Long non-coding RNA expression profiles in gallbladder carcinoma identified using microarray analysis

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Abstract. Gallbladder carcinoma (GBC) is the most common biliary tract cancer and exhibits poor patient prognosis. Previous studies have identified that long non-coding RNAs (lncRNAs) serve important regulatory roles in cancer biology. Alterations in lncRNAs are associated with several types of cancer. However, the contribution of lncRNAs to GBC remains unclear. To investigate the lncRNAs that are potentially involved in GBC, lncRNA profiles were identified in three pairs of human GBC and corresponding peri-carcinomatous tissue samples using microarray analysis. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to validate the microarray data. In order to elucidate potential functions, Gene Ontology, Kyoto Encyclopedia of Genes and Genomes analysis, and network analysis were used to determine relevant signaling pathways. Abundant RNA probes were used, and 1,758 lncRNAs and 1,254 mRNAs were detected to be differentially expressed by the microarray. Compared with para-carcinoma tissue, numerous lncRNAs were markedly upregulated or downregulated in GBC. The results demonstrated that the lncRNAs that were downregulated in GBC were more numerous compared with the lncRNAs that were upregulated. Among them, RP11-152P17.2-006 was the most upregulated, whereas CTA-941F9.9 was the most downregulated. The RT-qPCR results were consistent with the microarray data. Pathway analysis indicated that five pathways corresponded to the differentially expressed transcripts. It was demonstrated that lncRNA expression in GBC was markedly altered, and a series of novel lncRNAs associated with GBC were identified. The results of the present study suggest that the functions of lncRNAs are important in GBC development and progression.

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Abbreviations: GBC, gallbladder carcinoma; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCT, para-carcinoma tissue

Key words: computational biology, expression profile, gallbladder neoplasms, long non-coding RNA, gene microarray analysis

Introduction

Gallbladder carcinoma (GBC) is the most common biliary tract cancer and a common type of cancer of the gastrointestinal tract (1). The incidence and distribution of GBC differs by region (2,3); for example, the prevalence of GBC in South and East Asia is increased compared with that in Europe and the Americas (4). Surgical resection is the only treatment for GBC; however, the majority of patients are not candidates for curative resection when they are diagnosed, and patients with advanced stages of the disease are likely to exhibit recurrences following surgery (5). Although there have been advances in chemotherapy, the prognosis of GBC remains poor (6). The mean survival ranges between 5.2 and 24.4 months (7-10). The majority of patients with GBC succumb to metastasis and recurrence following surgery. Although previous studies have reported that accumulated genomic damage promotes GBC progression (11,12), the underlying molecular mechanisms of GBC progression remain unclear.

Previous studies have identified non-coding RNAs (ncRNAs) as principal components of the human transcriptome (13,14). According to their length, ncRNAs may be

divided into two types: Long ncRNAs (lncRNAs) and small regulatory RNAs. lncRNAs have lengths of between 200 bp and 100 kb (15). Increasing evidence in previous years has determined that ncRNAs serve important regulatory roles in cellular physiological processes and diseases (16), including Huntington's disease (17), Alzheimer's disease (18), and glioma, lung, colorectal, breast cancer and hepatocellular cancer (19-22). Accordingly, lncRNA dysregulation, including metastasis-associated lung adenocarcoma transcript 1, colon cancer-associated transcript 1, homeobox transcript antisense RNA (HOTAIR) and low expression in tumor, is also associated with GBC (23-26). Although numerous lncRNAs have been discovered over the previous decade, the biological functions of lncRNAs in GBC and their underlying molecular mechanisms remain unclear. Using microarray analysis, 654 lncRNAs and 1,057 mRNAs that were markedly aberrantly expressed in GBC and paired peri-carcinomatous tissue samples were identified. The results were validated using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and were consistent with the data analysis of the profiles. Co-expression networks of lncRNAs and mRNAs, as well as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, were used to determine the biological processes for lncRNAs. The results of the present study demonstrated that aberrantly expressed lncRNAs may serve a role in the occurrence and development of GBC.

Materials and methods

Ethics statement. All aspects of the present study were approved by the Zhongshan Hospital Research Ethics Committee (Zhongshan Hospital, Fudan University, Shanghai, China). All patients provided written informed consent for the use of their surgical specimens in the present study in accordance with the Committee's regulations. The clinical characteristics of the patients with GBC are presented in Table I. Tissue specimens were selected randomly from Zhongshan Hospital, Fudan University (Shanghai, China) between April and September 2014.

Extraction of total RNA and RT-qPCR. Paired tissues of GBC and peri-carcinomatous tissues from each patient were quick-frozen in liquid nitrogen immediately following resection and stored at -80˚C. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. A BioPhotometer® plus 6132 instrument (Eppendorf, Hamburg, Germany) was used to measure RNA concentrations. Total RNA (1 μg) was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Fermentas; Thermo Fisher Scientific, Inc.). The lncRNA expression levels were determined using RT-qPCR (27) with an Applied Biosystems® 7500 fast real-time PCR thermal cycler instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.), and three replicate PCRs were performed. The PCR kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China) and 250 ng cDNA was added to each PCR tube. The primers used in the present study are listed in Table II. β-Actin and 18s were used as an endogenous reference (the β-actin primers were purchased from Sangon Biotech Co., Ltd., Shanghai, China). The amplification conditions were as follows: Reverse transcription reaction at 42˚C for 30 min per cycle. The PCR cycling conditions were as follows: Enzyme activation at 95˚C for 10 sec per 40 cycles, and annealing and extension at 60˚C for 32 sec.

Table I. Clinical characteristics of 23 gallbladder carcinoma cases.

Patient no.	Age, years	Gender	T	N	M	TNM stage
P1	69	F	3	0	0	IIIA
P2	51	M	2	0	0	II
P3	62	M	3	1	0	IIIB
P3	50	F	3	0	0	IIIA
P4	40	M	1	0	0	I
P5	50	F	3	0	0	IIIA
P6	41	F	1b	0	0	I
P7	50	F	3	0	0	IIIA
P8	71	F	4	1	0	IVA
P9	60	F	4	2	1	IVB
P10	51	F	1b	0	0	I
P11	57	F	1b	0	0	I
P12	65	F	3	0	0	IIIA
P13	66	M	3	1	0	IIIB
P14	57	F	2	2	0	IVB
P15	78	F	1b	0	0	I
P16	68	F	2	0	0	II
P17	41	M	3	1	0	IIIB
P18	49	M	3	0	0	IIIA
P19	76	M	3	1	0	IIIB
P20	67	F	3	0	0	IIIA
P21	62	F	4	2	1	IVB
P22	66	M	3	0	0	IIIA
P23	75	M	3	0	0	IIIA

T, tumor; N, node; M, metastasis; P, patient; F, female; M, male.

lncRNA and mRNA microarray expression profiling. Microarray hybridization was carried out by the CapitalBio Corporation (Shanghai, China) on behalf of the present study. Ribosomal RNA was removed from total RNA using an mRNA-ONLY™ Eukaryotic mRNA Isolation kit (Epicentre; Illumina, Madison, WI, USA), according to the manufacturer's protocol, and discarded. Each mRNA sample was transcribed into complementary RNA (cRNA) containing cyanine-3-cytidine 5'-triphosphate fluorescent labels (Agilent Technologies, Inc., Santa Clara, CA, USA). Klenow enzyme labeling strategy was adopted following reverse transcription using CbcScript II reverse transcriptase. Labeled cDNA was produced by Eberwine's linear RNA amplification method and subsequent enzymatic reaction. This procedure has been previously described, and the procedure has been improved by using CapitalBio cRNA Amplification and Labeling kit

Table II. Primers used for reverse transcription-quantitative polymerase chain reaction.

lncRNA	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	CCAAGCAGCATGAAGATCAA	TCTGCTGGAAGGTGCTGAG
18s	TTGGTCTGTTTAGCGAGGTG	ACGCTGAGCCAGTCAGTGTA
CRNDE	CAAATGGAAGCCAGAGGAAA	ATTCAGCACAAGGCAAGGAT
CTA-941F9.9	CTCCGTTTCTTCTCTGAGACTTC	GAGGCACTTCCTTGTGACTT

lncRNA, long non-coding RNA; CRNDE, colorectal neoplasia differentially expressed.

(CapitalBio, Beijing, China) for producing higher yields of labeled cDNA (28). The labeled cRNAs were hybridized on the Human lncRNA array V4.0 (4x180K, Agilent Technologies, Inc.), which contained the global profiles of 108,458 transcripts (78,243 ncRNAs and 30,215 coding RNAs). The microarrays were washed with two consecutive solutions (0.2% SDS, 2xSSC at 42˚C for 5 min, and 0.2xSSC for 5 min at room temperature) and scanned using an Agilent G2505C Microarray Scanner system (Agilent Technologies, Inc.), according to the manufacturer's protocol. The raw data were analyzed using Feature Extraction software (version 10.7.1.1; Agilent Technologies, Inc.) and then normalized using percentile normalization. Aberrantly expressed lncRNAs and mRNAs (fold-change ≥2.0 or ≤0.5; P<0.05) were selected for further study. To identify lncRNAs and mRNAs expression patterns, hierarchical clustering was performed on six tissue samples using Cluster 3.0 (Stanford University School of Medicine, Stanford, CA, USA) and Treeview 2.0 (Baryshnikova Lab, Princeton University, NJ, USA). The results were compared with the microarray data deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) [gene sequence expression series (GSE) accession number GSE62335, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62335].

Functional group analysis. To identify the biological functions of the 1,057 aberrantly expressed mRNAs determined as described above, GO and KEGG analyses were used to determine the signaling pathways. The mRNA data were uploaded into the Database for Annotation, Visualization and Integrated Discovery (david.abcc.ncifcrf.gov/tools.jsp) prior to analyzing the enrichment. Fisher's exact test was used to determine the significance of the GO term enrichment for differentially expressed genes. P<0.05 was considered to indicate a statistically significant difference. A false discovery rate (FDR) <0.05 determined the significance of the association of the pathways with the conditions.

Co-expression network construction. An lncRNA-mRNA co-expression network was constructed to determine their association, and the algorithm was as follows: i) The data were preprocessed for median gene expression values of all transcripts from the same coding gene, without special treatment for lncRNAs; ii) data for aberrantly expressed lncRNAs and mRNAs were removed following screening; iii) Pearson's correlation coefficient (PCC) was calculated and the R value was used to calculate the PCC between lncRNAs and mRNAs; and iv) the data were screened and data for which the PCC was >0.99 were selected. The gene co-expression network was constructed using Cytoscape software version 3.1.1 (U.S. National Institute of General Medical Sciences, Washington, DC, USA).

In the network, yellow nodes represented the lncRNAs and green nodes represented the mRNAs. Circular nodes represented lncRNAs and diamond nodes represented the mRNAs. Continuous lines indicated a positive association and dashed lines indicated a negative association.

Cell culture. The human GBC cell line SGC-996 was provided by the Tumor Cytology Research Unit (Medical College, Tongji University, Shanghai, China). Human GBC cell line GBC-SD was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 units/ml penicillin. Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

siRNA transfection. RP5-899B16.2-specific siRNAs and non-silencing negative control siRNA were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China; product ID siP01001). The target sequences of RP5-899B16.2 were 5'-GGAUAGAUACAUUGACACUdTdT-3' (siRNA-1) and 5'-GGAUAGAAUCAGGUUCCAUdTdT-3' (siRNA-2). SGC-996 and GBC-SD cells were transfected with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Cell viability. Transfected and non-transfected SGC-996 cells and GBC-SD cells were seeded into 96-well plates at a concentration of $3x10^3$ cells/well and $2.5x10^3$ cells/well, respectively. Between days 1 and 5, 10 μl MTT solution with solvent PBS was added to each well of one plate, followed by incubation at 37˚C for 4 h. The absorbance at 490 nm was measured using a microplate reader. The assay was performed in triplicate.

Statistical analysis. All statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). For comparison, a two-tailed Student's t-test was performed, when appropriate. Data in Fig. 2 were presented as log2-transformed median fold-changes in expression ± standard error, and results in Fig. 5 were presented as the mean ± standard error of the mean. All histograms were constructed using Prism for Windows (version 5.0; GraphPad

Table III. Microarray analysis of the 30 most aberrantly regulated lncRNAs in three pairs of gallbladder carcinoma tissues.

Probe name	lncRNA ID	Gene symbol	P-value	FC	Regulation	Chromosome
p11051	ENST00000422971.1	CTA-941F9.9	0.017	15.00	Down	22(-): 46000311-46001501
p7473	ENST00000591222.1	NA	0.014	13.99	Down	17(+): 66186024-66188943
p3312	ENST00000551672.1	NA	0.021	13.56	Down	12(-): 80849274-80852604
p13773	ENST00000514158.1	CTC-454M9.1	0.009	13.45	Down	5(+): 88185276-88237187
p14911	ENST00000449672.1	AOAH-IT1	0.016	12.84	Down	7(-): 36637439-36639726
p21662	TCONS_00029197	NA	0.025	12.24	Down	21(+): 17979036-17979674
p20932	TCONS_00004443	NA	0.013	11.75	Down	2(-): 130324222-130351825
p35139_v4	ENST00000515376.1	NA	0.023	11.23	Down	4(+): 174451610-174512475
p35137_v4	ENST00000512246.1	NA	0.026	11.14	Down	4(+): 174451608-174462981
p2150	ENST00000436715.1	H19	0.019	11.02	Down	11(-): 2016668-2017801
p1587	ENST00000454837.1	ANTXRLP1	0.033	10.94	Down	10(-): 47620144-47640809
p15883	ENST00000520594.1	NA	0.039	10.57	Up	8(-): 106797231-107072695
p16513	ENST00000594708.1	NA	0.027	9.97	Down	9(-): 72808912-72873782
p36138_v4	TCONS_00010091	NA	0.025	9.44	Down	5(+): 130589969-130593098
p43626_v4	XR_430247.1	NA	0.040	9.30	Down	19(+): 42060081-42061684
p22857	TCONS_00009823	NA	0.042	9.29	Down	5(-): 178365677-178368084
p43270_v4	XR_429785.1	NA	0.000	9.12	Down	16(+): 85196766-85199924
p15702	ENST00000523786.1	NA	0.002	8.87	Down	8(-): 57432677-57472056
p15932	ENST00000517869.1	NA	0.033	8.87	Down	8(-): 126934766-126963394
p7444	ENST00000580515.1	BZRAP1-AS1	0.026	8.87	Down	17(+): 56406298-56429790
p35142_v4	ENST00000515345.1	NA	0.029	8.51	Down	4(+): 174451624-174458566
p33555	ENST00000460744.1	NA	0.022	8.06	Down	3(+): 111011565-111261149
RNA33675|snoRNA _scaRNA_271_77	RNA33675| snoRNA _scaRNA_271_77	NA	0.012	7.72	Down	NA
p38655_v4	ENST00000458974.1	NA	0.024	7.63	Down	14(+): 101364256-101364333
p12993	ENST00000509866.1	NA	0.036	7.62	Down	4(+): 174451612-174458842
p13228	ENST00000503568.1	NA	0.004	7.61	Down	5(-): 74343543-74348468
p34010_v4	ENST00000415582.1	NA	0.048	7.55	Up	1(-): 201969228-201970411
RNA143553|tRNA _470_66	RNA143553|tRNA _470_66	NA	0.018	7.45	Down	NA
p4096	ENST00000436329.1	GPC6-AS1	0.020	7.44	Down	13(-): 94806446-94840245
p40958_v4	XR_427898.1	NA	0.028	7.43	Down	6(-): 25015198-25036372

lncRNA, long non-coding RNA; ID, identification no.; FC, fold-change; NA, not annotated.

Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA and mRNA expression profiles in GBC. To analyze the expression profiles of lncRNAs between tumor tissue and PCT (para-carcinoma tissue), fold-changes (tumor tissue vs. PCT), P-values and FDRs were used to assess the normalized expression of genes. According to the microarray data, <17,032 lncRNAs and <22,848 mRNAs expressed in the three pairs of GBC and PCT were detected, which included 654 lncRNAs (229 upregulated and 425 downregulated) and 1,057 mRNAs (394 upregulated and 663 downregulated) with markedly different expression. In addition, 112 lncRNAs and 184 mRNAs were identified as consistently upregulated in all three GBC groups, whereas 283 lncRNAs and 427 mRNAs were consistently downregulated. The 30 most marked differentially expressed lncRNAs and mRNAs identified using microarray analysis are presented in Tables III and IV. The hierarchical clustering analysis dendrograms indicated that the samples exhibited associations among the lncRNA and mRNA expression patterns (Fig. 1A and B). Among the aberrantly expressed lncRNA transcripts, RP11-152P17.2-006 ($\log_2$ fold-change tumor/wild-type=10.57) was the most markedly upregulated, whereas the expression of CTA-941F9.9 ($\log_2$ fold-change tumor/wild-type=-15.00) was the most markedly downregulated. Subsequently, the results were compared with the microarray data deposited in the GEO, which demonstrated that 1,212 lncRNAs and 1,213 mRNAs were aberrantly expressed between five pairs of GBC and adjacent non-tumor samples (fold-change ≥1.25, P<0.05 and FDR<0.05). Three pairs of analogous samples were further analyzed, and 654 lncRNAs and 1,057 mRNAs aberrantly expressed in three

Table IV. Microarray analysis of the 30 most aberrantly regulated mRNAs in three pairs of gallbladder carcinoma tissues.

Probe name	Ensembl ID	Gene symbol	P-value	FC	Regulation	Chromosome
A_23_P390700	ENST00000550305	CNTN1	0.037	21.52	Down	12: 41414156-41414215
A_23_P134347	ENST00000542995	CPVL	0.003	18.15	Down	7: 29105730-29105671
A_33_P3265749	ENST00000370934	PTGER3	0.020	14.64	Down	1: 71478048-71477989
A_23_P168993	ENST00000345060	ADRB3	0.049	13.94	Down	8: 37821117-37821058
A_23_P150457	ENST00000438354	LYVE1	0.028	12.52	Down	11: 10580104-10580045
A_24_P236935	ENST00000424910	KLK6	0.006	12.00	Up	19: 51466784-51466725
A_33_P3397865	ENST00000291901	TNNT1	0.019	11.51	Up	19: 55644255-55644196
A_33_P3290239	NA	DUOXA1	0.017	11.51	Up	15: 45411363-45411304
A_33_P3275801	ENST00000373960	DES	0.031	11.51	Down	2: 220291400-220291459
A_24_P261760	ENST00000356986	KLRG1	0.007	11.49	Down	12: 9147774-9147833
A_33_P3254844	ENST00000401731	CEACAM7	0.005	11.48	Up	19: 42178513-42178454
A_33_P3256997	ENST00000398984	NA	0.029	11.05	Down	11: 59980755-59980696
A_33_P3265739	ENST00000306666	PTGER3	0.021	10.98	Down	1: 71436629-71436570
A_33_P3248405	ENST00000536164	NRK	0.038	10.96	Down	X: 105139249-105139308
A_23_P145718	ENST00000483864	AOAH	0.017	10.92	Down	7: 36570069-36561708
A_33_P3294533	ENST00000321728	PRKCB	0.040	10.62	Down	16: 24231495-24231554
A_23_P339588	ENST00000338313	TAGAP	0.011	10.37	Down	6: 159460015-159459956
A_24_P40626	ENST00000318160	GREM2	0.013	10.08	Down	1: 240654036-240653977
A_21_P0010449	ENST00000422971	XLOC_014399	0.012	10.05	Down	22: 46000392-46000333
A_33_P3406196	ENST00000344825	KLRD1	0.036	9.97	Down	12: 10462019-10462078
A_24_P156490	ENST00000286627	KCNMA1	0.006	9.84	Down	10: 78644826-78644767
A_33_P3225760	ENST00000412923	PCDH18	0.014	9.29	Down	4: 138453085-138453026
A_33_P3220015	ENST00000390341	NA	0.028	9.25	Down	7: 38339480-38339421
A_23_P64898	ENST00000538029	KLRG1	0.047	9.24	Down	12: 9162594-9162653
A_21_P0005574	NA	XLOC_006224	0.028	9.12	Down	7: 123284900-123284959
A_33_P3240512	ENST00000377474	KCTD12	0.043	8.94	Down	13: 77454410-77454351
A_33_P3249872	ENST00000262722	FBLN1	0.031	8.54	Down	22: 45959173-45959232
A_23_P145606	ENST00000320658	CHRM2	0.041	8.51	Down	7: 136700790-136700849
A_33_P3257027	ENST00000377614	FGF7	0.025	8.51	Down	15: 49776810-49776869
A_23_P342641	ENST00000536707	SLC44A5	0.021	8.42	Up	1: 75679412-75677201

ID, identification no.; FC, fold-change; NA, not annotated.

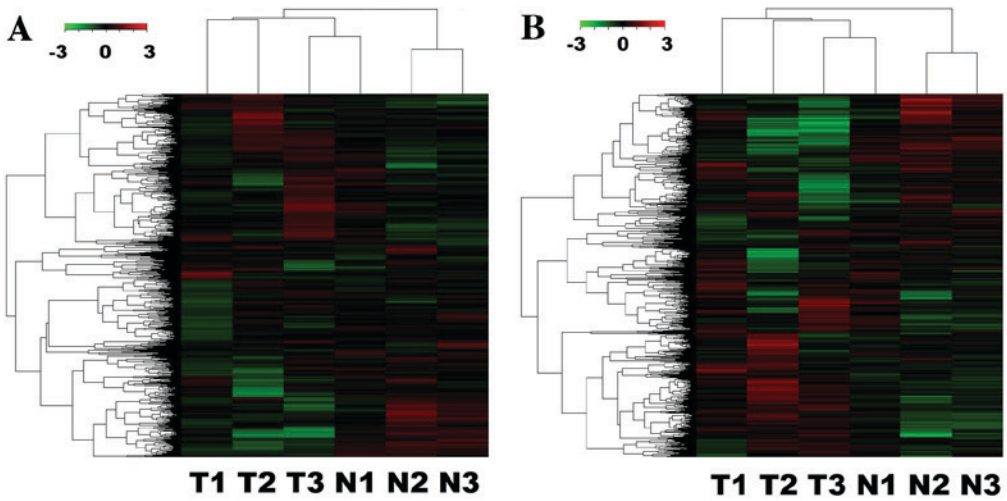

Figure 1. Differences in the lncRNA and mRNA expression profiles between gallbladder carcinoma and non-tumorous tissues. (A) Results from hierarchical clustering demonstrate distinguishable (A) lncRNA and (B) mRNA expression profiling among samples. Red indicates increased relative expression and green indicates decreased relative expression. lncRNA, long non-coding RNA. T, tumor; N, wild-type.

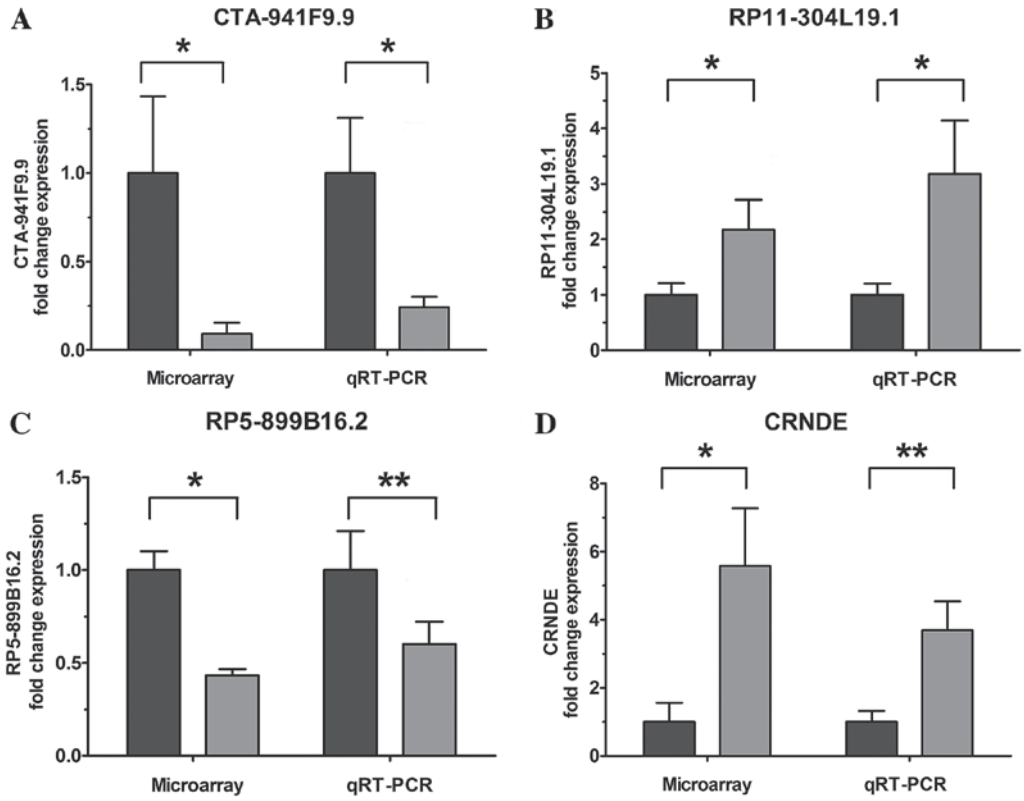

Figure 2. Comparison between the microarray data and RT-qPCR results. (A) CTA-941F9.9, (B) RP5-899B16.2, (C) RP11-304L19.1 and (D) CRNDE were determined to be differentially expressed in gallbladder carcinoma samples (grey bars) compared with wild-type samples (black bars) in three patients using microarray analysis, and validated using RT-qPCR in tissues of 23 patients. Results are presented as $\log_2$-transformed median fold-changes in expression ± standard error across patients for lncRNA validation. Validation of the lncRNAs indicated that the microarray data were consistent with the RT-qPCR results. *P<0.05, **P<0.01. RT-qPCR, reverse transcription-polymerase chain reaction; CRNDE, colorectal neoplasia differentially expressed.

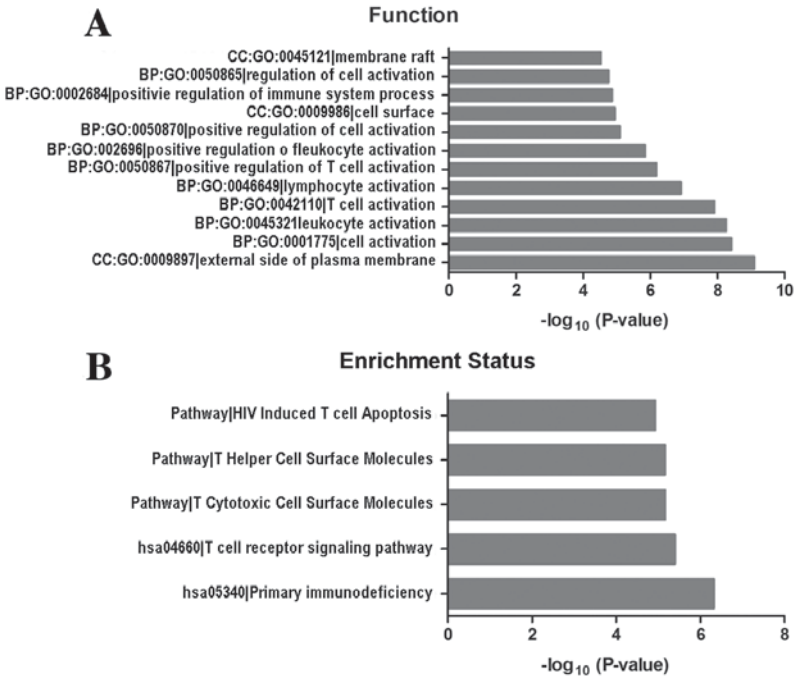

Figure 3. (A) GO analysis and (B) Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed long non-coding RNAs in gallbladder carcinoma. GO, Gene Ontology; HIV, human immunodeficiency virus; hsa, *Homo sapiens*.

GBC tissues compared with the adjacent non-tumor samples were identified (fold-change ≥2.0, P<0.05 and FDR<0.05), which used a different fold-change threshold compared with previous results. If the fold-change was defined to be ≥1.5, the

Figure 4. lncRNA-mRNA network constructed based on the correlation analysis between the differentially expressed lncRNAs and mRNAs. Yellow nodes represent lncRNAs and green nodes represent the target mRNAs. Red continuous lines represent a positive association, and blue dashed lines represent a negative association. lncRNA, long non-coding RNA.

aberrant expression gene numbers were 1,565 lncRNAs and 2,321 mRNAs.

Validation of the microarray data using RT-qPCR. To validate the microarray analysis results, four upregulated/downregulated lncRNAs were randomly selected for validation using RT-qPCR. The results demonstrated that lncRNAs RP11-304L19.1 and colorectal neoplasia differentially expressed (CRNDE) were upregulated and CTA-941F9.9 and RP5-899B16.2 were downregulated in the tumor samples compared with PCT samples (Fig. 2). These RT-qPCR results were consistent with the microarray data.

GO and KEGG pathway analyses. To investigate potential gene and gene product enrichment in biological processes, cellular components and molecular functions, GO analysis was performed with the differentially expressed mRNAs. An FDR ≤0.05 (Bonferroni correction) was used to determine significant changes in the differentially expressed gene list and the GO annotation list. The results suggested that a number of functional signaling pathways were enriched, including those involved in the regulation of cell activation, external side of the plasma membrane, membrane rafts and regulation of immune system processes. In addition, the cell surfaces exhibited the most increased enrichment of GO terms within GBC (Fig. 3A). Pathway analyses indicated that five pathways corresponded to differentially expressed transcripts and were the human immunodeficiency virus-induced T cell apoptosis, T cytotoxic cell-surface molecules, T helper cell-surface molecules, primary immunodeficiency and the T cell receptor signaling pathways (Fig. 3B).

Construction of a co-expression network. To explore the association between lncRNAs differentially expressed in GBC and targeted mRNAs, a co-expression network was constructed. Using PCC ≥0.99 and P<0.0001, 5,679 pairs of co-expressed lncRNAs and mRNAs composed of 922 mRNAs (87.2% of all differentially expressed mRNAs) and 602 lncRNAs (92.0% of all differentially expressed lncRNAs) were identified, and 3,366 pairs demonstrated a positive association (Fig. 4). The results suggested that the lncRNA-mRNA pairs exhibiting the most marked positive correlation coefficiency included XR_429508.1 and CD3γ. ENST00000451584.1 and transcription factor activator protein 2Aα exhibited the most marked negative correlation coefficiency. This co-expression network indicated that 1 lncRNA may target ≤29 coding genes and that one coding gene may associate with ≤3 lncRNAs. For example, this network indicated that

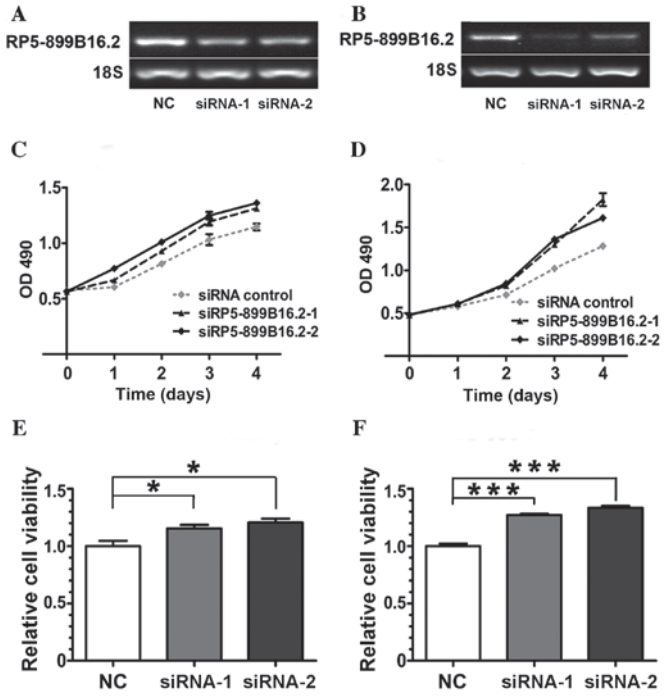

Figure 5. RP5-899B16.2 silencing affects the viability of gallbladder carcinoma cell lines. Expression of RP5-899B16.2 in (A) GBC-SD and (B) SGC-996 cell lines transfected with control siRNA or two siRNAs targeting RP5-899B16.2 (siRNA-1 and siRNA-2). 18S was used as the internal control. MTT assay of (C) GBC-SD and (D) SGC-996 cells transfected with NC, siRNA-1 and siRNA-2. Relative cell viability with respect to NC-treated cells (set as 1) of the (E) GBC-SD and (F) SGC-996 cell lines 96 h following siRNA transfection. siRNA-1: siRP5-899B16.2-1; siRNA-2: siRP5-899B16.2-2; *P<0.05; ***P<0.001. siRNA, small interfering RNA; NC, negative control; OD, optical density.

CRNDE was associated with cell division cycle-associated 7-like (CDCA7L) and solute carrier 44A5 (SLC44A5).

Silencing of RP5-899B16.2 increased GBC cell viability. To further confirm the functional relevance of lncRNA expression, SGC-996 and GBC-SD cell lines were transfected with RP5-899B16.2-specific siRNAs and non-silencing negative control siRNA. The RT-qPCR results indicated that RP5-899B16.2 expression was markedly downregulated at the mRNA level in the two transfected cells compared with the control (Fig. 5A and B).

The frequent dysregulation of RP5-899B16.2 in GBC tissues and cell lines suggested that this gene may serve an important role in GBC. To investigate the effects of STMN1 deficiency, an MTT assay was performed and cell viability curves were constructed. It was demonstrated that the viability of the GBC cell lines increased markedly following the silencing of RP5-899B16.2 (Fig. 5C-F).

Discussion

The carcinogenesis of GBC is a complex process (1). Despite advances in research in recent decades, its pathogenesis remains unclear, and further studies are required. The association between lncRNAs and tumors has been frequently investigated: Previous studies have indicated that lncRNAs serve important roles in regulating gene expression and are associated with cancer development (29,30), including HOTAIR in breast cancer (22), digestive system cancer (31) and urothelial cancer (32). The maternally expressed gene 3 was downregulated in several cancers and was demonstrated to inhibit tumor growth (33,34). CRNDE was upregulated in colorectal adenoma, adenocarcinoma and glioma (35,36). The majority of the aforementioned lncRNAs were also identified in the present study. However, studies on the association of lncRNA expression with GBC are limited (23-26). Therefore, it is important to understand the association between lncRNA expression and GBC to identify its pathogenesis. These results suggested that lncRNAs are potential targets for novel therapies.

In the present study, lncRNA and mRNA expression profiles in GBC were investigated using microarray chips. The results demonstrated that >100 lncRNA expression levels were changed compared with matched adjacent non-cancerous tissues. Currently, to the best of our knowledge, there are no studies on lncRNA expression profiles in GBC. The discrepancy between the data of the present study and GEO data may be due to differences in tumor tissues. Furthermore, the platform that was used was the Human lncRNA array V4.0 (4x180K), whereas the platform for the GEO data was the Affymetrix Human Gene 2.0 ST Array. The difference between the two platforms in array content, gene coverage availability, specific exon or splice junction probes, labeling systems and systematic lncRNA classification may lead to the difference in results. Therefore, novel lncRNAs were identified outside the scope of the GEO data. This avoided the elimination of lncRNAs that serve important roles in GBC.

In the previous decade, studies have suggested that a number of lncRNAs contribute to important functions, including the regulation of gene expression, serve an important role in cell development and metabolism, and are aberrant in a variety of diseases (13,37). To elucidate the underlying molecular mechanisms of lncRNA function, a co-expression network was further constructed by combining aberrantly expressed lncRNAs and mRNAs. Multiple lncRNAs were demonstrated to be markedly associated with mRNAs. CRNDE is an intergenic lncRNA located on chromosome 16, which is also overexpressed in colorectal carcinomas, gliomas and leukemias (35,38). CDCA7L is a target gene for cellular Myc proto-oncogene protein (c-Myc) that is involved in cell proliferative and apoptotic signaling pathways (39). However, SLC44A5 codes for a choline transporter-like protein that is associated with cell proliferation. Therefore, it was hypothesized that CRNDE may be a direct or indirect target gene of CDCA7L and SLC44A5. This lncRNA and mRNA co-expression network provides a strong foundation for predicting the function of lncRNAs. The malfunction of regulating this co-expression network may be an important step for the development and progression of GBC. The underlying molecular mechanisms of GBC progression remain unclear. Furthermore, GO analysis and pathway analysis were used to investigate the biological functions of lncRNA in the occurrence and development of GBC. More research is required to elucidate the functions of lncRNAs.

In conclusion, the expression profiles of lncRNAs and mRNAs in GBC with microarray analyses were determined, and 654 lncRNAs and 1,057 mRNAs were identified; the majority of which were novel identifications in GBC. Additionally, an lncRNA-mRNA co-expression network

was constructed and it was demonstrated that CRNDE was associated with the c-Myc protein CDCA7L. lncRNAs may function by interacting with mRNAs or proteins in GBC. Accurate signaling pathways warrant further study and are critical for identifying novel methods for the early diagnosis and treatment of GBC. Further functional studies may provide potential therapeutic targets or molecular biomarkers of GBC. The results of the present study suggest useful evidence for investigating potential therapeutic targets for GBC.

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