Expression and clinical significance of estrogen-regulated long non-coding RNAs in estrogen receptor α-positive ovarian cancer progression

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Abstract. Estrogen (E2) has long been implicated in epithelial ovarian cancer (EOC) progression. The effects of E2 on cancer progression can be mediated by numerous target genes, including coding RNAs and, more recently, non-coding RNAs (ncRNAs). Among the ncRNAs, long ncRNAs (lncRNAs) have emerged as new regulators in cancer progression; therefore, our aim was to determine whether the expression of any lncRNAs is regulated by E2 and, if so, whether a subset of these lncRNAs have some clinical significance in EOC progression. A microarray was performed to identify E2-regulated lncRNAs in E2 receptor (ER) α-positive EOC cells. Bioinformatics analyses of lncRNAs were conducted, focusing on Gene Ontology and pathway analyses. Quantitative real-time polymerase chain reactions were performed to confirm the expression of certain lncRNAs in ERα-positive EOC tissues. The correlation between certain lncRNA expression and clinicopathological factors as well as prognosis in ERα-positive EOC patients was then analyzed. We showed that 115 lncRNAs exhibited significant changes in E2-treated SKOV3 cells compared with untreated controls. Most of these lncRNAs were predicated to have potential to contribute to cancer progression. Notably, three candidates (TC0100223, TC0101686 and TC0101441) were aberrantly expressed in ERα-positive compared to ERα-negative EOC tissues, showing correlations with some malignant cancer phenotypes such as advanced FIGO stage and/or high histological grade. Furthermore, multivariate analysis indicated that TC0101441 was an independent prognostic factor for overall survival. Taken together, these results indicate for the first time that E2 can modulate lncRNA expression in ERα-positive EOC cells and that certain lncRNAs are correlated with advanced cancer progression and suggestive of a prognostic indicator in ERα-positive EOC patients. Knowledge of these E2-regulated lncRNAs could aid in the future understanding of the estrogenic effect on EOC progression and may assist in the clinical design of new target therapies based on a perspective of lncRNA.

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Introduction

Epithelial ovarian cancer (EOC) is the most deadly malignancy of the female reproductive tract in many countries (1,2). Involvement of steroid hormones, primarily estrogen, has been associated with EOC. Ample evidence from epidemiologic, clinical and experimental research has demonstrated that E2 is responsible for promoting EOC progression (3-9). Although the effects of E2 on EOC progression have been extensively studied, the underlying mechanisms remain unknown and the clinical response to steroid hormone therapy remains disappointing. Thus, fully identifying the contributions of E2 to EOC progression is urgently required.

Compelling data have demonstrated that the effects of E2 on EOC development are mediated by the regulation of target genes involved in the control of cancer progression. Previous studies, including ours, have identified a panel of aberrantly expressed E2-regulated protein-coding genes that are involved in cellular growth control, such as cyclin D1 and c-myc, and in cellular metastasis control, such as fibulin-1, cathepsin-D, HIF-1, nm23-H1, E-cadherin and MMP-2 (4-7,10,11). Despite these protein-coding genes, undoubtedly, the set of genes that directly mediate estrogenic effects on EOC progression has not been fully defined. Therefore, exploration of new E2-regulated genes is needed, which may help elucidate estrogenic effects on EOC progression and provide optional therapeutic targets.

The human transcriptome was found to be more complex than a collection of protein-coding genes, showing extensive non-coding RNA (ncRNA) expression (12). Long ncRNAs

(lncRNAs; >200 nt in length), initially argued to be spurious transcriptional noise (13), are emerging as new regulators in the cancer paradigm. Aberrant expression of lncRNAs has been reported to be associated with malignant phenotypes in various human tissues, and some lncRNAs, such as HOTAIR, MALAT-1, H19, HULC, lincRNA-p21 and MEG3, might also function as tumor suppressor genes or oncogenes (14-19). Although several published studies have reported lncRNAs such as lncRNA-LSINCT5 and HOST2 in EOC (20,21), to our knowledge, no studies have focused on E2-regulated lncRNAs in EOC.

Therefore, we sought to identify E2-regulated lncRNAs in EOC. We found that E2 stimulation of ERα-positive (ERα+) EOC cells resulted in a panel of differentially expressed lncRNAs, showing great potential to contribute to cancer progression based on bioinformatics analyses. Moreover, we found that some candidate lncRNAs were aberrantly expressed in ERα+ compared to ERα-negative (ERα-) EOC tissues, and their differential expression was associated with certain clinicopathological variables and poor prognosis of ERα+ EOC patients. Our results highlight for the first time the potential use of lncRNAs as causal link with estrogenic effects on EOC progression and as surrogate targets to hormone therapy.

Materials and methods

Cells and treatment. The ovarian cancer cell line SKOV3 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SKOV3 cells were routinely maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained at 37˚C with 5% CO_2. For the E2 induction experiments, cells (plated at 20-30% confluence) were grown for 3 days in phenol red-free RPMI-1640 (Gibco) containing 5% activated, charcoal-treated foetal bovine serum (Serana, Bunbury, Australia). Next, the cells were treated for 24 h with 10^{-8} M E2 or vehicle alone (DMSO, 0.01% of final volume) as a control.

RNA extraction and microarray. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from SKOV3 cells with or without 24-h treatment with 10^{-8} M E2. There were three replicates of each sample; they were purified using an RNeasy Micro kit (cat. #74004; Qiagen GmbH, Hilden, Germany). An Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to quantify the RNA and evaluate its integrity; the 28S:18S ratio was determined, and an RNA integrity number (RIN) was assigned to each sample. RNA with no evidence of degradation and no signs of DNA contamination (as indicated by an RIN ≥7.0 and a 28S:18S ratio ≥0.7) was processed for further analysis.

The lncRNA and mRNA expression profiles were obtained using the Glue Grant Human Transcriptome (GG-H) Array, which was manufactured by Affymetrix and Stanford University. This array contains 5,869 probes covering 730 non-coding, functional RNAs and 3,292,929 probes covering 27,670 coding genes collected from RefSeq, Ensembl and UCSC Known Genes, based on human genome assembly hg18 (22). The array experiments and computational analysis were performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Briefly, 0.2 μg of total RNA was amplified and labelled, and 20 μg of labelled cDNA was loaded onto the array. The array was hybridized and washed using the GeneChip® Hybridization, Wash and Stain kit (cat. #900720), Hybridization Oven 645 (cat. #00-0331-220V) and Fluidics Station 450 (cat. #00-0079). The slides were scanned in a GeneChip® Scanner 3000 (cat. #00-00212). The raw data were obtained using Command Console Software 3.1 with the default settings and were processed using Affymetrix Power Tools with Robust Multiarray Analysis (RMA) for background correction, normalization and summarization. Differentially expressed genes [defined as a fold-change ≥1.5 and a P-value <0.05 (t-test)] were selected for further study.

Bioinformatics functional analysis of E2-regulated lncRNAs
Identification of lncRNA-mRNA targeting pairs. Two procedures were performed to search for the target mRNAs of lncRNAs. First, UCSC hg18 (http://genome.ucsc.edu/) was used to predict lncRNA targets. Target genes under *cis*-regulatory control were defined as genes whose transcription was regulated by lncRNAs in nearby genomic locations (≤10 kbp upstream or downstream) (23). Based on mRNA sequence complementarity and RNA duplex energy prediction, *trans*-acting target genes were identified using BLAST software in the first round of screening (with the parameter e <1E-5) and RNAplex software for final verification (with the parameter -e -20) (24). Additionally, to improve the accuracy of the target prediction, the predicted lncRNA targets (both *cis* and *trans*) were combined with the differentially expressed mRNAs in the profile. The resultant overlapping mRNAs were considered the final putative targets of the differentially expressed lncRNAs. This information formed the basis for determining the lncRNA-mRNA targeting pairs.

Gene ontology (GO) and pathway analysis. For the GO and pathway analyses, the putative targets were initially inputted into the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/), which searched the GO terms to identify the molecular function represented in the gene profile (25), and then into the database of the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/) to analyze the roles of the targets in molecular pathways (26).

Tissue samples and patient data. The study included 95 patients who underwent surgery for primary ovarian cancer in the Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University between January 2006 and December 2008. Patients were included based on the availability of tissue and follow-up data. Patients with borderline ovarian tumors or with two or more different malignancies were excluded from the study. None of the patients had received preoperative radiotherapy, chemotherapy or hormonal therapy. All EOC tissue samples were frozen immediately after surgery and stored in liquid nitrogen until use.

Clinical and pathological data of EOC patients were evaluated by reviewing medical charts and the original pathology reports. Staging and grading were evaluated in accordance with the criteria of the International Federation of Gynecologists and Obstetricians (FIGO) and the World Health Organization

Table I. Primer sequences of the studied genes.

Gene	Primer	Sequence (5'-3')
TC1500845	F:	ACCACGACTCCCAAGAGGTA
	R:	CAGCTGCGATGGTGAGAACT
TC0101441	F:	CAAGGCAGGTGAGAACGAGT
	R:	CTCGACTTAGGGAGCTGCAC
TC0100223	F:	ATGAGGGCTCTGCTCTATGAATGG
	R:	GGCTTGTTCAGTGTCTGTTAAGGGT
TC0101686	F:	GGCTACTTACATGGTCCAGCA
	R:	TAGCATGGAAAGGACCACTGC
GAPDH	F:	TGACTTCAACAGCGACACCCA
	R:	CACCCTGTTGCTGTAGCCAAA

F, forward; R, reverse.

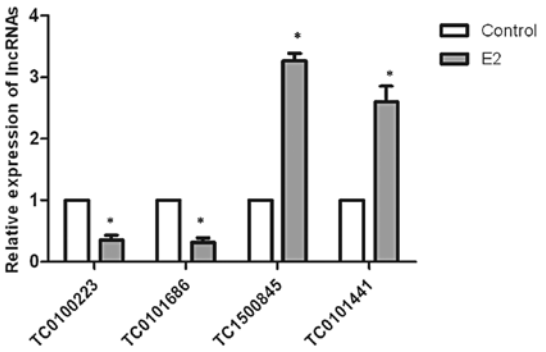

Figure 1. Relative expression of the four selected E2-regulated lncRNAs. The expression levels of four selected lncRNAs in the absence and presence of E2 in SKOV3 cells. *P<0.05 in comparison to E2- and vehicle-treated samples by qRT-PCR. Error bar denotes the standard deviation.

(WHO). Follow-up data were obtained by reviewing the out patient charts, contacting patients or correspondence. Overall survival (OS) was calculated from the date of surgery until the date of mortality or end of follow-up (January 2013). The present study was approved by the Research Ethics Committee of Fudan University, China. Informed consent was obtained from all the patients.

Immunohistochemistry. The immunohistochemical study of ERα was performed using a standard streptavidin-peroxidase method. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. For the antigen retrieval, slides were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 15 min in a microwave oven. Non-specific binding was blocked by 5% normal goat serum for 10 min. The slides were incubated with a 1:50 dilution of monoclonal antibody against ERα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4˚C overnight in a moist chamber. The slides were sequentially incubated with biotinylated goat anti-mouse IgG (1:100 dilution; Santa Cruz Biotechnology) and then streptavidin-peroxidase conjugate, each for 30 min at room temperature. Isotope-matched human IgG was used in each case as a negative control. Finally, the 3,5-diaminobenzidine (DAB) Substrate kit (Dako) was used for color development followed by Mayer's hematoxylin counterstaining. ERα+ cases were defined as tumors with >10% stained nuclei (27).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR analysis of lncRNA expression was performed using FastStart Universal SYBR-Green Master (Rox; Roche) and an ABI Prism 7900 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Briefly, total RNA was extracted from cells and tissues and converted to cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturer's protocol. The PCR amplifications were performed in a 10-μl (total volume) reaction that included 1 μl of cDNA template (~5 ng), 5 μl of FastStart Universal SYBR-Green Master (Rox), 3.6 μl of double-distilled water and 0.2 μl of each pair of forward and reverse primers (Table I; Sangon Biotech Co., Ltd., Shanghai, China). The PCR conditions included an initial denaturation step at 95˚C for 10 min and 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. All the experiments were performed in triplicate, and all the samples were normalized to GAPDH expression. The expression fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. The data were processed using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). Comparison of continuous data was analyzed using the Student's t-test, whereas categorical data was analyzed using the Chi-square test and Fisher's exact test where appropriate (when the expected frequency was <5). OS curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Variables were used in multivariate analysis on the basis of the Cox proportional hazards model. A P-value <0.05 was considered to indicate a statistically significant difference (P<0.05).

Results

Identification of E2-regulated lncRNAs in ERα+ ovarian cancer cells

Identification of E2-regulated lncRNAs in SKOV3 cells. As our previous studies provided evidence that E2 regulated some protein-coding genes in ERα+ ovarian cancer SKOV3 cells (5-7), we examined whether the expression of any lncRNAs is also regulated by E2 in SKOV3 cells. In the present study, SKOV3 cells were treated with 10^{-8} M E2 for 24 h, and changes in the lncRNA expression profile were analyzed by performing a microarray. The microarray data indicated that 115 lncRNAs were significantly dysregulated following E2 treatment, including 51 upregulated and 64 downregulated lncRNAs (fold-change ≥1.5, P<0.05; data not shown). The top ten relative increased and decreased E2-regulated lncRNAs are listed in Table II.

To confirm the microarray findings, we examined the expression of four lncRNAs selected from the top ten relative increased and decreased E2-regulated lncRNAs using qRT-PCR. The results revealed that the expression levels of TC0100223 and TC0101686 were significantly downregulated by E2, whereas TC1500845 and TC0101441 were significantly

Table II. The top ten relative increased and decreased E2-regulated lncRNAs in SKOV3 cells.

			Annotations			
Probeset_id	Regulated	Fold-change (E2/control)	Seqname	Start	End	Strand
TC0500815	Upregulated	3.419413635	chr5	1043143	1050457	-
TC0101441	Upregulated	3.275356271	chr1	202377159	202378011	+
TC0901107	Upregulated	3.269435942	chr9	89871170	89871958	-
TC0301101	Upregulated	3.258219523	chr3	37825199	37878275	-
TC1900181	Upregulated	3.247328231	chr19	10820078	10841404	+
TC1201706	Upregulated	3.232226492	chr12	131237621	131240193	-
TC0601086	Upregulated	3.186614845	chr6	29802359	29824805	-
TC1500845	Upregulated	3.183351441	chr15	38773376	38774597	-
TC0300769	Upregulated	3.143316689	chr3	169450147	169514658	+
TC0X00076	Upregulated	2.989272086	chrX	18821244	18823011	+
TC0501141	Downregulated	0.300418806	chr5	90642594	90645975	-
TC1201365	Downregulated	0.301520979	chr12	64556922	64561625	-
TC0101686	Downregulated	0.30390326	chr1	244341256	244343791	+
TC1200811	Downregulated	0.31213872	chr12	125146739	125152812	+
TC0300928	Downregulated	0.318626574	chr3	197148023	197150980	+
TC1900906	Downregulated	0.325966672	chr19	61499140	61513631	+
TC0801241	Downregulated	0.328971081	chr8	128289293	128300515	-
TC1400428	Downregulated	0.333717978	chr14	88886369	88900796	+
TC0201596	Downregulated	0.335402449	chr2	69870840	69879634	-
TC0100223	Downregulated	0.342972038	chr1	21465570	21466331	+

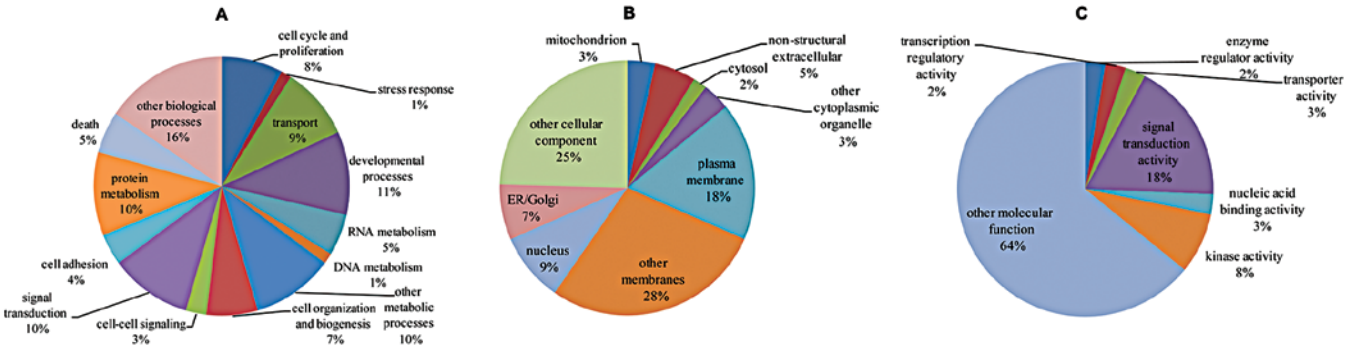

Figure 2. GO enrichment analysis of lncRNA targets. GO annotations of lncRNA targets categorized by (A) biological process, (B) cell component and (C) molecular function.

upregulated by E2 in SKOV3 cells, consistent with the microarray results (Fig. 1).

Putative targets of E2-regulated lncRNAs and their functional analysis. Based on the overlap between the targets predicted by bioinformatics and the differentially expressed mRNAs detected in the microarray, we constructed 55 E2-regulated lncRNA-target mRNA pairs (Table III). The GO (Fig. 2) and pathway (Table IV) analyses showed that a set of E2-regulated lncRNAs that mapped to the target mRNAs were correlated with several cellular processes and pathways known to be related to cancer progression, such as cell cycle and proliferation, developmental processes, cell adhesion, cell death, MAPK signaling, Hedgehog signaling, Jak-STAT signaling and cancer pathways, suggesting their great potential to contribute to cancer progression.

Expression of several candidate lncRNAs in ERα+ EOC tissues. In order to confirm the potential of some E2-regulated lncRNAs to contribute to cancer progression, we initially selected the four lncRNAs (TC0100223, TC0101686, TC1500845 and TC0101441) as candidates and tested their expression levels in EOC tissues. Considering the fact that ERα is the main form expressed in malignant ovarian tumors and as ERα has been reported to promote poor prognosis in EOC patients (28,29), we determined whether the expres-

Table III. lncRNA-target mRNA pairs regulated by E2 in SKOV3 cells.

Information of lncRNAs				lncRNA-mRNA pairs			Information of mRNAs			
Start	End	Strand	Seqname	lncRNA Probeset_id	mRNA Symbol	Type	Start	End	Strand	Seqname
43821718	43827655	+	chr20	TC2000321	CD40	cis	44180313	44366257	+	chr20
43821718	43827655	+	chr20	TC2000321	UBE2C	cis	43874662	43879003	+	chr20
63310787	63321606	-	chr19	TC1901868	ZNF544	cis	63432092	63480673	+	chr19
63310787	63321606	-	chr19	TC1901868	ZSCAN4	cis	62872115	62882317	+	chr19
63310787	63321606	-	chr19	TC1901868	ZNF417	cis	63110053	63119796	-	chr19
63310787	63321606	-	chr19	TC1901868	ZNF460	cis	62483670	62496635	+	chr19
61499140	61513631	+	chr19	TC1900906	ZNF460	cis	62483670	62496635	+	chr19
10820078	10841404	+	chr19	TC1900181	CDC37	cis	10362809	10375271	-	chr19
10820078	10841404	+	chr19	TC1900181	QTRT1	cis	10673106	10805160	+	chr19
75925981	75926392	+	chr17	TC1700826	KIAA1618	cis	75849262	75925295	+	chr17
29738362	29740074	-	chr16	TC1600989	QPRT	cis	29597859	29616810	+	chr16
70157455	70163889	+	chr16	TC1600575	LOC652737	cis	69398791	69457733	-	chr16
38773376	38774597	-	chr15	TC1500845	TYRO3	cis	39638524	39658826	+	chr15
38773376	38774597	-	chr15	TC1500845	IVD	cis	38484978	38515438	+	chr15
67751133	67757033	+	chr15	TC1500441	PAQR5	cis	67378348	67486098	+	chr15
21379262	21379796	+	chr14	TC1400062	ABHD4	cis	22136986	22151097	+	chr14
11599456	11603583	+	chr12	TC1200137	TAS2R7	cis	10845399	10846493	-	chr12
7154170	7159091	+	chr12	TC1200076	CDCA3	cis	6824224	6830686	-	chr12
7154170	7159091	+	chr12	TC1200076	CLSTN3	cis	7174234	7202795	+	chr12
7154170	7159091	+	chr12	TC1200076	ING4	cis	6629707	6642565	-	chr12
7154170	7159091	+	chr12	TC1200076	PTPN6	cis	6926001	6940741	+	chr12
6966612	7033762	+	chr12	TC1200074	CDCA3	cis	6824224	6830686	-	chr12
6966612	7033762	+	chr12	TC1200074	CLSTN3	cis	7174234	7202795	+	chr12
6966612	7033762	+	chr12	TC1200074	ING4	cis	6629707	6642565	-	chr12
6966612	7033762	+	chr12	TC1200074	PTPN6	cis	6926001	6940741	+	chr12
5383589	5384883	+	chr12	TC1200045	NTF3	cis	5473527	5474725	+	chr12
58457692	58582501	-	chr11	TC1101435	STX3	cis	59279108	59326752	+	chr11
18821244	18823011	+	chrX	TC0X00076	GPR64	cis	18917348	19050676	-	chrX
66450847	66456323	-	chr9	TC0900981	PIK3C2B	trans	2.03E+08	202730566	-	chr1
12264367	12270292	+	chr8	TC0800087	DLC1	cis	12985243	13506486	-	chr8
12264367	12270292	+	chr8	TC0800087	CTSB	cis	11737442	11763147	-	chr8
1.28E+08	1.28E+08	-	chr7	TC0701620	OPN1SW	cis	1.28E+08	128203087	-	chr7
1.28E+08	1.28E+08	-	chr7	TC0701620	TSPAN33	cis	1.29E+08	128595907	+	chr7
1.28E+08	1.28E+08	-	chr7	TC0701620	SMO	cis	1.29E+08	128640619	+	chr7
1.42E+08	1.42E+08	+	chr7	TC0700794	TAS2R5	cis	1.41E+08	141137635	+	chr7
1.4E+08	1.41E+08	+	chr7	TC0700753	TAS2R5	cis	1.41E+08	141137635	+	chr7
29802359	29824805	-	chr6	TC0601086	KIAA1949	cis	30752146	30763651	-	chr6
1.5E+08	1.5E+08	-	chr5	TC0501415	TNIP1	cis	1.5E+08	150446914	-	chr5
1.5E+08	1.5E+08	-	chr5	TC0501415	SLC36A1	cis	1.51E+08	150852132	+	chr5
1.5E+08	1.5E+08	-	chr5	TC0501415	ANXA6	cis	1.5E+08	150517636	-	chr5
1.5E+08	1.5E+08	-	chr5	TC0501415	CCDC69	cis	1.51E+08	150583899	-	chr5
1043143	1050457	-	chr5	TC0500815	LPCAT1	cis	1514544	1577092	-	chr5
1.15E+08	1.15E+08	-	chr4	TC0401208	ARSJ	cis	1.15E+08	115120306	-	chr4
37825199	37878275	-	chr3	TC0301101	XYLB	cis	38363244	38431471	+	chr3
1.35E+08	1.35E+08	+	chr3	TC0300635	AMOTL2	cis	1.36E+08	135576450	-	chr3
1.24E+08	1.24E+08	+	chr3	TC0300552	STXBP5L	cis	1.22E+08	122621336	+	chr3

Table III. Continued.

Information of lncRNAs				lncRNA-mRNA pairs			Information of mRNAs			
Start	End	Strand	Seqname	lncRNA Probeset_id	mRNA Symbol	Type	Start	End	Strand	Seqname
1.14E+08	1.14E+08	+	chr2	TC0200609	PAX8	cis	1.14E+08	113752969	-	chr2
2.46E+08	2.46E+08	-	chr1	TC0103436	ZNF670	cis	2.45E+08	245308738	-	chr1
2.44E+08	2.44E+08	+	chr1	TC0101686	ZNF670	cis	2.45E+08	245308738	-	chr1
2.02E+08	2.02E+08	+	chr1	TC0101441	PIK3C2B	cis	2.03E+08	202730566	-	chr1
2.02E+08	2.02E+08	+	chr1	TC0101441	ATP2B4	cis	2.02E+08	201979832	+	chr1
53566493	53569511	+	chr1	TC0100566	C1orf163	cis	52925096	52936964	-	chr1
53566493	53569511	+	chr1	TC0100566	CC2D1B	cis	52588855	52604453	-	chr1
21465570	21466331	+	chr1	TC0100223	ECE1	cis	21417664	21544621	-	chr1
21465570	21466331	+	chr1	TC0100223	RAP1GAP	cis	21795301	21868437	-	chr1

Table IV. Target mRNA-related pathways in SKOV3 cells.

Term	Count	Genes
Pentose and glucuronate interconversions	1	XYLB
Valine, leucine and isoleucine degradation	1	IVD
Inositol phosphate metabolism	1	PIK3C2B
Nicotinate and nicotinamide metabolism	1	QPRT
Metabolic pathways	4	IVD, QPRT, XYLB, PIK3C2B
MAPK signaling pathway	1	NTF3
Phosphatidylinositol signaling system	1	PIK3C2B
Ubiquitin mediated proteolysis	1	UBE2C
SNARE interactions in vesicular transport	1	STX3
Hedgehog signaling pathway	1	SMO
Adherens junction	1	PTPN6
Jak-STAT signaling pathway	1	PTPN6
Natural killer cell mediated cytotoxicity	1	PTPN6
T cell receptor signaling pathway	1	PTPN6
B cell receptor signaling pathway	1	PTPN6
Neurotrophin signaling pathway	1	NTF3
Pathways in cancer	1	SMO
Basal cell carcinoma	1	SMO

sion of *in vitro* E2-regulated lncRNAs, detected in ERα+ ovarian cancer cells, could discriminate between ERα+ and ERα- EOC tissues. Based on the qRT-PCR assay, we found that ERα+ tissues had lower expression of TC0100223 and TC0101686 and higher expression of TC0101441 (Fig. 3, ERα+, n=64 vs. ERα-, n=31, $P<0.01$; Fig. 3A shows the representative immunohistochemistry results of ERα expression in EOC tissues). In contrast, TC1500845 was not differentially expressed between ERα+ and ERα- EOC tissues. These results may be suggestive of the potential clinical significance of TC0100223, TC0101686 and TC0101441 in ERα+ EOC.

Association of lncRNA expression with clinicopathological characteristics in ERα+ EOC. According to the median value which was used as the cut-off (30), specific lncRNA expression in ERα+ EOC tissues, equal or more than median value was defined as high lncRNA group, and less than median value was defined as low lncRNA group. As shown in Table V, low-expression of TC0100223 and TC0101686 and high-expression of TC0101441 were closely related to ERα+ EOC tissues with advanced FIGO stage and/or high histological grade ($P<0.05$), suggesting that aberrant expression of the three candidate lncRNAs is associated with a more malignant ovarian cancer phenotype.

Table V. Association of lncRNA expression with clinicopathological variables in ERα-positive EOC patients.

Variables	Cases (N)	High TC0101441 expression		Low TC0100223 expression		Low TC0101686 expression	
		n (%)	P-value	n (%)	P-value	n (%)	P-value
Age (years)							
<50	25	14 (56.0)	0.442	10 (40.0)	0.2	15 (60.0)	0.2
≥50	39	18 (46.2)		22 (56.4)		17 (43.6)	
Histological subtype							
Serous	49	23 (46.9)	0.376	24 (49.0)	0.768	22 (44.9)	0.14
Other	15	9 (60.0)		8 (53.3)		10 (66.7)	
FIGO stage							
I-II	24	5 (20.8)	<0.001	8 (33.3)	0.039	7 (29.2)	0.01
III-IV	40	27 (67.5)		24 (60.0)		25 (62.5)	
Histological grade							
G1-G2	27	6 (22.2)	<0.001	9 (33.3)	0.023	11 (40.7)	0.206
G3	37	26 (70.3)		23 (62.2)		21 (56.8)	
Ascites							
>100	20	11 (55.0)	0.59	7 (35.0)	0.106	12 (60.0)	0.281
≥100	44	21 (47.7)		25 (56.8)		20 (45.5)	

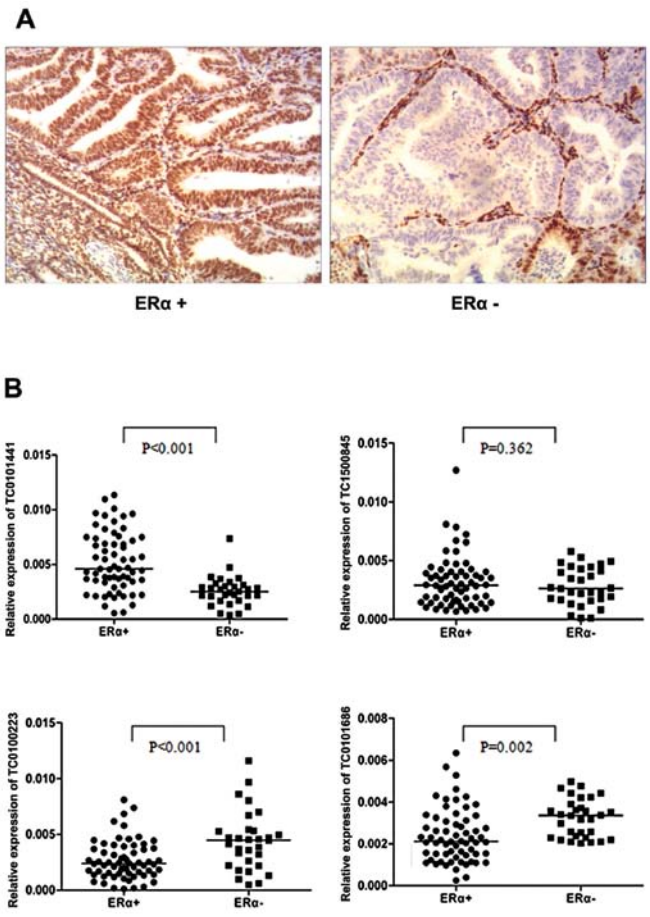

Figure 3. lncRNA expression in EOC tissues (ERα+ vs. ERα-). (A) Representative immunohistochemistry results of ERα expression in EOC tissues; original magnification, x200. (B) Relative expression levels of TC0101441, TC1500845, TC0100223 and TC0101686 in EOC tissues.

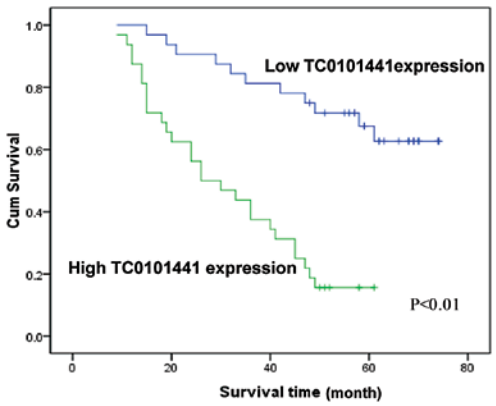

Figure 4. Survival curves of ERα+ EOC patients according to TC0101441 expression. Patients with high TC0101441 expression showed significantly poorer prognosis than those with low TC0101441 expression (P<0.01, log-rank test).

Association of lncRNA expression with prognosis of ERα+ EOC patients. We investigated whether the expression of TC0100223, TC0101686 and TC0101441 correlated with the postoperative survival of ERα+ EOC patients. Among the 64 ERα+ EOC patients, 38 died during follow-up. In univariate analysis, OS was associated with the FIGO stage, histological grade and expression of TC0100223 and TC0101441 (P<0.05; Table VI). Multivariate analysis further confirmed that high TC0101441 expression, advanced FIGO stage and high histological grade were independent factors for evaluation of OS in ERα+ EOC patients (P<0.05, Table VI; Fig. 4 shows the OS curves according to TC0101441 expression). Thus, it was concluded that of the three candidate lncRNAs, TC0101441 could be used as an independent prognostic factor for OS of ERα+ EOC patients.

Table VI. Univariate analysis for overall survival in ERα-positive EOC patients.

	Univariate analysis		Multivariate analysis					
	Overall survival		Overall survival					
Variables	Mean ± SE (months)	P-value	β	SE	Wald	P-value	Exp (β)	95% CI
Age (years)								
<50	45.52±4.21	0.939	-	-	-	-	-	-
≥50	47.49±3.98		-	-	-	-	-	-
Histological subtype								
Serous	45.99±3.53	0.496	-	-	-	-	-	-
Other	52.30±5.71		-	-	-	-	-	-
FIGO stage								
I-II	69.63±2.42		-	-	-	-	-	-
III-IV	33.03±2.73	<0.001	2.305	0.635	13.168	<0.001	10.022	2.886-34.803
Histological grade								
G1-G2	64.81±2.97		-	-	-	-	-	-
G3	32.62±2.90	<0.001	0.991	0.481	4.238	0.04	2.693	1.049-6.915
Ascites								
<100	44.71±5.95	0.77	-	-	-	-	-	-
≥100	48.67±3.48		-	-	-	-	-	-
TC0101686 expression								
Low	44.45±4.23	0.325	-	-	-	-	-	-
High	50.32±4.28		-	-	-	-	-	-
TC0100223 expression								
Low	37.48±3.48	0.018	-	-	-	-	-	-
High	54.40±3.99		0.019	0.352	0.003	0.957	0.981	0.492-1.956
TC0101441 expression								
Low	60.88±3.48		-	-	-	-	-	-
High	32.16±3.04	<0.001	0.817	0.402	4.122	0.042	2.263	1.029-4.979

β, regression coefficient; SE, standard error; CI, confidence interval.

Discussion

In the present study, we identified a series of differentially expressed E2-regulated lncRNAs in ERα+ ovarian cancer SKOV3 cells using a microarray. Bioinformatics functional analyses indicated that a fraction of these lncRNAs had the potential to contribute to cancer progression. Furthermore, in order to confirm that some E2-regulated lncRNAs are related to the development of ERα+ EOC, we tested the expression of several candidate lncRNAs in EOC tissues. The results showed that some candidate lncRNAs were aberrantly expressed in ERα+ compared to ERα- EOC tissues, and their differential expression was associated with certain clinicopathological variables and poor prognosis of ERα+ EOC. To the best of our knowledge, this is the first study to report E2-regulated lncRNAs in ERα+ EOC, the results of which may provide insight into the estrogenic effects on EOC progression and the design of new target therapies based on a perspective of lncRNA.

It is known that the ovary is a main source and target tissue of E2 in women. The action of E2 on ovarian tissue is believed to be mediated by the two ERs, ERα and ERβ. ERβ is highly expressed in normal epithelial ovarian cells and benign tumors, whereas ERα is expressed to a much greater extent in malignant ovarian tumors (28). Several studies thus far have revealed the contributions to EOC progression by multiple E2/ERα-regulated target protein-coding genes, such as cyclin D1 and c-myc (which are involved in cellular growth control) and fibulin-1 and cathepsin-D (which are involved in cellular motility and invasion) (10,11).Our previous studies also showed that E2 promoted metastasis and invasion in ERα+ ovarian cancer SKOV3 cells by regulating HIF-1, nm23-H1, E-cadherin and MMP-2 (5-7). Despite these protein-coding genes, however, the exact effects of E2 on EOC development remain unclear. In the present study, we identified 115 E2-regulated lncRNAs in ERα+ SKOV3 cells using a microarray, and subsequent bioinformatics analyses indicated that a subset of these lncRNAs had the potential to contribute to cancer progression. These findings may extend our current knowledge regarding E2 regulation of protein-coding genes in EOC progression to include lncRNAs.

lncRNAs, initially argued to be spurious transcriptional noise, are emerging as new regulators in the cancer paradigm. Misregulation of lncRNAs has been documented in many types of human cancer. For example, DDC and PCGEM are overexpressed in prostate cancer compared to normal prostate tissue, implicating their roles in tumorigenesis (31,32). Increased expression of MALAT1 indicates a poorer clinical outcome of lung cancer patients (15). HOTAIR is overexpressed in primary breast tumors and metastases, and elevated HOTAIR expression is an indispensable predictor of eventual metastasis and mortality (14). Inspired by these lines of evidence of lncRNA roles in cancer biology, we hypothesized that certain E2-regulated lncRNAs detected in SKOV3 cells in the current study may also have the potential to contribute to EOC progression. To address this hypothesis, we initially tested two upregulated (TC1500845 and TC0101441) and two downregulated lncRNAs (TC0100223 and TC0101686) in EOC tissues, the results of which showed a significant correlation of overexpressed TC0101441 and low-expressed TC0100223 and TC0101686 with ERα+ compared to ERα- EOC. Moreover, low-expression of TC0100223 and TC0101686 and overexpression of TC0101441 were found to be related to ERα+ EOC tissues with advanced FIGO stage and/or high histological grade. Most importantly, multivariate survival analysis revealed that TC0101441 was an independent prognostic factor for overall survival. Taken together, our findings suggest that the aberrant expression of certain E2-regulated lncRNAs is associated with malignant cancer phenotypes and poor clinical outcome of ERα+ EOC patients. Hence, these results may also lead us to consider that E2-regulated lncRNAs can be used as candidate biomarkers for EOC prognosis and therapy. Clearly, further studies are required to elucidate the roles and mechanisms by which these lncRNAs promote EOC development in detail.

In conclusion, the present study provided the first evidence that E2 can modulate a panel of lncRNAs in ERα+ EOC cells. Some aberrantly expressed lncRNAs, including TC0100223, TC0101686 and TC0101441, are correlated with the advanced cancer phenotypes. Of note, TC0101441 was an independent factor for poor prognosis of ERα+ EOC patients. Collectively, encouraged by the involvement of these E2-regulated lncRNAs in ERα+ EOC progression, our data highlight the utility of considering lncRNA expression in the future understanding of estrogenic effects on EOC progression and in the design of new target therapies.

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