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Decreased Expression of Wnt7a mRNA Is Inversely Associated with the Expression of Estrogen Receptor-α in Human Uterine Leiomyoma

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Abstract: Wnt-7a gene not only guides the development of the anterior-posterior axis in the female reproductive tract, but also plays a critical role in uterine smooth muscle pattering and maintenance of adult uterine function. This gene is also responsive to changes in the levels of sex steroid hormone in the female reproductive tract. To explore the molecular mechanisms underlying the pathogenesis of uterine leiomyoma, the expression of Wnt7a mRNA in the leiomyoma has been assessed. RT-PCR was performed on uterine leiomyomas and the adjacent myometria. Of 30 cases of leiomyomas studied, 67% showed a decreased mRNA level as compared to the paired myometria. On the other hand, estrogen receptor-α (ER-α) mRNA is hyper-expressed in 67% of the leiomyomas as compared to their paired myometrium. An inverse association at mRNA expression was found between Wnt7a and ER-α. Miller *et al* has shown that fetal exposure of DES results in de-regulation of Wnt7a during uterine morphogenesis. Referring to their results, we have postulated that hypersensitivity of leiomyoma cells to estrogen may deregulate the Wnt7a expression. Decreased expression of Wnt7a may lead to loss of control in patterning of the myometrium and result in development of leiomyoma.

Introduction

Leiomyomas, the most common uterine neoplasm, occur in more than 40% of women over the age of 35 (1, 2). Enlargement of leiomyoma causes serious gynecological problems, such as pelvic pain, menorrhagia, dysmenorrhea, reduced fertility, and recurrent miscarriage. In addition, this tumor is a major indication for hysterectomies, accounting for more than 200,000 costly procedures each year (3, 4).

The mechnism by which the uterine myometrium develops into leiomyoma is not yet fully understood. As described by pathologists, leiomyomas are composed of whorled, anastomosing fascicles of uniform, fusiform smooth muscles. The spindle-shaped cells have abundant fibrillar, eosinphilic cytoplasm as well as distinct borders. Nuclei are elongated with blunt or tapered ends, and have finely dispersed chromatin with small nuclei. The tumor generally remains benign, and only rarely transforms into malignant leiomyosarcoma (5). In general, leiomyomas are a mass of myometrial cells overgrown with poor differentiation which results in loss of normal direction and patterning. Biologically, leiomyoma cells express myometrial specific genes such as actins, myosins and desmins (5). A high level of steroid receptors, altered fibroblast growth factor, and transforming growth factor-beta has been found in leiomyoma cells (6, 7). Clonality studies have shown that multiple tumors in the same uterus develop a de novo mechanism (8). Although some chromosome changes such as translocations, duplications, and deletions have been identified in leiomyoma cells, these changes are considered as secondary events in tumor progression (9, 10).

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Considering the pathological appearance and biological characteristics of the leiomyoma, we postulate that the leiomyomas are a mass of uterine myometria which lose control of pattern/formality. However, no studies have been found postulating the concept that loss of control in patterning/formality of myometial cells can lead to the development of this tumor.

Wnt genes are a large family of highly conserved, developmentally-related genes (11). The vertebrate Wnt genes are homologous to *wingless*—the Dorosophila segment polarity gene that encodes a secreted molecule implicated in the patterning and establishment of cell boundaries during embryogenesis. The term "Wnt" is derived from the combination of *wingless* and *int-1*, which was found to be a common integration site of mouse mammary tumor virus (MMTV) in mammary epithelial carcinomas (12, 13). At least 16 family members have been identified in the vertebrate. Wnt genes are involved in cell-cell communication and patterning, and associated with oncogenesis, such as sex steroid hormone responsive cancers, including breast and uterine carcinoma (14-16). Wnt7a gene not only guides the development of the anterio-posterior axis in the female reproductive tract, but also plays a critical role in uterine smooth muscle patterning and maintenance of adult uterine function (17-19). In mice, loss of Wnt7a expression results in several notable abnormalities in the female reproductive tract. The uterus lacks glands and the epithelium is stratified compared to the normal cytoarchitecture of wide type female. In addition, an overgrown, poorly differentiated myometrium has been observed in Wnt7a knock-out mice (17). These features resemble pathological appearances in leiomyoma. Hence Wnt7a is a strong candidate for studying the patterning control of the uterine myometria. In addition, Wnt7a is also responsive to changes in the levels of sex steroid in the female reproductive tract (20-22). Such

evidence has persuaded us to explore the role of Wnt7a gene in the pathogenesis of human leiomyoma.

Materials and Methods

Tissue Sample Collection

Specimens were obtained from patients with fibroids undergoing hysterectomies at Medical Center of Louisiana at New Orleans. The adjacent uterine myometrium was obtained from the same patient to serve as a control (designated as "normal"). Each tissue block was divided into three parts for RNA and protein extraction, and histology examination.

RNA Extraction and RT-PCR

RNA was extracted using an Ultraspec™ RNA isolation kit (Biotecx Lab, TX). Frozen tissues were smashed and homogenized in RNA isolation solution. Then chloroform was added. After centrifugation, the colorless upper aqueous phase was removed to a new tube. An equal volume of isopropanol was added to precipitate RNA. The RNA pellet was washed with 75% ethanol and recovered in DEPC-treated water. Reverse transcription reactions were carried out using Gene Amp RNA PCR kit (Perkin Elmer, CA). Suggested methods of the manufacturer were followed with modification.

PCR reactions were carried out by adding 2μl of cDNA into 48 μl of PCR mixture. The PCR mixture contained 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH8.3, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin), 0.2 mM dNTP mixture (Promega, WI), 0.1 μg of each Wnt 7a primer or 0.017 μg of each GAPDH primer, 2 unit of Taq DNA polymerase pre-reacted with 0.44 μg of TaqStart Antibody (Clontech, CA). To avoid non-specific amplification and primer-dimer artifacts, TaqStart antibody was used. PCR reactions were performed on a DNA Thermal Cycler 960 (Perkin Elmer, CA). DNA was denatured at 94°C for 2 minutes and followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. The final extension of PCR was at 72°C for 7 minutes.

Primers were designed according to the published human Wnt-7a sequence (U53476 NCBI) (23) and were synthesized at Life technologies. Sense: 5'-GCCGTTCACGTGGAGCCT GTGCGTGC-3' (nucleotides 726-752); Antisense: 5'-AGCA TCCTGCCAGGGAGCCCGCAGCT-3' (nucleotides 1137-1163). Primers used to amplify the ER-α were: 5'-TGCCAA GGAGACTCGCTA-3'(nucleotides 894 to 912) and 5'-TCA ACATTCTCCCTCCTC-3'(nucleotides 1139 to 11570) (24).

Confirmation and Relative Quantification of RT-PCR

Target PCR bands were cloned into M13 vector (Invitrogen) and further proved by automatic sequencing. The PCR bands were scanned by Gel Doc 2000 using software "Quantity One Version 4.0" (Bio Rad). The relative expression level of each gene was derived by comparing the density of each target band to that of the housekeeping gene, GAPDH, amplified at the same time. The relative expression levels of Wnt-7a and ERα were further compared between leiomyoma and normal myometrium from the same patient. Then, ratios of leiomyoma to myometrium of Wnt7a and ER-α were achieved by comparing the relative expression of the genes. The expression was considered as increased if the ratio is greater than 1, equal if the ratio is 1, or less if the ratio is less than 1.

Results

Comparison of Wnt7a mRNA expression between leiomyoma and paired myometrium

Using our designed primers, the target band of Wnt7a was detected in all samples with molecular weight about 450 bp (Fig.1). In the comparison of leiomyoma and paired myometrium tissues, Wnt7a mRNA levels were lower in leiomyoma than in myometrium in 67% (20/30) of the samples; equal in 3% (1/30) of the samples; and higher in leiomyoma than in myometrium in 30% (9/30) of the samples (Fig. 2).

The effect of the menstrual cycle on Wnt7a mRNA expression

Pathological study of the endometrium provided further information on the specific phase of menstrual cycle in all 30 samples. There were 11 samples in the proliferative phase, 9 in the secretory phase, and the other 10 samples were grouped in a non-active phase with pathological changes such as atypia and atrophy. The expression level of Wnt7a mRNA between leiomyoma and paired myometrium in each phase of menstrual cycle was compared. Wnt7a mRNA levels were lower in leiomyoma compared to paired myometrium in 55% (6/11) of the samples in proliferative phase, and in 56% (5/9) of the samples in secretory phase (Fig. 3). In general, Wnt7a mRNA levels showed a similar trend, that is, lower in leiomyoma compared to paired myometrium, in all phases of the menstrual cycle.

Comparison of ER-α mRNA expression between leiomyoma and paired myometrium

Amplification of ER-α gives a product of 263bp in all samples (Fig. 1B). ER-α mRNA levels are higher in 67% (20/30) of the leiomyoma as compared to their paired myometrium; equal in 3% (1/30) of the samples; and lower in leiomyoma than in myometrium in 30% (9/30) of the samples (Fig. 4). An inverse association of mRNA expression was found between Wnt7a and ER-α.

The effect of menstrual cycle on ER-α mRNA expression

In samples from the proliferative phase, 64% (7/11) of the samples have a higher expression of ER-α mRNA expression in the leiomyoma than in the paired normal myometrium. The same result can be seen in 44% (4/9) of the samples from secretory phase (Fig. 5). In general, our data indicate that samples from the proliferative phase have a higher ER-α expression in leiomyoma than paired myometrium, but is not at the secretory phase of menstrual cycle.

Discussion

In the present study, we have shown a decreased expression of Wnt7a in leiomyoma compared to the paired myometrium. To avoid individual differences which may include menstrual status, hormone treatment and age difference, samples of the leiomyoma tissues and the adjacent myometrium were collected from the same patient. The relative expression of Wnt7a between leiomyoma and myometrium was achieved by comparing the relative expression of Wnt7a mRNA to the housekeeping gene, GAPDH, from the same patients. Wnt7a gene and GAPDH were amplified at the same time in the same condition, except at different cycles, to avoid time to time difference in PCR.

Studies from Wnt7a knock-out mice have shown that Wnt7a gene plays a critical role in uterine smooth muscle patterning and maintenance of adult uterine function (17-19).

Our data have shown decreased Wnt7a expression in leiomyoma compared to paired myometrium. Decreased Wnt7a expression may lead to loss of control in patterning of the myometial cells, which may be acountable to the development of leiomyoma.

Consistent with the previous studies, we have confirmed that the ERα mRNA is increased in the leiomyoma more than in the adjacent myometium (25) (6). The presence of a higher ER-α mRNA in leiomyoma correlates with an exacerbation of the hormone dependence of this tissue. The result that ERα expression is higher in the proiferative phase than in the secretory phase of the menstrual cycle may be due to the estrogen millieu. Similar hormone dependence of ERα expression has been reported by several studies (6, 26).

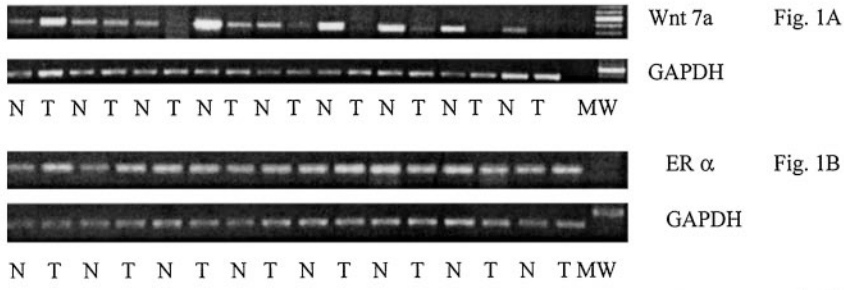

Fig. 1: Representative gels of RT/PCR. 1A: Wnt 7a and GAPDH gene; 1B: ER α gene and GAPDH. T: leiomyoma N: paired normal myometrium from the same patient.

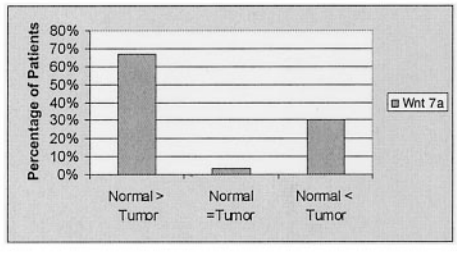

Fig. 2 Comparison of Wnt 7a mRNA expression between leiomyoma and paired myometrium

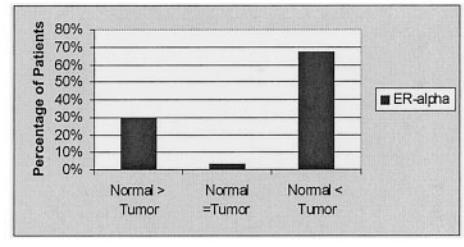

Fig. 4 Comparison of ERα mRNA expression between leiomyoma and paired myometrium

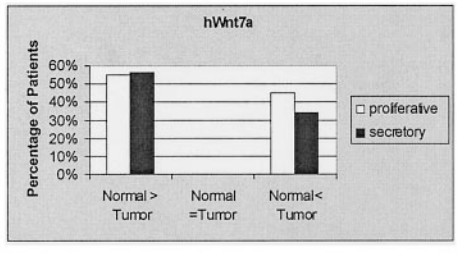

Fig. 3 Comparison of Wnt 7a mRNA expression in different phases of menstrual cycle between leiomyoma and paired myometrium

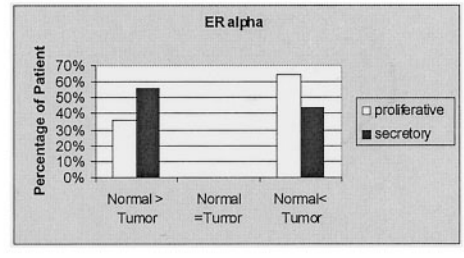

Fig. 5 Comparison of ERα mRNA expression in different phases of menstrual cycle between leiomyoma and paired myometrium

The inverse association of the Wnt7a and ER-α mRNA suggests a deregulation mechanism by which the steroid hormones regulate the expression of Wnt7a. Although the means by which estrogens mediate the expression of Wnt7a is unclear, studies have observed a suppression of Wnt7a by estrogens. Miller *et al* have shown that fetal exposure of diethylstilbestrol (DES), a synthetic estrogen, can result in deregulation of Wnt7a during murine uterine morphogenesis (20).

In conclusion, we have shown a decreased expression of Wnt7a in uterine leiomyoma as compared to that in the adjacent myometrium. The decreased expression has an inverse correlation with the ER-α. This is the first approach to exploring the etiology of the leiomyoma from the concept of pattern/formality control. Further investigation of the exact role of Wnt7a and its correlation with estrogen will initiate a new research avenue and provide informative evidence for prevention and treatment of the most widespread disease in women.

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