

Original Article

Exosomal miR-200 family as serum biomarkers for early detection and prognostic prediction of cholangiocarcinoma

Ling Shen¹, Gaoping Chen², Qunfeng Xia², Shijie Shao², Haixing Fang²

¹Zhejiang Provincial Key Laboratory of Ophthalmology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; ²Department of Oncological Surgery, The First People's Hospital of Fuyang, Hangzhou, Zhejiang, China

Received May 22, 2019; Accepted August 29, 2019; Epub October 1, 2019; Published October 15, 2019

Abstract: Cholangiocarcinoma (CCA) is a common and lethal disease but lacks of efficient biomarkers for early detection. A previous study using CCA cell lines showed a CCA-specific exosomal microRNA profile. We aimed to verify the results in CCA patients and evaluate the potential roles of these exosomal microRNAs as serum biomarkers. Peripheral blood samples were collected from 36 CCA patients and 12 healthy controls. Twenty exosomal microRNAs were compared between CCA and control group. The diagnostic value was assessed using area under receiver operating characteristic curve (AUC). Out of 20 exosomal microRNAs, 5 significantly differentially expressed between CCA and control group. Four microRNAs in miR-200 family (miR-141-3p, miR-200a-3p, miR-200b-3p, and miR-200c-3p) showed higher AUCs than CA19-9 (0.78). MiR-200c-3p presented the best diagnostic ability with the AUC of 0.93. Furthermore, miR-200a/c-3p was positively correlated with tumor stage ($P < 0.05$). Patients with advance tumor stage (III-IV) showed significantly higher serum exosomal miR-200a/c-3p levels than those with early stage (I-II) ($P < 0.05$). In conclusion, serum exosomal miR-200 family, particularly miR-200c-3p, could be efficient biomarkers for CCA. The serum levels of exosomal miR-200a/c-3p also represented the rate of CCA progression.

Keywords: Cholangiocarcinoma, exosome, microRNA, miR-200 family, serum biomarker

Introduction

Cholangiocarcinoma (CCA) is the second most common primary liver malignant tumor and accounts for about 10-15% all hepatobiliary malignancies [1]. It is a fatal malignancy with a 5-year survival of $< 10\%$ [1]. CCAs are often diagnosed at an advanced stage and only about 1/3 have the chance for curative treatment [1, 2]. The development of CCA is linked to some known (e.g., biliary inflammation, parasitic infections, toxins and hepatic virus) and potential risk factors (e.g., diabetes, obesity and alcohol). However, most CCAs arise sporadic, indicating the difficulty in epidemiological monitoring of the disease. Moreover, the mostly used tumor marker, CA19-9, lacks of diagnostic sensitivity and specificity, and also could be affected by bacterial cholangitis, cholestasis, and other malignancies [3]. Therefore, we need

better tumor markers for early detection of CCA and improvement of prognosis.

Exosomes, membranous structures measuring between 40 and 100 nm, are secreted by various types of living cells into body fluids, contain RNAs and proteins. Exosomes are closely associated with human physiological and pathological conditions. In recent years, increasing evidence demonstrated that tumor-derived exosomes could be a significant regulatory mechanism during cancer development, progression, and metastasis [4]. The signaling molecules (e.g., microRNA, lncRNA, circRNA, mRNA, and protein), carried by exosomes, have displayed promising value in cancer early diagnosis, treatment assessment, and prognosis prediction [4]. A previous study showed a CCA-specific exosomal microRNA profile by comparison between three different CCA cell lines and normal human cholangiocytes [5], suggesting a role of these

Exosomal miRNAs for CCA

Table 1. Subject characteristics

	CCA patients (n = 36)	Healthy subjects (n = 12)
Age (yr)	61.0±8.4	55.0±12.0
Male	17	6
Maximal tumor size (cm)	4.8±2.1	NA
Tumor number	1.8±1.6	NA
Tumor stage		
I-II	26	NA
III-IV	10	NA
Differentiation		
High	0	NA
Moderate	21	NA
Low	15	NA
Vascular invasion		
Macro-	2	NA
Micro-	14	NA
Location		
Intrahepatic	33	NA
Extrahepatic	3	NA
CA19-9 positive	21	0
HBV	3	0
HCV	0	0
Biliary parasite	1	0
Biliary stone	2	0
Overweight	1	0
Diabetes	1	0

Tumor stage was defined according to the TNM classification for CCA in the American Joint Committee on Cancer in the cancer staging manual (8th edition); CA19-9 positive: > 37 kU/L; CCA, cholangiocarcinoma; CA19-9, carbohydrate antigen 19-9; HBV, hepatitis B virus; and HCV, hepatitis C virus.

dysregulated exosome microRNAs in diagnosis of CCA.

In this study, we further verified the CCA-specific exosomal microRNA profile using patient samples and aimed to identify novel serum biomarkers for early detection and prognostic prediction of CCA.

Materials and methods

Clinical data and specimens

A total of 36 pathologically-confirmed CCA patients from the First People's Hospital of Fuyang Hangzhou were included. Another 12 healthy subjects, receiving a physical examination, were randomly enrolled as a control group. Subject characteristics are shown in **Table 1**.

Peripheral blood samples were collected before surgery. Informed consents were obtained. The study was approved by the Ethics Committee of People's Hospital of Fuyang and performed in accordance with the Declaration of Helsinki.

Exosomes isolation

Serum exosomes in CCA patients and healthy subjects were extracted by the miR-CURY Exosome Serum/Plasma Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Serum derived exosomes were detected and investigated on the scanning electron microscopy (Nova NANOSEM 450, FEI, USA). Briefly, exosomes were fixed with 4% paraformaldehyde. A drop of exosomes (20 ml) was pipetted onto a grid and stood for 5 min at room temperature. The sample was negatively stained with 3% (wt/vol) phosphotungstic acid (pH 6.8) for 5 min and analyzed after air drying under an electric incandescent lamp ([Figure S1](#)).

RNA extraction and detection

Total RNAs were extracted from exosomes using a kit (Qiagen). Then, RNA was reverse transcribed using SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsband, CA, USA). MicroRNA detection was performed using a SYBRPrimeScript microRNA RTPCR Kit (TaKaRa, Dalian, China) according to the standard protocol. TOP 10 up-(miR-205-5p, miR-200c-3p, miR-200b-3p, miR-141-3p, miR-200a-3p, miR-7843-5p, miR-429, miR-7705, miR-5585-3p, and miR-660-5p) and down-regulated microRNAs (miR-199a-5p, miR-199b-3p, miR-34c-5p, miR-214-3p, miR-424-5p, miR-4767, miR-6506-3p, miR-379-5p, miR-145-5p, and miR-342-5p) in CCA cell lines were included [5] and the primers were provided in [Table S1](#). Quantitative real-time polymerase chain reaction was performed using an SYBR kit in an Applied Biosystems 7900 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). The microRNA expression was analyzed using $\Delta\Delta CT$ method and normalized to *Caenorhabditis elegans* microRNA (*Cel-miR-39*) (Qiagen).

Statistical analysis

SPSS version 13.0 (SPSS Inc., Chicago, IL) was used to complete all the statistical analyses. A

Exosomal miRNAs for CCA

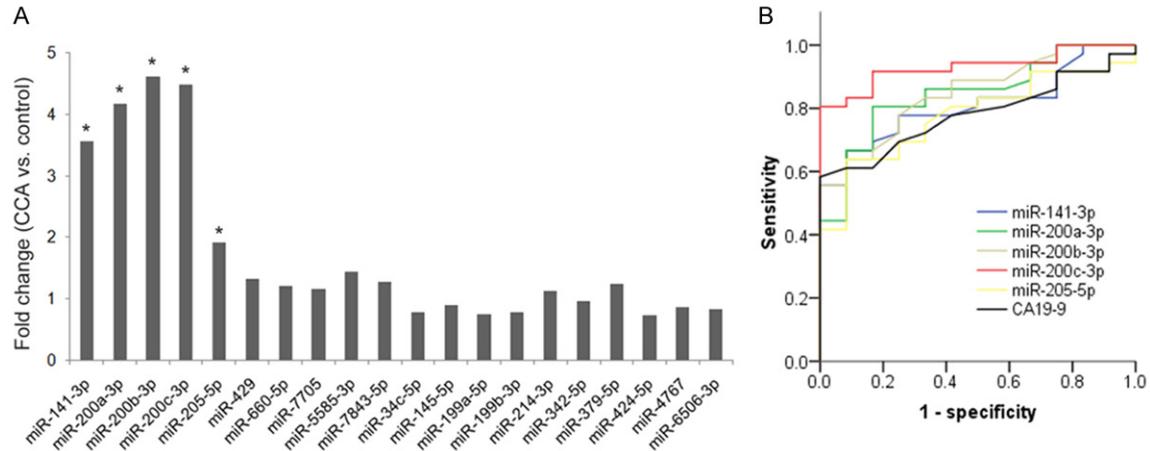


Figure 1. Serum exosomal microRNAs were detected for the diagnosis of cholangiocarcinoma. A. The fold change of serum exosomal microRNAs levels (36 CCA patients vs. 12 healthy controls); B. The AUC showing the diagnostic abilities of CA19-9 and 5 significantly dysregulated exosomal microRNAs between CCA patients and controls; CCA, cholangiocarcinoma; AUC, area under receiver operating characteristic curve.

P value of < 0.05 was considered statistically significant. Quantitative and categorical variables were described as the mean \pm standard deviation (SD) and values (percentages), and compared using an independent-samples *t* test and Pearson's χ^2 test, respectively. Area under receiver operating characteristic curve (AUC) was calculated to assess the diagnostic ability. Cutoff value was selected by considering both the sensitivity and specificity from the receiver operating characteristic curve. COX regression analysis was used to identify the recurrence or death risk. Kaplan-Meier analysis with log rank method was used to compare survival.

Results

Exosomal microRNAs could be serum biomarkers for the diagnosis of CCA

We compared 20 exosomal microRNAs, which were TOP 10 up- and down-regulated ones in CCA cell lines as compared to normal human cholangiocytes [5], between CCA group and control group. Out of the 20 exosomal microRNAs, 5 (miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p, and miR-205-5p) significantly differentially expressed between CCA group and control group (**Figure 1A**). Except miR-205-5p, the other 4 showed higher AUC (all > 0.80) than CA19-9 (0.78) in the diagnosis of CCA (**Figure 1B**). miR-200c-3p presented excellent diagnostic ability with the AUC of 0.93.

Serum exosomal miR-200 family was associated with tumor characteristics

We further correlated the expression of exosomal microRNAs (miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p, and miR-205-5p) with tumor characteristics (tumor size, number, differentiation, location, vascular invasion, lymph node metastasis, and CA19-9). We found significant positive correlations between miR-200a-3p and lymph node metastasis ($r = 0.410$, $P = 0.013$) and tumor stage ($r = 0.369$, $P = 0.027$). There were also positive correlations between miR-200c-3p and lymph node metastasis ($r = 0.457$, $P = 0.005$) and tumor stage ($r = 0.443$, $P = 0.007$). Patients with lymph node metastasis or advanced tumor stage showed significantly higher exosomal miR-200a-3p and miR-200c-3p levels (**Figure 2A** and **2B**).

Serum exosomal miR-200c-3p might have a prognostic value

Because miR-200a/c-3p correlated with tumor stage, which was associated with prognosis [6], we further assessed the impact of miR-200a/c-3p on patient outcome. We put serum exosomal miR-200a/c-3p levels along with several clinical parameters (patient baseline characteristics and tumor features) into a COX regression mode to evaluate the risk factors influencing recurrence. We found tumor stage, tumor number, lymph node metastasis, vascular invasion, and serum exosomal miR-200c-3p levels were

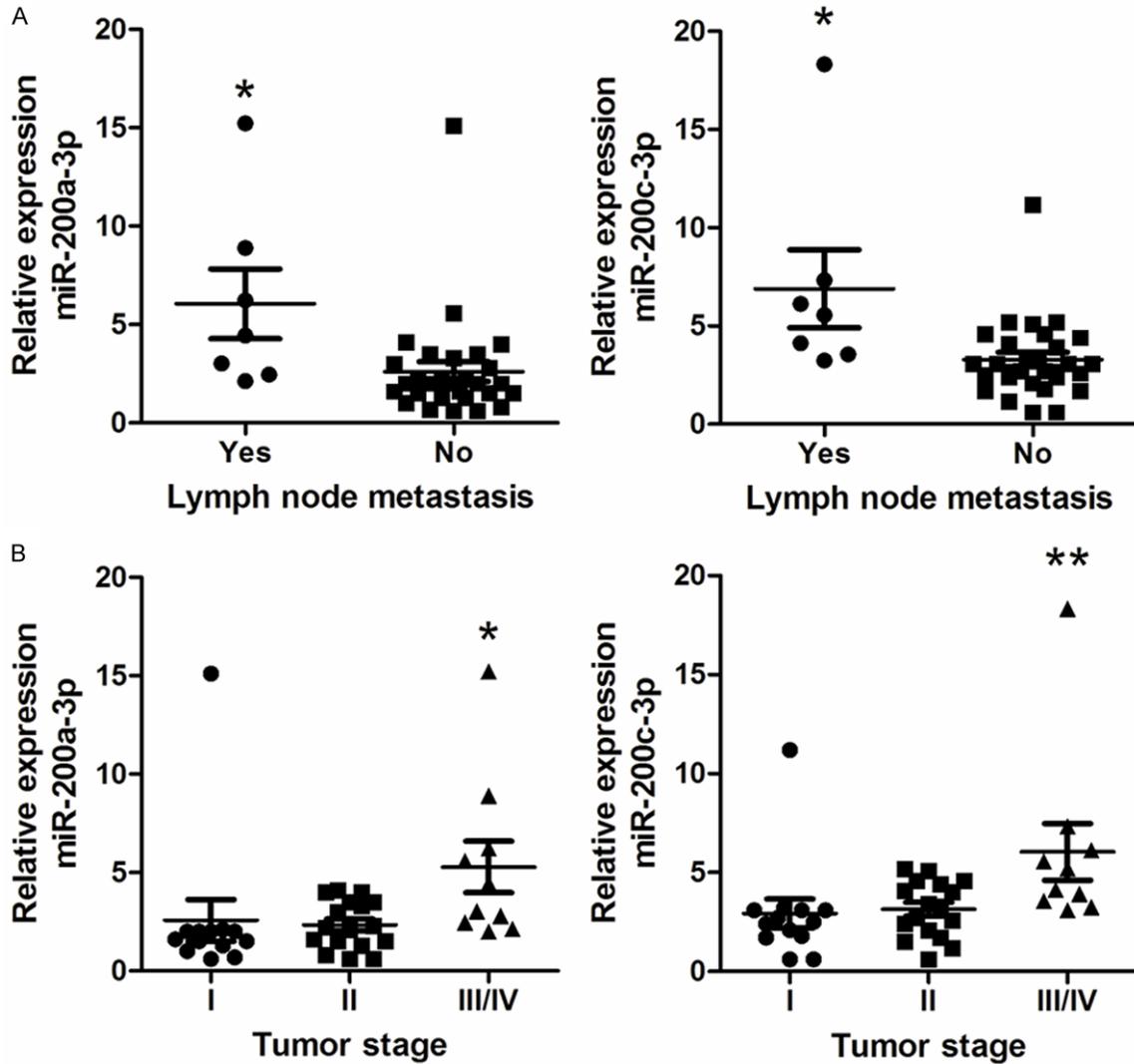


Figure 2. Serum exosomal miR-200 family was associated with tumor features. A. The expression of serum exosomal miR-200a/c-3p was significantly higher in patients with lymph node metastasis than those without lymph node metastasis; B. The expression of serum exosomal miR-200a/c-3p was significantly higher in patients with stage III-IV than those without stage I-II; *: $P < 0.05$, lymph node metastasis Yes vs. No, stage III-IV vs. stage I; **: $P < 0.05$, stage III-IV vs. stage I, stage III-IV vs. stage II.

potential influencing factors for CCA recurrence (all $P < 0.05$) (Table 2). Because tumor stage is a model including tumor number, vascular invasion, and lymph node metastasis [6], we performed the multivariate analyses either with tumor stage alone (model 1) or with tumor number, vascular invasion and lymph node metastasis (model 2). In model 1, tumor stage was the only independent risk factor for CCA recurrence (Table 2). In model 2, vascular invasion and elevated serum exosomal miR-200c-3p levels were independent risk factors for CCA recurrence (Table 2). In Kaplan-Meier survival

comparison, serum exosomal miR-200c-3p levels could stratify patients with low and high recurrence risk (Figure 3). However, miR-200a/c-3p was not found to be influencing factors of patient death in univariate analysis (Table 2). Therefore, we did not further perform multivariate analysis for patient death.

Discussion

To the best of our knowledge, this is the first study to evaluate the role of serum exosomal microRNAs in patients with CCA. Based on a previous study using CCA cell lines [5], we

Exosomal miRNAs for CCA

Table 2. The COX regression analysis for CCA recurrence or patient death

	Tumor recurrence		Patient death	
	HR (95% CI)	P	HR (95% CI)	P
Univariate				
Tumor stage (III-IV vs. I-II)	3.15 (1.74, 5.69)	< 0.001	3.60 (1.72, 7.51)	0.001
Tumor number (> 1 vs. 1)	1.44 (1.11, 1.88)	0.007	1.52 (1.14, 2.02)	0.005
Lymph node metastasis	6.87 (1.79, 26.4)	0.005	5.97 (1.31, 27.2)	0.021
Vascular invasion	5.30 (1.53, 18.3)	0.009	8.66 (1.81, 41.5)	0.007
Serum exosomal miR-200c-3p levels	1.11 (1.00, 1.23)	0.046	1.07 (0.95, 1.20)	0.298
Multivariate (model 1 ^a)				
Tumor stage (III-IV vs. I-II)	3.15 (1.74, 5.69)	< 0.001	NA	NA
Multivariate (model 2 ^b)				
Vascular invasion	5.29 (1.30, 21.5)	0.020	NA	NA
Serum exosomal miR-200c-3p levels	1.16 (1.02, 1.32)	0.021	NA	NA

a: Tumor stage and serum exosomal miR-200c-3p levels were entered into multivariate analysis; b: Other tumor characteristics except tumor stage and serum exosomal miR-200c-3p levels were entered into multivariate analysis.

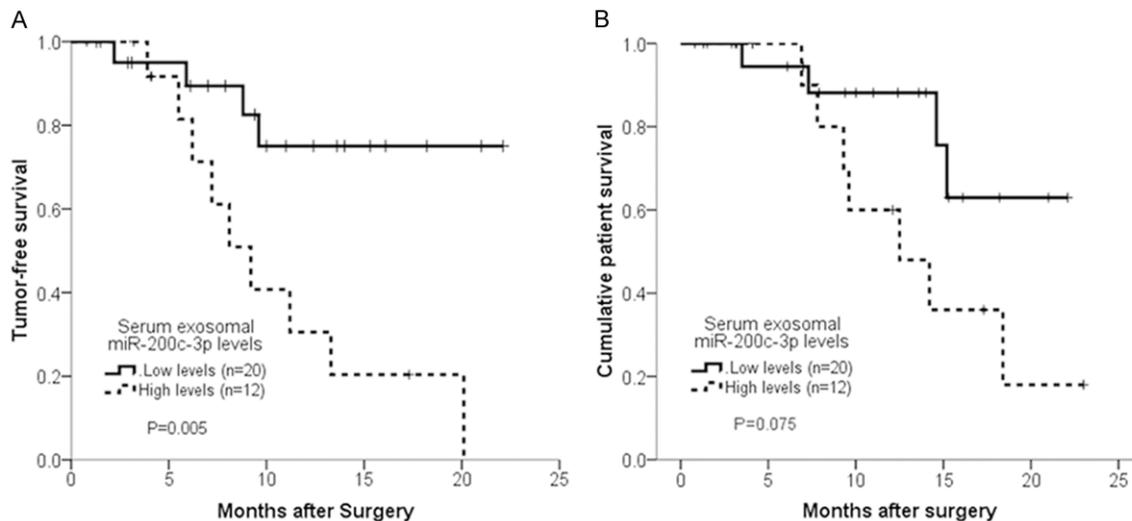


Figure 3. Patient survivals were compared according to serum exosomal miR-200c-3p levels. A. Patient tumor-free survival was significantly lower in patients with high serum exosomal miR-200c-3p levels as compared to those with low serum exosomal miR-200c-3p levels ($P = 0.005$); B. Patient overall survival was relatively lower in patients with high serum exosomal miR-200c-3p levels as compared to those with low serum exosomal miR-200c-3p levels ($P = 0.075$).

selected TOP 20 dysregulated microRNAs for verification in our cohort. Surprisingly, although all 20 microRNAs showed hundreds, even thousands, of fold change in the comparison of cell lines, most of them failed to be verified in the patient cohort. It indicated a significant difference between ‘bench’ and ‘bedside’. In addition, in the previous *in vitro* study, the 3 CCA cell lines were established from *O. viverrini* infected Thai patient and 1 was adenocarcinoma [5]. Therefore, race, histology, etiology, and heterogeneity of tumor may

also explain the discrepancy. Nevertheless, we found promising role of 4 exosomal microRNAs, all of which belong to the miR-200 family, as serum biomarkers for the diagnosis of CCA. Among them, miR-200c-3p displayed excellent diagnostic value with the AUC of > 0.9. It is better than CA19-9, which showed the AUC of 0.78 in our cohort and 0.83 in a meta-analysis including 3303 subjects [7] in the diagnosis of CCA. Whether serum exosomal miR-200c-3p by itself alone or integrated with traditional biomarkers could be an available tool for early

detection of CCA need to be further verified in large cohort with various ethics.

Moreover, we found that miR-200a/c-3p were not only diagnostic biomarkers but also signs representing the disease progression. There was a significantly positive correlation between miR-200a/c-3p and tumor stage. Patients with advance tumor stage (III-IV) showed remarkably higher serum exosomal miR-200a/c-3p levels than those with early stage (I-II). It seemed that serum exosomal miR-200a/c-3p levels were not correlated with tumor size, number, pathological differentiation, or vascular invasion, but closely linked with metastasis (lymph node). A previous study was consistent with our results. It showed that the increased serum levels of exosomal miR-200b and miR-200c mainly observed in patients with advanced epithelial ovarian cancer (stage III-IV and lymph node metastasis) [8]. In addition, miR-200c-3p might also have prognostic value, particularly for tumor recurrence, which was inconsistent with previous studies [9, 10]. In fact, miR-200 family members are proved to be tumor suppressors, which are significantly involved in inhibition of epithelial-to-mesenchymal transition (EMT) [11]. Among the family members, miR-200c is the most-frequently studied one, which involves in a large variety of biological processes and plays a key role in cancer progression including EMT, cell invasion, and metastasis [12]. Multiple molecules such as KRAS [13], PTEN [14], ZEB2 [15], and HIPK1/ β -Cateninaxis [16], have been found to be targets of miR-200c in regulating cancer progression. Taken together, it seems that cancer cells actively enhance their protumorigenic behavior by reprogramming gene expression [17]. They would exile the unfavorable molecules such as tumor suppressor miR-200 family as exosome cargo to promote tumorigenesis.

In conclusion, serum exosomal miR-200 family, particularly miR-200c-3p, was demonstrated to be efficient biomarkers for the early detection of CCA. Moreover, the serum levels of exosomal miR-200a/c-3p also represented the rate of CCA progression. The results need to be further verified in large samples.

Acknowledgements

This study was supported by the Research Program of Zhejiang Provincial Department of Health (No. 2017KY566).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Haixing Fang, Department of Oncological Surgery, The First People's Hospital of Fuyang, Beihuan Road 429#, Fuyang District, Hangzhou 311440, Zhejiang, China. Tel: +86 571 63157122; Fax: +86 571 63157122; E-mail: frankson79@hotmail.com

References

- [1] Bergquist A and von Seth E. Epidemiology of cholangiocarcinoma. *Best Pract Res Clin Gastroenterol* 2015; 29: 221-32.
- [2] Khan SA, Davidson BR, Goldin RD, Heaton N, Karani J, Pereira SP, Rosenberg WM, Tait P, Taylor-Robinson SD, Thillainayagam AV, Thomas HC, Wasan H; British Society of Gastroenterology. Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update. *Gut* 2012; 61: 1657-1669.
- [3] Sinakos E, Saenger AK, Keach J, Kim WR and Lindor KD. Many patients with primary sclerosing cholangitis and increased serum levels of carbohydrate antigen 19-9 do not have cholangiocarcinoma. *Clin Gastroenterol Hepatol* 2011; 9: 434-9.
- [4] Ruivo CF, Adem B, Silva M and Melo SA. The biology of cancer exosomes: insights and new perspectives. *Cancer Res* 2017; 77: 6480-6488.
- [5] Kitdumrongthum S, Metheetrairut C, Charoen-sawan V, Ounjai P, Janpipatkul K, Panvongsa W, Weerachayaphorn J, Piyachaturawat P and Chairoungdua A. Dysregulated microRNA expression profiles in cholangiocarcinoma cell-derived exosomes. *Life Sci* 2018; 210: 65-75.
- [6] Juntermanns B, Kaiser GM, Orth L, Reis H, Jaradat D, Sydor S, Buechter M, Kasper S, Mathe Z, Sotiropoulos GC, Baba HA, Canbay A, Paul A and Fingas CD. Comparison of the sixth and the seventh editions of the UICC classification for intrahepatic cholangiocarcinoma. *Eur J Med Res* 2018; 23: 29.
- [7] Liang B, Zhong L, He Q, Wang S, Pan Z, Wang T and Zhao Y. Diagnostic accuracy of serum CA19-9 in patients with cholangiocarcinoma: a systematic review and meta-analysis. *Med Sci Monit* 2015; 21: 3555-63.
- [8] Meng X, Muller V, Milde-Langosch K, Trillsch F, Pantel K and Schwarzenbach H. Diagnostic and prognostic relevance of circulating exosomal miR-373, miR-200a, miR-200b, and miR-200c in patients with epithelial ovarian cancer. *Oncotarget* 2016; 7: 16923-16935.
- [9] Roh MS, Lee HW, Jung SB, Kim K, Lee EH, Park MI, Lee JS and Kim MS. Expression of miR-

Exosomal miRNAs for CCA

- 200c and its clinicopathological significance in patients with colorectal cancer. *Pathol Res Pract* 2018; 214: 350-355.
- [10] Papadaki C, Stratigos M, Markakis G, Spiliotaki M, Mastrostamatis G, Nikolaou C, Mavroudis D and Agelaki S. Circulating microRNAs in the early prediction of disease recurrence in primary breast cancer. *Breast Cancer Res* 2018; 20: 72.
- [11] Feng X, Wang Z, Fillmore R and Xi Y. MiR-200, a new star miRNA in human cancer. *Cancer Lett* 2014; 344: 166-173.
- [12] Mutlu M, Raza U, Saatci O, Eyupoglu E, Yurdusev E and Sahin O. miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance. *J Mol Med (Berl)* 2016; 94: 629-44.
- [13] Song C, Liu LZ, Pei XQ, Liu X, Yang L, Ye F, Xie X, Chen J and Tang H. miR-200c inhibits breast cancer proliferation by targeting KRAS. *Oncotarget* 2015; 6: 34968-34978.
- [14] Chen R, Zhang M, Liu W, Chen H, Cai T, Xiong H, Sheng X, Liu S, Peng J, Wang F, Lin W, Xu X, Zheng W and Jiang Q. Estrogen affects the negative feedback loop of PTENP1-miR200c to inhibit PTEN expression in the development of endometrioid endometrial carcinoma. *Cell Death Dis* 2018; 10: 4.
- [15] Jiao A, Sui M, Zhang L, Sun P, Geng D, Zhang W, Wang X and Li J. MicroRNA-200c inhibits the metastasis of non-small cell lung cancer cells by targeting ZEB2, an epithelial-mesenchymal transition regulator. *Mol Med Rep* 2016; 13: 3349-55.
- [16] Liu B, Du R, Zhou L, Xu J, Chen S, Chen J, Yang X, Liu DX, Shao ZM, Zhang L, Yu Z, Xie N, Guan JL and Liu S. miR-200c/141 regulates breast cancer stem cell heterogeneity via targeting HIPK1/beta-catenin axis. *Theranostics* 2018; 8: 5801-5813.
- [17] Kanlikilicer P, Rashed MH, Bayraktar R, Mitra R, Ivan C, Aslan B, Zhang X, Filant J, Silva AM, Rodriguez-Aguayo C, Bayraktar E, Pichler M, Ozpolat B, Calin GA, Sood AK and Lopez-Berestein G. Ubiquitous release of exosomal tumor suppressor miR-6126 from ovarian cancer cells. *Cancer Res* 2016; 76: 7194-7207.

Exosomal miRNAs for CCA

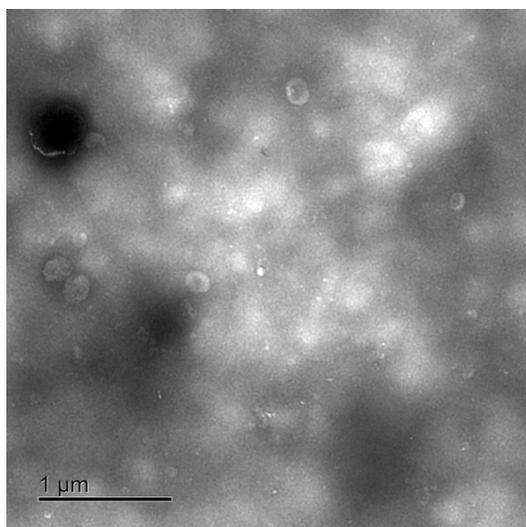


Figure S1. Electric incandescent lamp detection of exosomes.

Exosomal miRNAs for CCA

Table S1. The PCR primers

ID	Primers
hsa-miR-424-5p	F: 5'-GCCGGGCAGCAGCAATTCATGT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAAA-3'
hsa-miR-200b-3p	F: 5'-GCCGGGTAATACTGCCTGGTAA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCA-3'
hsa-miR-7843-5p	F: 5'-GCCGGGGAGGGCAGAGCCAGCT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGGA-3'
hsa-miR-342-5p	F: 5'-GCCGGGAGGGGTCTATCTGT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAATC-3'
hsa-miR-199b-3p	F: 5'-GCCGGGACAGTAGTCTGCACAT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCA-3'
hsa-miR-6506-3p	F: 5'-GCCGGGTCGTATCAGAGATTCC-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGTCT-3'
hsa-miR-4767	F: 5'-GCCGGGCGCGGGCGCTCCTGGCC-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCGGC-3'
hsa-miR-34c-5p	F: 5'-GCCGGGAGGCAGTGTAGTTAGCT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAATC-3'
hsa-miR-199a-5p	F: 5'-GCCGGGCCAGTGTTCAGACTAC-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAACAG-3'
hsa-miR-379-5p	F: 5'-GCCGGGTGGTAGACTATGGAA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTACG-3'
hsa-miR-205-5p	F: 5'-GCCGGGTCCTTCATTCCACCGG-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGACT-3'
hsa-miR-7705	F: 5'-GCCGGGAATAGCTCAGAATGTCA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGAAC-3'
hsa-miR-660-5p	F: 5'-GCCGGGTACCCATTGCATATCG-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAACTC-3'
hsa-miR-141-3p	F: 5'-GCCGGGTAACACTGTCTGGTAA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCCCT-3'
hsa-miR-200c-3p	F: 5'-GCCGGGTAATACTGCCGGTAA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCCATC-3'
hsa-miR-145-5p	F: 5'-GCCGGGTCCAGTTTTCCAGGA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAT-3'
hsa-miR-214-3p	F: 5'-GCCGGGACAGCAGGCACAGACA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGCC-3'
hsa-miR-429	F: 5'-GCCGGGTAATACTGTCTGGTAA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGGTT-3'
hsa-miR-5585-3p	F: 5'-GCCGGGCTGAATAGCTGGGACT-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCTGT-3'
hsa-miR-200a-3p	F: 5'-GCCGGGTAACACTGTCTGGTA-3' R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATCGT-3'