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Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers

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Uterine leiomyomas are common benign smooth muscle tumors that impose a major burden on women's health. Recent sequencing studies have revealed recurrent and mutually exclusive mutations in leiomyomas, suggesting the involvement of molecularly distinct pathways. In this study, we explored transcriptional differences among leiomyomas harboring different genetic drivers, including high mobility group AT-hook 2 (*HMGA2*) rearrangements, mediator complex subunit 12 (*MED12*) mutations, biallelic inactivation of fumarate hydratase (*FH*), and collagen, type IV, alpha 5 and collagen, type IV, alpha 6 (*COL4A5-COL4A6*) deletions. We also explored the transcriptional consequences of 7q22, 22q, and 1p deletions, aiming to identify possible target genes. We investigated 94 leiomyomas and 60 corresponding myometrial tissues using exon arrays, whole genome sequencing, and SNP arrays. This integrative approach revealed subtype-specific expression changes in key driver pathways, including Wnt/β-catenin, Prolactin, and insulin-like growth factor (IGF)1 signaling. Leiomyomas with *HMGA2* aberrations displayed highly significant up-regulation of the proto-oncogene pleomorphic adenoma gene 1 (*PLAG1*), suggesting that *HMGA2* promotes tumorigenesis through *PLAG1* activation. This was supported by the identification of genetic *PLAG1* alterations resulting in expression signatures as seen in leiomyomas with *HMGA2* aberrations. RAD51 paralog B (*RAD51B*), the preferential translocation partner of *HMGA2*, was up-regulated in *MED12* mutant lesions, suggesting a role for this gene in the genesis of leiomyomas. *FH*-deficient leiomyomas were uniquely characterized by activation of nuclear factor erythroid 2-related factor 2 (NRF2) target genes, supporting the hypothesis that accumulation of fumarate leads to activation of the oncogenic transcription factor NRF2. This study emphasizes the need for molecular stratification in leiomyoma research and possibly in clinical practice as well. Further research is needed to determine whether the candidate biomarkers presented herein can provide guidance for managing the millions of patients affected by these lesions.

uterine leiomyoma | transcriptional profiling | MED12 | HMGA2

Uterine leiomyomas, also known as fibroids, are highly common benign tumors arising from the smooth muscle cells of the myometrium. Leiomyomas can cause a variety of health complications and are the leading indication for hysterectomy (1). The clinical and scientific community widely regards leiomyomas as a single entity, although substantial evidence of heterogeneity exists at several different levels, including symptoms, histopathology, therapeutic requirements, and genetic changes (2).

In terms of genetics, recent high-throughput sequencing studies have identified recurrent and mutually exclusive mutations in a limited number of key genes (3, 4), indicating the existence of molecularly distinct subtypes of leiomyomas. The currently established driver changes include high mobility group AT-hook 2 (*HMGA2*) rearrangements, mediator complex subunit 12 (*MED12*) mutations, and biallelic inactivation of fumarate hydratase (*FH*) (5). Leiomyomas with deletions affecting collagen, type IV, alpha 5 and collagen, type IV, alpha 6 (*COL4A5-COL4A6*) may constitute a rare fourth subtype (4). *HMGA2* and *MED12* represent the two most common driver genes and together contribute to 80–90% of all leiomyomas (5).

Less frequently, leiomyomas harbor 6p21 rearrangements affecting high mobility group AT-hook 1 (*HMGA1*) (6). Some of these rearrangements have been reported to co-occur with *MED12* mutations, suggesting that a subset of *HMGA1* rearrangements are secondary events (7). Leiomyomas also may harbor recurrent deletions and rearrangements of 7q22, 22q, and 1p (8–11). These deletions co-occur with other genetic alterations and may be secondary driver events, often present only in a subpopulation of tumor cells (4, 7, 8, 11–13).

Although several genetic defects underlying the development of leiomyomas have been discovered, the mechanisms of tumorigenesis remain poorly understood, and whether these mutations affect the same or different driver pathways is unclear. Previous expression profiling studies have discovered that hundreds of genes are differentially expressed between leiomyomas and normal myometrial tissue (9, 14–20); however, the majority of these studies have not accounted for the genetic background of the lesions examined. Therefore, we sought to explore the transcriptional differences and similarities among 94 leiomyomas from 60 patients harboring different genetic driver alterations, including *HMGA2* rearrangements, *MED12* mutations, biallelic inactivation of *FH*, *COL4A5-COL4A6*

Significance

The clinical and scientific community widely regards uterine leiomyomas as a single entity, although evidence of genetic heterogeneity exists. The aim of this study was to explore transcriptional differences between leiomyomas harboring different genetic alterations, including high mobility group AT-hook 2 rearrangements, mediator complex subunit 12 mutations, biallelic inactivation of fumarate hydratase, and collagen, type IV, alpha 5-collagen, type IV, alpha 6 deletions. The evidence presented herein strongly suggests that specific driver mutations are the major determinants of expression changes in leiomyomas. Here we highlight subtype-specific expression differences in key driver pathways and emphasize the utility of stratification in leiomyoma research. Finally, we offer a set of candidate biomarkers that will facilitate the molecular classification of leiomyomas.

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deletions, and leiomyomas lacking these four driver changes, henceforth termed quadruple-negative leiomyomas. We also explored the transcriptional consequences of recurrent 7q22, 22q, and 1p deletions, aiming to identify the possible target genes.

Results

Unsupervised Hierarchical Clustering Reveals Distinct Expression Profiles Associated with Specific Driver Mutations. To identify possible expression patterns associated with driver mutations, we integrated genome-wide expression and genomic data of 94 leiomyomas. The selection of these leiomyomas is described in detail in *SI Materials and Methods*. As determined by the screening of known driver changes using whole genome sequencing (WGS), SNP arrays, and *MED12* sequencing, the 94 leiomyomas included 27 with an *HMGA2* rearrangement, 34 with a *MED12* mutation, 10 with biallelic loss of *FH*, and 4 with a *COL4A5-COL4A6* deletion (Dataset S1). Unsupervised hierarchical clustering analysis of exon array data revealed that the vast majority of leiomyomas clustered according to the mutation status of these four driver genes (Fig. 1).

Pathway Enrichment Analysis Using Differentially Expressed Genes. To identify genes differentially expressed in the complete set of leiomyomas, we compared all 94 leiomyomas against the corresponding 60 myometrium tissue specimens. This comparison identified 135 genes as significantly differentially expressed [q <0.05; –2> fold change (FC) >2] (Dataset S2). Zinc finger, matrin type 3 (*ZMAT3*) was the most significant gene, up-regulated in all leiomyomas regardless of subtype (Fig. 2*A*).

To identify genes differentially expressed in leiomyomas of the *HMGA2*, *MED12*, *FH*, and *COL4A5-COL4A6* subtypes, we compared leiomyomas of each subtype against all of the myometrium tissue specimens. This revealed 265 genes significantly differentially expressed in leiomyomas of the *HMGA2* subtype, 258 genes significantly differentially expressed in leiomyomas of the *MED12* subtype, 296 genes significantly differentially expressed in leiomyomas of the *FH* subtype, and 198 genes as significantly differentially expressed in leiomyomas of the *COL4A5-COL4A6* subtype (q <0.05; –2> FC >2) (Fig. S1 and Dataset S2). Differential expression analysis was not performed with the quadruple-negative leiomyomas, owing to the high transcriptional heterogeneity revealed by the clustering analysis.

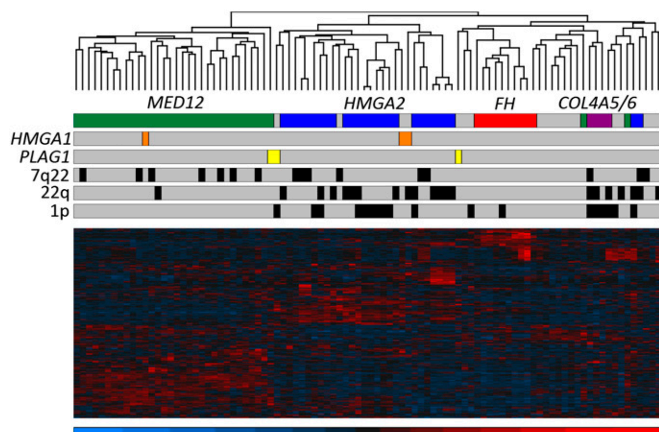

Fig. 1. Unsupervised hierarchical clustering of 94 leiomyomas from 60 patients. Hierarchical clustering using 1% most variable genes revealed that most leiomyomas grouped together according to the mutation status of *MED12* (green), *HMGA2* (blue), *FH* (red), and *COL4A5-COL4A6* (purple). The remaining quadruple-negative leiomyomas exhibited transcriptional heterogeneity and clustered into several unique branches; however, four of these clustered with leiomyomas of the *HMGA2* subtype, and two of them were found to harbor a genetic *HMGA1* alteration (orange). One leiomyoma (MY5008 m3) harbored both an *HMGA1* alteration and a *MED12* mutation, and consequently clustered with leiomyomas of the *MED12* subtype. We identified genetic *PLAG1* alterations (yellow) in three leiomyomas. Although one of these tumors (MY16 m1) also harbored a *MED12* mutation and clustered with the *MED12* subtypes, all three tumors also displayed expression signatures similar to those seen in leiomyomas with *HMGA2* or *HMGA1* alterations. Chromosomal deletions of 7q22, 22q, and 1p had no major influence on the clustering.

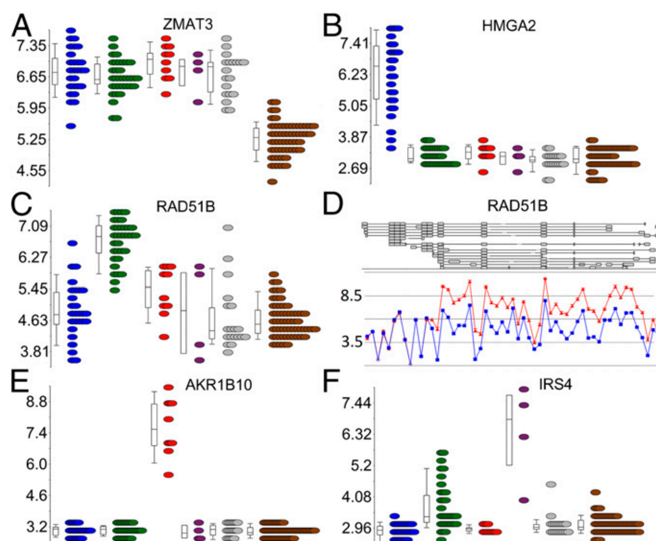

Fig. 2. Examples of shared and uniquely expressed genes. (*A*) *ZMAT3* was the most significantly differentially expressed gene in all leiomyomas compared with the normal myometrial tissue (brown). (*B*) *HMGA2* was the most uniquely expressed gene in leiomyomas of the *HMGA2* (blue) subtype. (*C*) *RAD51B* was the most uniquely expressed gene in leiomyomas of the *MED12* (green) subtype. (*E*) *AKR1B10* was the most uniquely expressed gene in leiomyomas of the *FH* (red) subtype. (*F*) *IRS4* was the most uniquely expressed gene in leiomyomas of the *COL4A5-COL4A6* (purple) subtype. (*D*) Exon-level analysis revealed that the overexpression of *RAD51B* in *MED12* mutant leiomyomas originated predominantly from a noncoding transcript (ENST00000492236).

Ingenuity Pathway Analysis (IPA) was performed with each of the five gene lists obtained from the differential expression analyses (Dataset S3). The most significant pathways among the different subgroups are compared in Table S1. The Wnt/β-catenin pathway was among the most significantly dysregulated pathways in the complete set of leiomyomas, and was predicted to be inhibited according to IPA. In addition, the Wnt antagonists Wnt inhibitory factor 1 (*WIF1*) and secreted frizzled-related protein 1 (*SFRP1*) (21) were significantly up-regulated in leiomyomas of the *HMGA2* and *MED12* subtypes, respectively (Dataset S2 and Fig. S2).

IPA also revealed a significant activation of prolactin signaling in leiomyomas. Indeed, prolactin (*PRL*) was one the most up-regulated genes in the complete set of leiomyomas (FC = 3.0; Dataset S2), and was significantly up-regulated in leiomyomas of the *HMGA2* (FC = 7.6), *MED12* (FC = 2.6), and *COL4A5-COL4A6* (FC = 9.9) subtypes (Dataset S2 and Fig. S3*A*). Prolactin-releasing hormone receptor (*PRLHR*) was also one the most up-regulated genes in the complete set (FC = 3.0; Dataset S2), and was significantly up-regulated in leiomyomas of the *HMGA2* (FC = 2.7) and *MED12* (FC = 9.0) subtypes (Dataset S2 and Fig. S3*B*).

Uniquely Expressed Genes in Different Leiomyoma Subtypes. To identify the most uniquely expressed genes for each leiomyoma subtype, we compared each subtype against the other leiomyomas and myometrium samples (Dataset S4). These genes represent candidate biomarkers of the different leiomyoma subtypes. The 20 most uniquely expressed (q <0.05; –2> FC >2) protein-coding genes for each subtype are presented in Table 1. Below we highlight some of these genes and their association with significantly dysregulated pathways.

Table 1. The 20 most uniquely expressed genes in each respective leiomyoma subtype

HMGA2: gene	*q*-value	FC	*MED12*: gene	*q*-value	FC	*FH*: gene	*q*-value	FC	*COL4A5/6*: gene	*q*-value	FC
HMGA2	5.0E-33	10.3	*RAD51B*	6.4E-22	3.8	*AKR1B10*	4.1E-42	27.1	*IRS4*	3.4E-08	10.5
IGF2BP2	6.0E-28	4.4	*PLP1*	3.5E-20	3.2	*TKT*	6.7E-35	4.4	*NSG1*	8.8E-08	2.2
CCND2	7.9E-18	2.5	*GARNL3*	2.4E-19	2.3	*PDK1*	2.8E-24	3.6	*MXRA8*	4.9E-05	−2.5
IL11RA	7.7E-17	2.7	*KIAA1199*	2.8E-18	5.7	*SLC7A11*	4.8E-24	7.2	*FBLN1*	4.9E-05	−3.8
C19orf38	1.3E-15	3.0	*LAMP5*	3.0E-18	5.1	*G6PD*	9.9E-22	3.9	*PCSK2*	2.1E-04	3.3
PLAG1	3.1E-15	8.2	*MMP11*	6.7E-18	5.5	*PIR*	1.7E-21	3.2	*DPYD*	5.7E-04	−2.7
GRPR	1.2E-13	8.3	*ADAM12*	8.7E-17	8.8	*GCLM*	4.1E-21	3.7	*SPATA6*	7.2E-04	−2.0
PAPPA2	7.4E-13	7.1	*POPDC2*	9.7E-17	3.2	*SRXN1*	4.6E-18	2.4	*CTNNA3*	7.7E-04	2.5
PLA2R1	7.4E-13	−4.3	*CPA3*	2.8E-15	−5.0	*ENTPD7*	1.1E-17	4.1	*TMEM55A*	6.9E-03	2.1
TBX3	3.1E-12	−2.4	*THSD4*	4.7E-15	2.5	*TNFRSF21*	3.1E-16	10.3	*PCDHB8*	9.3E-03	2.4
CBLN4	3.7E-12	3.1	*CACNA1C*	5.6E-15	2.1	*SLC6A6*	8.7E-15	4.8	*SCG2*	1.4E-02	8.7
GPR20	1.6E-11	2.7	*MMP16*	8.0E-15	4.0	*NQO1*	6.4E-13	7.3	*SLAIN1*	1.6E-02	−2.1
GPR22	4.6E-11	4.1	*CNTROB*	1.6E-14	2.2	*BNIP3*	9.4E-13	3.0	*PLAGL1*	1.8E-02	−2.5
QPRT	5.5E-11	2.0	*NHSL2*	1.6E-14	2.0	*RNF128*	1.2E-12	2.4	*PARM1*	1.9E-02	−3.0
PAWR	8.7E-11	−2.7	*KCNAB3*	1.9E-14	3.1	*MGAT5*	2.5E-12	2.5	*LIX1*	2.0E-02	2.4
MB21D2	1.1E-10	2.3	*UNC5D*	6.0E-14	2.8	*PGD*	2.7E-11	3.0	*RHOB*	2.0E-02	−2.0
CCND1	2.5E-10	3.6	*HPGDS*	9.1E-14	−2.4	*FAM46C*	2.7E-11	4.4	*TGFBR3*	2.3E-02	−2.0
WIF1	3.3E-10	5.0	*PCP4*	1.2E-13	3.3	*AEBP1*	4.2E-11	−3.9	*HIST1H4H*	3.1E-02	2.1
EGFR	4.2E-10	−2.2	*WBSCR17*	1.4E-13	2.2	*SESN3*	2.4E-10	4.0	*COL4A5*	3.6E-02	−3.7
AVPR1A	4.7E-10	−4.3	*RUNDC1*	1.4E-13	2.2	*ABCC3*	5.6E-10	2.1	*PCDHB2*	3.7E-02	4.4

Uniquely Expressed Genes in Leiomyomas of the *HMGA2* Subtype. We identified *HMGA2* itself as the most uniquely expressed gene (FC = 10.3) in leiomyomas of the *HMGA2* subtype (Table 1 and Fig. 2*B*). Insulin-like growth factor 2 mRNA-binding protein 2 (*IGF2BP2*), one of the few genes previously proven to be directly regulated by *HMGA2* (22), was the second-most significant gene (FC = 4.4; Fig. S4*A*). The proto-oncogene pleomorphic adenoma gene 1 (*PLAG1*) also was among the most uniquely expressed genes (FC = 8.2; Fig. S4*B*). Up-regulation (FC = 5.7) of *PLAG1* also was seen in three leiomyomas found to harbor an *HMGA1* alteration (Dataset S2). Only 2 out of 34 leiomyomas of the *MED12* subtype exhibited up-regulation of *PLAG1* (FC >2), and one of these harbored an *HMGA1* alteration (My5008 m3; FC = 2.6). The other tumor (My16 m1; FC = 17.5) was identified to harbor a balanced translocation, *t*(6, 8)(q13;q12), with breakpoints located ~2.3 kbp downstream of *PLAG1* and ~21.9 kbp downstream of *COL12A1* (Dataset S5). Further examination revealed up-regulation of *PLAG1* in two quadruple-negative leiomyomas (My5007 m2; FC = 11.3 and M51 m1; FC = 7.5) harboring a whole chromosome 8 duplication (Dataset S6). Leiomyomas with a genetic *PLAG1* alteration also displayed similar expression patterns as seen in leiomyomas with *HMGA2* or *HMGA1* alterations (Fig. 1). One of these (My5007 m2) also clustered with leiomyomas of the *HMGA2* subtype.

Insulin-like growth factor-2 (*IGF2*) has previously been shown to be directly regulated by *PLAG1* (23–25), and we detected a significant up-regulation of *IGF2* in leiomyomas of the *HMGA2* (FC = 3.0) and *MED12* (FC = 3.1) subtypes (Dataset S2 and Fig. S4*C*). A significant up-regulation (FC = 4.3) of *IGF2* was detected in the leiomyomas with a genetic *PLAG1* alteration as well (Dataset S2 and Fig. S4*C*). Leiomyomas of the *HMGA2* subtype also displayed a unique up-regulation of pappalysin 2 (*PAPPA2*) (FC = 7.1; Fig. S5*A*), a gene encoding for an insulin-like growth factor-binding protein 5 (IGFBP-5) protease (26). Interestingly, we found one quadruple-negative leiomyoma harboring a fusion gene joining exon 1 of *IGFBP5* to exon 11 of platelet-derived growth factor receptor, beta polypeptide (*PDGFRB*) (Dataset S5 and Fig. S6).

Uniquely Expressed Genes in Leiomyomas of the *MED12* Subtype. We identified RAD51 paralog B (*RAD51B*) as the most uniquely expressed gene (FC = 3.8) in leiomyomas of the *MED12* subtype (Table 1 and Fig. 2*C*). Exon-level analysis, confirmed by RNA sequencing, revealed that the up-regulation originated predominantly from a noncoding transcript of *RAD51B* (ENST00000492236; Fig. 2*D* and Fig. S7). Of note, expression of this noncoding transcript also was seen at lower levels in the corresponding myometrium samples. We also detected a unique up-regulation of ADAM metallopeptidase domain 12 (*ADAM12*) (FC = 8.8; Fig. S5*B*), another IGFBP-5 protease (27).

Uniquely Expressed Genes in Leiomyomas of the *FH* Subtype. We identified aldo-keto reductase family 1, member B10 (aldose reductase) (*AKR1B10*) as the most uniquely expressed gene (FC = 27.0) in leiomyomas of the *FH* subtype (Table 1). Expression of *AKR1B10* was not seen in any of the other leiomyoma or myometrium samples (Fig. 2*E*). The NRF2-mediated oxidative stress response was the most significantly dysregulated pathway in leiomyomas of the *FH* subtype (Table S1). Furthermore, 8 of the 20 most uniquely expressed genes (*AKR1B10*, *TKT*, *PIR*, *SLC7A11*, *NQO1*, *SRXN1*, *SLC6A6*, and *GCLM*) have previously been reported as targets of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) (28–31). The pentose phosphate pathway was the only other statistically significant pathway, and three (*TKT*, *PGD*, and *G6PD*) of the 20 most uniquely expressed genes encode for key enzymes of this pathway. None of the other leiomyoma subtypes displayed dysregulation of these two pathways (Table S1).

Uniquely Expressed Genes in Leiomyomas of the *COL4A5-COL4A6* Subtype. Although *COL4A5* and *COL4A6* are both affected by the characteristic *COL4A5-COL4A6* deletions, only *COL4A5* displayed a statistically significant down-regulation compared with the myometrium (FC = –3.3; Dataset S2). However, we identified insulin receptor substrate-4 (*IRS4*), a gene located adjacent to *COL4A5*, as the most uniquely expressed gene in these leiomyomas (FC = 10.5; Table 1 and Fig. 2*F*). No pathway reached statistical significance (Table S1).

Identification of Down-Regulated Genes Within Commonly Deleted Regions on Chromosomes 7q22, 22q, and 1p. To identify genes most significantly down-regulated by chromosome 7q22, 22q, and 1p deletions, we compared leiomyomas harboring these deletions against leiomyomas and myometrium tissue specimens lacking these aberrations. A total of 14 leiomyomas harbored a deletion spanning 7q22 (Fig. S8). In addition, two leiomyomas harbored chromosomal rearrangements affecting cut-like homeobox 1 (*CUX1*)

GENETICS

Table 2. **The 10 most significantly down-regulated genes by 7q22, 22q, and 1p deletions**

7q22: gene	*q*-value	FC	No. of samples	22q: gene	*q*-value	FC	No. of samples	1p: gene	*q*-value	FC	No. of samples
LMTK2	1.9E-04	−1.3	8	*FBXO7*	1.1E-12	−1.4	19	*UBE4B*	6.6E-11	−1.5	16
COPS6	7.5E-04	−1.3	9	*MTMR3*	7.6E-12	−1.4	19	*EXOSC10*	5.7E-08	−1.3	16
CUX1	7.9E-04	−1.5	13	*DEPDC5*	5.6E-08	−1.3	19	*DNAJC16*	5.7E-08	−1.2	15
MLL5	2.0E-03	−1.4	11	*RNF185*	1.6E-07	−1.5	19	*GNB1*	8.1E-08	−1.3	15
TNPO3	2.0E-03	−1.3	8	*EIF3D*	2.0E-07	−1.3	18	*PRDM2*	9.9E-08	−1.3	15
ZNF800	4.6E-03	−1.2	7	*DUSP18*	4.0E-07	−1.4	19	*FAM54B*	1.4E-07	−1.4	15
PNPLA8	1.9E-02	−1.4	13	*TTC28*	7.5E-07	−1.4	19	*VPS13D*	1.4E-07	−1.5	16
ZNF394	2.0E-02	−1.3	9	*EP300*	8.3E-07	−1.3	15	*RERE*	2.5E-07	−1.5	17
CADPS2	2.0E-02	−2	8	*MAPK1*	1.2E-06	−1.3	16	*KIF1B*	6.0E-07	−1.5	16
PMPCB	2.1E-02	−1.4	11	*MKL1*	1.3E-06	−1.4	16	*CLSTN1*	9.8E-07	−1.5	16

on 7q22 (Dataset S5 and Fig. S8). A total of 20 leiomyomas harbored a 22q deletion, including 5 leiomyomas harboring a "second hit" truncating mutation affecting DEP domain containing 5 (*DEPDC5*) (Fig. S9 and Dataset S1). We identified one additional leiomyoma (MY23 m4) harboring a chromosomal rearrangement with breakpoints located ∼14 kbp upstream of *DEPDC5* (Dataset S5). Another minimally deleted region was identified on 22q, and one leiomyoma (M9 m3) harbored an additional rearrangement within this region, resulting in a second hit loss of the SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1 (*SMARCB1*) gene (Fig. S9 and Dataset S5). A total of 18 leiomyomas harbored a 1p deletion, and the minimally deleted region contained only one protein-coding gene, nephronophthisis 4 (*NPHP4*) (Fig. S10). Table 2 presents the 10 most significantly down-regulated protein-coding genes ($q < 0.05$) within commonly deleted regions, and the number times that each gene was affected by a deletion.

Discussion

Recent high-throughput sequencing studies have underlined the genetic heterogeneity of leiomyomas (3, 4), suggesting the existence of molecularly distinct subtypes of leiomyomas. In this study, we identified global expression signatures associated with the mutation status of *HMGA2*, *MED12*, *FH*, and *COL4A5-COL4A6*, supporting the existence of molecularly distinct leiomyoma subtypes. In contrast, deletions of 7q22, 22q, and 1p frequently co-occurred with other genetic changes and had no major influence on the clustering, suggesting that these changes are involved in tumor progression rather than initiation. Leiomyomas with *HMGA1* or *HMGA2* alterations displayed similar global expression signatures, supporting the idea that these structurally and evolutionarily related transcription factors have similar functions in tumorigenesis (32). The majority of quadruple-negative leiomyomas clustered into several unique branches, indicating the presence of multiple rare and possibly novel subtypes.

Several recent studies have examined a potential role for Wnt/β-catenin signaling in the development of leiomyomas (7, 19, 33–35). Although our work confirms an aberrant expression of genes related to Wnt/β-catenin signaling, the pathway was unexpectedly predicted to be inhibited. Furthermore, we identified the Wnt pathway antagonists *WIF1* and *SFRP1* (21) as distinctly up-regulated in leiomyomas of the *HMGA2* and *MED12* subtypes, respectively. Interestingly, *WIF1* is located closely upstream of *HMGA2* and transcribed from the opposite strand. *WIF1* also is a recurrent translocation partner of *HMGA2* in pleomorphic adenomas of the salivary gland (36).

Previous studies have hypothesized that PRL may act as a mitogenic autocrine/paracrine growth factor in human tumorigenesis (37). We identified prolactin signaling as one of the most significantly activated pathways in the complete set of leiomyomas. Furthermore, *PRL* itself was one of the most highly expressed genes. Interestingly, transgenic mice overexpressing *HMGA2* have shown to develop pituitary adenomas secreting prolactin (38), and we detected a particularly high expression of *PRL* in leiomyomas of the *HMGA2* subtype.

The release of prolactin has been shown to be regulated by the prolactin-releasing peptide receptor (PrRPR) (39). We identified a high expression of *PRLHR*, the gene that encodes for this receptor in leiomyomas of the *MED12* subtype. A recent study showed that up-regulation of PrRPR stimulates the proliferation of cultured primary human leiomyoma cells, and that transgenic mice overexpressing *PRLHR* develop myometrial hyperplasia with excessive extracellular matrix deposition (40).

We identified *PLAG1* as one the most uniquely up-regulated genes in leiomyomas with *HMGA2* or *HMGA1* aberrations. Furthermore, we identified genetic *PLAG1* alterations in three leiomyomas, all of which exhibited expression signatures as seen in leiomyomas with *HMGA2* or *HMGA1* alterations. *PLAG1* encodes for a transcription factor whose ectopic expression can trigger the development of several benign mesenchymal tumors (41). Indeed, the overexpression of *PLAG1* is typically triggered by chromosomal translocations or, in rarer cases, by amplifications (42). *PLAG1* and *HMGA2* translocations are both frequent and mutually exclusive in pleomorphic adenomas of the salivary gland (43). *RAD51B* is the preferential translocation partner of *HMGA2* in leiomyomas, and *PLAG1* translocations also have been shown to involve the *RAD51B* loci in lipoblastomas (44). Taken together, these findings indicate that leiomyomas also harbor genetic *PLAG1* alterations, and suggest that *HMGA2* and *HMGA1* promote tumorigenesis through the activation of *PLAG1*.

Compatible with previous expression profiling studies (14), our pathway analysis revealed a significant dysregulation of IGF1 signaling in leiomyomas. After *HMGA2* itself, our statistical analysis identified *IGF2BP2* as the second-most uniquely expressed gene in leiomyomas of the *HMGA2* subtype. Previous studies have shown that *HMGA2* activates the expression of *IGF2BP2* by binding to an AT-rich regulatory region within its first intron (22). *IGF2BP2* encodes for a protein involved in promoting *IGF2* mRNA translation (45). Interestingly, several previous studies have demonstrated that *PLAG1* regulates the expression of *IGF2* by binding to its P3 promoter (23–25). In support of this, we detected up-regulation of *IGF2* in the majority of leiomyomas with an *HMGA2, HMGA1*, or *PLAG1* alteration. *IGF2* encodes for insulin-like growth factor 2 and exerts its growth-promoting effect by binding to the IGF1 receptor.

We identified *PAPPA2* and *ADAM12* as two highly uniquely overexpressed genes in leiomyomas of the *HMGA2* and *MED12* subtypes, respectively. Both of these genes are expressed at high levels during early placental development and encode for specific proteases of IGFBP-5 (26, 27). Previous studies have shown that IGFBP-5 inhibits IGF1-induced proliferation and migration of smooth muscle cells (46). We identified one quadruple-negative leiomyoma as harboring a fusion gene involving *IGFBP5* and *PDGFRB*. Although fusions involving *PDGFRB* are known to drive hematopoietic cancers (47), the disruption of *IGFBP5* may further enhance leiomyoma development. The exact role of IGFBP-5 in leiomyoma development remains to be resolved, given that IGFBP-5 has been found to both promote and inhibit cancer development (48).

We identified *IRS4*, a gene located adjacent to *COL4A5*, as the most uniquely expressed gene in leiomyomas of the *COL4A5-COL4A6* subtype. *IRS4* encodes for the insulin receptor substrate 4, which has been shown to enhance insulin-like growth factor 1–induced cell proliferation (49). Taken together, these observations support a central role for IGF1 signaling in leiomyomas of the *HMGA2*, *MED12*, and *COL4A5-COL4A6* subtypes.

The mechanism of tumorigenesis caused by *FH* mutations has remained unclear. The most extensively studied hypothesis is activation of the hypoxia pathway (50). Biallelic loss of *FH* results is accumulation of intracellular fumarate, which in turn may inhibit the degradation of hypoxia-inducible factor 1-alpha (HIF1α), leading to pseudohypoxia through aberrant accumulation of this key protein. More recently, two independent research groups demonstrated that KEAP1, a negative regulator of the oncogenic transcription factor NRF2, becomes succinated by high levels of fumarate, leading to accumulation and activation of NRF2 (30, 51). Activation of NRF2 has recently been identified as a common feature of many cancers (52). In this study, we found that the NRF2 signaling pathway was the single most significantly dysregulated pathway in leiomyomas of the *FH* subtype, whereas the HIF1α signaling pathway was not significantly altered. We detected *AKR1B10*, a known target of NRF2 (29), as a highly promising biomarker for *FH* deficiency. NRF2 activation has previously been shown to redirect glucose and glutamine into anabolic pathways, including the pentose phosphate pathway (31). In support of this, we identified the pentose phosphate pathway as the only other statistically significant pathway.

We previously reported *RAD51B* to be specifically up-regulated in leiomyomas with *MED12* mutations (4). In this study, we discovered that this up-regulation corresponds to a (long) non–protein-coding transcript of *RAD51B*. Remarkably, *RAD51B* is also the most common translocation partner of *HMGA2* in leiomyomas (4, 53). It is tempting to speculate that this noncoding transcript might have an unresolved tumor-promoting role.

Leiomyomas frequently harbor recurrent deletions affecting 7q22, 22q, and 1p, suggesting that these regions contain tumor suppressor genes (8–11). High-throughput sequencing studies have rarely detected second hit mutations within these regions, however (3, 4, 13), suggesting that the target genes may act in a haploinsufficient manner. Furthermore, these changes are often very complex, consisting of inversions, translocations, and deletions in various regions (4), suggesting that multiple genes are targeted simultaneously. In an effort to identify putative target genes, we explored the transcriptional consequences of genes located within these deletions.

We identified *CUX1* as the third-most significantly down-regulated gene of 7q22 deletions. *CUX1* is located within the minimally deleted region and has been shown to be disrupted by chromosomal rearrangements (4, 54); however, no point mutations have been found, and some 7q22 deletions do not span *CUX1* (55). Only one of our samples harbored a biallelic loss of *CUX1*, suggesting that *CUX1* is a haploinsufficent tumor suppressor. Indeed, *CUX1* was recently shown to have such a role in acute myeloid leukemia (56).

This study included five leiomyomas previously detected to harbor truncating *DEPDC5* point mutations, indicating that *DEPDC5* is a target gene on 22q (4). Compatible with this notion, our expression analysis identified *DEPDC5* as the third-most significantly down-regulated gene of 22q deletions. Interestingly, we identified another commonly deleted region on chromosome 22q. One leiomyoma harbored an additional chromosomal rearrangement within this region, resulting in biallelic loss of the tumor suppressor *SMARCB1*. *SMARCB1* is of special interest because a germline mutation in *SMARCB1*, typically causing schwannomatosis, was recently associated with the development of leiomyomas as well (57).

Deletions of 1p have been associated with distinct histopathological features and possible malignant progression of leiomyomas (11, 16). Interestingly, we identified *NPHP4* as the most commonly deleted gene on chromosome 1p. *NPHP4* has previously been highlighted as a putative target gene in leiomyomas, owing to recurrent translocation breakpoints located upstream of the *NPHP4* locus (10); however, we did not detect a significant down-regulation of *NPHP4* in leiomyomas with 1p deletions.

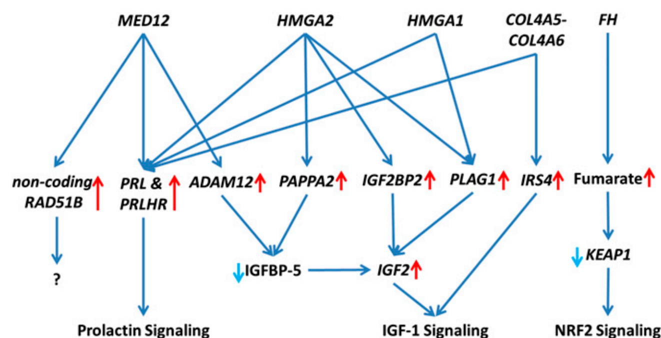

Fig. 3. Schematic of highlighted driver pathways in leiomyoma development and growth. Leiomyomas display subtype-specific differences in key driver pathways, including Prolactin, IGF1, and NRF2 signaling.

Conclusions

It is well known that uterine leiomyomas display significant heterogeneity in terms of symptoms, histopathology, therapeutic requirements, and genetic changes (2). The evidence presented in this study strongly suggests that specific driver mutations are the major determinants of expression changes in leiomyomas. The variability and inconsistencies frequently seen among samples and studies may be largely explained by different genetic factors driving the lesions. Here we highlight subtype-specific expression changes in key driver pathways, including Wnt/β-catenin, prolactin, IGF1, and NRF2 signaling (Fig. 3). Transcriptional differences in key driver genes and pathways also may explain the frequently seen differences in clinicopathological outcomes. The evidence presented in this study highlight the need for molecular stratification in uterine leiomyoma research, and possibly in clinical practice. This study offers a set of candidate biomarkers that will facilitate the classification of uterine leiomyomas in both contexts.

Materials and Methods

Detailed descriptions of the materials and methods used in this study are provided in *SI Materials and Methods*. The research was approved by the Ethics Review Board of Helsinki University Hospital. A total of 94 leiomyomas and 60 corresponding myometrium tissue specimens were investigated (Dataset S1). All tissue specimens were collected during hysterectomies and stored as fresh-frozen. The samples were derived from five tissue collections, one consisting of anonymous patients and the other four of patients who signed an informed consent before entering the study.

All leiomyomas were screened for *MED12* exon 1 and 2 mutations using Sanger sequencing with primers as reported previously (3). All specimens were screened for rearrangements and deletions affecting *HMGA2*, *HMGA1*, and *COL4A5-COL4A6* using WGS and/or SNP arrays. All specimens were also screened for 7q22, 22q, and 1p deletions, as well as for rearrangements located within the minimally deleted regions of these. Dataset S1 presents the mutational status of each lesion examined in this study.

WGS data were available for 63 leiomyomas and 31 corresponding myometrium tissue specimens. Genomic DNA libraries were prepared and sequenced according to Illumina and Complete Genomics paired-end sequencing service protocols. A total of 50 leiomyomas and 36 corresponding myometrium tissue specimens were prepared and analyzed using Illumina HumanOmni2.5-8 BeadChips version 1.1 or 1.2. Genome-wide somatic copy number alterations (SCNAs) were detected from Complete Genomics WGS as described previously (4). Both the SNP array data and the Illumina WGS data were analyzed for SCNAs using Partek Genomics Suite version 6.5. Genomic rearrangements were detected from Illumina and Complete Genomics WGS data as described previously (4). In short, genomic rearrangements were detected from Illumina BAM files using BreakDancer version 1.2.

Gene expression data were constructed using Affymetrix GeneChip Human Exon 1.0 ST arrays. Differential expression analyses were performed with Partek Genomics Suite version 6.5. Unsupervised hierarchical clustering analysis (cosine

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correlation) was performed using 1% most variable genes ($n = 372$), defined by the coefficient of variation calculated across all tumor samples. Pathway enrichment analysis was carried out with differentially expressed genes using Qiagen's IPA software. False discovery rate (FDR) control (Benjamini and Hochberg method) was used to correct for multiple testing. RNA sequencing libraries were prepared from rRNA-depleted (RiboMinus Transcriptome Isolation Kit; Life Technologies) samples using the Illumina TruSeq RNA Sample Preparation Kit A in accordance with the manufacturer's instructions.

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