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New potential regulators of uterine leiomyomata from DNA arrays: the ionotropic glutamate receptor GluR2☆

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Abstract

In the post-Genome era, new concepts emerge about the growth regulation of uterine leiomyomata. Screening of leiomyoma and myometrial tissues with DNA arrays revealed numerous genes up-regulated in leiomyomata that were not known to be expressed in the human uterus. GluR2, a subunit of a ligand-gated cation channel, is up-regulated in leiomyomata relative to myometrium by 15- to 30-fold at the protein and mRNA level and is localized in endothelial cells. GluR2 pre-mRNA in leiomyoma and myometrial tissues is nearly 100% edited at the Q/R site, indicative of low Ca^{2+} permeability of the ion channels. In spontaneous leiomyomata in women or leiomyomata induced in the guinea pig model, there is a likely synergism linking increased production of estradiol and all-*trans* retinoic acid with up-regulation of nuclear receptor PPARγ and RXRα proteins to support tumor growth. GluR2 might be coupled to this synergism directly or via interleukin-17B, kinesin KIF5 or related genes also up-regulated in leiomyomata. GluR antagonists should be tested as inhibitors of leiomyoma growth.

Keywords: AMPA receptors; Arrays; Glutamate receptors; Leiomyoma; Myometrium; Neovascularization; Uterine neoplasms

Uterine leiomyomata or fibroids are the most common tumors of the female genital tract (2–13 per 1000 person-years) [1] and the primary indication for a hysterectomy in the USA [2]. Leiomyomata grow slowly [3] and become a clinical problem mainly in the fourth decade of life. At greater risk for leiomyomata are women with increased body mass index and African American women [4,5]. At lower risk are women with increased age at menarche, greater number of live born children, and cigarette smokers; an association between incidence and history of nulliparity or infertility was found only with submucous leiomyomata [5]. Genetic factors could also affect leiomyomata [1,6].

Each leiomyoma is a benign, monoclonal, smooth muscle cell tumor that develops independently from other leiomyomata in the uterus. Chromosomal translocations, deletions, inversions, or breakpoints are found only in 40% of leiomyomata and may occur as secondary events [7,8]. In terms of sex steroid hormones, aromatase is higher in leiomyoma than in myometrium, supplying high estrogen levels to leiomyomata [9]; progesterone may also contribute to leiomyoma growth, since RU-486 is as effective as GnRH agonists in initiating leiomyoma regression [10].

Leiomyomata contain 4-fold higher levels of all-*trans*-retinoic acid and nuclear receptor RXRα and PPARγ proteins than myometrium, but only in the follicular phase of the menstrual cycle [11]; the latter suggests that gene expression in leiomyomata has not become independent but tracks the ovarian hormone cycle. In the guinea pig model, troglitazone (a PPARγ

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ligand) when combined with estradiol and all-*trans*-retinoic acid induced the largest uterine leiomyomata [11]. Estradiol, retinoic acids, RXRα, and PPARγ provide the framework of the "pre-array" leiomyoma mechanism, where "new" regulators from genome-wide array screening can be added.

Array experiments are comprehensive, unbiased, and fast and will help us establish the "necessary and sufficient" molecules controlling leiomyoma growth in the vast majority of women. The expression of up to 12,000 genes was measured in leiomyoma and matched myometrial tissues from a diverse group of nine patients using the HuGeneFL6800 and Hu95A arrays (Affymetrix Inc, Santa Clara, CA) [12]. Among the 60 genes up-regulated in leiomyomata relative to myometrium were three paternally expressed imprinted genes (IGF2, MEST, and dlk 1, which is distantly related to the Delta and Notch family of proteins), doublecortin, ionotropic glutamate receptor subunit 2 (GluR2 or GluRB), apolipoprotein E3, IGFBP-5, myelin proteolipid protein, kinesin KIF5B, frizzled-2, matrix metalloproteinase-14, TGFβ3, and CRABP-II. Among the 80 down-regulated genes were alcohol dehydrogenases 1α–γ, dermatopontin, thrombospondin-1 (an anti-angiogenic factor), coxsackievirus and adenovirus receptor (involved in cell–cell interactions and tight junctions), cyr61, nur77, and c-kit. Some down-regulated genes are more likely to belong to the myometrial-contraction apparatus, which is lost in leiomyomata, than to function as tumor suppressor genes.

Gene expression patterns in leiomyoma and myometrium may depend on the patient's biological profile, such as ethnicity, age, body mass index, and perhaps, adenomyosis. Since the myometrium and endometrium are not homogeneous anatomically or endocrinologically [13], the locations of the tissues sampled could affect the observed fold-change in expression (leiomyoma:myometrium or L:M) [14]. Walocha et al. showed by scanning electron microscopy that at the leiomyoma periphery there is an extremely dense vascular area that supplies and drains the tumor [15]; large avascular regions occur in the leiomyoma center and in smaller leiomyomata [16]. To allow comparisons of gene expression studies from different laboratories the site of leiomyoma and myometrial sampling should be specified. We had decided earlier to collect samples from the leiomyoma periphery for use in DNA array studies (as shown in Fig. 1 of Ref. [12]) and found high expression of GluR2 (gene symbol GRIA2) in leiomyomata.

Among the AMPA, kainate, and NMDA classes of ionotropic neurotransmitter receptors, the AMPA class consists of GluR1–GluR4 subunits; GluR2 dominantly confers low Ca^{2+} permeability to the channel [17]. GluR2 is known to undergo editing in its pre-mRNA changing an adenosine to an inosine, which results in the incorporation of an arginine rather than a glutamine at a critical position in the channel pore (Q/R site) [18,19]. This re-coding occurs nearly 100% in neuronally expressed GluR2 and is dominant in suppressing the Ca^{2+} permeability of the ion channel containing GluR2 subunits. Since impairment in the RNA editing of GluR2 has been linked to the progression of brain tumors [20], the RNA editing status of GluR2 transcripts was analyzed in leiomyoma and myometrial tissues. Because of the unprecedented high fold-change (L:M) of GluR2 mRNA levels in leiomyomata, we sought to confirm the array results by RT-PCR, Western blots and immunohistochemistry.

Materials and methods

GluR2 RT-PCR and mRNA editing. Human tissues were obtained according to a protocol approved by the University of South Florida Institutional Review Board. Total RNA was prepared as described [12] with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) using 300 mg tissue from 10 pairs of leiomyoma and myometrium collected at the follicular ($n = 4$) and luteal ($n = 6$) phases of the menstrual cycle, was digested with RNase-free DNase I (Roche Diagnostics, Indianapolis, IN, USA) and tested for integrity; 15 μg total RNA was used to synthesize cDNA with Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. All PCR tests were run including 'mock'-transcribed RNA as negative control. For amplification of GluR2, cDNA fragments covering the Q/R editing position, PCR with primers hRB1D 5′-TGCGGTACCTTTAGCCTA-TGA GATCTGGATGTGC-3′ (exon 11, sense, *Kpn*I) and hRB2U 5′-CA GAATTCGTGTAG-GAGGAGATTATGATCAGG-3′ (exon 12, antisense, *Eco*RI) was performed with 3 cycles of 94 °C for 20 s, 57 °C for 30 s, 72 °C for 30 s, and 32 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, as well as a final extension at 72 °C for 10 min [20]. RT-PCR amplicons from GluR2 were directly sequenced after gel purification. Dideoxy-sequencing was carried out at the MIT Cancer Center Sequencing Facility.

Western blots, immunohistochemistry, and calcium determination. Different leiomyoma and myometrial tissue samples from the nine uteri studied earlier using DNA arrays [12] were homogenized, as previously described [11], in a solution containing 50 mM sodium phosphate, pH 7.8, 120 mM NaCl, 100 mM NaF, 0.05% Nonidet P-40 detergent, PMSF (10 μg/ml), 20 μM each of proteasome inhibitor MG-132 (Peptide Institute Inc., Osaka, Japan), martix metalloprotease inhibitor MMP-200, cathepsin-L inhibitor Z-LLY-FMK, pan-calpase inhibitor FK-009, and 0.1 mg/ml each of calpain inhibitor I (FK-017) (all from Enzyme Systems Products, Livermore, CA, USA) and calpain inhibitor (Calbiochem, San Diego, CA, USA).

The homogenates were processed and used for Western blot analysis to detect GluR2 and α-desmin (monoclonal antibodies 60011 A to the N-terminal domain of GluR2, PharMingen International, San Diego, CA; and D-1033, Sigma Chemical Co., St. Louis, MO, USA, respectively), as described [11] except that samples were heated to 37 °C, not 100 °C, in the presence of dithiothreitol. The GluR2 blots were subsequently probed for α-desmin without stripping. Ca^{2+} levels [21] were measured in the same tissue extracts with the Roche/Hitachi Modular P system (Roche Diagnostics, Indianapolis, IN, USA).

Paraffin blocks were stained for GluR2 with polyclonal antibody AB1506 to the C-terminal domain (Chemicon International, Temecula, CA, USA) by the avidin-biotin technique using $LSAB^{+}$ kit (DAKO, Carpenteria, CA, USA) and DAB chromogen. For α-desmin staining, the monoclonal antibody M0760 and $LSAB^{+}$ kit from Dako and VIP chromogen (Vector, Burlingame, CA, USA) were used. For double staining, the GluR2 rabbit antibody was applied first at 1:50

dilution for 30 min and was detected with LSAB$^+$ kit and DAKO DAB$^+$ chromogen, followed by α-desmin as the second antibody at 1:50 for 30 min detected with LSAB2 kit and VIP chromogen. In negative controls for all experiments, the primary antibodies were replaced with rabbit or mouse IgG.

Results

GluR2 cDNA was amplified from random-primed reverse transcribed total RNA with PCR primers hRB1D and hRB2U [20]. The relative signal strength of the obtained amplicons from leiomyoma over myometrium confirmed the DNA array data [12], indicating that the expression of GluR2 is highly up-regulated in leiomyomata (data not shown).

Analysis of RNA editing at the Q/R position was performed by direct sequencing of the specific PCR amplicon and quantification of the A/G peak ratio at the editing site (Fig. 1) [20]. All leiomyoma specimens tested showed markedly increased GluR2 expression relative to myometrium and all but one leiomyoma sample showed 100% editing, which is the same value displayed by the control myometrial tissues; the leiomyoma from array patient 6 [12] showed 80% editing. These results indicate that the A-to-I editing machinery (ADAR2 [19]) is indeed active with the same specificity and efficiency in leiomyoma and myometrium as in the nervous system.

Based on known GluR2 function in the nervous system, individual GluR2 channels expressed in leiomyoma and myometrium possess low Ca^{2+} permeability. However, the macroscopic conductance mediated by the receptors might be significantly higher in leiomyoma due to strong GluR2 up-regulation. To test whether Ca^{2+} levels were higher in leiomyoma than in myometrium, presumably via increased GluR2 protein levels (see below), we measured Ca^{2+} in the tissue extracts used for GluR2 Western blots. Leiomyomata contained 0.16 ± 0.01 μg calcium per mg protein (mean ± SEM) whereas myometrium had 0.11 ± 0.01, respectively ($n = 9, p < 0.01$ by the paired t test). These values represent the "extractractable" Ca^{2+} during tissue homogenization, described under Methods, not total tissue Ca^{2+}.

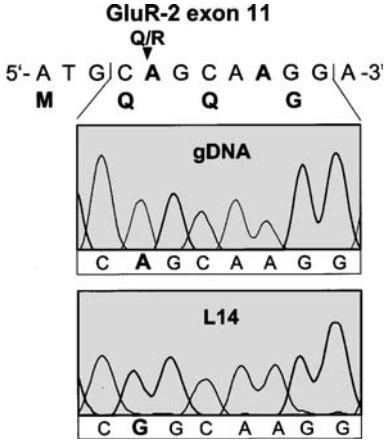

Fig. 1. Analysis of GluR-2 RNA editing status. The GluR-2 mRNA sequence around the Q/R editing site (arrow) is shown with the sequencing results (electropherograms from the automatic sequencer) for genomic DNA (zero editing control) and one characteristic example of a uterine leiomyoma (L14). Whereas the genomic DNA shows a single peak for A at the Q/R position, the analysis of the L14 derived GluR-2 mRNA indicates quantitative editing (pure G signal) at the Q/R site [20].

Western blots confirmed GluR2 up-regulation in leiomyomata at the protein level, as shown in Fig. 2 (top). A single GluR2 band at approximately 87 kDa (instead of 102 kDa in neurons) was found in leiomyomata and a less intense band in matched myometrial samples. Band intensities indicate that GluR2 is not a rare protein in leiomyomata. Fold-change (L:M) in GluR2 expression was calculated after band intensity was normalized to α-desmin (Fig. 2, bottom), marker for the majority of cells in leiomyomata and myometrium that correlates with total protein. L:M for patients 1–4 was 31.4, 8.1, 2.8, and 5.7, respectively. Mean L:M was 16.4 among the nine uteri tested with $p < 0.0001$ between leiomyoma and myometrium by the paired t test of log-transformed data. There is no correlation between fold-change for protein (Fig. 1 and patients 1–4) and mRNA (as shown in Table 1 of Ref. [12]). Lack of correlation could be due to technical limitations [14] and the fact that 0.3 g of different (adjacent) tissues was used for arrays than Western blots. Given the steep expression gradients that most likely exist within each leiomyoma [15] and along the length of the myometrium, the tissue samples used may be different affecting the fold-change for GluR2.

Immunohistochemical experiments revealed that GluR2 is localized not in smooth muscle cells but endothelial cells of the blood vessels in leiomyoma and myometrium (Fig. 3). These data strengthen Nowak's proposal [22] that leiomyomata are a disease of abnormal blood vessels and, in addition to smooth muscle

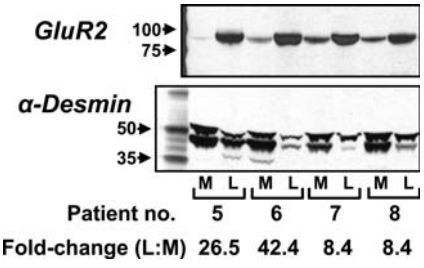

Fig. 2. Western blots with antibodies to GluR2 and α-desmin of leiomyoma (L) and matched myometrial (M) tissue extracts from patients 5–8, studied earlier with DNA arrays [12]. GluR2 band intensity was normalized to α-desmin, as a measure of protein loading, to derive the fold-change in GluR2 protein expression in leiomyoma relative to myometrium. Arrows point to the molecular mass markers.

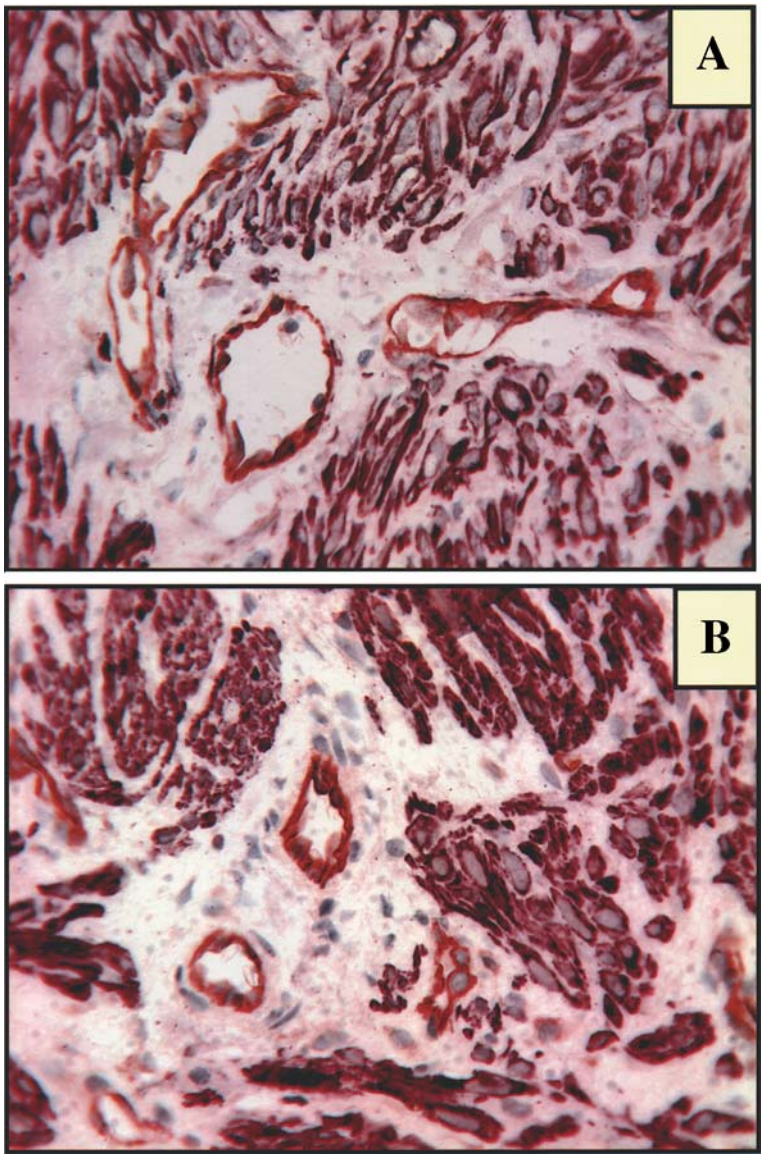

Fig. 3. Immunohistochemical staining of GluR2 (brown) and α-desmin (purple) in a leiomyoma (A) and myometrium (B) from patient no. 8, previously studied with DNA arrays [12]. Strong GluR2 staining is present in vascular endothelial cells and not smooth muscle cells (purple) of leiomyoma and myometrium. Original magnification ×63.

cells, endothelial cells [23] are important for leiomyoma growth.

Discussion

In the central nervous system, GluR2 binds to and signals through Lyn, a non-receptor protein tyrosine kinase [24]. Activation of Lyn, which is independent of Ca^{2+} and Na^{+} influx through GluR2, generates intracellular signals from the cell surface to the nucleus via the MAPK pathway and BDNF (brain-derived neurotrophic factor). Besides BDNF, the GluR2 gene promoter is also regulated by the glial cell line derived neurotrophic factor (GDNF) in a mechanism that involves the neuron-restrictive silencer element (REST/NRSF) [25].

The up-regulation of GluR2 in leiomyomata, and not of the other AMPA receptor subunits, predicts that any functional glutamate receptors would consist mostly or exclusively of GluR2 homomeric channels [26,27]. If higher glutamate concentrations in leiomyomata caused induction of glutamate receptors, why was the induction confined to GluR2 and not GluR1, GluR3, and GluR4? mRNA levels for GluR1, GluR3, GluR4, the metabotropic glutamate receptors, glutaminase and glutamate transporters, metabolizing enzymes were not different by more than twofold in leiomyoma compared to myometrium (array data in Ref. [12]; not shown).

The mechanism of GluR2 up-regulation in leiomyomata may have common elements with the incorporation of AMPA receptors, composed only of GluR2 subunits, in the synapses [17,28] where GluR2 function depends on a critical number of interactions of its C-terminus with cytosolic proteins such as GRIP, a glutamate receptor-interactive protein [28,29] that contains a PDZ-domain [30], PICK1, and kinesin KIF5 [17]. In leiomyomata, kinesin family members KIF5B and KIF5C were up-regulated by 9-fold [12] and 5-fold, respectively.

Is there a cause–effect relationship between GluR2 up-regulation and leiomyoma vascularization? One possibility is that increased Ca^{2+} influx into leiomyomata, facilitated by higher levels of GluR2 protein, could enhance tumor growth by supporting vascularization [31] mainly at the tumor periphery [15]. The 40% higher Ca^{2+} levels found in extracts of leiomyoma than in myometrium are compatible with this interpretation.

Angiogenic factors VEGF [32], bFGF, adrenomedullin (reviewed in [15]), and EGF and PDGF [33] are found in human leiomyomata and myometrium, but compared to GluR2 their expression is not as prominent in leiomyomata as in myometrium. Interleukin-17, recently shown to promote neovascularization in the retina [34], is up-regulated in leiomyomata by 30-fold as shown by U133A/B DNA arrays (Tsibris et al., unpublished data) and by 3-fold in a recent study [35].

In a newly developed mouse model for uterine leiomyosarcomas, GluR2 is up-regulated 12-fold relative to control [36]. If GluR2 up-regulation marks an event related to neovascularization, it would apply to growth regulation of malignant and benign solid tumors.

The structure of the GluR2 complex in leiomyomata can be studied with a variety of methods, such as chemical cross-linking, immunoprecipitation, and new mass spectroscopic analyses [37]. It is likely that some gene products co-induced in leiomyomata along with GluR2 (e.g., kinesin KIF5, calpain-6, and interleukin-17B) will be associated with the GluR2 complex. It is also possible that the GluR2 complex in leiomyomata would interact directly or indirectly with the "pre-array" leiomyoma inducers estradiol, retinoic acid, RXRα, and PPARγ [11].

GluR2 is found in other tissues outside the nervous system [38] and glutamate antagonists can limit the growth of colon, lung, and breast cancer cells [39]. Ionotropic glutamate receptor antagonists [39–41] should be tested as potential medications to relieve leiomyoma symptoms in perimenopausal women in combination therapy with selective estrogen receptor modulators (SERM), progesterone receptor modulators [42], and antagonists of RXRα and PPARγ. Combination therapy may be preferable to single-drug therapy if it provides better efficiency with lower drug doses, fewer side effects, and delay in developing drug resistance.

Laparoscopic or ultrasonically guided injections of drug cocktails into large leiomyomata that cause clinical symptoms may be the treatment of choice when a hysterectomy, myomectomy, or other procedures are contraindicated and before leiomyomata become calcified or necrotic. Ideally, these drugs should cause leiomyoma regression without adversely affecting the myometrium.

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