A novel three-miRNA signature predicts survival in cholangiocarcinoma based on RNA-Seq data

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Abstract. Accumulating evidence illustrates that many microRNAs (miRNAs) are abnormally expressed in cholangiocarcinoma and play important roles in tumorigenesis, tumor progression and metastasis. These miRNAs may serve as prognostic biomarkers and potential therapeutic targets. The aim of the present study was to identify the differentially expressed miRNAs in cholangiocarcinoma tissues vs. normal tissues by analyzing high-throughput data derived from The Cancer Genome Atlas (TCGA) database. Furthermore, we evaluated the prognostic performance of the differentially expressed miRNAs and developed a novel three-miRNA signature which predicted survival in cholangiocarcinoma patients. According to the cut-off criteria of $P<0.01$ and $|\log_2 FC|>1.0$, a total of 100 miRNAs (54 upregulated and 46 downregulated) were found to be differentially expressed and some of them were significantly associated with clinical features. Of the above 100 miRNAs, we obtained three miRNAs (miR-10b, miR-22 and miR-551b) which were markedly related to patient overall survival (OS). Subsequently, a novel three-miRNA signature was established and validated to be effective to predict survival. The results demonstrated that the survival rate, as well as the survival time of patients were obviously enhanced in relation to a lower miRNA signature index. Univariate and multivariate Cox regression analyses revealed that the three-miRNA signature was an independent prognostic factor in cholangiocarcinoma. The reliability of the three-miRNA signature was validated by an independent cohort from Gene Expression Omnibus (GEO). Furthermore, the functional enrichment analysis emphasized that the target genes of the aforementioned miRNAs may be involved in a variety of pathways and processes associated with cancer. Finally, these three miRNAs were detected for verification of expression using RT-qPCR, and miR-551b was selected for the verification of biological functions in cholangiocarcinoma cells. The results revealed that overexpression of miR-551b decreased cancer cell proliferation and promoted apoptosis. Collectively, the results of the present study indicated that a specific three-miRNA signature could be considered as an alternative prognostic marker in cholangiocarcinoma.

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Introduction

Cholangiocarcinoma, a highly aggressive tumor derived from bile duct epithelial cells, is one of the most severe forms of cancer with a 5-year survival rate <10% (1,2). During the last decades, the incidence and mortality rate of cholangiocarcinoma have been increasing globally (3,4). The survival quality and prognosis of patients with cholangiocarcinoma are poor as a result of early cancer cell invasion and metastasis (2). Radical surgery is the only curative treatment for cholangiocarcinoma, while patients gain little benefit, as they are usually diagnosed at an advanced stage (5,6). Thus, the exploration of powerful markers which may provide prognostic value for cholangiocarcinoma patients is of great significance. Currently, prognostic microRNA (miRNA) expression signatures in various cancers, such as colon cancer (7), clear cell renal cell carcinoma (8) and cervical cancer (9) have attracted the attention of researchers. Therefore, the miRNA signature has been regarded as an important change in cholangiocarcinoma progression and therapy (10).

miRNAs, a class of small non-coding RNAs of ~22-23 nucleotides in length, are considered to play pivotal roles in post-transcriptional gene regulation. It has been confirmed that miRNAs regulate malignancies by binding to the partially complementary recognition sequences in the 3-untranslated region of mRNAs, which causes target mRNA translation inhibition or degradation (11). A number of miRNAs play vital roles in tumorigenesis, such as cell proliferation, apoptosis, autophagy, migration, invasion and metastasis (12,13). Accordingly, miRNAs have a large potential to serve as markers in the diagnosis, prognosis and targeted therapies of cancers.

Although a large number of miRNAs have been identified in predicting the clinical outcome in cholangiocarcinoma, there are some limitations in previous studies. These may be due to molecular and clinical heterogeneity in different studies, relatively limited numbers of miRNAs, along with methodological differences in detection and analysis. The Cancer Genome Atlas (TCGA) is a National Cancer Institute effort to profile >20 different tumor types using genomic platforms and to make raw and processed data available to researchers worldwide (14). TCGA provides a collection of clinical data, RNA sequence, DNA copy number variations, DNA methylation and miRNA sequence profiles for cholangiocarcinoma. The aim of the present study was to identify the differentially expressed miRNAs between cholangiocarcinoma and normal tissues by analyzing high-throughput data downloaded from TCGA database. Furthermore, we evaluated the prognostic performance of the differentially expressed miRNAs and established a novel three-miRNA signature which could effectively predict survival in cholangiocarcinoma patients.

Materials and methods

TCGA dataset of cholangiocarcinoma. The miRNA sequencing data and corresponding clinical information for cholangiocarcinoma patients (up to June 29, 2017) were downloaded from TCGA data portal (https://portal.gdc.cancer.gov). The inclusion criteria were set as follows: i) samples with both miRNA sequencing data and clinical information; and ii) samples with detailed prognostic information. Finally, a total of 45 samples were enrolled in this study, including 36 cholangiocarcinoma tissues and 9 matched normal tissues.

Exploration of the differentially expressed miRNAs in cholangiocarcinoma. The RNA-Seq data of cholangiocarcinoma with 1,046 miRNAs were analyzed on the Illumina HiSeq miRNA Seq platform. Subsequently, the R language package 'edgeR' was used for the calculation of differentially expressed miRNAs (15). The expression difference of individual miRNAs was characterized by $\log_2$FC and adjusted P-value. LogFC indicates the fold change in expression of each miRNA between cholangiocarcinoma tissues and normal tissues. Upregulated and downregulated miRNAs were determined based on $\log_2$FC >1 and $\log_2$FC <-1 respectively, with adjusted P<0.01. The miRNAs which had expression mean value <1 were excluded.

Selection of the cut-off point for the Kaplan-Meier survival analysis. Cutoff Finder (http://molpath.charite.de/cutoff) was used to determine a cut-off point for patient stratification into two groups (16). Then, the differences in patient overall survival (OS) between the high-level and the low-level group were evaluated by Kaplan-Meier survival analysis (log-rank method). The miRNAs with a P-value <0.01 were regarded to display statistically significant differences between groups and were considered for further analysis.

Association between miRNA signature index and OS. A value of one or zero was assigned to patients according to each miRNA value. Subsequently, each miRNA value was scored in the signature. Thus, each patient would have a score, defined as miRNA signature index. We set index as high-risk and low-risk into two new groups according to the index value. Then, Kaplan-Meier survival analysis (log-rank method) was performed to evaluate the differences in patient OS between these two groups.

Information collection of the validation dataset. An independent cohort of cholangiocarcinoma patients (GSE53870) (17) downloaded from Gene Expression Omnibus (GEO) database was used for the prognostic signature validation. There consisted of 63 cholangiocarcinoma patients and corresponding prognostic information in the GSE53870 dataset.

Target gene prediction of three prognostic miRNAs and functional analysis. The target genes of the three prognostic miRNAs were predicted using TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/miRDB/), and miRanda (http://www.microrna.org/) online analysis tools. To further increase the bioinformatics analysis reliability, Venn diagram was carried out to identify the overlapping target genes. Furthermore, the Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of target genes were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (https://david.ncifcrf.gov/). The results were then presented in a bubble diagram.

RNA isolation and qRT-qPCR. Total RNA was extracted from cells using an HP Total RNA kit (Omega Biotech, Stamford, CT, USA) according to the manufacturer's protocol. Synthesis of cDNA with reverse transcriptase (RT) was performed with an M-MLV First Strand kit (Life Technologies; Thermo Fisher Scientific, Inc., Gaithersburg, MD, USA). Primer sequences for miR-10b, miR-22, miR-551b and U6 detection were obtained from RiboBio (Guangzhou, China). The RT primers for mature miRNAs and U6 were designed according to the concept of a stem-loop RT primer (18). RT-qPCR analysis was carried out using Platinum SYBR-Green qPCR SuperMix-UDG kits (Life Technologies) according to the manufacturer's protocol. Real-time PCR was performed on an Applied Biosystems ABI PRISM 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). Ct values of miRNAs were equilibrated to U6, which was used as an internal control. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

Cell culture and transfection. Human cholangiocarcinoma cell line HUCCT1 (cat. no. JCRB0425) was purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan) and human intrahepatic biliary epithelial cell line (HiBEC) (cat. no. 5100) was purchased from the ScienCell Research Laboratories (San Diego, CA, USA) and were cultured under standard conditions. When HUCCT1 cells reached 50-70% confluence, miR-551b mimics and negative control were transfected using Invitrogen™ Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. miR-551b mimics (sense, 5'-GCGACCCAUACUUGGUUUCAG-3' and antisense, 5'-GAAACCAAGUAUGGGUCGCUU-3') and corresponding negative control were purchased from GenePharma (Shanghai, China).

Proliferation assay. HUCCT1 cells were transfected with miR-551b mimics or negative control for 48 h, and then were plated into 96-well plates at a density of $5x10^3$ cells/well and then 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added and incubated for 4 h. The supernatant was then replaced with 100 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and read at 490 nm using a multifunction microplate reader (POLARstar OPTIMA; BMG, Offenburg, Germany). Concerning the colony formation assay, HUCCT1 cells ($1x10^3$ cells/dish) were seeded on 35-mm petri dishes. Cells were further cultured for two weeks to allow colonies to form. At the indicated time-point, colonies were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet solution and rinsed. Then, images were captured using a Nikon camera (Nikon Corp., Tokyo, Japan) and the colony number was counted.

Apoptosis assay. HUCCT1 cells were transfected with miR-551b mimics or negative control for 48 h, then were harvested by trypsinization in a tube and were washed twice in ice-cold phosphate-buffered saline (PBS). After staining using an Annexin V-FITC/7-AAD apoptosis detection kit (Becton-Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's protocol, the cell apoptosis rate was assessed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA).

Statistical analysis. Mann-Whitney U test was used to compare the expression levels of miRNAs between two different groups of each clinical characteristic. Univariate/multivariate Cox proportional hazard regression analyses were performed to compare each clinical parameter and prognostic miRNA signature (high-risk vs. low-risk). All statistical analysis was performed by SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as a two-sided P-value <0.05, unless specifically indicated.

Results

Exploration of differentially expressed miRNAs. A total of 45 samples were enrolled in our study, including 36 cholangiocarcinoma tissues and 9 matched normal tissues. The specific clinical characteristics included sex, age at diagnosis, stage, T stage, lymph node status, metastasis and histological type (Table I). According to the cut-off criteria of P<0.01 and $|\log_2 FC|>1.0$, a total of 100 miRNAs were found to be differentially expressed in cholangiocarcinoma vs. normal tissues, including 54 upregulated and 46 downregulated miRNAs (Table II). In order to show the above differentially expressed miRNA more clearly, we present the results as a Volcano plot (Fig. 1). Furthermore, it was obvious that cholangiocarcinoma tissues could be clearly discriminated from normal tissues in terms of differentially expressed miRNA patterns using unsupervised hierarchic cluster analysis (Fig. 2).

Association between differentially expressed miRNAs and clinical features. The differentially expressed miRNAs were further analyzed upon the expression level and clinical characteristics. Notably, miR-490 and miR-141 were found to be related with sex, whereas miR-615, miR-135b, miR-92b, miR-23c and miR-149 were found to be related with age at diagnosis. Furthermore, miR-301a was associated with stage, whereas miR-551b, miR-222 and miR-221 were associated with T stage, miR-92b and miR-615 were associated with lymph node status, miR-101-1 and miR-301a were associated with metastasis. In addition, we also found other 8 miRNAs which were linked to histological type (Table III).

Table I. Clinical characteristics of the cholangiocarcinoma patients (n=36).

Variables	Cases, n (%)
Sex	
Female	20 (55.56)
Male	16 (44.44)
Age at diagnosis (years)	
≤60	14 (38.89)
>60	22 (61.11)
Stage	
I+II	28 (77.78)
III+IV	8 (22.22)
T stage	
T1+T2	31 (86.11)
T3+T4	5 (13.89)
Lymph node status	
N0	26 (72.22)
N1	5 (13.89)
NX	5 (13.89)
Metastasis	
M0	28 (77.78)
M1	5 (13.89)
MX	3 (8.33)
Histological type	
Intrahepatic	30 (83.33)
Hilar+distal	6 (16.67)

Identification of three miRNAs associated with OS. Of the aforementioned 100 miRNAs, we used Cutoff Finder to determine a cut-off point and classified patients into two groups based on the miRNA expression level. Subsequently, the Kaplan-Meier survival analysis was performed to explore the association between miRNA expression and OS in cholangiocarcinoma patients. According to the survival analysis, three miRNAs were identified to be significantly associated with OS in cholangiocarcinoma patients. These three miRNAs were miR-10b, miR-22 and miR-551b. These results illustrated that high expression levels of miR-10b and miR-551b were considered as better prognostic markers vs. the low level group (Fig. 3A and C). In contrast, compared to the high-level group, low expression level of miR-22 revealed a longer survival rate and time (Fig. 3B).

Table II. Differentially expressed miRNAs between cholangiocarcinoma and normal tissues.

Upregulated miRNAs				Downregulated miRNAs			
miRNAs	logFC	P-value	FDR	miRNAs	logFC	P-value	FDR
hsa-mir-182	4.50	1.72E-26	8.03E-24	hsa-mir-148a	-2.67	1.12E-25	2.61E-23
hsa-mir-183	4.85	2.93E-25	4.56E-23	hsa-mir-1258	-4.77	3.35E-20	3.91E-18
hsa-mir-96	4.77	5.87E-19	3.92E-17	hsa-mir-378	-2.93	2.32E-19	2.17E-17
hsa-mir-21	2.53	1.09E-16	4.26E-15	hsa-mir-101-1	-2.02	3.13E-19	2.44E-17
hsa-mir-27a	1.93	2.97E-12	8.15E-11	hsa-let-7c	-2.10	1.39E-18	8.10E-17
hsa-mir-34c	4.83	3.39E-11	7.92E-10	hsa-mir-99a	-2.28	2.15E-18	1.11E-16
hsa-mir-200b	2.80	8.91E-11	1.89E-09	hsa-mir-505	-2.02	2.44E-18	1.14E-16
hsa-mir-92b	2.94	9.35E-11	1.90E-09	hsa-mir-139	-2.76	4.65E-18	1.97E-16
hsa-mir-200a	2.67	1.05E-09	2.04E-08	hsa-mir-125b-2	-2.25	5.65E-16	2.03E-14
hsa-mir-34b	6.26	1.28E-09	2.39E-08	hsa-mir-378c	-2.72	1.15E-14	3.83E-13
hsa-mir-181d	2.54	2.29E-09	3.97E-08	hsa-mir-490	-4.27	4.53E-14	1.41E-12
hsa-mir-23a	1.49	1.07E-08	1.49E-07	hsa-mir-675	-4.08	1.80E-13	5.25E-12
hsa-mir-222	1.98	1.26E-08	1.69E-07	hsa-mir-1468	-2.20	1.10E-11	2.84E-10
hsa-mir-181b-1	2.11	1.83E-08	2.37E-07	hsa-mir-483	-3.40	1.85E-11	4.55E-10
hsa-mir-429	2.75	3.28E-08	4.14E-07	hsa-mir-101-2	-1.80	8.32E-11	1.85E-09
hsa-mir-454	1.70	3.62E-08	4.45E-07	hsa-mir-424	-1.63	1.46E-09	2.62E-08
hsa-mir-221	1.82	5.94E-08	6.93E-07	hsa-mir-122	-4.09	2.42E-09	4.04E-08
hsa-mir-330	1.76	7.15E-08	8.14E-07	hsa-mir-885	-3.80	3.21E-09	5.18E-08
hsa-mir-135b	4.88	2.04E-07	2.11E-06	hsa-mir-22	-1.27	5.24E-09	8.15E-08
hsa-let-7e	1.36	3.31E-07	3.36E-06	hsa-mir-383	-2.81	6.81E-09	1.02E-07
hsa-mir-181c	1.83	4.60E-07	4.57E-06	hsa-mir-551b	-2.54	7.01E-09	1.02E-07
hsa-mir-708	2.62	2.01E-06	1.77E-05	hsa-mir-574	-1.36	1.09E-08	1.49E-07
hsa-mir-99b	1.26	7.07E-06	5.88E-05	hsa-mir-192	-1.91	4.51E-08	5.40E-07
hsa-mir-181b-2	2.00	7.17E-06	5.88E-05	hsa-mir-624	-1.48	8.71E-08	9.69E-07
hsa-mir-301a	1.27	1.39E-05	0.000108	hsa-mir-455	-1.71	1.62E-07	1.76E-06
hsa-mir-181a-1	1.36	1.91E-05	0.000144	hsa-mir-152	-1.17	1.82E-07	1.93E-06
hsa-mir-1301	1.50	2.29E-05	0.00017	hsa-mir-194-2	-1.67	4.97E-07	4.84E-06
hsa-mir-196b	4.30	2.38E-05	0.000174	hsa-mir-194-1	-1.65	7.29E-07	6.95E-06
hsa-mir-1266	2.18	2.70E-05	0.000194	hsa-mir-618	-1.96	1.08E-06	1.01E-05
hsa-mir-561	3.32	2.91E-05	0.000206	hsa-mir-144	-2.25	1.25E-06	1.14E-05
hsa-mir-200c	5.13	3.64E-05	0.000254	hsa-mir-1228	-1.55	1.38E-06	1.24E-05
hsa-mir-218-2	1.70	4.09E-05	0.000277	hsa-mir-511-2	-1.69	3.88E-06	3.35E-05
hsa-mir-149	1.89	7.53E-05	0.000488	hsa-mir-511-1	-1.73	4.10E-06	3.48E-05
hsa-mir-141	4.73	9.43E-05	0.000603	hsa-mir-125b-1	-1.17	7.87E-06	6.34E-05
hsa-mir-625	1.69	0.00011	0.000683	hsa-mir-548b	-2.35	1.28E-05	0.000102
hsa-mir-10b	2.38	0.000126	0.000774	hsa-mir-3614	-1.31	3.91E-05	0.000269
hsa-mir-196a-1	5.03	0.000151	0.000883	hsa-mir-33b	-1.48	6.25E-05	0.000417
hsa-mir-1224	4.63	0.000177	0.001009	hsa-mir-3648	-1.79	7.18E-05	0.000472
hsa-mir-3934	2.08	0.000189	0.00105	hsa-mir-486	-1.64	0.000107	0.000672
hsa-mir-615	5.88	0.000218	0.001184	hsa-mir-23c	-1.46	0.000151	0.000883
hsa-mir-598	1.37	0.000332	0.00176	hsa-mir-548j	-1.48	0.000184	0.001033
hsa-mir-187	2.69	0.000544	0.002704	hsa-mir-328	-1.06	0.000206	0.001135
hsa-mir-3200	1.82	0.000662	0.003252	hsa-mir-365-1	-1.03	0.000687	0.003343
hsa-mir-320b-2	1.66	0.00071	0.00342	hsa-mir-211	-2.16	0.001105	0.005009
hsa-mir-1180	1.15	0.000753	0.003587	hsa-mir-451	-1.44	0.001677	0.007107
hsa-mir-526b	6.47	0.000834	0.003895	hsa-mir-3944	-1.78	0.002201	0.00871
hsa-mir-1304	2.47	0.000859	0.003972				
hsa-mir-31	4.13	0.001327	0.005901				
hsa-mir-3691	2.94	0.001532	0.006748				
hsa-mir-218-1	2.09	0.001665	0.007107				

Table II. Continued.

Upregulated miRNAs				Downregulated miRNAs			
miRNAs	logFC	P-value	FDR	miRNAs	logFC	P-value	FDR
hsa-mir-577	3.92	0.001689	0.007107				
hsa-mir-137	4.94	0.001893	0.007687				
hsa-mir-196a-2	4.62	0.002038	0.008205				
hsa-mir-30d	1.13	0.00219	0.00871				

FDR, false discovery rate.

Table III. Differentially expressed miRNAs associated with clinical features.

Variables	Upregulated miRNAs identified in TCGA	Downregulated miRNAs identified in TCGA
Sex (female vs. male)	miR-141	miR-490
Age at diagnosis (≤60 vs. >60)	miR-615, miR-135b, miR-92b, miR-149	miR-23c
Stage (I+II vs. III+IV)	miR-301a	
T stage (T1+T2 vs. T3+T4)	miR-222, miR-221	miR-551b
Lymph node status (N0 vs. N1)	miR-92b, miR-615	
Metastasis (M0 vs. M1)	miR-301a	miR-101-1
Histological type (intrahepatic vs. hilar+distal)	miR-23a, miR-196a-1, miR-27a, miR-598, miR-31, mir-181c	miR-365-1, miR-383

Definition of three-miRNA signature index for cholangiocarcinoma prognosis. In order to establish the miRNA signature index, we assigned a score for each patient. To be specific, patients who belonged to the shorter survival group received one score for each miRNA, while those who belonged to the longer survival group received a 0 score for each miRNA. Subsequently, we calculated the total score for each patient. Subsequently, we calculated the score for each patient. According to these criteria, the highest score was 3 and the lowest score was 0. Subsequently, we ranked 36 cholangiocarcinoma patients based on their miRNA signature index and divided them into two new groups (Table IV). The high-risk group miRNA signature index score was 2-3, while the score in the low-risk group was 0-1. Markedly, Kaplan-Meier survival analysis illustrated that these two groups were significantly associated with patient OS (Fig. 4). In the low-risk group, ~80% patients showed 5-year survival, while none of the patients survived >5 years in the high-risk group. Furthermore, the median survival of the low-risk group was markedly longer than that of the high-risk group (63.75 vs. 14.63 months). As a result, our findings demonstrated that the survival rate, as well as the survival time of patients were obviously enhanced in relation to a lower miRNA signature index.

Taking into account the following clinical parameters: Sex, age at diagnosis, stage, T stage, lymph node status, metastasis and histological type, univariate and multivariate Cox regression analysis was used to test the effect of the three-miRNA signature (high-risk vs. low-risk) on OS. In univariate analysis, stage [hazard ratio (HR)=3.104, P=0.040] and three-miRNA signature (HR=6.013, P<0.0001) were associated with OS in cholangiocarcinoma patients. In multivariate analysis, three-miRNA signature (HR=6.124, P<0.0001) was revealed to be an independent prognostic factor in cholangiocarcinoma patients (Table V).

Three-miRNA signature verification in the validation cohort. In order to validate the performance of the prognostic miRNA signature, it was tested on the GSE53870 dataset derived from GEO database. As displayed in Fig. 5, miR-22 and miR-551b were observed to be markedly associated with OS in cholangiocarcinoma patients (P<0.05), while miR-10b was found to be marginally significant with patient OS (P=0.0511).

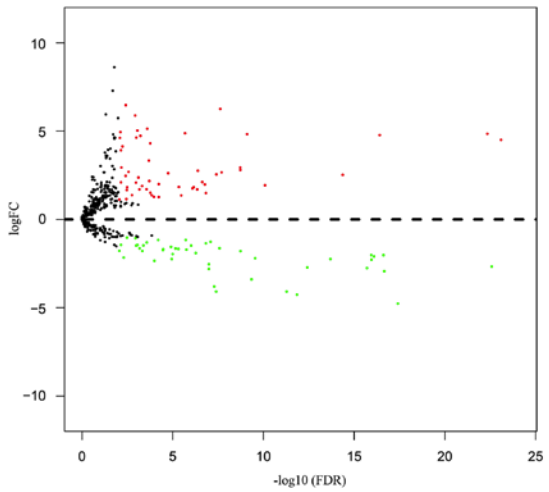

Figure 1. Volcano plot of differentially expressed miRNAs. The red dots represent upregulated miRNAs, and the green dots represent downregulated miRNAs. miRNAs, microRNAs; FDR, false discovery rate.

Subsequently, we used the same risk-score formula to calculate three-miRNA signature index for each of the 63 patients and dichotomized them into low-risk and high-risk group. Notably, these two groups were surprisingly associated with patient OS. Based on the three-miRNA signature index, >40% patients in the low-risk group showed 5-year survival, while none of the patients survived >5 years in the high-risk group. In addition, the median survival of the low-risk group was significantly longer than that of high-risk group (34.20 vs. 12.80 months). These findings were similar to what was noted in TCGA data. Collectively, these results proved the accuracy of the prognostic miRNAs signature.

KEGG pathway enrichment and GO annotation of three miRNA predicted genes. TargetScan, miRDB and miRanda online analysis tools were used to predict the target genes of these three miRNAs (miR-10b, miR-22 and miR-551b). A total of 110 overlapping genes of miR-10b, 222 overlapping genes of miR-22, and 2 overlapping genes of miR-551b were identified (Fig. 6). Then, enrichment analysis was performed to elucidate the biological function of consensus target genes. Notably, cancer-related pathways were found to be intensely activated according to KEGG results, including small cell lung cancer, chronic myeloid leukemia, prostate cancer, glioma, miRNAs in cancer and proteoglycans in cancer. We hypothesized that these target genes play pivotal roles in various types of cancers. Furthermore, target genes were significantly enriched in the mTOR, FoxO and HIF-1 signaling

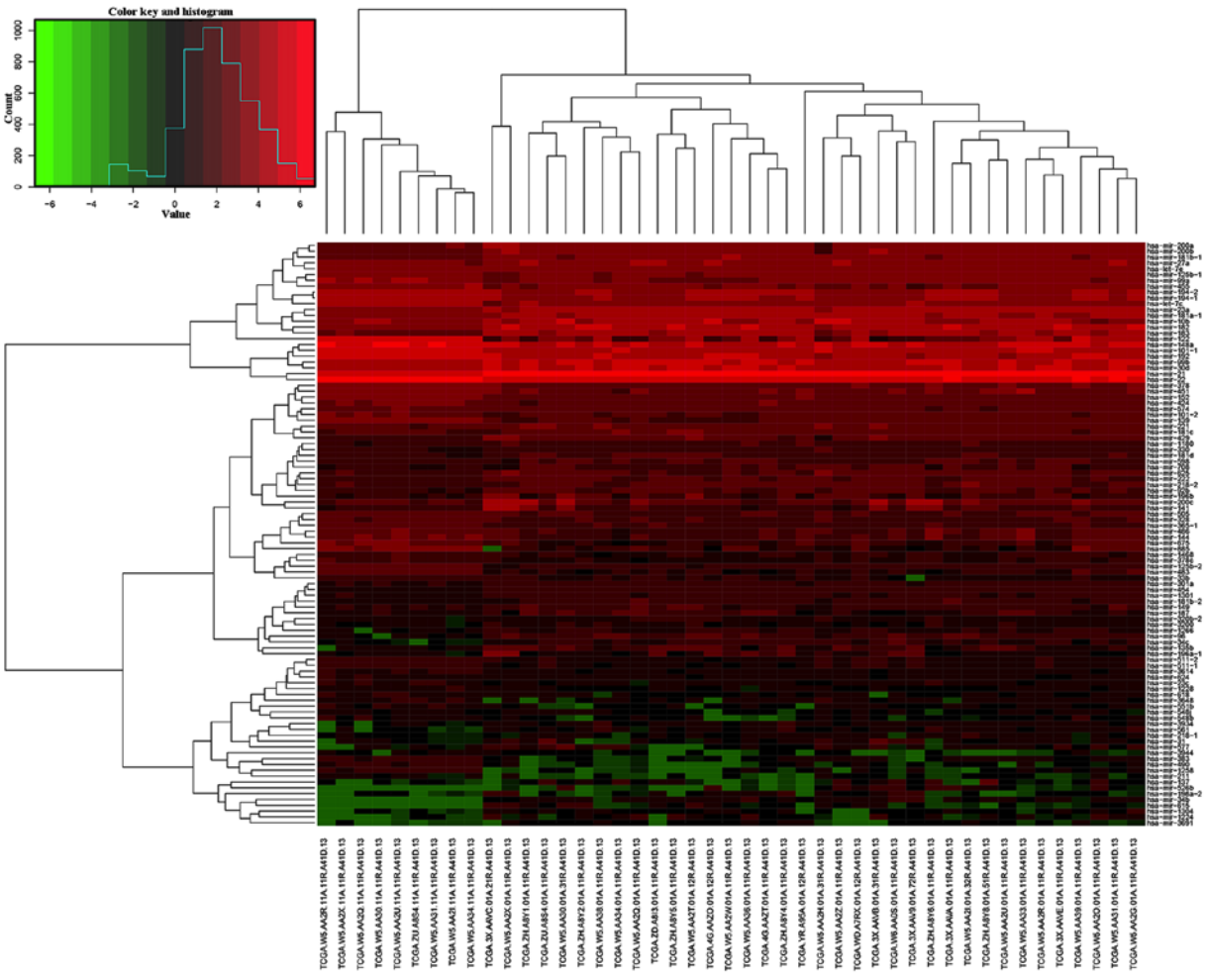

Figure 2. Hierarchical clustering of cancer tissues and non-cancer tissues by differentially expressed miRNAs. The heatmap consist of 9 normal tissues (left panel) and 36 cholangiocarcinoma tissues (right panel). Each row represents the expression level of a miRNA, and each column represents a sample. miRNAs, microRNAs.

Table IV. Three-miRNA signature index for survival analysis.

Patient ID	Survival status	Overall survival (months)	miR-10b	miR-22	miR-551b	miRNA index
TCGA-3X-AAVB	Alive	13.22	0	0	0	0
TCGA-3X-AAVE	Alive	21.37	0	0	0	0
TCGA-3X-AAVC	Alive	23.31	0	0	0	0
TCGA-W5-AA2H	Alive	35.41	0	0	0	0
TCGA-W5-AA33	Alive	47.64	0	0	0	0
TCGA-W5-AA2R	Alive	50.70	0	0	0	0
TCGA-W5-AA2Q	Alive	1.64	0	0	1	1
TCGA-4G-AAZT	Alive	13.81	0	0	1	1
TCGA-ZH-A8Y8	Alive	19.79	0	0	1	1
TCGA-ZH-A8Y4	Dead	24.36	0	0	1	1
TCGA-W5-AA30	Alive	37.91	0	0	1	1
TCGA-4G-AAZO	Alive	38.70	0	0	1	1
TCGA-ZH-A8Y5	Alive	40.41	0	0	1	1
TCGA-W5-AA36	Dead	46.09	1	0	0	1
TCGA-W5-AA38	Alive	48.36	0	0	1	1
TCGA-W5-AA2Z	Alive	53.06	0	0	1	1
TCGA-W5-AA2I	Dead	63.75	1	0	0	1
TCGA-W5-AA2G	Alive	64.96	1	0	0	1
TCGA-W5-AA31	Alive	0.33	1	1	0	2
TCGA-WD-A7RX	Dead	0.69	1	0	1	2
TCGA-ZU-A8S4	Dead	3.22	0	1	1	2
TCGA-ZD-A8I3	Dead	5.56	0	1	1	2
TCGA-W5-AA2X	Dead	8.91	1	0	1	2
TCGA-3X-AAV9	Dead	11.15	0	1	1	2
TCGA-ZH-A8Y1	Dead	12.66	1	0	1	2
TCGA-W5-AA34	Dead	18.25	1	0	1	2
TCGA-W5-AA2U	Dead	20.61	0	1	1	2
TCGA-W5-AA2O	Dead	21.04	1	0	1	2
TCGA-ZH-A8Y2	Dead	23.05	1	0	1	2
TCGA-W6-AA0S	Alive	26.56	0	1	1	2
TCGA-W5-AA2W	Dead	30.38	1	0	1	2
TCGA-W5-AA2T	Dead	40.11	1	0	1	2
TCGA-YR-A95A	Dead	0.85	1	1	1	3
TCGA-W5-AA39	Dead	5.59	1	1	1	3
TCGA-3X-AAVA	Dead	14.63	1	1	1	3
TCGA-ZH-A8Y6	Alive	17.06	1	1	1	3

TCGA, The Cancer Genome Atlas.

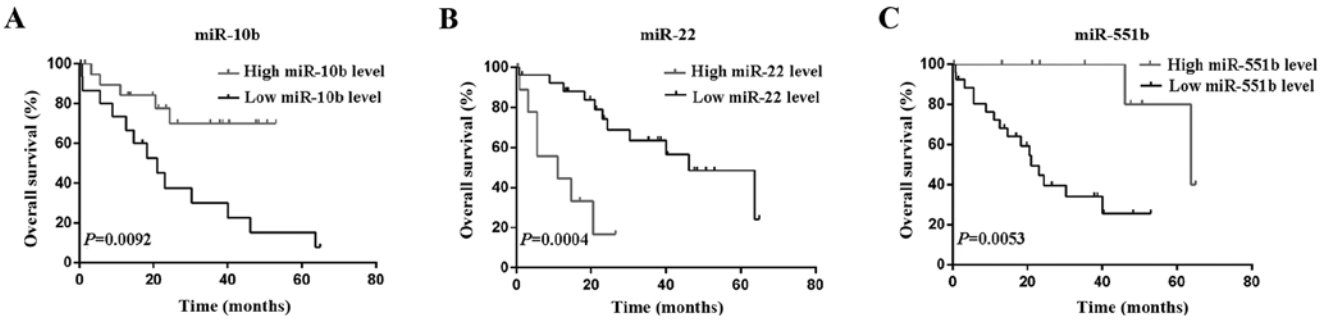

Figure 3. Kaplan-Meier survival curves for three miRNAs, (A) miR-10b, (B) miR-22 and (C) miR-551b, associated with OS in cholangiocarcinoma patients. miRNAs, microRNAs; OS, overall survival.

Table V. Univariate and multivariate analyses of parameters associated with overall survival.

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Sex (male vs. female)	1.387 (0.544-3.534)	0.494		
Age at diagnosis (>60 vs. ≤60)	0.911 (0.350-2.375)	0.849		
Stage (III+IV vs. I+II)	3.104 (1.051-9.169)	0.040	2.983 (0.981-9.072)	0.054
T stage (T3+T4 vs. T1+T2)	0.660 (0.150-2.896)	0.582		
Lymph node status (N1 vs. N0)	2.289 (0.602-8.700)	0.224		
Metastasis (M1 vs. M0)	1.650 (0.462-5.891)	0.440		
Histological type (hilar+distal vs. intrahepatic)	1.197 (0.343-4.172)	0.778		
Three-miRNA signature (high risk vs. low risk)	6.013 (2.621-13.796)	<0.0001	6.124 (2.582-14.525)	<0.0001

HR, hazard ratio; CI, confidence interval.

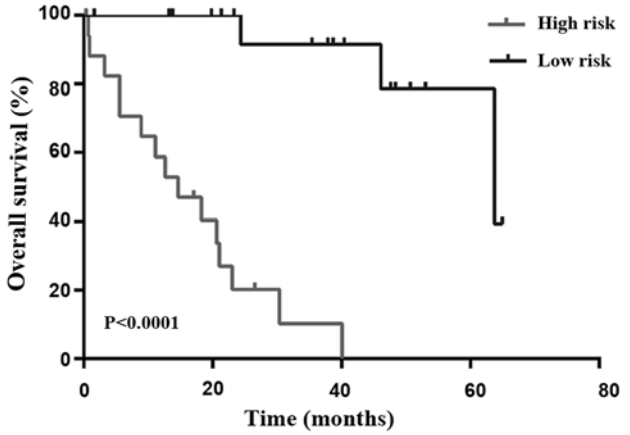

Figure 4. Kaplan-Meier survival curve for the three-miRNA signature in cholangiocarcinoma patients. miRNAs, microRNAs.

pathways (Fig. 7A). The GO biological process terms were mainly enriched in the regulation of metabolism, modification and transcription (Fig. 7B), which indicated that these three miRNAs may be closely associated with biological function and gene expression.

Effect of miR-551b overexpression on the proliferation and apoptosis in HUCCT1 cells. To further validate this conclusion, we first detected the expression of three miRNAs by RT-qPCR. According to the data in Fig. 8, two of the three tested miRNAs (miR-22 and miR-551b) yielded results quite similar to those of the TCGA data. Although the performance of miR-10b was not well-repeated, the direction of change was similar to that noted in the TCGA data. These results indicated that the differentially expressed miRNAs we identified by RNA-Seq data were reliable. As miR-551b is the least studied member among these three miRNAs in cancer, we here focused on its biological function in cholangiocarcinoma. miR-551b mimics were transfected into HUCCT1 cells to upregulate the expression of miR-551b. Successful overexpression of miR-551b in HUCCT1 cells was confirmed by RT-qPCR (Fig. 9A). MTT and colony formation assays revealed that overexpression of miR-551b significantly inhibited proliferation (Fig. 9B) and colony formation in HUCCT1 cells (Fig. 9C). In addition, flow cytometry analysis showed that overexpression of miR-551b significantly induced apoptosis in the HUCCT1 cells (Fig. 9D).

Discussion

In the last decade, miRNAs, as the master modulators of multiple biological and pathological processes, are a 'hot' research topic in the field of cancer development. miRNAs are regarded as a novel group of disease biomarkers for the stability and universality in human tissues (19). Currently, growing investigations have demonstrated specific miRNA profiles in multiple cancers, emphasizing the pivotal roles of miRNAs in the initiation and progression of cancer, including cholangiocarcinoma. Previous studies have demonstrated that many miRNAs are crucial for the initiation, progression, and metastasis of cholangiocarcinoma by regulating various processes, including cancer cell proliferation, apoptosis,

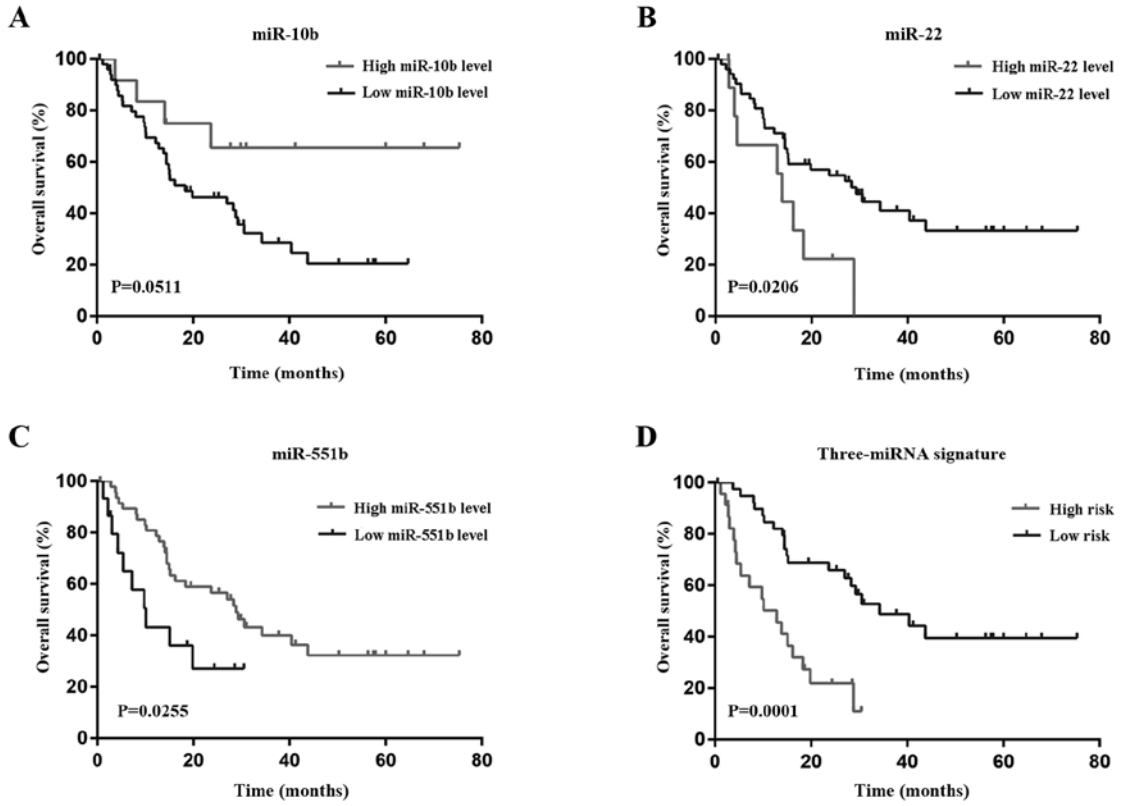

Figure 5. Validations of three miRNAs and three-miRNA signature in an independent cohort. (A) miR-10b was found to be marginally significant with patient OS (P=0.0511). (B and C) miR-22 and miR-551b were markedly associated with OS in cholangiocarcinoma patients (P<0.05). (D) Based on the three-miRNA signature index, >40% patients in the low-risk group showed 5-year survival, while none of the patients survived >5 years in the high-risk group. In addition, the median survival of the low-risk group was significantly longer than that of high-risk group (34.20 vs. 12.80 months) miRNAs, microRNAs; OS, overall survival.

Figure 6. Target gene prediction of three prognostic miRNAs. The overlapping target genes of the three miRNAs, (A) miR-10b, (B) miR-22 and (C) miR-551b were predicted using TargetScan, miRDB and miRanda online analysis tools. miRNAs, microRNAs.

adhesion, cell cycle arrest, migration and invasion (20-23). To date, several studies have identified a number of miRNAs with prognostic value in cholangiocarcinoma, such as miR-126 (24), miR-192 (25), miR-26a (26), miR-203 (27), miR-106a (28) and miR-29a (29). Unfortunately, due to molecular and clinical heterogeneity in different studies, the relatively limited numbers of miRNAs to investigate, as well as the methodological differences in detection and analysis, there still exist some restrictions for applying the above specific miRNAs for prognosis.

TCGA was constructed to contain a wide assortment of high-throughput experimental data, which are available and will be valuable to researchers worldwide. In our study, we analyzed high-throughput data downloaded from TCGA database, and eventually obtained 100 differentially expressed miRNAs between cholangiocarcinoma and normal tissues, of which 54 were upregulated and 46 were downregulated. Subsequently, we evaluated the prognostic value of each differentially expressed miRNA. According to previous research, the performance of a single biomarker in predicting

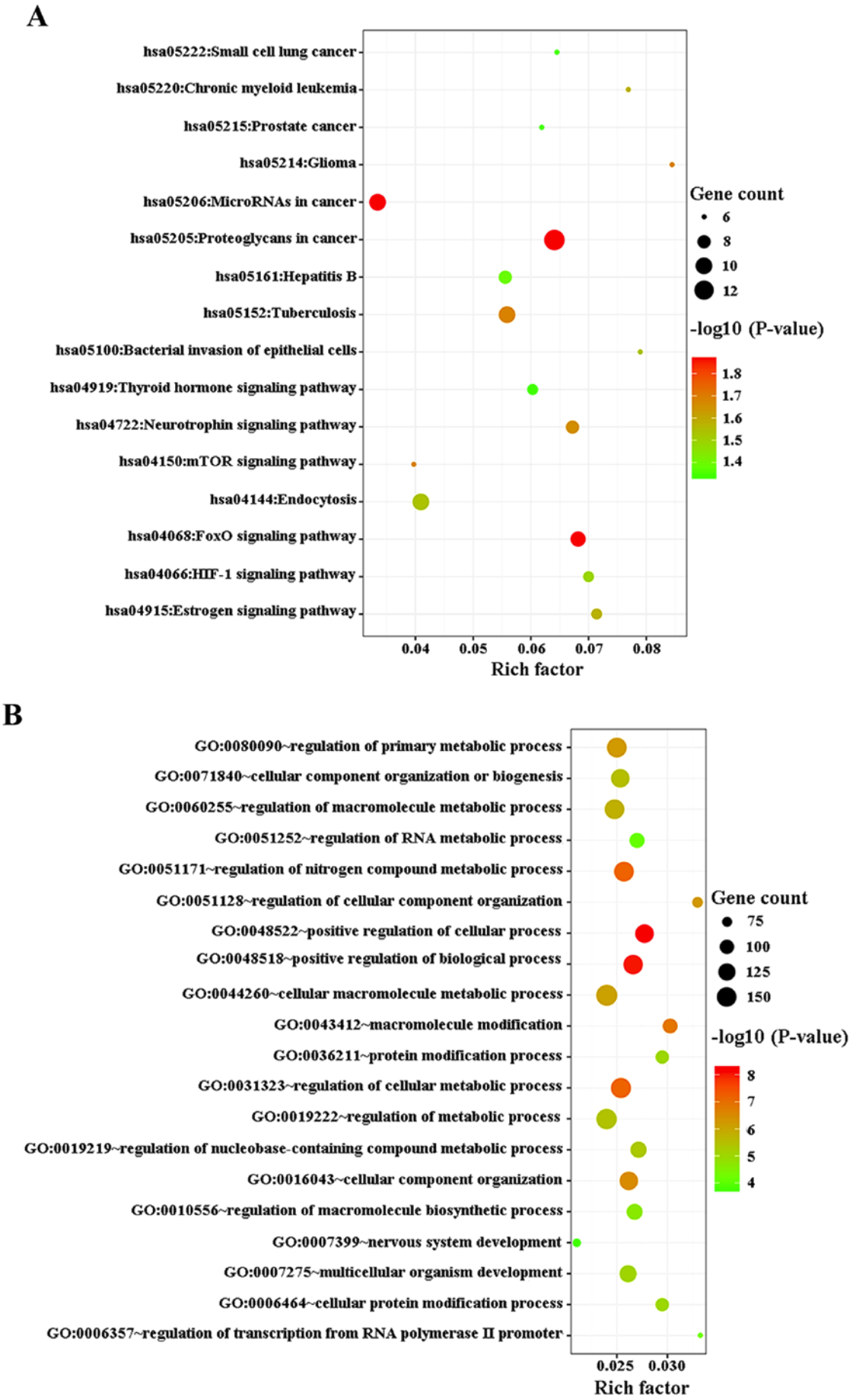

Figure 7. Functional analysis of target genes. (A) The significant enriched KEGG pathways of target genes. (B) The significant enriched GO biological processes of target genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO Gene ontology.

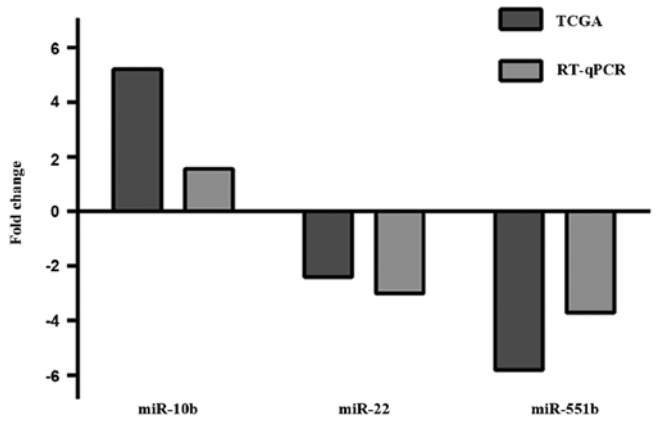

Figure 8. RT-qPCR validation of three miRNAs. Comparison of fold change of miRNAs between TCGA and RT-qPCR results. miRNAs, microRNAs; TCGA, The Cancer Genome Atlas.

survival across the datasets is unstable, while the combination of biomarkers increases the performance (30). Therefore, we established a novel three-miRNA signature (high-risk vs. low-risk) with excellent prognostic performance for cholangiocarcinoma patients. Although no statistically significant associations were observed between our three-miRNA signature and other clinical parameters (data not shown), it was then identified to be an independent prognostic factor and was successfully validated in an independent cohort from the GEO database.

Emerging evidence has demonstrated that numerous miRNAs are aberrantly expressed (upregulated or downregulated) in various cancers (31). Markedly, miRNAs show differential effects in multiple cancers, that is, they serve not only as tumor suppressors, but also as oncogenic promoters to hinder or aggravate cancer formation and malignant transformation. miR-10b, first reported as an oncogene in breast cancer, was found to induce the invasion and metastasis of breast cancer cells (32). Notably, a previous study also suggested that miR-10b may be a tumor suppressor in patients with gastric cancer and the lower level of miR-10b was detected in advanced stage small-cell carcinoma of the cervix patients compared to the early ones (33). Furthermore, miR-22, located on chromosome 17p13.3 (34), was reported to retard cellular growth, invasion and metastasis in cervical and

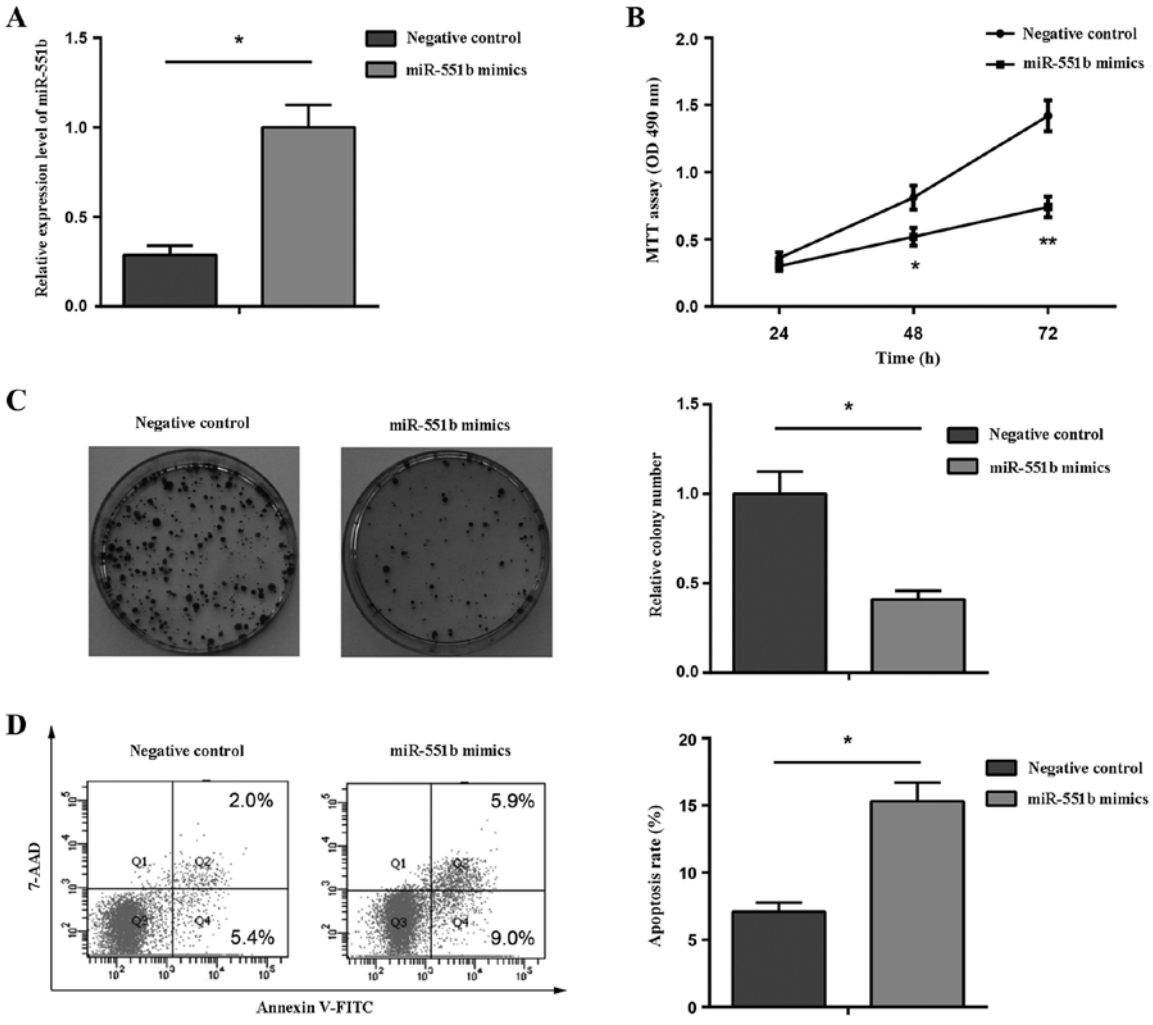

Figure 9. Overexpression of miR-551b inhibits proliferation and induces apoptosis in cholangiocarcinoma cells. (A) Overexpression of miR-551b using miR-551b mimics in cholangiocarcinoma cells. (B) Cell proliferation was determined in cholangiocarcinoma cells transfected with miR-551b mimics or negative control. (C) Cell colony formation was determined in cholangiocarcinoma cells transfected with miR-551b mimics or negative control. (D) Cell apoptosis was determined in cholangiocarcinoma cells transfected with miR-551b mimics or negative control. *P<0.05, **P<0.01.

breast cancer through inducing p53 expression and concurrently targeting SIRT1, CDK6 as well as Sp1 to activate pRb signaling pathway (35). However, Budd *et al* (36) reported that miR-22 in prostate cancer was overexpressed and promoted prostate cancer tumorigenesis by directly targeting PTEN. As for miR-551b, Lin *et al* (37) reported that it was upregulated in lung adenocarcinoma tissues compared to normal tissues and high level of miR-551b predicted a longer survival. Conversely, Song *et al* (38) found that miR-551b was downregulated in gastric cancer and suppressed EMT and metastasis in gastric cancer by inhibiting ERBB4. Furthermore, the functions of these three miRNAs in cholangiocarcinoma were poorly investigated. The expression and clinical information have provided us some clues to investigate the roles of these miRNAs in cholangiocarcinoma. Hence, we explored the biological functions of miR-551b in HUCCT1 cells and found that miR-551b overexpression inhibited proliferation and induced apoptosis, which was similar to that noted in Yuan *et al* recent study of gastric cancer (39).

To gain a deep insight into the molecular mechanisms of these three miRNAs, we predicted their target genes and analyzed the related pathways and GO annotations. Abnormal signaling pathways play crucial roles in the pathogenesis and progression of cholangiocarcinoma. We found that three miRNAs regulated several key signaling pathways, including mTOR, FoxO and HIF-1 signaling pathway. It has been well acknowledged that the PI3K/Akt/mTOR signaling pathway plays an important role in cholangiocarcinoma, and inhibition of mTOR kinase activity may be a viable approach for future application in patients with cholangiocarcinoma (40). FoxO transcription factors have been reported to play vital roles in tumorigenesis and drug resistance. Guan *et al* (41) reported that FoxO3 inactivation promoted human cholangiocarcinoma tumorigenesis and chemoresistance through Keap1-Nrf2 signaling. As for hypoxia inducible factor-1 (HIF-1), a family of heterodimeric proteins which includes HIF-1α and HIF-1β subunits, has been identified to play an important role in initiation and progression of multiple cancers. Thongchot *et al* (42) reported that positive expression of HIF-1α enhanced metastasis and predicted a poor prognosis of cholangiocarcinoma. Therefore, further molecular investigations are needed to confirm these predictions, and may provide new therapeutic interventions in cholangiocarcinoma.

However, there are some limitations in interpreting the above results. Firstly, a larger sample size was required to validate our findings. Secondly, the miRNA expression profiles were detected from bile duct tissues, which may not accurately reflect the levels of miRNAs in saliva, serum, urine or stool. Hence, we may need to explore the miRNAs signature in the above-mentioned samples since they are conveniently available for monitoring.

In conclusion, through performing an integrative analysis for differentially expressed miRNAs accompanied with relevant clinical data, we established a novel three-miRNA signature as an alternative prognostic predictor for cholangiocarcinoma patients. Further investigations are required to validate our findings and further functional studies are also needed to explore the potential molecular mechanisms of these miRNAs in cholangiocarcinoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JC, QM and WD designed the study. JC, LS and JL collected the data. JC performed the bioinformatical analysis. CZ and LC performed the statistical analysis. JC wrote the main manuscript and prepared all figures. KC, BY and WQ contributed to the revision of the manuscript and were also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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