Microarray analysis reveals differentially expressed lncRNAs in benign epithelial ovarian cysts and normal ovaries

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Abstract. Recent studies suggest that long non-coding RNAs (lncRNAs) play crucial roles in many types of human malignant cancers. However, the function of lncRNAs in benign tumors remains poorly understood. In the present study, to explored the potential roles of lncRNAs in benign epithelial ovarian cysts (BEOCs) which commonly occur in young women and possess malignant potential, we described the expression profile of the lncRNAs between BEOC and normal ovarian tissues using lncRNA microarray techniques. The results showed that 1,325 transcripts of lncRNAs (1,014 upregulated and 311 downregulated) were differentially expressed in the BEOCs compared with the normal controls [absolute fold-change ≥2, false discovery rate (FDR) <0.05]. We also conducted quantitative real-time PCR (qPCR) to confirm the microarray data. The results of qPCR revealed that the expression trend of 6 randomly selected lncRNAs was consistent with the microarray data. Furthermore, candidate lncRNAs were characterized by pathway analysis and Gene Ontology (GO). The present study is the first to demonstrate different expression profiles of lncRNAs between BEOCs and normal ovarian tissues. These lncRNAs may play a crucial role in the pathological process of BEOCs.

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Introduction

Ovarian tumors are the leading cause of death among all gynecologic tumors (1). In the US, ~22,280 cases of ovarian cancer were estimated to be diagnosed and it was estimated that 14,240 women died from ovarian cancer in 2016 (2). Despite modern surgical techniques and chemotherapy, the prognosis of ovarian cancer remains poor (3). Ovarian tumors are a common type of neoplasm in women and the histological type is various and complex. Epithelial ovarian tumors are the most common type of ovarian tumor and represents 50-70% of all primary ovarian tumors. According to the characteristics of the tumor cells and the severity to health, epithelial ovarian tumors can be divided into three types: benign, borderline and malignant ovarian tumors. In various cases, benign tumors can develop into a malignant tumor (4-7), which suggests that benign ovarian tumors have an increased risk to transform into malignant tumors due to the changes of various genes (8) or proteins (9). However, extensive research has mainly focused on malignant epithelial ovarian cancer, and benign ovarian tumors have not been a principal focus of research. In fact, there are far more benign lesions occurring in the epithelial ovary, and these are commonly diagnosed during pregnancy (10). In addition, these are also associated with an increased risk of malignant epithelial ovarian cancer. Therefore, a deeper understanding of benign epithelial ovarian cysts (BEOCs) may not only provide more effective treatments for BEOCs, but may also reduce the incidence of malignant epithelial ovarian cancer.

Long non-coding RNAs (lncRNAs) are a type of RNAs that are longer than 200 nucleotides in length and without coding protein capacity (11). lncRNAs have been previously considered as 'transcriptional noise' for a long time (12). Recently, studies have confirmed that lncRNAs play a critical role in the development of cancer, including ovarian cancer. Emerging

evidence suggests that lncRNAs are associated with ovarian cancer biological behaviors such as cell proliferation (13,14), apoptosis (15,16), and invasion and metastasis (17,18). Moreover, due to tissue-specificity, some lncRNAs may served as potential biomarkers for cancer prognosis, including ovarian cancer (19). However, these studies have only focused on the regulation of lncRNAs in malignant ovarian cancer, and little research has been carried out on the relationship between lncRNAs and benign ovarian tumors. Therefore, lncRNA expression profiles in BEOCs may help us to better understand BEOC pathogenesis.

In the present study, we described the distinct expression profiles of lncRNAs in BEOC and normal ovarian epithelial tissues. In total, 1,014 transcripts of lncRNAs were upregulated and 311 transcripts of lncRNAs were downregulated in BEOCs compared with normal controls [absolute fold-change ≥2, false discovery rate (FDR) <0.05]. Moreover, we also examined the Gene Ontology (GO) enrichment of their associated protein-coding genes and performed pathway analyses to analyze the potential function of these differentially expressed lncRNAs. The present study may aid in elucidating the tumorigenesis of ovarian epithelial tissue and decrease the incidence of malignant transformation in regards to BEOCs.

Materials and methods

Tissue collection. Samples of BEOCs and normal ovarian tissues were collected from the Gynecologic Oncology Department of Nanjing Maternal and Child Health Hospital (Nanjing, China) from 2014 to 2016. No patient received chemotherapy or radiotherapy before surgery. Informed consent for the use of the tissues was obtained from all patients. Finally, 10 cases of normal ovarian tissues and 17 cases of BEOCs were collected and immediately stored in RNAsafety™ and frozen at -80˚C before the experiments. All the tissues were confirmed through histopathological diagnoses. The present study was approved by the Ethics Review Committee of Nanjing Maternity and Child Health Care Hospital.

Total RNA extraction. Frozen tissues were dissolved in TRIzol reagent (Life Technologies, Grand Island, NY, USA). Total RNA was extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). NanoDrop and the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) were used to check the quantification and quality of the extracted RNA, respectively. The extracted RNA samples were stored at -80˚C for further experiments. Complementary DNA (cDNA) was synthesized from 1 μg of total-RNA using a PrimesScript™ RT Master Mix kit (Applied Takara, Dalian, China) with random hexamer primers in a final volume of 20 ml. The condition of reverse-transcription reaction was as follows: 37˚C for 15 min, 85˚C for 5 sec and 4˚C for 10 min.

Microarray analysis. To screen the global profiling of human lncRNAs and protein-coding transcripts, we profiled three BEOC tissues and three normal ovarian epithelial tissues with ArrayStar Human Microarray V3.0. The lncRNAs were searched for using authoritative databases such as RefSeq, Ensembl and UCSC Known Genes and related studies while the mRNAs were collected from RefSeq and GENCODE. To recognize every individual transcript exactly, each transcript was described with a specific exon or splice junction probe. Both positive probes (the housekeeping genes) and negative probes were printed onto the array for hybridization quality control. The sample processing and microarray hybridization were performed in terms of the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Briefly, 1 mg of total RNA was obtained for purification by removing the rRNA (mRNA-ONLY Eukaryotic mRNA Isolation kit; Epicentre, Madison, WI, USA). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method. Agilent Quick Amp Labeling kit was employed to normalize the values, and then, lncRNAs and mRNAs, for which at least one out of two groups had flags in present or marginal, were chosen for further data analysis. Additionally, hierarchical clustering and combined analyses were performed using homemade scripts.

Quantitative real-time PCR (qPCR). qPCR was performed to detect the relative gene expression using Power SYBR-Green PCR Master Mix (2X Applied Biosystems) according to the standard protocol. GAPDH was taken as an internal reference. The qPCR reaction conditions were set as follows: an initial denaturation at 95˚C for 30 sec, followed by 40 PCR cycles at 95˚C for 5 sec and 60˚C for 34 sec. Finally annealing and extension at 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. Each sample was detected in triplicates. The fold-change was calculated with the ΔCT method to describe the relative gene expression in BEOC samples relative to the normal ovarian tissue samples. All of the primers are presented in Table I.

GO and pathway analyses. Pathway and GO analyses were applied to determine the potential roles of differentially expressed lncRNAs in biological pathways or GO terms. The predicted target genes of the differentially expressed lncRNAs were mapped to GO terms in the Database for Annotation Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/). Fisher's exact test was used to ascertain whether true differences existed between the groups. In addition, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/) to confirm the pathway enrichment analysis. The ontology covers three domains: biological process (BP), cellular component (CC) and molecular function (MF). The threshold of significance was defined by FDR.

Statistical analysis. Differential expression levels of lncRNAs were selected by fold-change filtering (absolute fold-change >2.0). Independent samples t-test between two groups was used, and Fisher's exact test was used in GO and pathway analyses. A value of FDR <0.05 was considered statistically significant. Computer-based calculations were conducted using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA).

Results

Differentially expressed lncRNAs and mRNAs in BEOCs compared with normal ovarian tissues. Firstly, to explore the

Table I. qPCR primers used in the present study.

lncRNAs	Forward (5'-3')	Reverse (5'-3')
LOC339166	GCCTCTCTGGAGCTGAATCG	CGTGCCAGTGGGTTTCCTAA
LOC440214	CCACCCCAAAGAAGATGCTG	ACAGGAGACAAAGCCTTCGC
LOC644656	AATTAGTTGTGGCCGTTGCG	ATCTTTAGTCGGCCTGGTGC
NENF	CAGGAGCAGGTTCTTGGGAG	CCAAGGACAACAGGAGGCAT
RP11-471J12.1	TCAGCCCACCTGCTCCAA	TGATCTGTGCCTTCCTGGTACA
MEG3	CTGCCCATCTACACCTCACG	CTCTCCGCCGTCTGCGCTAGGGGCT
GAPDH	CCAGAACATCATCCCTGCCT	CCTGCTTCACCACCTTCTTG

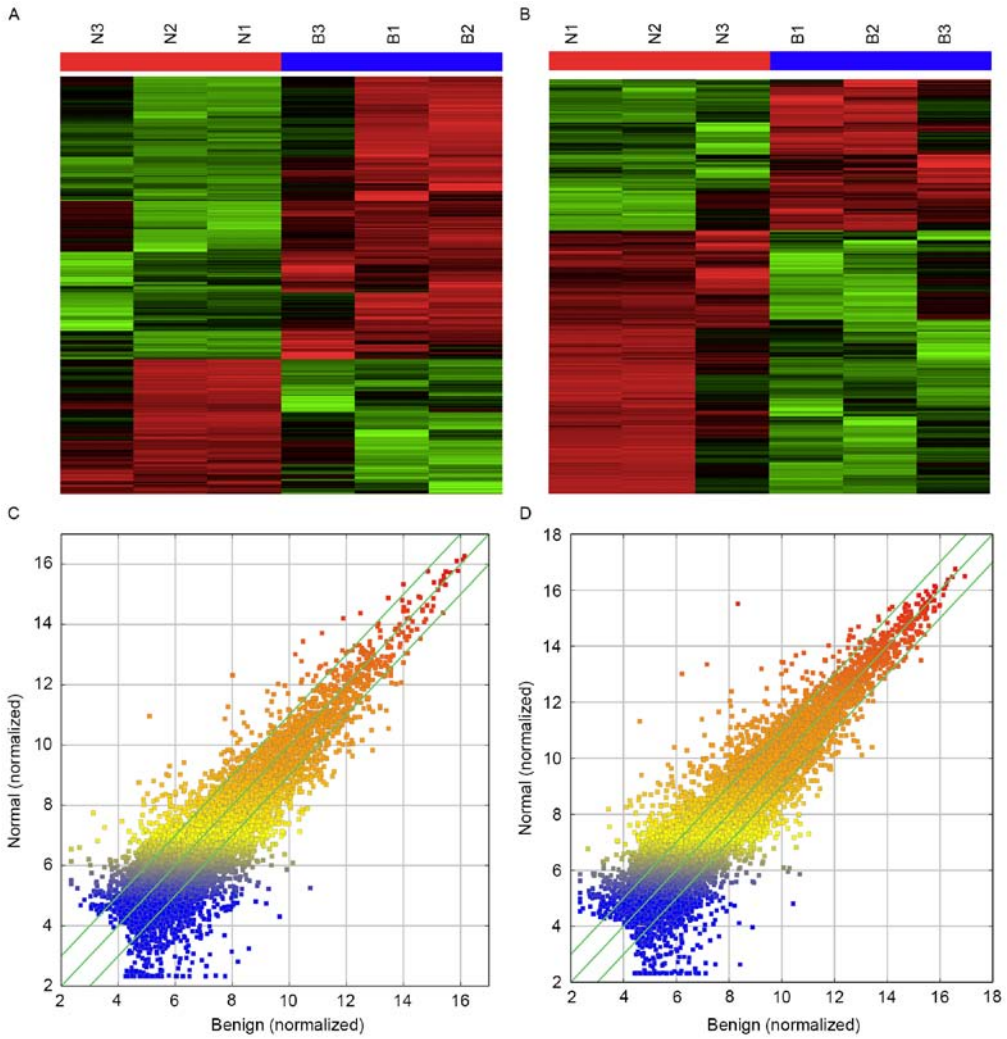

Figure 1. Differentially expressed lncRNAs in BEOCs compared with normal ovarian tissues. (A and B) Differentially expressed lncRNAs and mRNAs between BEOCs and normal ovarian tissues were analyzed using hierarchical clustering; 'red' indicates high relative expression, and 'green' indicates low relative expression. (C and D) Scatter plots were used to assess expression variations of lncRNAs and mRNAs between BEOCs and normal ovaries. lncRNAs and mRNAs above the top green line and below the green line exhibit a >2.0 fold-change.

altered lncRNAs in the BEOCs, we determined the lncRNA and mRNA expression profiles using microarray analyses of normal ovarian and BEOC tissues. Heatmaps and scatter-plots were used to describe the variation in lncRNA expression among normal ovarian, benign ovarian cysts and malignant epithelial ovarian cancer tissues (Fig. 1). All lncRNAs and mRNAs with a signal altered by 2-fold and with a false discovery rate (FDR) <0.05 were identified as statistically altered. Finally, 1,325 transcripts of lncRNAs (1,014 upregulated and 311 downregulated) and 1,563 mRNAs (613 upregulated and 950 downregulated) were found to be differentially expressed in BEOC tissues compared with normal controls. Lists of the top 20 upregulated and down-regulated lncRNAs identified in the microarray analyses are presented in Tables II and III.

Table II. The top 20 upregulated lncRNAs in the BEOCs compared with the normal ovarian tissues.

Seqname	GeneSymbol	FDR	Fold-change	Source	Associated-gene	Mean expression	
						Normal	Benign
ENST00000502882	RP11-158J3.2	0.017	44.223	GENCODE	HTR1A	38.428	1699.407
ENST00000534866	TAS2R64P	0.022	42.733	GENCODE		5.029	214.918
uc010ciy.1	BC160930	0.038	41.621	UCSC_knowngene	RP11-566K11.2	7.028	292.506
AK021689		0.017	40.678	NRED		19.848	807.381
ENST00000500487	RP11-32B5.7	0.031	40.156	GENCODE		9.524	382.452
ENST00000419463	AC019117.1	0.018	33.387	GENCODE		5.110	170.614
ENST00000526388	CTC-497E21.4	0.010	28.260	GENCODE		8.881	250.981
ENST00000563752	SLC25A3P1	0.019	26.511	GENCODE		5.029	133.333
ENST00000566892	RP11-1081M5.2	0.027	21.685	GENCODE		26.929	583.973
NR_040017	RNF157-AS1	0.020	21.544	RefSeq	FOXJ1	6.342	136.631
uc001gzl.3	BC034684	0.005	17.922	UCSC_knowngene	CHI3L1	5.029	90.138
ENST00000450480	RP4-797C5.2	0.018	17.793	GENCODE	KCND2	8.340	148.387
TCONS_00026830	XLOC_013047	0.034	16.688	LincRNAs identified by Cabili *et al* (12)		7.306	121.921
NR_040033	LOC729950	0.023	16.490	RefSeq		37.444	617.446
ENST00000381181	AP000569.2	0.034	16.383	GENCODE		12.908	211.472
NR_027309	LOC148824	0.016	16.354	RefSeq	OR2C3	68.207	1115.456
ENST00000521558	RP11-1081M5.1	0.025	16.292	GENCODE		58.657	955.648
NR_038883	LINC00649	0.017	16.086	RefSeq		28.062	451.399
TCONS_00018520	XLOC_008826	0.021	15.539	LincRNAs identified by Cabili *et al* (12)		5.811	90.301
chr14:84031800-84050525+	chr14:84031800-84050525	0.024	15.300	LincRNAs identified by Khalil *et al* (26)		5.029	76.949

lncRNAs, long non-coding RNAs; FDR, false discovery rate.

Table III. The top 20 downregulated lncRNAs in the BEOCs compared with the normal ovarian tissues.

Seqname	GeneSymbol	FDR	Fold-change	Source	Associated-gene	Mean expression	
						Normal	Benign
uc002ejp.1	MT1JP	0.016	58.627	UCSC_knowngene		1999.563	34.107
uc003xxw.1	AX747593	0.020	24.817	UCSC_knowngene	RP11-664D7.4	215.542	8.685
ENST00000584923	SNORD3A	0.014	20.002	GENCODE		5117.821	255.872
ENST00000437593	RP11-500G22.2	0.012	18.427	GENCODE	ATE1	93.894	5.095
TCONS_00023858	XLOC_011173	0.014	16.438	LincRNAs identified by Cabili *et al* (12)		432.820	26.330
ENST00000542078	RP11-392P7.8	0.014	15.472	GENCODE	HEBP1	96.912	6.264
ENST00000417522	RP11-38J22.6	0.017	13.893	GENCODE	C1orf186	70.787	5.095
ENST00000580684	RP11-835E18.2	0.048	13.632	GENCODE		154.759	11.353
ENST00000536029	RP11-392P7.8	0.020	11.776	GENCODE	HEBP1	113.762	9.660
TCONS_00017618	XLOC_008306	0.011	11.503	LincRNAs identified by Cabili *et al* (12)		635.853	55.276
uc002lch.1	AK095045	0.018	10.279	UCSC_knowngene		335.169	32.609
TCONS_00014161	XLOC_006144	0.023	10.191	LincRNAs identified by Cabili *et al* (12)		178.103	17.477
ENST00000499314	RP11-1277A3.2	0.021	10.179	GENCODE		522.572	51.336
TCONS_00029245	XLOC_013980	0.031	10.076	LincRNAs identified by Cabili *et al* (12)		294.817	29.260
ENST00000417089	H19	0.017	9.329	GENCODE		508.334	54.491
ENST00000555882	DIO3OS	0.005	9.060	GENCODE		46.162	5.095
NR_026860	LINC00473	0.020	8.979	RefSeq		410.373	45.702
uc010rog.2	NEAT1	0.044	8.809	UCSC_knowngene		261.236	29.656
ENST00000520913	PVT1	0.010	8.783	GENCODE		1881.694	214.238
ENST00000447298	H19	0.017	8.718	GENCODE		688.447	78.972

lncRNAs, long non-coding RNAs; FDR, false discovery rate.

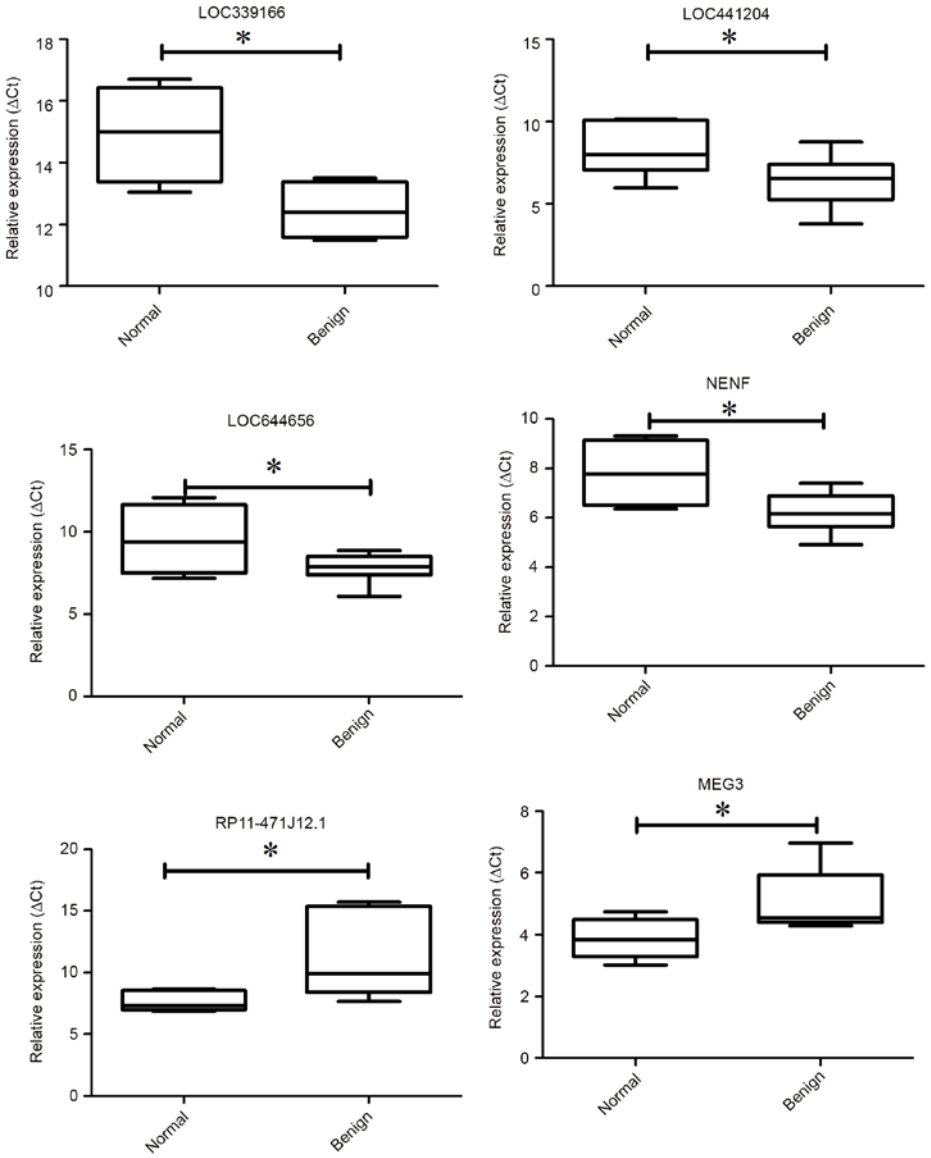

Figure 2. Validation by qPCR of candidate lncRNAs in BEOCs and normal ovarian tissues. The relative expression levels of candidate lncRNAs were detected by qPCR in 8 normal ovarian, 17 benign cyst and 15 malignant EOC samples. The ΔCt values of the lncRNAs were determined by subtracting the GAPDH ΔCt value. A smaller ΔCt value indicates higher expression levels. Data are presented as the relative expression level in tumor tissues (shown as ΔCt); all $^{*}P<0.05$.

Validation of candidate lncRNAs by qPCR. To confirm the validity of the microarray data, we next conducted qPCR to detect the expression of the lncRNAs. We randomly selected 6 differentially expressed lncRNAs. Among the 6 lncRNAs, lncRNAs LOC339166, LOC441204, LOC644656 and NENF were upregulated whereas lncRNAs RP11-471J12.1 and MEG3 were downregulated in the BEOCs compared with the normal controls (Fig. 2). The result of qPCR confirmed that the expression trend of the 6 selected lncRNAs was consistent with the microarray data.

GO and pathway analyses of the differentially expressed lncRNAs. To investigate the function of altered lncRNAs in the BEOCs, we performed GO analysis which covered the following three domains: biological processes (BP), cellular components (CC) and molecular functions (MF). We found that the highest GO classifications targeted by the upregulated lncRNAs were single-organism process (Fig. 3A), membrane (Fig. 3B) and signal transducer activity (Fig. 3C). However, the highest GO classifications targeted by downregulated lncRNAs were cellular process (Fig. 3D), cell part (Fig. 3E) and binding, particularly protein binding (Fig. 3F). To map these lncRNAs to pathways, we also performed pathway analysis. The result indicated that the 9 main pathways corresponding to the upregulated transcripts and the most enriched network was 'neuroactive ligand-receptor interaction' (Fig. 4A). The 9 main pathways are shown: i) neuroactive ligand-receptor interaction; ii) axon guidance; iii) mucin type O-glycan biosynthesis; iv) *Staphylococcus areus* infection; v) transcriptional misregulation in cancer; vi) GABAergic synapse; vii) carbohydrate digestion and absorption; viii) serotonergic synapse; ix) glutamatergic synapse. We also observed 10 main pathways corresponding to the downregulated transcripts (Fig. 4B): i) focal adhesion; ii) bacterial invasion of epithelial cells; iii) phagosome; iv) leukocyte transendothelial migration;

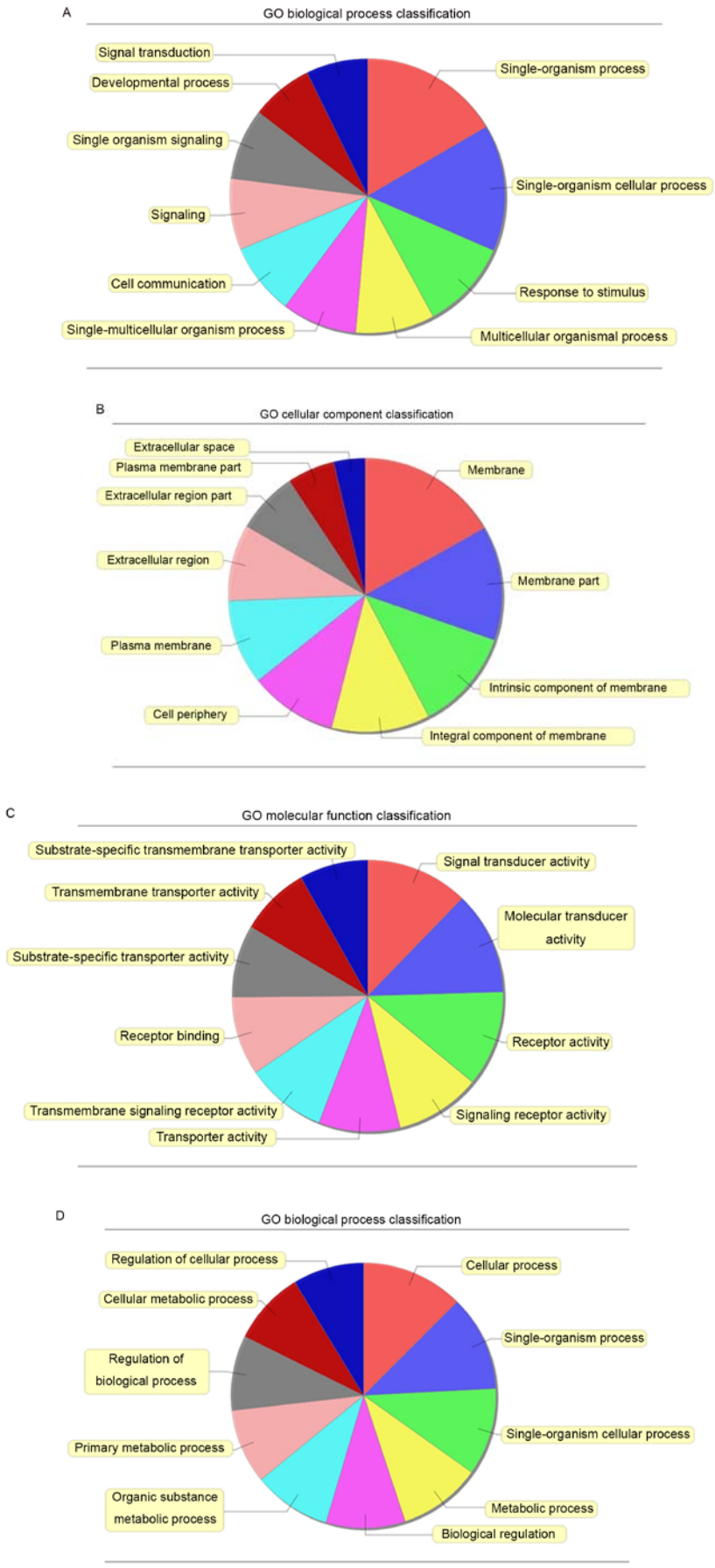

Figure 3. GO analysis of the differentially expressed lncRNAs. GO analysis results show the differentially expressed lncRNAs associated with biological processes (BP), cellular components (CC) and molecular functions (MF). The most frequent fold enrichment BP associated with (A) upregulated lncRNAs and (D) downregulated lncRNAs in BEOCs. The most frequent fold enrichment CC for (B) lncRNAs upregulated in BEOCs compared with normal control. The top 10 GO terms of MF associated with (C) upregulated lncRNAs.

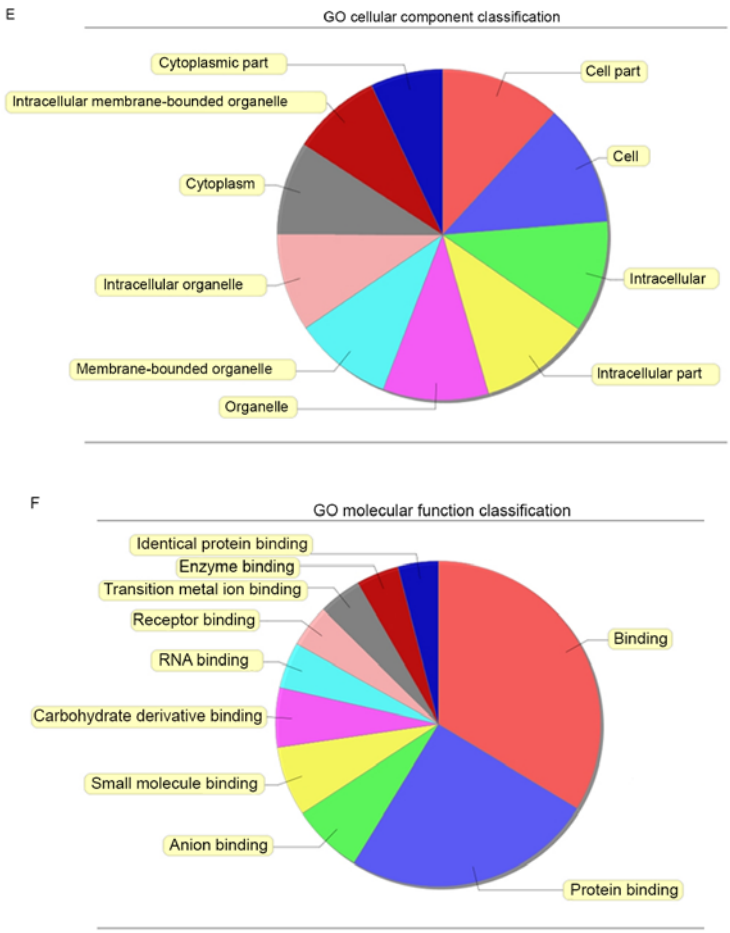

Figure 3. Continued. The most frequent fold enrichment for CC for (E) downregulated lncRNAs in BEOCs compared with normal control. The top 10 GO terms of MF associated with (F) downregulated lncRNAs.

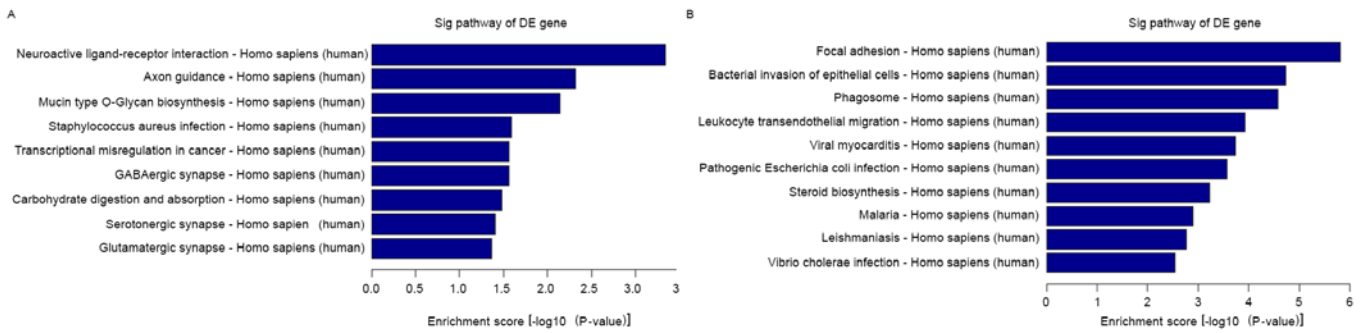

Figure 4. Pathway analysis of the differentially expressed lncRNAs. (A) The most frequent enrichment pathways corresponding to the lncRNAs upregulated in BEOC compared with normal tissues. (B) Top 10 pathways corresponding to the lncRNAs downregulated in BEOC tissues.

v) viral myocarditis; vi) pathogenic *Escherichia coli* infection; vii) steroid biosynthesis; viii) malaria; ix) leishmaniasis; x) *Vibrio cholera* infection. The most enriched network was 'focal adhesion' with 37 transcripts annotated with this term (Fisher P-value=1.53367E-06).

Discussion

BEOCs are the most common form of ovarian tumors in women, accounting for ~80% of all ovarian masses (20). However, the molecular mechanisms related to the tumorigenesis of ovarian epithelial cells remains largely unknown. Increasing studies have claimed that lncRNAs are highly functional and have a crucial role in malignat ovarian cancer (13,17,18,21,22). In our previous study, we identified dysregulation of many lncRNAs in malignant ovarian cancer compared with benign ovarian cysts and normal ovary. However, we also found that several lncRNAs, such as LEMD1-AS1 and AK 125532 were differentially expressed in BEOCs compared with these in the normal control (23). Thus, we next identified differentially expressed lncRNAs in BEOCs compared with normal ovarian tissues. To the best of our knowledge, the present study is the

first to investigate the lncRNA expression profiling in BEOCs compared with normal ovarian tissues. The present study provides a better understanding of the molecular mechanisms of BEOCs.

On the basis of the GO analysis, we found that the BP of upregulated and downregulated lncRNAs was both tightly associated with single-organism process and single-organism cellular process. These GO terms are also associated with death and cell proliferation (24). The destiny of BEOCs is critically regulated by the cell cycle and apoptosis process in which lncRNAs may play an important role. For CC, the top GO term of the upregulated lncRNAs was membrane part and in downregulated lncRNAs this was cell part. These results indicate that lncRNAs may primarily regulate various mRNAs which are located in the cell or on the membrane and exercise their function. It can also be observed that the highest frequency of the MF GO terms in the upregulated lncRNAs was molecular transducer activity. Many studies have reported that some lncRNAs act as a molecular transducer. For example, lncRNA-TUG1 is overexpressed in non-small cell lung cancer, and can be regulated by p53 and affect cell proliferation through HOXB7 expression (25). However, the top MF GO term in the downregulated lncRNAs was binding, particularly protein binding. Increasing studies suggest that the primary function of lncRNAs is the epigenetic regulation of coding gene through proteins or microRNAs. Many lncRNAs have been reported to recruit and bind to PRC2 (26) or other chromatin-associated proteins (27). In addition, accumulating evidence indicates that lncRNAs act as competing endogenous RNAs (ceRNAs) by 'sponging' microRNAs (22,28,29).

In addition, pathway analysis displayed that the upregulated lncRNAs were mainly correlated with neuroactive ligand-receptor interaction and axon guidance. Various studies have reported that the sympathetic nervous system is important in the tumor microenvironment (30,31), and autonomic nerve development promotes cancer progression (32). Therefore, we speculated that there may be a relationship between ovarian tumorigenesis and the dysregulation of neural signaling pathways. In contrast, the downregulated lncRNAs were mainly correlated with focal adhesion. Focal adhesion is important between two cells or between a cell and the extracellular matrix. Various coding genes such as E-cadherin play critical roles in focal adhesion and tumor metastasis when malignant potential is increased in ovarian cancer cells. Moreover, some lncRNAs such as H19 (33), HOTAIR and MALAT1 (34) were found to promote cancer aggression by regulating E-cadherin.

Research has shown that the expression of H19 is upregulated in many types of cancers including ovarian cancer. The ectopic expression of H19 was found to promote cancer cell proliferation and invasion, suggesting that H19 may function as an oncogene. In our microarray, the transcript of H19 (ENST00000422826) was overexpressed ~3-fold in BEOCs compared with that in normal tissues. The results also indicated that BEOCs may have malignancy potential. However, the transcripts of H19 (ENST00000417089, ENST00000447298, uc001lva.4, uc021qbz.1, ENST00000442037 and ENST00000446406) were reduced in the BEOCs. The results of PVT1 were also the same as H19; different transcripts had differential expression in the BEOCs. The different transcripts of lncRNAs had differential expression and function. However, confirmation and elucidation of this finding require further study.

Due to the important roles of lncRNAs in cancer, a growing number of studies have focused on the potential lncRNA-related biomarkers for tumors. lncRNA-related biomarkers aid clinicians to make accurate diagnoses and treatment decisions. Crea *et al* reported that PCAT18, the lncNRA which is specifically expressed in the prostate, could be a potential therapeutic target and biomarker for metastatic prostate cancer (35). In addition, similar lncRNA-related biomarkers with prognostic value have also been identified in other types of cancers such as breast (36), pancreatic (37) and lung cancer (38,39). In ovarian cancer, various lncRNAs have been shown to have potential as prognostic biomarkers, such as HOTAIR (18), H19 (40) and HOXA11AS (41). However, lncRNA-related biomarkers in benign tumors still have not been reported. The present study may aid in the identification of lncRNA-related biomarkers for benign ovarian cysts.

In summary, we profiled the differential expression of lncRNAs and mRNAs between BEOCs and normal ovarian tissues. In total, 1,325 transcripts of lncRNAs and 1,563 mRNAs were found to be differentially expressed between BEOCs and normal ovarian control tissues (absolute fold-change ≥2, FDR <0.05). Furthermore, dysregulated lncRNAs were characterized by a comprehensive examination of GO enrichment and pathway analysis by their associated protein-coding genes. Collectively, our findings suggest that lncRNAs play a critical role in the pathological process of BEOCs.

Acknowledgements

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