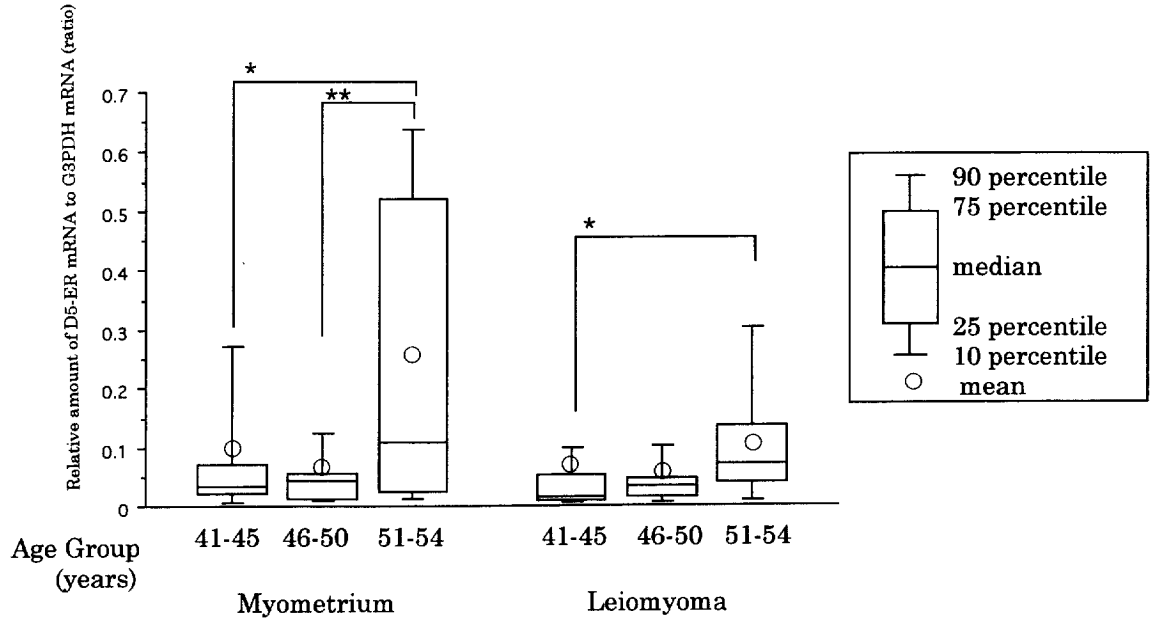

Fig. 4. Relative amount of D5-ER mRNA to G3PDH mRNA measured by a quantitative RT-PCR. Age groups are the same as in Fig. 4. *$p < 0.05$, **$p < 0.01$; one-way ANOVA (post hoc test: Fisher's PLSD)

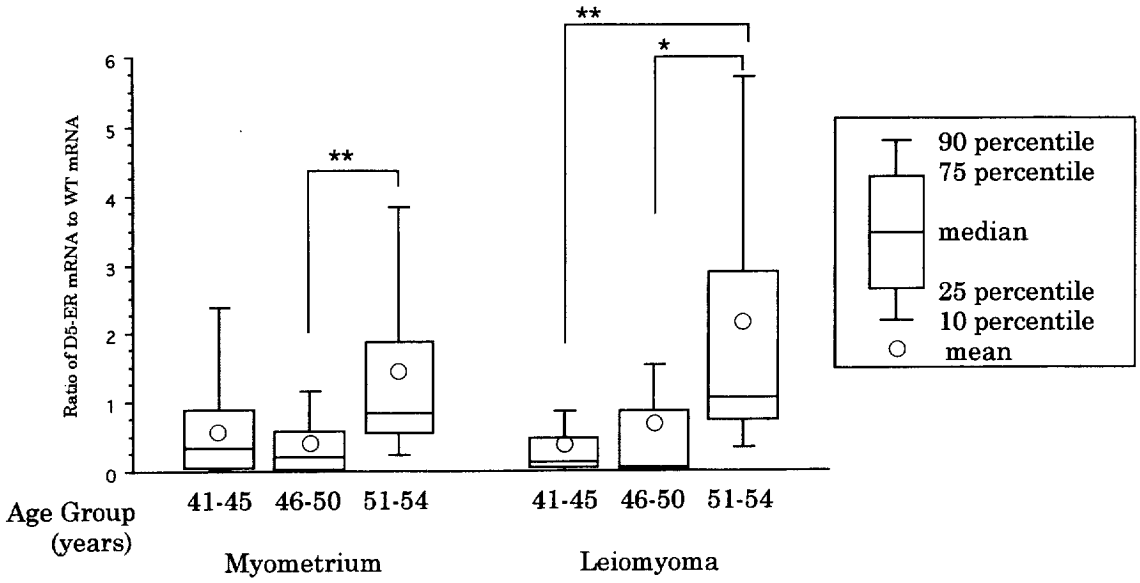

Fig. 5. D5-ER mRNA to WT mRNA ratio measured by the quantitative RT-PCR method. Age groups are the same as in Fig. 4. *$p < 0.05$, **$p < 0.01$; ANOVA

($p < 0.05$; one-way ANOVA) (Fig. 3). The amount of WT was higher in myometrium than in leiomyoma in groups A and C, but vice versa in group B (not significant; unpaired *T*-test) (Fig. 3).

The relative amount of D5-ER mRNA was highest in group C in both the myometrium and leiomyoma, but individual differences were large (Fig. 4). No significant difference was observed between myometrium and leiomyoma (unpaired *T*-test), but in group C, D5-ER was expressed higher in myometrium (Fig. 4).

D5/WT ratio was significantly highest in group C both in myometrium and leiomyoma (one-way ANOVA) (Fig. 5). In group C, about 50% of the specimens showed a D5/WT exceeding 1.0 which mean a higher expression of D5-ER mRNA than WT mRNA (Fig. 5).

The D5/WT ratio in leiomyoma to that in myometrium (L/M ratio) was determined in each individual (Fig. 6). An L/M ratio>1.0 means that D5-ER may play a relatively more active role in leiomyoma than in myometrium. The specimens in which the L/M ratio>1.0 increased markedly with age comprising 20, 52 and 80% of the specimens in groups A, B and C, respectively.

DISCUSSION

D5-ER mRNA was first identified by Fuqua *et al.* [12] in paradoxically ER – /PR+ breast cancer and

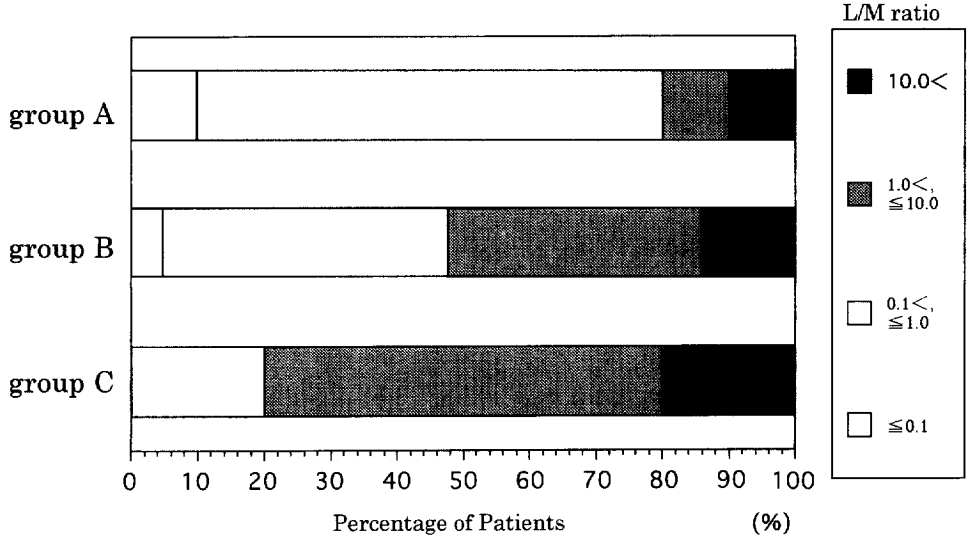

Fig. 6. The percentage of the patients classified according to the D5-ER mRNA to WT mRNA ratio in leiomyoma to that in myometrium in the same individuals in each age group

has also been detected in uterine endometrial cancer [18, 19], hepatocellular cancer [15], and meningiomas [16, 17], but the D5-ER protein itself has not been detected *in vivo*. ER has five different functional domains [22]; A/B, C, D, E and F. ER has two transcriptional activation functions [22–24], AF-1 in the A/B domain and AF-2 in the E domain, which can act both independently and synergistically in a promoter- and cell-specific manner in animal cells [25]. Exon 5 deletion by alternative splicing leads stop codon in exon 6 with a frame shift at the mRNA level and it should be translated into truncated protein with a shortened E domain of the ligand binding site but full length of DNA-binding site. D5-ER reportedly loses the AF-2 site in E domain leaving the full length of AF-1 site intact. D5-ER possesses constitutive transcriptional regulatory activity independent of ligand in the yeast expression system [12, 13, 26]. 4-Hydroxy tamoxifen, an estrogen antagonist, has partial AF-1-dependent agonistic activity in certain cells [21, 24] and in human uterus [27–29]. These studies suggested that AF-1 on ER has an important role in human uterus. Since D5-ER mRNA is expressed widely in estrogen-dependent cells and tissues, D5-ER may play significant roles in uterine tissue.

According to Daffada *et al.* [30], the expression of D5-ER mRNA was detected in 88% of breast cancer tissue specimens and according to Zhang *et al.* [31] in 66%. In this study, all uterine myometrium and leiomyoma specimens expressed both D5-ER and WT mRNAs simultaneously. The amount of D5-ER was 29% of that of WT in myometrium and 18% of that in leiomyoma. Thus D5-ER was not a transient or rare variant in uterine tissues. Daffada *et al.* [30] reported that the median D5/WT ratio in primary breast cancer tissue was 0.15. In our study, it was 0.40 in myometrium and 0.23 in leiomyoma [Fig. 2(c)] and higher than that in breast cancer tissue. Zhang *et al.* [31] reported that D5-ER exceeded corresponding WT in 18% of breast cancer specimens which expressed D5-ER mRNA. In our study, 22% of myometrium and 24% of leiomyoma showed D5/WT ratio>1.0. Moreover, in group C, 40 and 50% of myometrium and leiomyoma samples had a D5/WT ratio>1.0. Although it is very difficult to compare the expression ratio of mRNAs in different tissues and different methods, the inversion can occur in a certain ratio both in benign uterine tumor and malignant breast tumor.

Daffada *et al.* [30] first measured the D5/WT ratio using RT-PCR-Southern blotting, but the amounts of both WT and D5-ER can not be measured by this method. In this study, we measured the relative amount of WT and D5-ER mRNAs versus G3PDH mRNA precisely by a quantitative RT-PCR. The standard templates of the cloned plasmid DNA were amplified on PCR using the same condition as RT product.

To our knowledge, the change of ER expression with aging has not been discussed previously. In this study, we found an age-related change in the expression of WT mRNA and its variant in uterine tissue. Moreover, the expression changed differently in myometrium and leiomyoma. The decrease in WT mRNA in myometrium seems very reasonable toward menopause and the high expression of WT mRNA in leiomyoma in group B may be one reason for worsening symptoms in this age group. Actually, 40.5% of the patients who underwent hysterectomy in our university hospital during 1994–1996 were in group B. The increase in the relative amount of D5-ER and D5/WT ratio in the patients over 50 years old suggests that D5-ER may have transcriptional activity without ligands *in vivo*. D5-ER may increase to supplement the decreasing amount of WT to maintain homeostasis in uterus. The percentage of the patients whose L/M ratio was over 1.0 increased with age, which may be related to the higher hysterectomy rate in pre- or peri-menopausal patients. Clinically, gynecologists know that the symptoms caused by leiomyomas can differ with patient age. Most of the patients in group A had regular menstrual cycles of about 28–32 days and complained of hypermenorrhea and dysmenorrhea, while patients in group B had regular but shorter cycles with prolonged duration and complained of hypermenorrhea, and the patients in group C complained of bleeding which was not distinguishible from menstruation. The growth velocity and symptoms of uterine leiomyoma have not been of major concern because the tumors themselves are not fatal and they are completely curedby hysterectomy. As shown in Fig. 6, our findings support the theory that autonomous local estrogenic effects in uterine leiomyoma increase in pre-/peri-menopausal age.

We found no relationship between the size of leiomyoma and the amount of ER mRNAs (data not shown). The size of leiomyoma may not represent the estrogen effect on the leiomyoma.

Various other factors have also been reported as growth promoting factors of uterine leiomyoma. Of these factors, IGF-I, EGF and TGF-alpha [32–34], also modulate ER transcriptional activity with cross-talk between the A/B domain [35–37]. D5-ER may show cross-talk on this domain and affect tumor growth. Larger amounts of PR in leiomyoma than in myometrium have been reported both at the protein level and mRNA level, which suggests that PR acts as a growth promoting factor [7, 8, 38–40]. If D5-ER has transcriptional activity on PR mRNA expression as suggested previously [41, 12] [50], although opposed by others [43], D5-ER may be more important for the growth of leiomyoma.

A conclusion on the role of ER in uterine leiomyoma has not been reached yet. Brandon *et al.* [5]

found higher ER in leiomyoma than in myometrium by Northern blotting (mRNA), EIA, Western blotting and ligand-binding assay. Vollenhoven *et al.* [8] reported that the amount of ER was higher in leiomyoma than in myometrium by a ligand-binding assay, but the expression of ER mRNA in leiomyoma was not different. Fernandez-Montoli *et al.* [38] reported that the amount of ER measured by EIA was significantly higher than that measured by a ligand-binding assay both in myometrium and leiomyoma, and that ER was significantly higher in leiomyoma by a ligand-binding assay but not significantly different by EIA as Chrapsta *et al.* [7] reported. These differences can be explained by the modification of the amount of ER by D5-ER and other ER variants. The present finding of age-related changes of ERs in myometrium and leiomyoma may increase the complexity of this problem as well as post-transcriptional process [8] or menstrual cycle [8, 44, 45] reported previously. No clear relationship with menstrual cycle was observed in our study because many patients in groups B and C had irregular menstrual cycles.

In conclusion, higher D5-ER mRNA expression in uterine tissue of patients over 50 years old suggests the importance of this mutant and explains the inconsistency of previous reports about the relationship between ER and leiomyoma. The changes in the amount of WT and D5-ER with age and the differences between myometrium and leiomyoma during the pre-/peri-menopausal age, may affect the symptoms of leiomyoma with modulation of other factors and may have caused the inconsistency of the previous findings on the expression of ER.

REFERENCES

1. Puzigaca Z., Prelevic G. M. and Sretenovic Z., Differential reduction in the volume of leiomyoma and uterus during buserelin treatment. *Gynecol. Endocrinol.* **8** (1994) 39–43.
2. Otubu J. A., Buttram V. C. and Besch P. K., Unconjugated steroids in leiomyomas and tumor-bearing myometrium. *Am. J. Obstet. Gynecol.* **143** (1982) 130–133.
3. Yamamoto T., Takamori K. and Okada H., Estrogen biosynthesis in leiomyomas and myometrium of the uterus. *Horm. Metab. Res.* **16** (1984) 678–679.
4. Lumsden M. A., West C. P., Hawkins R. A., Bramley T. A. and Rumgay L., The binding of steroids to myometrium and leiomyomata(fibroids) in women treated with the gonadotropin-releasing hormone agonist Zoladex (ICI 118630). *J. Endocrinol.* **121** (1989) 389–396.
5. Brandon D. D., Erickson T. E., Keenan E. J., Strawn E. Y., Novy M. J., Burry K. A., Warner C. and Clinton G. M., Estrogen receptor gene expression in human uterine leiomyomata. *J. Clin. Endocrinol. Metab.* **80** (1995) 1876–1881.
6. Sadan O., van Iddekinge B., van Gelderen C. J., Savage N., Becker P. J., van der Walt L. A. and Robinson M., Oestrogen and progesterone receptor concentrations in leiomyoma and normal myometrium. *Ann. Clin. Biochem.* **24** (1987) 263–267.
7. Chrapusta S., Konopka B., Paszko Z., Sieinski W. and Szamborski J., Immunoreactive and estrogen-binding estrogen receptors, and progestin receptor levels in uterine leiomyomata and their parental myometrium. *Eur. J. Gynaecol. Oncol.* **11** (1990) 275–281.
8. Vollenhoven B. J., Pearce P., Herington A. C. and Heary D. L., Steroid receptor binding and messenger RNA expression in fibroids from untreated and gonadotrophin-releasing hormone agonist pretreated women. *Clin. Endocrinol. (Oxf.)* **40** (1994) 537–544.
9. Kornyei J., Csermery T., Szekely J. A. and Vertes M., Two types of nuclear oestradiol binding sites in human myometrium and leiomyoma during the menstrual cycle. *Exp. Clin. Endocrinol. Biol.* **87** (1986) 256–264.
10. Sluyser M., Mutations in the estrogen receptor gene. *Human Mutat.* **6** (1995) 97–103.
11. Pink J. J., Wu S.-Q., Woplf D. M., Bilimoria M. M. and Jordan V. C., A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. *Nucl. Acids Res.* **24** (1996) 962–969.
12. Fuqua S. A. W., Fitzgerald S. D., Chamness G. C., Tandon A. K., McNonnell D. P., Nawaz Z., O'Maalley B. W. and McGuire W. L., Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.* **51** (1991) 105–109.
13. Castles C. G., Fuqua S. A., Klotz D. M. and Hill S. M., Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. *Cancer Res.* **53** (1993) 5934–5939.
14. Koehorst S. G. A., Cox J. J., Donker G. H., Lopes da Silva S., Burbach J. P. H., Thijssen J. H. H. and Blankenstein M. A., Functional analysis of an alternatively spliced estrogen receptor lacking exon 4 isolated from MCF-7 breast cancer cells and meningioma tissue. *Mol. Cell. Endocrinol.* **101** (1994) 237–245.
15. Villa E., Camellini L., Dugani A., Zucchi F., Grottola A., Merighi A., Buttafoco P., Losi L. and Manenti F., Variant estrogen receptor messenger RNA species detected in human primary hepatocellular carcinoma. *Cancer Res.* **55** (1995) 498–500.
16. Koehorst S. G. A., Jacobs H. M., Tilanus M. G. J., Bouwens A. G. M., Thijssen J. H. H. and Blankenstein M. A., Aberrant oestrogen receptor species in human meningioma tissue. *J. Steroid Biochem. Mol. Biol.* **43** (1992) 57–61.
17. Blankenstein M. A., Koehorst S. G., van der Kallen C. J., Jacobs H. M., van Spriel A. B., Donker G. H., van't Verlaat J. W., Blaauw G. and Thijssen J. H., Oestrogen receptor independent expression of progestin receptors in human meningioma: a review. *J. Steroid Biochem. Mol. Biol.* **53** (1995) 361–365.
18. Hirata S., Yamada-Mouri N., Nara M., Takizawa M., Ito H. and Kato J., Presence of alternatively spliced-estrogen receptor mRNA variants in normal human uterine endometrium and endometrial cancer. *Endocrinol. J.* **42** (1995) 289–293.
19. Fujimoto J., Ichigo S., Hirose R., Hori M. and Tamaya T., Expression of estrogen receptor exon 5 splicing variant (ER E5SV) mRNA in gynaecological cancers. *J. Steroid Biochem. Mol. Biol.* **60** (1997) 25–30.
20. Greene G. L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J., Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** (1986) 1150–1154.
21. Tsi J. Y., Sun X. H., Kao T. H., Reece K. S. and Wu R., Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucl. Acids Res.* **13** (1985) 2485–2502.
22. Kumar V., Green Stack G., Berry M., Jin J. R. and Chambon P., Functional domains of the human estrogen receptor. *Cell* **51** (1987) 941–951.
23. Kumar V. and Chambon P., The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55** (1988) 145–156.
24. Ponglikitmongkol M., Green S. and Chambon P., Genomic organization of the human oestrogen receptor gene. *EMBO J.* **7** (1988) 3385–3388.
25. Tora L., White J., Brou C., Tasset D., Webster N., Scheer E. and Chambon P., The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59** (1989) 477–487.
26. Limieux P. and Fuqua S., The role of the estrogen receptor in tumor progression. *J. Steroid Biochem. Mol. Biol.* **56** (1996) 87–91.
27. Kedar, R. P., Bourne, T. H., Powles, T. J., Collins, W. P., Ashley, S. E., Cosgrove, D. O. and Campell, S., Effects of tamoxifen on uterus and ovaries of postmenopausal women in

a randomised breast cancer prevention trial. *Lancet*, 1994, **343**, 1318–21–752.
28. Jamil A., Croxtall J. D. and White J. O., The effect of antioestrogens on cell growth and progesterone receptor concentration in human endometrial cancer cells (Ishikawa). *J. Mol. Endocrinol.* **6** (1991) 215–221.
29. Huynh H. T. and Pollak M., Insulin-like growth factor I gene expression in the uterus is stimulated by tamoxifen and inhibited by the pure antiestrogen ICI 182780. *Cancer Res.* **53** (1993) 5585–5588.
30. Daffada A. A. I., Johonston S. R. D., Nicolls J. and Dowsett M., Detention of wild type and exon-5 deleted splice variant oestrogen receptor (ER) mRNA in ER-positive and negative breast cancer cell lines by reverse transcription/polymerase chain reaction. *J. Mol. Endocrinol.* **13** (1994) 265–273.
31. Zhang Q. X., Hilsenbeck S. G., Fuqua S. A. W. and Borg A., Multiple splicing variants of the estrogen receptor are present in individual human breast tumors. *J. Steroid Biochem. Mol. Biol.* **59** (1996) 251–260.
32. Strawn E. Y. Jr., Novy M. J., Burry K. A. and Bethea C. L., Insulin-like growth factor I promotes leiomyoma cell growth *in vitro*. *Am. J. Obstet. Gynecol.* **172** (1995) 1837–1844.
33. Vollenhoven B. J., Herington A. C. and Healy D. L., Epidermal growth factor and transforming growth factor-beta in uterine fibroids and myometrium. *Gynecol. Obstet. Invest.* **40** (1995) 120–124.
34. Dou Q., Zhao Y., Tarnuzzer R. W., Rong H., Williams R. S., Schultz G. S. and Chegini N., Suppression of transforming growth factor-beta (TGF beta) and TGF beta receptor messenger ribonucleic acid and protein expression in leiomyomata in women receiving gonadotropin-releasing hormone agonist therapy. *J. Clin. Endocrinol. Metab.* **81** (1996) 3222–3230.
35. Ignar-Trowbridge D. M., Pimentel M., Parker M. G., McLachlan J. A. and Korach K. S., Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* **137** (1996) 1735–1744.
36. Kato S., Endoh H., Masuhiro Y., Kitamoto T., Uchiyama S., Sasaki H., Masusige S., Gotoh Y., Nishida E., Kawashima H., Metzger D. and Chambon P., Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** (1995) 1491–1494.
37. Bunone G., Briand P. A., Miksicek R. J. and Picard D., Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* **15** (1996) 2174–2183.
38. Fernandez-Montoli M. E., Diez-Gibert O., Samanniego J. M., Balaguero L. and Navarro M. A., Total and unbound cytosolic estrogen and progesterone receptors in myometrium and fibroid after gonadotropin-releasing hormone agonist treatment. *Fertil. Steril.* **63** (1995) 522–527.
39. Vij U., Murugesan K., Laumas K. R. and Farooq A., Progestin and antiprogestin interactions with progesterone receptors in human myomas. *Int. J. Gynaecol. Obstet.* **31** (1990) 347–353.
40. Murphy A. A., Kettel L. M., Morales A. J., Roberts V. J. and Yen S. S., Regression of uterine leiomyomata in response to the antiprogesterone RU 486. *J. Clin. Endocrinol. Metab.* **76** (1993) 513–517.
41. Zhang Q. X., Borg A. and Fuqua S. A. W., An exon-5 deletion variant of the estrogen receptor frequently coexpressed with wild-type estrogen receptor in human breast cancer. *Cancer Res.* **53** (1993) 5882–5884.
42. McGuire W. L., Chamness G. C. and Fqua S. A., Estrogen receptor variants in clinical breast cancer. *Mol. Endocrinol.* **5** (1991) 1571–1577.
43. Rea D. and Parker M. G., Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cell. *Cancer Res.* **56** (1996) 1556–1563.
44. Soules M. R. and McCarty K. S., Leiomyomas: Steroid receptor content. Variation within normal menstrual cycles. *Am. J. Obstet. Gynecol.* **143** (1982) 6–11.
45. Ochiai K., Cyclic variation and distribution in the concentration of cytosol estrogen and progesterone receptors in the normal human uterus and myoma. *Acta Obst. Gynaec. Jpn.* **32** (1980) 945–952.
46. Murphy L. C., Dotzlaw H., Leygue E., Douglas D., Coutts A. and Watson P. H., Estrogen receptor variants and mutations. *J. Steroid Biochem. Mol. Biol.* **62** (1997) 363–372.