Next-generation sequencing reveals differentially expressed small noncoding RNAs in uterine leiomyoma

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Objective: To determine the expression profile of small noncoding RNAs (sncRNAs) in leiomyoma, which has not been investigated to date.
Design: Laboratory-based investigation.
Setting: Academic center.
Patient(s): Women undergoing hysterectomy for benign indications.
Intervention(s): Next-generation sequencing and screening of an sncRNA database with confirmatory analysis by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).
Main Outcome Measure(s): Expression profile of sncRNAs in leiomyoma and matched myometrium.
Result(s): Screening our previously determined RNA sequencing data with the sncRNA database resulted in identification of 15 small nuclear (sn) RNAs, 284 small nucleolar (sno) RNAs, 98 Piwi-interacting (pi) RNAs, 152 transfer (t) RNAs, and 45 ribosomal (r) RNAs, of which 15 snoRNAs, 24 piRNAs, 7 tRNAs, and 6 rRNAs were differentially expressed at a 1.5-fold change cutoff in leiomyoma compared with myometrium. We selected 5 snoRNAs, 4 piRNAs, 1 tRNA, and 1 rRNA that were differentially expressed and confirmed their expression in paired tissues (n = 20) from both phases of the menstrual cycle with the use of qRT-PCR. The results indicated up-regulation of the snoRNAs (SNORD30, SNORD27, SNORA16A, SNORD46, and SNORD56) and down-regulation of the piRNAs (piR-1311, piR-16677, piR-20365, piR-4153), tRNA (TRG-GCC5–1), and rRNA (RNA5SP202) expression in leiomyoma compared with myometrium ($P<.05$). The pattern of expression of these sncRNAs was similar to RNA sequencing analysis, with no menstrual cycle–dependent differences detected except for SNORD30. Because Argonaute 2 (AGO2) is required for sncRNA-mediated gene silencing, we determined its expression and found greater abundance in leiomyoma.
Conclusion(s): Our results provide the first evidence for the differential expression of additional classes of sncRNAs and AGO2 in leiomyoma, implicating their roles as a gene regulatory mechanism. (Fertil Steril® 2018;109:919–29. ©2018 by American Society for Reproductive Medicine.)
Key Words: sncRNA, piRNA, snoRNA, AGO2, leiomyoma

Leiomyomas are benign uterine tumors with an unknown etiology affecting 40%–70% of women during their reproductive years (1). Symptomatic tumors are a major cause of chronic pelvic pain, abnormal uterine bleeding, and associated infertility and account for more than 30% of all hysterectomies performed in the United States annually (1). Although ovarian steroids are known to be key regulators of leiomyoma growth, altered expression of many protein-coding genes (1–3) as well as genetic heterogeneity associated with chromosomal rearrangements and mutation in a number of genes also have been associated with their development and growth progression (1). In addition to protein-coding genes, conventional, microarray, and recent high-throughput

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sequencing have provided further evidence for the expression of a large number of non–protein-coding RNAs (ncRNAs) in leiomyoma (4–6).

To date, tens of thousands of ncRNAs derived from different genomic loci have been identified. These RNAs are classified as small (17–120 nucleotides long) and long (>200 nucleotides long) noncoding RNAs (sncRNAs and lncRNAs) (7). sncRNAs are transcribed as precursors of 60–300 nucleotides length, and in their mature form they are further classified as microRNAs (miRNAs; 17–22 nucleotides), small nuclear and nucleolar RNAs (snRNA and snoRNAs; 70–120 nucleotides), and Piwi-interacting RNAs (piRNAs; 26–33 nucleotides). snoRNAs are highly conserved and classified as C/D box (SNORDs) or H/ACA box (SNORAs) based on their sequence and structure (8). miRNAs have been the most intensively investigated in the past few years, and accumulated evidence supports their regulatory function on a vast number of protein-coding genes involved in various normal cellular activities. Aberrant expression of miRNAs is associated with a wide range of disorders, including tumorigenesis and tissue fibrosis (9, 10). In previous studies, including our own, high-throughput sequencing identified the expression profile of a large number of miRNAs in leiomyoma and myometrium and provided support for altered expression and regulatory function of a number of them, including let7, miR-21, miR-29, miR-200, and miR-25/93/106 cluster in leiomyoma and leiomyoma smooth muscle cells (6,11–15).

Far less is known about the function and expression profile of other members of sncRNAs. Functionally, snoRNAs play a central role in modifying and processing snRNAs and ribosomal (rRNA) and transfer (tRNA) RNA (16). piRNAs are the largest class of sncRNA family and functionally regulate epigenetic post-transcriptional gene expression and degradation (17). tRNAs are derived from pre-tRNA by endonuclease RNase Z and in their mature form play a well defined role in protein translation (18). However, shorter tRNA fragments (tRFs) of 30–35 nucleotides length are also generated through cleavage of mature tRNAs at both 5′ and 3′ ends at the anticodon loop by angiogenin (19). In addition, snRNAs, snoRNAs, tRNAs, and rRNAs can be further processed into miRNAs or small interfering RNAs (siRNAs) and incorporated into Argonaute proteins to form RNA-induced silencing complex (RISC) for cleavage, degradation, or inhibition of translation of downstream target messenger RNA (mRNA) (20).

Argonaute proteins are the active part of RISC and play a central role in RNA silencing processes, resulting in RNA degradation or translation inhibition (21). In humans, there are eight Argonaute family members separated into two subfamilies: 1) four members of Argonaute-like subfamily interact with miRNAs and siRNAs; and 2) four members of Piwi-like subfamily bind to piRNAs (20). The Argonaute family members are composed of four characteristic domains: the N-terminal domain, the Piwi-Argonaute-Zwille (PAZ) domain, the middle (MID) domain, and the C-terminal Piwi domain (20). Among them, the Piwi domain, structurally resembling RNase H, is essential for target RNA cleavage through an active catalytic motif "Asp-Asp-Asp/His/Glu/Lys" that harbors a divalent metal ion and is necessary for catalysis. The Piwi domain also mediates protein-protein interaction with Dicer at one of its RNase III domains (20). Although four Argonaute-like proteins are capable of loading miRNAs or siRNAs, only Argonaute 2 (AGO2) has endonuclease activity in humans, thus playing a key role in gene silencing (22).

The identity and pattern of expression of snRNAs, snoRNAs, piRNAs, tRNAs, and rRNAs, as well as AGO2, in leiomyoma and myometrium has not been evaluated to date. Therefore, in the present study, with the use of our existing high-throughput sequencing dataset generated from leiomyoma and paired myometrium, we carried out an in-depth data analysis to identify the expression profile of snRNAs, snoRNAs, piRNAs, tRNAs, and rRNAs. With the use of quantitative reverse-transcription polymerase chain reaction (qRT-PCR), we selected and confirmed the expression of a number of differentially expressed sncRNAs along with detection of AGO2 protein in 20 pairs of leiomyoma and matched myometrium tissues from both phases of the menstrual cycle.

MATERIALS AND METHODS

Myometrium and Leiomyoma Tissue Collection

Leiomyoma (3–5 cm in diameter) and paired myometrium (n = 20) were collected at Harbor-UCLA Medical Center with prior approval obtained from the Institutional Review Board at LA-Biomed Research Institute, Harbor-UCLA Medical Center. The paired tissues were from white Hispanic (n = 15) and African-American (n = 5) women aged 30–49 years (mean 42 ± 5.6 y). This sample set included three paired tissues previously used for high-throughput sequencing (4). Based on endometrial histology, the paired leiomyoma and myometrium were from follicular (n = 10) and luteal (n = 10) phases of the menstrual cycle. The tissues were snap-frozen and stored in liquid nitrogen for further analysis.

Total RNA Isolation and RNA Sequencing

Total RNA was isolated from leiomyoma and matched myometrium with the use of miRNeasy Mini Kit (Qiagen), and RNA concentration and integrity were determined as previously described (4). Samples with RNA integrity numbers ≥9 were used for library preparation. The RNA sequencing was carried out at the UCLA Clinical Microarray Core Facility (http://pathology.ucla.edu/tcgb) as previously described (4). Briefly, for the production of small RNA sequencing libraries, 500 ng total RNA from each tissue was used and libraries were prepared according to manufacturer's instructions for the Truseq small-RNA kit (Illumina). Each library was then pooled and sequenced on a MiSeq single-ended 35-bp run (Illumina) to 10 million reads with 80%–90% alignment per library. Our sequencing GEO accession number is GSE100338.

Assembly of snRNA, snoRNA, piRNA, tRNA, and rRNA Annotations

For data quality control, FastQC was used to check the raw FastQ data quality and Trimmomatic to remove adaptors and trim quality bases. After adapter clipping, we removed leading and trailing ambiguous or low-quality bases (below