Distinct expression profile of lncRNA in endometrial carcinoma

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Abstract. Endometrial carcinoma (EC) is the most common malignancy in women. Dispite its prevalence, the prognosis of endometrial carcinoma still relies on conventional histological type, grade and invasion information. Its morbidity is still increasing and the outcome is very poor. To the best of our knowledge, hormonal imbalance and/or molecular genetic alterations are the main cause of EC. However, the alterations of lncRNAs which accounts for approximately 4/5 of human transcripts are still poorly understood. In the present study, using the RiboArray™ Custom Array, we studied the expression profiles of lncRNA in EC as compared to normal endometrium (NE) to find potential core lncRNAs for the diagnosis of EC. We found the potential core lncRNA by GO, KEGG, lncRNA and mRNA co-expression network. The potential functional lncRNAs were further detected by qPCR to validate the microarray results. A total of 172 lncRNAs and 188 mRNAs were found to be differentially expressed between type I EC and the NE samples (fold change >1.5). qPCR validation showed good consistency with the microarray data. GO, pathway analysis, the lncRNA and mRNA co-expression network as well as the TCGA data revealed that 6 lncRNAs (KIAA0087, RP11-501O2, FAM212B-AS1, LOC102723552, RP11-140I24 and RP11-600K151) may be the core regulators of endometrial carcinogenesis. The potential core lncRNAs revealed by the mRNA and lncRNA co-expression network might be helpful to explore potential early diagnostic and therapeutic targets for EC.

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Abbreviations: EC, endometrial carcinoma; EEC, endometrioid carcinoma; lncRNA, long non-coding RNA; mRNA, messenger RNA; GAPDH, glyceradehyde 3-phosphate dehydrogenase; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; UCSC, University of California Santa Cruz

Key words: endometrial carcinoma, microarray, lncRNA, GO, KEGG, co-expression network

Introduction

Endometrial carcinoma is the most frequent malignancy in female genital tract accounting for 1-2% mortality rate of all cancer in the western world (1). According to the statistic data collected from 2000 to 2011 in China, the morbidity has increased by 3.7% every year (2). Lack of an early diagnostic marker and therapeutic targets is the main cause of EC deaths.

In general, EC is mainly classified into two major types (type I and type II). Type I ECs comprise 80% of new cases (3). They are histologically well and moderately differentiated, estrogen-dependent and less prone to be aggressive. Type II ECs are mostly low differentiated, estrogen-independent and with a tendency to recur, even at early stage. The hormonal imbalance and the genetic alterations are proved to be the main cause of EC (4,5). Most of EC cases are diagnosed after menopause. The use of estrogen only replacement therapy and late menopause onset can increase the risk of EC. Genetic alteration, such as MLH1 (6), MSH2 (7), PMS2 (8), MSH6 (9), ARID1A (10), PTEN (11), KRAS2 (12), CTNNB1 (13), RB1 (11), TP53 (12), c-Kit (14) gene mutations or gene loss have been demonstrated to be associated with the increased risk of EC, ATR mutation in endometrioid carcinoma (EEC) is associated with aggressiveness of the EEC (15). Thus, we believe that new molecular-based subtype classification can help for the early diagnosis and treatment decision.

Recently, the non-coding RNAs, including miRNA, lncRNAs (longer than 200 bp, not encoding proteins, transcribed by RNA polymerase II) which account for approximately 4/5 of all human transcripts are proved to be critical regulators of cancers, including EC. They are involved in nearly all of the metastatic processes of cancer, such as proliferation, invasion and metastasis through regulating the protein coding gene expression transcriptionally, post-transcriptionally or by functioning as a scaffold of the lncRNA, miRNA and protein interaction (16). Besides, a variety of lncRNAs have been found to be the prognostic marker of diseases, such as breast (17), lung (18) and ovarian cancer (19).

Table I. Details of primer pairs used in analysis of lncRNA and mRNA expression by qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
KIAA0087	TCGGGGGACCGAGAAATACT	CCAGTCCAAGAGAAGCAGCA
RP11-508N22	TTAGCAGCACATGCTCACCA	GGTAAGTAGCTGGGCTGTGG
RP11-501O2	ACGATGAGACTTGGTGCTGAA	TGGTCCTGTTTCTCGCTGAC
FAM212B-AS1	CCAGAGAAGAAAGGCAGCGA	ACTCCTTCGCGTGTTCAGTT
LOC102723552	ACGAGATGCAGAAGATTACGC	TGAAATCTTACCTGACTGCAGATT
RP11-140I24	TCAGTCGTCTTCACGCTTCC	CAGATTAGCTGGCAGAGGGG
RP11-600K151	AGGGCTCAGTAGATTTGCCC	AGGCAGCATCTCACCTAACG
ALCAM	CTGGAGTACAAGACAACCAAGG	GTCACCTGCTCTGTAGGATAG
POLR3E	TTTCAGTACCCTGTGCGTCC	TGGGCTTGATCTTGGCTGAG
TDRD10	GGAGATCCTGTTGGAAAGCA	GCCAACATACACCTCTGTCTC

Thus, the exploration of lncRNA expression profile is essential for the discovery of early diagnostic markers and the potential therapeutic candidates. Notably, Zhai *et al* (20), reported the differentially lncRNA in randomly selected in EC samples and the adjacent non-tumor tissues. However, the mechanism of the type I EC and type II EC is quite different, the differential lncRNAs in type I EC and the normal endometrium (NE) from the uterine leiomyoma patients which will give us the important information of the potential early diagnostic marker is poorly understood. In the present study, we aimed to find the potential early diagnostic and therapeutic candidates of lncRNAs for EC. We first utilized the high throughput lncRNA microarray analysis to find the differentially expressed lncRNAs. Then, we further analyzed the lncRNA function by Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), mRNA-lncRNA co-expression network as well as the TCGA database to find the potential core lncRNAs that may be involved in EC. This would be helpful for exploring the mechanism of EC development as well as for prediction of clinical outcomes.

Materials and methods

Preparation of samples. Samples of human endometrial carcinoma (EC) and the NE (from the patients with hysterectomy) were collected immediately after surgery at the Obstetrics and Gynecology Hospital, affiliated to Nanjing Medical University. Tissue samples were collected with the consent of the patients and approved by the Ethics Committees in the hospital. In addition, all samples were pathologically confirmed. All samples were placed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -80˚C. RNA for RT-PCR was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA samples for microarray were extracted by Guangzhou RiboBio Co., Ltd., (Guangzhou, China). Total RNA (1 μg) was reversely transcribed using the Thermo Scientific ReverseAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Wilmington, DE, USA).

Microarray. RNA preparation and microarray hybridization were performed by Guangzhou RiboBio Co., Ltd., according to their manufacturer's standard protocols. Briefly, RNA was purified from total RNA after removal of rRNA. Approximately 17899 lncRNAs and 26363 mRNAs are detected using the lncRNA microarray.

Quantitative real-time (qRT) PCR. qRT-PCR was performed using SYBR-Green (Applied Biosystems, Shanghai, China) on an Applied Biosystems ViiA™ 7 DX machine (Life Technologies, Carlsbad, CA, USA). Assays were run in triplicate or quadruplicate and values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers for each gene and lncRNA were designed according to the NCBI online primer-blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and sequences are listed in Table I. The PCR conditions were 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 31 sec.

Statistical analysis. The data were analyzed using the Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). The values are presented as mean ± SEM. Student's t-test was used for the comparison of two independent groups ($P<0.05$, $P<0.01$ and $P<0.001$).

Results

Clinical characteristics of patient samples. EC samples were collected from 12 patients and the control endometrium were collected from at least 18 patients who underwent hysterectomy after having been diagnosed with uterine leiomyoma. According to the clinicopathological study, all the patients are type I EC at stage I/II. Pathology analysis showed that all EC patients are PR^+;ER^+;Ki-67^+ (30-80%) with middle/high differentiated tissue morphology.

lncRNA microarray profiling. To find the potential roles of lncRNA function in EC, we first examined the differentially expressed lncRNA by microarray profiling (Fig. 1). lncRNAs were collected from the authoritative database NCBI-GRCh38, ENA, VEGA, RNAcentral, HANAVA, ENSEMBL, RFAM (Fig. 1A). In the data, a total of 17899 lncRNA and 26363 mRNA were detected with 172 lncRNA and 188 mRNA differentially expressed between type I EC (stage I/II) and their relative control endometrium (fold >1.5 and $P<0.05$) as

Table II. Dysregulated lncRNAs in the EC compared with the NE filtered by a fold-change >5.0 and P<0.05

Transcript:seqname	Regulation	Ratio	Chromosome	Strand	Database
URS0000781FB6:URS0000781FB6	Downregulation	47.28	chr13	-	RNAcentral
ENST00000624452:RP1-41C23	Downregulation	61.15	chr17	-	HAVANA
URS00004EDFF6:AC013461	Downregulation	89.16	chr2	-	VEGA
XR_243449;XR_243450;XR_243448: RP11-7O141	Downregulation	33.50	chr16	+	NCBI_GRCh38
XR_430290:LOC102723552	Downregulation	11.26	chr20	-	NCBI_GRCh38
ENST00000624279:RP11-140I24	Downregulation	42.61	chr16	-	HAVANA
XR_427960:TRDN	Downregulation	19.50	chr6	-	NCBI_GRCh38
URS0000415D28:RP11-311B18	Downregulation	39.35	chr10	-	VEGA
URS0000506C5B:AC003090	Downregulation	54.03	chr7	-	VEGA
XR_426628:ANKRD13C	Downregulation	27.04	chr1	-	NCBI_GRCh38
URS000034FDBD:RP11-508N22	Downregulation	45.24	chr10	+	VEGA
XR_427604:LOC102724353	Downregulation	27.03	chr4	-	NCBI_GRCh38
NR_002319:PIPSL	Downregulation	20.52	chr10	-	NCBI_GRCh38
URS00004A7EA2:CTD-2410N18	Downregulation	31.52	chr5	+	VEGA
URS00001B388A:AC097517	Downregulation	15.44	chr2	-	VEGA
ENST00000620339:KLF3-AS1	Downregulation	35.30	chr4	-	HAVANA
URS00000000FD:AC113618	Downregulation	30.04	chr2	+	ENA
URS0000258264:RP11-501O2	Downregulation	18.88	chr3	+	ENA
NR_022006:KIAA0087	Downregulation	9.25	chr7	-	NCBI_GRCh38
NR_039986:RP11-600K151	Downregulation	18.06	chr8	-	NCBI_GRCh38
NR_038951;NR_038952:FAM212B-AS1	Downregulation	51.75	chr1	+	NCBI_GRCh38
URS000049614A:RP11-140I24	Downregulation	10.28	chr16	+	ENA
URS000058EAF9:RP11-680F8	Downregulation	8.16	chr15	+	ENA
URS000041DE60:RP1-35C21	Upregulation	12.36	chr1	+	ENA
URS000075B1F5:ROR1-AS1	Upregulation	10.60	chr1	-	RNAcentral
NR_029671:MIR125B1	Upregulation	21.10	chr11	-	NCBI_GRCh38
URS00004B2E02:RP11-573J24	Upregulation	18.64	chr8	+	ENA
URS00005369A5:RP11-474P2	Upregulation	32.39	chr12	-	VEGA
URS00001CE0A9:AC098828	Upregulation	15.07	chr2	-	VEGA
XR_243832:RP11-856M71	Upregulation	78.22	chr18	+	NCBI_GRCh38
ENST00000623186:RP11-449M6	Upregulation	19.09	chr8	+	HAVANA
URS0000610163:RP11-539G18	Upregulation	7.00	chr4	+	ENA
NR_027333:GPR158-AS1	Upregulation	7.01	chr10	-	NCBI_GRCh38
URS000032778C:RP11-1143G9	Upregulation	9.89	chr12	-	VEGA

shown in the scatter plot data (Fig. 1B and D) (the red dots indicate the differentially expressed lncRNA and mRNA with fold >1.5 and P<0.05) and clustering data (Fig. 1C and E; fold >1.5 and P<0.05). The detailed information of the differentially lncRNAs and mRNAs are presented in Tables II and III with the fold change >5 and P<0.05.

Expression signature of dysregulated lncRNAs in EC compared to NE. To investigate the signature of the dysregulated lncRNAs in EC, we studied the dysregulated lncRNA classification, lengths and chromosome distribution (Fig. 2). In the present study, the majority of the lncRNAs that were differentially expressed in EC were intergenic lncRNAs, antisense lncRNAs as well as the TEC (to be experimentally confirmed) lncRNAs (Fig. 2A). The lncRNA lengths are mainly between 400 and 64000 bp (Fig. 2B). The upregulated lncRNAs and the downregulated lncRNAs are distributed in different chromosomes (Fig. 2C and D).

GO and pathway analysis. To explore the potential function of the dysregulated lncRNAs in EC, we predicted the lncRNA target genes according to their chromosome location. In

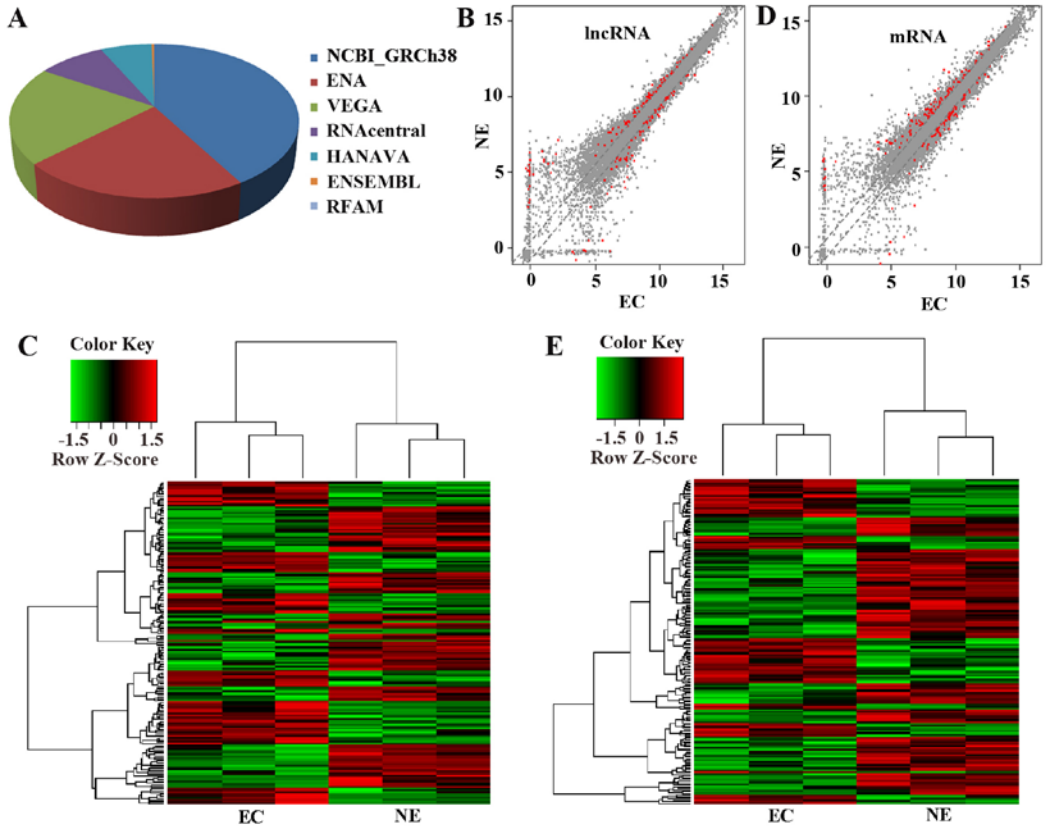

Figure 1. lncRNA and mRNA microarray profiling data of endometrial carcinoma (EC) and normal endometrium (NE). Using second generation microarray, 17899 lncRNAs were identified. The pie chart shows the relative numbers of lncRNAs from the authoritative databases (A). The red points above the top green line indicate >1.5-fold upregulated lncRNAs (B) and mRNAs (D) in EC and the red points below the top green line indicate >1.5 fold-downregulated lncRNAs (B) and mRNAs (D) in EC (P<0.05). Hierarchical clustering shows a remarkable lncRNA (C) and mRNA (E) expression profile between EC and NE (>1.5 fold and P<0.05).

Table III. Dysregulated mRNAs in the EC compared with the NE filtered by a fold-change >5.0 and P<0.05.

Name	Regulation	Ratio
CLDN23	Downregulation	64.81788
NEIL3	Downregulation	60.71055
ALCAM	Downregulation	27.00711
TMEM68	Downregulation	68.43251
POLR3E	Downregulation	18.53199
TDRD10	Downregulation	18.66308
SELP	Downregulation	40.2663
FGFR2	Downregulation	15.83808
DISP1	Downregulation	32.15958
TBC1D22A	Downregulation	27.66319
GALK2	Downregulation	19.62079
RTKN2	Upregulation	39.20362
CADPS	Upregulation	40.45117
NLRP10	Upregulation	16.82022
UPP1	Upregulation	5.53434
EPC2	Upregulation	23.74738
NXNL2	Upregulation	37.67642
TXNL4B	Downregulation	5.196587
PCDH17	Downregulation	8.172227
HSD17B2	Downregulation	7.949501
LRRC1	Downregulation	5.796955
STK25	Downregulation	5.488636

addition, we carried out GO and pathway analysis of these lncRNAs. The Gene Ontology consortium which incorporate many databases and developed three ontologies (biological processes, cellular components and molecular functions) (http://www.geneontology.org) revealed that numerous biological processes, cellular components and molecular function are altered in EC compared to control (Fig. 3A-C). To find which pathways are altered, we also mapped the genes to KEGG pathways. It is noteworthy that the plasma components which are the main components during cell response to the intracellular or extracellular signals, such as the cell adhesion molecules are the most dysregulated molecules in EC as shown in the result of biological pathway analysis, the cellular component and KEGG pathway analyses (Fig. 3A, C and D). Besides, the structure specific DNA binding components which may function during transcription and the oxidoreductase component which may be involved in cell metabolism and other processes are also dysregulated in EC (Fig. 3B).

Cell adhesion molecules are important for tissue integrity and also cell migration in tumor cells or other developmental cells (16,21). E-cadherin, N-cadherin and integrins which are the most famous adhesion molecules have been demonstrated to be associated with cell invasion of various cancer types (22-24), the transmembrane glycoprotein ALCAM (also known as CD166) which belongs to the immunoglobulin superfamily has been considered as the marker of breast cancer (25). In the

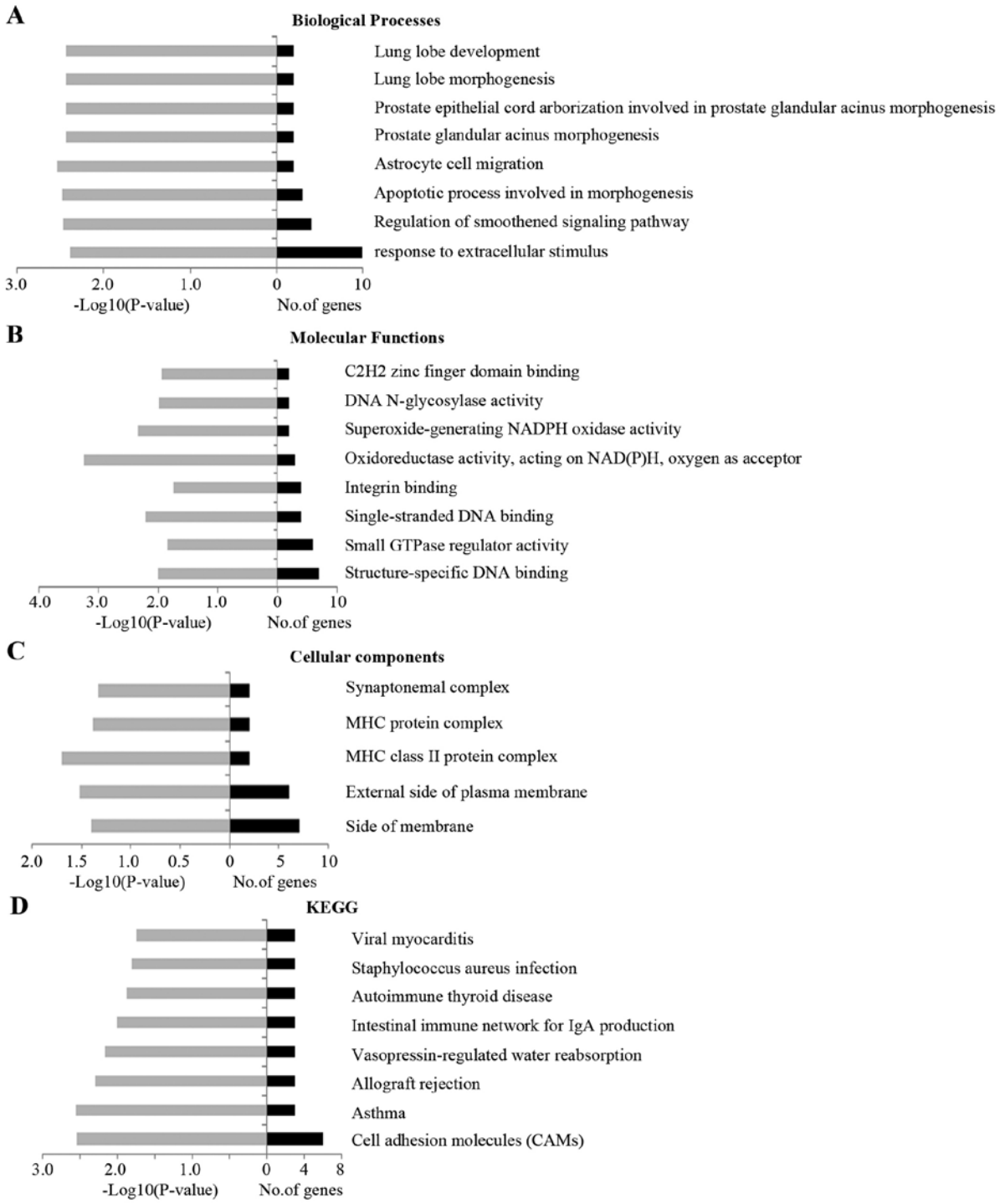

Figure 2. Distribution of differentially expressed lncRNAs. According to the NCBI database, the differentially expressed lncRNA (fold >1.5, P<0.05) include 62 intergenic (35 upregulated and 27 downregulated), 48 antisense (18 upregulated and 30 downregulated), 8 TEC (4 upregulated and 4 downregulated) and 54 other classes (such as processed transcript, sense overlapping or the lncRNAs whose classification are not very clear, 24 upregulated and 30 downregulated) (A). Most of the differentially expressed lncRNAs are 401-64000 bp in length (B). Chromosome distribution of the upregulated (C) and downregulated (D) lncRNAs show that the upregulated lncRNAs and the downregulated lncRNAs are distributed in different chromosomes.

present study, the cell adhesion molecules, such as ALCAM are also the most dysregulated mRNA.

mRNA-lncRNA co-expression network. lncRNA function has been implicated in several steps of carcinogenesis by acting as structural, catalytic or regulatory RNA or interacting with DNA, RNA and protein (16). However, only a few lncRNA functions have been elucidated. mRNA and lncRNA co-expression network, which can provide evidence for the involvement of new genes or lncRNAs in core biological functions, is very important for the prediction of the lncRNA function preliminarily (19,26). Thus, we carried out the lncRNA and mRNA co-expression network in Guangzhou RiboBio Co. The co-expression network in Fig. 4A shows that ALCAM is co-expressed with 5 lncRNAs (ENST00000624279:RP11-140I24, LOC102723552, KIAA0087, RP11-600K151 and

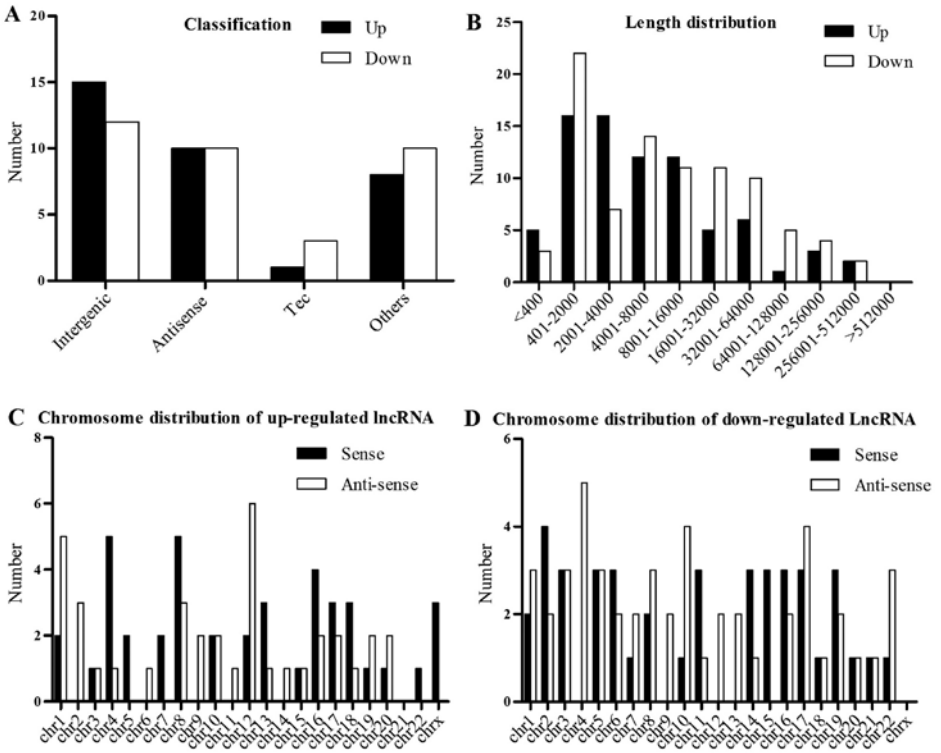

Figure 3. GO and KEGG analysis of differentially expressed lncRNAs. GO (A-C) and the KEGG pathway analysis (D) of the differentially expressed lncRNAs indicated that the plasma components such as the cell adhesion molecules which response to the intracellular or extracellular signals, the structure-specific DNA binding components as well as the oxidoreductases involved in metabolism are the most dysregulated molecules in EC compared to NE.

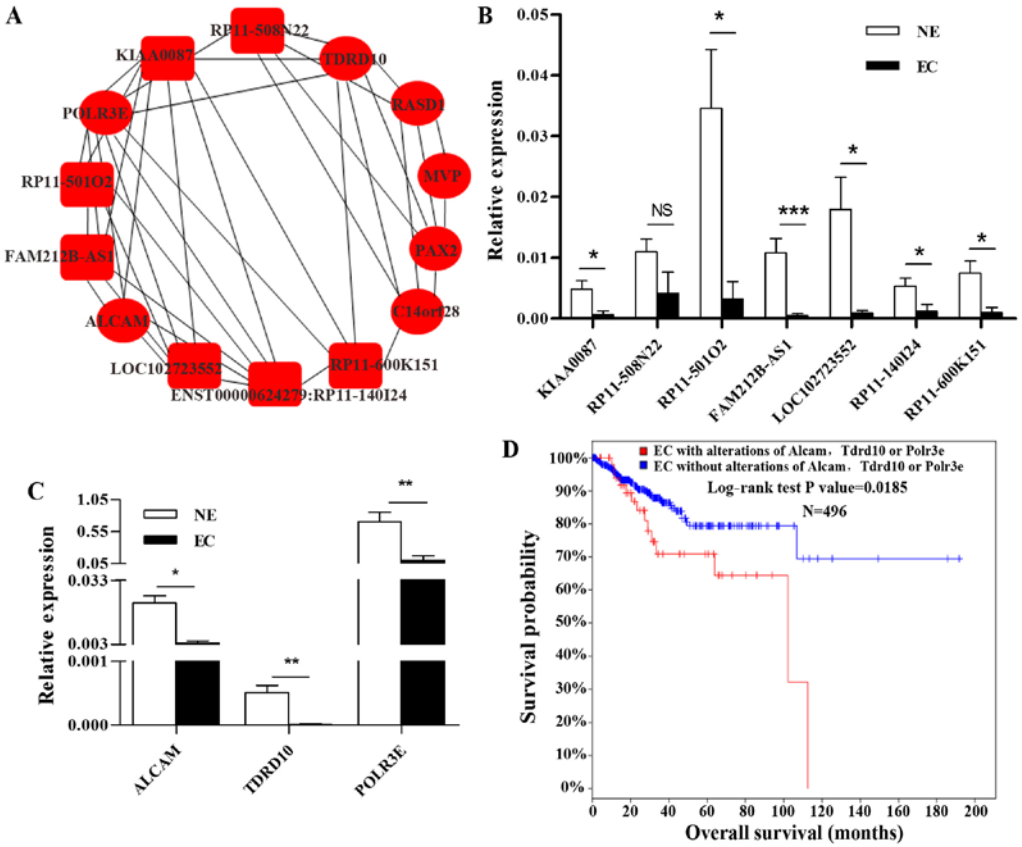

Figure 4. lncRNA and mRNA co-expression network showed a variety of co-expressed lncRNAs and mRNAs that are downregulated in EC. (A) A diagram of the differentially expressed lncRNA and mRNA co-expression network (red indicated the downregulated lncRNAs and mRNAs in EC). (B and C) Real-time PCR verification of those dysregulated lncRNAs (B) and mRNAs (C) that were co-expressed (fold >5, P<0.05). (D) Survival analysis of ALCAM, TDRD10 and POLR3E status shows divergent outcome of the gene altered cases (with lower overall survival rate) than the unaltered group according to the TCGA data presented in cBioPortal.

FAM212B-AS1) and 1 mRNA (POLR3E), POLR3E is co-expressed with 6 lncRNAs (FAM212B-AS1, RP11-140I24, LOC102723552, RP11-508N22, RP11-501O2 and KIAA0087) and 2 mRNAs (ALCAM and TDRD10), TDRD10 is co-expressed with 3 lncRNAs (RP11-600K151, RP11-508N22 and KIAA0087) and 1 mRNA (POLR3E) which are differentially expressed in EC (>5-fold, P<0.05). Real-time PCR verified that 6 of the 7 lncRNAs and all of the 3 mRNAs are dramatically downregulated in EC, which is consistent with the microarray data (Fig. 4B and C). Interestingly, most of these lncRNAs are relatively conserved between human, rhesus, rat and mouse as analyzed using UCSC website (http://genome.ucsc.edu/) (data not shown).

The Cancer Genome Atlas (TCGA) database includes most of the tumor types and information on each case for community research use. cBioPortal for Cancer Genomics provides visualization and analysis of the TCGA datasets. Notably, the results presented in the cBioPortal of cancer genomics (http://www.cbioportal.org/) showed that EC cases with ALCAM, TDRD10 and/or POLR3E alterations showed poorer overall survival rate as compared with cases without alterations of these genes (Fig. 4D).

Discussion

According to the ENCODE Project Consortium, the protein coding genes only account for 1% of the human genome (27). Most of the regions are not transcribed, such as a large class of the non-coding RNA. lncRNA functions as structural, catalytic, regulatory RNAs have been found to be involved in a variety of processes, such as development (18), tumorigenesis (16), cardiovascular disease (28) and neurodegenerative diseases (29). Similarly to the protein coding genes, lncRNAs which are distributed in the cytoplasm, nucleus and also transported to the extracellular matrix can be identified as the biomarkers of various diseases. For example, studies found that lncRNA-DANCR can be used as a prognostic biomarker of hepatocellular carcinoma (30). The circulating lncRNA-LIPCAR can be used to predict survival of patients with heart failure (31). lncRNAs are also known as biomarkers or therapeutic target of cancers, such as breast (32), lung (33) and ovarian cancer (19). Since most of the lncRNAs function through the regulation of the mRNAs the lncRNA-mRNA co-expression network provides us with a powerful platform for the prediction of the lncRNA function (19). For example, RP11-284N8.3.1 and AC104699.1.1 which were identified using the lncRNA-mRNA co-expression network are found to be the independent predictive biomarkers of ovarian cancer (19). In this study, we first used microarray analysis to find the differentially expressed lncRNAs in EC, and then by using GO and KEGG as well as the lncRNA-mRNA co-expression network, we predicted that the 6 lncRNAs and 3 mRNAs are probably the early biomarkers of EC.

The glycolysis which produces ATP more quickly is strongly enhanced in tumor cells (34,35). Here, we used the GO analysis to prove that the main molecular function involved is the oxidoreductase activity, emphasizing the difference between the metabolism between the tumor and normal tissue (Fig. 3B).

The adhesion molecules have been demonstrated to be the important regulators of endometrial epithelium integrity, its decreased expression will disrupt the integrity of the NE and may be involved in the pathology of the endometrium (22). Asthma which affects 5-10% of the population may increase the patients' risk of cancer is also altered in EC as compared with control (36). The chronic inflammatory disease associated gene has been found to help cancers metastasize and may be the predictor of brain cancer in children (37,38). Notably, the KEGG analysis showed that the adhesion molecules and asthma associated genes are the top two differentially altered pathways in EC.

Endometrial carcinoma which is a common malignancy of female reproductive tract accounts for ~1-2% of the tumor deaths in the western world (1). It is also becoming more prevalent in China each year. Thus, it is very urgent for us to find the predictive biomarkers of EC. Here, in order to find whether lncRNAs can be used as EC biomarkers, we performed the lncRNA microarray. By using the microarray data and the lncRNA-mRNA co-expression network, we found 6 lncRNAs (KIAA0087, RP11-501O2, FAM212B-AS1, LOC102723552, RP11-140I24 and RP11-600K151) and 3 mRNAs (ALCAM, TDRD10 and POLR3E) that are functionally related in EC. Interestingly, ALCAM is highly expressed in the endometrium glandular cells and its expression was decreased in ~50% of the endometrial cancer tissues while POLR3E is moderately expressed in the endometrium glandular cells and its expression was decreased in ~50% of EC according to the human protein atlas website which is consistent with our data (http://www.proteinatlas.org/). The TCGA data also confirmed that these three gene alterations indicated poorer overall survival as compared with the cases without these gene alterations. We also validated that the lncRNAs which are co-expressed with these three mRNAs are downregulated in EC.

Since the mechanism of the type I EC and type II EC is quite different, distinguish the different type of EC is quite important. Different from the differentially expressed lncRNAs between the randomly selected EC samples and the adjacent non-tumor tissue reported before (20), we reported the differentially expressed lncRNAs in type I EC as compared with NE using the microarray and the lncRNAs and mRNA co-expression network for the first time. The differentially expressed lncRNAs may be indicators of the type I EC and may provide the potential information for the evaluation of EC.

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