SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 6079-6084, 2017

Transcriptome analysis reveals dysregulated long non-coding RNAs and mRNAs associated with extrahepatic cholangiocarcinoma progression

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Received February 26, 2016; Accepted February 7, 2017

DOI: 10.3892/ol.2017.6987

Abstract. The incidence of extrahepatic cholangiocarcinoma (ECC) is the highest of all the cholangiocarcinoma cases. However, the molecular mechanism of ECC genesis and progression remains unclear. Long non-coding RNAs (lncRNAs) have been revealed to perform critical regulatory roles in cancer biology. In order to understand lncRNA expression patterns and their potential function in ECC, a transcriptome analysis of lncRNA and mRNA expression was performed in ECC and paired adjacent non-cancerous tissues using Agilent human lncRNA + mRNA arrayV4.0 (4x180 K format). It was identified that 268 lncRNAs and 459 mRNAs were differentially expressed in ECC. Among these, 78 lncRNAs and 66 mRNAs were upregulated >2-fold compared with adjacent non-cancerous tissues, and 190 lncRNAs and 393 mRNAs were downregulated in the ECC samples. Differences in lncRNA expression between ECC and paired adjacent non-cancerous tissues were confirmed using reverse transcription-quantitative polymerase chain reactionas proof of principle. Functional analysis of co-expressed mRNAs with lncRNAs indicated that these dysregulated lncRNAsmay be involved in known ECC-associated biological processes and pathways. The present findings indicated that mRNAs and lncRNAs perform important roles in the development and progression of ECC. The present findings may lay the foundation for future efforts to understand the role of lncRNAs and develop novel biomarkers in ECC

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Key words: extrahepatic cholangiocarcinoma, gene expression, long non-coding RNAs, microarray

Introduction

Extrahepatic cholangiocarcinoma (ECC) is a highly malignant cancer, representing ~80% of all cholangiocarcinoma clinical cases. In previous years, the incidence and mortality of ECC has continued to increase worldwide (1). Although continued advances in surgical techniques and treatment strategies have been achieved, the 5-year survival rate for patients who undergo surgical resection has been reported to be only 20-40% (2,3). The main reasons for the poor prognosis of ECC are a low rate of early diagnosis, fast progression and a high rate of recurrence. There is currently no effective method to improve the diagnosis and treatment of ECC, and the major cause of the molecular pathogenesis of oncogenesis and the progression of ECC remains largely unclear.

Long non-coding RNAs (lncRNAs) are a large class of ncRNAs. It has been revealed that lncRNAs are involved in the development and progression of tumors, and that their abnormal expression is associated with tumor proliferation, apoptosis, the cell cycle, angiogenesis, recurrence and metastasis in numerous different types of cancer (4,5). Previous studies have demonstrated the potential roles of lncRNAs to serve as diagnostic markers and therapeutic targets for cancers (6-14). However, the role and mechanism of lncRNAs in ECC remains largely unknown. Via transcriptome analysis, the present study aimed to investigate lncRNA and mRNA expression that is up- or downregulated in ECC tissues compared with paired peritumoral tissues. Additional bioinformatics analysis and validation studies were performed to reveal an association between clinical characteristics and lncRNA expression levels. These analyses and observations indicated that alterations in lncRNA expression may become a novel biomarker or therapeutic target for ECC diagnosis and treatment.

Materials and methods

Patients and tissue samples. A total of 42 patients with ECC who underwent surgical resection at the Second Affiliated Hospital of Harbin Medical University (between January 2013 and October 2015) were included in the present study. All patients provided their written informed consent for inclusion in this study prior to surgery. Patients who were treated with preoperative radiotherapy or chemotherapy were excluded. ECC tissues and paired adjacent non-cancerous tissues were collected and immediately frozen in liquid nitrogen. Matched non-cancerous tissues were obtained from regions of at least 3 cm distant from the tumor borders (China National Genebank v1.00). A total of 3 pairs of samples were used for microarray analysis, and all samples were subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

lncRNA and mRNA microarrays. The Agilent human lncRNA + mRNA Array v4.0 was designed with four identical arrays per slide (4x180 K format), with each array containing probes interrogating ~41,000 human lncRNAs and ~34,000 human mRNAs (Agilent Technologies, Inc., Santa Clara, CA, USA). Those lncRNA and mRNA target sequences were mergedfrom multiple databases: 23,898 from GENCODE (http://www.gencodegenes.org/)/ENSEMBL(http://www.ensembl.org); 14,353 from Human LincRNA Catalog (15); 7,760 from RefSeq (https://www.ncbi.nlm.nih.gov/refseq/); 5,627 from UCSC (https://genome.ucsc.edu/); 13,701 from ncRNA Expression Database; 21,488 from LNCipedia; 1,038 from H-InvDB; 3,019 from lncRNAs-a (Enhancer-like); 1,053 from antisense ncRNA pipeline; and 407 Hox ncRNAs, 962 upstream conserved regions (UCRs) and 848 lncRNAs from the Chen Ruisheng lab (Institute of Biophysics, Chinese Academy of Science, Shanghai, China). Each RNA was detected by probes, and experiments were repeated twice. The array also contained 4,974 Agilent control probes (Agilent Technologies, Inc.).

RNA extraction and quality control. Total RNA was extracted from 42 pairs of frozen ECC tissues and matched non-cancerous tissues using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Tissue (50-100 mg) was homogenized with 1 ml TRIzol reagent in a round-bottomed tube using a glass Teflon homogenizer, and the homogenized sample was incubated for 10 min at room temperature. The sample was then centrifuged at 12,000 x g for 10 min at 4˚C and the cleared supernatant was transferred to a new tube. Chloroform (0.3 ml per 1 ml TRIzol) was added to the tube, and the tube was agitated vigorously by hand for 15 sec, and then incubated for 2-3 min at room temperature. The sample was then centrifuged at 12,000 x g for 15 min at 4˚C. The aqueous phase was removed to a fresh tube, 0.5 ml of 100% isopropanol per 1 ml TRIzol was added to the aqueous phase, and the mixture was incubated at room temperature for 10 min. Subsequently, the sample was centrifuged at 12,000 x g for 10 min at 4˚C, the supernatant was removed, and the RNA pellet was washed with 75% (v/v) ethyl alcohol (EtOH) and vortexed. This was followed by centrifugation at 7,500 x g for 5 min at 4˚C, removal of the supernatant and subsequent removal of the remaining EtOH by air drying for 5 min. Finally, diethyl pyrocarbonate water (20-50 μl) was added to resuspend the RNA pellets in the tube by passing the solution up and down several times through a pipette tip. The RNA concentrations were assessed by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

RT-qPCR. The expression of lncRNAs in ECC and adjacent non-cancerous tissues was measured by RT-qPCR using SYBR Premix Ex TaqÔ (Bioneer Corporation, Daejeon, Korea) and using the following cycling parameters: Initial denaturation at 94˚C for 5 min; followed by 40 cycles of 94˚C for 30 sec; 60˚C for 30 sec and 72˚C for 30 sec; and 72˚C for 5 min. Primers were designed by Sangon Biotech Co., Ltd. (Shanghai, China). GAPDH was used as a control. Experiments were performed in triplicate. The median in each triplicate was used to calculate relative lncRNA concentrations using the formula: $\Delta Cq=Cq_{median}$ lncRNAs-Cq_{median} GAPDH. Expression fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (16). Primer sequences: ENST00000508732 forward, 5'-ACAGAGATAGCGGAAGGACA-3' and reverse, 5'-AATGGAGGACTGGAGGGATT-3'; ENST00000519319 forward, 5'-AATGGCATGAACCTGGGAGGCG-3' and reverse, 5'-GGCTTTGGGAAGTGCTTTGGAG-3'; UC022BVT forward, 5'-TGCTAAAGCATCAGAGAAGAGAAG-3' and reverse, 5'-GGACGTTCAACCTCATTCCC-3'; ENST00000438290 forward, 5'-GAGGGTTAAACCTGGAGAAGGG-3' and reverse, 5'-GCAAGAAAATGCGAGAAGCCT-3'; ENST00000593604 forward, 5'-CATGAGGACTGAGCGCATGA-3' and reverse, 5'-TGCAGTTCCTGTAGGTCAGA-3'; and GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3'.

Microarray analysis. The lncRNA and mRNA microarray data were analyzed for data summarization, normalization and quality control using the GeneSpring software version 13.0 (Agilent Technologies, Inc.). Differentially-expressed lncRNAs and mRNAs were determined based on $P<0.05$, following Benjamini-Hochberg correction and a fold-change difference of ≥2.0. The raw microarray data was Log_2-transformed and median-centered. The hierarchical clustering with average linkage was performed for genes and samples using CLUSTER 3.0 software (17). Finally, tree visualization was performed using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA).

Construction of the lncRNA-mRNA co-expression network. The lncRNA-mRNA co-expression network was constructed based on association analysis between the differentially-expressed lncRNAs and mRNAs. For each pair of genes, Pearson's correlation coefficient was calculated and the significantly correlated pairs were selected to construct the network. LncRNAs and mRNAs with Pearson's correlation coefficients >0.99 were selected to draw the network.

Functional enrichment analysis. To investigate the potential functional roles of lncRNAs, functional enrichment analysis was performed at the gene ontology (GO; http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes

Table I. List of 10 differentially-expressed lncRNAs in ECC identified using a microarray screening in ECC and adjacent non-cancerous tissues (fold change, >2.0; P<0.05).

lncRNAs	Chr	Strand	Genomic coordinates	Expression	Fold-change
ENST00000508732.2	15	-	95822513-95870329	Upregulated	21.486
TCONS_00004225	2	-	43199538-43228604	Downregulated	7.547
ENST00000438290.1	13	+	94712716-94716246	Downregulated	6.386
ENST00000423943.1	1	+	159931014-159948851	Downregulated	5.770
TCONS_00014813	8	+	102326509-102328921	Upregulated	5.134
ENST00000515485.1	4	+	165675216-165722606	Downregulated	4.172
ENST00000437097.1	9	+	128329858-128335302	Upregulated	4.079
TCONS_00008571	4	-	128015586-128017878	Downregulated	4.043
ENST00000606993.1	1	+	1104737-1105723	Downregulated	4.042
ENST00000550334.1	12	-	72255681-72271991	Downregulated	3.954

lncRNA, long non-coding RNA; Chr, chromosome; ECC, extrahepatic cholangiocarcinoma.

(KEGG; http://www.genome.jp/kegg/) levels using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov/). P<0.05 was considered to indicate statistically significant functional annotations.

Computational predictions of lncRNA targets. The regulatory roles of lncRNA on target genes were mediated by cis- and trans-acting mechanisms. The trans-prediction was conducted using BLAT tools to compare the full sequence of the lncRNA with the 3'UTR of its co-expression mRNAs, with the default parameter setting. For cis-acting lncRNAs, the regulatory RNAs were transcribed from the same locus as that which encodes the target gene and which was performed by their tight association (Pearson's association coefficient >0.99) to a group of expressed protein-coding genes. The lncRNA resided at genomic loci where a protein-coding gene and an lncRNA gene were within 10 kb of each other along the genome (18,19); cis therefore refers to same-locus (not necessarily same-allele) regulatory mechanisms, which include antisense-mediated regulation by lncRNAs of protein-coding genes that are encoded in the same locus.

Statistical analysis. Statistical analysis was performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± standard deviation of three separate assays. Differences between groups were assessed using the two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of differentially-expressed lncRNAs and mRNAs in ECC. Differential gene expression analysis in ECC and adjacent non-cancerous samples was performed to identify dysregulated lncRNAs and mRNAs in ECC. Among the 41,000 lncRNAs and 34,000 mRNAs transcripts accessed in the present microarray, it was identified that 268 lncRNAs and 459 mRNAs were differentially expressed (fold change >2.0) between tumor and adjacent non-cancerous samples. Among them, 78 lncRNAs and 66 mRNAs were upregulated (>2-fold in ECC versus adjacent non-cancerous samples), and 190 lncRNAs and 393 mRNAs were downregulated in ECC samples. The 10 most differentially-expressed lncRNAs and mRNAs between ECC and adjacent non-cancerous tissue are listed in Table I. The data was Log_2-transformed and median-centered by genes using Adjust Data function of CLUSTER 3.0 software. Hierachial clustering analysis was then performed, and it was revealed that the expression profiles of differentially-expressed lncRNAs and mRNAs were able to distinguish ECC samples from normal tissue samples (Fig. 1A and B).

Functional analysis of differentially-expressed lncRNAs. To reveal the potential roles of lncRNAs in ECC, the association between lncRNAs and mRNAs was investigated, and a coding-non-coding gene co-expression (CNC) network was constructed by examining the association between the expression values of lncRNAs and those of the mRNAs. A total of 270 network nodes were associated with 5,788 network pairs of co-expressed lncRNAs and mRNA. The number of positively-associated pairs was greater than the number of negatively-associated pairs. The CNC network indicated that mRNAs may be associated with one or numerous lncRNAs. Similarly, lncRNAs may be associated with one or numerous mRNAs. *XLOC_002797* had 38 neighbors, whereas collagen a-3 (type VI) mRNA had 29 neighbors. The CNC networkrevealed the inter-regulation of lncRNAs and mRNAs in ECC.

GO and KEGG function enrichment analysis was then performed for mRNAs co-expressed with lncRNAs to identify biological processes and signaling pathways affected by differentially-expressed lncRNAs. GO analysis revealed that the differentially-expressed mRNAs between ECC and adjacent non-cancerous tissue were significantly enriched in cellular response to ultraviolet-A rays, the sensory perception of pain, the creatinine metabolic process and protein processing. KEGG analysis indicated that the deregulated mRNAs between ECC and adjacent non-cancerous tissue were mainly involved in drug metabolism-cytochrome P450, nitrotoluene degradation, caffeine metabolism, the mitogen-activated

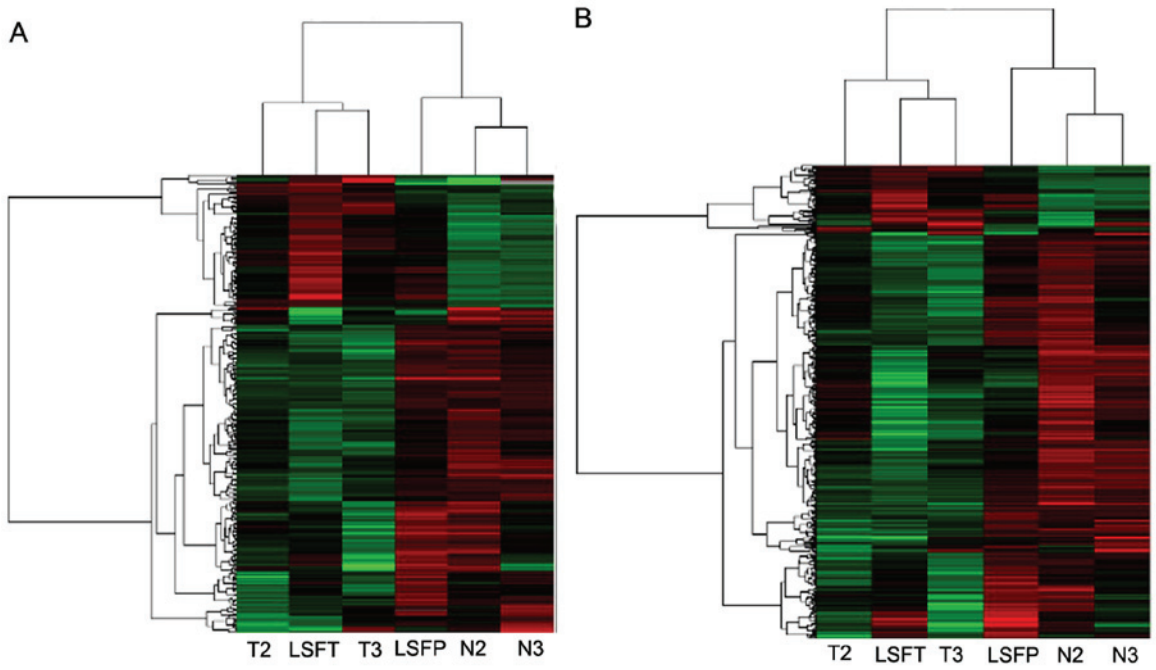

Figure 1. (A) Hierarchical clustering analysis of 268 differentially expressed long non-coding RNAs and (B) 459 differentially expressed mRNAs. Red and green colors indicate high and low expression, respectively. In the heat map, columns represent samples and rows represent each gene. T2, LSFT and T3 denote extrahepatic cholangiocarcinoma tissue samples; LSFP, N2 and N3 denote paired adjacent non-cancerous tissues.

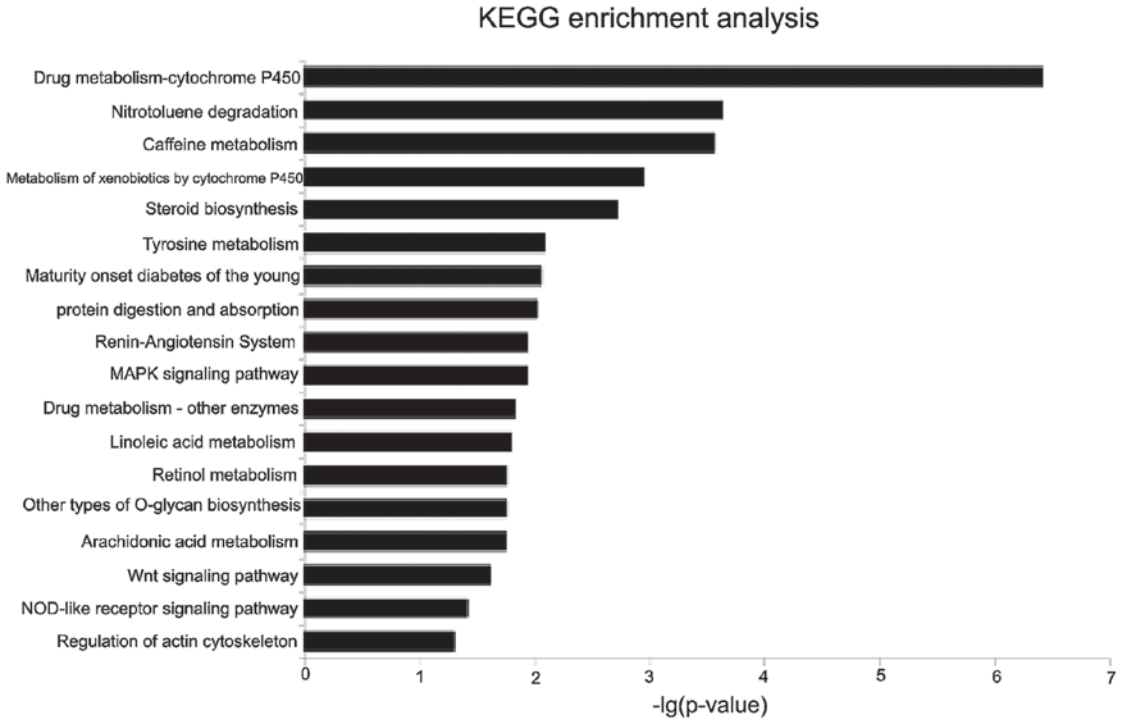

Figure 2. Significantly enriched KEGG pathways of mRNAs co-expressed with lncRNAs in the coding-non-coding gene co-expression network. KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; NOD, nucleotide oligomerization domain.

protein kinase (MAPK) signaling pathway, the peroxisome proliferator-activated receptor (PPAR) signaling pathway, protein digestion and absorption, the Wnt signaling pathway and the nucleotide oligomerization domain-like receptor signaling pathway ($P<0.05$, following multiple testing correction; Fig. 2).

Validation of microarray results by RT-qPCR. In order to confirm aberrant lncRNA expression, 5 of the differentially-expressed lncRNAs in ECC were randomly selected for subsequent analysis to ensure the validity of microarray results using RT-qPCR in 42 pairs of ECC and adjacent non-cancerous tissues. The results revealed that

Table II. Target prediction from lncRNAs to mRNAs.

lncRNA	mRNA	Correlation	P-value	Cis-regulation	Trans-regulation
p14589	A_P186342	0.99929	$2.387x10^{-2}$		miRNA sequestration
p29152	A_P186342	-0.99977	$1.353x10^{-2}$		miRNA sequestration
p21976	A_P0002916	0.99992	$7.638x10^{-3}$	Sense	
p17770	A_P328023	-0.99848	$3.508x10^{-2}$		miRNA sequestration
p20598	A_P132317	-0.99908	$2.722x10^{-2}$		miRNA sequestration
p6091	A_P3414127	0.99851	$3.469x10^{-2}$		miRNA sequestration
p9680	A_P0001828	0.99999	$6.390x10^{-5}$	Sense	
p41956	A_P3221253	-0.99772	$4.293x10^{-2}$		miRNA sequestration

lncRNA, long non-coding RNA; miRNA, microRNA.

ENST00000508732, ENST00000519319 and UC022BVT were upregulated, while ENST00000438290 and ENST00000593604 were downregulated in ECC compared with adjacent non-cancerous tissue (Fig. 3). Overall, the present results demonstrated an association between RT-qPCR and microarray findings.

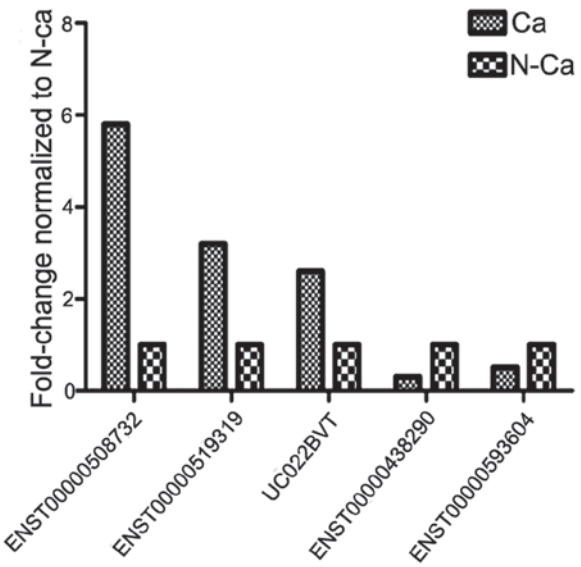

Figure 3. Selected lncRNA expression in microarray, and validation by reverse transcription-quantitative polymerase chain reaction.Ca, ECC tissues samples; N-Ca, paired adjacent non-cancerous tissues.

Discussion

lncRNAs are important regulators of gene expression during biological information processing and major cellular pathways, including proliferation, differentiation and apoptosis in living cells. Therefore, lncRNAs are involved in carcinogenesis or the antitumor effects of numerous human malignancies (20). However, thus far, knowledge about lncRNA expression in ECC is largely unknown.

A number of the differentially expressed lncRNAs and mRNAs that were identified in the present study are known to perform important roles in ECC. Differential expression of 268 lncRNA transcripts (defined as expression differences >2-fold) was observed. The expression was successfully validated for upregulated (ENST00000508732, ENST00000519319 and UC022BVT) and downregulated (ENST00000438290 and ENST00000593604) lncRNAs using qPCR in 42 pairs of ECC and adjacent non-cancerous frozen specimen. Thus, the specificity of the microarray results was confirmed.

If expression differences of lncRNAs are validated by independent researchers, these lncRNAs may represent diagnostic biomarkers or therapeutic targets in ECC. A previous study reported that lncRNA may be a potential diagnostic and prognostic biomarker for intrahepatic cholangiocarcinoma (ICC), using lncRNA and mRNA microarrays, and also considered that the expression of lncRNA and mRNA may predict the survival of patients with ICC (21), although, as is commonlyunderstood, the embryogenesis, anatomy and biological behavior of ECC and ICC differ (22). Identification of diagnostic biomarkers or therapeutic targets in bodily fluids may assist to improve patient outcome and understanding of the molecular mechanismof cancer progression,for example,lncRNA *PCA3* in urine is used as a diagnostic biomarker for prostate cancer (23). CertainlncRNAs, including H19 (imprinted maternally-expressed transcript) and FENDRR (adjacent non-coding developmental regulatory RNA), have been revealed to be differentially expressed in certain tumors (24,25); this dysregulationwas also observedin ECC tissues by microarray detection in the present study. The role and mechanism of certain known lncRNAs in ECC require additional investigations.

However, little is known on the function of lncRNAs and how to research them. Therefore, microarrays of lncRNAs and mRNAs may assist to elucidate this through certain bioinformatics methods, including CNC network and target gene predictions (cis and trans). The present results may provide clues for additional basic studies (Table II). The theory of target gene predictions may reveal that lncRNA functions via lncRNA-mRNA-protein interactions (26). The majority of these proteins code genes that function in the splicing, binding, transport, localization, transcription, translation and processing of RNA, according to GO function prediction.

It has also been reported that lncRNAs may act as a microRNA sponge by binding specific microRNAs and thereby disrupting microRNA regulation of mRNA 3'UTRs (27). In the present study, 39 lncRNAswere predicted

to target mRNAs by comparingsequences of lncRNAs with the 3'UTR of mRNAs. The miRTarget2 algorithm (28), starBase(http://starbase.sysu.edu.cn/) (29) and miRcode (www.mircode.org/) (30) were used to predict miRNA seeds within the validated lncRNA transcripts, which may assist in constructing the lncRNA-miRNA-mRNA axis. Prensner *et al* showed that prostate cancer-associated transcript 1 is able to abrogate the downregulation of cMyc by downregulating the expression of miR-34a in prostate cancer (4).

KEGG analysis has revealed that drug metabolism-cytochrome P450 (31), nitrotoluene degradation and caffeine metabolism induced by N-acetyltransferase (NAT) 1 and NAT2 (32) have significant association with the genesis and development of ECC, with the exception of classical proliferation and apoptotic pathways (such as the PPAR and MAPK pathways).

In summary, the present study revealed that dysregulation of ~4% of the lncRNA transcripts occurs in ECC, and altered lncRNA expression may modulate fundamental cellular processes. lncRNA profiles were able to accurately distinguish between ECC and adjacent non-cancer tissue. Thus, lncRNAs may be used as biomarkers and therapeutic targets for patients with ECC.

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