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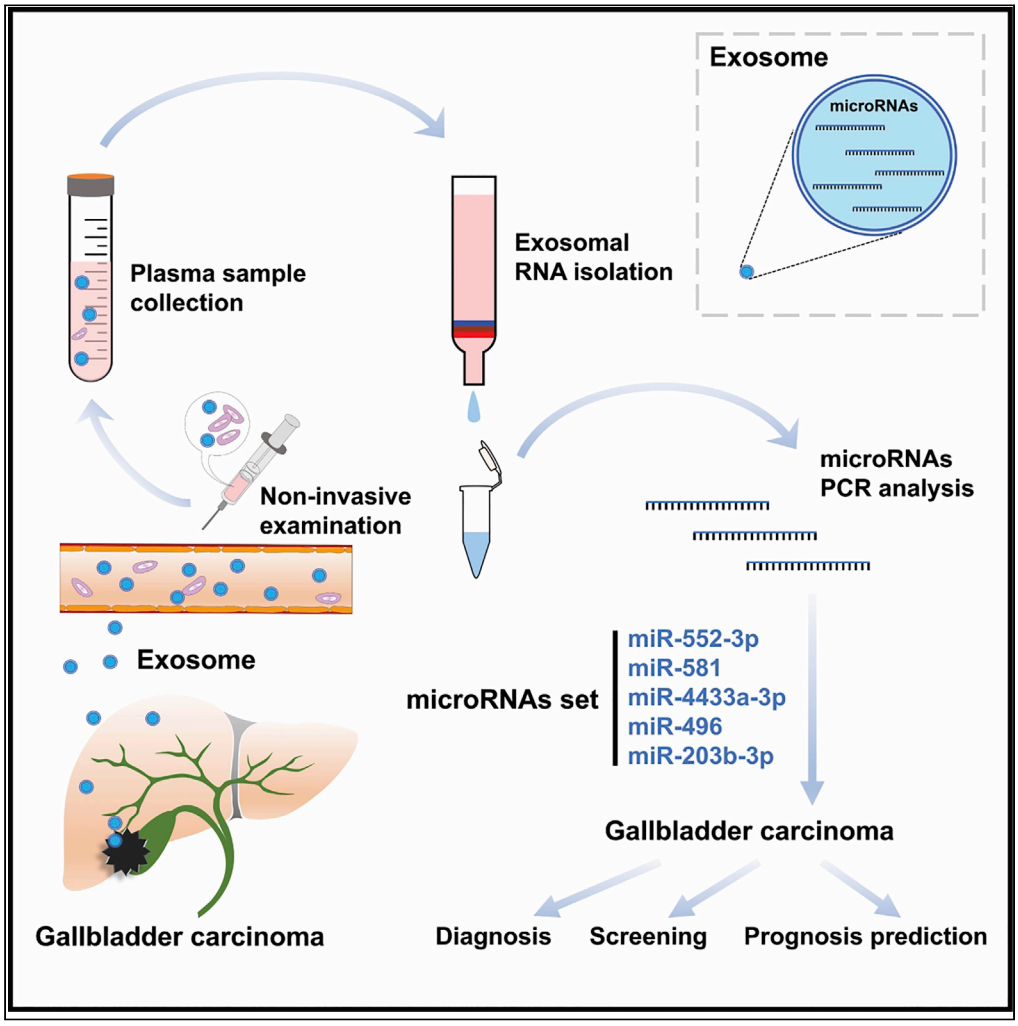

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Highlights

A five exosomal miRNAs-set is identified to diagnose GBC through a three-step study

The efficacy of this noninvasive biomarker is superior to that of conventional ones

This biomarker is correlated with multiple GBC clinical features and the prognosis

The functions and signal pathways that this biomarker may affect were estimated

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Exosomal MicroRNA signature acts as an efficient biomarker for non-invasive diagnosis of gallbladder carcinoma

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SUMMARY

Through a three-step study that relies on biomarker discovery, training, and validation, we identified a set of five exosomal microRNAs (miRNAs) that can be used to evaluate the risk of gallbladder carcinoma (GBC), including miR-552-3p, miR-581, miR-4433a-3p, miR-496, and miR-203b-3p. When validated in 102 GBC patients and 112 chronic cholecystitis patients from multiple medical centers, the AUC of this combinatorial biomarker was 0.905, with a sensitivity of 81.37% and a specificity of 86.61%. The performance of this biomarker is superior to that of the standard biomarkers CA199 and CEA and is suited for GBC early diagnosis. The multi-clinicopathological features and prognosis of GBC patients were significantly associated with this biomarker. After building a miRNA-target gene regulation network, cell functions and signaling pathways regulated by these five miRNAs were examined. This biomarker signature can be used in the development of a noninvasive tool for GBC diagnosis, screening and prognosis prediction.

INTRODUCTION

Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract, accounting for 80–95% of all biliary tract cancers, and it is the fifth most common gastrointestinal cancer (Rakić et al., 2014; Wu et al., 2014). In recent years, the incidence of GBC has rapidly increased, and GBC is considered one of the most aggressive and lethal malignant tumors. Globally, there were 219,420 newly diagnosed cases and 165,087 deaths from gallbladder cancer in 2018 according to global cancer statistics (Bray et al., 2018). Unfortunately, due to the high levels of imperceptibility and heterogeneity, most GBC patients are diagnosed at an advanced stage, with local invasion of adjacent organs or distant metastasis, and have an overall 5-year survival rate of less than 5% (Dasari et al., 2018). Moreover, there are currently no reliable biomarkers for the early diagnosis of GBC or reliable serum markers to improve the potential efficacy of screening strategies in high-risk individuals, such as patients with gallbladder stones and polyps (Hundal and Shaffer, 2014). Better GBC diagnostic and prognostic biomarkers can improve the poor rate of disease diagnosis and allow more patients to undergo radical surgical resection, which is the most effective GBC treatment at this stage. In addition, the discovery of potential biomarkers may provide new therapeutic targets for postoperative treatment and improvement of GBC patient survival.

Exosomes are small homogeneous membrane vesicles with a diameter of 40–100 nm that can be secreted by a variety of cell types in the body. Exosomes are widely distributed in intercellular spaces, extracellular fluids and even in bodily fluids, such as saliva, plasma, and milk (Kalluri and LeBleu, 2020). Depending on their cellular and tissue origin, exosomes carry functional biomolecules, including proteins, lipids, and nucleic acids, such as DNA, mRNAs, and microRNAs (miRNAs) (Raposo and Stoorvogel, 2013). Accumulating evidence has demonstrated that exosomes function as key players in intercellular communication between cancer cells and their microenvironment via the horizontal transfer of information through cargo miRNAs (Huang and Deng, 2019). Cancer cell-derived exosomes are closely related to tumor occurrence, development, and prognosis and can affect multiple tumorigenic and developmental processes, including tumor proliferation, extracellular matrix remodeling, invasion, metastasis, angiogenesis, immune escape, and

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drug resistance (Chung et al., 2020). For instance, glypican-1-positive circulating exosomes can serve as a potential noninvasive diagnostic and screening tool for the detection of early stage pancreatic cancer and allow patients to undergo potential curative surgical therapy with absolute specificity and sensitivity (Melo et al., 2015). Furthermore, the levels of glypican-1-positive circulating exosomes are correlated with tumor burden and overall patient survival before and after surgery (Wu et al., 2019). The presence of the integrin family of proteins on circulating exosomes was shown to predict the organotropic metastatic characteristics of tumors, which explained the phenomenon that tumors-specific metastasis has an organ preference and strongly complemented the theory of "seed and soil" (Hoshino et al., 2015). Exosomal mRNA components isolated from the blood, saliva, urine, and other bodily fluids of cancer patients have also been considered diagnostic markers of certain tumors (Xu et al., 2018; Barile and Vassalli, 2017). To date, exosomes have been proposed to be a potential source of biomarkers in various types of cancer (Properzi et al., 2013; Brock et al., 2015; Jin et al., 2017).

MiRNAs are endogenous small noncoding RNAs with a length of approximately 19–25 nt. MiRNAs exert their biological effects through the negative regulation of target gene expression via specific binding to the 3′ untranslated regions (UTRs) of target mRNAs (Wei et al., 2021). Recent studies have shown that miRNAs, a new kind of signaling molecule involved in intercellular communication, can be packaged into exosomes and transported to recipient cells to regulate their biological functions (Wang et al., 2020; Moradi-Chaleshtori et al., 2019). The intercellular transmission of information that is mediated by exosomal miRNAs plays a vital role in tumor occurrence and development (Pourhanifeh et al., 2020; Mills et al., 2019). Previous research demonstrated that exosomal miRNAs can affect HPV-mediated inflammation and cervical cancer through intercellular communication (Sadri Nahand et al., 2020). Exosomes secreted from IL-4-activated macrophages shuttle miR-223 to breast cancer cells and further promote breast cancer cell invasion (Yang et al., 2011). Preventing malignant breast epithelium-macrophage communication may inhibit the metastatic cascade during cancer progression; thus, the molecules involved in this communication may be important targets for breast cancer therapy. The results from early research suggested that AZ-P7a metastatic gastric cancer cells release let-7 miRNAs into the extracellular environment via exosomes to maintain their oncogenesis (Ohshima et al., 2010). Exosomes containing miR-141-3p that are released by epithelial ovarian cancer cells can promote endothelial cell angiogenesis and tumor metastasis by activating the JAK/STAT3 and NF-κB signaling pathways (Masoumi-Dehghi et al., 2020). A recent study showed that FAK ablation in cancer-associated fibroblasts impaired their ability to promote cancer cell migration and other abilities due to alterations in exosomal miRNAs (Wu et al., 2020). Another study showed that exosomes from highly metastatic colorectal cancer (CRC) cells can deliver miR-106b-3p to surrounding cells, which promotes their metastatic ability and inhibits DLC-1 expression in recipient cells (Liu et al., 2020).

Unlike circulating miRNAs, exosomal miRNAs can be enriched in the circulatory system and are stable enough to avoid degradation (Gallo et al., 2012). These characteristics make them a potential source of biomarkers for complex diseases, including malignancies (Nik Mohamed Kamal and Shahidan, 2019). Many studies have proven that exosomal miRNAs could function as potential diagnostic biomarkers in tumor screening, diagnosis and monitoring (Li et al., 2019; Kalishwaralal et al., 2019). For example, circulating exosomal miR-146b-5p and miR-222-3p levels have been shown to be indicators for the lymph node metastasis of papillary thyroid carcinoma (Jiang et al., 2020). Serum exosomal miR-484 levels could serve as a reliable and noninvasive marker for predicting the prognosis of ovarian cancer (Zhang et al., 2020). Serum exosomal miR-378 has strong potential for use as a promising noninvasive biomarker for screening and monitoring non-small cell lung cancer (Zhang and Xu, 2020).

Exosomal miRNAs play important roles in tumor occurrence and development. As reported, some previous studies have performed useful investigations of the use of extracellular vesicle-shuttled miRNAs as GBC biomarkers (Ueta et al., 2021; Xue et al., 2020). However, there are still some limitations in this area that have not been thoroughly addressed, such as small study sizes, single-center study designs, and no independent validation. Therefore, we designed a large-scale, multicenter validation study to assess the diagnostic accuracy of an exosomal miRNA signature as a noninvasive biomarker for early- and late-stage GBC, and as part of the National Cancer Institute's Early Detection Research Network (EDRN)-defined biomarker study (Pepe et al., 2001). Moreover, the clinical value of the exosomal miRNA signature in predicting the overall and disease-free survival after surgery was evaluated to improve the prognostic prediction of GBC.

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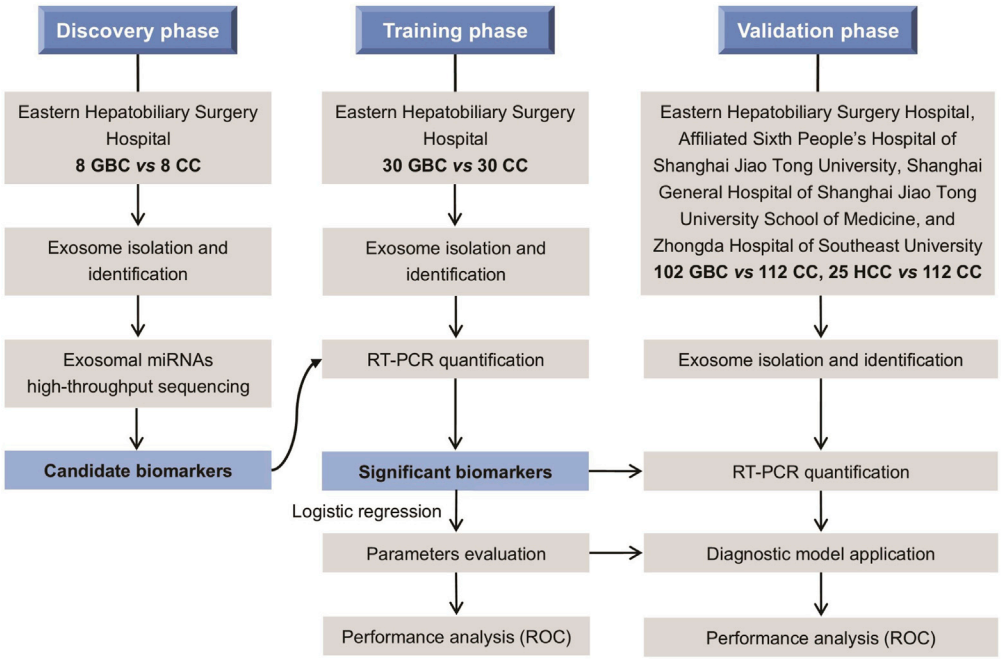


Figure 1. Study design

The experimental procedure and number of patients included. GBC: gallbladder carcinoma, CC: chronic cholecystitis, RT–PCR: reverse transcriptase polymerase chain reaction, ROC, receiver operating characteristics.

RESULTS

Discovering differentially expressed exosomal miRNAs

The three-step biomarker study procedure (Figure 1) based on biomarker discovery, training and validation is described in detail in "STAR Methods" section. An exosome extraction kit (Qiagen, Germany) was used to isolate exosomes from plasma samples of eight gallbladder cancer (GBC) patients and eight chronic cholecystitis (CC) patients (see Table S1 for general clinical data) in discovery phase. After observation and identification by electron microscopy, the volume and morphology of the separated membrane structures was found to be consistent with general exosome characteristics (Figure S1A). The purified exosomes exhibited similar sizes (diameters ranging from 20 to 200 nm) (Figure S1B). Furthermore, the exosome identity was confirmed by the presence of the exosomal markers CD63 and CD81 (Figure S1C).

Small RNA high-throughput sequencing technology was used to measure the expression of single-stranded small molecular RNAs that were 18–40 bases in length in the exosomes from patients with GBC and CC in discovery phase. After data analysis, we found that in the plasma exosomes from both GBC and CC patients, the predominant RNA components were miRNAs, and the minor components were Y RNAs (Figure 2A). Moreover, the proportions of plasma exosomal miRNAs and Y RNAs in GBC patients were not significantly different from the proportions of these two indicators in CC patients, respectively (Figure 2B). There were 22 types of downregulated and 24 types of upregulated miRNAs in the plasma exosomes of GBC patients compared to those of CC patients (Figure 2C). Using hierarchical cluster analysis, we found that GBC can be effectively distinguished from CC by these sets of upregulated and downregulated miRNAs (Figure 2D).

Biomarker training and diagnostic model construction

Furthermore, we selected the top 10 upregulated and 10 downregulated plasma exosomal miRNAs (GBC compared to CC) as candidate biomarkers and expanded the sample size to independently execute the training. miRNA levels in plasma exosomes were measured in samples from a cohort of 30 GBC and 30 CC patients using real-time qPCR. Among the top 10 candidates, the expression of miR-552-3p, miR-581, miR-4433a-3p, and miR-372-3p still showed increasing trends in the training cohort (Figure 3A). The

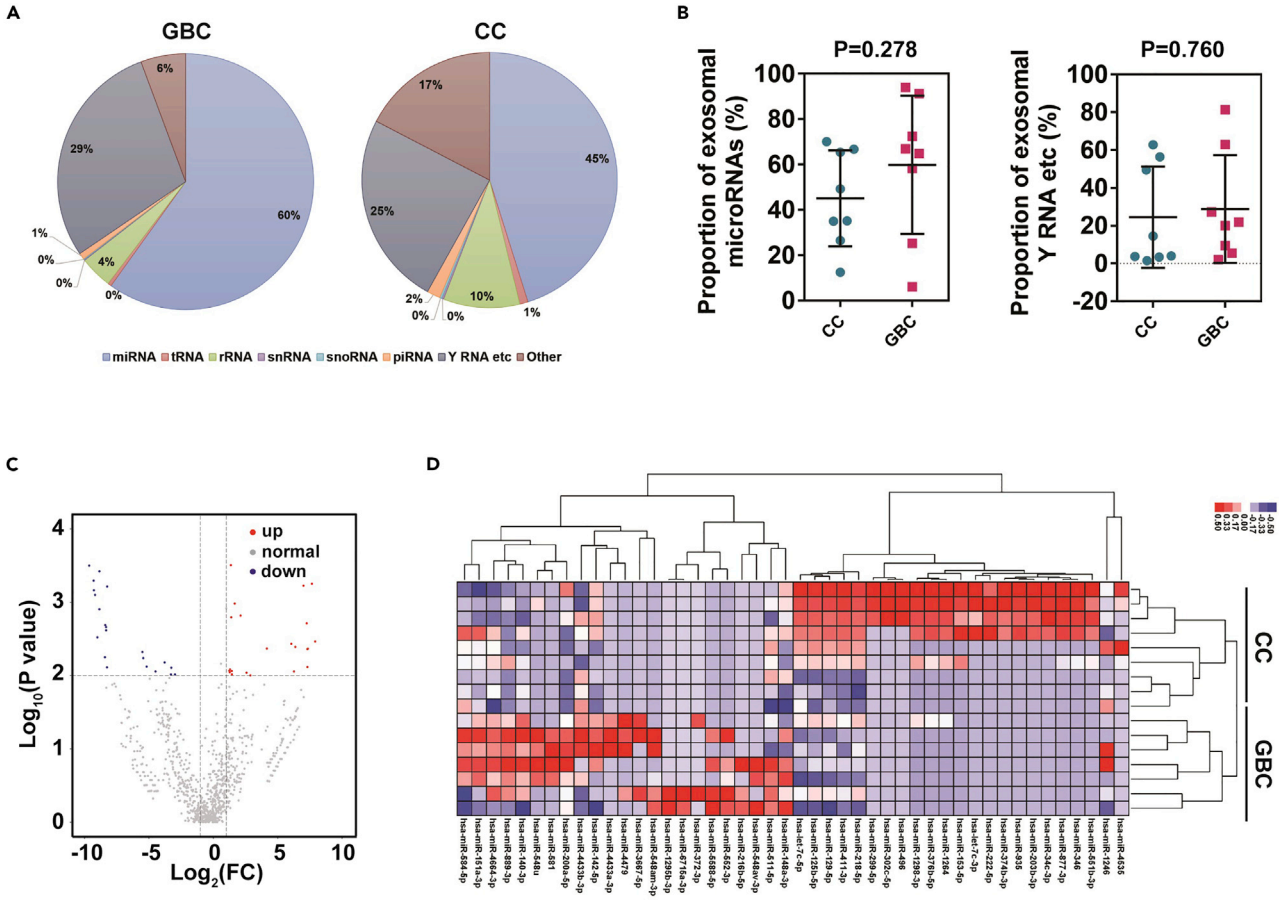


Figure 2. Plasma exosomal miRNA profiles of GBC and CC patients

(A) Display of the proportions of various small RNAs in plasma exosomes from GBC and CC patients.

(B) The percentage of exosomal miRNAs and Y RNAs in GBC and CC patients. Data are represented as mean ± SD.

(C) In the discovery cohort, unique miRNAs that were either upregulated or downregulated in GBC patients relative to CC patients (fold-change ≥ 2 and $p \leq 0.01$) are shown in the volcano plot graph.

(D) Hierarchical clustering analysis of exosomal miRNAs that were differentially expressed with fold-change ≥ 2 and $p \leq 0.01$ between the GBC and CC groups.

expression levels of miR-496, miR-551b-3p and miR-203b-3p were still downregulated in the training cohort (Figure 3B). The non-differentially expressed miRNAs in the training cohort are shown in Figures S2A and S2B. To compare the performance of the identified exosomal miRNA biomarkers with that of traditional tumor markers, we also measured the expression levels of a range of traditional markers. The carbohydrate antigen 199 (CA199) and carcinoembryonic antigen (CEA) levels in GBC patients were higher than those in CC patients (Figure 3C), but the AFP, CA15, CA125, CA242 and CA724 levels were not significantly different (Figure S2C).

Receiver operating characteristic (ROC) analysis of all seven differentially expressed miRNAs that have been passed the large samples verification, showed that the corresponding AUCs were 0.678 for miR-552-3p, 0.655 for miR-581, 0.612 for miR-4433a-3p, 0.680 for miR-496, 0.654 for miR-203b-3p, 0.644 for miR-551b-3p, and 0.678 for miR-372-3p, respectively, indicating that they can be further estimated by the establishment of logistic regression equations. Sensitivity and specificity values and likelihood ratios for the seven exosomal miRNAs, CA199, and CEA in GBC diagnosis are shown in Table 1. When combining the expression data of the seven miRNAs to establish the logistic regression equation and investigate the diagnostic ability of this miRNAs-set, five of the seven miRNAs (miR-552-3p, miR-581, miR-4433a-3p, miR-496, and miR-203b-3p) were flowed through the analysis (Figure S3A). MiR-551b-3p and miR-372-3p were removed from this training cohort (Figure S3B).

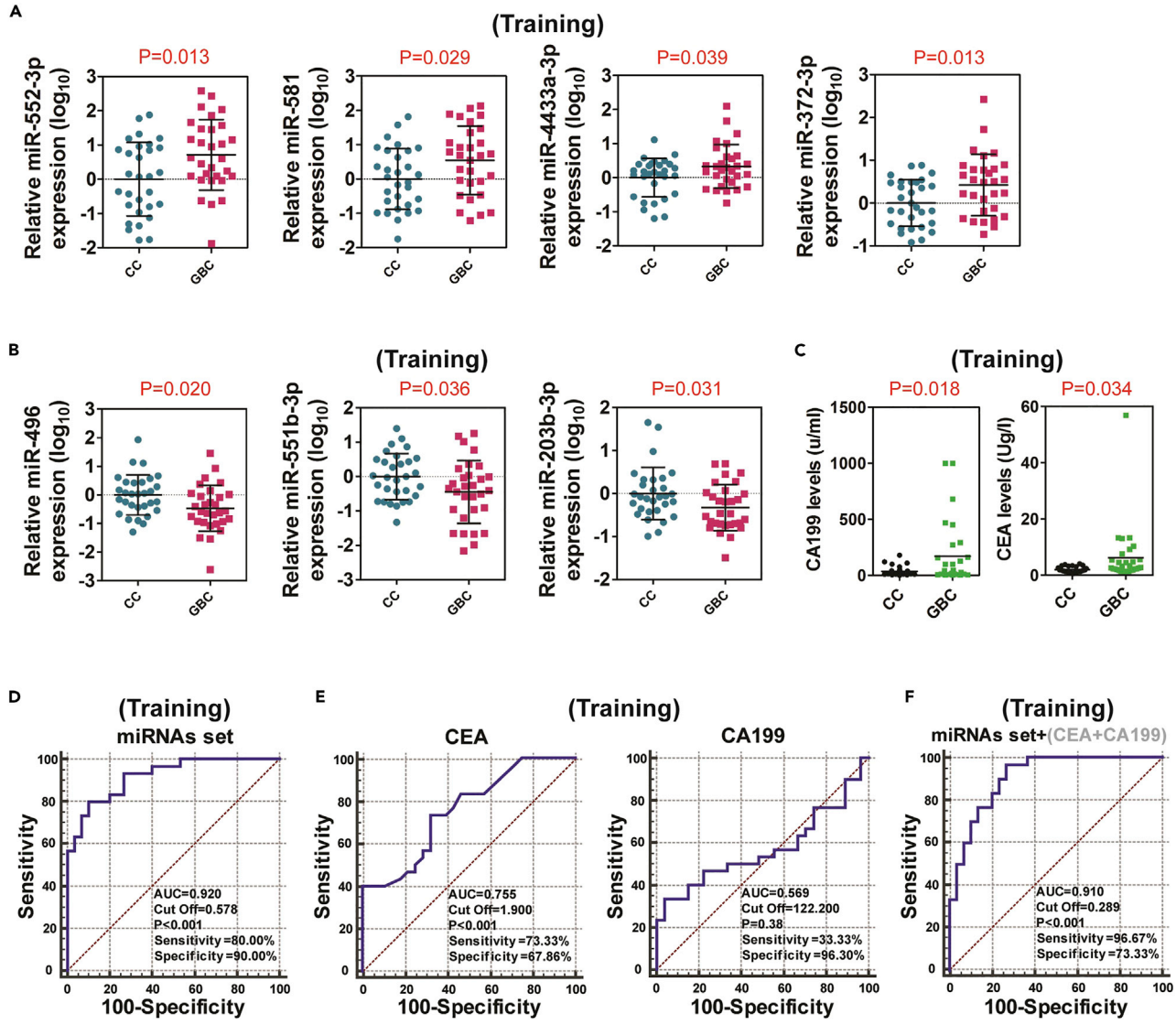


Figure 3. A set of five exosomal miRNAs acted as an effective diagnostic biomarker for GBC in the training cohort

(A and B) Differentially expressed exosomal miRNAs in the training cohort. miRNA expression was measured by RT–PCR. Data are represented as mean ± SD.

(C) CA199 and CEA levels in GBC patients in the training cohort. Data are represented as mean ± SD.

(D–F) ROC curve analysis was performed for GBC (n = 30) and CC (n = 30) patients in the training cohort. AUC: area under the curve.

As shown in Table 1 and Figure 3D, combining the above five exosomal miRNAs markedly enhanced the AUC to 0.920 (95% CI: 0.820–0.974), with a sensitivity of 80% and a specificity of 90% at the optimal cutoff value of 0.578. Logistic regression analysis also indicated that this five exosomal miRNAs-set is an independent marker for discriminating GBC patients with an odds ratio (OR) of 36 (95% CI: 8.105–159.894, Table 1). Intriguingly, the performance of this biomarker set was markedly better than that of the traditional tumor markers CA199 and CEA, which are abnormally expressed in the circulation of GBC patients. In this training cohort (Table 1 and Figure 3E), CA199 had a sensitivity of 33.33% and specificity of 96.30% at the optimal cutoff value of 122.20 (U/mL), and CEA had a sensitivity of 73.33% and specificity of 67.86% at the optimal cutoff value of 1.90 (μg/L). Noteworthy, when combining the analysis of this five exosomal miRNAs-set with CA199 and CEA, there was no significant improvement in the AUC (0.910) with an observed decrease in specificity (73.33%) and an increase in sensitivity (96.67%), compared with those of the exosomal miRNAs-set alone (Figure 3F).

Table 1. The ROC analysis of different variates

Variate	AUC (95% CI)	Optimal Cut Off	SE (%)	SP (%)	PPV (%)	NPV (%)	PLR	NLR	OR (95% CI)
miR-552-3p	0.678 (0.545,0.793)	−0.362	86.67	46.67	61.90	77.78	1.62	0.29	5.688 (1.591,20.330)
miR-581	0.655(0.521,0.773)	0.362	56.67	70.00	65.38	61.76	1.89	0.62	3.051(1.053,8.839)
miR-372-3p	0.678(0.545,0.793)	0.542	46.67	86.67	77.78	61.90	3.50	0.62	5.688(1.591,20.330)
miR-4433a-3p	0.612(0.477,0.735)	−0.476	96.67	26.67	56.86	88.89	1.32	0.13	10.545(1.227,90.662)
miR-551b-3p	0.644(0.510,0.763)	−0.908	33.33	96.67	90.91	59.18	10.00	0.69	14.500(1.718,122.395)
miR-496	0.680(0.547,0.795)	−0.559	53.33	80.00	72.73	63.16	2.67	0.58	4.571(1.452,14.389)
miR-203b-3p	0.654(0.521,0.773)	−0.466	53.33	80.00	72.73	63.16	2.67	0.58	4.571(1.452,14.389)
CEA (μg/L)	0.755(0.624,0.858)	1.900	73.33	67.86	70.97	70.37	2.28	0.39	5.806(1.870,18.027)
CA199 (U/mL)	0.569(0.431,0.700)	122.200	33.33	96.30	90.91	56.52	6.30	0.80	13.000(1.535,110.127)
miRNAs-set	0.920(0.820,0.974)	0.578	80.00	90.00	88.89	81.82	8.00	0.22	36.000(8.105,159.894)
miRNAs-set + CEA + CA199	0.910(0.808,0.968)	0.289	96.67	73.33	78.38	95.65	3.62	0.045	79.750(9.276,685.633)

Taken together, by analyzing the training cohort, we obtained a diagnostic biomarker for GBC that included five exosomal miRNAs. This biomarker set exhibits better performance than the traditional tumor markers CA199 and CEA. The diagnostic parameters and diagnostic performance that were achieved with the training cohort required further validation in larger independent samples.

Biomarker validation and diagnostic parameter application

Next, in the validation cohort, independent GBC (n = 102) and CC (n = 112) samples from four medical centers were enrolled to verify the performance of the parameters of the logistic regression model from the training cohort. We found that the levels of the five exosomal miRNAs in the GBC samples in the validation cohort were completely consistent with their expression patterns in the training cohort (Figure 4A). Unsurprisingly, the two traditional tumor diagnostic markers, CEA and CA199, were also abnormally expressed in the GBC validation samples (Figure 4B). To investigate whether these five exosomal miRNAs were specific to GBC or secreted from GBC tumor tissue, we first verified the expression levels of these molecules in hepatocellular carcinoma (HCC), a common tumor of the hepatobiliary system. However, three miRNAs were not abnormally expressed in the exosomes of HCC patients, and the expression trends of the other two exosomal miRNAs were opposite to of those observed in GBC (Figure S4A). Second, we determined the levels of these five miRNAs in plasma exosomes of patients before and after surgery and in solid tumor tissues. After testing with existing samples, we found significantly decreased exosomal miR-552-3p, miR-581, and miR-4433a-3p levels and significantly increased exosomal miR-203b-3p levels in GBC patients who underwent surgery. There was no statistically significant change in exosomal miR-496 levels between the preoperative and postoperative samples (Figure S5A). After estimating the expression levels of these five miRNAs in existing GBC tumor tissues and paratumor tissues, we found that tumor tissues have higher miR-552-3p, miR-581, and miR-4433a-3p levels than paratumor tissues. In addition, miR-496 and miR-203b-3p did not show statistically significant differences between tumor and paratumor samples (Figure S5B). These results indicated that exosomal miR-552-3p, miR-581, and miR-4433a-3p may be specific in GBC and derived from GBC tumor tissue. Meanwhile, we need to further investigate the complex sources of exosomal miR-203b-3p and miR-496.

Subsequently, we utilized ROC analysis to evaluate the efficacy of plasma exosomal miRNAs, individually (Figures S6A and S6B) or in combination (Figure 4C), together with the traditional diagnostic markers (Figure 4D) in GBC diagnosis. Similar to the training cohort samples, the AUC of the five exosomal miRNAs-set in the multicenter validation samples was 0.905 when using the cutoff value (0.578) identified in the biomarker training phase, and the sensitivity was 81.37% and the specificity was 86.61% (Figure 4C). For CA199, the AUC was 0.723 with a sensitivity of 27.45% and a specificity of 100% when using the threshold (122.2 U/mL) that was acquired in the training phase. The AUC of CEA was 0.759 when the cutoff value (1.9 μg/L) from the training phase was used, and the sensitivity and specificity of GBC diagnosis were 74.51% and 54.55%, respectively. The specificity + sensitivity of

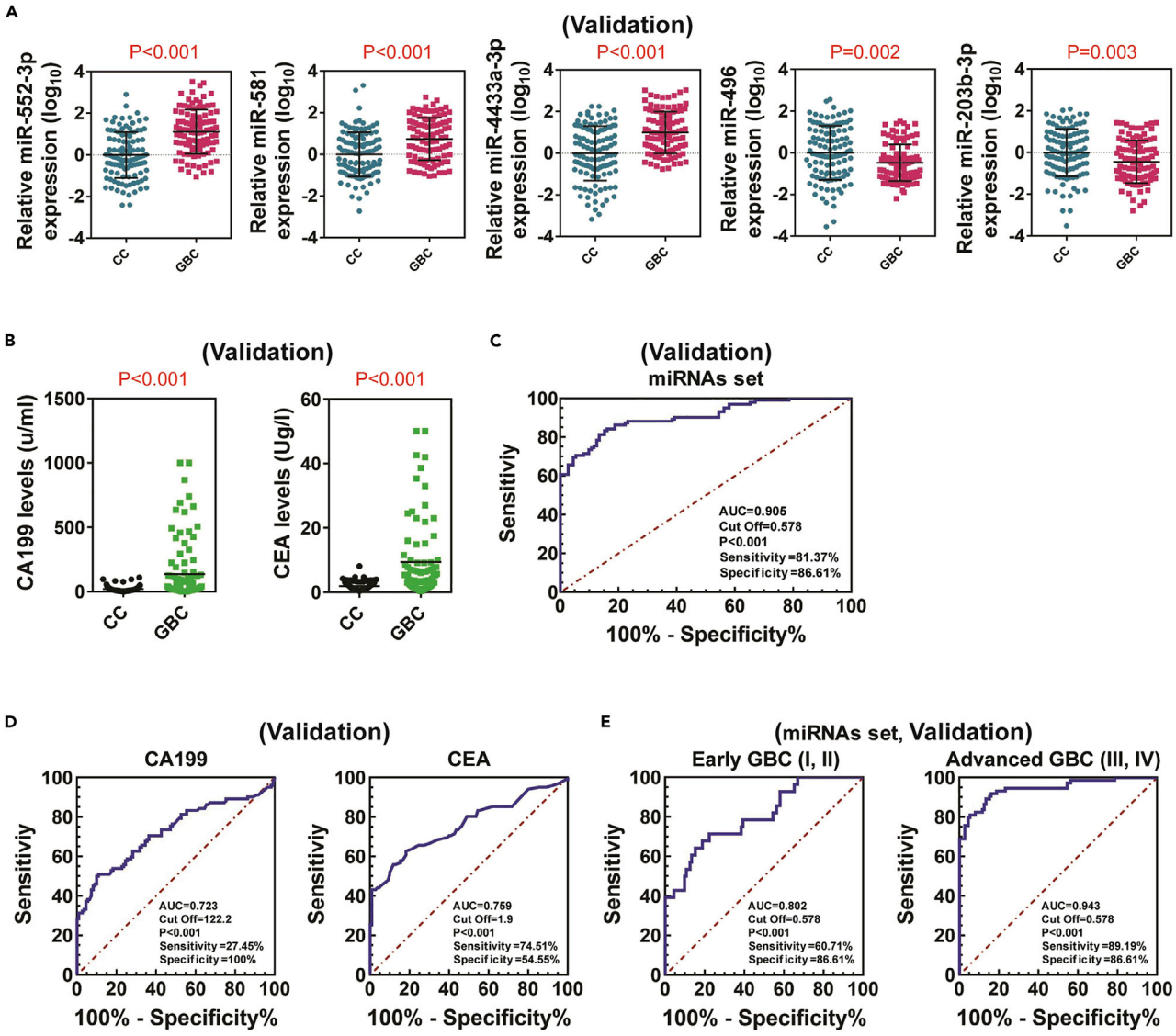


Figure 4. Outcomes of the use of exosomal miRNAs in the diagnosis of GBC patients in the validation cohort

(A) Differentially expressed exosomal miRNAs in the validation cohort, n = 102 for GBC patients, n = 112 for CC patients, n = 25 for HCC patients. miRNA expression was measured by RT–PCR. Data are represented as mean ± SD.

(B) CA199 and CEA levels in GBC patients in the validation cohort. Data are represented as mean ± SD.

(C) The logistic regression equation and parameters created in the training cohort were used to construct the ROC curve in the validation cohort.

(D) ROC curve analysis was performed to analyze the performance of CA199 and CEA in the validation cohort.

(E) Patients with TNM stage I or II were classified as having early GBC. Patients with TNM stage III or IV were classified as having advanced GBC.

the five exosomal miRNAs-set in GBC diagnosis was better than that of the traditional tumor diagnostic indicators (Figures 4C and 4D).

Importantly, the possibility of using this five exosomal miRNAs-set for the early diagnosis of GBC was further explored. We found that this biomarker set have good efficiency in diagnosing early-stage GBC, although this efficiency was moderately lower than that of advanced GBC (AUC = 0.802 with a sensitivity of 60.71% and a specificity of 86.61% for early GBC; AUC = 0.943 with a sensitivity of 89.19% and a specificity of 86.61% for advanced GBC). The five exosomal miRNAs-set may act as a potential biomarker for the early diagnosis of GBC or as a biomarker for screening GBC in high-risk populations (Figure 4E).

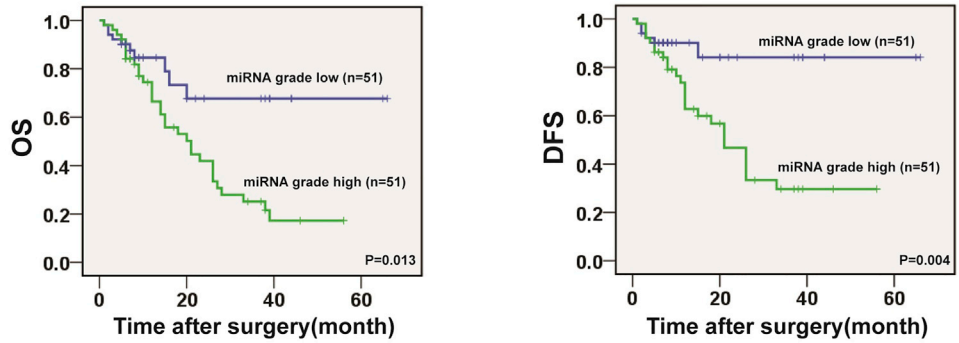
A

Relation between exosomal miRNA signature and the clinicopathologic characteristics of GBC patients

Variable	Exosomal miRNA grade (logit (P))		P value
	Low (n=51)	High (n=51)	
Age (years) *	63 (24-78)	62 (26-76)	0.3841 #
Sex, M:F	25:26	20:31	0.3187
Diameter (cm), <3: 3-5: >5	25:14:12	12:18:21	0.0233
Differentiation, low: medium: high	5:44:2	15:34:2	0.0432
Lymph-node metastasis, yes: no	17:34	27:24	0.0456
Liver metastasis, yes: no	19:32	30:21	0.0293
Distant metastasis, yes: no	4:47	12:39	0.0294
TNM, I:II:III:IV	7:12:26:6	1:7:30:13	0.0339
CEA (μg/L), <10:≥10	39:12	41:10	0.6302
CA199 (U/ml), <39:≥39	22:29	30:21	0.1131

Note: * Median (range). # Mann-Whitney test. P<0.05 by Chi-square test or Student t test.

B



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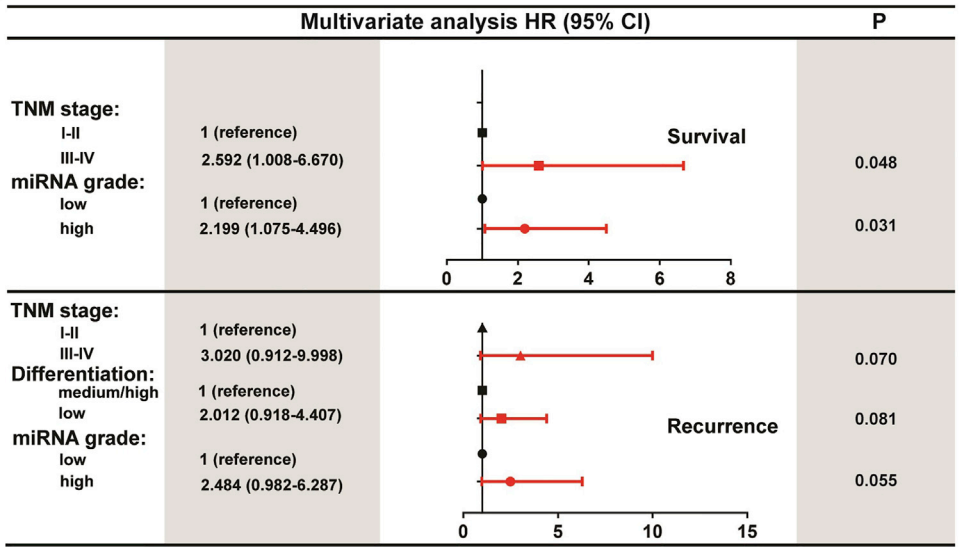

Figure 5. The five exosomal miRNAs-set is a significant prognostic factor of GBC

(A) Associations between exosomal miRNA grade and the clinicopathological characteristics of GBC patients. GBC patients were stratified into low-grade or high-grade groups with the median cutoff based on the calculated exosomal miRNA levels.

Figure 5. *Continued*
(B) Kaplan–Meier analysis of the correlation between exosomal miRNA grade and overall survival (OS) or disease-free survival (DFS) in patients with GBC.
(C) Multivariate analysis of HRs for overall survival and tumor recurrence. HR: hazard ratio, CI: confidence interval.

Exosomal miRNA signature correlated with multiple clinicopathological characteristics and survival in GBC patients

To comprehensively explore the clinical value of the exosomal miRNA signature in GBC, we first stratified GBC patients into low-grade and high-grade groups with a median cutoff that was based on the calculated levels of exosomal miRNA expression, and we determined the relationship between the clinical characteristics in the two groups. We found that the grade of the five exosomal miRNAs-set was significantly correlated with the multiple clinicopathological characteristics of GBC patients (Figure 5A). Compared with GBC patients with a low exosomal miRNA grade, the high-grade patients had larger tumor diameters, poor differentiation, lymph node metastasis, liver metastasis, distant metastasis, and later TNM stages (Figure 5A).

Moreover, Kaplan–Meier survival analysis showed that the exosomal miRNA grades were negatively correlated with the overall survival and disease-free survival of GBC patients from four medical centers (Figure 5B). Eleven survival- and recurrence-related clinicopathological variables were analyzed by univariate analysis (Table 2), which showed that differentiation, TNM stage and exosomal miRNA grades were statistically correlated with overall survival. In addition, differentiation, lymph node metastasis, distant metastasis, TNM stage, and exosomal miRNA grades were statistically correlated with recurrence (Table 2). Each individual parameter was further subjected to multivariate Cox proportional hazards analysis, which showed that TNM stage and exosomal miRNA grade were independent and significant factors affecting the survival of GBC patients and that differentiation, TNM stage and exosomal miRNA grade were independent factors affecting the recurrence of GBC (Figure 5C).

Downstream target prediction and signal transduction pathway enrichment

As miRNAs function by inhibiting downstream target genes, we aimed to reveal the potential function of the up- or downregulated exosomal miRNAs in GBC patients. To this end, we further analyzed the potential impact of these miRNAs on downstream targets. Using bioinformatics analysis that was based on overlapping information from the Miranda and RNAhybrid databases, we established a miRNA-target gene regulation network (Figure 6A). This network revealed that 1633 mRNAs were possibly targeted by the five differentially expressed exosomal miRNAs. Using GO functional annotation, we found that the five differentially expressed exosomal miRNAs may mainly influence transcription (Figure 6B). KEGG pathway enrichment analysis was further conducted with the candidate target genes, and the top 20 enriched pathways are shown in Figure 6C. According to the results of KEGG pathway enrichment, some cancer-related pathways, including the T cell receptor signaling pathway, NOD-like receptor signaling pathway, FoxO signaling pathway, calcium signaling pathway and estrogen signaling pathway, were identified.

In conclusion, by analyzing the target genes of the five differentially expressed exosomal miRNAs and the cell functions and signaling pathways that may be affected by these miRNAs, it is possible to use some references to study the mechanism of action of those biomarkers.

DISCUSSION

Gallbladder carcinoma is the most common malignancy of the biliary tract, which has a poor prognosis and is associated with local invasion, lymph node metastasis, and local vascular invasion (Liu et al., 2021). In recent years, an increasing number of molecular diagnostic and prognostic biomarkers and therapeutic targets have been identified for different types of cancer, providing an opportunity for accurate diagnostic and prognostic evaluations of cancer patients and the development of innovative cancer drugs. However, few systematic studies on the identification of diagnostic and population screening biomarkers of GBC have been conducted.

To systematic evaluate and verify the feasibility of the use of plasma exosomes as a noninvasive method for the diagnosis and screening of GBC, we conducted a multicenter study to explore potential biomarkers that distinguish GBC patients from high-risk populations. Through a three-step study that included biomarker discovery, training, and validation, we identified a set of five exosomal miRNAs that was used to evaluate the risk of GBC. This set included exosomal miR-552-3p, miR-581, miR-4433a-3p, miR-496,

Table 2. Univariate analysis of the variables associated with prognosis

		OS		DFS	
Variables	No	mean time to event	*P*	mean time to event	*P*
Age					
≤50 years	12	32.775 ± 8.107	0.693	33.021 ± 8.169	0.974
>50 years	90	32.556 ± 3.435		39.572 ± 3.831	
Sex					
Male	45	33.363 ± 5.485	0.856	36.561 ± 5.716	0.470
female	57	32.440 ± 3.862		40.185 ± 4.497	
Diameter					
≤5 cm	67	34.175 ± 4.160	0.371	42.907 ± 4.489	0.068
>5 cm	35	29.222 ± 4.865		31.038 ± 5.456	
Differentiation					
medium/high	82	34.503 ± 3.570	0.020	41.140 ± 4.010	0.005
Low	20	19.977 ± 4.698		21.668 ± 5.061	
Lymph-node metastasis					
No	58	39.833 ± 4.525	0.054	48.109 ± 4.504	0.011
Yes	44	21.499 ± 2.406		22.185 ± 2.767	
Liver metastasis					
No	53	40.787 ± 5.159	0.094	46.325 ± 5.027	0.194
Yes	49	22.687 ± 2.681		25.135 ± 2.662	
Distant metastasis					
No	86	34.512 ± 3.772	0.499	43.587 ± 4.050	0.019
Yes	16	24.417 ± 3.651		20.708 ± 3.860	
TNM stage					
I-II	27	47.067 ± 7.063	0.021	53.422 ± 6.747	0.022
III-IV	75	23.663 ± 2.169		26.454 ± 2.454	
CEA					
<10 μg/L	80	35.827 ± 3.740	0.060	41.943 ± 4.013	0.056
≥10 μg/L	22	18.802 ± 2.927		19.251 ± 3.072	
CA199					
<39 U/mL	52	32.864 ± 4.215	0.808	38.086 ± 4.546	0.852
≥39 U/mL	50	32.235 ± 4.875		39.902 ± 5.706	
Exosomal miRNA grade (logit (P))					
Low	51	48.221 ± 5.130	0.013	56.669 ± 3.887	0.004
High	51	24.308 ± 2.788		27.312 ± 3.343	

and miR-203b-3p. The calculated cutoff value for GBC diagnosis based on the exosomal miRNA panel was 0.578. A calculated value higher than 0.578 suggested a high risk of GBC occurrence.

CA199 level is elevated in patients with pancreatic, gastric, and bile duct cancers, while CEA level is observed in gastrointestinal cancer and in the normal embryonic gut, pancreas, and biliary tract. Although the increased expression of these two broad-spectrum tumor markers may reflect the presence of a variety of tumors, their specificity and sensitivity for the diagnosis of bile duct tumors are unsatisfactory (Grunnet and Mau-Sørensen, 2014; Wen et al., 2017). It has been reported that in the diagnosis of GBC, the sensitivity of CEA is only 11.5% and that of CA199 is 71.7% (Wang et al., 2014). In another study, the AUCs of CEA and CA199 were 0.770 (sensitivity = 60%, specificity = 83.3%) and 0.729 (sensitivity = 58%, specificity = 92.6%), respectively (Ueta et al., 2021). Notably, the efficacy of this five exosomal miRNAs-set in GBC diagnosis was

Figure 6. Analysis of the potential function of the five exosomal miRNAs in GBC patients

(A) miRNA-target gene regulation network consisting of the five exosomal miRNAs.

(B and C) GO and KEGG analyses of the functions of the potential target genes of differentially expressed exosomal miRNAs. The X axis represents the rich factor. The Y axis represents the top 20 functions or signaling pathways associated with each term.

better than that of the traditional biomarkers CA199 and CEA when the data from this study and previous studies were compared (Ueta et al., 2021; Wang et al., 2014). Here, when validated in multicenter GBC samples, the AUC of the plasma exosomal miRNA set was 0.905, with a sensitivity of 81.37% and a specificity of 86.61%. In this study, the AUC of CA199 was 0.723 (sensitivity = 27.45%, specificity = 100%), and the AUC of CEA was 0.759 (sensitivity = 74.51%, specificity = 54.55%). In addition, the set of exosomal miRNAs we identified has advantages compared to other extracellular vesicle-shuttled miRNA biomarkers. For example, this set is superior to CEA, CA199 and the miR-1246 combinatorial biomarker (AUC = 0.816) and the exosomal miR-151a-5p biomarker (AUC = 0.5955) (Ueta et al., 2021; Xue et al., 2020). More importantly, we verified that this exosomal miRNA biomarker in our study has good efficiency for diagnosing early stage GBC, indicating that it may act as a potential early diagnostic biomarker for GBC or as a biomarker for screening GBC in high-risk populations.

Interestingly, the grade of this identified exosomal miRNA biomarker significantly correlated with the multiple clinicopathological characteristics of GBC patients, including tumor diameter, differentiation, lymph node metastasis, liver metastasis, distant metastasis, and TNM stages. Moreover, it also negatively correlated with the overall survival and disease-free survival of GBC patients. These results indicated that this exosomal miRNA biomarker could be used not only as a diagnostic marker for GBC but also as a prognostic marker.

Mechanistically, a miRNA-target gene regulatory network was constructed using bioinformatic analysis. The cell functions and signaling transduction pathways that may be affected by these five exosomal miRNAs were further been speculated. Interestingly, epidemical studies have demonstrated that the incidence of GBC has a distinct gender bias, suggesting critical roles of the estrogen signaling pathway (Gabbi et al., 2010). These results provide a basis for exploring the underlying mechanisms and therapeutic possibilities of GBC.

After experimental optimization, it would take only approximately 5 h and less than 2 mL of plasma to complete the evaluation. It is believed that this exosomal miRNA biomarker may enable the development of a noninvasive and efficient tool for GBC diagnosis. Moreover, this approach also offers the possibility of early screening and prognostic assessment of GBC patients.

Limitations of the study

The limitations of this study are mainly the retrospective study design, which resulted in limited information available, unavoidable selection bias, and partial absence of samples and information. Further investigation with a larger pool of prospective patients is warranted to assess the performance of this exosomal miRNA biomarker. With that information, this biomarker will soon be available for clinical application.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104816.

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AUTHOR CONTRIBUTIONS

P.H.Y. and F.L.S. contributed to experiments, writing-original draft, and validation; X.W.Y. and X.Z.Y. contributed to clinical sample resources, writing-review & editing, validation; X.Y.H., Z.J.Q., Z.Q.L., H.C.W., and S.J.X. contributed to clinical sample resources; Z.J.W. and C.L. contributed to experiments; X.L.X. and K.C.Z. contributed to methodology; J.Y. and H.L. contributed to software analysis; L.L. contributed to data curation, formal analysis, funding acquisition, project administration; B.H.Z. contributed to conceptualization, funding acquisition, supervision, formal analysis; H.Y.W. contributed to conceptualization, data curation, funding acquisition, supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Small RNA high throughput sequencing data	GEO database	Accession number: GSE176159
Critical commercial assays		
exoRNeasy Serum/Plasma Midi Kit	Qiagen	Cat. No. 77044
miScript II RT Kit	Qiagen	Cat. No. 218161
miScript SYBR Green PCR Kit	Qiagen	Cat. No. 218073

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hongyang Wang (hywangk@vip.sina.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The Small RNA high throughput sequencing data are available in GEO database (www.ncbi.nlm.nih.gov/geo/) with accession number GSE176159. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients

A total of 140 plasma samples from gallbladder cancer (GBC) patients(median age: 63 years old, range: 24–78; Male: Female = 66:74), 150 plasma samples from chronic cholecystitis (CC) patients (median age: 57 years old, range: 18–88; Male: Female = 84:66) and 25 plasma samples from hepatocellular carcinoma (HCC) patients (median age: 50 years old, range: 40–75; Male: Female = 23:2) were collected between March 2015 and January 2020 from Eastern Hepatobiliary Surgery Hospital of Second Military Medical University, Affiliated Sixth People's Hospital of Shanghai Jiao Tong University, Shanghai General Hospital of Shanghai Jiao Tong University School of Medicine, and Zhongda Hospital of Southeast University, China. These samples were distributed through a three stages study. The "3-step" study procedure (Figure 1) was approved by all four local institutional review boards. All participants signed a consent form for participation in the survey, with a permission for sample collection, utilization, and data analysis.

METHOD DETAILS

Three-step biomarker study procedure

Discovery phase

Small RNA high throughput sequencing technology was performed to identify plasma exosomal miRNAs expression between 8 GBC and 8 CC patients. GBC and CC patients were matched according to the enrollment date, specimen collection protocol, age, and sex (Table S1). A total of 22 types of miRNAs in plasma exosomes of GBC patients were down-regulated and 24 types of up-regulation when compared with CC.

Training phase

By gathering independent set of samples, a total of 30 participants in the GBC group and 30 participants in the CC group were randomly selected as a training set to construct the logistic regression model. Using real-time qPCR, the top 10 up-regulated and top 10 down-regulated miRNAs in the GBC group were verified at the training stage. Seven miRNAs were differentially expressed between GBC and CC and five in these (miR-552-3p, miR-581, miR-4433a-3p, miR-496 and miR-203b-3p) were flowed through the logistic regression analysis in this phase. The values of the traditional diagnostic index were provided by the local institution.

Validation phase

The logistic regression model and cut off values from the training cohort were applied to an independent set of samples that were collected from multiple centers in the validation cohort (102 participants in the GBC group, 112 in participants in the CC group and 25 participants in the HCC group) to identify the performance of the selected five miRNAs-set. The expressions of the five miRNAs that were flowed through training stage, were determined by real-time qPCR.

Exosomal small RNA high-throughput sequencing

Small RNA high throughput sequencing detected 18–40 base single-stranded small RNA in the plasma exosomes of patients with gallbladder cancer and control cholecystitis, including miRNA, piRNA, snoRNA, snRNA, tRNA, etc. The high throughput sequencing was completed by Guangzhou RiboBio Co., Ltd. The Small RNA high throughput sequencing data are available in GEO database (www.ncbi.nlm.nih.gov/geo/) with accession number GSE176159.

From the total RNA extraction to final cDNA library preparation mainly includes the following steps: total RNA is attached to 5′and 3′ connectors respectively; The first strand cDNA is synthesized; PCR amplification; The cDNA libraries with inserted fragments of about 18–40nt were obtained by gel electrophoresis, and then sequenced by sequencing machine. The information analysis process is as follows: the 50nt raw reads set obtained from Illumina Hiseq^{TM}2500 sequencing was filtered through removing the joints at both ends of reads, removing low-quality reads, decontamination and other processes to obtain clean reads. The sequence length distribution and common sequences among samples were statistically analyzed. Clean reads were classified and annotated to obtain the composition and expression levels information of all kinds of small RNA in the sample. After all small RNA fragments were annotated, the remaining small RNA fragments were used to predict new small RNA.

Collection of plasma samples

For plasma-derived exosome purification, a total of 10 mL venous blood samples from GBC and CC patients were collected using anticoagulation tubes (containing EDTA) (GD050EK2, Gongdong). To harvest plasma, blood samples were centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was collected. Next, centrifugation was repeated, and the supernatant was collected again. Plasma samples were stored at −80°C before use.

Exosomal RNA isolation and PCR analysis

Exosomal total RNA was extracted and purified from 2 mL of plasma using an exoRNeasy Serum/Plasma Midi Kit (77044, Qiagen) following the manufacturer's instructions. The same amount of *Caenorhabditis elegans* cel-miR-39-3p microRNA was spiked into each sample as an external calibration for RNA extraction, reverse transcription, and microRNA amplification. The total amount of RNA for each reverse transcription reaction was 100 ng, and the complementary strand template was prepared using a miScript II RT Kit (218161, Qiagen) according to the manufacturer's protocol. The exosomal microRNA quantification was obtained with qPCR using specific microRNA primers (Qiagen), miScript SYBR Green PCR Kit (218073, Qiagen), and LightCycler® 96 Real-Time PCR System (Roche) according to the manufacturer's instructions. All samples were normalized by the initial liquid input volume used for RNA extraction and calibrated by the spike-in cel-miR-39-3p microRNA to rule out the minute bias caused by different RNA isolation efficiencies and PCR efficiencies among samples.

Flow cytometry for exosomes

Flow cytometry was performed to analyze the exosome phenotype using fluorescein-conjugated monoclonal antibodies (CD81, CD63, BD Biosciences) according to the manufacturer's recommendations. Briefly, exosomes purified from plasma were incubated for 30 min with 0.5% bovine serum albumin (BSA, GIBCO) in PBS to block nonspecific antigens. Then, conjugated monoclonal antibodies were added according to the manufacturer's recommendations, and the mixtures were incubated at 4°C in the dark overnight.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Data analyses were undertaken by independent statisticians. The performance of exosomal miRNAs as diagnostic biomarkers was examined by estimating a receiver operating characteristic (ROC) curve. The area under the curve (AUC) was then calculated for measuring the diagnostic accuracy. The optimal cutoff value was determined by the following criteria: (1) Maximizing the sum of sensitivity and specificity; (2) minimizing the overall error (square root of the sum $[1\text{-sensitivity}]^2 + [1\text{-specificity}]^2$); (3) minimizing the distance of the cutoff value to the top-left corner of the ROC curve. A logistic regression model was used to estimate the exosomal miRNAs set based on comparing GBC samples with CC samples in the training dataset. The MedCalc (version 10.4.7.0; MedCalc, Mariakerke, Belgium) software was used to perform the ROC and regression analyses. A new variable predicted probability (p) for diagnosis GBC was constructed based on the equation that was created by the logistic regression model: logit(P) = 1.628×miR-552-3p +1.543×miR-581 + 2.485×miR-4433a-3p-3.228×miR-496-3.127×miR-203b-3p-2.254 for exosomal miRNAs-set biomarker. The values of the new variable were used as one marker subjecting to ROC analysis.

Qualitative variables were analyzed by chi-square test or Fisher exact test. For the data obtained by real-time qPCR, differences between the two groups were compared using analysis of student t test when applicable or the nonparametric test. Statistical analyses were performed with the SPSS 18.0 software. Unless otherwise specified, the results are presented as the means ± standard deviation (SD). In addition to the specifically stated, statistical tests were two-sided, and $p < 0.05$ was considered statistically significant. Experiment-specific statistical information can be found in the figure legends, figures, and results. N = the number of samples utilized to determine statistical significance.