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Authors
McGuire, Megan M
Yatsenko, Alexander
Hoffner, Lori
et al.

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Whole Exome Sequencing in a Random Sample of North American Women with Leiomyomas Identifies MED12 Mutations in Majority of Uterine Leiomyomas

Megan M. McGuire¹, Alexander Yatsenko¹, Lori Hoffner¹, Mirka Jones², Urvashi Surti¹, Aleksandar Rajkovic¹*

¹Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, ²Department of Gynecologic Pathology, Magee-Womens Hospital of UPMC, Pittsburgh, Pennsylvania, United States of America

Abstract

Uterine leiomyomas (uterine fibroids) arise from smooth muscle tissue in the majority of women by age 45. It is common for these clonal tumors to develop from multiple locations within the uterus, leading to a variety of symptoms such as pelvic pain, abnormal uterine bleeding, and infertility. We performed whole exome sequencing on genomic DNA from five pairs of leiomyomas and corresponding normal myometrium to determine genetic variations unique to leiomyomas. Whole exome sequencing revealed that the gene encoding transcription factor MED12 (Mediator complex subunit 12) harbored heterozygous missense mutations caused by single nucleotide variants in highly conserved codon 44 of exon 2 in two of five leiomyomas. Sanger re-sequencing of MED12 among these five leiomyomas confirmed the two single nucleotide variants and detected a 42 base-pair deletion within exon 2 of MED12 in a third leiomyoma. MED12 was sequenced in an additional 143 leiomyomas and 73 normal myometrial tissues. Overall, MED12 was mutated in 100/148 (67%) of the genotyped leiomyomas: 79/148 (53%) leiomyomas exhibited heterozygous missense single nucleotide variants, 17/148 (11%) leiomyomas exhibited heterozygous in-frame deletions/insertion-deletions, 2/148 (1%) leiomyomas exhibited intronic heterozygous single nucleotide variants affecting splicing, and 2/148 (1%) leiomyomas exhibited heterozygous deletions/insertion-deletions spanning the intron 1-exon 2 boundary which affected the splice acceptor site. Mutations were not detected in MED12 in normal myometrial tissue. MED12 mutations were equally distributed among karyotypically normal and normal uterine leiomyomas and were identified in leiomyomas from both black and white American women. Our studies show an association between MED12 mutations and leiomyomas in ethnically and racially diverse American women.

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* E-mail: rajkovic@upmc.edu

Introduction

Uterine leiomyomas, better known as fibroid tumors, are clinically apparent in nearly 25% of women by age 45, and they cause major morbidity in American women. More than 200,000 surgeries are performed each year to either remove the leiomyomatous tumors (myomectomy) or the entire uterus (hysterectomy) [1,2]. These tumors are responsive to steroid hormones such as estrogen and progesterone, and they often shrink following menopause [3]. Approximately half of all leiomyomas are asymptomatic, while the rest cause pelvic pressure and pain, menometrorrhagia, anemia, premature labor and infertility. These symptoms are intensified by the common occurrence of multiple tumors within a single uterus, often necessitating surgical intervention [4].

Previous genetic analyses of leiomyomas have established that approximately one half of these tumors have an abnormal karyotype, while the other half are karyotypically normal. We recently showed that a subset of karyotypically normal leiomyomas contained microdeletions and microduplications, but there is still a large proportion of karyotypically normal leiomyomas that have no known lesions [5]. In karyotypically abnormal leiomyomas, cytogenetic aberrations commonly include deletions in 7q, trisomy of chromosome 12, and various translocations between chromosomes 12 and 14 involving the high mobility group AT-hook 2 (HMG2) gene at 12q15, which encodes a transcriptional regulator [6,7,8,9]. Cytogenetic abnormalities are likely a reflection of general genomic instability as is true for other tumors. Mutations in leiomyomas that cause such genomic instabilities are unknown. Furthermore, heterozygous germline mutations in fumarate hydratase (FH) can cause a rare disorder of hereditary leiomyomatosis and renal cell carcinoma (HLRCC [MIM 150800]) [10,11]. However, fumarate hydratase does not appear to play an important role in the non-syndromic forms of uterine leiomyomas [12].

Methods

Patient information

This study was approved by the Institutional Review Board of the University of Pittsburgh as an exempt study (Pittsburgh, PA;
IRB # PRO111201699, and informed consent was not obtained. All leiomyoma and myometrial samples were derived from specimens that were originally obtained for clinical treatment or pathology purposes only, and specimens did not contain any personal identifiers or linkage codes. Samples were collected from women who were diagnosed with uterine leiomyomas and underwent medically indicated abdominal hysterectomy. Chromosome analysis was performed using standard G-bandning technique. Whole exome sequencing on the initial five pairs of samples from five different individuals was performed on randomly selected, karyotypically normal leiomyomas. Subsequent DNA sequencing was performed on 143 randomly selected leiomyomas from our biobank of more than 500 samples. The histology of all samples was reviewed by a board certified gynecologic pathologist.

Nucleic acid extraction and preparation
Genomic DNAs from all leiomyomas and corresponding myometrial samples were extracted from 100 mg of freshly frozen tissue using the Gentra Puregene Blood Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s protocol. RNA was extracted from a randomly selected group of leiomyoma samples which harbored MED12 mutations. Tissue samples stored at −80°C were placed in chilled RNAiso+®-ICE (Invitrogen, Carlsbad, CA, USA) for at least 16 hrs at −20°C to allow for tissue thawing under preservative conditions prior to RNA isolation. TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was used to carry out RNA isolations according to the manufacturer’s protocol. RNA samples were converted to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol.

Whole exome capture and DNA sequencing
Ten genomic DNA samples (five leiomyomas and five corresponding myometrial samples) were subjected to in-solution exome enrichment via the SureSelect Human All Exon Kit v2 (Agilent, Santa Clara, CA, USA). Following exome capture, the samples were submitted for single read high-throughput sequencing on the Genome Analyzer IIx (Illumina Inc., San Diego, CA). Following exome capture, the samples were subjected to in-solution exome enrichment using the SureSelect Human All Exon Kit v2 (Agilent, Santa Clara, CA, USA). Following exome capture, the samples were submitted for single read high-throughput sequencing on the Genome Analyzer IIx (Illumina Inc., San Diego, CA, USA).

Sequencing data analysis
Exome sequencing raw data files were received in FASTQ format and converted to FASTA format using default settings for NextGENe v2.16 software (SoftGenetics, State College, PA, USA). NextGENe was also used for sequence alignment against reference human genome assembly GRCh37/hg19. Minimum coverage of 5 sequencing reads per base-pair (bp) was required for variant calling and variants in less than 20% of the sequencing reads were considered sequencing artifacts. Nucleotides in exons and within 10 bps of the exon-intron junctions were the focus of further variant analysis.

Variant filtering
The initial steps of variant filtering for the exome sequencing data were carried out using NextGENe software; later filtering steps were performed on exported NextGENe files using the functions available in a spreadsheet. Each pair of leiomyoma and corresponding normal myometrial tissue was compared independently. Our focus was limited to DNA variants which were present in the leiomyoma but absent in the corresponding normal myometrium. Minimum coverage of 20 sequencing reads per bp was required for this stage of variant filtering. Due to the presence of common variants in both samples, we required the frequency of the reference allele in the leiomyoma sample to be at least 25% less than the frequency of the reference allele in the corresponding myometrial sample in order for the variant to pass the first filtering step. Variants outside of the exons and exon-intron junctions (10 bps flanking each exon) were excluded from further analysis. The variant lists for each tissue type (leiomyoma/myometrium) were compared against each other to elucidate variants which were unique to the leiomyoma. DNA variants present in the Single Nucleotide Polymorphism database v132 (dbSNP132) were removed (http://www.ncbi.nlm.nih.gov/projects/SNP/). Non-synonymous single nucleotide variants (SNVs) were also removed. Finally, DNA variants were evaluated with protein prediction tools, PolyPhen-2 and SIFT (http://sift.jcvi.org/), to reveal variants which were predicted to have an impact on protein function. The final variant lists were compared across samples to identify genes commonly mutated in multiple leiomyomas as a means to focus further study.

MED12 variant validation
Exome sequence data analysis identified one nucleotide in codon 44 of exon 2 of MED12 which was uniquely mutated in two of five leiomyomas. To validate the presence of the SNVs, the five pairs of leiomyoma and corresponding myometrial samples underwent Sanger re-sequencing of MED12. Oligonucleotide primers were designed against MED12 (NCBI Gene ID: 9968; chrX:70,338,406–70,362,304 [GRCh37/hg19]) using Primer3 (http://frodo.wi.mit.edu/primer3/). Genomic DNA was amplified with amfisure PCR Master Mix (GenDEPOT, Barker, TX, USA) under the following conditions: 94°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec; and 72°C for 2 min. DNA sequences were evaluated using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA). Upon confirmation of the SNVs in two leiomyomas and identification of a deletion missed by whole exome sequencing (due to length) in a third leiomyoma, an additional 143 leiomyomas, 73 of which had corresponding myometrial samples, were screened for variants in MED12 via Sanger sequencing, as described above. MED12 variant data was deposited in the Single Nucleotide Polymorphism database (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP/).

cDNA sequencing
MED12 cDNAs of exon 2 and flanking regions (exons 1 and 3) in ten randomly selected leiomyomas with MED12 variants were sequenced to verify that the mutated allele was actively expressed in each leiomyoma. Oligonucleotide primers were designed using Primer3. cDNA was amplified with amfisure PCR Master Mix under the previously specified conditions. PCR products were gel purified using the QIAGEN Gel Extraction Kit (QIAGEN, Valencia, CA, USA) prior to Sanger sequencing.

Statistical analysis
A one-tailed Fisher’s exact test was performed to determine statistical significance of associations between categorical variables, and a p value < 0.05 was considered to be significant.

Results
To search for gene(s) that associate with leiomyomas, we examined the genomic DNA of leiomyomas and corresponding myometrial samples from five individuals via whole exome enrichment and high-throughput DNA sequencing. We analyzed DNA variants in exons and within 10 base-pairs (bps) of exon-intron junctions, excluding single nucleotide variants (SNVs) that populate the Single Nucleotide Polymorphism database v132.
MED12 Is Mutated in Leiomyomas

Genomic DNAs from five pairs of leiomyomas and corresponding myometrial samples underwent whole exome enrichment and sequencing. After sequence alignment, each pair of leiomyoma and corresponding myometrium was independently analyzed using NextGENe software (represented by each row in the table; SoftGenetics, State College, PA). The DNA variants in each tissue (leiomyoma/normal myometrium) were compared to reveal mutations which were unique to the leiomyoma. After sequence filtering, all of the DNA variants that we observed in uterine leiomyomas were heterozygous. Since random X chromosome inactivation allows either the wild-type or the mutant allele to be expressed, we examined whether mutated MED12 transcripts were expressed in leiomyomas harboring MED12 DNA variants [13]. We isolated RNA and generated cDNA from a random selection of ten leiomyomas known to carry MED12 DNA variants. Sanger sequencing of cDNAs from these leiomyomas revealed that all of the transcripts derived solely from the mutant MED12 alleles. These results are consistent with the interpretation that leiomyomatous tumors with MED12 DNA variants express a mutant form of the MED12 protein.

Karyotype data was available for 134 leiomyomas: 85 (63%) were karyotypically normal, while 49 (37%) had various cytogenetic abnormalities (Table S1). The proportions of karyotypically normal and abnormal leiomyomas were consistent with previous studies [14,15]. Fifty-nine of 85 leiomyomas (69%) with a normal karyotype harbored MED12 variants, while 31/49

### Table 1. Whole exome data filtering schema for DNA variants.

| Sample ID | Variants in exons and exon-intron junctions | Variants unique to leiomyoma | Filtered variants | Filtered SNVs | Filtered dels/indels | Damaging variants |
|-----------|-----------------------------------------------|-------------------------------|-------------------|---------------|---------------------|------------------|
| L1        | 13,654                                        | 355                           | 13                | 9             | 2                   | 2                |
| L2        | 13,477                                        | 456                           | 18                | 14            | 1                   | 1                |
| L3        | 13,976                                        | 342                           | 18                | 12            | 1                   | 1                |
| L4        | 12,799                                        | 387                           | 8                 | 6             | -                   | 1                |
| L5        | 13,513                                        | 373                           | 18                | 11            | 2                   | 5                |

*All DNA variants in exons and within 10 base-pairs (bps) of exon-intron junctions in introns.
*DNA variants unique to the leiomyoma in exons and within 10 bps of exon-intron junctions in introns.
*DNA variants unique to the leiomyoma in exons and within 10 bps of exon-intron junctions in introns which were not found in dbSNP132.
*Exonic single nucleotide variants (SNVs) unique to the leiomyoma which caused a change in the protein sequence and were not found in dbSNP132.
*SNVs unique to the leiomyoma located in introns within 10 bps of exon-intron junctions which were not found in dbSNP132.
*Exonic deletions/insertion-deletions (dels/indels) unique to the leiomyoma which were not found in dbSNP132.
*DNA variants unique to the leiomyoma in exons and within 10 bps of exon-intron junctions in introns which were predicted to be damaging to the protein, not found in dbSNP132.
*Leiomyomas L2 and L3 harbored missense SNVs in exon 2 of MED12 which were detected by exome sequencing and predicted to be damaging. L1 contained a 42 bp deletion in exon 2 of MED12 which was undetected by exome sequencing but revealed via Sanger sequencing.

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leiomyomas (63%) with an abnormal karyotype had MED12 variants. These results were not statistically significant ($p = 0.29$). We also had racial information on 143 leiomyomas. Leiomyomas under analysis were obtained from 23 black American women and 120 white American women. The frequency of MED12 mutation was 78% (18/23) in black American women and 66% (79/120) in white American women, but the association between race and MED12 status was not statistically significant ($p = 0.36$). The frequency of MED12 mutation was higher when multiple tumors were detected in a single uterus (72.5%; 87/120) as opposed to a single tumor (41%; 9/22) ($p < 0.01$).

**Discussion**

Uterine leiomyomas are benign smooth muscle tumors that can emanate from anywhere in the uterus and distort uterine anatomy and function. Leiomyomas are the leading cause of dysfunctional uterine bleeding and hysterectomies. The clonal origin of leiomyomas makes them genetically homogenous. However, karyotypes between individual leiomyomas differ, and abnormal karyotypes are identified in approximately 40% of cases [14,13,16]. Rare hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC [MIM 150800]) is caused by heterozygous germline mutations in the gene encoding fumarate hydratase, but the role(s) of individual genes in common leiomyomas is not well understood [10,11]. The presence of MED12 variants in nearly 70% of leiomyomas suggests that MED12 plays an important role in the genesis of these tumors in both white and black American women.

**Table 2. DNA variants in leiomyomas were confined to exon 2 of MED12.**

| Variant location* | Variant typeb | Nucleotide change | Protein change | Mutated leiomyomasc |
|-------------------|---------------|------------------|----------------|---------------------|
| Splice site       | SNV           | IVS1-8T>A        | p.E33_D34insPQ | 2 (1.4%/2.0%)       |
|                   | Deletion/Indel| IVS1-1, 139del41 | Splice acceptor loss | 1 (0.7%/1.0%)     |
|                   |               | IVS1-2, 141del44insAG | Splice acceptor loss | 1 (0.7%/1.0%)   |
| Exon 2            | SNV           | c.107T>G         | p.L36R         | 3 (2.0%/3.0%)       |
|                   |               | c.128A>C         | p.Q43P         | 5 (3.4%/5.0%)       |
|                   |               | c.130G>C         | p.G44R         | 7 (4.7%/7.0%)       |
|                   |               | c.130G>A         | p.G44S         | 9 (6.1%/9.0%)       |
|                   |               | c.130G>T         | p.G44C         | 8 (5.4%/8.0%)       |
|                   |               | c.131G>C         | p.G44A         | 5 (3.4%/5.0%)       |
|                   |               | c.131G>A         | p.G44D         | 32 (21.6%/32.0%)    |
|                   |               | c.131G>T         | p.G44V         | 6 (9.1%/9.0%)       |
|                   |               | c.130G>T:c.131G>T | p.G44F         | 1 (0.7%/1.0%)       |
|                   | Deletion/Indel| c.103_138del36   | p.E35_N46del   | 1 (0.7%/1.0%)       |
|                   |               | c.107_111del5insGC | p.L36_T37delinsR | 1 (0.7%/1.0%)      |
|                   |               | c.111_155del45   | p.A38_S52del   | 1 (0.7%/1.0%)       |
|                   |               | c.113_121del19   | p.A38_N40del   | 1 (0.7%/1.0%)       |
|                   |               | c.117_122del6    | p.N40_V41del   | 1 (0.7%/1.0%)       |
|                   |               | c.118_132del15   | p.N40_G44del   | 1 (0.7%/1.0%)       |
|                   |               | c.118_134del17insTA | p.N40_F45delinsY | 1 (0.7%/1.0%)      |
|                   |               | c.118_146del29insTT | p.N40_P49delinsF | 1 (0.7%/1.0%)      |
|                   |               | c.122_148del27   | p.V41_P49del   | 1 (0.7%/1.0%)       |
|                   |               | c.122_163del42   | p.V41_D54del   | 1 (0.7%/1.0%)       |
|                   |               | c.123_152del30   | p.K42_V51del   | 1 (0.7%/1.0%)       |
|                   |               | c.126_131del6    | p.K42_G44delinsN | 1 (0.7%/1.0%)     |
|                   |               | c.126_140del15   | p.K42_F45del   | 1 (0.7%/1.0%)       |
|                   |               | c.129_137del19   | p.Q43_N46delinsH | 1 (0.7%/1.0%)     |
|                   |               | c.129_143del15   | p.G44_Q48del   | 1 (0.7%/1.0%)       |
|                   |               | c.133_144del12   | p.F45_Q48del   | 1 (0.7%/1.0%)       |
|                   |               | c.149_163del15   | p.A50_D54del   | 1 (0.7%/1.0%)       |

*Splice site variants were located in intron 1 within 10 base-pairs of the intron 1-exon 2 junction.

Variants were classified as a single nucleotide variant (SNV) or a deletion/insertion-deletion (indel).

The number of leiomyomas with each specific variant is followed in parentheses by the percentage of the total number of leiomyomas under study (148) and the percentage of the total number of mutated leiomyomas (100), respectively.

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MED12 Is Mutated in Leiomyomas

Figure 1. Variants in codon 44 in exon 2 of MED12 accounted for 71% of mutations in uterine leiomyomas. This schema depicts the full-length human MED12 transcript which contains 45 coding exons (6,531 base-pairs/2,177 amino acids). Exon 2, denoted by the asterisk, is located near the N terminus and contains 105 base-pairs/35 amino acids. Codon 44, which encodes glycine (boxed G), harbored single nucleotide variants in 71/100 (71%) mutated leiomyomas. These variants replaced guanines at nucleotide positions 130 and 131. All variants changed the amino acid encoded by codon 44, and these variants were predicted to be damaging. The percentage of the total number of mutated leiomyomas (100) harboring each variant is noted in parentheses. * One leiomyoma exhibited two consecutive single nucleotide variants at positions 130 and 131.

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activation [18]; through this interaction, MED12 indirectly aids in transcriptional regulation, as CDK8/Cyclin C is capable of phosphorylating the carboxy-terminal domain (CTD) of RNAPII leading to subsequent transcription of genes with certain types of promoters [19]. CDK8 is a colorectal cancer oncogene and likely plays a role in tumorigenesis [20]. MED12 is also important in chromatin modification and transcriptional repression, independent of its role in CDK8 activation [21]. If the current association is causative, MED12 is a tumor suppressor gene, leading to abnormal leiomyomatous growth when mutated.

The variants described here are not germline mutations; rather, they are solely restricted to the tumor. Since MED12 is located on the X chromosome, random X chromosome inactivation results in sole expression of the mutated allele with consequent tumorigenesis. Interestingly, there was no association between karyotype and MED12 mutation, in our study. Numerous karyotype abnormalities associate with leiomyomas, and it is likely that such chromosomal imbalances are a secondary consequence of mutations that derail normal cell cycle controls. We hypothesize that MED12 variants deregulate the cell cycle in the normal myometrium, resulting in abnormal growth and genomic instability.

Germline MED12 mutations are responsible for at least two forms of X-linked mental retardation: Opitz-Kaveggia syndrome (FG syndrome [MIM 305450]) and Lujan-Fryns syndrome (MIM 309520). The SNVs implicated in these syndromes are located in exons 21 and 22, affecting the carboxy-terminus of MED12 (p.R961W and p.N1000S, respectively) [22,23]. These two syndromes are not associated with tumorigenesis and carry female are reported to be “normal”. None of the syndromic mutations were present in the leiomyomas under study. The concentration of variants within exon 2 of MED12 in a significant subset of leiomyomas indicates that this region of the protein is involved in cell cycle control and tumor repression.

MED12 mutations were recently described in leiomyomas from a group of Finnish women [24]. In Finnish women, MED12 was found to be mutated in 70% (159/225) of tumors from a total of 80 individuals. Remarkably and similar to our study, they also found that all of the variants were located in exon 2, and a high percentage of the SNVs were localized to codon 44 (110/225; 49%). MED12 variants were not limited to Finnish women, as 14/28 (50%) leiomyomas from eighteen black/colored South African women carried MED12 variants [25]. These results are in agreement with our findings that 78% of black American women carried MED12 variants. A recent study reported that rearrangements involving the 12q14~15 region, and presumably associated with disruption of the HMGA2 gene, are not associated with MED12 mutations [26]. Among our cases, only two leiomyomas exhibited 12q14~15 rearrangements, and neither of these tumors harbored MED12 variants. Therefore, our sample size was too small to support or refute a hypothesis that rearrangements involving 12q14~15 represent a separate genetic pathway.

Our study on outbred American women, including both white and black individuals, further extends the association of MED12 variants and leiomyomas in various ethnic and racial groups. Further studies are needed to determine whether this association is causative or a reflection of other underlying variants in the myometrial genome which increase susceptibility to leiomyoma development.

Supporting Information

Table S1 Karyotype and demographic data for genotyped uterine leiomyoma. A single tumor (leiomyoma) was genotyped from 148 patients. We have provided the karyotype of the genotyped tumor, size of the genotyped tumor, number of tumors detected (one/multiple), race, age at treatment, and MED12 status of the genotyped tumor for each patient, where available. * Race is denoted by B for black American and W for white American. * MED12 status is indicated by the specific mutation detected or WT, which denotes wild-type.

Author Contributions

Conceived and designed the experiments: AR MM AY. Performed the experiments: MM. Analyzed the data: MM AR AY MJ. Contributed reagents/materials/analysis tools: US LH. Wrote the paper: MM AR.
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