Combined differential gene expression profile and pathway enrichment analyses to elucidate the molecular mechanisms of uterine leiomyoma after gonadotropin-releasing hormone treatment

Composite regulatory signature database (CRSD), a self-developed comprehensive Web server for composite regulatory signature discovery, used to compare the published microarray data with our data on patients with uterine leiomyoma treated with or without GnRH analogue (GnRH-a), revealed that the focal adhesion, mitogen-activated protein kinase (MAPK), CXC chemokine receptor 4/stromal-derived factor-1 (CXCR4/SDF-1), T-cell receptor, integrin, vascular endothelial growth factor (VEGF), GnRH, and transforming growth factor-β (TGF-β) signaling pathways are highly expressed in uterine leiomyoma and significantly down-regulated after GnRH-a treatment. According to the results these signaling pathways could be involved in inflammation, proliferation, and remodeling processes of leiomyoma development and possibly in the regression of leiomyoma after GnRH-a treatment, which might improve our understanding of the mechanisms of leiomyoma formation and help us to find novel drug targets or specific markers for diagnosis and prognosis in uterine leiomyoma. (Fertil Steril® 2008;90:1219–25. ©2008 by American Society for Reproductive Medicine.)

Myomas are benign, monoclonal tumors from the smooth muscle cells of the myometrium, which is one of the most common gynecological diseases and might cause infertility (1–3). The mechanisms that could cause the pathogenesis of leiomyoma and affect reproductive ability remain unclear. Several genetic and epidemiological studies have shown that genetic alterations, including estrogen (E) receptor-α polymorphism, might play an important role in leiomyoma development and could be a target for gene therapy (4–6).

Cytogenetic studies also indicated that 40% of leiomyoma are chromosomally abnormal and some of the candidate genes in these chromosome regions showed some relationship in uterine leiomyoma, including *CUTL1, ORC5L, DLX5, 6, PCOLCE* genes on chromosome 7q22q23, high mobility group (HMG) *HMGIY* gene on chromosome 6p21, *HMGIC* on chromosome 12q15, E receptor β *(ESR2)* on chromosome 14q22, and *RAD51L1* gene on chromosome 14q23 (7–9). However, the other 60% of leiomyoma might have undetected mutations. To identify the critical genes involved in uterine leiomyoma, a cDNA microarray screening method was used in more than a dozen previous studies (7, 10, 11). After comparing the gene expression of the eight published studies on uterine leiomyoma, the overlapping gene alterations have shown that *ADH1, ATF3, CRABP2, CYR61, DPT, GRIA2, IGF2, MEST* are the highest ranked candidate genes (7). However, the gene regulations and potential signaling pathways in leiomyoma were still unknown.

In this study, to compare the published data with ours on differentially expressed profiles in uterine leiomyoma, some potential signaling pathways have been identified by carefully examining the published lists of differentially expressed genes and the overlapping pathways by the composite regulatory signature database (CRSD), a self-developed comprehensive Web server for composite regulatory signature discovery (12, 13). These results might provide helpful information on the hypothesized maps of the leiomyoma-related signaling pathways.

This study was approved by the Institutional Review Board (IRB) committee of the Taipei Medical University Hospital (Taipei, Taiwan). Myoma tissue samples with or without GnRH analogue (GnRH-a) treatment ($n > 8$ in each group) for cDNA microarray were obtained from women who were undergoing surgery and the samples were then stored in liquid nitrogen for mRNA extraction. Five micrograms of the mRNAs derived from tissues were labeled with biotin during reverse transcription (RT) and proceeded to cDNA microarray (carrying 9,600 polymerase chain reaction [PCR]-amplified cDNA fragments) analysis, which was described in our previous reports (14, 15). The microarray images were processed by commercial image processing programs to convert the true-color images into gray-scale images, and then the image analysis and spot quantification were done by the GenePix 3.0 (Axon, Union City, CA). The significance analysis of microarrays was performed by the fold-change analysis, which was calculated by comparing the gene expression levels in leiomyoma samples relative to leiomyoma with GnRH-a treatment. To compare our data

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TABLE 1

Potential pathways selected by the CRSD analysis in leiomyoma.

Pathway	Name	Total	Found	*P* value	*Q*-value	Associated genes (UniGene)	Ref.
KEGG	Focal adhesion	193	16	6.8E-6	9.3E-3	Hs.127897 (RAPGEF1) Hs.459691 (PDPK1) Hs.145442 (MAP2K1) Hs.247077 (RHOA) Hs.21 3861 (LAMA4) Hs.488293 (EGFR) Hs.395482 (PTK2) Hs.267659 (VAV3) Hs.474053 (COL6A1) Hs.390567 (FYN) Hs.591 600 (ROCK2) Hs.556600 (MYLK) Hs.505654 (ITGA5) Hs.645355 (ILK) Hs.643896 (VCL) Hs.645250 (COL1A1)	
			9	1.6E-8	7.4E-6	Hs.517601 (RAC2) Hs.474053 (COL6A1) Hs.203717 (FN1) Hs.143250 (TNC) Hs.247077 (RHOA) Hs.21 3861 (LAMA4) Hs.556600 (MYLK) Hs.446336 (PXN) Hs.490415 (ZYX)	28
			4	4.1E-3	3.8E-2	Hs.474053 (COL6A1) Hs.444356 (GRB2) Hs.395482 (PTK2) Hs.534951 (PIK3R3)	29
			24	1.3E-8	2.3E-5	Hs.861 (MAPK3) Hs.125503 (MAPK10) Hs.133397 (ITGA6) Hs.211426 (THBS4) Hs.525704 (JUN) Hs.631 564 (PRKCG) Hs.143250 (TNC) Hs.247077 (RHOA) Hs.443625 (COL3A1) Hs.534951 (PIK3R3) Hs.233240 (COL6A3) Hs.49041 5 (ZYX) Hs.509765 (ACTN1) Hs.517601 (RAC2) Hs.371147 (THBS2) Hs.474053 (COL6A1) Hs.508716 (COL4A2) Hs.558371 (RELN) Hs.444356 (GRB2) Hs.41 9815 (EGF) Hs.556600 (MYLK) Hs.445827 (COL5A2) Hs.645250 (COL1A1) Hs.78781 (VEGFB)	7[a]
KEGG	MAPK signal pathway	269	15	9.1E-4	7.3E-2	Hs.435512 (PPP3CA) Hs.145442 (MAP2K1) Hs.78846 (HSPB2) Hs.488293 (EGFR) Hs.291623 (TAOK2) Hs.531754 (MAP2K7) Hs.43505 (IKBKG) Hs.209983 (STMN1) Hs.466804 (PLA2G2A) Hs.150136 (MAPK7) Hs.111 (FGF9) Hs.505033 (KRAS) Hs.459642 (CACNA1H) Hs.184233 (HSPA9B) Hs.468239 (MAP4K3)	[b]
			6	1.3E-2	9.7E-2	Hs.284244 (FGF2) Hs.444356 (GRB2) Hs.514681 (MAP2K4) Hs.244139 (FAS) Hs.502875 (RELA) Hs.81328 (NFKBIA)	29

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TABLE 1

Continued.

Pathway	Name	Total	Found	P value	Q-value	Associated genes (UniGene)	Ref.
			27	1.3E-7	7.3E-5	Hs.443417 (MINK1) Hs.525704 (JUN) Hs.1183 (DUSP2) Hs.631564 (PRKCG) Hs.244139 (FAS) Hs.336916 (DAXX) Hs.171695 (DUSP1) Hs.517601 (RAC2) Hs.524430 (NR4A1) Hs.444356 (GRB2) Hs.463978 (MAP2K6) Hs.284244 (FGF2) Hs.861 (MAPK3) Hs.125503 (MAPK10) Hs.285354 (MAX) Hs.515032 (MKNK2) Hs.2780 (JUND) Hs.138378 (CASP4) Hs.514681 (MAP2K4) Hs.2561 (NGFB) Hs.186486 (MAP3K5) Hs.500067 (PPP3CB) Hs.1420 (FGFR3) Hs.645227 (TGFB1) Hs.153752 (CDC25B) Hs.419815 (EGF) Hs.5353 (CASP10)	7[a]
BioCarta	CXCR4 signaling pathway	24	4	1.9E-3	1.0E-1	Hs.522891 (CXCL12) Hs.145442 (MAP2K1) Hs.430425 (GNB1) Hs.395482 (PTK2)	[b]
			2	2.8E-3	5.5E-2	Hs.446336 (PXN) Hs.491 322 (PTK2B)	28
			2	3.0E-3	3.3E-2	Hs.502875 (RELA) Hs.395482 (PTK2)	29
			3	3.8E-2	3.9E-1	Hs.861 (MAPK3) Hs.430425 (GNB1) Hs.502875 (RELA)	7[a]
KEGG	T-cell receptor signaling pathway	92	10	4.0E-5	2.7E-2	Hs.267659 (VAV3) Hs.591 629 (CD28) Hs.517499 (GRAP2) Hs.435512 (PPP3CA) Hs.43505 (IKBKG) Hs.390567 (FYN) Hs.3003 (CD3E) Hs.458276 (NFKBIE) Hs.247077 (RHOA) Hs.505033 (KRAS)	[b]
			2	3.6E-2	3.0E-1	Hs.193717 (IL10) Hs.247077 (RHOA)	28
			4	1.6E-5	1.0E-3	Hs.444356 (GRB2) Hs.51 4681 (MAP2K4) Hs.502875 (RELA) Hs.81 328 (NFKBIA)	29
			12	3.6E-5	3.1E-3	Hs.193717 (IL10) Hs.500067 (PPP3CB) Hs.504096 (CBL) Hs.73917 (IL4) Hs.525704 (JUN) Hs.371 987 (NFAT5) Hs.444356 (GRB2) Hs.304475 (LCP2) Hs.247077 (RHOA) Hs.193516 (BCL10) Hs.81328 (NFKBIA) Hs.534951 (PIK3R3)	7
BioCarta	Integrin signaling pathway	36	6	1.5E-4	2.5E-2	Hs.127897 (RAPGEF1) Hs.390567 (FYN) Hs.145442 (MAP2K1) Hs.247077 (RHOA) Hs.643896 (VCL) Hs.395482 (PTK2)	[b]
			4	7.9E-4	2.3E-2	Hs.76206 (CDH5) Hs.520048 (HLA-DRA) Hs.77961 (HLA-B) Hs.503878 (NCAM1)	28
			2	6.6E-3	5.6E-2	Hs.444356 (GRB2) Hs.395482 (PTK2)	29

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TABLE 1

Continued.

Pathway	Name	Total	Found	*P* value	*Q*-value	Associated genes (UniGene)	Ref.
			7	1.4E-4	8.9E-3	Hs.861 (MAPK3) Hs.509765 (ACTN1) Hs.525704 (JUN) Hs.444356 (GRB2) Hs.247077 (RHOA) Hs.77793 (CSK) Hs.490415 (ZYX)	7[a]
KEGG	VEGF signaling pathway	69	7	8.5E-4	7.2E-2	Hs.435512 (PPP3CA) Hs.466804 (PLA2G2A) Hs.145442 (MAP2K1) Hs.438823 (NOS3) Hs.505033 (KRAS) Hs.78846 (HSPB2) Hs.395482 (PTK2)	[b]
			2	2.1 E-2	2.7E-1	Hs.51 7601 (RAC2) Hs.446336 (PXN)	28
			3	1.6E-3	2.5E-2	Hs.438823 (NOS3) Hs.395482 (PTK2) Hs.534951 (PIK3R3)	29
			7	5.8E-3	1.0E-1	Hs.861 (MAPK3) Hs.500067 (PPP3CB) Hs.51 7601 (RAC2) Hs.371987 (NFAT5) Hs.631564 (PRKCG) Hs.438823 (NOS3) Hs.534951 (PIK3R3)	7[a]
KEGG	GnRH signaling pathway	96	7	5.2E-3	1.8E-1	Hs.531754 (MAP2K7) Hs.443428 (ADCY4) Hs.466804 (PLA2G2A) Hs.145442 (MAP2K1) Hs.150136 (MAPK7) Hs.505033 (KRAS) Hs.488293 (EGFR)	[b]
			2	4.1E-2	2.0E-1	Hs.444356 (GRB2) Hs.51 4681 (MAP2K4)	29
			9	3.1E-3	7.5E-2	Hs.861 (MAPK3) Hs.2399 (MMP14) Hs.125503 (MAPK10) Hs.567295 (ITPR1) Hs.525704 (JUN) Hs.444356 (GRB2) Hs.463978 (MAP2K6) Hs.514681 (MAP2K4) Hs.799 (HBEGF)	7[a]
KEGG	TGF-β signaling pathway	81	2	2.9E-2	2.9E-1	Hs.247077 (RHOA) Hs.471119 (BMPR2)	28
			13	1.9E-6	5.4E-4	Hs.861 (MAPK3) Hs.36915 (SMAD3) Hs.211426 (THBS4) Hs.247077 (RHOA) Hs.79353 (TFDP1) Hs.371147 (THBS2) Hs.49787 (LTBP1) Hs.491440 (PPP2CB) Hs.645227 (TGFB1) Hs.445758 (E2F5) Hs.146806 (CUL1) Hs.519601 (ID4) Hs.471119 (BMPR2)	7[a]

Note: The potential signaling pathways have been identified by carefully examining the published lists of differentially expressed genes and the overlapping pathways by the self-developed composite regulatory signature database (CRSD) Web server (http://140.120.213.10:8080/crsd/) based on KEGG (http://www.genome.ad.jp/kegg/pathway.html) and BioCarta (http://www.biocarta.com/index.asp) pathway databases.

[a] Reference 7 contains the data from references 16 to 27. *P* value for a multiple hypothesis test, the false discovery rate (*Q*-value) was estimated in a previous study (12).

[b] Our current study.

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with other microarray studies of uterine leiomyoma, we have carefully examined the published lists of differentially expressed genes and identified the overlapping pathways by the self-developed CRSD Web server (12, 13).

According to cDNA microarray analysis, 172 genes were up-regulated (>3 times) in myoma without GnRH-a treatment, whereas 29 genes were highly up-regulated (>5 times) when compared with the gene expression levels in GnRH-a treated myoma. In contrast, 70 genes were down-regulated (>3 times) in myoma without GnRH-a treatment, whereas 17 genes were dramatically down-regulated (>5 times) when compared with the gene expression levels in the GnRH-a treatment group. The differentially expressed gene lists were carefully compared with previous reports on leiomyoma versus normal myometrium (7, 16–27) and leiomyoma with or without GnRH-a treatment (10, 19, 28, 29). These differentially expressed gene profiles were uploaded to the Website of CRSD (http://biochip.nchu.edu.tw/crsd1/) and compared. This comparison resulted in the identification of only a few genes in common. We confirmed the previous discussions in the reports by Arslan et al. (7) and Chegini et al. (19). The tissues collection from different phases of the menstrual cycle, differences in leiomyoma size, experimental process and different microarray platforms, and the method of data acquisition and analysis in these studies might lead to the expected variation in gene expression profiles (19). These limited amount of common genes might be helpful as specific markers for leiomyoma diagnostics; however, it still could not give us the perspective of knowing the pathogenesis of leiomyoma.

To broaden the scope on leiomyoma, we compared the common pathways, not just to match the single gene, from these studies and our data. Interestingly, although intrinsic individual genes are different in each study they may contribute to the same pathways, and common pathways could be identified. Table 1 shows overlapping common signaling pathways, including several reported pathways (mitogen-activated protein kinase [MAPK] signal pathway, vascular endothelial growth factor [VEGF] signaling pathway, GnRH signaling pathway, and transforming growth factor-β [TGF-β] signaling pathway) and some novel signaling pathways (CXCR4 signaling pathway, T-cell receptor signaling pathway, focal adhesion, and integrin signaling pathway). The most significant pathways should be focal adhesion (P=1.395 $\times$ 10^{-8}) (supplement data, Fig. S1) and MAPK signaling (P=1.322 $\times$ 10^{-7}) pathways, which are important in cell proliferation and migration. Furthermore, many kinase-related signaling pathways are also significantly increased in leiomyoma, including Wnt signaling pathway (P=6.298 $\times$ 10^{-5}), Jak-STAT signaling pathway (P=9.864 $\times$ 10^{-5}), and epidermal growth factor receptor (EGFR) tyrosine kinase signaling (P=2.921 $\times$ 10^{-5}) (supplement data, Table S1). These should be important in cell proliferation, migration, survival, and differentiation in leiomyoma.

Interestingly, many cytokines and chemokines are significantly increased in leiomyoma ($P = 3.835 \times 10^{-5}$; supplement data, Table S1). There are seven important genes in the CXCR4/SDF1 signaling pathway, including SDF-1, CXCR4, MAPK3, GNB1, PIK3R1, PTK2, and RELA (supplement data, Fig. S2, B). SDF-1 and CXCR4 are two important chemokine ligand/receptor pairs that play a crucial role in numerous biological processes, including hematopoiesis, cardiogenesis, vasculogenesis, neuronal development, immune cell trafficking, cell migration, and epithelial–mesenchymal transition. This has been suggested as a new therapeutic target of several diseases (30, 31). Recent studies also indicated that the SDF-1alpha/CXCL12-CXCR4 chemokine played an important role in the muscular infiltration of endometrial cancer through the activation of the PI3K-Akt signaling pathway and promotes VEGF-mediated tumor angiogenesis through the Akt signaling pathway (32, 33). Suppression of this pathway by CXCR4 monoclonal antibody (mAb) (12G5) or CXCR4 antagonist (AMD3 100) could be an effective target for the treatment of early uterine cancer (32). Our preliminary data also showed that patients with leiomyoma have higher serum levels of CXCL12/SDF-1 than patients treated with GnRH-a. These results and our novel finding indicated that the SDF-1/CXCR4 signaling might play a role in the pathogenesis of leiomyoma and could be a potential target for further gene therapy. Other chemokine/cytokine-related factors, leukemia inhibitory factor receptor (LIFR), interferon gamma receptor (IFNGR), Proto-oncogene tyrosine-protein kinase Kit (c-KIT), tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10), transforming growth factor-beta 1 (TGFB1), leptin receptor (LEPR), interleukin 10 (IL-10), tumor necrosis factor receptor superfamily, member 25 (TNFRSF25), chemokine (C-C motif) ligand 8 (CCL8), chemokine (C-C motif) ligand 15 (CCL15), interleukin 11 receptor, alpha (IL11RA), colony stimulating factor 2 receptor-alpha (CSF2RA), vascular endothelial growth factor B (VEGFB), bone morphogenetic protein receptor type II (BMPR2), fms-related tyrosine kinase 3 ligand (FLT3LG), interleukin 4 (IL4), chemokine (C-C motif) ligand 11 (CCL11), chemokine (C-C motif) ligand 21 (CCL21), interleukin 13 receptor, alpha 1 (IL13RA1), tumor necrosis factor receptor superfamily, member 11 b (osteoprotegerin, TNFRSF11B), epidermal growth factor (EGF), interferon-alpha receptor 1(IFNAR1), chemokine (C-X-C motif) ligand 5 (CXCL5), interleukin-15 receptor alpha (IL15RA), were highly regulated in leiomyoma, which suggest that these factors might play some important roles in the pathogenesis of leiomyoma (supplement data Table S1) (34, 35).

According to the pathway analysis, several kinases and intracellular signalings are highly expressed in leiomyoma (supplement data Table S1), including growth factor-related receptor tyrosine kinase signaling, E-dependent cyclin-dependent kinases, MAPK, nuclear factor-κB (NF-κB), and Jak-STAT signaling pathways. These data suggest and

illustrate that the newly developed kinases target therapies (e.g., receptor tyrosine kinase- I, PI3KI) and anti-inflammatory drugs (e.g., COX2 inhibitor, mTOR inhibitor) could shed some light in treating the cell proliferation in leiomyoma (36–38). Furthermore, these pathways from gene expression profiling provided more candidate targets for new drugs or other therapeutic intervention development.

By integrating multiple microarray datasets this approach suggested that several signaling pathways could play important roles in human uterine leiomyoma. Unlike the previous studies comparing the limited genes with expected variations, the pathway-predicting system provides a broader scope in studying leiomyoma and showed that these significantly regulated signaling pathways might be common and universal, as shown in these studies by different research groups. Further confirmation and validation of these genes and pathways in leiomyoma are necessary and undergoing, which can be used to treat and define their roles in the uterine fibroid development. To understand the maps and exact molecular interactions among these gene products in uterine leiomyoma provides new directions for therapeutic intervention in this disease.

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