LncRNA and transcriptomic analysis of fetal membrane reveal potential targets involved in oligohydramnios

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Research article

Keywords: pathophysiology; transcriptomic; fetal membrane; oligohydramnios

Posted Date: January 3rd, 2020

DOI: https://doi.org/10.21203/rs.2.13689/v2

Version of Record: A version of this preprint was published on September 18th, 2020. See the published version at https://doi.org/10.1186/s12920-020-00792-z.

Abstract

Background: The multiple causes of oligohydramnios make it challenging to study. Long noncoding RNAs (lncRNAs) are sets of RNAs that have been proven to function in multiple biological processes. The purpose of this study is to study expression level and possible role of lncRNAs in oligohydramnios. Methods: In this study, total RNA was isolated from fetal membranes resected from oligohydramnios pregnant women (OP) and normal amount of amniotic fluid pregnant women (Normal). LncRNA microarray was used to analyze the differentially expressed lncRNAs and mRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to analyze the main enrichment pathways of differentially expressed mRNAs. Real-time quantitative PCR (qPCR) was used to validate the lncRNA expression level. Results: LncRNA microarray analysis revealed that a total of 801 lncRNAs and 367 mRNAs were differentially expressed in OP; in these results, 638 lncRNAs and 189 mRNAs were upregulated, and 163 lncRNAs and 178 mRNAs were downregulated. Of the lncRNAs, 566 were intergenic lncRNA, 351 were intronic antisense lncRNA, and 300 were natural antisense lncRNA. The differentially expressed lncRNAs were primarily located in chromosomes 2, 1, and 11. KEGG enrichment pathways revealed that the differentially expressed mRNAs were enriched in focal adhesion as well as in the signaling pathways of Ras, TNF, estrogen, and chemokine. The qPCR results confirmed that LINC00515 and RP11-388P9.2 were upregulated in OP. Furthermore, the constructed lncRNA–miRNA–mRNA regulatory network revealed TNR, CFTR, ABCA12, and COL9A2 as the candidate targets of LINC00515 and RP11-388P9.2. Conclusions: In summary, we revealed the profiles of lncRNA and mRNA in OP. These results may offer potential targets for biological prevention for pregnant women with oligohydramnios detected before delivery and provide a reliable basis for clinical biological treatment in OP.

Background

Amniotic fluid is critical for a healthy pregnancy because it allows for fetal movements and it protects the fetus from trauma by acting as a physical cushion. It also plays an important role in fetal lung and limb development [1]. The volume of amniotic fluid varies at different stages of pregnancy [2, 3], and the average amniotic fluid volume is 400 mL at term [4].

Oligohydramnios is generally defined as a reduced amount of amniotic fluid. The normal range of amniotic fluid has an amniotic fluid index (AFI) of 5–24 cm and a single deepest pocket (SDP) of 2–8 cm. AFI < 5 cm or SDP ≤ 2 cm [5, 6] are the commonly used parameters for diagnosis. An amniotic fluid volume in the third trimester of pregnancy is 800 mL. Oligohydramnios is a common complication during pregnancy; it can increase delivery rates and labor induction rates in pregnant women and can significantly increase the mortality rate of perinatal children [7, 8]. In addition, oligohydramnios is associated with intrauterine fetal growth restriction, meconium-stained liquor, and prolonged labor [9].

Currently, the mechanisms underlying oligohydramnios remain unclear. Long noncoding RNA (lncRNA) is a class of transcripts that contains more than 200 nucleotides, but it cannot encode proteins. LncRNAs have been proven to be expressed in a wide range of diseases, and they are involved in regulating cancer

development and metastasis [10], heart diseases [11, 12], and autoimmune diseases [13]. There is also evidence suggesting that lncRNA is involved in pregnancy-associated events. For example, placental lncRNA expression is alert in response to phthalate exposure during pregnancy [14]. Furthermore, lncRNA uc003fir suppresses the proliferation and migration of trophoblast cells, which might contribute to preeclampsia development [15]. However, little is known about the association between lncRNAs and oligohydramnios.

Therefore, in the present study, we perform lncRNA and mRNA microarray analyses to explore the lncRNA and mRNA expression profile in response to oligohydramnios in pregnant women. Both lncRNAs and mRNAs are sequenced for lncRNA–miRNA–mRNA integrated analysis. In this study, we provide the first evidence that lncRNAs and mRNAs are differentially expressed in the fetal membrane in oligohydramnios pregnant women (OP); based on the lncRNA–miRNA–mRNA network, we predict the potential role of lncRNAs and mRNAs in OP.

Methods

Patient recruitment

Fetal membranes, collected for microarray analysis, were obtained from OP and normal amount of amniotic fluid pregnant women (Normal). Pregnant women who meet the following standard criteria are diagnosed with oligohydramnios: an SDP of ≤2 cm or an AFI of ≤5 cm [5, 6]. The pregnant women in this study did not take any drugs that may have affected their amniotic fluid volume, such as angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, or non-steroidal anti-inflammatory drugs. The mean values of the AFI and the SDP for pregnant women with oligohydramnios were 53.81 ± 13.82 and 26.95 ± 7.51 mm, respectively, and the content of amniotic fluid in pregnant women with oligohydramnios during delivery was about 194.29 ± 50.06 mL.

Tissue collection and RNA isolation

The fetal membrane of a woman who had just given birth was placed in liquid nitrogen within 30 min. The membranes were washed prior to being homogenized. Approximately 1 cm^3 of the tissue block was resected for grinding. Samples were ground in a motor-driven homogenizer. Trizol (Invitrogen, CA, USA) was used to extract total RNA from the tissues in accordance with the manufacturer's protocol. The concentration and qualification of the isolated total RNA was assessed by a Nanodrop 2001 spectrophotometer (Thermo Fisher Scientific, MA, USA).

LncRNA and mRNA microarray analysis

Total RNA from the fetal membranes, which were obtained from five OP and five Normal, was used for microarray analysis. The Human LncRNA Array V4.0 (8 × 60k) was performed by KangChen Bio-tech Inc. (Shanghai, China). The microarray analyses included 40,173 lncRNAs and 20,730 mRNAs. mRNA was purified from the total RNA after the removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit,

Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts, without 3' bias and utilizing a random priming method. The labeled cRNAs were purified by a RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by a NanoDrop ND-1000. Of each labeled cRNA, 1 μg was fragmented by adding 11 μL 10 × Blocking Agent and 2.2 μL of 25 × Fragmentation Buffer; the mixture was then heated at 60 °C for 30 min, and finally 55 μL 2 × GE Hybridization Buffer was added to dilute the labeled cRNA. Additionally, 100 μL of hybridization solution was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed, and scanned with an Agilent DNA Microarray Scanner (part number G2505C).

Bioinformatics

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed with the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that had flags in Present ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs between two conditions were identified through fold change filtering. Heatmaps and scatter plots were generated for differentially expressed genes using the R package (version 3.1.0) [16]; taking the RPKM value of different genes under different experimental conditions as the expression level, R package-pheatmap software was used to do hierarchical clustering analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using an online tool (http://www.genome.jp/kegg/). KEGG pathways that met the requirement of FDR ≤ 0.001 were considered to be significantly enriched.

To explore the potential role of lncRNA, a lncRNA–miRNA–mRNA interaction network was constructed. We used miRNA target gene prediction software (miRanda) to predict miRNA targets on lncRNA. The overlap miRNAs that harbored both lncRNA and mRNA binding targets were used to construct the lncRNA–miRNA–mRNA interaction network. The sub-network that contained predicted targets of lncRNA and was differentially expressed in OP was included. The network was visualized using Cytoscape_V2_8_3 (https://www.innatedb.ca/cytoscape-v2.8.3/plugins/) software.

Real-time quantitative PCR (qPCR)

The relative expression of lncRNA between 20 OP and 19 Normal was measured by qPCR. Primers targeting LINC00515 and RP11-388P92 are listed in Table 1. Total RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Takara, Dalian, China). cDNAs were then amplified and quantified on an ABI 7500 real-time PCR system (Applied Biosystems, CA, USA) with a SYBR Realtime PCR Master Mix Kit (TOYOBO, Osaka, Japan). The program for cDNA amplification was as follows: the first step, 95 °C for 120 s; the second step, 95 °C for 15 s and 60 °C for 30 s, for 40 cycles; the third step, for melting curve

generation, 60 °C to 95 °C. The relative expression of lncRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control. The primers are shown in Table 1.

Statistical analysis

Student's t-test was used to analyze the significant differences by the SPSS 18.0 software package in the study. Three biological replicates were performed in the study. A P-value of <0.05 defined the significant differences between the two groups.

Results

Overview of lncRNA and mRNA profiles in OP

To explore the profile of lncRNA and mRNA in the fetal membranes of five OP and five Normal were obtained and subjected to microarray analysis. As shown in Tables 2 and 3, the cesarean section rate, recurrent abortion rate, and placental weight of pregnant women with oligohydramnios was about 55%, 25%, and 0.47 kg, respectively. Recurrent abortion rate and placental weight of OP were significantly different from those of pregnant women with a normal amount of amniotic fluid. As shown in Figure 1, the profiles of lncRNA and mRNA in OP were different from those in Normal. A sequence of lncRNAs and mRNAs were alert in OP, compared with Normal (Figure 1A&B). Among the differentially expressed lncRNAs, 638 were upregulated and 163 were downregulated. Of the differentially expressed mRNAs, 189 were upregulated and 178 were downregulated, as shown in the volcano plot (Figure 1C&D and Supplemental Table 1&2).

Characteristics of the differentially expressed lncRNAs and mRNAs in OP

To further observe the expression characteristics of these differentially expressed lncRNAs and mRNAs, there was an analysis of the genomic location distribution as well as the length and type distribution. The statistical results of the differentially expressed lncRNA showed that the differentially expressed lncRNA was mainly distributed on chromosomes 2, 1, and 11, with the least distribution on the Y chromosome; this indicates that the lncRNAs that play a role in oligohydramnios are mainly located on chromosomes 2, 1 and 11 (Figure 2A). The length distribution showed that most lncRNAs were distributed within 1 kb, whereas the mRNAs were mainly distributed at 2–3 kb in length (Supplemental Figure 1). Analysis for differential lncRNA type revealed the largest number of intergenic lncRNAs, followed by intronic antisense and natural antisense, indicating that the lncRNAs that play a role in oligohydramnios are mainly intergenic lncRNAs (Figure 2B). KEGG results revealed that differential mRNAs mainly enrich in focal adhesion as well as in the signaling pathways of Ras, TNF, estrogen, and chemokine (Figure 3). Other top pathways are shown in Figure 3.

The expression of lncRNAs and the potential regulatory network

In order to explore the function of differentially expressed lncRNA in OP, specific analysis was conducted for the differentially expressed lncRNAs and their regulatory network. Table 3 listed the lncRNAs in the top 10 of upregulated and downregulated expression in OP women. As shown in Table 4, the highest differential expression was G017197, and the upregulation fold change was 6.99 times; the highest downregulation was G083088, and the expression was downregulated to 0.15-fold. We verified the differential expression of two specific lncRNAs, LINC00515 and RP11-388P9.2, by using qPCR (Figure 4). As shown in Figure 4, both LINC00515 and RP11-388P9.2 showed increased expression in OP. Furthermore, a lncRNA–miRNA–mRNA interaction network based on LINC00515 and RP11-388P9.2 was generated. Potential miRNA targets of LINC00515 and RP11-388P9.2 were predicted and then screened for consistency with mRNAs that were expressed as upregulated in OP. As revealed in Figure 5, a regulatory network of LINC00515 and RP11-388P9.2 was obtained. The network included 27 miRNAs and 5 mRNAs (Figure 5 and Supplemental Table 4). The mRNAs that were finally captured were TNR, CFTR, ABCA12, and COL9A2.

Discussion

Oligohydramnios is one of the common obstetric complications. The etiology of oligohydramnios mainly includes the fetal factor, placental membrane factor, maternal factor, and drug factor. For example, in the prolonged pregnant women with oligohydramnios, resistance index in the fetal renal artery is higher than it is in the controls, which are without oligohydramnios [17]; additionally, cyclooxygenase-2 inhibitor nimesulide and long-term diclofenac exposure are associated with oligohydramnios [18, 19]. In our study, the possible causes of oligohydramnios in pregnant women may be due to an increased number of abortions and abnormal transport functions of fetal membranes; still, the underlying molecular mechanism for oligohydramnios remains unknown. Therefore, we conducted microarray analysis and unveiled the expression profile of lncRNA and mRNA in OP. These results provide the first overview of the lncRNA profile in the fetal membrane in OP.

The fetal membrane is a vital tissue for communication between mother and fetus. There is a hypothesis that the resorption pathway that crosses the amnion to the fetal circulation may keep the balance of normal amniotic fluid volume [20]. Therefore, it is reasonable to speculate that changes in the expression of molecules in the membrane tissue are response to changes in the microenvironment. Many studies have previously provided the molecular information [20-23] of the fetal membrane tissue. However, studies focused on this in the context of oligohydramnios are still limited. Here we showed that 638 lncRNAs and 189 mRNAs were upregulated and that 163 lncRNAs and 178 mRNAs were downregulated. Moreover, we found that the differentially expressed mRNAs were mainly enriched in focal adhesion as well as in the signaling pathways of Ras, TNF, estrogen, and chemokine. Research shows that TNF-α and IL-6 play an essential role in the inflammatory process in pregnant women with spontaneous preterm births [24]. Under the induction of ferutinin, human amniotic fluid stem cells are differentiated into osteoblasts through estrogen receptor α and the PI3K/Akt signal pathway [25]. The TNF signaling pathway and the estrogen signaling pathway may be involved in the regulation of OP.

We also confirmed the upregulation of two lncRNAs, LINC00515 and RP11-388P9.2, in OP. Also, a lncRNA–miRNA–mRNA interaction network was constructed to illustrate the possible function of LINC00515 and RP11-388P9.2 involved in oligohydramnios. In the network, the role of almost all miRNAs in oligohydramnios remains unknown. In addition, four mRNAs, cystic fibrosis transmembrane conductance regulator (CFTR), tenascin R (TNR), ATP-binding cassette sub-family A member 12 (ABCA12), and collagen 9A2 (COL9A2) were predicted to be included in the regulatory network of LINC00515 and RP11-388P9.2. Of these mRNAs, CFTR, a small conductance Cl^- channel, is regulated by intracellular ATP and cAMP-dependent phosphorylation, predominantly located in the apical membrane of organ epithelial cells. The study of endometrial epithelia shows that CFTR is involved in the secretory effects of ovarian hormone regulation [26], showing that CFTR plays a vital role in female reproduction. It can be inferred that RP11-388P9.2 might affect the CFTR expression by regulating has-miR-114-3p, further affecting the amniotic fluid content of pregnant women. Moreover, TNR can interact with fibronectin 1, which can be involved in cell migration and adhesion biological processes including embryogenesis [27]. Tenascin is also produced in the extracellular matrix of cultured amnion epithelial cells [28]. It can be inferred that RP11-388P9.2 might affect the TNR expression by regulating the has-miR-508-5p, has-miR-3667-3p, has-miR-574-5p, and has-miR-6734-3p, further affecting the amniotic fluid content of pregnant women. Compared with the amniotic fluid cells of a healthy human, the cells of fetuses with neural tube defects do not deposit type I collagen [29]. It can be inferred that LINC00515 might affect the COL9A2 expression by regulating the has-miR-186-3p and has-miR-664a-5p, further affecting the amniotic fluid content of pregnant women. However, their mechanism is still unclear and needs further study.

Conclusions

In summary, we revealed the profiles of lncRNA and mRNA in OP, validated the upregulation of LINC00515 and RP11-388P9.2, and suggested a lncRNA–miRNA–mRNA network that might be involved in the pathogenesis of oligohydramnios. These results may offer potential targets for biological prevention for pregnant women with oligohydramnios detected before delivery and provide a reliable basis for clinical biological treatment in OP.

Declarations

Ethics approval and consent to participate

All pregnant women gave informed consent. This study was approved by the Ethics Committee of Sun Yat-sen University.

Consent to publish

Not applicable

Availability of data and materials

The datasets generated during the current study are available in the GEO Database Series GSE142701 repository, [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142701].

Competing interests

The authors declare no conflict of interest.

Funding

This study was supported by the National Nature Science Foundation of China (81771660 and 81741017), the Science and Technology Planning Project of Guangdong Province (2017A020214018 and 2017A020214003), Guangdong Natural Science Foundation (2018A030313023 and 2018A030310162), the Science and Technology Planning Project of Guangzhou City Central Universities (201704020034), 5010 projects at Sun Yat-Sen University (2012006), and
Major Program of the National Natural Science Foundation of China (2019YFA0801403).

Authors' Contributions

Yuhua Ou and Yukun Liu wrote the manuscript, Hui Chen and Jianping Zhang were responsible for the study design. Jianping Zhang edited and corrected the manuscript. Yuhua Ou, Yukun Liu and Liqiong Zhu were responsible for the experimental studies. Yuhua Ou and Hui Chen were responsible for the integrity of the data and the accuracy of the data analysis. Manqi Chen and Xiaochun Yi were responsible for participant recruitment and clinical follow-up data.

Acknowledgments

The authors thank Jianping Tan, Yinglin Liu and Rui Zhang for helpful discussion. The authors are grateful to Song Guo, Huixiang Liu, Linxiang Wu and Yuanping Wu for assistance with sample collecting. The authors also thank all the volunteers for participating in the study.

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Tables

Table 1. Primers used in this study.

Primer name	Sequence (5’ to 3’)	Production size
LINC00515F	TCAAGGCAGCAGTGGCAGAG	142
LINC00515R	AGTCACAGGCGTGGAGGTCA	
RP11-388P92F	ATTTGCCAGCTTCTCCTTTGA	145
RP11-388P92R	TTGGCAGAATGAGACATCAAG	
GAPDHF	GAGTCAACGGATTTGGTCGT	185
GAPDHR	GAGTCAACGGATTTGGTCGT	

Table 2. Demographic information.

Characteristics	Oligohydramnios (n = 20)	Normal control (n = 19)	P-value
Age (years)	32.63 ± 5.23	30.8 ± 4.17	0.233
Gestational age (weeks)	37.34 ± 2.01	39.84 ± 2.88	0.003*
BMI	26.21 ± 2.48	26.23 ± 2.81	0.977
Number of pregnancy	2.48 ± 1.50	2.68 ± 1.62	0.68
Number of productions	1.38 ± 0.50	1.47 ± 0.88	0.695
Total number of abortions	1.09 ± 1.34	1.21 ± 1.15	0.775
Spontaneous abortion	0.16 ± 0.37	0.7 ± 0.92	0.023*
IVF (%)	2 (9.5)	1 (5.2)	0.538
Cesarean section (%)	11 (55)	6 (31.6)	0.157
Apgar 1 min	9.65 ± 0.74	9.89 ± 0.31	0.131
Baby gender			
Male	8	8	-
Female	12	11	-
Birth weight (kg)	2.67 ± 0.57	3.31 ± 0.23	3.89E-5*
Placental weight (kg)	0.47 ± 0.04	0.51 ± 0.02	0.003*

BMI: Body Mass Index; IVF: In-vitro fertilization. *P < 0.05 (Student's t-test). Variable is mean ± standard deviation.

Table 3. Pregnancy complications.

Characteristics	Oligohydramnios (n = 20)	Normal control (n = 19)	P-value
FGR (%)	7□35□	0	0.005#
RSA (%)	5 (25)	0	0.027#
Premature birth (%)	6 (30)	0	0.012#
GDM (%)	1 (5)	2 (10.5)	0.48
UCTD (%)	1 (5)	0	0.513
Mild anemia (%)	2 (10)	0	0.256
Hypothyroidism (%)	2 (10)	4 (21.1)	0.305
Thalassemia (%)	2 (10)	0	0.256

FGR: Fetal growth restriction; RSA: Recurrent abortion; GDM: Gestational diabetes mellitus; UCTD: Undifferentiated connective tissue disease. *P < 0.05 (Student's t-test). Classification variable is calculated by ratio (%). #Fisher was used to accurately test P-value < 0.05

Table 4. Information of the top-10 most upregulated and downregualted lncRNAs in OP women.

NA_ID	Fold Change	Regulation	P-value	FDR
7197	6.992997551	up	0.022330367	0.323992752
3960	6.156767122	up	0.007011989	0.231007727
5426	5.925028116	up	0.014297859	0.282223415
61474_XLOC_033346	5.875790672	up	0.018248847	0.30550168
9353	5.369031987	up	0.022024259	0.322125587
91729.7	5.280611483	up	0.015183552	0.286476988
C00515	5.265016308	up	0.000229591	0.092301652
4752	5.123561472	up	0.039183093	0.394165858
l-388P9.2	5.0709114	up	0.01273173	0.271540086
10-AS1	5.0609152	up	0.003061877	0.185019347
3088	0.156292992	down	0.024924406	0.335128165
C00501	0.192207162	down	0.019901778	0.31149042
5291	0.213060492	down	0.039570275	0.395804997
C01510	0.231630157	down	0.002294572	0.176676707
l-1399P15.1	0.273437307	down	0.021416751	0.321541985
l-150O12.1	0.286446378	down	0.000781865	0.143720717
C74B	0.292328015	down	0.010723383	0.257909493
l-567G11.1	0.292488539	down	0.012877268	0.272737873
5402	0.301039562	down	0.007261346	0.232753219
3293	0.302661694	down	0.019071938	0.309549149

Supplemental Figure Legend

Supplemental Figure 1. Distribution of lncRNA length in oligohydramnios pregnant women (OP).

Figures

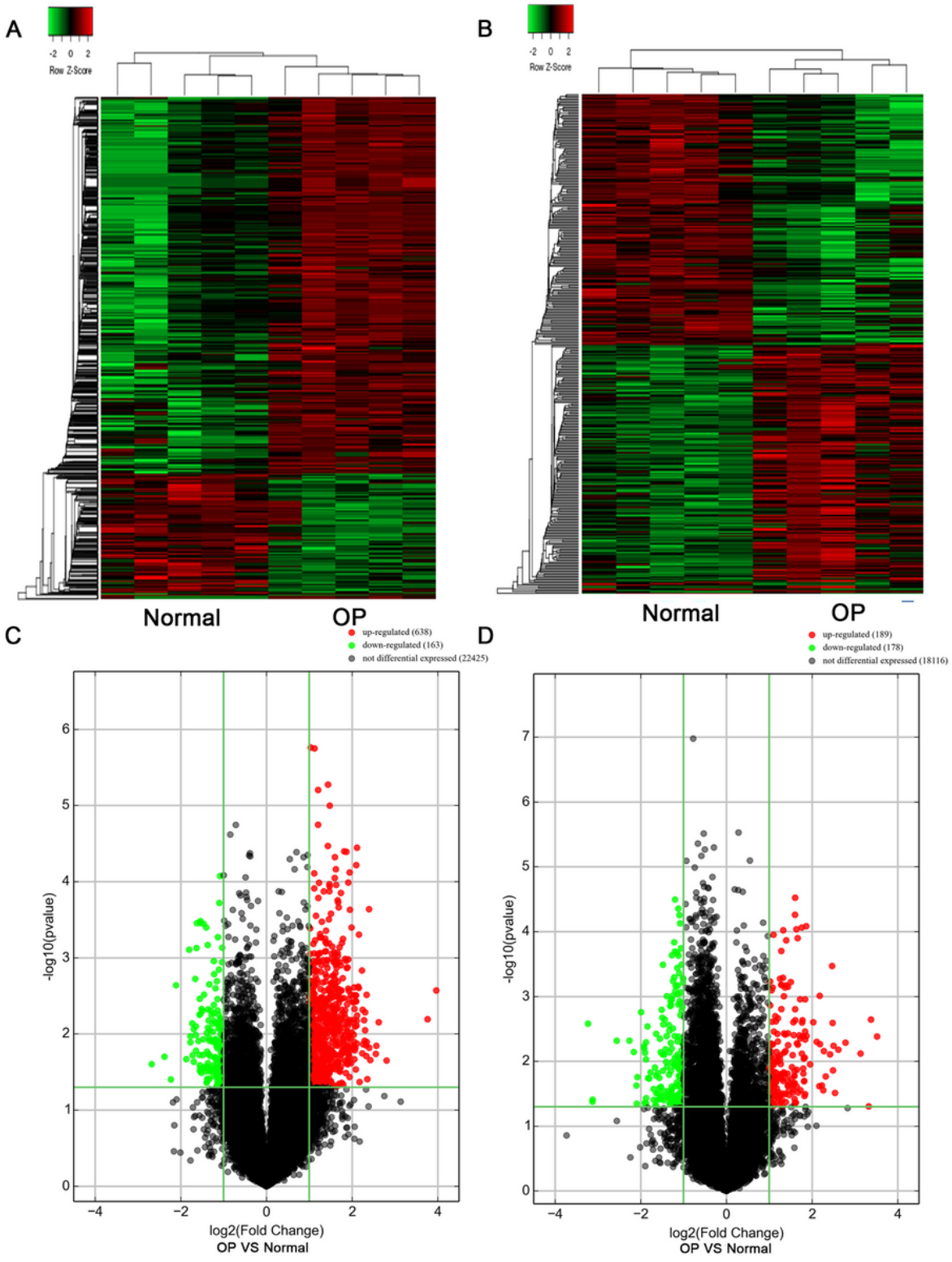

Figure 1

Microarray analysis revealed differential lncRNA profiles in fetal membranes resected from five oligohydramnios pregnant women (OP) and five normal amount of amniotic fluid pregnant women (Normal). The heatmap shows the profile of lncRNA (A) and mRNAs (B) in OP and Normal. The volcano

plot shows the overall change in expression of lncRNAs (C) and mRNAs (D). The upregulated RNA is labeled in red, whereas the downregulated RNA is labeled in green.

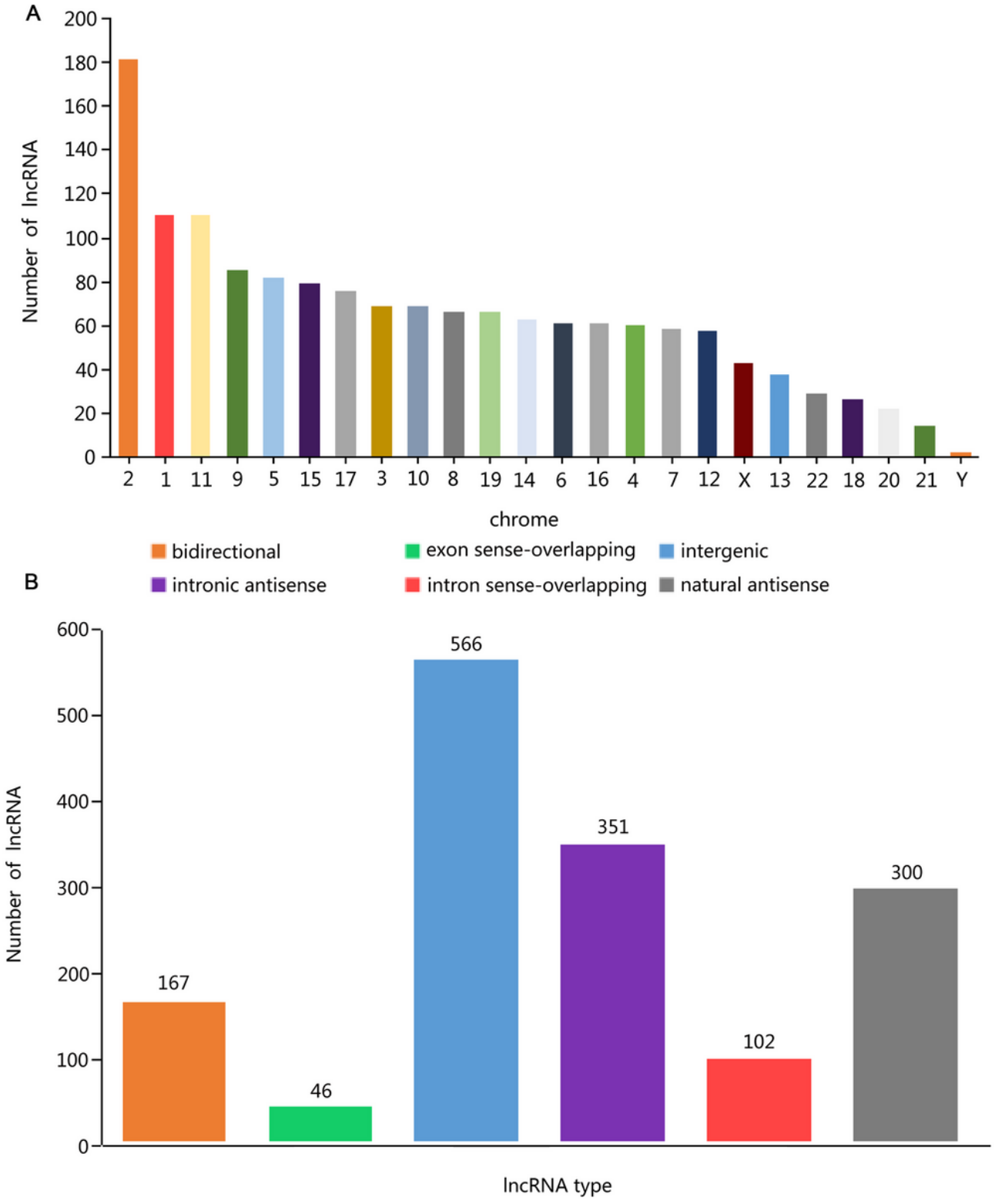

Figure 2

Distribution of lncRNA genomic location (A) and type of the differentially expressed lncRNA (B) in oligohydramnios pregnant women (OP).

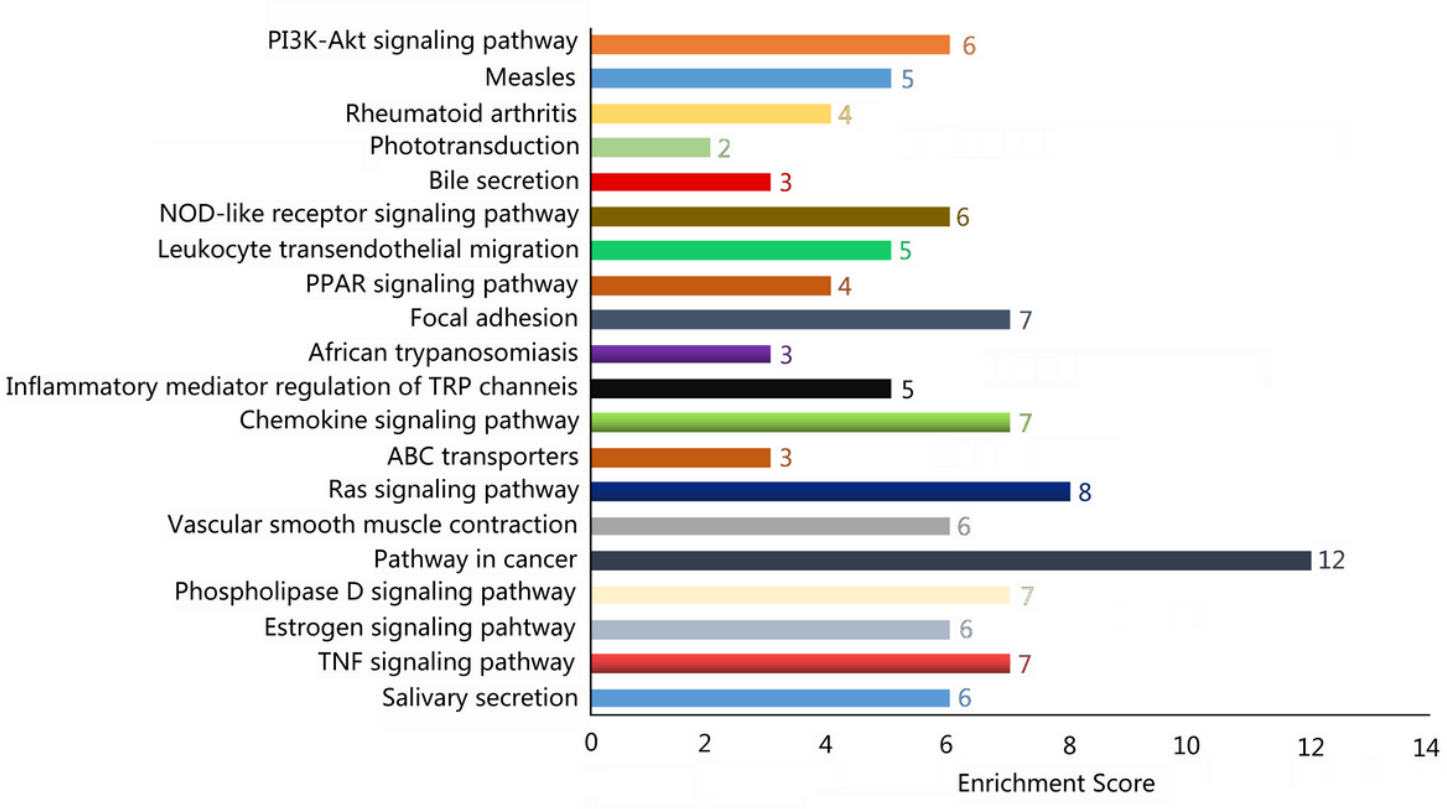

Figure 3

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showing the most enriched pathways for the differentially expressed mRNAs in oligohydramnios pregnant women (OP).

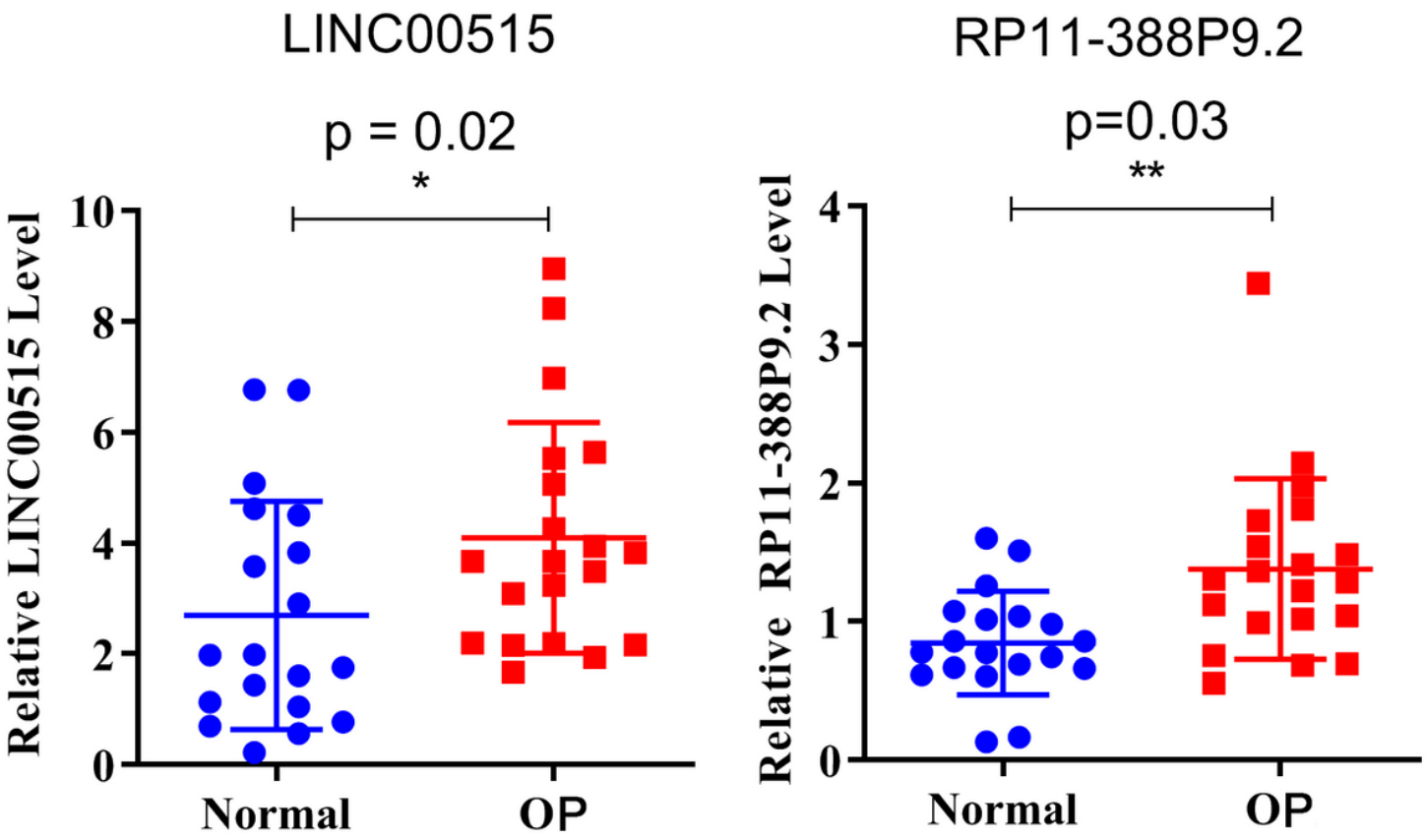

Figure 4

The expression of LINC00515 and RP11-388P9.2 in oligohydramnios pregnant women (OP) and normal amount of amniotic fluid pregnant women (Normal). *P < 0.05 represents the significant difference.

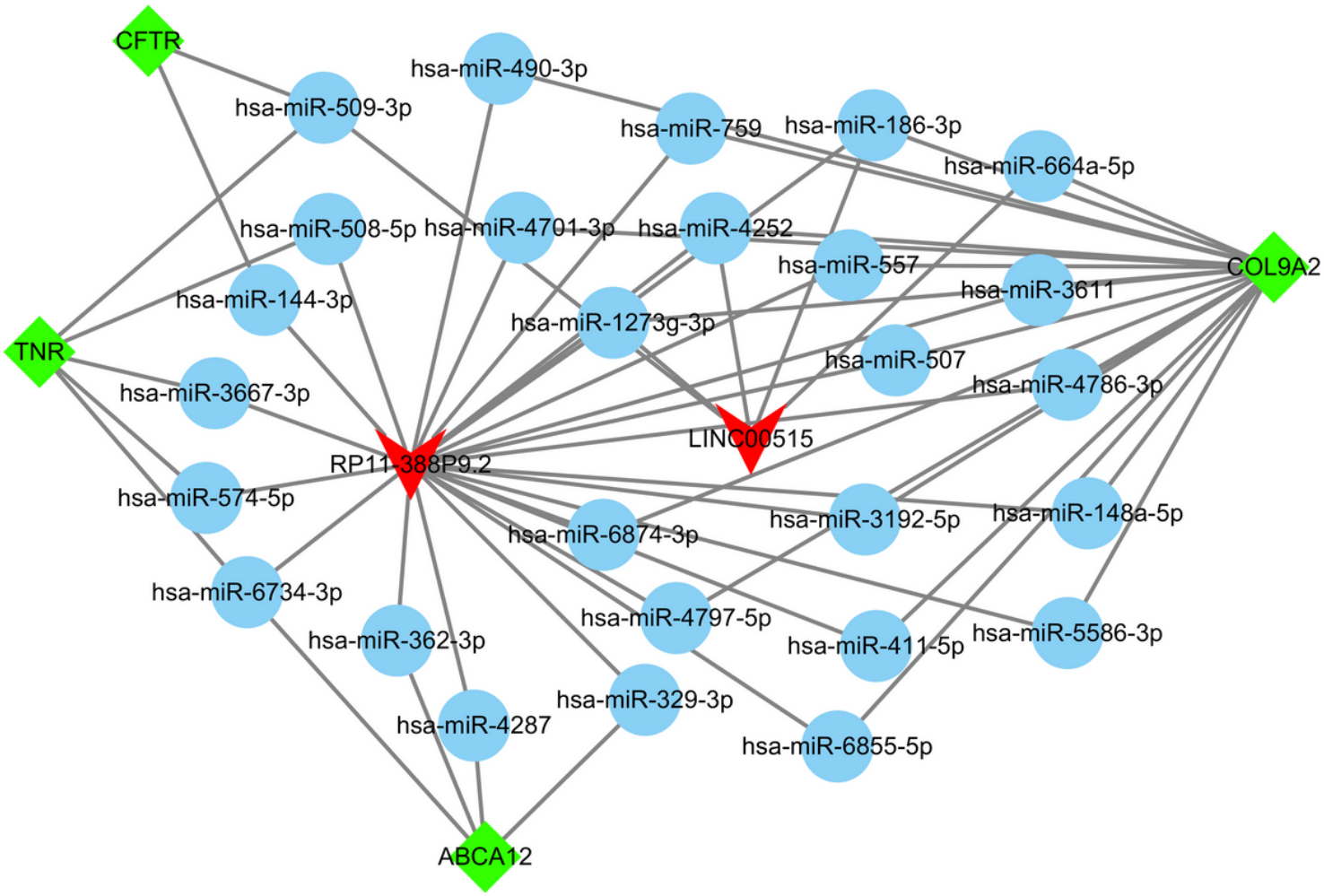

Figure 5

The lncRNA–miRNA–mRNA interaction network. The miRNAs are also potential targets of LINC00515 and RP11-388P9.2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFigure1.tif
- SupplementalTable1.xls
- SupplementalTable2.xls