Identification of Differentially Expressed mRNAs and lncRNAs in Uterine Leiomyomas with RNA Sequencing

Xinyu Chen (1870230507@qq.com)
Lianyungang maternal and child health hospital
Fanfei Meng
Lianyungang maternal and child health hospital
Yijing Ji
Lianyungang maternal and child health hospital
Yuan Wang
Lianyungang maternal and child health hospital
Maofang Hua
Lianyungang maternal and child health hospital

Research Article

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Identification of differentially expressed mRNAs and lncRNAs in uterine leiomyomas with RNA sequencing

Fanfei Meng[1], Yijing Ji[1], Xinyu Chen[1], Yuan Wang[1], Maofang Hua[1*]

[1]Department of Gynecology, Lianyungang maternal and child health hospital, NO.669 Qindongmen Road, Lianyungang, 222001, Jiangsu Province, China.

*Corresponding author:
Maofang Hua
Department of Gynecology, Lianyungang maternal and child health hospital, NO.669 Qindongmen Road, Lianyungang, 222001, Jiangsu Province, China.
E-mail: 18705130566@163.com

Abstract

Objective: To explore the functions of mRNAs and lncRNAs in the occurrence of uterine leiomyomas (UL) and further clarify the pathogenesis of UL by detecting the differential expression of mRNAs and lncRNA in 10 cases of UL tissues and surrounding normal myometrial tissues by high-throughput RNA sequencing.

Methods: The tissue samples of 10 patients who underwent hysterectomy for UL in Lianyungang maternal and child health hospital from January 2016 to December 2021 were collected. The differentially expressed mRNAs (DEmRNAs) and lncRNAs (DElncRNAs) were identified, and further analyzed by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis. The protein–protein interaction network (PPI) was constructed in Cytoscape software. Functional annotation of the nearby target cis-DEmRNAs of DElncRNAs was performed with DAVID. Meanwhile, the co-expression network of DElncRNA-DEmRNA was constructed in Cytoscape software.

Results: A total of 553 DElncRNAs (283 up-regulated DElncRNAs and 270 down-regulated DElncRNAs) and 3,293 DEmRNAs (1,632 up-regulated DEmRNAs and 1,661 down-regulated DEmRNAs) were obtained. GO pathways enrichment analysis revealed that several important pathways were significantly enriched in UL such as blood vessel development, regulation of ion transport and external encapsulating structure organization. In addition, Cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction and complement and coagulation cascades were significantly enriched in KEGG pathways enrichment analysis. A total of 409 DElncRNAs-nearby-targeted DEmRNA pairs were detected, which included 118 DElncRNAs and 136 DEmRNAs. Finally, we found that top two DElncRNAs with most nearby DEmRNAs were BISPR and AC012531.1.

Conclusions: These results suggested that 3293 DEmRNAs and 553 DElncRNAs were differentially expressed in UL tissue and normal myometrium tissue, which might be candidate identified therapeutic and prognostic targets for UL and be considered as offering several possible mechanisms and pathogenesis of UL in the future.

Keywords: Uterine leiomyomas; Long noncoding RNA; mRNAs; High-throughput-sequencing.

Introduction

Uterine leiomyomas (UL), as one of the most common benign tumor in women, is arising from the non-neoplastic proliferation of smooth muscle cells within the myometrium[1,2]. UL is more than 70% of women suffered before menopause, with an incidence of up to 20% to 25% in women of reproductive age[3,4]. Accumulating clinical evidences have demonstrated that UL has caused a lot of health problems, including uterine bleeding, dysmenorrhoea, repetitive pregnancy loss, menorrhagia, infertility, pelvic pressure, pain and so on[5, 6]. Therefore, it is urgent to find new etiological and pathological features of UL, to improve the diagnostic efficiency of UL[1]. To date, little is known about the pathogenesis of UL, leading to the lack of a valid medical treatment[7]. However, there is considerable evidence that estrogen plays a major role in the proliferation of these benign tumors[8]. While a strong correlation between ovarian hormone levels or age and the development of UL was found, recent studies of UL have identified novel genes or pathways suggesting the differential expression of mRNAs and lncRNA of this disease progression, in order to improve the early detection of tumor and early intervention and treatment of UL[9,10].

With the development of high-throughput sequencing, novel bioinformatics approaches and corresponding experimental validation, a broad spectrum of mRNAs and lncRNAs have been suggested to play an important participation in various biological processes[11,12]. Long non-coding RNAs (lncRNAs) are a type of long non-coding RNA molecules, more than 200 nucleotides in length, which limited protein-encoding potential[13]. Functionally, accumulating evidences have implicated that lncRNAs are involved in various physiological processes, such as human genome imprinting, chromatin remodeling, X chromosome silencing, epigenetic regulation, cell cycle and differentiation, and the abnormal expression and dysfunction of lncRNAs play an functional role in the occurrence and development of a variety of diseases[14,15]. Mechanistically, more and more studies have shown that lncRNAs could participate in gene expression by recruiting chromosome remodeling complex into gene promoter[16-18]. And several lncRNAs fulfill their roles by serving as sponges to arrest miRNA function[17]. Besides, they also directly

interact with some important proteins to augment or attenuate their function[18]. In addition, the abnormal expression or functional changes in lncRNA expression has become important biological regulators for understanding the molecular mechanisms of these diseases and identifying effective diagnostic biomarkers and therapeutic targets[19,20]. However, little information available in literatures about the association between lncRNAs and the progression of UL has yet been elucidated.

In the present study, the differentially expressed lncRNAs (DElncRNA) and mRNAs (DEmRNAs) in 10 cases of UL tissues and surrounding normal myometrial tissues by high-throughput RNA sequencing. The results found that a total of 553 DElncRNAs (283 up-regulated DElncRNAs and 270 down-regulated DElncRNAs) and 3,293 DEmRNAs (1,632 up-regulated DEmRNAs and 1,661 down-regulated DEmRNAs) were obtained. This study could represent a novel proposal to increase the understanding of the pathogenesis and molecular targeted therapy of UL.

Materials and Methods

Ethical compliance

The experiment complied with the Ethics Committee of Lianyungang maternal and child health hospital, and the written informed consent was obtained from each participant before surgical procedures.

Patients and samples

A total of 10 UL pairs with tumour samples and surrounding normal myometrial tissue samples were obtained, and chosen for high-throughput RNA sequencing at random from UL patients who underwent hysterectomy in Lianyungang maternal and child health hospital from January 2016 to December 2021. All of the patients with UL were free of treatment before surgery. Pathological diagnostics for UL were independently diagnosed by three experienced pathologists. Fresh tissue samples were immediately frozen in liquid nitrogen after resection from UL patients to protect the protein or RNA away from degradation.

RNA extraction and RNA sequencing

According to the manufacturer's protocol, the total RNA was isolated and extracted from frozen UL tissues and surrounding normal myometrial tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed to high-quality cDNA using a PrimeScript RT Master Mix (Vazyme Biotech, Nanjing, China). With Agilent 2100 Bioanalyzer, the concentration, integrity and RNA integrity number (RIN) values of RNA were assessed. RNA sequencing was performed based on the Illumina Hiseq platform (Illumina, Inc., San Diego, CA, USA) with 151 bp sequencing mode. The RNA sequencing was performed with paired-ends and 10G depth. With Base Calling version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the FASTQ sequence data were acquired from the RNA sequencing data. Read QC tool in FastQC version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/ fastqc/) was used for the quality control of FASTQ data with Q > 30. Trimming of raw data was performed with cutadapt version 1.16 (http://cutadapt.readthedocs.io). Reads with low quality were removed to obtain the clean reads.

Identification of DEmRNAs and DElncRNAs

In order to align the clean reads with the human reference genome, Ensemble GRCh38.p7, HISAT2 version 2.1.0 was applied. The expression levels of mRNAs and lncRNAs were normalized and outputted with StringTie version 1.3.3b (http://ccb.jhu. edu/software/stringtie/). Fragments per Kilobase of exon per million fragments mapped (FPKM) of lncRNAs and mRNAs were calculated with StringTie. With edgeR version 3.24 (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html), both DEmRNAs and DElncRNAs were obtained with $|\log2FC| > 1$ and p-value < 0.05. By using R package "pheatmap", hierarchical clustering analysis of DElncRNAs and DEmRNAs were conducted.

Functional annotation of DEmRNAs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed with DAVID (https://david.ncifcrf.gov/). Statistical significance was assumed where $p < 0.05$.

Construction of Protein-protein interaction (PPI) networks

Top 100 up-regulated DEmRNAs and 100 down-regulated DEmRNAs were submitted to the STRING (https://www.string-db.org/). Then, PPI networks were visualized by Cytoscape software (version 3.5.0, http://www.cytoscape. org).

Cis nearby-targeted DEmRNAs of the DElncRNAs

DEmRNAs transcribed within a 100-kb window upstream or downstream of DElncRNAs were searched, which were defined as cis nearby-targeted DEmRNAs of DElncRNAs, to obtain the targeted DEmRNAs of DElncRNAs with cis-regulatory effects. The networks were visualized by Cytoscape software. Functional annotation of the cis nearby-targeted DEmRNAs of the DElncRNAs was conducted with DAVID.

DElncRNA-DEmRNA co-expression networks

To further examine the potential roles of DElncRNAs and DEmRNAs in UL, the DElncRNA-DEmRNA co-expression networks were constructed. DElncRNA-DEmRNA pairs with an absolute value of PCC > 0.95 and $p < 0.01$ were defined as co-expressed DElncRNA-DEmRNA pairs. By using Cytoscape, the co-expressed DElncRNA-DEmRNA networks were visualized. Functional annotation of the DEmRNAs co-expressed with DElncRNAs was performed with DAVID. A value of $p < 0.05$ was set as the cut-off for significance.

Results

DElncRNAs and DEmRNAs expression profiling in uterine leiomyomas

A total of 553 DElncRNAs (283 up-regulated DElncRNAs and 270 down-regulated DElncRNAs) and 3293 DEmRNAs (1,632 up-regulated and 1,661 down-regulated DEmRNAs) were found in UL tissue samples. Furthermore, the top 10 up-regulated and down-regulated DElncRNAs and DEmRNAs were shown in **Table 1** and **Table 2**, respectively. Hierarchical clustering analysis of top 100 up-regulated and down-regulated DElncRNAs and DEmRNAs between UL samples and surrounding normal myometrial tissue samples were presented in **Figure**

1A and **Figure 1B**, respectively. The volcano and scatter plots displayed the variation in DElncRNAs and DEmRNAs expression between UL groups and surrounding normal myometrial tissue groups (**Figure 1C** and **Figure 1D**).

Functional annotation analysis

GO and KEGG pathways enrichment analyses were performed for the DEmRNAs. GO analysis revealed that mRNAs were deregulated in UL, and mainly involved in a number of signaling pathways, including blood vessel development (p=1.21 E-21), regulation of ion transport (p=1.94E-16) and external encapsulating structure organization (p=8.05E-16) (**Figure 2A**). KEGG pathway analysis revealed that the deregulated mRNAs were primarily enriched in signaling pathways associated with cytokine-cytokine receptor interaction (p=3.10E-21), neuroactive ligand-receptor interaction (p=2.67E-21) and complement and coagulation cascades (CAMs) (p=1.50E-18) (**Figure 2B**).

Protein-protein interaction (PPI) networks

PPI networks were constructed and included 313 nodes and 347 edges. NR3C1 (degree = 57), BIRC3 (degree = 27), ARRB1 (degree = 24), KRT8 (degree = 20) and APOA1 (degree = 15) were top five hub proteins in the PPI networks (**Figure 3**).

Cis-nearby-targeted DEmRNAs of DElncRNAs

There were 409 DElncRNAs-nearby-targeted DEmRNA pairs, including 118 DElncRNAs and 136 DEmRNAs. (**Figure 4**). The top two DElncRNAs with most nearby DEmRNAs were BISPR and AC012531.1, which contained 4 DElncRNAs and 4 nearby DEmRNAs, respectively. Blood vessel development (p=6.61E-10), ventricular septum development (p=2.21E-5) and skeletal system development (p=3.08E-06) were significantly enriched GO terms (**Figure 5A**). Hypertrophic cardiomyopathy (p=6.54E-04), TNF signaling pathway (p=1.75E-03) and chemical carcinogenesis (p=6.95E-03) were significantly enriched KEGG pathways (**Figure 5B**).

Discussion

UL, as a common disease in women, occurs in women of reproductive age[2,5]. Most of patients with UL do not affect the physical health of women, but there is a need for surgery for UL patients with fertility requirements, which cause female menorrhagia, pelvic pain, and compression symptoms. However, the pathogenesis of UL is not fully clear. Therefore, it is necessary to identify the molecular mechanisms of disease progression that is useful in the diagnosis and treatment of UL[1, 2, 8]. Recently, the abnormal expression of lncRNAs and mRNAs has been verified in various diseases, and the growing evidences indicated that lncRNAs and mRNAs were involved in the progression of diseases[10,11,15]. LncRNAs are a class of non-coding RNAs with longer than 200 nucleotides that are considered to be a transcriptional by-product and do not encode protein[14]. In these years, lncRNAs, which was considered as byproducts of RNA polymerase II, have been emerging the important roles in regulating various physiological and pathological progresses[11,15]. In addition, more and more studies have demonstrated that lncRNAs are closely associated with the occurrence and development of tumors, aging, and inflammation, with the rapid development of molecular biology and genetic diagnostic techniques[12,13]. A great number of studies have found the dysregulation of lncRNAs and mRNAs involved in many cell-signaling pathways and participated in the occurrence and development of UL[10, 11]. For example, the increased lncRNA XIST in UL could lead to reduced expression of miR-29c and miR-200c by targeting COL1A1, COL3A1, and FN1[21]. Besides, lncRNA AL445665.1-4 was found to be involved in the development of UL through interacting with miR-146b-5p[22]. At last, lncRNA APTR promoted the proliferation of UL cell through the Wnt pathway by targeting ERα[23]. All this indicated that lncRNAs and mRNAs could be used as a novel biomarkers for the diagnosis and treatment of UL.

Generally speaking, lncRNAs activate or inhibit gene expression through recruiting various remodeling complexes to gene promoter, ultimately affecting epigenetics[11, 12]. Accumulating evidences showed that different biological functions of lncRNAs mainly depend on their clearly different subcellular localization[10]. Cytoplasmic lncRNAs could function as decoys for miRNAs, leading to the regulation of mRNA stability or translation, and finally influence signaling pathways[13, 21]. In this study, a total of 553 DElncRNAs (283 up-regulated DElncRNAs and 270 down-regulated DElncRNAs) and 3,293 DEmRNAs (1,632 up-regulated and 1,661 down-regulated DEmRNAs) were found in UL tissue samples. To further dissect the

function of those target genes contained in the DElncRNAs and DEmRNAs networks, GO analysis revealed that mRNAs were deregulated in UL were mainly involved in a number of signaling pathways, including blood vessel development, regulation of ion transport and external encapsulating structure organization. KEGG pathway analysis revealed that the deregulated mRNAs were primarily enriched in signaling pathways associated with cytokine-cytokine receptor interaction, neuroactive ligand-receptor interaction and complement and coagulation cascades. The main blood supply of uterine fibroids is from the uterine artery and its branches. Blood vessels deliver oxygen and nutrients to every part of the body. The dysfunction of blood-vessel formation were responsible for the initiation and progression of diseases including cancer[24]. Elevated vascular endothelial-derived growth factor (VEGF) was found in UL compared with uterine myometrial layer, suggesting that blood vessel development may participate in the development and growth of UL[24].

In this study, we speculated that some lncRNAs might regulate genes in a cis-regulatory fashion. For example, LncRNA BST2 interferon-stimulated positive regulator (BISPR) may regulate the production of inflammatory mediators, and promote tumorigenesis[27]. Many studies reported that BISPR was involved in the multiple malignant behaviors for tumor development, including renal cell carcinoma and oral cavity cancer[27, 28]. Furthermore, BISPR increased the propagation of cancer cells in thyroid papillary carcinoma by inhibiting miR-21-5p[29]. In this study, we found that the top two DElncRNAs with most nearby DEmRNAs were BISPR and AC012531.1, which contained 4 DElncRNAs and 4 nearby DEmRNAs, respectively. Blood vessel development, ventricular septum development and skeletal system development were significantly enriched GO terms. Hypertrophic cardiomyopathy, TNF signaling pathway and chemical carcinogenesis were significantly enriched KEGG pathways. Taken together, our findings revealed that lncRNAs might act as ceRNAs to impact blood vessel development, Hypertrophic cardiomyopathy and so on during the development of UL.

However, our study has several limitations. First, RNA-seq and mRNA-seq were performed to predict the potential roles of lncRNAs and mRNAs by bioinformatics analysis. Although some experimentally validated gene regulatory networks appeared in our analysis, it is still necessary to validate these results with related experiments. In addition, it remain to be further validated because of the relatively small sample size, contributing to potentially limiting the statistical

power to investigate the real association. Hence, a larger sample size is needed to verify our results. In this study, high-throughput RNA sequencing technology was applied to screen lncRNAs specifically expressed in UL tissues, and bioinformatics methods were used to analyze the key pathways enriched by specific lncRNAs in GO terms and KEGG pathways. In the next step, we will still expand the sample size of clinical studies, further verify the differential expression of lncRNAs and their target genes between UL tissues and surrounding normal myometrial tissues, and verify the regulation of lncRNAs on their target genes by *in vitro* cell experiments. To discuss the pathogenesis of UL from the perspective of epigenetics and provide a preliminary theory for the prevention and treatment of UL.

Conclusion

In summary, these research results suggested that 3,293 DEmRNAs and 553 DElncRNAs are differentially expressed in UL tissues and surrounding normal myometrial tissues, which might be candidate identified therapeutic and prognostic targets for UL and be considered as offering several possible mechanisms and pathogenesis of UL in the future.

Ethics approval and consent to participate

Institutional Review Board approval and informed consent was waived.

Consent for publication

The patient gave an informed consent for this publication.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All the authors clinically managed the patient. HMF and WY conducted the literature research. MFF, JYJ, and CXY compiled the manuscript. All the authors read and approved the final manuscript.

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None.

Figure legends

Figure 1. DElncRNAs and DEmRNAs expression patterns in uterine leiomyoma tissues relative to those in surrounding normal myometrial tissues.

(A). Hierarchical cluster analysis of all differentially expressed DElncRNAs; (B). Hierarchical cluster analysis of all differentially expressed DEmRNAs; Each column represents a sample and each row represents a lncRNA and mRNA. The color scale indicates relative expression, upregulation (red), and downregulation (blue). A represents uterine leiomyoma tissue group and B represents surrounding normal myometrial tissue group; (C). Volcano plots and Scatter plots demonstrate differential expression of DElncRNAs between two different conditions; (D). Volcano plots and Scatter plots demonstrate differential expression of DEmRNAs between two different conditions; Red points indicated upregulated while the green points indicated downregulated expression. The values plotted on X and Y axes are the averaged normalized signal values of each group (log2 scaled). FC $\geq$ 2 and P $\leq$ 0.05 were regarded as the differentially expressed DElncRNAs and DEmRNAs.

Figure 2. Functional annotation analysis for the validated DEmRNAs gathering genes.

(A). GO analysis; (B). KEGG pathway analysis; The vertical axis shows the annotated functions of the target genes. The horizontal axes show the enrichment score (−log10 transformed p-value)

and the gene number of each cluster, respectively. Only the top 10 significantly enriched clusters are included.

Figure 3. Protein-protein interaction (PPI) networks analysis.

The network comprising the top upregulated (red triangles) and downregulated circRNAs (green inverted arrows) and their target mRNAs are presented.

Figure 4. Cis-nearby-targeted DEmRNAs of DElncRNAs.

There were 409 DElncRNAs-nearby-targeted DEmRNA pairs, including 118 DElncRNAs and 136 DEmRNAs.

Figure 5. Functional annotation analysis for the validated cis-nearby-targeted DEmRNAs of DElncRNAs gathering genes.

(A). GO analysis; (B). KEGG pathway analysis; The vertical axis shows the annotated functions of the target genes. The horizontal axes show the enrichment score (−log10 transformed p-value) and the gene number of each cluster, respectively. Only the top 10 significantly enriched clusters are included.

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Figures

Figure 1

DElncRNAs and DEmRNAs expression patterns in uterine leiomyoma tissues relative to those in surrounding normal myometrial tissues. (A). Hierarchical cluster analysis of all differentially expressed DElncRNAs; (B). Hierarchical cluster analysis of all differentially expressed DEmRNAs; Each column represents a sample and each row represents a lncRNA and mRNA. The color scale indicates relative expression, upregulation (red), and downregulation (blue). A represents uterine leiomyoma tissue group and B represents surrounding normal myometrial tissue group; (C). Volcano plots and Scatter plots demonstrate differential expression of DElncRNAs between two different conditions; (D). Volcano plots and Scatter plots demonstrate differential expression of DEmRNAs between two different conditions; Red points indicated upregulated while the green points indicated downregulated expression. The values plotted on X and Y axes are the averaged normalized signal values of each group (log2 scaled). FC ≥ 2 and P ≤ 0.05 were regarded as the differentially expressed DElncRNAs

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Functional annotation analysis for the validated DEmRNAs gathering genes. (A). GO analysis; (B). KEGG pathway analysis; The vertical axis shows the annotated functions of the target genes. The horizontal axes show the enrichment score (−log10 transformed p-value) and the gene number of each cluster, respectively. Only the top 10 significantly enriched clusters are included

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