Expression of insulin-like growth factors (IGFs) and IGF signaling: molecular complexity in uterine leiomyomas

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Objective: To study whether dysregulation of insulin-like growth factors (IGFs) and IGF signaling are common molecular changes in symptomatic leiomyomas (fibroids) and whether IGFs are associated with large fibroids.

Design: Examination of IGFs and IGF pathway genes in a large cohort of fibroids at transcriptional and translational levels. Mechanisms leading to alterations of IGFs and related genes were also analyzed.

Setting: University clinical research laboratory.

Patient(s): Hysterectomies for symptomatic fibroids were collected: 180 cases from paraffin-embedded tissues and 50 cases from fresh-frozen tissues.

Intervention(s): Tissue microarray and immunohistochemistry, DNA methylation analysis, reverse-transcriptase polymerase chain reaction, and Western blot.

Main Outcome Measurement(s): Transcription and translation analyses of IGF-1/2, p-AKT, p-S6K, and TSC1/2 in fibroids and matched myometrium.

Result(s): Insulin-like growth factors and downstream effectors were dysregulated in approximately one third of fibroids. All except for IGF-2 seemed to be abnormally regulated at translation levels. Up-regulation of IGF-2 messenger RNAs was contributed by all four alternating slicing promoters. There was a positive correlation of IGF-1 and p-AKT over-expression with fibroid size. Insulin-like growth factor 1 but not IGF-2 levels directly correlated with activation of p-AKT and p-S6K.

Conclusion(s): Altered expressions of IGFs and their related downstream proteins were found in one third of fibroids. Large fibroids show high levels of IGF-1 and p-AKT activity compared with small ones. (Fertil Steril® 2009;91:2664–75. ©2009 by American Society for Reproductive Medicine.)

Key Words: IGF-1, IGF-2, IGF pathway, tissue microarray, promoter, leiomyomas

Uterine leiomyomas (fibroids) are the most common smooth muscle tumors in reproductive-aged women (1). Approximately 20% of women with fibroids experience symptoms and seek medical treatment (2). Symptoms can be caused by large tumor sizes, numerous tumors, and/or tumor location. Large fibroids often cause severe symptoms and require invasive management, including hysterectomy. In the United States approximately 200,000 hysterectomies for symptomatic fibroids are performed annually (2).

Fibroids have a high incidence (1) but are not usually symptomatic unless they are large or submucosal. The important issue regarding leiomyomata may not be the etiology per se but rather why some fibroids grow faster and larger whereas others remain small. The factors that determine the differential growth rate among leiomyomata are poorly understood. Studies on the natural history of fibroids reveal a wide range of fibroid growth: some grow faster, whereas others remain small (3, 4).

Ovarian steroid hormones are the major driving force behind fibroid growth, as suggested by the appearance of symptomatic fibroids in premenopausal women that regress in hypoestrogenic status, like postmenopause and GnRHa treatment. In addition to sex steroid hormones, the molecular pathogenesis of fibroid growth involves many genes that regulate cell proliferation, differentiation, and the extracellular matrix (5). Many local growth factors are associated with fibroids, such as insulin-like growth factors (IGFs) (6). Insulin-like growth factor signaling activity seems to be one of the most important pathways responsible for fibroid growth. Insulin-like growth factors are over-expressed in a paracrine or autocrine fashion in fibroids (7–10). There are two IGFs, IGF-1 and IGF-2. The mitogenic role of IGF-1 in fibroid growth
has been reported both in vitro and in vivo (10–12). As a potent mitogenic factor (13), IGF-2 was reported to be over-expressed in fibroids in 1990 (14), and recent global gene transcription profiling analyses have supported the speculation that it is one of few genes constantly over-expressed in fibroids (15–21). However, the function and regulation (22–24) of IGF-2 in fibroids are largely unknown. Some studies evaluating the activity of IGF signaling activity in fibroids have been reported (5–7, 10, 12, 25, 26). An attempt to treat fibroids by administration of somatostatin (27) in a small trial led to a reduction of tumor size. However, additional molecular studies are required to further characterize the role of IGFs and IGF signaling activity in response to fibroid growth.

In this study we examined IGFs and related genes in a large cohort of fibroid samples. We examined the levels of IGFs and related gene expression at the transcriptional and translational levels and compared gene expression of each fibroid with its respective sex hormonal status and tumor size. The functional protein products of protein kinase B (AKT), ribosomal S6 protein kinase (S6K), tuberous sclerosis complex genes (TSC), and their phosphorylation statuses were also analyzed. Our findings reveal that IGFs and their signaling pathways are activated in approximately one third of fibroids and, more importantly, that IGF signaling is positively associated with large and actively growing fibroids.

MATERIALS AND METHODS

Patients and Specimens

Tissue samples for this study were collected from hysterectomies for symptomatic fibroids. A total of 50 cases were collected from fresh-frozen tissue samples (Table 1). Each case included small (<3 cm in diameter) and large (≥10 cm in diameter) fibroids and matched myometrium. Another 180 cases were collected from formalin-fixed paraffin-embedded tissues. Tissue samples from these cases were included in our established tissue microarrays (TMA) in TMA1 and TMA2 (TMA1 = 60 cases, TMA2 = 120 cases). Detailed information about these two TMAs has been previously published (28, 29). All leiomyomas selected for the study were histologically the usual type. All patients had well-documented clinical histories for age, cycling phase, tumor size, and other endometrial or myometrial diseases at the time of their hysterectomies. Patient and tissue information is summarized in Table 1. This study was approved for use of human materials by our institutional review boards.

Primers

To examine the overall IGF-2 messenger RNA (mRNA) levels, an IGF-2 forward primer from exon 7 (Exon7F) and a reverse primer from exon 8 (Exon8R) were used. Exon 7 and exon 8 are the common exons used by all alternatively spliced IGF-2 mRNAs. To examine the IGF-2 transcripts from each of four promoters, forward primers were designed from Exon1F and Exon2F (P1), Exon4F (P2), Exon5F (P3), and Exon6F (P4); reverse primers were designed from Exon7R and Exon8R (Table 2). Information regarding IGF-2 sequences from alternating transcripts and exons can be found at http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/ and in Mineo et al. (30). Primers for all other genes used in this study are summarized in Table 2. The primer sequences were designed by OligoPerfect Designer (Invitrogen, Carlsbad, CA).

RNA and RT-PCR

Fresh tissue from both tumors and matched myometrium were collected <4 hours after hysterectomy for fibroid uteri. The tissues were immediately immersed into RNA later solution (Ambion, Austin, TX). Total RNA was extracted by a modified one-step RNA extraction with TRizol (Invitrogen). One to five micrograms of total RNA were used for complementary DNA (cDNA) synthesis using the cDNA synthesis kit from BD Biosciences (Mountain View, CA). One twentieth of the cDNA was used for reverse-transcriptase polymerase chain reaction (RT-PCR) in a P200 cycler (MJ Research, Waltham, MA). To optimize RT-PCR for a semi-quantitation in single and multiplex PCR, all primer pairs were tested in 28, 32, and 35 cycles. Reverse-transcriptase PCR with 32 cycles (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes) was optimal in detecting IGF-2 cDNA products from P2 to P4, and 35 cycles were required to detect P1 cDNA product. Both positive (cloned IGF-2 cDNA) and negative controls (no template) were also used in each reaction. The RT-PCR products were size-fractionated by 1% regular agarose gel electrophoresis and photographed. Reverse-transcriptase PCR cycles for

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>No. of cases (no. of tumors)</th>
<th>Mean age (range) (y)</th>
<th>Mean tumor size (range) (cm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMA1</td>
<td>60 (60)</td>
<td>49.2 (37–82)</td>
<td>5.77 (1.5–19.0)</td>
<td>Wei et al. 2005 (28)</td>
</tr>
<tr>
<td>TMA2</td>
<td>120 (120)</td>
<td>46.8 (35–59)</td>
<td>6.83 (1.0–22.0)</td>
<td>Wei et al. 2006 (29)</td>
</tr>
<tr>
<td>Fresh-frozen tissue</td>
<td>50 (100)</td>
<td>45.8 (35–52)</td>
<td>≤3 (n = 50)</td>
<td>Present study</td>
</tr>
</tbody>
</table>

other genes ranged from 28 to 35, depending on the intensity of cDNA in the testing run.

Methylation Analysis
Deoxyribonucleic acid methylation analysis was performed with the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the protocol recommended by the manufacturer. In brief, 2 μg of genomic DNA from five fibroids and five matched myometrium were mixed with M-Dilution Buffer and incubated at 37°C for 15 minutes, after which 100 μL of the prepared CT Conversion Reagent was added. The mix/solution was left in the dark at 50°C for 12–16 hours. The DNA samples were transferred on ice for 10 minutes, and 400 μL of M-Binding Buffer was subsequently added. The DNA samples were purified through the Zymo-Spin I Column, washed by M-Wash Buffer, and treated with M-Desulphonation Buffer. The DNA was then eluted with 10 μL of M-Elution Buffer. Bisulfide-modified DNA was then digested with the selected restriction enzymes purified and amplified with IGF-2 promoter-specific primers (Table 2).

Antibodies
The antibodies used in this study are summarized in Table 3. The concentrations of each antibody used in immunohistochemical stains or Western blot analyses follow the recommended concentrations provided by the manufacturers. To minimize the variation between cases, internal normal myometrial controls were selected from each case.

Western Blot Analysis
Total protein was isolated from the tissues with protein extraction buffer (Boston Bioproducts, Ashland, MA). The protein content was determined according to the Bradford method (Bio-Rad, Hercules, CA), with bovine serum albumin used as a standard. Protein samples (30 μg) were boiled, separated by molecular weight on 8% or 12% sodium dodecyl sulfide polyacrylamide gel, and transferred to a polyvinylidene fluoride transfer membrane (NEW Research Products, Boston, MA). The membrane was hybridized with a dilution of the primary antibodies. Signals were detected by enhanced chemiluminescence (Amersham Life Science,

#### Table 2

<table>
<thead>
<tr>
<th>Primers for IGF-2 transcripts</th>
<th>Sequences (5′ – 3′)</th>
<th>cDNA product (bp)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>All</th>
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</thead>
<tbody>
<tr>
<td>IGF-1 Forward</td>
<td>TGGATGCCTCTTCAGTTG</td>
<td>280</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 Reverse</td>
<td>ACTCGTGAGGCAAAAGAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-2b Exon1F</td>
<td>AGAGTCACACCCGAAGCTT</td>
<td>570</td>
<td>X</td>
<td></td>
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<td></td>
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<tr>
<td>IGF-2b Exon2F</td>
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<td>X</td>
<td></td>
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<tr>
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<td>X</td>
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<td></td>
</tr>
<tr>
<td>IGF-2b Exon6F</td>
<td>AAGTCGATGGTGCTTTCTT</td>
<td>280</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IGF-2b Exon7F</td>
<td>CGTGGGACATCGGAGGACT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
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<td>IGF-2b Exon8R</td>
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<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>IGF-2 promoter 1 (P1)</td>
<td>TCCTCTTCATCATCTCCA</td>
<td>518</td>
<td></td>
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<tr>
<td>IGF-2 promoter 2 (P2)</td>
<td>GATGCACATGCTCTGTAG</td>
<td>485</td>
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<tr>
<td>TSC1 Forward</td>
<td>GAATGGCCCAACACAGCA</td>
<td>840</td>
<td></td>
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<tr>
<td>TSC1 Reverse</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TSC2 Forward</td>
<td>CACCATGGCCAAACCAAC</td>
<td>960</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSC2 Reverse</td>
<td>AGAATGTCGACGGCACC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a: X: each pair of forward and reverse primers.

b: Location of IGF-2 primers can be seen in Figure 2B.

Little Chalfont, UK). The housekeeping protein ERK or Actin were used as a loading control.

Statistical Analysis

The immunoscore was determined by visual semiquantitative (optical density of the immunoreactivity) assessment according to the following scale: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), as previously described(29). The intensities of the RT-PCR products were scored by density photometry (National Institutes of Health free software, Scion Image). The density scores of the PCR products of IGFs and other gene products were normalized by calculating the ratio of tested cDNA/G3PDH (glyceraldehyde-3-phosphate dehydrogenase). Mean values, standard errors, and pair-wise t-test values were calculated. P values of < .05 were considered statistically significant. The amount of proteins detected by Western blot analysis was also quantified by density photometry and normalized with loading controls, such as Erk or Actin.

RESULTS

The IGF signaling pathway regulates cell proliferation, differentiation, and cell death (apoptosis). Insulin-like growth factor–mediated signaling involves multiple genes modulated by either transcriptional regulation and/or post-translational modification of functional forms of the existing gene products (i.e., phosphorylation). To characterize the role of the IGF signaling pathway in uterine fibroids, we examined IGFs and genes in its pathway at the transcriptional and translational levels, as well as their functional status.

IGF-1 Expression in Fibroids

We first examined IGF-1 mRNA expression in 33 randomly selected fibroids in 33 premenopausal women. With appropriate RNA loading control of G3PDH, semiquantitative RT-PCR revealed a moderate expression of IGF-1 mRNA in both fibroids and matched myometrium (Fig. 1A). Overall, the abundance of IGF-1 cDNA varied slightly, but insignificantly, between the fibroids and matched myometrium. The mean values of IGF-1 mRNA in the fibroids and matched myometrium were calculated according to the intensity of the cDNA band by density-photometry analysis. As illustrated in Figure 1B, there was no difference in the IGF-1 mRNA expression between the fibroids and normal myometrium (P > .05).

We then analyzed the possible association of IGF-1 mRNA expression with patient age, hormonal status, and tumor size. Among 33 cases at the time of hysterectomy, 15 were in the secretory endometrial phase (SE), and 18 were in the proliferative endometrial phase (PE). Insulin-like growth factor-1 mRNA levels were significantly higher in the PE phase than in the SE phase (P < .05), both in fibroids and matched myometrium (Fig. 1C and D). The findings of higher levels of IGF-1 in the proliferating phase of endometrium further supported a suggested mechanism in which E2, acting through an estrogen receptor stimulates the local synthesis of IGF-1 in the uterus(31). However, our data suggested that there were similar estrogen effects on IGF-1 mRNA expression in both fibroids and myometrium (Fig. 1D). In addition, there were no differences between IGF-1 mRNA expression and tumor size or patient age (data not shown).

To compare the differential expression of IGF-1 proteins between fibroids and matched myometrium, we examined the levels of IGF-1 protein in a total of 180 fibroids (TMA1 and TMA2) by immunohistochemistry (Fig. 1E). The levels of IGF-1 protein were scored semiquantitatively by optical immunointensity (see Materials and Methods). More than one third of fibroids (67 of 180) had a higher IGF-1 immunoreactivity compared with the matched myometrium. When tumors were classified by size (1–3 cm [n = 47], 4–6 cm [n = 53], 7–9 cm [n = 50], and ≥ 10 cm [n = 30]), the mean immunoreactivity for IGF-1 was significantly higher in large fibroids than in small fibroids (Fig. 1F).

### Table 3

Antibodies, vendor, and titration.

<table>
<thead>
<tr>
<th>No.</th>
<th>Markers</th>
<th>Vendor/Source</th>
<th>Titer IHC (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IGF-1</td>
<td>Santa Cruz</td>
<td>1:40</td>
</tr>
<tr>
<td>2</td>
<td>IGF-2</td>
<td>Neo Markers</td>
<td>1:40</td>
</tr>
<tr>
<td>3</td>
<td>AKT</td>
<td>Cell Signaling</td>
<td>1:20 (1:100)</td>
</tr>
<tr>
<td>4</td>
<td>pAKT (Ser473)</td>
<td>Cell Signaling</td>
<td>1:20 (1:100)</td>
</tr>
<tr>
<td>5</td>
<td>p70 S6K (Thr389)</td>
<td>Cell Signaling</td>
<td>1:20 (1:100)</td>
</tr>
<tr>
<td>6</td>
<td>Hamartin</td>
<td>Mizuguchi M</td>
<td>1:500 (1:200)</td>
</tr>
<tr>
<td>7</td>
<td>Tuberin</td>
<td>Mizuguchi M</td>
<td>1:700 (1:250)</td>
</tr>
<tr>
<td>8</td>
<td>pTSC2 (Ser664)</td>
<td>P. P. Pandolfi</td>
<td>1:50</td>
</tr>
<tr>
<td>9</td>
<td>ERK</td>
<td>Cell Signaling</td>
<td>(1:100)</td>
</tr>
<tr>
<td>10</td>
<td>Actin</td>
<td>Santa Cruz</td>
<td>(1:200)</td>
</tr>
</tbody>
</table>

Note: IHC = immunohistochemistry; W = Western blot.

Expression of IGF-2 and its Isoforms in Fibroids

To validate the gene profiling data for over-expression of IGF-2, we first examined IGF-2 mRNA expression in a total of 64 fibroids from 35 patients (35 large [≥ 10 cm] and 29 small [≤ 3 cm]) and matched myometrium. The expression levels of IGF-2 mRNA were semiquantitated by RT-PCR, normalized with control G3PDH, and scored by density photometry (see Materials and Methods). The overall expression of
IGF-2 mRNA was detected and determined by RT-PCR products from exons 7 and 8 (exons shared by all IGF-2 isoforms) (Fig. 2B). Of 64 fibroids, over-expression of IGF-2 mRNA was found in 77% of them (49 of 64), minimal or no change of IGF-2 mRNA was found in 22% (14 of 64), and down-regulation of IGF-2 mRNA was found in only one tumor (1 of 64). The level of IGF-2 mRNA was found to be in the range of two- to fivefold over-expressed in most fibroids. There was no significant difference of IGF-2 expression between large (≥ 10 cm) and small (< 3 cm) fibroids. The level of over-expression of IGF-2 mRNA was inversely associated with patient age. The level of IGF-2 up-regulation in uterine fibroids of the
that from the different endometrial phases, immunoscores for IGF-2 seemed to be higher in the luteal phase and lower in the inactive endometrium (data not shown). The immunoreactivity for IGF-2 seemed to be positively associated with fibroid size, but no statistical significance was observed ($P > 0.05$) (Fig. 2E).

P1 in IGF-2 is the only promoter transcribed biallelically, whereas the other three promoters are tightly controlled by maternal imprinting (32). There was no detectable P1 transcript in myometrium and a significant increase of P2 transcript in fibroids compared with myometrium (Fig. 2A, 3A). To investigate whether the differential usage of IGF-2 promoters was associated with the different methylation status, we examined methylation in IGF-2 promoters P1 and P2. We selected approximately 500 base pairs of genomic sequences from immediately upstream of the P1 and P2 transcription start site (Fig. 3B), which is enriched in CpG content. Purified genomic DNA from fibroids and normal myometrium with or without bisulfide treatments (see Materials and Methods) was amplified by PCR (primers in Table 2). The amplified IGF-2 promoters P1 and P2 DNA were digested by restriction enzymes Hae III (CCGG) and Smal (CCCCCCG) (Fig. 3B and C), respectively. Both IGF-2 promoters P1 and P2 exhibited significant methylation in one allele (Fig. 3C). The results indicated that transcription is likely occurring from one allele only. However, the molecular mechanism responsible for IGF-2 mRNA over-expression in fibroids remains unknown.

Activity of p-AKT and p-S6K in Fibroids
To study the activity of p-AKT and p-S6K in fibroids, we examined total AKT, phosphorylated AKT (p-AKT [Ser473]), and p-S6K (p70 S6K [Thr389]) by immunohistochemistry. Both phosphorylated AKT and S6K showed characteristic cytoplasmic immunoreactivity (Fig. 4A). Among 180 cases, 30% and 31% of fibroids had detectable immunoreactivity for p-AKT and p-S6K, respectively (Fig. 4B). Because the immunostains for these two proteins were patched, the positive rate in fibroids could be underscored by tissue microarray of the sampling errors. Nevertheless, only <5% of myometria were immunopositive for p-AKT and p-S6K. There was a statistically significant difference in the mean gene expression between normal and fibroid tissues ($P < 0.001$). There was no significant association between the immunoreactivity for p-AKT and p-S6K with tumor size (Fig. 4E and F), patient age, and endometrial phase (data not shown). No significant difference of nonphosphorylated AKT was found between fibroids and matched myometrium.

The activations of the phosphorylated p-AKT in fibroids were confirmed by Western blot. As illustrated in Figure 4E, immunostain-positive p-AKT forms in fibroids showed much higher phosphorylated p-AKT in fibroid than matched myometrium, whereas no difference of nonphosphorylated AKT between fibroid and myometrium was noted.

Correlation analysis demonstrated that there was a moderate correlation between the immunoreactivity of p-AKT and that of p-S6K ($r = 0.64$), and a weak to moderate correlation between IGF-1 and both p-AKT and p-S6K ($r = 0.31$ and
Because not all fibroids with IGF-1 over-expression had p-AKT and p-S6K over-expression, it is possible that a negative feedback loop of hyperactive IGF signaling may reduce the levels of p-AKT and p-S6K activity (33).

In contrast, over-expression of IGF-2 was present in more than two thirds of fibroids (both in protein and mRNA levels). Correlations of IGF-2 with p-AKT and p-S6K were very weak (r < 0.20). The role of IGF-2 in activating IGF signaling in fibroids remains to be established.

**Differential Expression of TSC Genes and Gene Products in Fibroids**

To evaluate whether under-expression of the TSC2 gene product in fibroids occurred at the transcriptional level, we performed RT-PCR (primers in Table 2) in a total of 30 fibroids and matched myometrium. There was no significant difference of the TSC2 mRNA expression between fibroids and matched myometrium (P>0.05, data not shown). To confirm our previous findings of down-regulation of the TSC2 gene product tuberin, we examined the immunoreactivity for tuberin in an additional 120 cases (TMA2) and found a reduction of immunoreactivity for tuberin, as previously reported (Fig. 5A and B). To validate the immunohistochemistry results, we examined tuberin by Western blot in five randomly selected patients, from each of whom large (≥10 cm) and small (≤3 cm) fibroids and matched myometrium were selected. With an appropriate loading control (Erk), reduction of tuberin was evident in most fibroids (Fig. 5C). Therefore, the reduction of tuberin may be an important molecular defect and may play a role in the tumorigenesis of uterine leiomyomas. It has been shown that activated p-AKT inactivates tuberin and results in its down-regulation (34, 35). In our large cohort of 180 fibroids, over-expression of p-AKT was found in approximately 30% of tumors, whereas under-expression of tuberin was detected in >50% of tumors. By correlation analysis, a lower correlation index (r < −0.2) was established between the down-regulation of tuberin and the increase of p-AKT.

*FIGURE 3*

Methylation analysis of IGF-2 promoter 1 and 2 in fibroids and myometrium. (A) Two cases with significant over-expression of IGF-2 in both large (L) and small (S) fibroids (using IGF-2 P1 and P2) were selected for methylation analysis. (B) Sketch diagram of the genomic DNA immediately upstream of IGF-2 P1 (518 base pairs) and P2 (485 base pairs) selected for methylation analysis. The sites of restriction enzyme SmaI and HaellII were indicated by arrows. Arrow of SmaI is a polymorphic site. (C) Methylation analysis of IGF-2 P1 and P2 in fibroids and matched myometrium (MM). Insulin-like growth factor-2 promoter DNAs with and without bismuth treatment were amplified by PCR, then digested with SmaI (P1) and HaellII (P2), and size-fractionated in agarose gels. The methylation patterns of P1 and P2 in fibroids were identical to that in myometrium, in which only one allele had high levels of methylation.
Additional in vitro studies may be necessary to characterize the role of p-AKT in regulating tuberin in fibroids.

To test whether activated mitogen-activated protein kinase phosphorylates TSC2, contributing to TSC2/TSC1 complex disruption, we examined p-TSC2 (Ser664, epitope specifically phosphorylated by p-ERK, developed by Dr. Pandolfi’s laboratory [46]). Among all 180 fibroids, no immunoreactivity for p-TSC2 was found, indicating that this mechanism does not contribute to the inactivation of the TSC complex.

We found similar levels of $TSC1$ mRNA expression in both fibroids and matched myometrium ($n = 30$) according to semiquantitative RT-PCR (data not shown). There was a slight, but significant, increase of immunoreactivity for hamartin in fibroids compared with the matched myometrium ($P < .05$; Fig. 5A and B). This finding was further confirmed by Western blot analysis (Fig. 5C and D). There were no significant differences in TSC1 expression linked to tumor size (Fig. 5), patient age, and sex steroid hormonal status (data not shown).

**DISCUSSION**

Englund et al. (10) reported a significant increase of IGF-1 mRNA and protein expression in fibroids by a small cohort...
Differential expressions of IGF-1 been reported in fibroids from human and Eker rat (7, 10, 36). However, significant over-expression of IGF-1 mRNA was identified in only 1 of 12 studies by a global gene expression profiling analysis (5). To investigate whether over-expression of IGF-1 is a common feature in fibroids, we examined IGF-1 expression at transcriptional and translational levels. We found that differential expression of IGF-1 in fibroids was only apparent at the protein, not at the mRNA level. The findings suggested a post-translational regulation of IGF-1 in fibroids, particularly in those of larger size.

A potential mechanism controlling translational regulation of IGF-1 in fibroids may be microRNA (miRNA) deregulation. We recently found that miR-29b was significantly down-regulated in fibroids compared with matched myometrium. The level of miR-29b down-regulation was linearly correlated to tumor size (37). By computer prediction, there are at least two miRNA regulation elements (miREs) of miR-29b at IGF-1 3′UTR (3′ untranslated region). We proposed that a loss of miR-29b in fibroids may rescue the negative regulation of IGF-1 translation in fibroids.

To test our hypothesis, we conducted transient transfection in triplicate, in which we transfected miR-29b mimics (Dharmacon, Chicago, IL) into fibroid primary cell cultures. After 48 hours, miR-29b-treated cells and control cells were harvested, and the IGF-1 protein was measured by ELISA analysis (Roche, Indianapolis, IN). We found that there were no significant IGF-1 protein changes in fibroid primary cell cultures either with or without the miR-29b addition. Our results suggest that translational modifications of IGF-1 may be regulated by other as-yet identified mechanism other than by miR-29b.
Over-expression of IGF-2 in fibroids has been identified in many independent global gene profiling analyses (15–21). Insulin-like growth factor-2 is one of a few growth factors identified by global transcription profiling as up-regulated, and the level of its over-expression is generally in the range of a two- to threefold increase in fibroids compared with matched myometrium (5). In contrast to IGF-1, over-expression of IGF-2 occurs at the transcriptional level. We found that more than two thirds of fibroids have an over-expression of IGF-2 mRNA. Insulin-like growth factor-2 is one of a few genes that are regulated by imprinting. Previous studies found no evidence of a loss of imprinting of IGF-2 in fibroids (22–24). High usage of promoters of all isoforms, rather than a change in methylation or imprinting, is the major contribution to the over-expression of IGF-2 in fibroids.

We found a significant association between IGF-1 levels and the follicular phase or estrogen status. The molecular relationship between up-regulation of IGF-2 with the sex steroid hormone status is of great interest. Although there are a few observations of the molecular interaction between IGF-2 and estrogen in certain types of tissues (38, 39), this interaction has not been established in fibroids (8). The IGF-2 regulatory elements contain binding domains specific to some nuclear receptors, such as RXRα, and ERβ (40), suggesting that these receptors may have the potential to regulate IGF-2.

The IGF-2 gene contains multiple promoters that transcribe different IGF-2 mRNA isoforms in human tissue (30). All published studies support the maintenance of imprinting of IGF-2 in fibroids (22–24). Therefore, a different mechanism must be responsible for the increased expression of IGF-2 in fibroids. Because use of different promoters is a mechanism that regulates gene expression levels (41), we examined whether different IGF-2 promoters may be responsible for an up-regulation of IGF-2 in fibroids. It has been shown that among four alternating initial exons of IGF-2, only P1 is not regulated by the imprinting mechanism in normal tissue (32). P1 has its minimal activity in myometrium. In contrast, P1 is substantially over-expressed in fibroids. Although P2–P4 are tightly controlled by maintenance of imprinting in both uterine fibroids and myometrium, over-expressions of IGF-2 from P2 and P3 are also evident. Our findings indicate that using different IGF-2 promoters contribute to its up-regulation, at least in part, in human uterine fibroids. Therefore, IGF-2 P1 is susceptible to regulation at the transcriptional level.

Although IGF-1 transcription can be promoted by estrogen in both normal and tumor tissue (Fig. 1C), only a portion of fibroids (approximately one third) have increased IGF-1 protein production, and therefore the over-expression of IGF-1, particularly in large fibroids (Fig. 1F), may contribute to its mitogenic role in tumor growth. Future studies that identify how fibroids gain the over-expression of IGF-1 at the translational level may eventually help in developing specific targets of IGF-1 gene products in fibroids.

AKT and S6K are key downstream molecules in the IGF signaling pathway that regulate protein synthesis and gene transcription. The activities of AKT and S6K are largely dependent on their phosphorylation status. The most significant finding is that p-AKT and p-S6K are relatively specific to fibroids. Because IGF-1 can be detected in both tumor tissue and matched myometrium, evaluation of the net gain or loss of IGF-1 may introduce human errors. p-AKT and p-S6K can be used as markers to determine which fibroids are likely associated with activation of the IGF signaling pathway.

It has been well recognized that TSC1 and TSC2, as tumor suppressors, negatively regulate IGF-AKT-mTOR signaling in tumorigenesis (42–44). In human, loss of TSCs induces many benign and malignant neoplasms. It remains uncertain whether a genetic alteration of TSC genes can produce human uterine fibroids. In our previous study we found that nearly 50% of fibroids displayed either a reduction or absence of tuberin expression (28). A genetic defect of TSC2 gene in Eker rats has been found to be a likely causative factor inducing fibroid development through loss of heterozygosity (45). Loss or reduction of the TSC2 gene product tuberin in human fibroids identified by our study could be an important molecular defect associated with human fibroid development. Future studies will focus on exploring the cause of how tuberin is lost and whether loss of tuberin can promote fibroid growth. Additional in vitro assays may be needed to characterize whether IGF-AKT activation can destabilize tuberin in fibroids.

This is the first attempt to examine IGFs and related gene expression at both the transcriptional and translational levels in a large cohort of fibroids. In addition to our findings supported by previous studies, we provide quantitative data regarding the extent to which differential expression of IGFs and their signaling activities contribute to the molecular pathogenesis of fibroids. The findings will be valuable in our understanding of the role of IGF signaling and will be further used to guide case selection for symptomatic patients who may benefit from anti-IGF treatments.

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