

# Microarray expression profile of lncRNAs and the upregulated ASLNC04080 lncRNA in human endometrial carcinoma

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**Abstract.** Long non-coding RNAs (lncRNAs) have been recognized as a regulator of gene expression, and the deregulation of lncRNAs have been reported to be correlated with carcinogenesis and cancer progression. To explore the function of lncRNA in endometrial carcinoma, we analyzed the expression profiles of lncRNAs and coding genes in 3 paired endometrial carcinoma and adjacent non-tumor tissues, using a microarray. The results of microarray analysis indicated a significant difference in lncRNA and coding gene expression between endometrial carcinoma and their paired adjacent non-tumor tissues. A total of 53 lncRNAs (fold change >2.0, p-value <0.05) were found to be differently expressed in endometrial carcinoma compared to the normal controls. Among these ASLNC04080 was the most significantly upregulated lncRNA in microarray data, highly expressed in 22 out of 24 endometrial carcinoma tissues and HEC-1-B cell line. ASLNC04080 is 1867nt in length, consist of 6 exons, and locates at 1 p35.3(chr1:-28905061 - -28909492). In addition, 46 coding gene transcripts were differentially expressed (fold change >2.0, p-value <0.05) between endometrial carcinoma and adjacent non-tumor tissues. Pathway and gene ontology analysis demonstrated that these deregulated transcripts were involved in multiple signal pathways, biological processes, cellular components and molecular functions. Moreover, the ASLNC04080 lncRNA expression was correlated with 19 coding genes, and may contribute to endometrial carcinoma genesis and progression by co-regulating with coding gene. Expression inhibition of lncRNA ASLNC04080 in HEC-1-B cells caused repression of cell proliferation, increased cell apoptosis, and G1 phase arrest. These results suggested a potential function of ASLNC04080 in endometrial carcinoma genesis and progression.

## Introduction

Endometrial carcinoma, comprising of several types of malignancies arise from the endometrium or lining of the uterus, is the most common gynecologic malignancy among women in the United States, with an estimated 52,630 new case and 8,590 deaths in 2014 (1). Endometrial carcinoma cases in Chinese women increased in the last decade. Based on clinical features and pathogenesis endometrial carcinomas have been classified into two types (2). Type I endometrial carcinomas occur commonly in perimenopausal women, with low grade, related to obesity and estrogen exposure. Type II endometrial carcinomas are more common in older women, unrelated to hormone excess, with a worse outcome than Type I. Previous studies have reported that various gene mutations, and expression deregulation are related to endometrial carcinoma genesis. PTEN and K-ras mutations occur in Type I endometrial carcinomas, often with Wnt, AKT and PI3KCA deregulation. TP53 and PPP2R1A mutations are frequent in Type II endometrial carcinomas, with HER2/neu overexpression and p16 inactivation (3,4).

lncRNAs are a spectrum of RNAs transcribed by RNA polymerase II, but not translated into proteins, with more than 200 nucleotides in length. lncRNAs which have previously been identified as transcription noise are proved to be involved in the process of multiple gene expression regulation (5,6). Increasing evidence suggests that the deregulation of lncRNAs is linked to disease, especially carcinoma genesis. UCA1 (7-10) upregulated in bladder carcinoma, is a functional lncRNA molecule involved in cell growth, cell cycle, cell invasion and tumorigenesis. lncRNA AFAP1-AS1 (11) is hypomethylated and upregulated in Barrett's esophagus (BE), esophageal adenocarcinoma (EAC) tissues and cell lines. Inhibition of its expression in EAC cells was able to diminish cell growth, migration, and invasion, as well as increase apoptosis. MALAT1 (12) is first discovered in non-small cell lung cancer and its over-expression associates with high metastatic potential and poor patient prognosis in variety of cancer. A recent report shows that the promoter hypermethylation silencing of tumor suppressor PCDH10 contributed to MALAT1 upregulation in endometrioid endometrial cancer (EEC) (13).

The mechanism of endometrial carcinoma genesis is complex and related with multiple gene mutation and deregulation. The exploration of gene expression profile, especially the

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potential functional lncRNA will help to gain better knowledge and to discover new therapeutic candidates for endometrial carcinoma. We performed a microarray analysis to study the expression of lncRNAs and coding genes in 3 endometrial carcinomas and their paired adjacent non-tumor tissues. Our result demonstrated that the expression profile of lncRNAs is significantly different between endometrial carcinomas and non-tumor endometrium. The novel ASLNC04080 lncRNA is upregulated in endometrial carcinomas and HEC-1-B cell line. Additionally the deregulation of coding genes was also detected in endometrial carcinomas, and these deregulated coding transcripts were involved in multiple biological processes, cellular components, molecular functions and pathways which were related to carcinogenesis and cancer progression. We constructed a co-expression network of lncRNAs and coding gene transcripts based on the expression level relation between lncRNAs and coding gene transcripts. According to the co-expression network ASLNC04080's expression was correlated to 19 coding transcripts, showing a potential co-regulation function of lncRNA ASLNC04080. Taken together, these results suggest the altered expression levels of lncRNAs may contribute to endometrial carcinomas genesis and multiple molecular processes. In addition, ASLNC04080 could be a functional lncRNA molecule with potential use as biomarker or therapeutic target.

## Materials and methods

**Patient samples.** All human endometrial carcinomas and their paired adjacent nontumor tissues were obtained from patients of the First Affiliated Hospital, School of Medicine of Xi'an Jiaotong University. Tissue samples were collected with informed consent from patients, as approved by the Hospital Ethics Committees. All tissue samples were pathologically confirmed. Three pairs of these patient samples were randomly selected for human lncRNA microarray analysis.

**DNA microarray.** RNA quality was assessed by Nanodrop ND-1000 and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. The RNA extraction was performed by KangChen Bio-tech, Shanghai, China.

The Human 12x135 k Long Non-coding RNA Array was manufactured by Roche NimbleGen. Each array represents all long transcripts, both protein coding mRNAs and lncRNAs (long non-coding RNAs) in the human genome. More than 23000 lncRNAs are collected from the authoritative data sources including NCBI RefSeq, UCSC, RNAdb, lncRNAs from literature and UCRs. The microarray analysis was performed by KangChen Bio-tech.

**RNA labeling and array hybridization.** Double-strand cDNA (ds-cDNA) was synthesized from 5  $\mu$ g of total RNA using an Invitrogen SuperScript ds-cDNA synthesis kit in the presence of 100 pmol oligo dT primers. ds-cDNA was cleaned and labeled in accordance with the Nimblegen Gene Expression Analysis protocol (Nimblegen Systems, Inc., Madison, WI, USA). Briefly, ds-cDNA was incubated with 4  $\mu$ g RNase A at 37°C for 10 min and cleaned using phenol:chloroform:isoamyl alcohol, followed by ice-cold absolute ethanol precipitation.

The purified cDNA was quantified using a nanodrop ND-1000. For Cy3 labeling of cDNA, the Nimblegen One-Color DNA labeling kit was used according to the manufacturer's guideline detailed in the Gene Expression Analysis protocol (Nimblegen Systems, Inc.). ds-cDNA (1  $\mu$ g) was incubated for 10 min at 98°C with 1 OD of Cy3-9mer primer. Then, 100 pmol of deoxynucleoside triphosphates and 100 units of the Klenow fragment (New England Biolabs, Ipswich, MA, USA) were added and the mix incubated at 37°C for 2 h. The reaction was stopped by adding 0.1 volume of 0.5 M EDTA, and the labeled ds-cDNA was purified by isopropanol/ethanol precipitation. Microarrays were hybridized at 42°C for 16–20 h with 4  $\mu$ g of Cy3 labelled ds-cDNA in Nimblegen hybridization buffer/hybridization component A in a hybridization chamber (Hybridization System - Nimblegen Systems, Inc.). Following hybridization, washing was performed using the Nimblegen Wash Buffer kit (Nimblegen Systems, Inc.). After being washed in an ozone-free environment, the slides were scanned using the Axon GenePix 4000B microarray scanner. The microarray analysis was performed by KangChen Bio-tech.

**Data analysis.** Slides were scanned at 5  $\mu$ m/pixel resolution using an Axon GenePix 4000 B scanner (Molecular Devices Corp.) piloted by GenePix Pro 6.0 software (Axon). Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level (\*\_norm\_RMA.pair) files and mRNA level (\*\_RMA.calls) files were generated after normalization. All mRNA level files were imported into Agilent GeneSpring Software (version 11.0) for further analysis. Differentially expressed lncRNAs and mRNAs were identified through Fold Change filtering. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.0). GO analysis and Pathway analysis was performed using the standard enrichment computation method. The analysis was performed by KangChen Bio-tech.

**GO analysis.** The Gene Ontology project provides a controlled vocabulary to describe gene and gene product attributes in any organism (<http://www.geneontology.org>). The ontology covers three domains: Biological Process, Cellular Component and Molecular Function. Fisher's exact test is used to find if there is more overlap between the DE list and the GO annotation list than would be expected by chance. The p-value denotes the significance of GO terms enrichment in the DE genes. The lower the p-value, the more significant the GO term ( $p \geq 0.05$  is recommended).

**Pathway analysis.** Pathway analysis is a functional analysis mapping genes to KEGG pathways. The p-value (EASE-score, Fisher p-value or Hypergeometric-p-value) denotes the significance of the Pathway correlated to the conditions. The lower the p-value, the more significant the pathway is (the recommend p-value cut-off is 0.05).

**Co-expression network.** We constructed a coding-noncoding gene co-expression network to investigate the relation between

lncRNAs and their coding genes, 6 lncRNAs up- or down-regulated in endometrial carcinoma tissues were selected to draw the network. i) The data were preprocessed by using the median gene expression value of all transcripts expressed from the same coding gene, without special treatment of the lncRNA expression value. ii) Then the data were screened for differentially expressed lncRNAs and mRNAs and removed from the dataset. iii) The R-value was used to calculate the correlation coefficient of the PCC between lncRNA and coding genes (only lncRNA-coding PCC, not including lncRNA-lncRNA or coding-coding PCC). iv) Based on Pearson's correlation coefficient selecting PCC  $\geq 0.95$  as meaningful related pair v) The co-expression network was drawn using Cytoscape. In the network, a round node represents the coding gene, a box node represents the lncRNA, a red node represents an upregulated lncRNA/mRNA and a green node represents an under-regulated lncRNA/mRNA. A red solid line indicates a positive correlation, and a blue dashed line indicates a negative correlation.

**RNA extraction and cell culture.** Total RNA from tissue samples, whole blood and cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and integrity were determined by spectrophotometry and standard RNA gel electrophoresis.

The human endometrial carcinoma (HEC-1-B); cervical carcinoma (Siha HeLa) and ovarian cancer (3AO SKOV3) cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% bovine calf serum. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Expression analysis of ASLNC04080 by RT-PCR and qRT-PCR.** Total RNA isolated from tissue samples, whole blood and cells was reverse transcribed by using PrimeScript™ RT Reagent kit with gDNA Eraser (Takara, Dalian, China). Takara Taq™ (Takara) was used for 32 PCR cycles, annealing temperature was 65°C. SYBR® Premix Ex Taq™ was used for real-time PCR, annealing temperature was 61°C. Primer sequences were as follows: ASLNC04080: forward primer, 5'-CGCTATGTGTGGTGCCTGGGGTG-3' and reverse primer, 5'-CAGCGCCTGAGTGGGTTTCGG-3'; 18S: forward primer, 5'-GCTCAGCGTGTGCCTACCCTAC-3' and reverse primer, 5'-GTAGTAGCGACGGGCGGTGTGTA-3'.

**Rapid amplification of cDNA ends (5'- and 3'-RACE).** One microgram of total RNA of whole blood was purified further by treating with RNase-Free DNaseI (Takara), then reverse transcribed with the SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to manufacturer's instructions. Specific 5'- and 3'-RACE cDNA ends were amplified with the universal primer mix provided in the kit and gene specific primers (GSPs) with the advantage 2 PCR polymerase mix (Clontech). The PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and several recombinant clones were isolated for sequencing. The GSP sequences: 5'RACE Gene-Specific Primers (Out Primer 5'-AGCGCCTGAGTGGGTTTCGG-3'; Inner Primer 5'-GGG GGTCTACTCCCCGAAAGG-3'). 3'RACE Gene-Specific Primers (Out Primer 5'-TTAATAGATTAGGCACAGGA

TGGGT-3'; Inner Primer 5'-CAGGATGGGTGTTTAATTC TCGGCA-3').

**Amplification of truncated full length cDNA sequence.** Total RNA isolated from whole blood and cells was reverse transcribed by using PrimeScript II 1st Strand cDNA Synthesis kit (Takara). The Takara LA Taq® (Takara) was used for 30 cycles PCR amplification, annealing temperature was 62°C. The truncated full length cDNA sequence primers: forward primer 5'-ACCGCACCCGGCAGTAGTAC-3', reverse primer 5'-ATT GATCACCTCTGAAGTTCAGTAGCA-3'. The PCR products were subcloned into pGEM-T Easy vector (Promega) and several recombinant clones were isolated for sequencing.

**Small interfering RNA transfection.** Three different small interfering RNAs (siRNA) targeted ASLNC04080 (si-RNA616, si-RNA1315, si-RNA1535; (Gene Pharma, Shanghai, China) and nonsense siRNA control were transected to HEC-1-B cells by X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany). The sequences of the 3 siRNAs were as follows: si-RAN616: 5'-CAGGGUUCAUUCCAGACU-3'; si-RNA1315: 5'-GGCGUACUUAAGCAGAUGA-3'; si-RNA1535: 5'-GGAGUUGGGACAAUCUCUA-3'. The knockdown effect of siRNA was detected by qRT-PCR.

**Cell proliferation assays.** HEC-1-B cells were plated 3000 cells per well onto 96-well plates, after 24 h siRNA1535 and nonsense siRNA control were transfected. Every 24 h cell proliferation reagent CCK8 (Dojindo, Kumamoto, Japan) was added 10 µl per well and then incubated at 37°C for 2 h. Optical density was measured at 450 nm using a microplate reader (EnSpire, USA), and the proliferation activity curve was drawn.

**Cell apoptosis assays.** HEC-1-B cells were plated at 15x10<sup>4</sup> cells per well into 6-well plates. After 48 h of treatment with siRNA-1535 and nonsense siRNA control, HEC-1-B cells were harvested and stained with Annexin V and PI using Annexin V-FITC/PI apoptosis detection kits (Beyotime, Haimen, China) and then examined by flow cytometry (BD FACSCantoII, BD Biosciences, San Jose, CA, USA). Cellular proteins were extracted 48 h after siRNA transfection. Caspase-3 (Cell Signaling Technology, Danvers, MA) expression was detected by western blotting.

**Cell cycle analysis.** HEC-1-B cells were plated at 15x10<sup>4</sup> cells per well into 6-well plates. After 48 h of treatment with siRNA1535 and nonsense siRNA control, HEC-1-B cells were harvested, washed with ice-cold phosphate-buffered saline, fixed with 70% ethanol overnight, and pretreated with 5 mg/ml ribonuclease for 30 min at 37°C and then stained with PI (100 µg/ml). Cell cycle profile was determined by flow cytometry (BD FACSCanto II, BD Biosciences) analysis of DNA content of cell nuclei.

## Results

**ASLNC04080 is upregulated in endometrial carcinoma and cell lines.** We performed a microarray analysis of lncRNA in 3 paired endometrial carcinoma and adjacent non-tumor

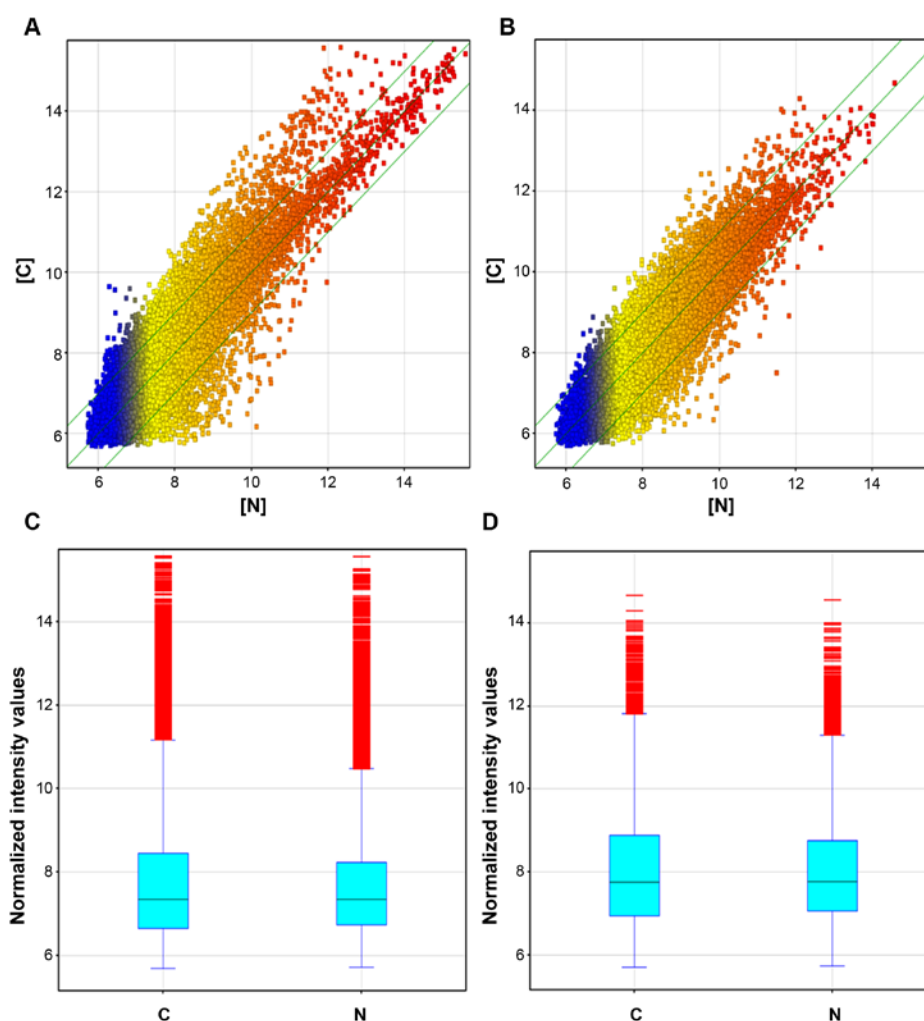


Figure 1. The gene expression profile difference in 3 paired endometrial carcinoma [C] and adjacent nontumor tissues [N]. The scatter plot is a visualization method used for assessing the lncRNA (A) and mRNA (B) expression variations between endometrial carcinoma [C] and paired adjacent nontumor tissues [N]. The values of the X and Y axes in the scatter plot are the averaged normalized signal values of the group (log2 scaled). The green lines represent fold-change (the default fold change given is 2.0). The box plot is a convenient way to quickly visualize the distribution of a dataset for the lncRNAs (C) and the mRNAs (D) profiles. After normalization, the distributions of log2 ratios among the groups are nearly the same.

tissues. In the lncRNA expression profiling data, a total of 23,837 lncRNAs expressed in endometrial carcinoma were detected. A comparison of lncRNA expression level between endometrial carcinoma and adjacent non-tumor tissues identified 53 lncRNAs which were significantly differentially expressed (fold change  $\geq 2.0$ ,  $p \leq 0.05$ ) (Fig. 1A and C and Table I). ASLNC04080 was the most upregulated lncRNA (fold change = 3.38,  $p = 0.038$ ), and CD109474 was the most downregulated lncRNA (fold change = 4.72,  $p = 0.034$ ) in the endometrial carcinoma group.

The RT-PCR result showed ASLNC04080 expression difference in 3 paired endometrial carcinoma and adjacent non-tumor tissues which we used for microarray analysis (Fig. 2B). We evaluated the expression level of lncRNA ASLNC04080 in 24 paired endometrial carcinoma and adjacent non-tumor tissues as well as gynecological cancer cell lines. In our result ASLNC04080 expression level in endometrial carcinoma tissues was upregulated in 22 out of 24 paired samples (Fig. 2A). The ASLNC04080 transcripts were also detected in endometrial carcinoma (HEC-1-B), cervical

carcinoma (Siha HeLa) and ovarian cancer (3AO SKOV3) cell lines (Fig. 2C). Additionally, according to UCSC information, ASLNC04080 was expressed in a series of lymphocytes (Fig. 2D). We detected the expression of ASLNC04080 in whole blood from both endometrial carcinoma patients as well as healthy people (Fig. 2E).

**Sequence structure of ASLNC04080.** The 5'RACE and 3'RACE were performed to acquire 5'-end and 3'-end cDNA sequence of ASLNC04080 (Fig. 3A). According to the overlapping region, we spliced 5'-end (744 bp) and 3'-end (1488 bp) sequence forming the full length cDNA sequence (1867 bp) (Fig. 3B). The sequence message was submitted to NCBI, GeneBank, Accession no. KJ782215. The ASLNC04080 cDNA sequence identity is 99.5% of the part human chromosome 1 cosmid (chr1: -28905061 - -28909492) sequence, by mapping with Blat search program from UCSC. The full length ASLNC04080 cDNA contained 6 exons mapped with the 1 p35.3 (Fig. 3C), and the identity is 99% (Query cover 39%) of the SNHG12.

Table I. Differentially expressed lncRNAs with &gt;2-fold change in 3 paired endometrial carcinoma tissues (C) vs. adjacent non-tumor tissues (N).

SEQ_ID	Log2 fold change C/N	Regulation
ASLNC00294	2.5359635	Up
ASLNC02239	2.0272071	Up
ASLNC04080	3.3796694	Up
ASLNC06245	2.5081081	Up
ASLNC06537	2.5098338	Up
ASLNC08884	2.236056	Up
ASLNC09130	2.2931793	Up
ASLNC09507	2.4517279	Up
ASLNC10247	2.355146	Up
ASLNC12496	2.2451406	Up
ASLNC14462	2.097873	Up
ASLNC14832	2.020886	Up
ASLNC15527	2.5785253	Up
ASLNC17047	2.3866124	Up
ASLNC17464	2.1540515	Up
ASLNC23065	2.1494098	Up
ASLNC23333	2.0054722	Up
ASLNC23414	2.4249542	Up
ASLNC23671	2.4036572	Up
AV732045	2.1128297	Up
BC146594	2.3366451	Up
BF448178	2.5298557	Up
BF958740	2.079252	Up
BI046482	2.8131595	Up
BI520265	2.009207	Up
BX642924	3.1237326	Up
BX648912	2.0242465	Up
DA195606	2.1546383	Up
DB269443	2.336296	Up
DB335254	2.1803222	Up
DB341724	2.82081	Up
DB349701	2.6501746	Up
H44233	2.1850893	Up
exon2439	2.012377	Up
exon2817	2.0926008	Up
exon3497	2.1918285	Up
exon418	2.4487195	Up
exon4652	2.4781675	Up
exon844	2.45636	Up
ASLNC00038	2.559887	Down
ASLNC00087	2.1803436	Down
ASLNC04117	2.09159	Down
ASLNC05193	2.5719202	Down
ASLNC05485	2.8765132	Down
ASLNC09591	2.8102934	Down
ASLNC10223	2.6993935	Down
ASLNC10915	2.2386236	Down
ASLNC14186	2.162685	Down
ASLNC18394	2.2218604	Down
ASLNC21513	2.0064554	Down
BF507708	2.3711028	Down
CD109474	4.7178984	Down
HMLincRNA1454	2.032924	Down

P-values &lt;0.05 were considered significant.

Table II. Differentially expressed mRNAs with &gt;2-fold change in 3 paired endometrial carcinoma tissues (C) vs. adjacent non-tumor tissues (N).

Gene symbol	Log2 fold change C/N	Regulation
COL1A2	2.1063402	Up
PLA2G1B	2.4415922	Up
OR1S1	2.391917	Up
GEMIN7	2.4388766	Up
C10orf114	2.1364949	Up
FIGNL2	3.4194834	Up
IL31	2.0702891	Up
FAM23A	2.0272226	Up
ACOX3	2.8613539	Up
NKPD1	2.0944095	Up
FAM148C	2.1054876	Up
AKR1C1	2.4084988	Up
TLE2	2.0259166	Up
CDH7	2.0604987	Up
HOXC6	2.1476102	Up
C9orf61	2.0307186	Up
KIR2DL1	2.1328676	Up
TINAG	2.2404068	Up
MGRN1	2.1519182	Up
CCR10	3.6094117	Up
EMID2	2.6075573	Up
BAX	2.5370033	Up
DAND5	2.2088263	Up
HOXC6	2.1748328	Up
ZBTB12	2.3850377	Up
SCN1B	2.1365902	Up
GCH1	2.2663758	Down
H2BFWT	2.1060047	Down
DDAH1	2.0587702	Down
LOC120376	2.1094232	Down
CRYM	3.8512769	Down
RDH5	2.0616512	Down
SLC4A4	2.5983744	Down
EGR3	2.0082827	Down
TSPAN8	2.9661324	Down
APBA3	2.10935	Down
XBP1	2.010461	Down
SERPINB3	2.4469104	Down
KRT23	2.4278355	Down
SERTAD4	2.100912	Down
KIAA1324	3.5217378	Down
SLC38A5	2.3809822	Down
PARP15	2.4587605	Down
CACNB2	3.3419204	Down
ZNF320	2.033858	Down
PRSS12	2.3177733	Down

P-values &lt;0.05 were considered significant.

The truncated full length cDNA sequence (184-1838, 1655 bp) was acquired from human whole blood and endometrial carcinoma cell line HEC-1-B (Fig. 3D), and identity

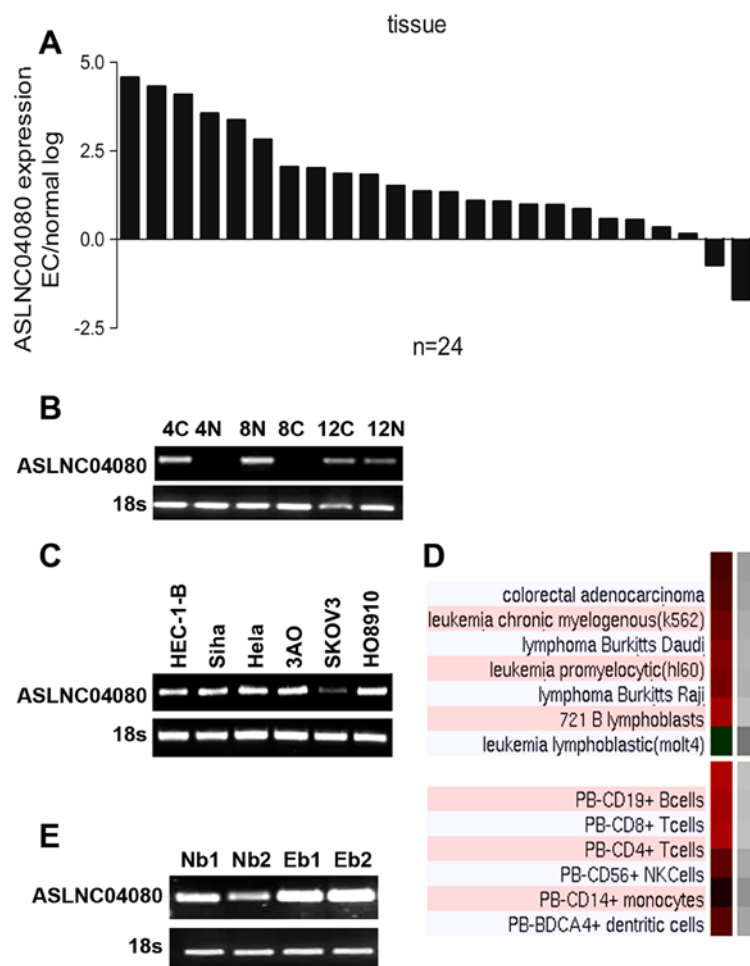


Figure 2. ASLNC04080 was upregulated in endometrial carcinoma tissues. (A) ASLNC04080 expression was increased in 92% endometrial carcinoma tissues (EC) (22/24) vs. adjacent nontumor tissues (normal), qRT-PCR. (B) ASLNC04080 expression RT-PCR result conformed to the microarray data. (C) ASLNC04080 was expressed in endometrial carcinoma cell line (HEC-1-B), cervical carcinoma cell lines (Siha, HeLa) and ovarian cancer cell lines (3AO, SKOV3, HO8910), RT-PCR. (D) ASLNC04080 expression profile information from UCSC microarray expression data. (E) ASLNC04080 expression was detected in whole blood of endometrial carcinoma patients (Eb) and healthy people (Nb), RT-PCR.

of 99% (Query cover 88%) of the full length ASLNC04080 cDNA (KJ782215).

**Coding gene expression profile in endometrial carcinoma.** The microarray analysis also included information on the coding gene. A total of 18,738 coding transcripts (mRNA) could be detected in the 3 paired tissue samples. Compared the mRNA expression level between endometrial carcinoma and adjacent non-tumor tissues, there were 46 mRNA differentially expressed; of those 26 were upregulated and 20 were downregulated in the endometrial carcinoma group (fold change  $\geq 2.0$ ,  $p \leq 0.05$ ) (Fig. 1B and D; and Table II).

With Pathway and Gene Ontology analysis we found that these deregulated coding genes were involved in multiple pathways and gene ontology. Pathway analysis indicated that 12 pathways corresponded to upregulated transcripts (Table III), and 12 pathways corresponded to downregulated transcripts (Table IV). Ras signaling pathway corresponded to 15 transcripts from both up- and down-regulated data (Tables III and IV).

Gene ontology (GO) analysis was preformed to show that the differently expressed mRNA transcripts were associated with biological processes (BP), cellular components (CC) and

molecular function (MF) (Fig. 4). Progesterone metabolic process is one of the most frequent fold enrichment biological processes. There were 13 upregulated and 18 downregulated transcripts involved in biological process of wound healing. Deregulated transcripts were involved in the molecular function voltage-gated channel activity, including voltage-gated ion channel activity, voltage-gated channel activity, voltage-gated cation channel activity and voltage-gated calcium channel activity

**Expression of ASLNC04080 correlates with the coding genes.** Based on the correlation analysis between differently expressed lncRNAs and mRNAs, we constructed a coding-non-coding gene co-expression network. Four upregulated and 2 downregulated lncRNAs in endometrial carcinoma tissues were selected to draw the network. The expression of 289 mRNAs was related (Pearson's correlation coefficients:  $PCC \geq 0.95$ ) to these 6 lncRNAs (Fig. 5).

ASLNC04080 expression level was correlated with 19 coding gene transcripts (Fig. 5). Besides, these coding genes were involved in multiple pathways and gene ontology. For example CCR10 was upregulated in endometrial carcinoma and positively correlated with ASLNC04080 expression





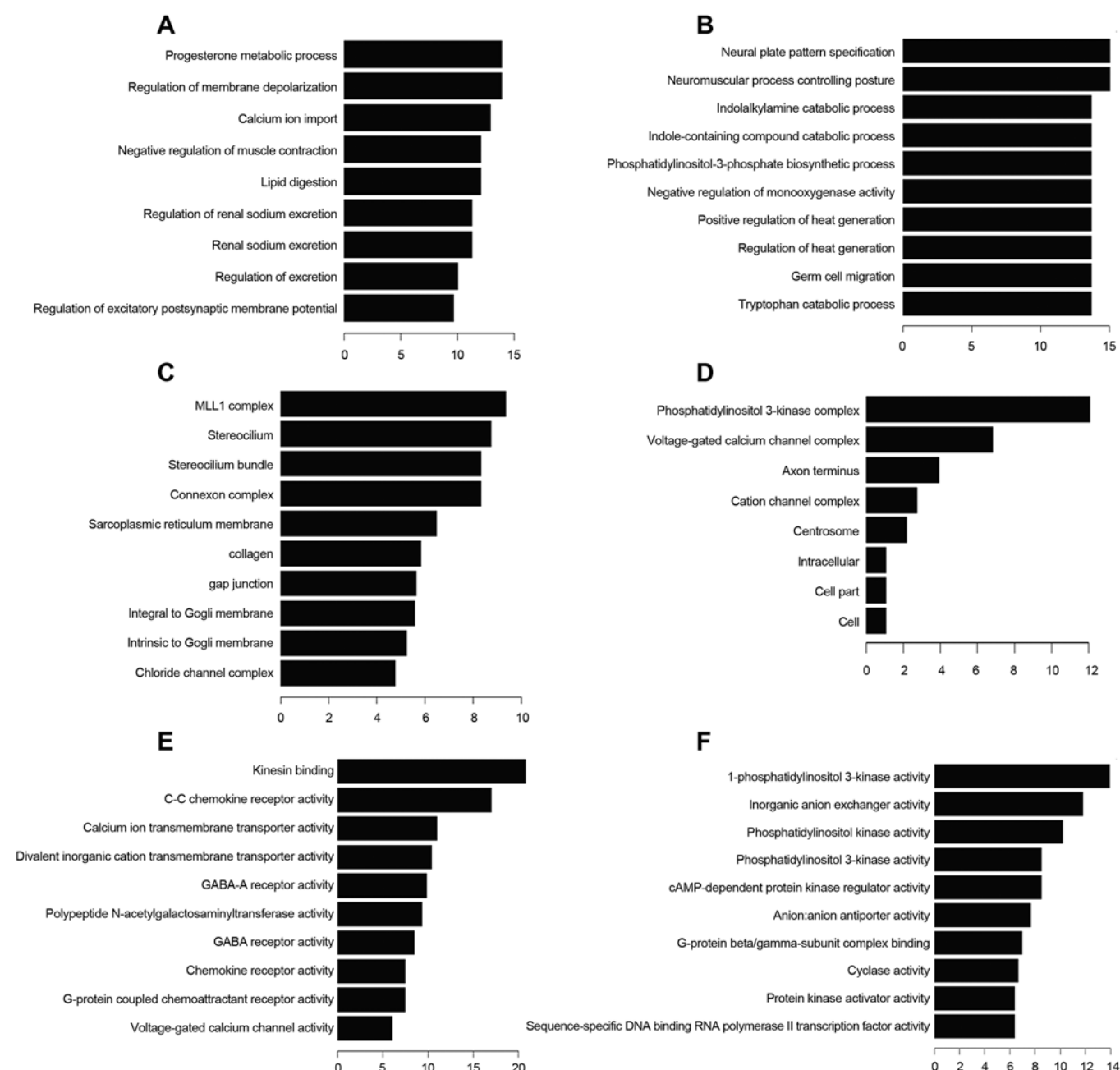


Figure 4. Gene ontology analysis results show the differently expressed coding gene transcripts associated with biological process (BP), cellular components (CC) and molecular function (MF). The most frequent fold enrichment biological processes (BP) associated with upregulated (A) and downregulated (B) coding gene transcripts. The most frequent fold enrichment cellular components (CC) associated with upregulated (C) and downregulated (D) coding gene transcripts. The most frequent fold enrichment molecular functions (MF) associated with upregulated (E) and downregulated (F) coding gene transcripts.

cytometry after HEC-1-B transfection with ASLNC04080 and negative control siRNA. Knockdown of ASLNC04080 expression increased apoptosis in HEC-1-B cells (NC vs. siRNA-1535:  $15.9 \pm 0.66\%$  vs.  $26.9 \pm 1.27\%$ , T-test  $P < 0.05$ ) (Fig. 6C). Moreover, caspase-3 protein cleavage was detected only in siRNA-1535 transfected cells (Fig. 6D). We performed cell cycle assays after siRNA transfection 48 h. Knockdown of ASLNC04080 expression induced G1 phase arrest (NC vs. siRNA-1535:  $41.14 \pm 1.10\%$  vs.  $49.85 \pm 0.77\%$ , t-test  $P < 0.05$ ) (Fig. 6E).

These findings suggest that the ASLNC04080 lncRNA could regulate endometrial carcinoma cell HEC-1-B proliferation, apoptosis and cell cycle.

## Discussion

Molecular alterations in endometrial carcinoma have been studied for many years (14,15). Microsatellite instability (MI), and gene mutations (PTEN, K-RAS and PIK3CA) have been shown involved in type I endometrial carcinoma. Type II endometrial carcinoma exhibits mutations of p53, loss of heterozygosity (LOH), and molecular alterations (STK1 5, p16, and c-erb-B2). Recently a comprehensive, multiplatform analysis of 373 endometrial carcinomas identified new hotspot mutations in POLE, and based on the genomic characterization they classified endometrial cancers into four categories: POLE ultramutated, microsatellite instability hypermutated, copy-



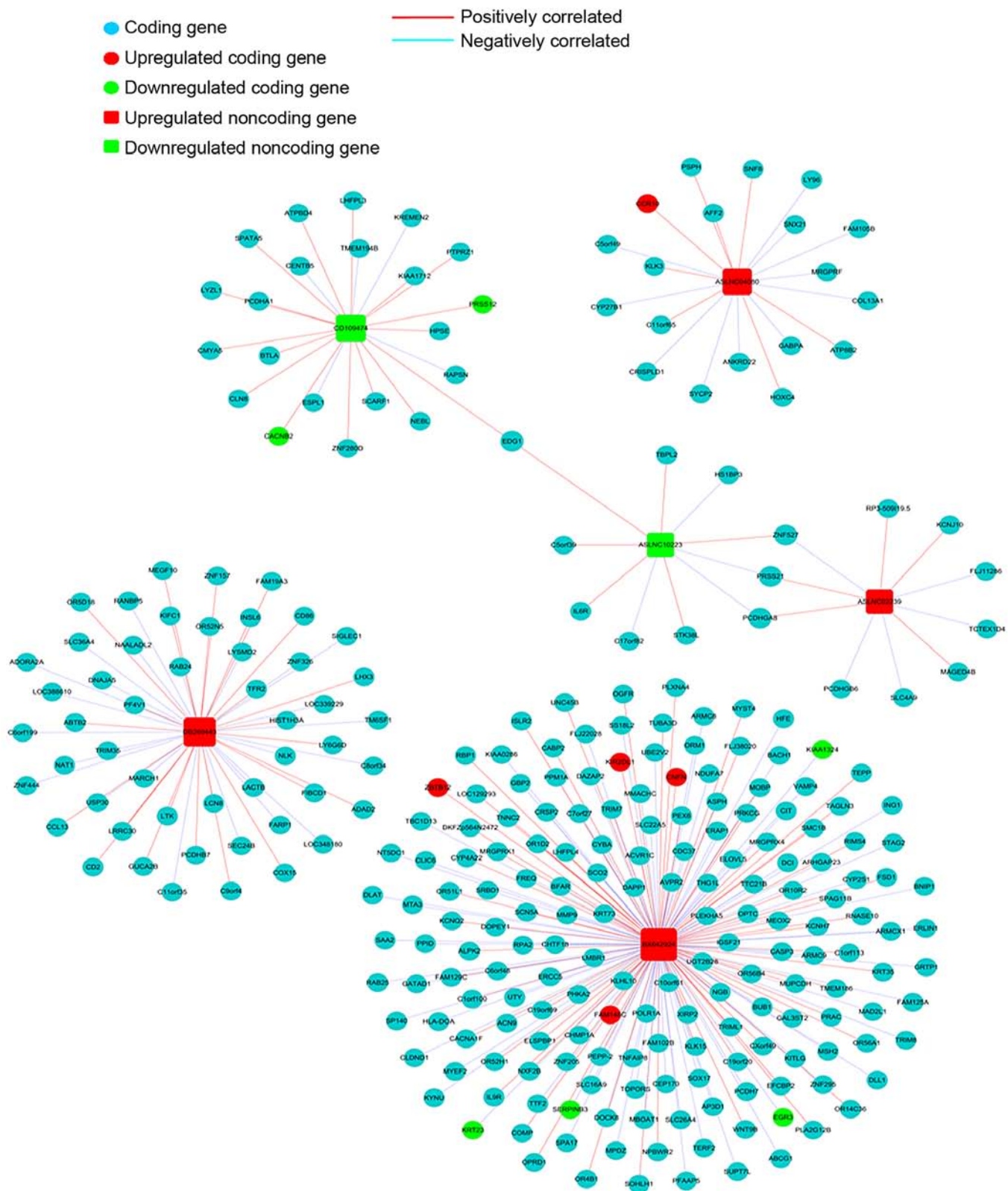


Figure 5. The co-expression network construction. Six selected lncRNAs were co-expression with 298 coding gene transcripts. ASLN04080 expression correlates with 19 coding gene transcripts (PCC >0.95).

number low, and copy-number high (16). The efforts on the integrated analysis of molecules not only help us to construct new tumor classifications, but also affect treatment recommendations for patients, provides opportunities for genome-guided clinical trials and drug development. Besides the known

coding genes, noncoding RNAs act as a new hallmark for endometrial carcinoma diagnosis and therapy, have attracted wide attention. A large quantity of miRNAs target important genes in tumor development and progression has been identified in endometrial carcinoma (17). Hiroki *et al* (18), identified

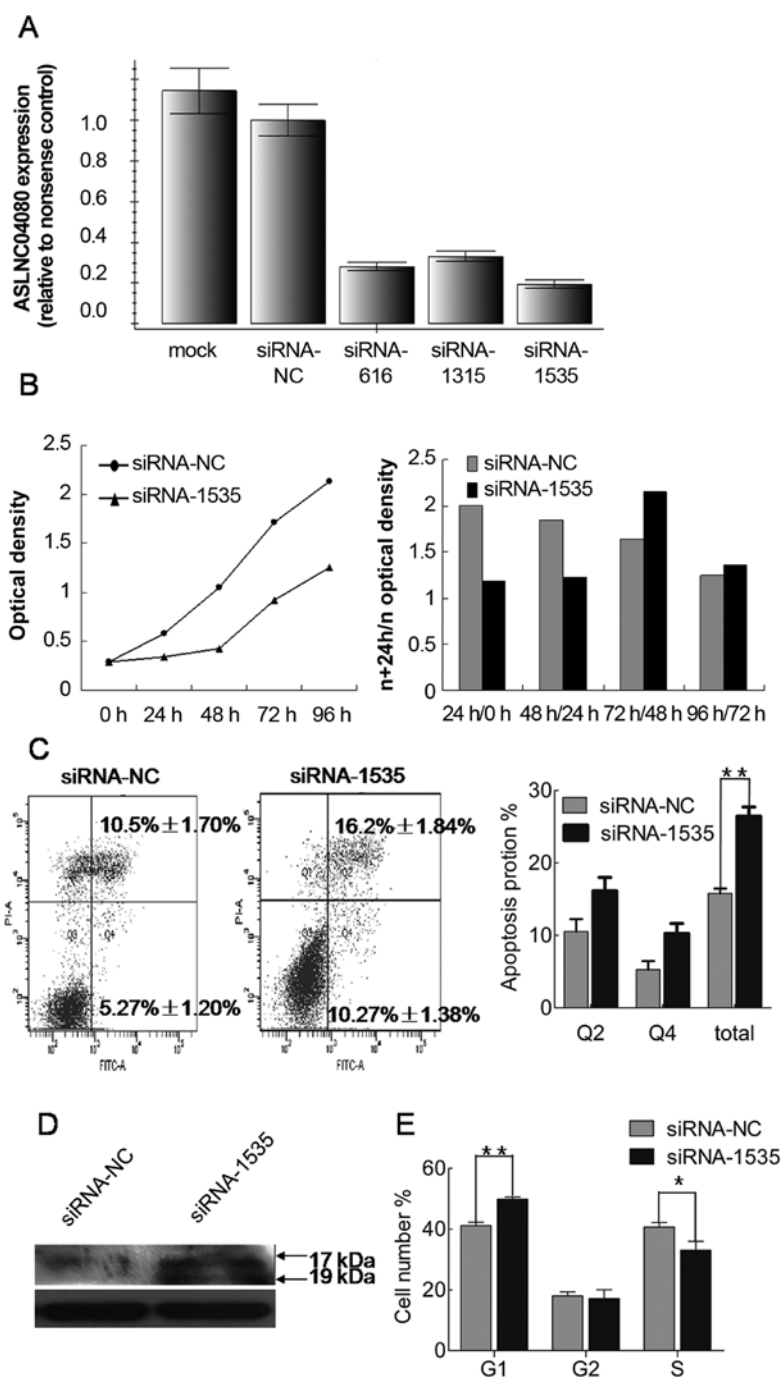


Figure 6. Inhibition of the ASLNC04080 expression suppressed proliferation, increased apoptosis and induced G1 phase arrest in HEC-1-B cells. (A) Knockdown efficiency of ASLNC04080 specific siRNAs in HEC-1-B cells. All 3 siRNA molecules induced >50% inhibition of ASLNC04080 expression, and siRNA-1535 caused >80% reduction in efficiency of ASLNC04080 expression. (B) CCK8 cell proliferation assays in HEC-1-B cells. The siRNA-1535 transfection induced lower growth rate. Especially in the first 48 h the cell growth rate was inhibited in ASLNC04080 expression knockdown cells. (C) Transfection of siRNA-1535 significantly induced HEC-1-B cell apoptosis (NC vs. siRNA-1535:  $15.9 \pm 0.66\%$  vs.  $26.9 \pm 1.27\%$ , t-test  $p < 0.001$ ). (D) Western blotting revealed cleaved caspase-3 only in ASLNC04080 expression knockdown cells. (E) Knockdown of ASLNC04080 expression induced G1 phase arrest in HEC-1-B cells (NC vs. siRNA-1535:  $41.14 \pm 1.10\%$  vs.  $49.85 \pm 0.77\%$ , t-test  $p < 0.001$ ).

120 miRNAs deregulated in endometrial serous carcinoma. Moreover, their results showed that microRNA expression is associated with clinical pathology and prognosis of patients with endometrial serous adenocarcinoma.

lncRNA has been confirmed to regulate gene expression in histone modification (19), regulation of transcription (20) and splicing, and plays an essential role in cellular proliferation, development and metabolism (21). The deregulation of lncRNA

is associated with physiological disorders and disease development (22). Increased lncRNAs correlated with carcinogenesis and tumor progression have been discovered accompanied with sequencing and microarray technological development. A series of differentially expressed lncRNAs (lncRNA-HEIH, lncRNA-MVIH, lncRNA-LALR1, lncRNA-LET and lncRNA-Dreh) have been identified between HBV-related HCC and paired peritumoral tissues by microarray (23-25). With

Table III. Upregulated coding gene transcripts corresponding to 12 pathways.

Pathway ID	Definition	Fisher - P-value	Genes
hsa05032	Morphine addiction - <i>Homo sapiens</i> (human)	0.004254111	ADORA1//GABRB1//GABRQ//PDE3A//PRKCG
hsa00512	Mucin type O-Glycan biosynthesis - <i>Homo sapiens</i> (human)	0.00528077	GALNT13//GALNTL6//ST3GAL2
hsa04972	Pancreatic secretion - <i>Homo sapiens</i> (human)	0.005316287	ATP2A1//ATP2B3//PLA2G1B//PRKCG//PRSS2
hsa04974	Protein digestion and absorption - <i>Homo sapiens</i> (human)	0.01848372	COL12A1//COL1A2//COL4A6//PRSS2
hsa04672	Intestinal immune network for IgA production - <i>Homo sapiens</i> (human)	0.01969566	CCR10//CCR9//CD86
hsa00140	Steroid hormone biosynthesis - <i>Homo sapiens</i> (human)	0.02776873	AKR1C1//CYP19A1//UGT2B28
hsa04510	Focal adhesion - <i>Homo sapiens</i> (human)	0.03121468	COL1A2//COL4A6//MYLPP//PAK4//PRKCG//VEGFC
hsa00592	$\alpha$ -Linolenic acid metabolism - <i>Homo sapiens</i> (human)	0.03328378	ACOX3//PLA2G1B
hsa04950	Maturity onset diabetes of the young - <i>Homo sapiens</i> (human)	0.03328378	HNF4A//NKX2-2
hsa05211	Renal cell carcinoma - <i>Homo sapiens</i> (human)	0.04036226	GAB1//PAK4//TCEB2
hsa00514	Other types of O-glycan biosynthesis - <i>Homo sapiens</i> (human)	0.04651509	GLT25D2//RFNG
hsa04014	Ras signaling pathway - <i>Homo sapiens</i> (human)	0.0466148	ANGPT4//GAB1//PAK4//PLA2G1B//PRKCG//VEGFC

further studies, these lncRNAs were verified to be functional molecules contributing to HCC tumor growth, angiogenesis, metastasis and serving as a predictor for HCC patients' poor recurrence-free survival after hepatectomy. Although a systematic study of lncRNA in endometrial carcinoma is lacking, a few functional lncRNAs have been shown deregulated in endometrial carcinoma. lncRNA NCT25 mutations were observed in endometrial tumor specimens (in 23 of 48 of the samples), and could be a mutational target specifically in endometrial cancer. MALAT1 upregulation is the result of tumor suppressor PCDH10 silencing in endometrioid cancer (13). Therefore, more functional lncRNA could be related to endometrial carcinoma genesis and progression, and act as potential hallmark for endometrial carcinoma diagnosis and therapy.

To investigate the lncRNA expression profile of endometrial carcinoma, we performed a microarray analysis containing lncRNA and coding gene information in 3 paired endometrial carcinoma and adjacent non-tumor tissues. The microarray data showed a significant difference of lncRNA expression pattern between endometrial carcinoma and adjacent non-tumor tissues. A total of 53 lncRNAs (C vs. N: 39 up-, 14 down-regulation, fold change >2.0,  $p < 0.05$ ) were deregulated in endometrial carcinoma. ASLNC04080 is the most upregulated lncRNA in endometrial carcinoma (fold change: 3.379,  $p = 0.03778$ ). The expression level of ASLNC04080 is higher in endometrial carcinoma tissues (22/24) compared with non-tumor tissues. ASLNC04080 expression could also

be detected in endometrial carcinoma cell line HEC-1-B as well as other gynecological cancer cell lines (Siha, HeLa, 3AO and SKOV3). ASLNC04080 is transcribed from the chr1: -28905061 - -28909492 loci (1 p35.3), consisting 6 exons 1,867nt in length.

According to the coding gene microarray data 46 mRNAs (C vs. N: 26 up-, 20 down-regulation, fold change >2.0,  $p < 0.05$ ) exhibited a different expression between paired endometrial carcinoma and adjacent non-tumor tissues. Previous studies demonstrated that PTEN mutations occur early in endometrial carcinogenesis and co-exist frequently with other deregulated molecules targeting AKT-PI3K-mTOR pathway. Through pathway analysis, we have found 6 up- and 9 down-regulated coding gene transcripts in endometrial carcinoma targeting Ras signaling pathway. Estrogen and progesterone accession and metabolism are related to endometrial carcinoma genesis and progression (26). In our data progesterone metabolic process is one of the most frequent fold enrichment biological processes. Two endometrial carcinoma upregulated coding gene transcripts CYP19A1 and AKR1C1 participated in this processes, and correlated with endometrial carcinoma genesis (27,28). Besides, 31 deregulated coding transcripts target the wound healing process. Growth evidence shows that voltage-gated channel activity alteration of carcinoma cell membrane is a characteristic of carcinoma cells (29-31). In our results, the molecules target voltage-gated ion channel activity, voltage-gated channel activity, voltage-gated cation

Table IV. Downregulated coding gene transcripts corresponding to 12 pathways.

Pathway ID	Definition	Fisher - P-value	Genes
hsa04015	Rap1 signaling pathway - <i>Homo sapiens</i> (human)	0.002010251	CSF1//FGF13//GNAQ//HGF//ITGAM//ITGB1//KITLG//MLLT4//PIK3CB
hsa05152	Tuberculosis - <i>Homo sapiens</i> (human)	0.00262324	ARHGEF12//ATP6V0A2//CYP27B1//HLA-DOA//IL10//ITGAM//NFYC//PIK3C3
hsa04014	Ras signaling pathway - <i>Homo sapiens</i> (human)	0.003095239	CSF1//ETS1//FGF13//HGF//KITLG//MLLT4//PIK3CB//PLA2G4A//SHC1
hsa05146	Amoebiasis - <i>Homo sapiens</i> (human)	0.003198103	COL5A2//GNAQ//IL10//ITGAM//PIK3CB//SERPINB3
hsa00380	Tryptophan metabolism - <i>Homo sapiens</i> (human)	0.0158507	ACMSD//KYNU//OGDHL
hsa05140	Leishmaniasis - <i>Homo sapiens</i> (human)	0.01747982	HLA-DOA//IL10//ITGAM//ITGB1
hsa04270	Vascular smooth muscle contraction - <i>Homo sapiens</i> (human)	0.03014367	ARHGEF12//GNAQ//GUCY1A3//KCNU1//PLA2G4A
hsa04970	Salivary secretion - <i>Homo sapiens</i> (human)	0.03164653	ATP1A2//GNAQ//GUCY1A3//STATH
hsa04912	GnRH signaling pathway - <i>Homo sapiens</i> (human)	0.03392592	GNAQ//GNRH1//MAP3K3//PLA2G4A
hsa04964	Proximal tubule bicarbonate reclamation - <i>Homo sapiens</i> (human)	0.03706998	ATP1A2//SLC4A4
hsa05150	Staphylococcus aureus infection - <i>Homo sapiens</i> (human)	0.04175273	HLA-DOA//IL10//ITGAM
hsa04730	Long-term depression - <i>Homo sapiens</i> (human)	0.04544242	GNAQ//GUCY1A3//PLA2G4A

channel activity and voltage-gated calcium channel activity, displaying deregulation in endometrial carcinoma.

We constructed a coding-non-coding gene co-expression network (CNC), by analyzing the correlation of lncRNAs and the expression level of the coding gene transcripts. The expression of six selected lncRNAs were showed to be related to 289 coding gene transcripts. The BX642924 expression was correlated with 177 coding gene transcripts (Fig. 5), especially positively correlated to PRKCG (upregulated in endometrial carcinoma, PCC >0.98) and negatively correlated to KITLG (downregulated in endometrial carcinoma, PCC >0.95) expression. Both PRKCG and KITLG were involved in Ras signaling pathway, and have been showed related to tumor genesis and progression (32,33). According to these analyses we speculate that BX642924 may correlate with the Ras signaling pathway. The expression of three coding gene transcripts (ZNF275, PCDHGA8, PRSS21) was correlated with ASLNC02239 (upregulated in endometrial carcinoma) and ASLNC10223 (downregulated in endometrial carcinoma), showing a possible relation among these genes. More work is needed to confirm the relations and the underlying regulation mechanism of these coding and non-coding genes.

ASLNC04080 expression is correlated with 19 coding gene transcripts. KLK3 is the most positively related transcript with ASLNC04080 (PCC >0.97). Increasing evidence indicates that KLK3 (kallikrein-related peptidase 3) is implicated in carcinogenesis and acts as a biomarker or a diagnosis target in multiple types of cancer (34). Moreover, SYCP2 negatively

correlates with ASLNC04080 expression (PCC >0.95), and have been shown to participate in the cell cycle pathway (35). Inhibition of ASLNC04080 expression in HEC-1-B cells, resulted in decreased cell proliferation, increased cell apoptosis and G1 phase arrest. Taking together, these findings suggest that ASLNC04080 is a functional lncRNA in human endometrial carcinoma and may contribute to carcinogenesis via interaction with other coding genes. More exploration of the function mechanism of ASLNC04080 is required.

To our knowledge, this is the first systematic research project of lncRNA expression profile in endometrial carcinoma. We found several lncRNAs expressed in endometrial carcinoma and correlated with multiple Gene Ontology and pathways involved in carcinogenesis. These findings could help us enrich the knowledge on the mechanism of endometrial carcinogenesis and find new diagnostic or therapeutic targets for endometrial carcinoma.

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