Microarray expression profiling of long non-coding RNAs in epithelial ovarian cancer

YE DING, DA-ZHENG YANG, YONG-NING ZHAI, KAI XUE, FENG XU, XIAO-YAN GU and SU-MIN WANG

State Key Laboratory of Reproductive Medicine, Department of Endoscopic Diagnostic and Treatment Center, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, Nanjing, Jiangsu 210004, P.R. China

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Abstract. Although numerous long non-coding RNAs (lncRNAs) have been identified to be important in human cancer, their potential regulatory roles in epithelial tumorigenesis and tumor progression in ovarian cancer remain unclear. The purpose of the present study was to investigate lncRNAs that were differentially expressed (DE) in epithelial ovarian cancer and to explore their potential functions. The lncRNA profiles in five pairs of human epithelial ovarian cancer tissues and their adjacent normal tissues were described using microarrays. The results of the microarray analysis revealed that 672 upregulated and 549 downregulated (fold-change ≥2.0) lncRNAs were DE between the cancerous and normal tissues. Reverse transcription-quantitative polymerase chain reaction was used to validate the microarray results using four upregulated (RP11-1C1.7, XLOC_003286, growth arrest-specific 5 and ZNF295-AS1) and four downregulated (protein tyrosine kinase 7, maternally expressed gene 3, AC079776.2 and ribosomal protein lateral stalk subunit P0 pseudogene 2) lncRNAs. Furthermore, gene ontology and pathway analyses were used to carry out functional analyses of the candidate genes of DE lncRNAs. The results identified lncRNAs with significantly altered expression profiles in human epithelial ovarian cancer cells compared with those in adjacent normal cells. These data offer new insights into the occurrence and development of epithelial ovarian cancer, and these lncRNAs may provide novel molecular biomarkers for further research on epithelial ovarian cancer.

Correspondence to: Dr Su-Min Wang or Dr Xiao-Yan Gu, State Key Laboratory of Reproductive Medicine, Department of Endoscopic Diagnostic and Treatment Center, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, 123, Tianfeixiang, Mochou Road, Nanjing, Jiangsu 210004, P.R. China
E-mail: 115657929@qq.com
E-mail: njguxiaoyan@163.com

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Introduction

Ovarian cancer is the most common cause of mortality from gynecological tumors in women worldwide (1). The 5-year survival rate for patients with advanced ovarian cancer has been reported to be ~30% (2). The incidence of ovarian cancer in Asian countries is considerably lower than that in developed countries, but the difference is reducing (3). In China, the estimated incidence of ovarian cancer during 1999-2010 was 7.91 per 100,000 people (4). Epithelial ovarian cancer accounts for nearly 90% of all ovarian tumors (5). The high mortality of epithelial ovarian cancer is attributed to late-stage diagnosis in >70% of the patients (6). Constant damage and repair of ovarian surface epithelial cells, use of gonadotropin-releasing hormone and steroid hormones, inflammation, genetic factors, and environmental factors have been previously shown to be associated with epithelial ovarian cancer (7-9); however, the exact molecular mechanisms of its occurrence and development remain to be fully identified.

For more than half a century, the concept of gene was limited to the messenger RNA (mRNA) coding region of the genome. With progress in life science research in the post-genome era, numerous studies have demonstrated the involvement of non-coding RNAs (ncRNAs) at various levels in the cell, including transcription, and post-transcriptional regulation of nuclear internal and external signal communication (10). In addition, these RNAs have been demonstrated to be closely associated with the pathological processes of numerous serious diseases (11). Long ncRNAs (lncRNAs) are non-coding RNAs >200 nt in length. Accumulating evidence indicates that lncRNAs serve an important role in various biological processes such as genomic imprinting, transcription activation and inhibition, chromosome recombination, intranuclear transportation, and organ development (12,13). Certain studies have indicated that aberrant regulation of lncRNAs is associated with various types of human cancer (14). Furthermore, lncRNAs are often used as a potential biomarker in the diagnosis and prognosis of tumors (15). Although a few lncRNAs have been implicated in the progression of epithelial ovarian cancer, the functions of the majority of lncRNAs remain to be investigated.

Therefore, the present study used an lncRNA microarray to identify lncRNAs that are differentially expressed (DE) in epithelial ovarian cancer. The microarray results were verified by reverse transcription-quantitative polymerase chain

reaction (RT-qPCR) for specific DE lncRNAs. The present data may provide a molecular basis for understanding the pathogenesis of epithelial ovarian cancer.

Materials and methods

Tissue collection. For tissue collection, five patients with epithelial ovarian cancer were recruited between May and July 2014 at the Department of Gynecology, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University (Nanjing, China). The patients were pathologically confirmed as having epithelial ovarian cancer. Epithelial ovarian cancer tissues and surrounding normal tissues were collected following surgery, snap frozen in liquid nitrogen, and stored at -80˚C. Written informed consent was obtained from all patients and the study was approved by the ethics committee of Nanjing Medical University.

RNA extraction. Total RNA was extracted from five pairs of epithelial ovarian cancer and adjacent normal tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol, and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The RNA integrity of each sample was assessed using standard denaturing gel electrophoresis, as previously described (16).

Microarray and data analysis. Microarray analysis was performed by Kangchen Biotech Co., Ltd. (Shanghai, China). Arraystar Human LncRNA Microarray V3.0 (Arraystar Inc., Rockville, MD, USA) is designed for the global profiling of human lncRNAs and protein-coding transcripts. This software is capable of detecting ~30,586 lncRNAs and 26,109 coding transcripts (17). Briefly, mRNA was purified from total RNA upon removal of ribosomal RNA using the mRNA-ONLY™ Eukaryotic mRNA Isolation kit (Epicentre, Madison, WI, USA). Then, each sample was amplified and transcribed into fluorescent complementary RNA (cRNA) along the entire length of the transcripts without 3'-bias using the Quick Amp Labeling kit, One-Color (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The labeled cRNAs were purified using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). The concentration and specific activity of the labeled cRNAs (pmol cyanine 3/μg cRNA) were measured by the NanoDrop ND-1000. First, 1 μg of each labeled cRNA was fragmented by adding 5 μl of 10X blocking agent and 1 μl of 25X fragmentation buffer (both Agilent Technologies, Inc.). The mixture was then heated at 60˚C for 30 min, and subsequently, 25 μl of 2X hybridization buffer (GE Healthcare Life Sciences, Little Chalfont, UK) was added to dilute the labeled cRNA. For microarray analysis, 50 μl of the hybridization solution was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65˚C in a Microarray Hybridization Oven (Agilent Technologies, Inc.). The hybridized arrays were washed with Gene Expression Wash Buffer (Agilent Technologies, Inc.) and scanned with using the G2505C Microarray Scanner System (Agilent Technologies, Inc.). Feature Extraction software version 11.0.1.1 (Agilent Technologies, Inc.) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies, Inc.).

Gene ontology (GO) and pathway analyses. GO and pathway analyses were used to determine the roles of DE mRNAs in biological pathways or GO terms. Differentially regulated mRNAs were uploaded into the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/), which utilized GO terms to identify the molecular function represented in the gene profile. Pathway analysis was carried out based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.ad.jp/kegg/).

RT-qPCR validation. Total RNA was reverse transcribed into complementary DNA (cDNA) using the AMV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR Sequence Detection System (Thermo Fisher Scientific, Inc.). RT-qPCR was conducted using 1 μl of cDNA, 12.5 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 10.5 μl of diethyl pyrocarbonate-treated water, and 0.5 μl of 10 μM forward and reverse primers, in a total volume of 25 μl. The following specific primers were used for PCR: RP11-1C1.7 forward, 5'-CTC AGG CTT GGC TCA GAC AC-3' and reverse, 5'-GCA AAC AGC CTT GGA GAA GC-3'; XLOC_003286 forward, 5'-AAG GGA TCT GGT CTT CAA CA-3' and reverse, 5'-TTC CAC CAT GTA ATG GGT CC-3'; growth arrest specific 5 (GAS5) forward, 5'-TGA AGT CCT AAA GAG CAA GCC-3' and reverse, 5'-ACC AGG AGC AGA ACC ATTA AG-3'; ZNF295-AS1 forward, 5'-CCC AGG AGG GAG GTG ATA CT-3' and reverse, 5'-TGG GTA GCT TGT GAA CCA CC-3'; protein tyrosine kinase 7 (PTK7) forward, 5'-GGA AGC CAC ACT TCA CCT AGC AG-3' and reverse, 5'-CTG CCA CAG TGA GCT GGA CAT GG-3'; maternally expressed gene 3 (MEG3) forward, 5'-GCT CTA CTC CGT GGA AGC AC-3' and reverse, 5'-CAA ACC AGG AAG GAG ACG AG-3'; AC079776.2, forward, 5'-GCC GAT GGT AGA GAA GAC CG-3' and reverse, 5'-GGG GCT CAG AAG CCA TCT TT-3'; and ribosomal protein lateral stalk subunit P0 pseudogene 2 (RPLP0P2) forward, 5'-AAA AAC GAT CAA CGA ACC TT-3' and reverse, 5'-AAT CGT CTC TGC TTT TCT TG-3'. The PCR conditions were as follows: Denaturation at 95˚C for 10 min, followed by 40 cycles of amplification and quantification at 95˚C for 15 sec and 60˚C for 1 min. GAPDH (forward, 5'-CCG GGA AAC TGT GGC GTG ATG G-3' and reverse, 5'-AGG TGG AGG TAT GGG TGT CGC TGT T-3') was used as the internal control. The experiments were performed in triplicate. The relative fold-change was calculated using the $2^{-\Delta\Delta Cq}$ method (18).

Statistical analysis. The lncRNAs and mRNAs that exhibited significantly different expression levels between the two groups were identified through P-value/false discovery rate filtering. DE lncRNAs and mRNAs were identified

Table I. Screening of differentially expressed lncRNAs (tumor vs. normal).

Regulation	lncRNA	Fold-change	Chromosomal localization	RNA length, bp
Up	RP5-857K21.3	91.6369032	Chr1	437
Up	uc001zjx.1	64.7598797	Chr15	641
Up	DQ573539	39.8247748	Chr9	1,713
Up	RP11-1C1.7	38.8887511	Chr5	483
Up	XLOC_004134	25.2266495	Chr4	261
Up	RP11-872J21.3	21.4447620	Chr14	1,512
Up	LOC338817	18.7987392	Chr12	3,684
Up	CDKN2B-AS1	15.7325039	Chr9	1,067
Up	HLA-DRB6	15.1244408	Chr6	715
Up	UCA1	12.9894370	Chr19	1,413
Up	BX004987.5	11.7750069	Chr1	736
Up	FOLH1B	10.8238534	Chr11	2,163
Up	ZNF295-AS1	9.3852619	Chr21	1,073
Up	AK054990	9.1453539	Chr2	2,070
Up	AP001615.9	8.1669081	Chr21	461
Up	GAS5	7.8179616	Chr1	822
Up	LINC00152	7.0158480	Chr2	455
Up	XLOC_003286	6.5502125	Chr3	409
Up	DPY19L2P2	4.4375165	Chr7	3,433
Up	AL833634	2.2275523	Chr11	1,885
Down	CTD-2536I1.1	58.1029053	Chr15	614
Down	BC071789	46.6526362	Chr3	2,730
Down	RP11-548O1.3	41.2599738	Chr3	483
Down	MEG3	35.0543457	Chr14	1,351
Down	RP11-471J12.1	30.7697326	Chr4	892
Down	LEMD1-AS1	24.3438594	Chr1	2,781
Down	CLCN6	20.5708229	Chr1	5,697
Down	AL132709.5	19.7389918	Chr14	644
Down	XLOC_010463	17.3764962	Chr13	9,590
Down	CACNA1G-AS1	15.5318244	Chr17	1,450
Down	AC079776.2	12.6763061	Chr2	400
Down	RP11-998D10.2	10.6026574	Chr14	548
Down	LOC253044	7.5169687	Chr15	1,735
Down	PVT1	4.8097586	Chr8	654
Down	AX747026	4.3736710	Chr1	2,133
Down	OPA1-AS1	3.4889195	Chr3	513
Down	PTK7	3.1639252	Chr6	4,040
Down	RP11-799B12.4	2.5604262	Chr18	735
Down	RPLP0P2	2.4997850	Chr11	573
Down	HOTAIR	2.1863176	Chr12	2,370

lncRNA, long non-coding RNA; Chr, chromosome.

by fold-change filtering and Student's t test. All data were expressed as means ± standard deviation. Statistical analysis was performed using SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

DE lncRNAs and mRNAs. A total of 1221 lncRNAs were significantly DE between the tumor and control groups (fold-change ≥2.0), among which, 672 were upregulated and

Table II. Screening of differentially expressed mRNAs (tumor vs. normal).

Regulation	mRNA	Fold-change	Chromosomal localization	RNA length, bp
Up	GAL	112.8379148	Chr11	778
Up	LAMC2	94.8845376	Chr1	5,623
Up	CCNA1	80.0032110	Chr13	1,841
Up	MUC1	62.3494142	Chr1	878
Up	WDR69	54.5549954	Chr2	1,669
Up	ENKUR	47.3980040	Chr10	3,382
Up	STOML3	32.8593469	Chr13	1,936
Up	KIAA0101	27.1783242	Chr15	1,345
Up	CCNB2	20.5538621	Chr15	1,566
Up	SLC1A3	18.5310330	Chr5	3,670
Up	SAA2	16.4134886	Chr11	594
Up	FGF18	14.3701010	Chr5	1,999
Up	UBE2C	12.9501868	Chr20	520
Up	NAA16	9.5211755	Chr13	1,833
Up	KCNIP4	7.5399784	Chr4	2,371
Up	SLITRK6	6.5738521	Chr13	4,199
Up	CEP44	4.4520673	Chr4	3,290
Up	C20orf201	3.2842057	Chr20	868
Up	DHCR7	2.3597491	Chr11	2,665
Up	RNLS	2.0603828	Chr10	2,420
Down	ITM2A	110.4209953	ChrX	1,719
Down	ZBTB16	82.7721198	Chr11	2,417
Down	CPXM1	80.2367909	Chr20	2,409
Down	GATA4	69.2038646	Chr8	3,419
Down	APOD	54.0064083	Chr3	1,130
Down	DCN	48.0233786	Chr12	1,336
Down	GNG11	37.6068614	Chr7	964
Down	DHRS2	32.5328881	Chr14	1,709
Down	ACADL	28.4221424	Chr2	2,565
Down	LCE1C	24.4961395	Chr1	695
Down	MATN2	18.1700061	Chr8	4065
Down	LCE2C	16.5275274	Chr1	614
Down	PPP1R14A	10.4409979	Chr19	782
Down	OSR2	8.4374819	Chr8	1,907
Down	AKT3	6.6042407	Chr1	7,091
Down	IL28RA	5.1905507	Chr1	4,432
Down	PIK3IP1	3.6901547	Chr22	2,478
Down	SULF1	3.4824364	Chr8	5,716
Down	DCAF4L2	2.8399696	Chr8	3,339
Down	MARK3	2.6464566	Chr14	3,519

mRNA, messenger RNA; Chr, chromosome.

549 were downregulated. Among the DE mRNAs between the two groups, 525 were upregulated and 418 were downregulated. Partial results for the DE lncRNAs and mRNAs are listed in Tables I and II, respectively.

Validation of de lncRNAs. The results of the microarray analysis were confirmed by RT-qPCR of eight randomly selected lncRNAs. GAPDH was used as a normalization control. Of these randomly selected lncRNAs, four (RP11-1C1.7,

XLOC_003286, GAS5 and ZNF295-AS1) were upregulated and the other four (PTK7, MEG3, AC079776.2 and RPLP0P2) were downregulated in epithelial ovarian cancer samples compared with their expression levels in adjacent normal tissues of the same individual. As the results of RT-qPCR and microarray analyses are consistent (Fig. 1), these data can be used with confidence in further research.

Pathway analysis. Pathway analysis is a functional method of mapping genes to KEGG pathways (19). Based on the KEGG database (http://www.genome.jp/kegg), KEGG pathway analysis was employed for DE mRNAs. Each P-value denoted the significance of the corresponding pathway, while the EASE Score, Fisher's P-value or hypergeometric P-value denoted the significance of the pathway correlated to the conditions. A low P-value indicated a marked significance of the pathway (P-value cut-off, 0.05). The bar plots in Fig. 2 show the top 10 enrichment scores [-$\log_{10}$ (P-value)] of the significant enrichment pathway. Fig. 2 presents the results of the KEGG pathway analysis for the upregulated and downregulated mRNAs.

GO analysis. The GO 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 processes, cellular components and molecular function. Fisher's exact test is used to determine if there are any more overlaps between the DE gene list and the GO annotation list than what is expected by chance. The P-value denotes the significance of enrichment of GO terms in the DE genes. The lower the P-value, the more significant is the GO term (P≤0.05 is recommended) (20). The bar plots in Fig. 3 show the 10 most significant enrichment terms with the most number of DE genes.

Discussion

As increasing research has focused on the function of lncRNAs in epithelial ovarian cancer, an increasing number of lncRNAs have been identified. For example, Gao *et al* demonstrated that the lncRNA human ovarian cancer-specific transcript 2 promotes tumor cell migration, invasion and proliferation in epithelial ovarian cancer by modulating microRNA let-7b availability (21). lncRNA H19 expression was inhibited by histone H1.3, which contributes to the suppression of epithelial ovarian carcinogenesis (22). However, the genome-wide expression and the biological functional significance of lncRNAs in epithelial ovarian cancer remain unknown.

In the present study, microarray analysis was used to compare lncRNA expression in epithelial ovarian cancer cells and adjacent normal tissues, and 1221 DE lncRNAs (672 upregulated and 549 downregulated) were identified. These results were further confirmed via RT-qPCR for eight randomly selected lncRNAs.

A previous study has reported that Hox transcript antisense intergenic RNA (HOTAIR) is a 2.2-kb lncRNA located at the HOXC locus (23).It has been reported that suppression of HOTAIR expression in highly metastatic epithelial ovarian cancer cell lines significantly reduced cell invasion, and the HOTAIR expression levels were highly positively correlated

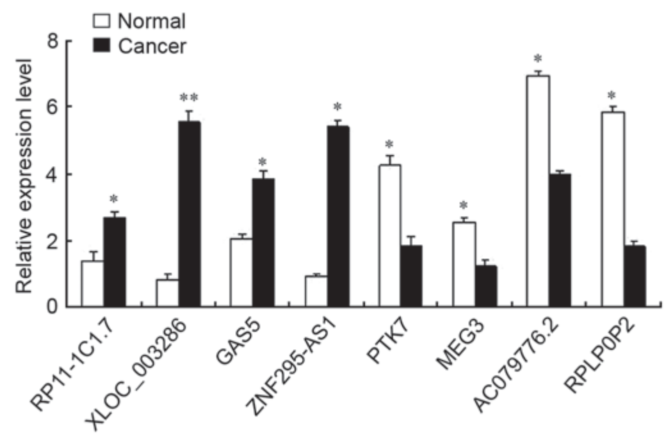

Figure 1. Validation of differentially expressed long non-coding RNAs using reverse transcription-quantitative polymerase chain reaction. *P<0.05, **P<0.01 vs. either normal or cancer.

with the International Federation of Gynecology and Obstetrics stage (24). The MEG3 gene is located in chromosome 14q32 (25), and is expressed in numerous normal tissues, but its expression level has been reported by various previous studies to be either downregulated or absent in a variety of tumor tissues, including ovarian cancer cells and epithelial ovarian cancer tissues (26-28). In the present study, HOTAIR was upregulated and MEG3 was downregulated in epithelial ovarian cancer vs. normal tissues. These results confirmed that HOTAIR and MEG3 serve a critical role in the occurrence, development and invasion of epithelial ovarian cancer.

GAS5 is encoded at chromosome 1q25, and was originally isolated from NIH-3T3 cells by subtractive hybridization (29). Several recent studies have shown that GAS5 is an lncRNA that functions as a tumor suppressor. For example, Cao *et al* noticed that patients with cervical cancer with reduced expression of GAS5 have significantly poorer overall survival than those with higher GAS5 expression (30). Shi *et al* reported that GAS5 expression was downregulated in non-small cell lung cancer tissues compared with that in noncancerous tissues, and was highly associated with tumor size and tumor-node-metastasis stage (31). However, in the present study, it was observed that the expression of GAS5 was upregulated in epithelial ovarian cancer compared with that in adjacent healthy tissues. The majority of scholars agree that glucocorticoids serve an important role in the regulation of ovarian epithelial function, and they are closely associated with the occurrence and development of ovarian cancer (32,33). In another study, glucocorticoids were demonstrated to significantly inhibit the proliferation of human ovarian cancer cells (34). Therefore, it can be hypothesized that, as a glucocorticoid receptor response element (GRE) analogue, GAS5 may be able to inhibit glucocorticoid production by competing with GRE to associate with the DNA-binding domain of the glucocorticoid receptor (35).

To understand the function of the targets of DE lncRNAs, GO terms and KEGG pathway annotation were applied in the present study to the target gene pool. The GO analysis revealed that the DE genes were associated with mitogen-activated protein kinase phosphatase activity, major

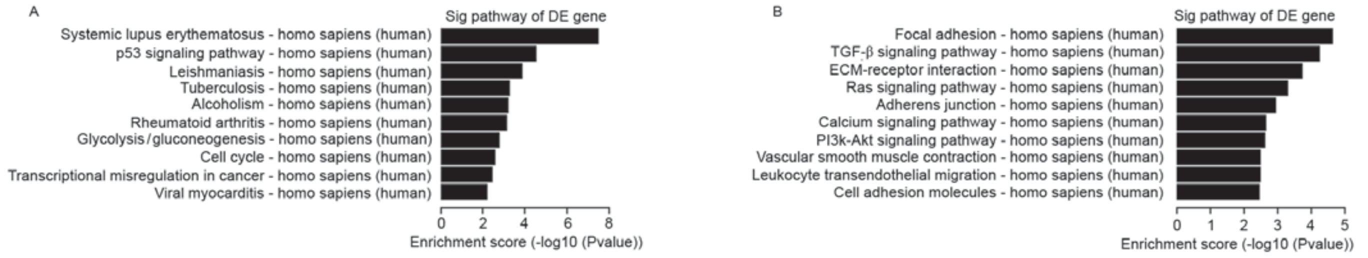

Figure 2. Pathway analysis. The bar plots show the top 10 enrichment scores [-$\log_{10}$ (P-value)] of the most significant enrichment pathways. (A) Upregulation in cancer vs. normal cells. (B) Downregulation in cancer vs. normal cells. TGF, transforming growth factor; ECM, extracellular matrix; PI3k, phosphoinositide 3 kinase; Sig, significant; DE, differentially expressed.

Figure 3. Gene ontology analysis. The bar plots show the 10 most significant enrichment terms with the most number of differentially expressed genes. The ontology covers three domains: BP, CC and MF. (A-C) Upregulation in cancer vs. normal cells. (D-F) Downregulation in cancer vs. normal cells. MAP, mitogen-activated protein; MHC, major histocompatibility complex; BP, biological processes; CC, cellular components; MF, molecular function.

histocompatibility complex class II receptor activity and DNA metabolic processes, which is consistent with previous research (36-38). Previous studies have demonstrated that signaling pathways, including the Ras, p53 and transforming growth factor-β signaling pathways, serve a critical role in the regulation of pathophysiological processes in ovarian cancer (39-41). In addition to these signaling pathways, the present study also demonstrated that focal adhesion, extracellular matrix-receptor interaction, cell adhesion molecules, cell cycle, transcriptional misregulation in cancer and other

signaling pathways were involved in the pathogenesis of epithelial ovarian cancer.

In summary, the present study identified lncRNAs that were aberrantly expressed in epithelial ovarian cancer compared with their expression in matched normal tissue. Further studies are required to reveal the possible biological functions and mechanism of these lncRNAs.

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