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

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Abstract

The challenge of oligohydramnios study is multiple causes of oligohydramnios. Long noncoding RNAs (lncRNAs) is a set of RNAs that has been proved to function in multiple biological process. Currently, little is know about their expression and possible role in oligohydramnios. Total RNA was isolated from fetal membranes ressected from oligohydramnios pregnant women (OR) and normal amniotic fluid control (Normal).RNA-sequencing (RNA-seq) obtain that a total of 801 lncRNAs and 367 mRNAs were differentially expressed in OR. Of which, 638 lncRNAs and 189 mRNAs were upregulated, and 163 lncRNAs and 178 mRNAs were downregulated. Of these lncRNAs, 566 of them were intergenic lncRNA, 351 were intronic antisense lncRNA and 300 natural antisense. The differentially expressed lncRNA were primary located in chromosome 2, 1 and 11. KEGG enrichment pathways revealed the differential expressed mRNAs were enriched in pathway in cancer, Ras signaling pathway, TNF signaling pathway, focal adhesion, and chemokine signaling pathway. The qRT-PCR result confirmed that LINC00515 and RP11-388P9.2 were upregulated in OR. Furthermore, the constructed lncRNA-miRNA-mRNA regulatory network revealed TNR, CFTR, ABCC2, ABCA12, and COL9A2 as the candidate targets of LINC00515 and RP11-388P9.2. A wide range of lncRNAs were alert in OR, in particularly, LINC00515 and RP11-388P9.2 were confirmed to be uprgulated in OR, and their predicted downstream targets were transport and tissue growth and development associated. Further study focused on the role of differential expressed lncRNAs such as LINC00515 and RP11-388P9.2 would provide more insight into the pathophysiology in OR.

1. Introduction

Amniotic fluid is critical for a healthy pregnancy, it allows fetal movements and protect the fetus from trauma by acting as a physical cushion. It also play an important role in fetal lungs and limbs 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 defied as a reduced amount of amniotic fluid, amniotic fluid index (AFI) < 5 cm or single deepest pool < 2 cm[5, 6] were the commonly used parameters for diagnosis. It is a common complication during pregnancy, complicates 0.5% to 8% of pregnancies, which can cause increased delivery rate and labor induction rate in pregnant women, and significantly increase the mortality rate of perinatal children[7, 8]. In addition, oligohydramnios is considered to be associated with intrauterine fetal growth restriction, meconium stained liquor, and prolonged labour[9].

Currently, the mechanism underlying oligohydramnios remain unclear. LncRNA is a class of transcripts that contains more than 200 nucleotides but cannot encode proteins. lncRNAs have been proved to be expressed in a wide range of diseases and involved in regulating cancer development and metastasis [10], heart disease[11, 12], and autoimmune diseases [13]. There are also evidences suggested that lncRNA involved in pregnancy associated events. For example, placental lncRNA expression is alert in response to phthalate exposure during pregnant[14]. LncRNA uc003fir suppress proliferation and

migration of trophoblast cells, which might contribute to preeclampsia development[15]. However, little is know about the association between lncRNAs and oligohydramnios.

Therefore, in the present study, we perform RNA-sequencing (RNA-seq) to explore the lncRNA and mRNA expression profile in response to oligohydramnios in pregnant women. Both of lncRNAs and mRNAs were sequencing for lncRNA-miRNA-mRNA integrated analysis. In this study, we provide the first evidence that lncRNAs and mRNAs were differential expressed in fetal membrane in oligohydramnios women and predict their potential role based on lncRNA-miRNA-mRNA network.

2. Results

2.1. The overview of lncRNA and mRNA profiles in OR women

To explore the profile of lncRNA and mRNA in the OR women, fetal membrane of five OR women and five normal women were obtained and subjected to microarray. The characteristics of women inclued in this study was matched in age and BMI. The OR women has higher spontaneous abortion rate (Table 1).

As shown in Figure 1, the profile of lncRNA and mRNA in OR women were different from that of the Normal, and the expression of lncRNA were reproducible within OR or Normal. A sequence of lncRNAs and mRNAs were alert in the OR women (Figure 1A, 1B). Among the differential expressed lncRNAs, 638 were upregulated and 163 were downregulated. Of the differential expressed mRNAs, 189 were upregulated and 178 were downregulated, which can be evidenced by the volcano plot (Figure 1C, 1D).

2.2. Characteristics of the differential expressed lncRNAs and mRNAs in OR women

To further observe the expression characteristics of these differentially expressed lncRNAs and mRNAs, the genomic location distribution, length characteristics and type distribution of these differentially expressed lncRNAs were further analyzed. In addition, differentially expressed mRNAs are enriched for their functionally related pathways.

The statistical results of 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 (Figure 2A). The length distribution showed that the lncRNA and mRNA in differential expression had the most lncRNA within 1 kb; while the mRNA was distributed at 2–3 kb in length (Figure 2B). Analysis of the type of differential lncRNA revealed the largest number of intergenic lncRNAs, followed by intronic antisense and natural antisense (Figure 2C).

KEGG result revealed differential mRNAs mainly enrich in Ras signaling pathway, TNF signaling pathway, focal adhesion, and chemokine signaling pathway (Figure 3). Other top pathways were also showed in

Figure 3.

2.3. The expression of lncRNAs and potential regulatory network

In order to explore the function of differential expressed lncRNA in OR, specific analysis were conducted for the differential expressed lncRNAs and their regulatory network. Table 2 lists the lncRNAs in the top 10 positions of up-regulated and down-regulated expression in OR women. As shown in Table 2, the highest differential expression was G017197, the upregulation fold change was 6.99 times; the highest downregulation was G083088, the expression was downregulated to 0.15-fold.

Specifically, we verified the differential expression of two lncRNAs, LINC00515 and RP11–388P9.2, by qPCR (Figure 4). As shown in Figure 4, both LINC00515 and RP11–388P9.2 showed increased expression in the OR women. 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 merged with those miRNAs targeting mRNAs that were upregulated expressed in OR women in the current study. 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). The mRNAs finally captured were TNR, CFTR, ABCC2, ABCA12, and COL9A2.

3. Discussion

Little is known about the disease mechanism of oligohydramnios, and severe depression is associated with oligohydramnios in pregnant women. In pregnant women with oligohydramnios, depression scores and anxiety are significantly higher than those with normal amniotic fluid volume[16]. In the prolonged pregnant women with oligohydramnios, resistance index in fetal renal artery is higher than the controls that without oligohydramnios, suggestion an association between resistance index in fetal renal artery and oligohydramnios[17]. Drug induction can also cause oligohydramnios, for example, cyclooxygenase–2 inhibitor nimesulide and long-term diclofenac exposure is associated with oligohydramnios, however, recovery of amniotic fluid volume is observed after discontinuation[18, 19].

At the molecular level, studies have suggested that aquaporin is associated with oligohydramnios[1]. Aquaporin 11 is identified to be expressed in pregnant women's amnion, chorion and placenta and its expression is negatively correlated with amniotic fluid amount[20]. Aquaporin 1, aquaporins 8 and 9 expression was decreased in amnion of OR women, indicating their involvement in oligohydramnios[21, 22]. Survivin and Caspase–3 and N-terminal pro-brain natriuretic peptide are also reported to be associated with oligohydramnios[23–25]. However, there is still large amount molecule that are oligohydramnios relevant remains unknown. In the present study, we conducted microarray and unveiled the expression profile of lncRNA and mRNA in the OR women. These results provide the first overview of lncRNA profile in the fetal membrane of OR women.

Fetal membrane is an important tissues for the communication between the maternal and the fetus. There is a hypothesis that resorption pathway that across 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[26–29] in the fetal membrane tissue. However, studies focused on the context of oligohydramnios is still limit. Here, we showed 638 lncRNAs and 189 of mRNAs were upregulated, and 163 lncRNAs and 178 mRNAs were downregulated. Moreover, we found the differential expressed mRNAs are mainly enrich in pathway in cancer, Ras signaling pathway, TNF signaling pathway, focal adhesion, and chemokine signaling pathway, indicating the potential involved pathways in oligohydramnios. We specifically confirm the upregulation of two lncRNAs, LINC00515 and RP11–388P9.2, in OR women, although precise function of them remains to be investigated. We further draw a lncRNA-miRNA-mRNA to illustrate their possible function of these two lncRNAs involved in oligohydramnios.

In the network, the role of almost all miRNAs in oligohydramnios remains unknown. However, based on previous research, mir–509–3p[30], mir–490–3p [31]mir–508–5p[32]mir–362–3p[33], mir–557 [34], mir–411–5p [35], mir–507 [36], most of them are down-regulated in tumor cells/tissues and have anti-tumor effects. It has the effect of inhibiting cell proliferation and migration and promoting apoptosis. Mir–144–3p [37] and mir–4287[38] have a regulatory role in osteogenesis and cartilage differentiation and formation, and the function of his miRNA has not been reported. In addition, five mRNAs, TNR, cystic fibrosis transmembrane conductance regulator (CFTR), ABC transporters (ABCC2), ATP-binding cassette sub-family A member 12 (ABCA12), and COL9A2 were predicted to be included in the regulatory network of LINC00515 and RP11–388P9.2. Of these mRNAs, As a chloride channel, CFTR considered is associated with cell-based cystic fibrosis[39]. ABCC2 is found to mediate the transport of various organic anions including drug and toxicants[40]. ABCA12 is reported to be associated with copper toxicosis[41] and transport of lipids[42]. Mutation in COL9A2 is associated with th development of intervertebral disc disease[43]. The relevant reports suggesting these five mRNAs were transport and growth and development function associated. These indicate candidate downstream targets of LINC00515 and RP11–388P9.2 may contribute to affecting fetus nutrition and development. Especially, a previous research suggested the involvement of copper in oligohydramnios[25], further study focus on the metal transport related ABCA12 function in oligohydramnios would provide more information.

In summary, we revealed the profiles of lncRNA and mRNA in oligohydramnios, validated the upregulation of LINC00515 and RP11–388P9.2, and suggested a lncRNA-miRNA-mRNA network that might involved in the pathogenesis of oligohydramnios. Since there have been evidence of the presence of cell-free DNA and RNA in the amniotic fluid[44–46], it would be great important to investigate the correlation between the expression of amniotic fluid cell-free RNA and fetal membrane RNA for oligohydramnios prognosis and therapy.

4. Material And Methods

4.1. Patient recruitment

Fetal membranes were obtained from pregnant women with oligohydramnios (OR) and amniotic fluid normal delivery (Normal) were collected. Pregnant women who meet the following standard criteria are diagnosed with oligohydramnios: SDP ≤ 2 cm, or AFI ≤ 5 cm[5, 6]. Patients who experience multiple gestations, eclampsia, and presented fetal anomalies were excluded.

4.2. Ethnic statement

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

4.3. Tissue collection and RNA isolation

The fetal membrane of a woman who had just given birth was quickly placed in liquid nitrogen in 30 minutes. Approximate $1cm^3$ of tissue block were ressected for grinding. Samples were grinded in a motor-driven homogenizer. Trizol (Invitrogen, CA, USA) was used to extract total RNA from tissues following the standard protocol of manufacture's protocol. Concentration and qualification of the isolated total RNA was assessed by Nanodrop 2001 spectrophotometer (Thermo Fisher Scientific, MA, USA).

4.4. lncRNA and mRNA microarray

The Human Human LncRNA Array V4.0 (8×60k) was performed by KangChen Bio-tech, Shanghai, China Company. The microarrays included 40173 lncRNAs and 20730 mRNAs.

4.5. Relative expression of lncRNA assessment

The relative expression of lncRNA between OR and Normal was measured by Quantitative real time PCR (qRT-PCR). Primers targeting LINC00515 and RP11–388P92 were list in Table 3. 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 SYBR Real time PCR Master Mix kit (TOYOBO, Osaka, Japan). Programme for cDNA amplification was as follows, the first step, 95℃, 120 s; the second step, 95℃, 15 s, 60℃, 30 s, for 40 cycles; the third step for melting curve generation, 60 to 95℃. The relative expression of lncRNA was analyzed using $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal control.

4.6. Bioinformatics

Cluster analysis were carried out. Heatmaps and scatter plots were generated for differentially expressed genes using the R package. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using an online tool (http://www.genome.jp/kegg/). KEGG pathway ways meet FDR≤0.001were considered as significantly enriched.

To explore the potential role of lncRNA, a lncRNA-miRNA-mRNA interaction network was constructed. Briefly, miRNAs that harboring lncRNA and mRNA biding sites were predicted by R package. The overlap miRNAs that both harboring lncRNA and mRNA binding targets were used to constructed lncRNA-miRNA-mRNA interaction network. Sub-network that contain predicted targets of lncRNA and were differentially expressed in OR were included. The network was visualized using Cytoscape_V2_8_3 (https://www.innatedb.ca/cytoscape-v2.8.3/plugins/) software.

Declarations

Ethics approval and consent to participate

Not applicable

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Author 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.

Conflicts of Interest

The authors declare no conflict of interest.

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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. Clinical characteristics of women included in this study.

characteristics	Oligohydramnios	Normal control	P-value
Age(years)	32.63±5.23	30.8±4.17	0.233
BMI	26.21±2.48	26.23±2.81	0.977
Spontaneous abortion	0.16±0.37	0.7±0.92	0.023*

Table 3. Information of the top-10 most upregulated and downregualted lncRNAs in OR women.

Transcript_id	lncRNA_id	Fold Change	Regulation	P-value	FDR
T074729	G017197	6.992997551	up	0.022330367	0.323992752
T122682	G028960	6.156767122	up	0.007011989	0.231007727
T245792	G056426	5.925028116	up	0.014297859	0.282223415
GSE61474_TCONS_00183926	GSE61474_XLOC_033346	5.875790672	up	0.018248847	0.30550168
T257490	G059353	5.369031987	up	0.022024259	0.322125587
ENST00000449721	AC091729.7	5.280611483	up	0.015183552	0.286476988
NR_024092	LINC00515	5.265016308	up	0.000229591	0.092301652
T104828	G024752	5.123561472	up	0.039183093	0.394165858
ENST00000414383	RP11-388P9.2	4.567856614	up	0.003180925	0.185628546
NR_036580	DPP10-AS1	4.5185482	up	0.042740897	0.403301473
T352051	G083088	0.156292992	down	0.024924406	0.335128165
NR_047465	LINC00501	0.192207162	down	0.019901778	0.31149042
T196519	G045291	0.213060492	down	0.039570275	0.395804997
NR_120506	LINC01510	0.231630157	down	0.002294572	0.176676707
ENST00000608142	RP11-1399P15.1	0.273437307	down	0.021416751	0.321541985
ENST00000522718	RP11-150O12.1	0.286446378	down	0.000781865	0.143720717
NR_110991	LRRC74B	0.292328015	down	0.010723383	0.257909493
ENST00000440726	RP11-567G11.1	0.292488539	down	0.012877268	0.272737873
T070890	G016402	0.301039562	down	0.007261346	0.232753219
T188489	G043293	0.302661694	down	0.019071938	0.309549149

Figures

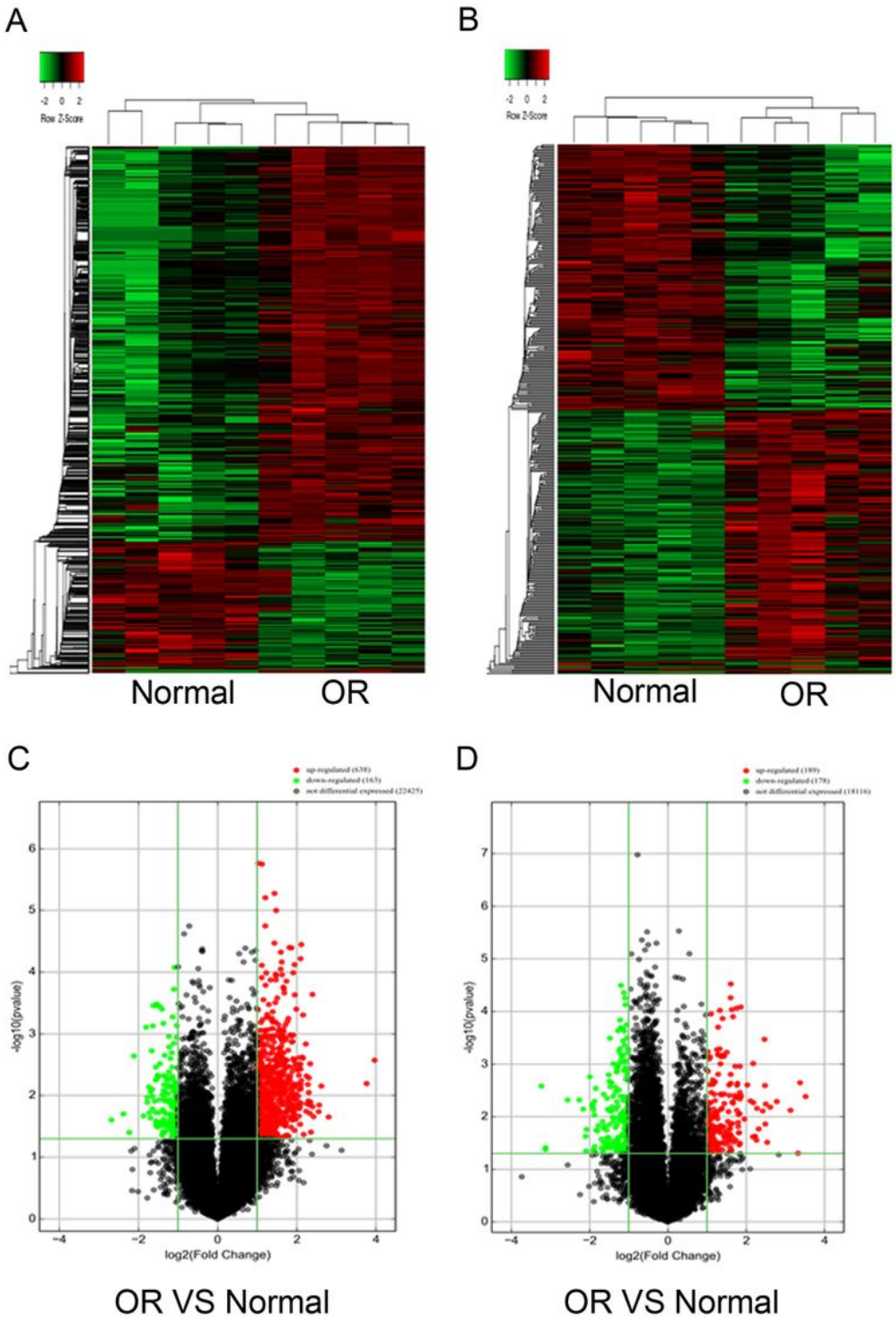

Figure 1

Microarray revealed differential lncRNA profiles between pregnant woman with normal amniotic fluid volume (Normal) and those with oligohydramnios (OR). N = 5. Heatmap showing the profile of lncRNA (A) and (B) in OR and normal women. Volcano plot showing the overall change in expression of lncRNAs (C) and mRNAs (D). The upregualted RNA were labeled in red while the downregulate RNA were labeled in green.

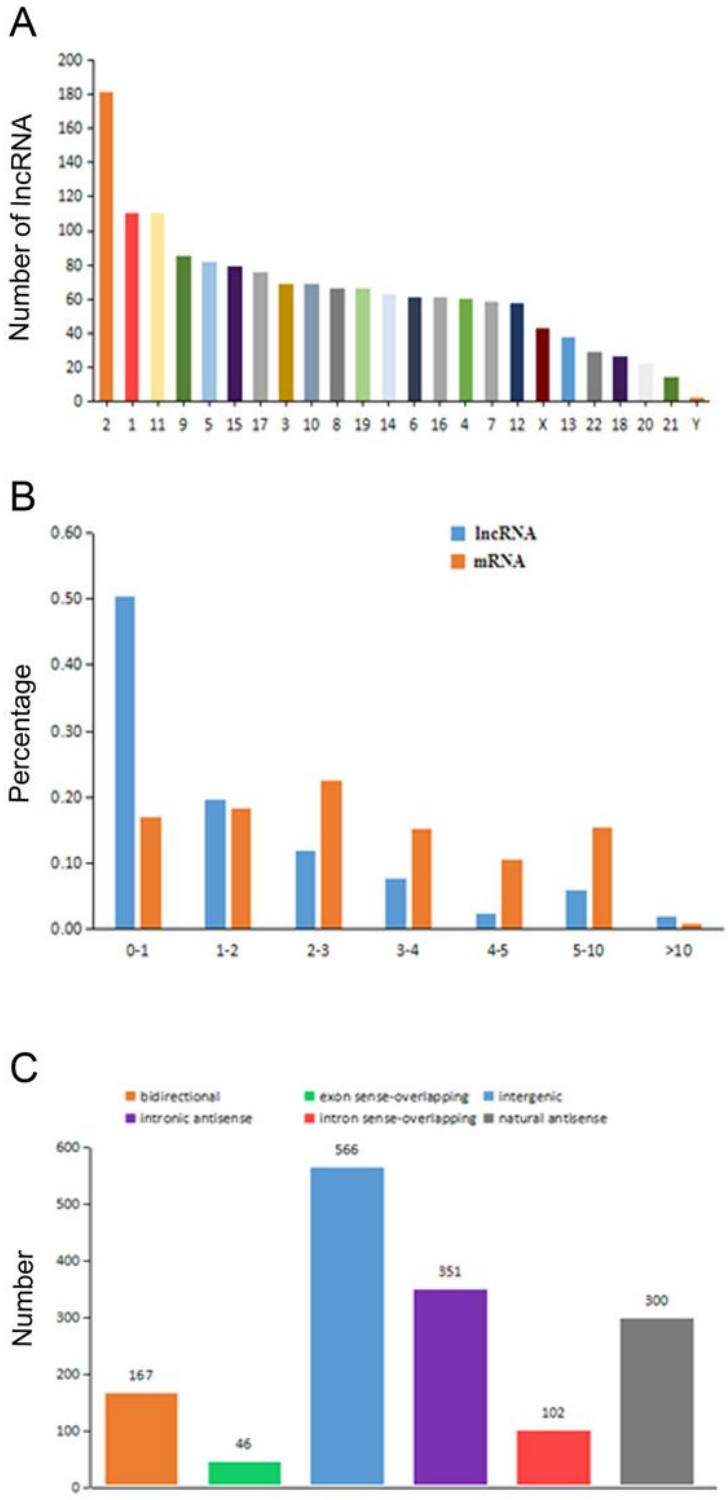

Figure 2

Distribution of lncRNA genomic location (A), lncRNA length (B) and type of the differential expressed lnRNA in OR women.

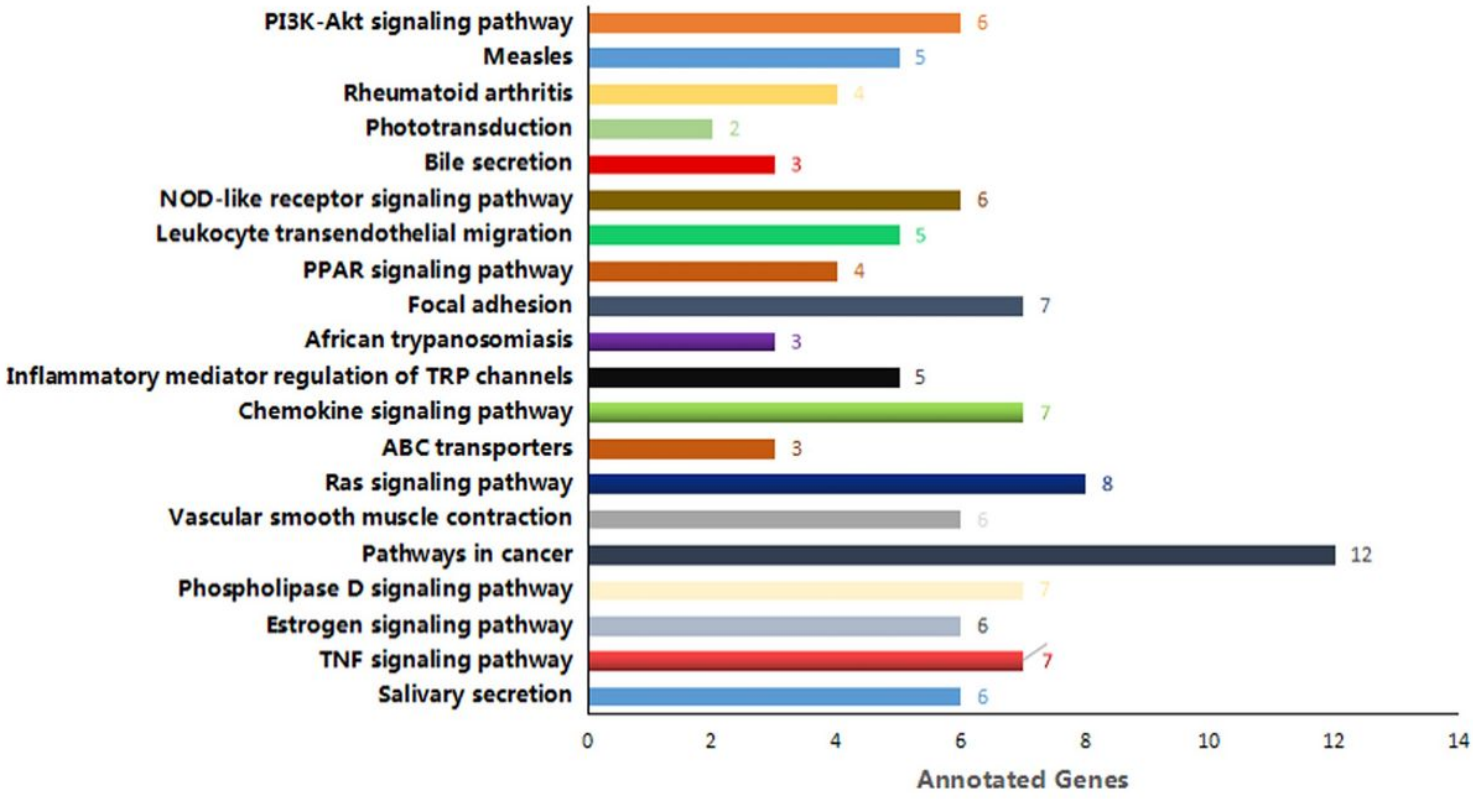

Figure 3

KEGG enrichment analysis showing the most enrich pathways for the differential expressed mRNAs in OR.

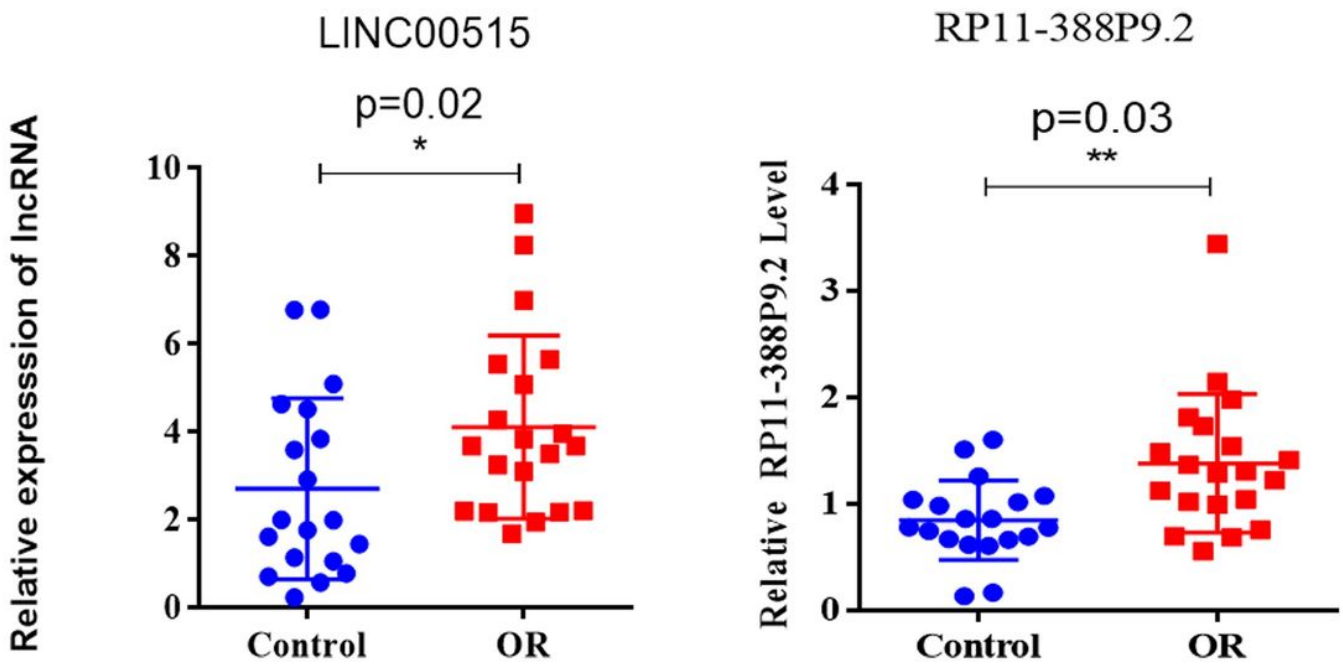

Figure 4

The expression of LINC00515 and RP11-388P9.2 in OR women relative to Normal women.

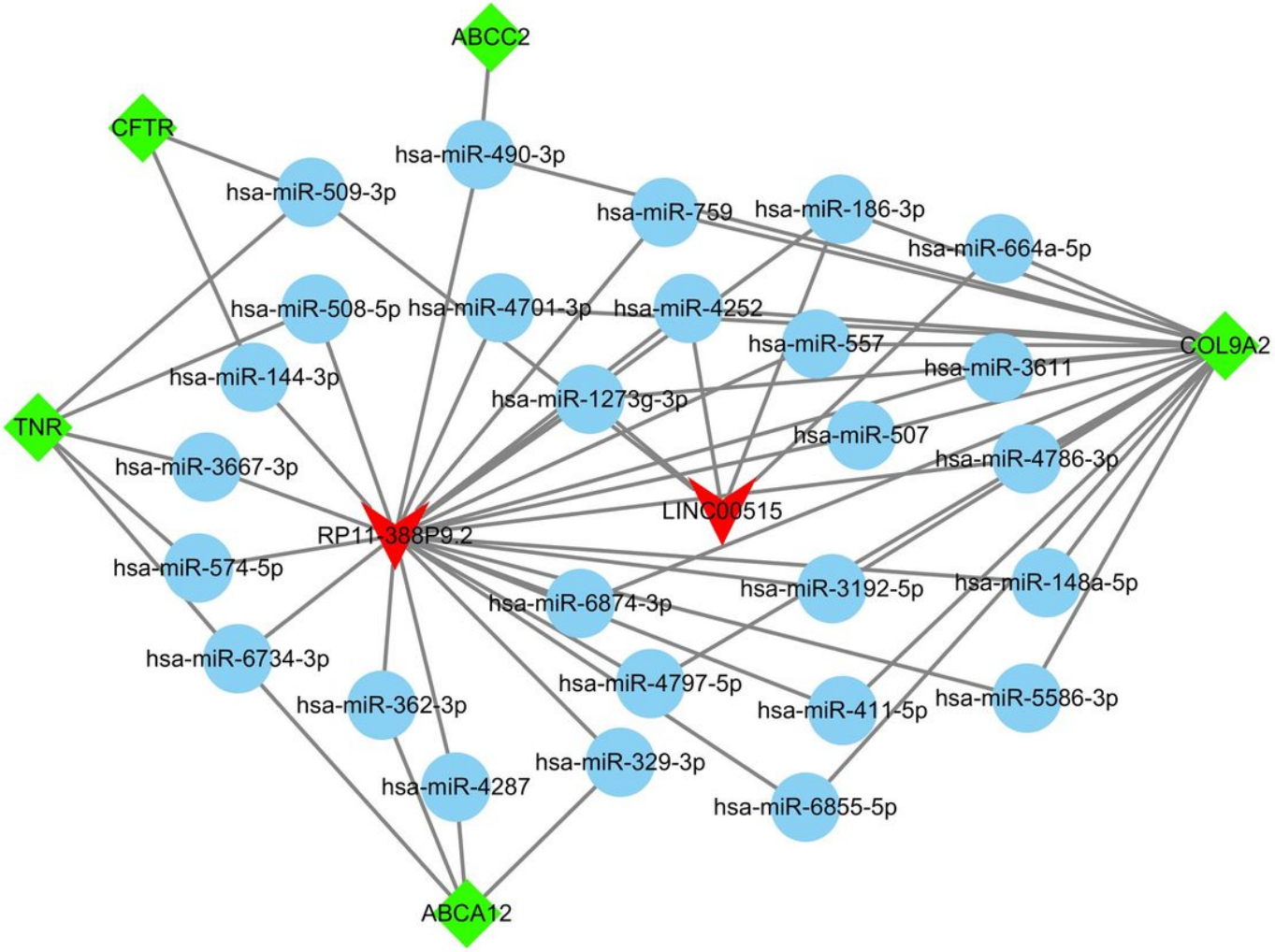

Figure 5

The lncRNA-miRNA-mRNA interaction network. mRNAs are differential expressed in OR women and contain biding sites of miRNAs. The miRNAs are also potential targets of LINC00515 and RP11-388P9.2.