Comprehensive analysis of competing endogenous RNA networks associated with cholangiocarcinoma

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Abstract. Cholangiocarcinoma (CCA) is the second most common type of primary malignancy of the liver. Certain long non-coding RNAs (lncRNAs) have been demonstrated to have key roles in tumor pathogenesis by binding to microRNAs (miRNAs). However, the competing endogenous RNA (ceRNA) network of CCA remains to be fully determined. In the present study, the RNA expression profiles for CCA were downloaded from The Cancer Genome Atlas and further analyzed. A total of 318 differentially expressed (DE) lncRNAs, 87 DE miRNAs and 3,851 DE mRNAs were identified from 36 CCA samples and 9 adjacent non-tumor samples (for lncRNAs and miRNAs, fold change ≥2.5 and P<0.01; for mRNAs, fold change ≥2 and P<0.01). Further bioinformatics analyses were performed and the ceRNA network for CCA was constructed, which included 16 lncRNAs, 55 miRNAs and 373 mRNAs. Survival analysis of all genes in the network revealed that high expression of the mRNAs fucosyltransferase 4 (P<0.005) and huntingtin-interacting protein 1 related (P<0.001) has a positive impact on the overall survival of patients with CAA. Furthermore, the lncRNAs H19 and PVT1, and the miRNAs *Homo sapiens* (hsa)-miR-16-5p and hsa-miR-424-5p, together with peroxisome proliferator-activated receptors, may also have important roles in the pathogenesis of CCA. The present study provided data to further the understanding of and research into the molecular mechanisms implicated in CCA.

Introduction

Cholangiocarcinoma (CCA) is a deadly malignancy of the biliary tree. Due to the high frequency of diagnosis at a late stage, metastasis and recurrence, surgical treatment for CCA is associated with poor outcomes. The 5-year survival rate is 5-10% in patients with CCA (1). Therefore, understanding the molecular mechanisms involved in the tumorigenesis of CCA is a critical step for improving early diagnosis, reducing mortality, and developing effective targeted therapies. Long non-coding RNAs (lncRNAs) are autonomously transcribed non-coding RNAs of >200 nt in length, which have an important role in the regulation of gene transcription through the recruitment of chromatin-modifying enzymes (2). Accumulating studies have indicated that lncRNAs may interact with microRNAs (miRNAs/miRs) as competing endogenous RNAs (ceRNAs), and regulate the expression of target genes, which may have a role in tumor occurrence and progression (3). Therefore, lncRNAs are considered to be potential diagnostic and prognostic biomarkers of malignancy (4,5). Based on these facts, Salmena *et al* (3) provided the ceRNA hypothesis and constructed a large-scale ceRNA regulatory network, which may explain tumor processes and present opportunities for novel therapies.

In previous studies, regulatory ceRNA networks composed of lncRNAs, miRNAs and mRNAs have been used to study molecular mechanisms of tumor occurrence and progression. Numerous studies have indicated that ceRNA regulatory lncRNA-miRNA-mRNA networks are implicated in the occurrence and progression of gastric, breast, pancreatic and liver cancer (6-9). Recently, lncRNA actin filament associated protein 1 (AFAP1)-antisense (AS)1 was reported to promote the growth and metastasis of CAA (10). AFAP1-AS1 expression was upregulated in CCA tumor samples and its knockdown reduced cell stress filament integrity, suggesting that it may be a diagnostic and prognostic biomarker for CCA. Another study indicated that the expression of lncRNA colon cancer-associated transcript 1 (CCAT1) was significantly upregulated in CAA samples, and promoted cell migration and invasion by suppressing miR-152 (11). Based on the aforementioned studies, it may be hypothesized that dysregulation of certain lncRNAs may promote CAA by regulating key pathways. Therefore, identification of a CAA-associated ceRNA network may be useful for understanding the role of ceRNAs in the genesis of CAA and therapeutic outcomes (12). In the present study, a ceRNA network of CAA was constructed, including 16 lncRNAs, 55 miRNAs and 373 mRNAs. Based on the network, survival analysis of all genes suggested that

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fucosyltransferase 4 (FUT4) and huntingtin-interacting protein 1 related (HIP1R) were associated with overall survival. These results provided further insight into the mechanisms of the pathogenesis of CAA, and may provide potential novel therapeutic markers for CAA treatment.

Materials and methods

Patients and The Cancer Genome Atlas (TCGA) data retrieval. The microarray data for 45 CCA samples were obtained from TCGA data portal (https://portal.gdc.cancer.gov/), with search results up to October 14th, 2018 included. The RNA and miRNA sequencing (seq) data were obtained from the IlluminaHiseq_RNASeq and the IlluminaHiSeq_miRNASeq sequencing platforms. All data are open access and free to download. The sequencing data included the corresponding RNA-seq and miRNA-seq data. The human samples were divided into 2 groups: The CCA samples (n=36) and adjacent non-tumor samples (n=9). All of the protocols were in accordance with the guidelines of TCGA and no further ethical approval was required, since all of the data were collected from TCGA (https://cancergenome.nih.gov/publications/publicationguidelines).

Identification of differentially expressed (DE) lncRNAs, miRNAs and miRNAs. The raw data of the microarray datasets were preprocessed via background correction and normalization. Prior to the analysis, all unexpressed RNAs were filtered out by using R language (version 3.2.5; https://cran.r-project.org/). According to the R language results, those genes with mean read count ≤1 were deleted. The lncRNAs and mRNAs were identified using the Ensembl database (version 89; http://www.ensembl.org/index.html). Subsequently, the DE mRNAs, lncRNAs and miRNAs of the two groups were obtained using edgeR software (13). The false discovery rate (FDR) was used for multiple comparisons of statistically significant P-values. The fold change (FC) was used for measuring the differential expression levels of genes, where |log2FC|≥2 and FDR adjusted to P<0.01 were considered to indicate a significant DE mRNA or lncRNA; the standard for miRNAs was a fold change ≥2.5 and an FDR adjusted to P<0.01. Finally, all of the DE RNAs were analyzed and a volcano map was generated using the R platform.

Generation of the ceRNA regulatory network of CCA. To investigate the association between ceRNAs in patients with CAA, the lncRNA-miRNA-mRNA regulatory network was constructed, which was established through the following procedures based on the results of the DE analysis. First, the regulatory interactions between lncRNAs and miRNAs were predicted using the miRcode database (14). Subsequently, miRNA-targeted mRNAs were extracted from the miRTarBase (15), miRDB (16) and TargetScan (17) databases. Finally, using Cytoscape 3.5.1 (http://www.cytoscape.org/), the ceRNA regulatory network was generated and visualized.

Functional enrichment analysis. In order to better understand the mechanisms of CCA tumorigenesis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of DE mRNAs were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://www.david.abcc.ncifcrf.gov/). A GO term or KEGG pathway with FDR <0.05 was considered statistically significant. The enriched GO terms and pathways of the DE mRNAs with the most significant P-values were ranked by their enrichment score (-log P-value).

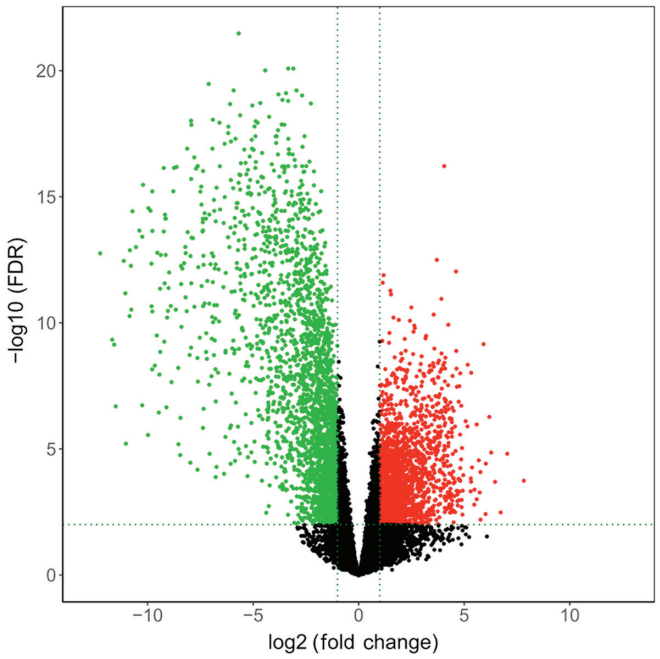

Figure 1. Volcano plot displaying the distribution of differentially expressed mRNAs. Green spots represent downregulated genes and red spots represent upregulated genes. FDR, false discovery rate.

Survival analysis. The R survival package (version 2.41-3; https://CRAN.R-project.org/package=survival) was used for the survival analysis. The Kaplan-Meier method was used to estimate cumulative survival rates and the log-rank test was then used to compare the differences in overall survival between the different groups. P<0.05 was considered to indicate statistical significance.

Results

DE lncRNAs, mRNAs and miRNAs in CCA. The present study investigated the differential RNA expression in 36 CCA tissues and 9 adjacent non-tumor tissues. The integrated analysis identified 318 DE lncRNAs, 3,851 DE mRNAs and 87 DE miRNAs using the edgeR package. Of the 318 DE lncRNAs, 205 were upregulated (64.5%) and 113 (35.5%) were downregulated. A total of 1,549 (40.2%) mRNAs were identified to be upregulated and 2,302 (59.8%) were downregulated out of the 3,851 DE mRNAs. For the 87 DE miRNAs, 41 (47.1%) were upregulated and the remaining 46 (52.9%) were downregulated. The top 20 upregulated and downregulated lncRNAs, mRNAs and miRNAs are listed in Tables I-III, respectively. In addition, the distributions of all DE mRNAs are presented in a volcano plot in Fig. 1.

Construction of a ceRNA regulatory network in CCA. To better understand the characteristics of the lncRNAs and to further clarify the interactions between the lncRNAs and

Table I. The top 20 up- and downregulated lncRNAs (ranked by P-values).

A, Top 20 upregulated lncRNAs

Ensembl ID	logFC	P-value	FDR
ENSG00000261659	3.198316385	$1.55x10^{-11}$	$3.30x10^{-10}$
ENSG00000253210	2.985738665	$1.21x10^{-10}$	$2.13x10^{-09}$
ENSG00000261183	4.001322806	$2.92x10^{-10}$	$4.81x10^{-09}$
ENSG00000228109	3.369141828	$3.07x10^{-10}$	$5.02x10^{-09}$
ENSG00000265688	3.446807939	$4.91x10^{-10}$	$7.64x10^{-09}$
ENSG00000261068	4.766573772	$2.45x10^{-09}$	$3.24x10^{-08}$
ENSG00000172965	2.575378258	$3.51x10^{-09}$	$4.50x10^{-08}$
ENSG00000273230	2.560329476	$4.77x10^{-09}$	$5.97x10^{-08}$
ENSG00000234741	2.018672373	$1.11x10^{-08}$	$1.30x10^{-07}$
ENSG00000261801	3.691796251	$1.53x10^{-08}$	$1.74x10^{-07}$
ENSG00000285255	4.217290775	$1.65x10^{-08}$	$1.86x10^{-07}$
ENSG00000226711	2.650291782	$3.45x10^{-08}$	$3.56x10^{-07}$
ENSG00000261437	4.32349391	$6.22x10^{-08}$	$6.02x10^{-07}$
ENSG00000277283	1.914981951	$1.10x10^{-07}$	$1.01x10^{-06}$
ENSG00000243479	5.595465468	$1.18x10^{-07}$	$1.08x10^{-06}$
ENSG00000244041	1.90448619	$1.32x10^{-07}$	$1.20x10^{-06}$
ENSG00000269680	3.185294307	$1.55x10^{-07}$	$1.39x10^{-06}$
ENSG00000273759	2.435273695	$1.66x10^{-07}$	$1.47x10^{-06}$
ENSG00000257556	2.979912742	$1.96x10^{-07}$	$1.71x10^{-06}$
ENSG00000255381	1.597506825	$2.13x10^{-07}$	$1.85x10^{-06}$

B, Top 20 downregulated lncRNAs

Ensembl ID	logFC	P-value	FDR
ENSG00000225756	-4.439300193	$5.12x10^{-18}$	$6.55x10^{-16}$
ENSG00000251165	-5.214981183	$6.12x10^{-17}$	$5.09x10^{-15}$
ENSG00000261572	-3.875298917	$1.23x10^{-15}$	$6.95x10^{-14}$
ENSG00000263400	-4.480906234	$1.29x10^{-15}$	$7.23x10^{-14}$
ENSG00000215386	-3.687757377	$1.76x10^{-15}$	$9.49x10^{-14}$
ENSG00000264575	-2.497423285	$2.22x10^{-15}$	$1.17x10^{-13}$
ENSG00000267390	-3.142413979	$2.31x10^{-15}$	$1.20x10^{-13}$
ENSG00000234456	-3.123344646	$5.80x10^{-15}$	$2.68x10^{-13}$
ENSG00000261012	-7.173215127	$6.10x10^{-14}$	$2.19x10^{-12}$
ENSG00000235609	-2.872714774	$1.54x10^{-13}$	$4.97x10^{-12}$
ENSG00000261578	-4.233216461	$1.78x10^{-13}$	$5.66x10^{-12}$
ENSG00000228794	-1.905394222	$8.41x10^{-13}$	$2.31x10^{-11}$
ENSG00000259370	-4.369092089	$3.65x10^{-12}$	$8.74x10^{-11}$
ENSG00000215256	-2.110042475	$3.88x10^{-12}$	$9.20x10^{-11}$
ENSG00000223797	-2.072567868	$5.02x10^{-12}$	$1.17x10^{-10}$
ENSG00000275494	-2.27756684	$1.10x10^{-11}$	$2.41x10^{-10}$
ENSG00000269386	-2.361574508	$2.36x10^{-11}$	$4.81x10^{-10}$
ENSG00000273616	-2.942297335	$3.18x10^{-11}$	$6.25x10^{-10}$
ENSG00000267675	-5.990122102	$5.41x10^{-11}$	$1.03x10^{-09}$
ENSG00000260274	-1.989229751	$5.54x10^{-11}$	$1.05x10^{-09}$

lncRNA, long non-coding RNA; ID, identification; FC, fold change; FDR, false discovery rate.

miRNAs, an lncRNA-miRNA-mRNA-associated regulatory network was constructed. First, the 318 DE lncRNAs derived from the miRcode database were used; these were applied to the Perl program, which identified 56 pairs of interacting

Table II. The top 20 up- and down-regulated mRNAs (ranked by P-values).

A, Top 20 upregulated mRNAs

Ensembl ID	logFC	P-value	FDR
ENSG00000067225	4.055367983	$2.56996x10^{-19}$	$6.10345x10^{-17}$
ENSG00000167642	3.701088077	$7.00796x10^{-15}$	$3.17737x10^{-13}$
ENSG00000166145	4.613180178	$2.32377x10^{-14}$	$9.14954x10^{-13}$
ENSG00000080603	1.181826005	$3.47222x10^{-14}$	$1.28677x10^{-12}$
ENSG00000129351	1.139806521	$7.36096x10^{-14}$	$2.57927x10^{-12}$
ENSG00000127616	1.503804232	$1.66381x10^{-13}$	$5.31921x10^{-12}$
ENSG00000102317	1.536020573	$2.42826x10^{-13}$	$7.47564x10^{-12}$
ENSG00000186765	3.918721626	$3.7811x10^{-13}$	$1.11584x10^{-11}$
ENSG00000149639	2.497348198	$8.96987x10^{-13}$	$2.46252x10^{-11}$
ENSG00000272398	3.551356872	$1.82259x10^{-12}$	$4.72611x10^{-11}$
ENSG00000179051	1.650897428	$2.44245x10^{-12}$	$6.15218x10^{-11}$
ENSG00000112118	1.902906765	$3.13164x10^{-12}$	$7.63689x10^{-11}$
ENSG00000164221	2.428188979	$3.41876x10^{-12}$	$8.25024x10^{-11}$
ENSG00000139734	4.246825211	$5.12237x10^{-12}$	$1.18639x10^{-10}$
ENSG00000166557	2.635202192	$5.60838x10^{-12}$	$1.28503x10^{-10}$
ENSG00000106012	2.659102098	$7.13445x10^{-12}$	$1.60899x10^{-10}$
ENSG00000034510	2.598284344	$1.03483x10^{-11}$	$2.27358x10^{-10}$
ENSG00000115561	1.462267513	$1.14603x10^{-11}$	$2.51053x10^{-10}$
ENSG00000187642	3.168721529	$1.99786x10^{-11}$	$4.13444x10^{-10}$
ENSG00000130702	2.181520136	$2.11404x10^{-11}$	$4.35678x10^{-10}$

B, Top 20 downregulated mRNAs

Ensembl ID	logFC	P-value	FDR
ENSG00000080709	-5.686469859	$2.2178x10^{-26}$	$3.31827x10^{-22}$
ENSG00000105607	-3.33705124	$1.59062x10^{-24}$	$8.19986x10^{-21}$
ENSG00000059769	-3.096941246	$1.64414x10^{-24}$	$8.19986x10^{-21}$
ENSG00000155666	-4.429426553	$2.5976x10^{-24}$	$9.71631x10^{-21}$
ENSG00000143257	-7.106918488	$1.12249x10^{-23}$	$3.35895x10^{-20}$
ENSG00000091831	-5.926384811	$2.4347x10^{-23}$	$6.04595x10^{-20}$
ENSG00000115841	-2.951203464	$2.82861x10^{-23}$	$6.04595x10^{-20}$
ENSG00000160323	-3.440732637	$4.13922x10^{-23}$	$7.74137x10^{-20}$
ENSG00000120158	-3.801441126	$5.20254x10^{-23}$	$8.64894x10^{-20}$
ENSG00000205707	-2.678156335	$6.41175x10^{-23}$	$9.59327x10^{-20}$
ENSG00000133027	-3.608581255	$1.0559x10^{-22}$	$1.43621x10^{-19}$
ENSG00000119673	-3.336686032	$1.26151x10^{-22}$	$1.57289x10^{-19}$
ENSG00000118777	-4.659839514	$1.66311x10^{-22}$	$1.91412x10^{-19}$
ENSG00000179152	-2.262072095	$1.86674x10^{-22}$	$1.99501x10^{-19}$
ENSG00000117009	-6.093754472	$2.11114x10^{-22}$	$2.1058x10^{-19}$
ENSG00000213398	-5.036902246	$2.55547x10^{-22}$	$2.38968x10^{-19}$
ENSG00000116761	-5.616242901	$6.69097x10^{-22}$	$5.88884x10^{-19}$
ENSG00000109929	-4.242818901	$8.11342x10^{-22}$	$6.74406x10^{-19}$
ENSG00000168306	-6.910609036	$1.11334x10^{-21}$	$8.76728x10^{-19}$
ENSG00000124713	-7.940751186	$1.28333x10^{-21}$	$9.6006x10^{-19}$

ID, identification; FC, fold change; FDR, false discovery rate.

lncRNAs and miRNAs. From the 87 DE miRNAs retrieved, it was predicted that 55 of them were able to interact with 16 DE lncRNAs. mRNAs targeted by these 55 DE miRNAs were selected in all three databases (miRTarBase, miRDB and TargetScan). The targeted mRNAs with DE miRNAs retrieved from the miRcode database were cross-checked. The 373 DE

Table III. The top 20 up- and downregulated miRNAs (ranked by P-values).

A, Top 20 upregulated miRNAs

miRNA	logFC	P-value	FDR
hsa-miR-183-5p	4.836903706	6.38×10^{-17}	1.40×10^{-14}
hsa-miR-182-5p	4.483302702	6.43×10^{-17}	1.40×10^{-14}
hsa-miR-21-5p	2.485307292	6.28×10^{-14}	6.52×10^{-12}
hsa-miR-96-5p	5.172417117	1.81×10^{-13}	1.31×10^{-11}
hsa-miR-27a-3p	1.975273987	3.22×10^{-13}	1.99×10^{-11}
hsa-miR-34c-3p	3.911770715	2.54×10^{-10}	4.76×10^{-09}
hsa-miR-222-3p	2.267762864	3.08×10^{-10}	5.34×10^{-09}
hsa-miR-23a-3p	1.494080752	4.43×10^{-10}	7.12×10^{-09}
hsa-miR-34c-5p	4.181782951	1.10×10^{-08}	1.45×10^{-07}
hsa-miR-92b-3p	2.769232681	1.75×10^{-08}	2.23×10^{-07}
hsa-miR-330-5p	1.866060112	2.30×10^{-08}	2.78×10^{-07}
hsa-miR-454-3p	1.608154597	2.67×10^{-08}	3.13×10^{-07}
hsa-miR-181d-5p	2.43995135	3.65×10^{-08}	4.17×10^{-07}
hsa-let-7e-5p	1.391443094	4.99×10^{-08}	5.42×10^{-07}
hsa-miR-221-3p	1.752600485	8.05×10^{-08}	8.27×10^{-07}
hsa-miR-181b-5p	1.952571231	9.66×10^{-08}	9.26×10^{-07}
hsa-miR-200b-3p	2.634730289	9.82×10^{-08}	9.26×10^{-07}
hsa-miR-24-2-5p	1.597889448	1.14×10^{-07}	1.05×10^{-06}
hsa-miR-301a-3p	1.537288068	2.14×10^{-07}	1.94×10^{-06}
hsa-miR-99b-3p	1.648708541	2.45×10^{-07}	2.13×10^{-06}

B, Top 20 downregulated miRNAs

miRNA	logFC	P-value	FDR
hsa-miR-148a-3p	-2.8329	5.07×10^{-15}	7.33×10^{-13}
hsa-miR-4662a-5p	-3.27785	7.51×10^{-14}	6.52×10^{-12}
hsa-miR-101-3p	-2.08208	4.85×10^{-13}	2.63×10^{-11}
hsa-miR-505-3p	-2.01987	2.01×10^{-12}	9.68×10^{-11}
hsa-miR-378a-3p	-3.24209	3.83×10^{-12}	1.66×10^{-10}
hsa-miR-148a-5p	-2.53305	7.56×10^{-12}	2.98×10^{-10}
hsa-miR-378a-5p	-2.94829	1.74×10^{-11}	6.30×10^{-10}
hsa-miR-125b-2-3p	-2.4123	2.24×10^{-11}	7.48×10^{-10}
hsa-miR-483-3p	-4.78428	2.72×10^{-11}	8.43×10^{-10}
hsa-miR-122-3p	-7.01304	3.26×10^{-11}	9.43×10^{-10}
hsa-miR-194-3p	-3.2353	3.93×10^{-11}	1.07×10^{-09}
hsa-miR-139-3p	-2.76339	5.01×10^{-11}	1.28×10^{-09}
hsa-let-7c-5p	-2.24221	6.46×10^{-11}	1.56×10^{-09}
hsa-miR-99a-3p	-2.34474	8.98×10^{-11}	1.97×10^{-09}
hsa-miR-675-3p	-5.17096	9.09×10^{-11}	1.97×10^{-09}
hsa-miR-139-5p	-3.13233	1.08×10^{-10}	2.24×10^{-09}
hsa-miR-378c	-2.82711	2.22×10^{-10}	4.38×10^{-09}
hsa-miR-885-5p	-6.05348	2.63×10^{-10}	4.76×10^{-09}
hsa-miR-99a-5p	-2.41861	3.84×10^{-10}	6.42×10^{-09}
hsa-miR-483-5p	-4.28222	4.93×10^{-10}	7.65×10^{-09}

miR/miRNA, microRNA; hsa, *Homo sapiens*; FC, fold change; FDR, false discovery rate.

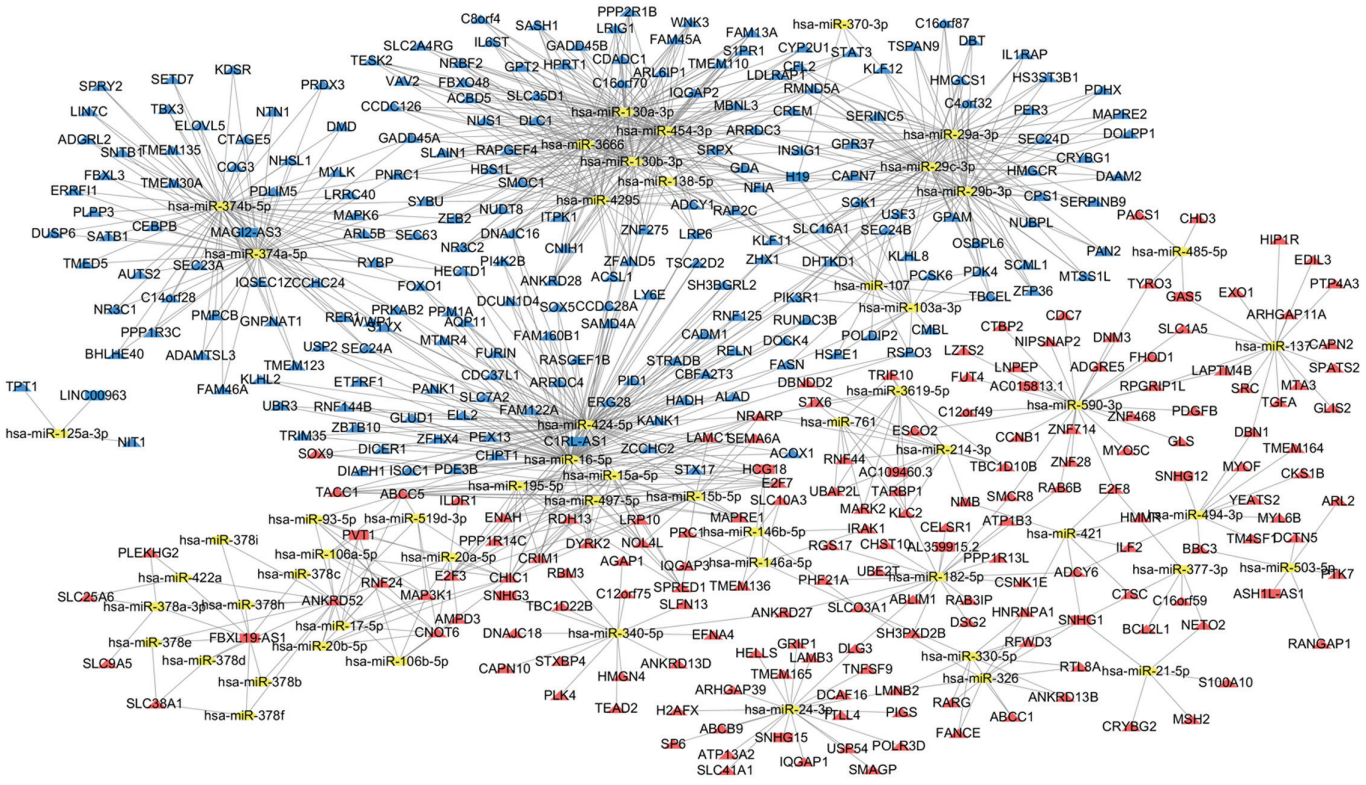

Figure 2. Competing endogenous RNA regulatory network in cholangiocarcinoma. The squares represent long non-coding RNAs, triangles indicate mRNAs and yellow circles represent miRs. Blue indicates downregulated RNAs and red represents upregulated RNAs. miR, microRNA; Hsa, *Homo sapiens*.

targeted mRNAs were then selected. Finally, the ceRNA regulatory network for CCA was created by incorporating 16 DE lncRNAs, 55 DE miRNAs and 373 DE mRNAs (Fig. 2).

Functional enrichment analysis. GO analysis revealed that there were 427 enriched GO terms with statistical significance in the category biological process (BP), 44 in the category cellular component (CC) and 105 in the category molecular function (MF). The top enriched terms for BP, CC and MF are small molecule catabolic process, mitochondrial matrix and coenzyme binding, respectively (Fig. 3). KEGG analysis revealed 49 pathways associated with mRNAs, including 'cell metabolism', 'proliferation' and 'sustained angiogenesis'. The top 15 pathways are provided in Fig. 4 and most of them are associated with metabolism.

Survival analysis and the ceRNA network. To identify the prognostic characteristics of the ceRNA network, each gene of the 16 lncRNAs, 55 miRNAs and 373 mRNAs were analyzed using Kaplan-Meier survival analysis separately. The results revealed that only two DE mRNAs in the network (FUT4 and HIP1R) were associated with overall survival. The Kaplan-Meier survival analysis indicated that high levels of FUT4 ($P<0.005$) and HIP1R ($P<0.001$) were positively associated with overall survival (Fig. 5).

Discussion

CCA is the second most common type of primary liver malignancy. Although surgical resection remains the only potential treatment, it is frequently unfeasible due to the advanced tumor stage at the initial diagnosis, with a 5-year survival rate of <5-10% (18). Diagnostic and therapeutic biomarkers are urgently required for patients with CCA. In recent years, accumulating evidence has indicated that lncRNAs have a key role in cancer. It has been suggested that lncRNAs may serve as sponges for miRNA, reducing their regulatory effect on mRNAs (19). This function introduces an extra layer of complexity in the miRNA-target interaction network, the dysregulation of which may contribute to the development and progression of multiple diseases (19). Although recent studies have reported that certain lncRNAs, including AFAP1-AS1, CCAT1, nuclear paraspeckle assembly transcript 1 and metastasis associated lung adenocarcinoma transcript 1, may be associated with CCA, it remains necessary to perform a more comprehensive analysis of the ceRNA regulatory networks in CCA (20).

The present study systematically analyzed and constructed a ceRNA regulatory network containing 16 DE lncRNAs, 55 DE miRNAs and 373 DE mRNAs, in an attempt to better understand the pathogenesis of CCA. In this network, the lncRNAs primarily bound to *Homo sapiens* (hsa)-miR-16-5p, hsa-miR-424-5p, hsa-miR-130a-3p, hsa-miR-130b-3p and hsa-miR-454-3p. In addition, hsa-miR-16-5p and hsa-miR-424-5p were the largest nodes that interacted with 82 non-coding and coding RNAs in the current network, suggesting that these two miRNAs may be critical to the pathogenesis of CCA. The enriched GO terms of the DE mRNAs were strongly linked to metabolism and included 'small molecule catabolic process', 'organic acid catabolic process' and 'carboxylic acid catabolic process'. Pathway analysis revealed that 'carbon metabolism', 'peroxisome proliferator-activated receptor (PPAR) signaling pathway', 'bile secretion' and 'fat

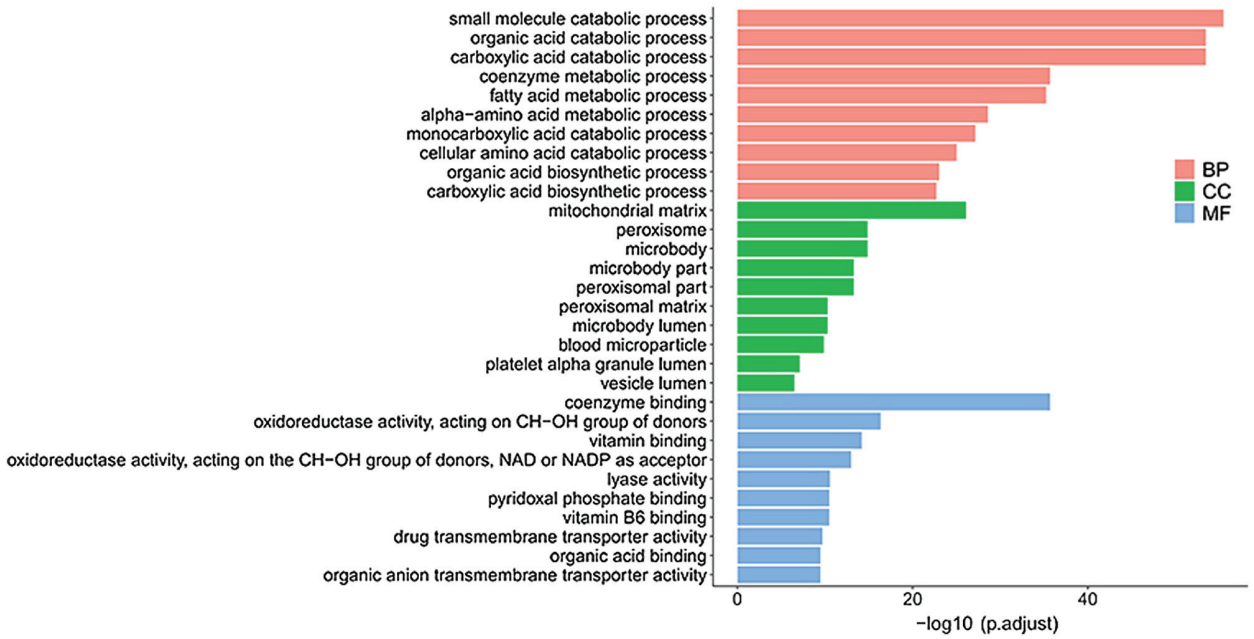

Figure 3. Functional enrichment analysis of differentially expressed mRNAs. The top 10 significantly enriched Gene Ontology terms in the categories BP, CC and MF are provided. BP, biological process; CC, cellular component; MF, molecular function.

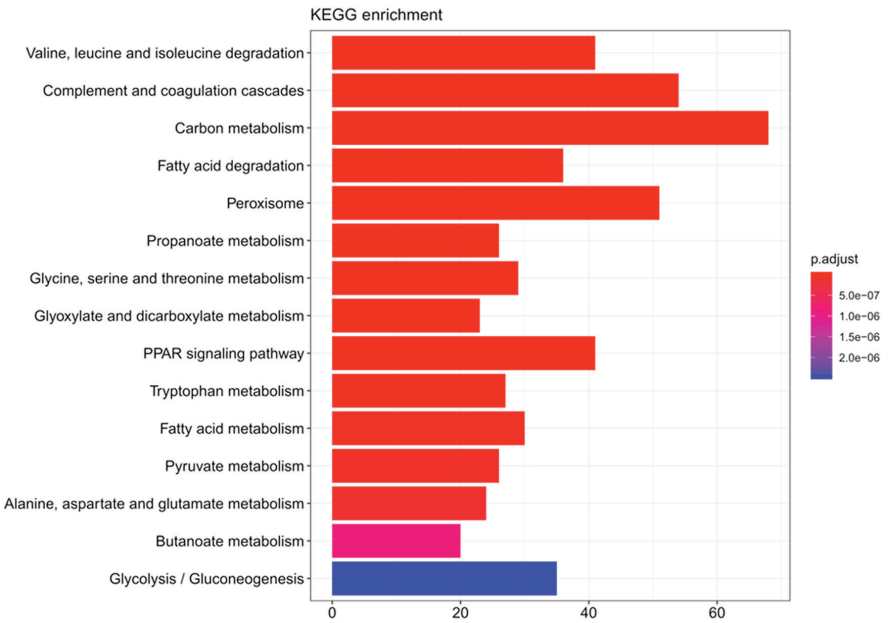

Figure 4. The top 15 KEGG pathways with the most significant P-values. The x-axis represents the number of differentially expressed mRNAs involved in the respective pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR, peroxisome proliferator-activated receptor.

digestion' were significantly enriched, and these have been reported to exert a pivotal influence on CCA (21,22).

Of the 16 DE lncRNAs identified in the ceRNA network, the complementary mRNAs mainly bound to H19, F-box and leucine rich repeat protein 19-AS1, HLA complex group 18, PVT1 and small nucleolar RNA host gene 1. H19 and PVT1 are the most widely studied lncRNAs among the 16 DE lncRNAs. A growing number of studies have highlighted the fact that the oncofetal lncRNA gene H19 is a critical factor in embryonic development, fibrosis and tumorigenesis (23,24). Accumulating evidence has revealed that the tumor suppressor protein and cell cycle regulator p53 negatively regulates H19 in tumor cells. Yang *et al* (25) reported that H19 was associated with p53 inactivation, which may contribute to suppression of apoptosis and increased cell proliferation in gastric cancer. Furthermore, it was reported that H19-derived miR-675 has an important role in inhibiting p53 and p53-dependent protein expression in bladder cancer cells *in vivo* (26). H19 also has crucial roles in tumor metastasis through the regulation of epithelial to mesenchymal transition (EMT), by functioning as an miRNA sponge in colorectal cancer (27,28). A study has indicated that oxidative stress caused by infection is linked

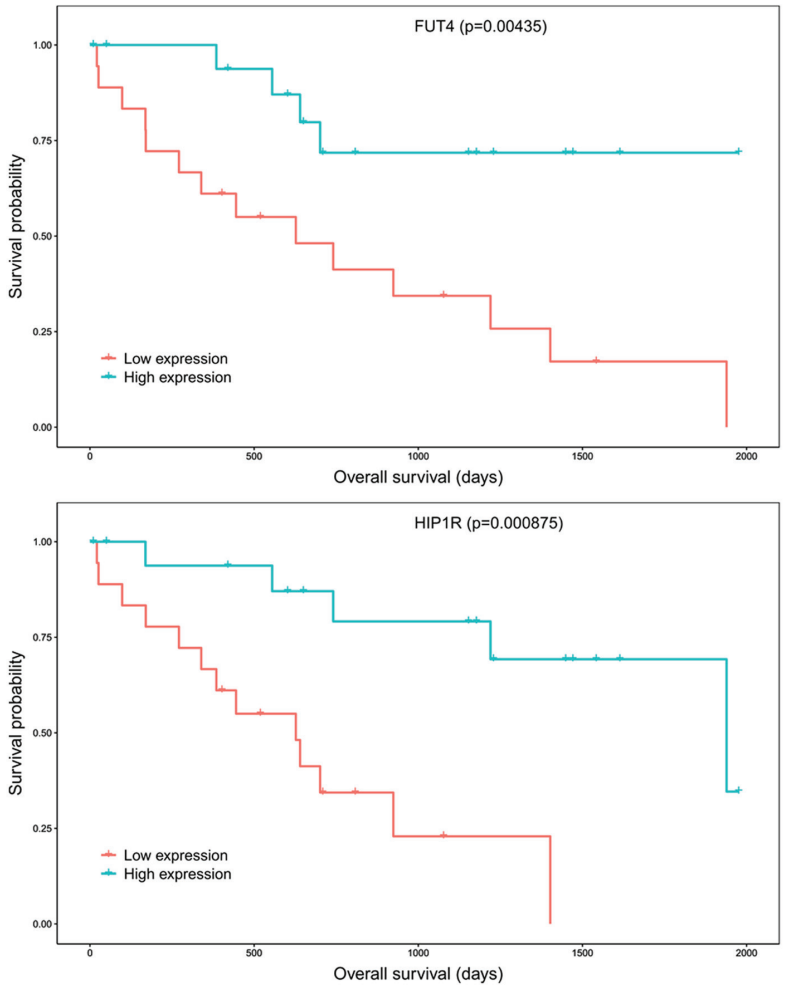

Figure 5. Kaplan-Meier analysis of overall survival associated with the expression status of the mRNAs FUT4 and HIP1R from the competing endogenous RNA network of cholangiocarcinoma patients. Other RNAs in the network analyzed using K-M methods with P<0.05 were not shown in the figure. FUT4, fucosyltransferase 4; HIP1R, huntingtin-interacting protein 1 related.

to inflammation and the occurrence of CAA (29). In addition, H19 is considered to be involved in the progression of CCA by causing partial inactivation of interleukin-6 and downstream inflammatory responses triggered by oxidative stress (30). Similar to H19, overexpression of PVT1 has also been indicated to be associated with poor prognosis in a variety of human malignancies (31). In non-small cell lung cancer, PVT1 contributes to lung adenocarcinoma cell proliferation, and knockdown markedly reduces cell proliferation and induces apoptosis *in vitro* and *in vivo* (32). This phenomenon may be partly mediated through enhancer of zeste 2 polycomb repressive complex 2 subunit-associated suppression of the large tumor suppressor kinase 2/MDM2/p53 pathway. PVT1 has been further observed to be involved in the regulation of EMT, which is a vital step in the progression, invasion and metastasis of esophageal cancer (33). Furthermore, PVT1 was reported to promote liver fibrosis by downregulating patched 1 expression via binding to miR-152 and contributing to EMT (34). Huang *et al* (35) reported that increased expression of PVT1 is associated with tumor progression, and suggested that PVT1 may be an independent prognostic factor for poor prognosis in pancreatic cancer patients. Furthermore, a meta-analysis reported that increased PVT1 expression was significantly associated with factors of poor prognosis, including positive lymph node metastasis, positive distant metastasis, advanced tumor-nodes-metastasis stage and poor degree of differentiation, suggesting that PVT1 may be a potential biomarker in various cancer types (36). However, studies on the association between H19 or PVT1 and CCA have rarely been performed.

PPARs belong to the ligand-inducible nuclear hormone receptor superfamily (37). By forming heterodimers with retinoid X receptor, PPARs modulate the expression of lipid metabolism-, adipogenesis-, inflammation- and anti-cancer-associated genes in various human cancer types (37). In the present study, pathway analysis revealed that the PPAR signaling pathway was significantly enriched and exerted a pivotal influence in patients with CCA. Of all PPAR isoforms, PPARγ is considered to be most associated with tumors through the activation of different pathways. PPAR ligands have been reported to promote differentiation and apoptosis in numerous malignancies, including CCA, breast cancer and ovarian cancer (38-40). Han *et al* (41) reported that ligands of PPARγ inhibit the proliferation of CCA cells through p53-dependent mechanisms. Furthermore, the PPARγ ligand 15-deoxy-δ-12,14-PGJ2 may induce apoptosis in CCA cell lines, although apoptosis-associated protein expression varies between different cell lines (41). In addition, accumulating

evidence suggests that the mechanisms of the EMT promote the occurrence of CCA (42). PPARγ is able to upregulate the expression of Sprouty 4 through Wnt7A/Fzs9 signaling, and suppresses EMT (42). On the contrary, ectopic PPARγ expression in CCA cell lines may promote cell proliferation via the Smad pathway, which is a vital step in EMT (43,44). Suzuki *et al* (45) reported on the administration of the PPARγ agonist pioglitazone in a 73-year-old male patient diagnosed with diabetes, CCA and CCA-induced cholangiohepatitis. In this case, not only the diabetes was controlled, but also the cholangiohepatitis, which was thought to be linked to CCA progression. Furthermore, Asukai *et at* (46) reported that pioglitazone had a synergistic effect with gemcitabine and alleviated gemcitabine resistance in CCA gemcitabine-resistant cells by reducing miR-130a-3p expression *in vitro*. However, further studies of how PPARs are involved in CCA pathogenesis will be required.

The prognostic value of the ceRNA network was also analyzed. The results revealed that high expression of FUT4 ($P<0.005$) and HIP1R ($P<0.001$) was positively correlated with overall survival. However, according to previous studies, increased FUT4 expression was observed to be associated with poor prognosis in breast cancer, lung adenocarcinoma and colorectal cancer, which was contrary to the present results (47-49). Another study suggested that lower HIP1R protein expression is associated with a poor overall survival rate and progression-free survival in patients with diffuse large B-cell lymphoma (50). However, the opposite appears to be the case for prostate cancer: HIP1R was reported to increase the migratory and invasive properties of prostate cancer cells, and this may be suppressed by the miRNA-23b/27b cluster (51). To the best of our knowledge, the roles of FUT4 or HIP1R in CCA have not been assessed by any previous study.

The present study had several limitations. In the present study, only the data from TCGA database were considered to create the network. Furthermore, the network was not validated by any other database or *in vitro* analysis. Therefore, further confirmation of key RNAs and investigation of the possible molecular biological mechanisms of CCA in human CCA tissues will be performed in the future. Furthermore, the ceRNA network established in the present study requires broad validation by future studies.

In conclusion, in the present study, an integrated analysis of DE lncRNAs, miRNAs and mRNAs in CCA as performed and a ceRNA network was constructed. This ceRNA network may provide novel insight for further mechanistic investigation and may lead to improvements in the survival and prognosis of patients with CAA. Further experimental verification is required.

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Availability of data and materials

All data were collected from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/)

Authors' contributions

WG concieved and designed the study. FX designed the experiments and wrote the first draft of the manuscript. YZ participated in data analysis, intepreted the data and revised the manuscript. GQ collected and analyzed the data. YH and LL prepared all figures and tables; WG and LL revised the manuscript and approved the final version. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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