WNT Signaling and HBP1-Associated Functional Analysis in Uterine Leiomyoma: Potential for a Pharmacological Approach to Disease Treatment

A thesis submitted by

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In partial fulfillment of the requirement for the degree of

Master of Science

in

Pharmacology and Drug Development

Tufts University

Sackler School of Graduate Biomedical Sciences

May 2018

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Abstract

Uterine Leiomyomas (UL) are the most common tumors in women of reproductive age, which leads to approximately 200,000 hysterectomies annually in the United States. Although over 95% of UL tumors are benign, they destroy the function of the uterus and cause abnormal uterine bleeding, anemia, pelvic pain, and recurrent pregnancy loss. Up-regulation of the WNT pathway is well established to associate with cell proliferation and tumorigenesis. In UL, cytogenetic deletion studies have shown that the chromosomal region 7q22.3 is associated with UL in around 40% of patients. Furthermore, the tumor suppressor HBP1, an inhibitor of Wnt signaling, is one of the 15 genes located by mapping these deletions. Because HBP1, a repressor of Wnt signaling, lies in this minimally deleted region, we hypothesized that WNT signaling may play a role in UL. Using patient samples obtained from hysterectomy, the protein levels of AXINII and β-catenin increased in leiomyoma compared to that in myometrium, suggesting activation of WNT signaling. Furthermore, HBP1 also decreased in a number ofleiomyomas, consistent with the deletion studies. These results were sufficient to attempt an unbiased screen of UL patients using RNAseq and bioinformatics analysis to identify global and HBP1-dependent changes in UL. As predicted by previous published work, an ESR1-activated proliferation gene expression signature was common in all leiomyoma samples. In tumors with normal HBP1 levels, cell cycle and GPCR signaling, cholesterol biosynthesis related gene signatures were upregulated compared to HBP1 low leiomyomas. In contrast, in the HBP1-low tumors, WNT signaling, mRNA metabolism and translation are present, which are consistent with qPCR and Western Blotting results.

Furthermore, cell-cell junction and proteasome are common present in both groups, indicating the association between ECM and pathogenesis of fibroids. In conclusion, these data demonstrate that the WNT signaling pathway, and in particular the Wnt repressor HBP1, may play a critical role in pathogenesis of leiomyoma.

Acknowledgements

I express my sincere gratitude to Professor Amy Yee, Professor Eric Paulson and Professor John Castellot from Sackler Graduate School of Biomedical Sciences, for their enlightening instruction, scientific guidance and continuous encouragement. I thank Professor Cynthia Morton and C. Scott Gallagher from Brigham and Women's Hospital and Harvard Medical School, for cytogenetic analyses and genetic association study in uterine leiomyoma. And I thank Maria Ibrahim, previous master student at Pharmacology department, and Professor Eric Paulson, for their important results of western blot, qPCR and bioinformatic analyses in uterine leiomyoma from different patients' samples, which have contributed to our overall statistical analysis.

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Hodge, J. C., Park, P. J., Dreyfuss, J. M., Assil-Kishawi, I., Somasundaram, P., Semere, L. G., . . . Morton, C. C. (2009). Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer, 48(10), 865-885. doi:10.1002/gcc.20692

Chapter1: Introduction

1.1 Definition and Pathobiology of Uterine Leiomyoma

Uterine Leiomyomas (UL) are the most common pelvic tumors in women of reproductive age. UL are benign, monoclonal tumors that arise from uterine myometrium and typically are comprised of fascicles of smooth muscle cells with abundant pink cytoplasm and uniform spindle-shaped nuclei, buried in abundant quantities of extracellular matrix. (Bulun et al, 2013) Nearly 35% of reproductive-aged women experience significant morbidity and require medical intervention due to associated symptoms that include urinary incontinence, pelvic pain, infertility, increased risk for cesarean section, and heavy bleeding. (Eggert et al., 2012)

Hysterectomy is the only essentially curative treatment currently available for UL and is often for loss of the ability to bear children. (Eggert et al., 2012) An alternative surgical method of treatment is myomectomy, which involves resection of the tumorous tissue from the affected uterus. (Eggert et al., 2012) Approximately 200,000 hysterectomies, 30,000 myomectomies are performed annually in the United States, and the annual economic burden of these tumors is estimated to be between $5.9 billion and $34.4 billion. (Bulun, 2013)

1.2 Canonical WNT Pathway in Uterine Leiomyoma

Recently, very little is known about the etiology and progression of UL. However, it is well-established that up-regulation of the WNT pathway is often associated with cell proliferation and tumorigenesis. Mangrioni's lab demonstrated that the Wnt5b gene is

overexpressed in uterine leiomyomas, suggesting a possible role in pathogenesis (Mangrioni S, et al., 2005). Selective overexpression of constitutively activated β-catenin in uterine mesenchyme during embryonic development and in adults gives rise to leiomyoma-like tumors in the uterus of all female mice, suggesting that signaling by WNT/β-catenin seems to play a role in somatic stem cell function in the myometrium and uterine leiomyoma tissue (Hodge et al., 2009). Besides, the role of WNT signaling in carcinogenesis in other tissues is well-documented, previous research in Dr. Yee's lab has shown that WNT signaling is up-regulated in breast cancer. Constitutive Wnt signaling has been linked to increased proliferation and invasiveness in breast and other cancers. In breast cancer, excessiveβ-catenin levels are correlated to breast cancers of poor prognosis (Tanwar et al., 2009), whereas the overall percentage of tumors with high β-catenin expression is estimated to be 50% (Tanwar et al., 2009).

In canonical Wnt signaling, absence of Wnt ligands leads to phosphorylation of β-catenin by the destruction complex, which contains the scaffold protein Axin, APC and the kinases GSK3β and casein kinase (CK1α). In this state, β-catenin is phosphorylated by GSK3β, ubiquitinated by β-TrCP200 and targeted for proteasomal degradation. When WNT binds Frizzled receptor and LRP5/6 co-receptor, the destruction complex function becomes disrupted. The formation of a Wnt-Fz-LRP6 complex together with the recruitment of the scaffolding protein Dishevelled (Dvl) results in LRP6 phosphorylation and the recruitment of the destruction complex to the receptors. In this way, β-catenin escapes from degradation and translocates to the nucleus to interact with TCF/LEF transcription factors. Increased β-catenin can initiate transcriptional activation of cyclin

D1 and c-myc, which control the G1 to S phase transition in the cell cycle, promoting cell proliferation. (Stamos & Weis 2013)

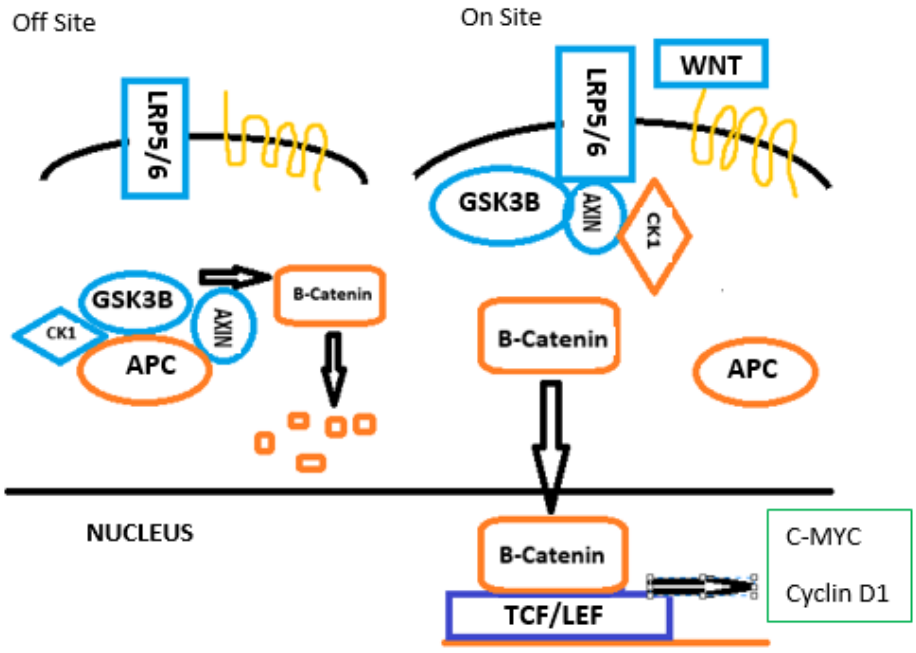

Figure 1.1 WNT Signaling Pathway. The pathway is maintained in the off state by the β-catenin degradation complex and the proteasome. When a Wnt ligand is present, the complex cannot assemble and β-catenin translocates to the nucleus and initiates transcription of its target genes.

1.3 Transcriptional Repressor for WNT----HBP1

Furthermore, about 40% of ULs contain cytogenic alterations including simple and recurrent deletions, inversions and translocations. (Nibert and Heim, 1990; Meloni et al., 1992). These abnormalities were used to classify UL into subgroups and provide landmarks for gene discovery. One of the largest UL subgroups is defined by the presence of chromosome 7 long arm abnormalities, most commonly the interstitial deletion del (7) (q22q32), which represents approximately 15% of all UL (Gallagher & Morton, 2016). According to Cynthia Morton's lab, the smallest commonly deleted

region on chromosome 7 in all samples spanned approximately 9.5 megabases from 7q22.1-q31.1 (Fig. 1.2). A del(7q) UL-specific gene list that is highly enriched for genes in 7q22, including seven genes (MML5, ZNF498, TRAF3IP1, MGC39821, SSR2, MARCKS, and HBP1), which are identified as being significant in decreased expressionTable1. (Hodge et al., 2009) Previous work in Dr. Yee's lab reported that HBP1 at 7q22.3 is a negative regulator of the Wnt pathway, which is implicated in breast and other cancers (Tanwar et al., 2009); and the molecular mechanism of HBP1 in Wnt signaling results from direct inhibition of transcriptional activation of Wnt target genes (such as cyclin D1 and c-MYC). Because HBP1, a repressor of Wnt signaling, lies in this minimally deleted region, we hypothesized that WNT signaling may play a role in UL. Determining related mutations and genes involved in UL tumorigenesis will provide targets for novel screening methods and therapeutic avenues.

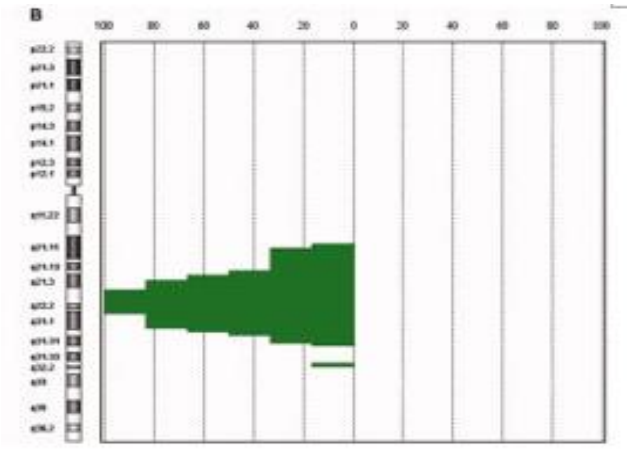

Figure 1.2: Genomic Penetrance Summary of Chromosome 7. Genomic penetrance summary of chromosome 7. showed the affected regions and in what percentage of the six cases they were found to be abnormal. The common region of genomic loss for the six cases, as indicated by the 100% line, spans approximately 9.5 megabases from 7q22.1-q31.1 (Adapted with permission from [Hodge, J. C., Park, P. J., Dreyfuss, J. M., Assil-Kishawi, I., Somasundaram, P., Semere, L. G., . . . Morton, C. C. (2009). Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer, 48(10), 865-885. doi:10.1002/gcc.20692]. Changes include cropping of commonly deleted region in chromosome 7 in six cases.)

Gene Symbol	Gene Name	Probe Set	Ref Seq	Fold Change	P-value	Q-value	Chromosome
ZNF498	zinc finger protein 498	228138_at	NM_145115	-1.42	9.56E-07	0.0239	7q22.1
TRAF3IP1	TNF receptor-associated factor 3 interacting protein 1	238494_at	NM_015650	-1.23	9.49E-06	0.0753	2q37.3
MGC39821	hypothetical protein MGC39821	1555363_s_at	XR_041448	-1.67	1.23E-05	0.0753	19p13.11
MLL5	myeloid/lymphoid or mixed-lineage leukemia 5	226100_at	NM_018682	-1.37	1.48E-05	0.0753	7q22.1
SSR2	signal sequence receptor, beta	200652_at	NM_003145	1.26	1.52E-05	0.0753	1q21-q23
MARCKS	myristoylated alanine-rich protein kinase C substrate	225897_at	NM_002356	1.32	1.81E-05	0.0753	6q22.2
HBP1	HMG-box transcription factor 1	209102_s_at	NM_012257	-1.29	2.96E-05	0.1060	7q22-q31

Table 1.1: Significant Genes in Cytogenic Analysis. Seven most significant genes identified by weighting the microarray data for the level of del(7q) cell mosaicism in each UL. The significance of these seven genes is reflected by a Q-value ≤ 0.10, and three of the genes are in the region of interest at 7q22. (Adapted with permission from [Hodge, J. C., Park, P. J., Dreyfuss, J. M., Assil-Kishawi, I., Somasundaram, P., Semere, L. G., . . . Morton, C. C. (2009). Identifying the molecular signature of the interstitial deletion 7q subgroup of uterine leiomyomata using a paired analysis. Genes Chromosomes Cancer, 48(10), 865-885. doi:10.1002/gcc.20692]. Changes include cropping of the seven most significant genes identified by weighting the microarray data for the level of del(7q) cell mosaicism in each UL.)

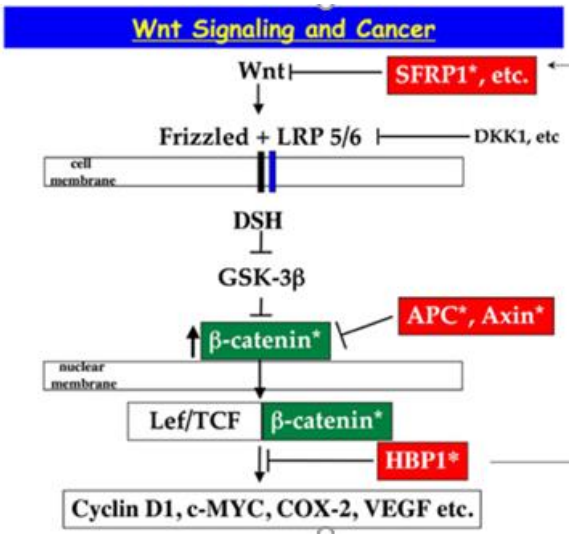

Figure 1.3: HBP1 in WNT Signaling Pathway. This figure shows that HBP1 is a negative regulator of Wnt signaling pathway and prevents the expression of downstream Wnt targets through interactions with TCF/LEF. (Yee Lab Figure)

Chapter 2: Materials and Methods

2.1 Myometrium and leiomyoma patient samples

Human fibroid and the adjacent myometrium samples were collected from patients who underwent hysterectomies at the Brigham and Women Hospital, including 18 uterine ULMs of usual type from 6 patients. Fresh samples were transported immediately to the laboratory on ice and frozen at -80 °C. Among them, samples from patient1, patient16 and patient17 were selected for gene expression analysis.

2.2 Western Blot

Human leiomyoma tissue samples were homogenized in ice-cold radioimmunoprecipitation assay buffer (RIPA) supplemented with protease and phosphatase inhibitors. Equal amounts of proteins were loaded equally onto 10% Mini-PROTEAN TGX Precast Protein Gels (BioRad). Membranes were incubated with a primary antibody overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies against mouse and rabbit IgG in room temperature for 30 minutes. The band of Western blot was quantified using NIH ImageJ. Following are the primary and secondary antibodies used in this study:

Primary Antibody	anti-Actin 1:2000	AntiAxinII 1:2000	Anti- β-catenin 1:2000	Anti-P-GSK3b 1:5000	Anti-T-GSK3b 1:5000
Secondary Antibody	anti-rabbit 1:20000	anti-rabbit 1:20000	Anti-mouse 1:20000	Anti-rabbit 1:20000	Anti-rabbit 1:20000

Table 2.1: Antibody used in this study.

2.3 Quantitative Real-time RT-PCR

Total RNA was extracted using TRIzol RNA Isolation Reagents and assessed for purity and quantity on a Nanodrop spectrophotometer. Complementary DNA (cDNA) was synthesized using qScript cDNA SuperMix advanced kit. Quantitative real-time RT-PCR was performed using the ABI 7900HT and Power SYBR Green PCR Master Mix for 40 cycles, with each cycle consisting of a 15-sec denaturation at 95.0°C, followed by 1 min of annealing at 60.0°C. For any sample, the expression level, normalized to the housekeeping gene 18s. Expression data with Ct-values >35 were excluded from analysis because expression levels are assumed to be out of accurate range.

Primer	Sequence
hB-Catenin Forward	ATGGCTTGGAATGAGACTGCTG
hB-Catenin Reverse	ACTGGATAGTCAGCACCAGGG
hCDH11 Forward	ACCATGAGAAGGGCAAGGAGG
hCDH11 Reverse	AATGTTCCCATCACCAGAGTCAA
hCYP1B1 Forward	AACGTACCGGCCACTATCAC
hCYP1B1 Reverse	TCACCCATACAAGGCAGACG

Table 2.2: Primers and Sequence in this Study.

2.4 Ingenuity Pathways Analysis

Functional analyses of the top 12472 probe sets from six leiomyomas and matched myometrial samples were performed using Ingenuity Pathways Analysis through uploading of the Affymetrix probe set identifiers and fold changes. Fisher's exact test was used to assign statistical significance, which is displayed as a score based on log(P-value). A score greater than two indicates less than a one in 100 likelihood (P –value <0.01) that genes are assembled into a network by random chance.

2.5 Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) in ULMs with HBP1 high level versus ULMs with HBP1 normal level was performed according to the authors' guidelines published at the Broad Institute web pages(http://www.broadinstitute.org/gsea/index.jsp), using Reactome and KEGG pathway. GSEA was performed to determine whether a priori defined set of genes shows statistically significant, consistent differences between two biological states.

Chapter 3: Results

3.1 Elevated AxinII and β-catenin protein levels in human uterine leiomyoma

To examine whether WNT signaling was increased in human uterine leiomyomas, western blot analyses were used to compare leiomyomas and adjacent normal myometrium from two patients. From each patient, we analyzed three fibroids and one adjacent normal tissue sample. The protein expression levels of AxinII and β-catenin are higher than normal myometrium B in patient1's uterine leiomyoma. (Figure 3.1) Myometrium A of patient1 showed increased AxinII and β-catenin levels compared to fibroids, thus this adjacent "myometrium" tissue may be histologically similar to fibroids and excluded from the study. Similarly, in patient2, the protein expression levels of AxinII and β-catenin are increased in fibroids compared to normal myometrium A & B. (Figure 3.4). Quantification of protein bands revealed significant differences in AxinII, β-catenin protein expression between myometrium and fibroids (Figure 3.2-3.3 & 3.5-3.6). Altogether, these results support the activation of Wnt/β-catenin signaling in human uterine leiomyomas.

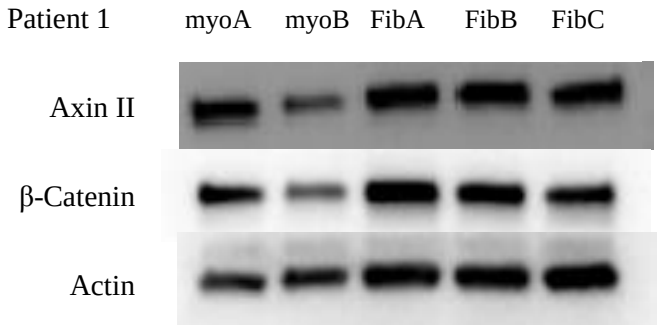

Figure 3.1: Western Blot for AxinII and β-Catenin in patient1. Fibroids A, B, C have more intensive bands in AxinII and β-catenin protein expression in patient1.

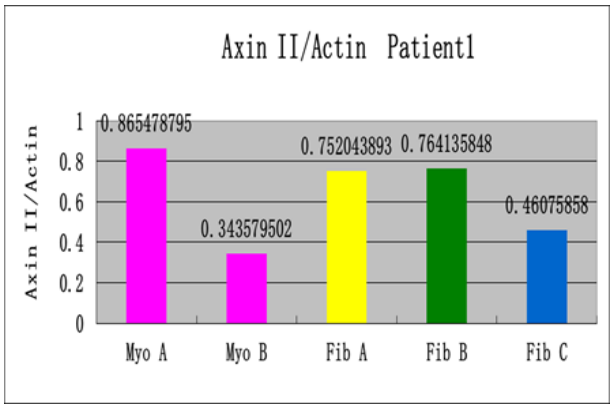

Figure 3.2: AxinII Protein Quantification in Patient1. Quantification of Axin II in western blot showed higher expression in Fibroids.

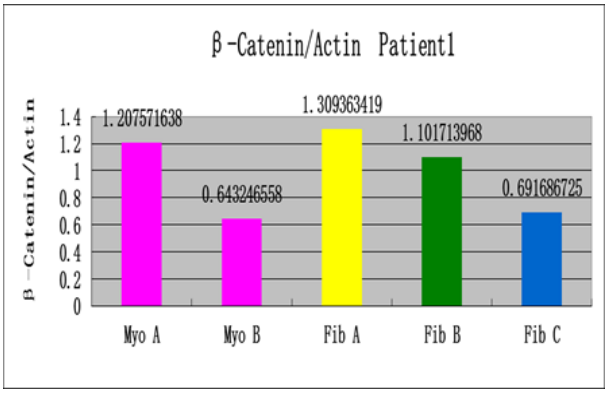

Figure 3.3: β-Catenin Protein Quantification in Patient2. Quantification of β-catenin in western blot showed higher expression in fibroids

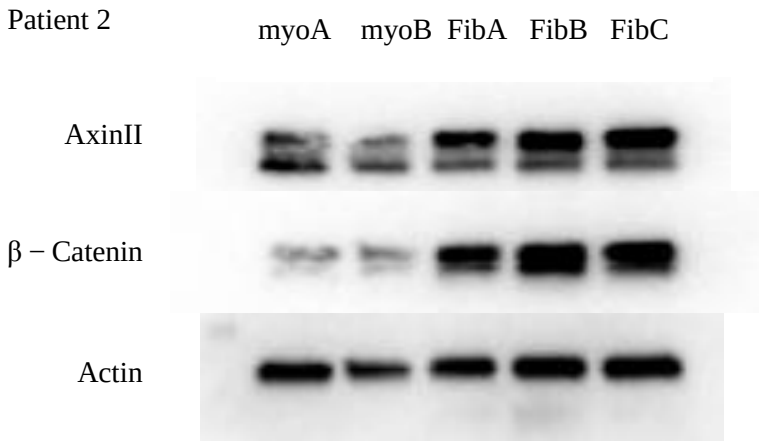

Figure 3.4: Western Blot for AxinII and β-catenin in Pateint2. The intensity of AxinII and β − Catenin protein in fibroids was stronger than the adjacent normal myometrium in patient2. Protein quantification of western blot used NIH Image J. The intensity of AxinII and β − Catenin protein was normalized with the corresponding control Actin.

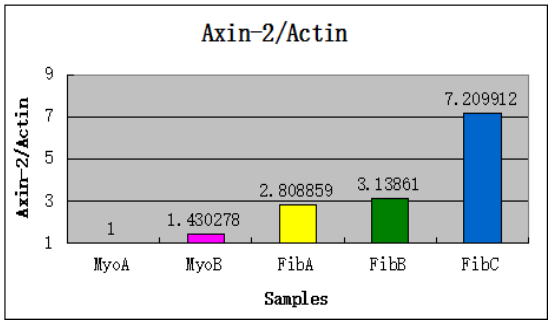

Figure 3.5: AxinII Protein Quantification in Patient2. AxinII protein expression was increased in fibroids than in myometrium.

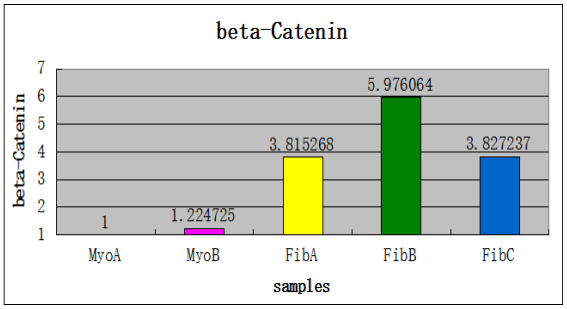

Figure 3.6: β-Catenin Protein Quantification in Patient2. AxinII protein expression was increased in fibroids than in myometrium.

3.2 AxinII mRNA Levels in Leiomyomas and Matched Myometrial as Detected by Real-time Quantitative PCR

To validate the downstream Wnt signaling activity, real-time quantitative PCR was used to compare fibroids and myometrium in AxinII mRNA level. AxinII is one of the components of destruction complex and the target genes of Wnt signaling pathway. In accordance with our western blot results (Figure 3.1-3.6), the AxinII mRNA level was elevated in all three fibroids compared to the myometrium B in patient1. (Figure 3.7). However, in patient2, AxinII mRNA level decreased in Fibroid C, AxinII mRNA level was elevated in Fibroid A and Fibroid B compared to the myometrium A. (Figure 3.8) Because uterine fibroid is the monoclonal tumor, multiple signaling pathways may regulate the tumorigenesis cooperatively, fibroid tumors showed individual difference. But overall, up-regulation in WNT signaling pathway were shown in fibroids universally.

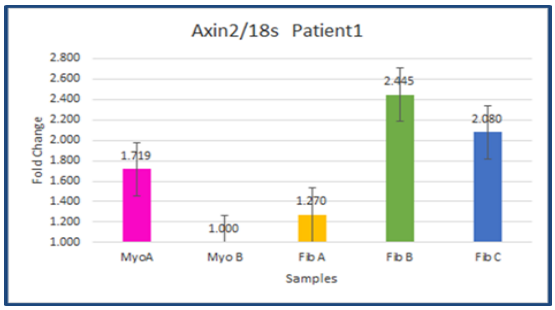

Figure 3.7: AxinII RNA Expression in Patient1. AxinII mRNA levels in leiomyoma and in their correspondent myometrium cells. Total RNA isolated from human fibroid tissues was quantified by real-time quantitative PCR analysis using the 18s an endogenous control. The increased RNA levels in AxinII of fibroids is consistent with the protein level in Axin II

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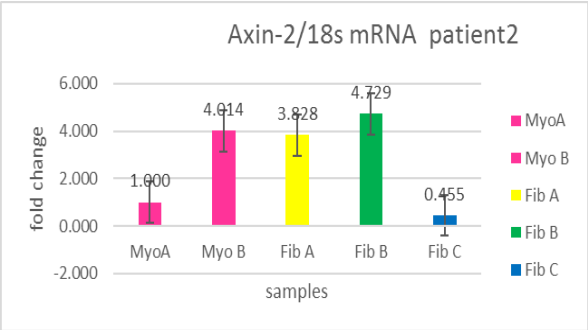

Figure 3.8: AxinII RNA Expression in Patient2. The RNA levels in AxinII of fibroids A and B are higher than that in myometriumA. Fibroid c showed a decreased RNA level in AxinII.

3.3 HBP1 Expression Levels in Leiomyomas and Matched Myometrial as Detected by Real-time Quantitative PCR

According to previous research, the loss of HBP1 expression has been associated with invasive breast cancer, suggesting the decreased expression found in del(7q) UL may contribute to the proliferative capacity of these tumors (Paulson et al., 2007). Therefore, to test whether decreased HBP1 expression levels in UL, real-time quantitative PCR was used to compare fibroids and matched myometrium. As shown in Figure 3.9, surprisingly, HBP1 mRNA levels were higher than or as normal as myometrial in all fibroids in patient1. Additionally, HBP1 RNA levels were decreased in Fibroid A and Fibroid C compared to myometrium A in patient2, consistent with the cytogenic studies. (Figure 3.10) These results provided insight into an inverse HBP1 expression patterns and Wnt activation independent of HBP1 expression levels in UL.

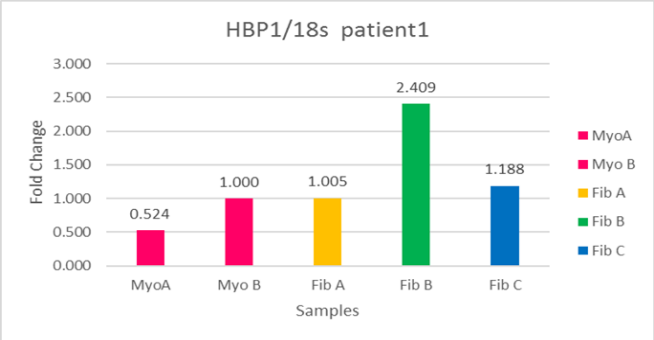

Figure 3.9: HBP1 RNA Expression in Patient1. HBP1 RNA levels were increased in three fibroids compared to myometrium.

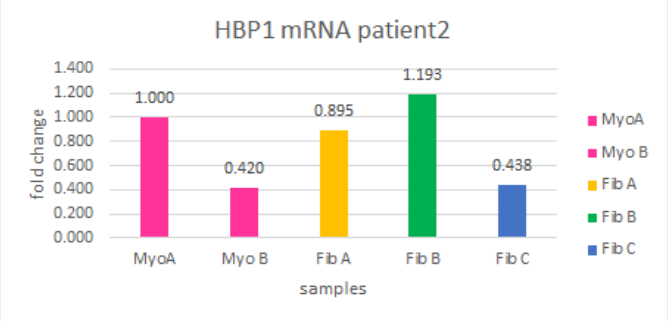

Figure 3.10: HBP1 RNA Expression in Patient2. Fibroid A and Fibroid C had lower levels in HBP1 compared to Fibroid B.

Furthermore, we then combined my data with previous experiments by Maria Ibrahim and Eric Paulson and showed that there is a clear subset of fibroid tumors with near-normal myometrium HBP1 levels, and a subset with significantly lower HBP1 levels. (Figure 3.11). To determine whether HBP1 levels correlated with Wnt signaling in UL, we did a pairwise plot of HBP1 expression levels with B-catenin and Axin II. As shown in Figure 3.12, decreased HBP1 correlated with increased b-catenin and Axin II,

suggesting that indeed, HBP1 levels influence the amount of Wnt signaling in myometrial and fibroid tissue.

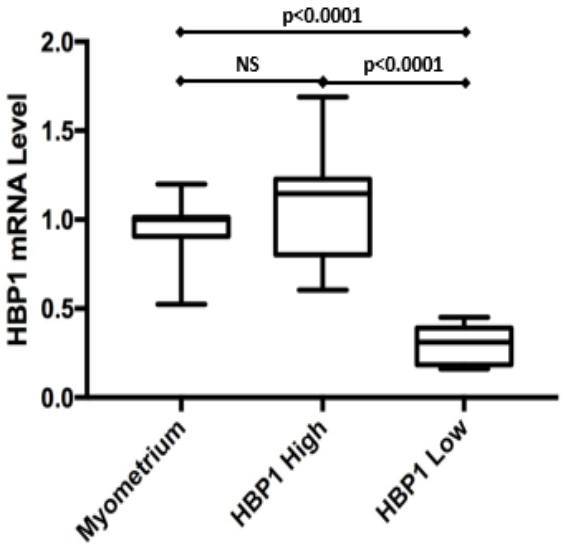

Figure 3.11: HBP1-low/-high Groups. HBP1-high group has no statistically significant difference with normal myometrium. HBP1 mRNA level in HBP1-low group is significantly lower than myometrium and HBP1-high group.

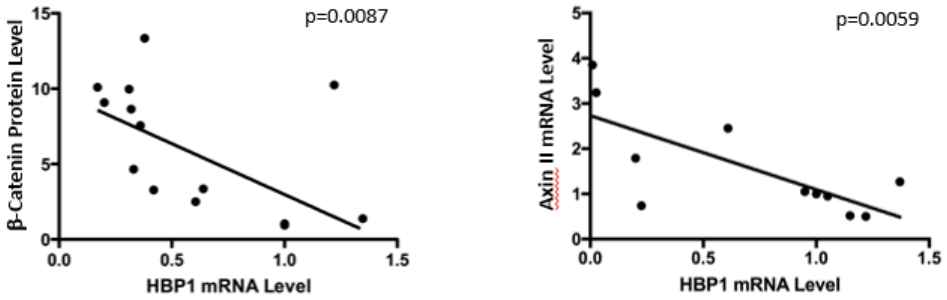

Figure 3.12: Correlation of HBP1 Expression and AxinII, β-Catenin Expression. HBP1 RNA level is inversely correlated to AxinII and b-Catenin RNA levels.

3.4 Bioinformatic Analysis of Human Uterine Leiomyomas

To examine whether a priori defined set of genes shows statistically significant, concordant differences between HBP1-normal and HBP1-low groups, we implemented gene set enrichment analysis (GSEA, Figure 3.13 & 3.14) using REACTOME and KEGG pathways. A number of themes showed overlap, such as proteomic degradation, cell cycle, diabetes pathway and modulation of cellular processes associated with migratory properties of the tumors (cell-cell junction and cytoskeleton). In general, more gene sets were enriched in HBP1-low group, indicating that HBP1 loss can be regulated in multiples genetic ways. Moreover, in HBP1 low-group, genes were mainly enriched in WNT signaling, in agreement with our western blot and qPCR results. Other enrichments were in PI3K cascade, mRNA processing, splicing and metabolism. In HBP1 high group, genes were enriched in cholesterol synthesis, neuronal systems and GPCR ligand binding. (Figure 3.13)

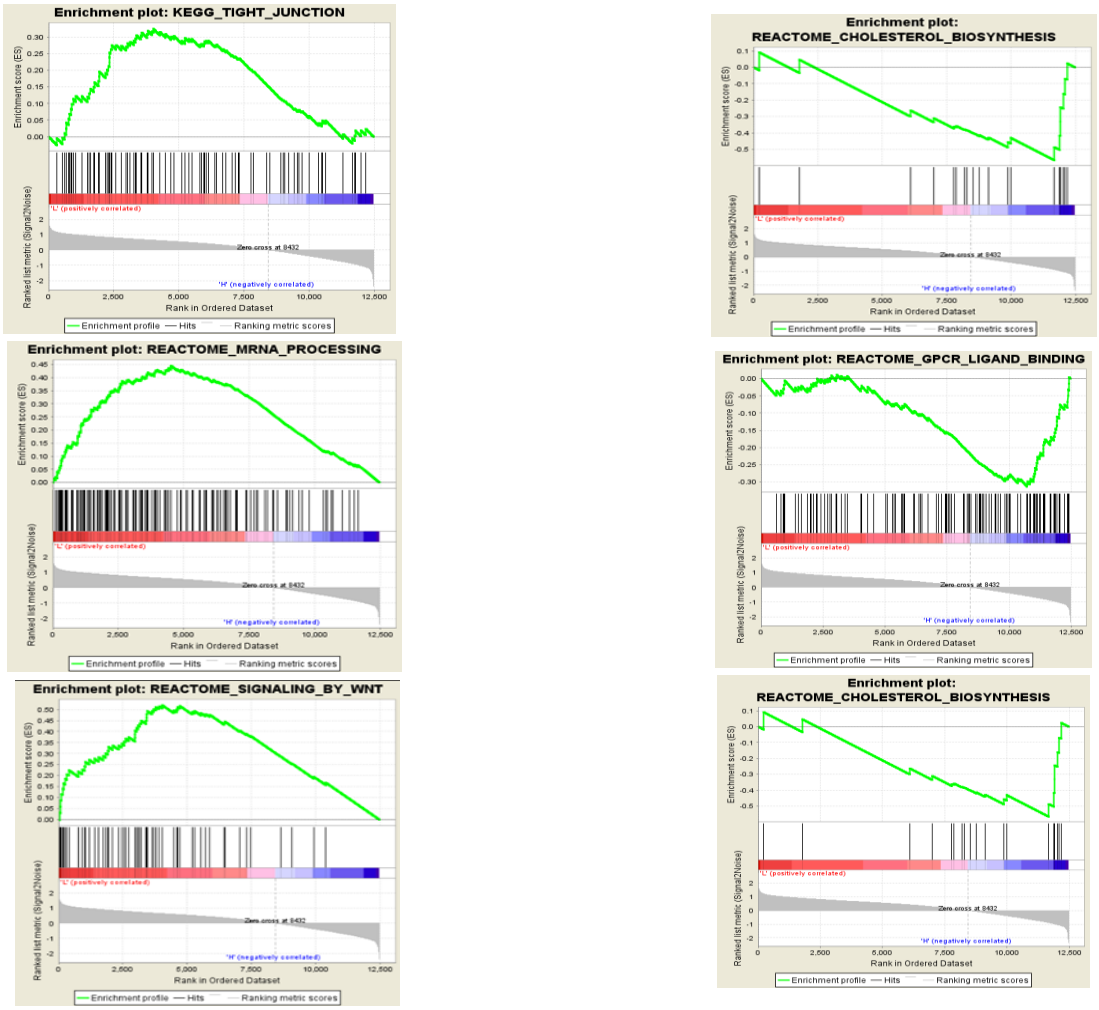

Figure 3.13: GSEA Snapshots. Gene sets enriched in HBP1-low group are at left lane. Gene sets enriched in HBP1-high group are at right lane. HBP1-low group: patient17 Fibroid A&B, patient16 Fibroid B. HBP1-high group: patient 16 Fibroid C, patient1 Fibroid A&B.

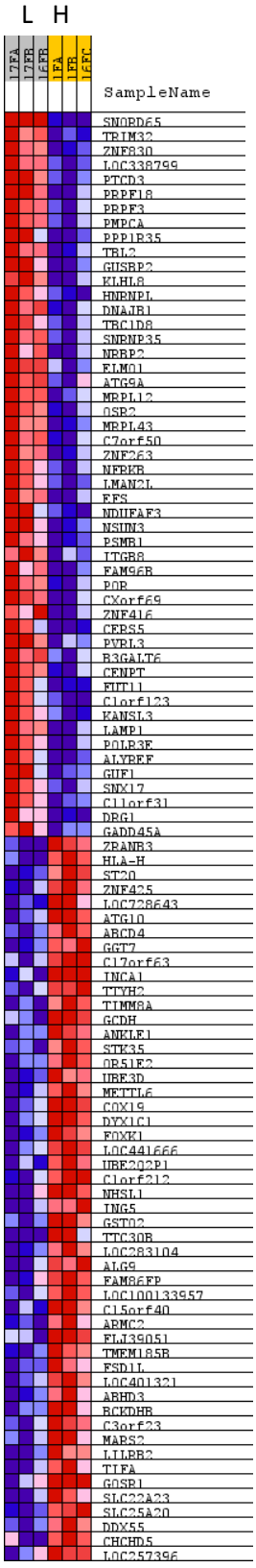

Figure 3.14: GSEA Heatmap. Heat map of the top 50 features for each phenotype and a plot showing the correlation between the ranked genes and the phenotypes. In a heat map, expression values are represented as colors, where the range of colors (red, pink, light blue, dark blue) shows the range of expression values (high, moderate, low, lowest).

L: HBP1-low group H: HBP1-high group

3.5 IPA analysis for Leiomyoma RNA samples

Our qPCR results showed HBP1 does not have a dominant role in fibroid tumor growth or Wnt signaling. Therefore, to validate our GSEA results, we examined 3 tumors with normal levels of HBP1 (relative to myometrium) and 3 tumors with low HBP1 using RNAseq and Ingenuity Pathway Analysis (IPA). As shown by the arrows, there were clearly two upstream regulatory pathways activated in all tumors; estrogen receptor (ESR1) and cMyc (MYC). ESR1 activation is consistent with tumors driven by estrogen, while cMyc is a classic indicator of tumor growth. However, the HBP1 low vs HBP1 normal tumors showed a massive difference in signaling pathway activation or inhibition. (Figure 3.15)

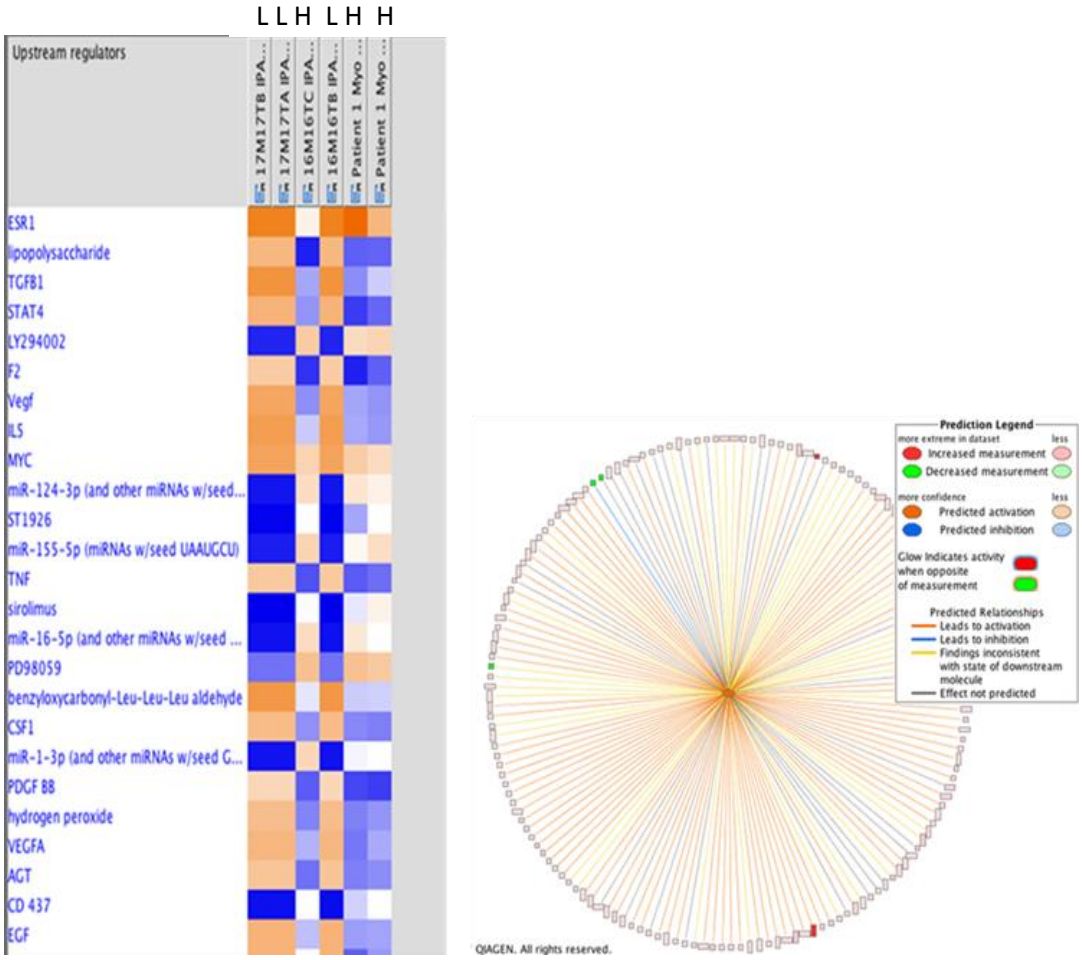

Figure 3.15: IPA Analysis. Heatmap (left) showed top 50 pathways that are upregulated in HBP1-low group as shown in orange, down-regulated pathways shown in blue in HBP1-high group. The panel (right) showed the specific induced genes or inhibited genes in MYC pathway.

L: HBP1-low group H: HBP1-high group

3.6 Quantitative PCR (Q-PCR) Confirmation of Genes Expression in IPA

To confirm that genes upregulated in GSEA and IPA analyses were also increased in transcriptional level, Q-PCR was performed to evaluate the expression of β-catenin, CDH11, CYP1B1 and SFRP4, which had increased expression value in bioinformatic analysis. The increased fold change in β-catenin, CDH11, CYP1B1 and SFRP4 are shown in table4. Q-PCR for CDH11 confirmed the IPA data of 1.4 to 3.9 fold induction by showing at least 7 to 16 fold increase in RNA expression in UL compared to myometrium after normalization to 18s. (Figure 3.15) Similarly, the mRNA expression of CYP1B1 (Figure 3.16), B-Catenin (Figure 3.17) and SFRP4 (Figure 3.18) showed higher levels in UL compared to myometrium.

According previous researches, CDHII encodes type II classical cadherin. The action of cadherins involves both cell-cell adhesion and interference with intracellular signaling, and in particular the Wnt pathway. Cadherin-11 binds to β-catenin and modulate its cytoplasmic pools and transcriptional activity. (Marie et al., 2014) And CYP1B1 enhances cell proliferation and metastasis through induction of EMT and activation of Wnt signaling via Sp1 upregulation. (Kwon et al., 2016) It is known that β-Catenin can accumulate and translocate to the nucleus in the presence of WNT ligands. Here, our results showed that the transcriptional levels of β-catenin also increased in fibroids. Plus, overexpression of sFRP4 is a robust, progesterone-regulated feature of leiomyomas that increases smooth muscle proliferation which was found by Meaghan's lab. (Meaghan et al., 2017)

In accordance to our bioinformatic analyses, these researches and our results indicate that the important role of CDH11, CYP1B1, B-Catenin, SFRP4 of participating in the pathogenesis of UL, particularly in ECM remodeling.

Furthermore, by IPA analysis, ESR1 pathway was also induced among six fibroids samples with RNAseq database. But surprisingly, not all fibroids showed increased ESR1 transcriptional levels, which may indicate a multiple regulation by hormonal stimulation and signaling transduction. (Figure 3.19)

Fold Change	Patient1 Fibroid A	Patient1 Fibroid B	Patient16 Fibroid B	Patient16 Fibroid C	Patient17 Fibroid A	Patient17 Fibroid B
CDH11	4.0	3.7	5.4	3.8	5.4	2.7
CYP1B1	1	1.5	1.1	1.2	3.9	3.2
CTNNB1	1	1	1.4	1.2	4	3
SFRP4	5.4	3.8	5.4	2.7	5	3.5

Table 3.1: Fold Change of Genes Expression in IPA. Six fibroids showed 1 to 5.4 fold changes in transcriptional levels of induced genes.

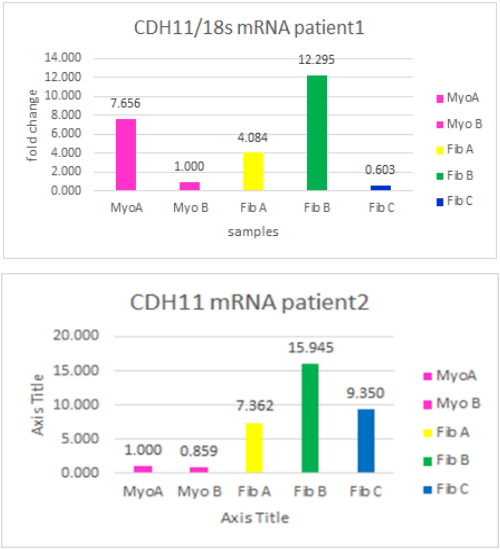

Figure 3.16: CDH11 RNA Expression. qPCR showed CDH11 RNA level is higher in fibroids than in myometrium.

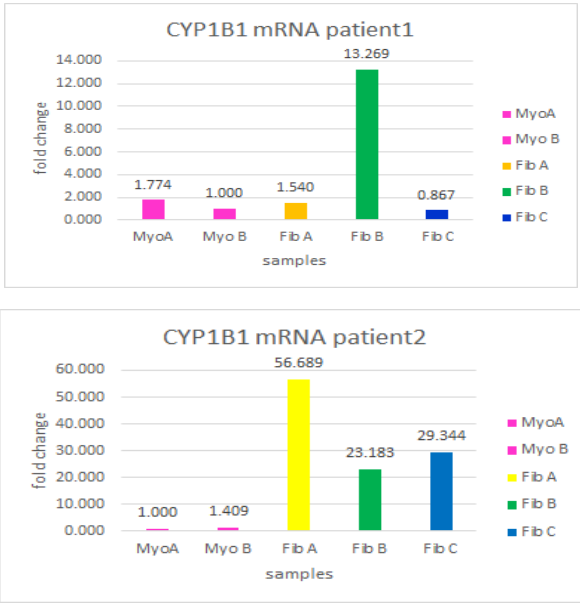

Figure 3.17: CYP1B1 RNA Expression. Q-PCR showed CYP1B1 RNA levels was higher in fibroids than in myometrium.

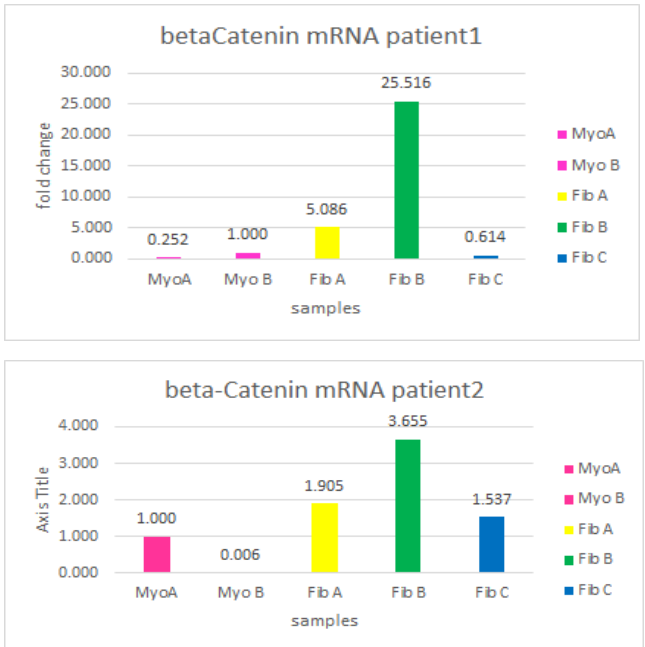

Figure 3.18: β-Catenin RNA Expression. B-Catenin mRNA levels were increased in fibroids than in adjacent myometrial except for Fibroid C of patient1, decreased in B-Catenin Expression.

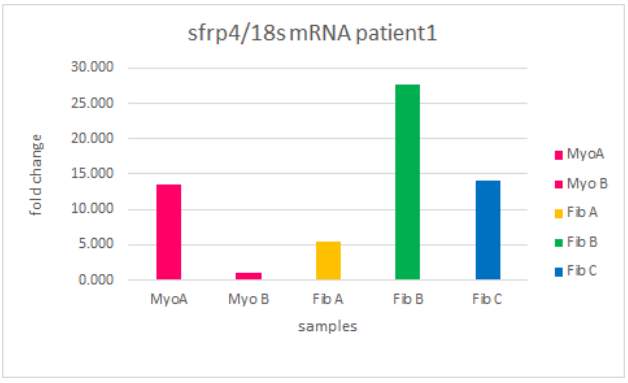

Figure 3.19: SFRP4 RNA Expression. In patient1, SFRP4 mRNA levels were increased in fibroids than in myometrium B.

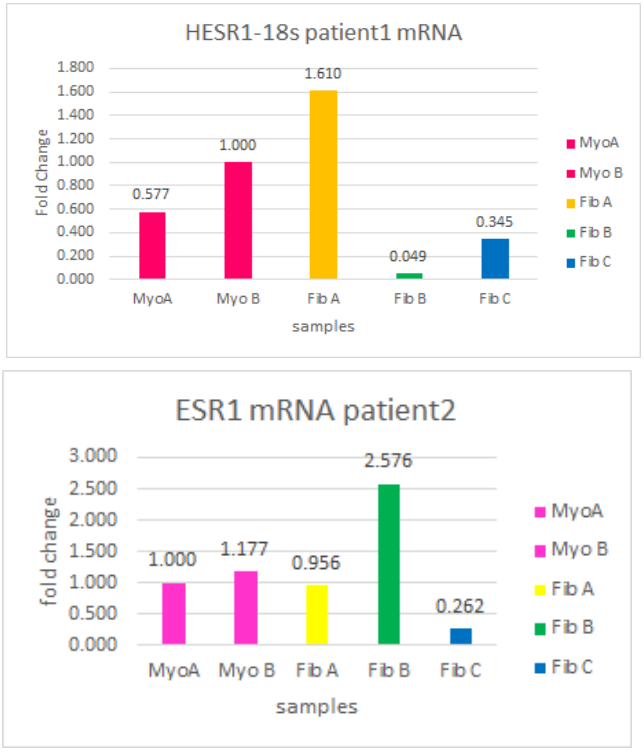

Figure 3.20: ESR1 RNA expression. Two fibroids showed increased level in ESR1 RNA transcription, but four of them showed decreased ESR1 levels.

Discussion

In the present study, we report that Wnt/β-catenin pathway activation in uterine leiomyoma demonstrated by the increased expression of AxinII, β-catenin at RNA and protein levels. Furthermore, an inverse HBP1 expression in different fibroids in the same patient and different patients may indicate HBP1-dependent subtypes of ULs (HBP1-low and HBP1-high). This classification was based on the inverse HBP1 expression levels showed by qPCR and bioinformatic analyses. Bioinformatic analyses showed gene sets of different signatures and functions were well-enriched in two distinct groups but a number of gene sets were commonly expressed in both, such as cell cycle, cell-cell junction, proteasome and diabetes pathway. Additionally, qPCR well confirmed the increased expression of genes (CDH11, CYP1B1, B-Catenin, SFRP4), which were showed high-fold changes in bioinformatic analyses. However, it is still uncertain that how these genes function in genetic regulation in the pathobiology of UL. More patients' data are needed to further validate this phenomenon.

Regarding HBP1-related subtypes of uterine leiomyoma, recent studies have also showed four UL subtypes with distinct driver pathways and biomarkers. 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) (Mehine et al., 2014). Leiomyomas with deletions affecting collagen, type IV, alpha 5 and collagen, type IV, alpha 6 (COL4A5-COL4A6) may constitute a rare fourth subtype (Mehine et al., 2013). HMGA2 and MED12 represent the two most common driver genes and together contribute to 80–90%

of all leiomyomas (Mehine et al., 2014). It is well known that uterine leiomyomas display significant heterogeneity in terms of symptoms, histopathology, therapeutic requirements, and genetic changes. The 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 (Mehine et al., 2016). Here we highlight subtype-specific expression changes in key driver pathways, including Wnt/β-catenin, IGF1, and PI3/AKT signaling. 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.

Additionally, leiomyomas are thought to be monoclonal tumors arising from the myometrium; however, it is not known what cell population in the myometrium gives rise to these tumors. As mentioned before, several recurrent genetic aberrations, such as trisomy of chromosome 12, deletions in 7q, and mutation affecting MED12 or HMGA2 gene were reported in uterine leiomyomas. As in other diseases, these genetic abnormalities and tumor stem cells are considered to play pivotal roles in the tumorigenesis in the leiomyoma. Intriguingly, some studies found that leiomyoma-derived side population (LMSP) have tumorigenic capacity under Estrogen+Progesterone stimulation but LMSP are negative for ESR1 and PGR. In mammary structures of humans and mice, mammary stem cells, despite being void of ESR1 and PGR themselves, are subject to regulation in both number and repopulating ability by steroid

hormones, particularly progesterone (Lydon JP 2010) (Fernandez-Valdivia R, Lydon JP 2011) (Asselin-Labat ML et al., 2011) (Joshi PA et al., 2011). Thus, paracrine factors are hypothesized to mediate signals from steroid receptor-positive adjacent to LMSP and the characterization of LMSP is necessary to understand the complex mechanisms underlying the pathogenesis of leiomyoma.

In recent years, several classes of small molecules have been shown to act as WNT inhibitors, which exert their inhibitory effects at various levels of the WNT signaling pathway. Three small molecules—Inhibitor of β-Catenin And TCF, niclosamide, and XAV939—have been shown to strongly and specifically reduce levels of active β-catenin in vitro by inhibiting β-catenin stabilization and downstream β-catenin signaling. The mechanism of action of niclosamide is thought to be through the internalization of Fzd1 and downregulation of WNT pathway intermediaries (Ono et al., 2014). It is known that the function of β-catenin is controlled by ubiquitination and ultimately degradation by the proteolytic pathways and our GSEA analysis also showed proteolytic pathways were enriched in fibroids; a possible mechanism of niclosamide function might be its effect on either the ubiquitination or a proteolytic pathway to cause degradation of β-catenin (Ono et al., 2014). Recent studies found that XAV939 inhibits the PARP domain of Tankyrase 1 and 2 and destabilizes AXIN1 and 2 by PARsylation (Ulsamer et al., 2006). Besides, WNT974, an orally available inhibitor of porcupine (PORCN), with potential antineoplastic activity. WNT974 binds to and inhibits PORCN in the endoplasmic reticulum (ER), which blocks post-translational acylation of Wnt ligands and inhibits their secretion (Cancer.gov). Recently a Phase II Trial is evaluating WNT974 in patients with metastatic head and neck squamous cell carcinoma (ClinicalTrials.gov).

Furthermore, epidemiological studies have associated green tea consumption with reduced recurrence of invasive and other breast cancers. EGCG ((-)-epigallocatechin3-gallate), the major phytochemical in green tea, emerged as an inhibitor, which can inhibit Wnt signaling in a dose-dependent manner in breast cancer cells. (Kim et al., 2006) The mechanism targeted the HBP1 transcriptional repressor, which we had previously characterized as a suppressor of Wnt signaling. The EGCG study provides a potential for combining EGCG with other small molecule inhibitors of Wnt signaling, including Cox-2 selective inhibitors, which were used to prevent colon cancer in familial adenematous polyposis patients. (Kim et al., 2006) Therefore, our future study will test EGCG and other pharmacological compounds for inhibiting Wnt signaling in annimal models, which may help us explore potential in uterine leiomyoma prevention or therapeutics.

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