ORIGINAL ARTICLE

Retrospective Study

Detecting plasma SHOX2, HOXA9, SEPTIN9, and RASSF1A methylation and circulating cancer cells for cholangiocarcinoma clinical diagnosis and monitoring

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Abstract

BACKGROUND
Cholangiocarcinoma (CCA), also known as bile duct cancer, is a devastating malignancy primarily affecting the biliary tract.

AIM
To assess their performance in clinical diagnosis and monitoring of CCA, plasma methylation and circulating tumor cells were detected.

METHODS
Plasma samples were collected from Hubei Cancer Hospital (*n* = 156). Plasma

DNA was tested to detect SHOX2, HOXA9, SEPTIN9, and RASSF1A methylation using TaqMan PCR. Circulating tumor cells (CTCs) were detected in the peripheral blood of patients using the United States Food and Drug Administration-approved cell search system before and after clinical therapy. The CCA diagnostic value was estimated using the area under the curve. The independent prognosis risk factors for patients with CCA were estimated using Cox and logistic regression analyses.

RESULTS
The sensitivity and specificity of the four DNA plasma methylations exhibited 64.74% sensitivity and 93.88% specificity for detecting CCA. The receiver operating characteristic curve of the combined value for CCA diagnosis in plasma was 0.828 ± 0.032. RASSF1A plasma methylation was related to the prognosis of patients with CCA. We determined the prognostic hazard ratio for CCA using CTC count, tumor stage, methylation, and carbohydrate antigen 19-9 levels as key factors. Our overall survival nomogram achieved a C-index of 0.705 (0.605-0.805).

CONCLUSION
SHOX2, HOXA9, SEPTIN9, and RASSF1A plasma methylation demonstrated increased sensitivity for diagnosing CCA. RASSF1A plasma methylation and CTCs were valuable predictors to assess CCA prognosis and recurrence.

Key Words: Cholangiocarcinoma; Methylation; Circulating cancer cells; Diagnosis; Prognosis

Core Tip: This study first analyzed the clinical diagnosis and monitoring value of detecting plasma SHOX2, HOXA9, SEPTIN9, and RASSF1A methylation for cholangiocarcinoma (CCA). We determined that the four DNA plasma methylations exhibited 64.58% sensitivity and 94% specificity for detecting CCA. The hazard ratio of prognosis for the risk of CCA risk was identified using the Circulating tumor cells (CTCs) count, tumor stage, methylation, and carbohydrate antigen 19-9 (CA199) levels as independent prognostic factors. We developed a predictive nomogram for CCA overall survival, age, stage, CTCs, methylation, and CA199, with a C-index of 0.705 (95%CI: 0.605-0.805). This model evaluates risk factors.

INTRODUCTION
Cholangiocarcinoma (CCA), also known as bile duct cancer, is a devastating malignancy primarily affecting the biliary tract. CCA encompasses the intrahepatic, perihilar, and distal CCA subtypes. The most recent Chinese statistics from 2022 ranked CCA as the second most common primary hepatobiliary malignancy in terms of morbidity and mortality, underscoring its significant clinical burden[1,2]. Unfortunately, the non-specific early symptoms and lack of sensitive diagnostic tools mean that most patients with CCA are diagnosed at an advanced stage, when curative surgical resection is no longer feasible. Furthermore, such patients are prone to frequent metastasis and poor prognosis[3,4]. As a result, the 5-year overall survival (OS) rates are notably low, with patients having a median survival duration of roughly 24 months and facing a staggering 90% mortality rate within five years[3,4].

Identifying CCA early and initiating prompt treatment is essential for enhancing patient prognosis. However, traditional diagnostic methods, such as imaging and tumor marker assessments, have limited sensitivity and specificity, often failing to detect the disease at an early stage[5]. Chronic hepatitis B virus infection is a well-established CCA risk factor with a pivotal etiological role in its development[6]. Despite advances in chemotherapy regimens centered on cisplatin and gemcitabine, CCA remains largely insensitive to chemoradiotherapy and exhibits drug resistance, highlighting the urgent need for more effective diagnostic and monitoring strategies[3].

The role of epigenetic alterations, particularly DNA methylation, in cancer development and progression has garnered significant attention recently[7,8]. Cancer cell genes undergo methylation changes early in carcinogenesis, which persist and dynamically evolve throughout tumor progression. Tumor suppressor genes are frequently silenced by the methylation of CpG islands located upstream of their promoters, disrupting normal cellular processes and promoting malignancy[9,10]. The CpG island hypermethylation of specific genes has been closely linked to cancer initiation and progression, serving as a promising biomarker for early cancer detection and prognosis assessment[11,12].

Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) have emerged as non-invasive biomarkers for cancer screening and monitoring[13]. ctDNA is shed by tumor cells into the circulation and contains numerous cancer-specific genetic signatures that reflect the genomic landscape of the primary tumor[14]. CTCs represent micrometastatic dissemination and can be used as real-time indicators of disease status and therapeutic response[15]. The United States Food and Drug Administration (FDA)-approved CellSearch system, which is based on positive immunoselection of

epithelial cell adhesion molecule (EpCAM) and negative selection of CD45, enables CTC enumeration and characterization into distinct phenotypes (epithelial, mesenchymal, and hybrid)[16].

Among the myriad of genes undergoing methylation changes in cancer, SHOX2, HOXA9, SEPTIN9, and RASSF1A were specifically chosen for this study due to their established roles in cancer development and progression. SHOX2 and RASSF1A are tumor suppressor genes frequently silenced by methylation in various cancers, and are crucial in the initiation and progression of malignancy[17,18]. The homeobox gene HOXA9 plays a crucial role in modulating mechanisms associated with cancer progression[19]. SEPTIN9 is involved in cytoskeleton organization and cell division and exhibits methylation changes associated with cancer development, particularly in colorectal cancer[20].

Several genes have emerged as potential epigenetic markers in CCA. RASSF1A is a tumor suppressor gene involved in diverse biological processes and is pivotal in tumor development. RASSF1A promoter hypermethylation has been frequently observed in CCA and other tumor types, implicating it as a potential molecular marker[13,18]. Similarly, SEPTIN9, a protein-coding gene critical for cytoskeleton organization and cell division, undergoes methylation changes that affect gene expression and contribute to abnormal cell function, fostering tumor formation and growth in pre-colorectal cancer diseases[20].

Recent studies have further underscored the diagnostic and prognostic value of specific methylation markers in various cancers, including CCA. For example, RASSF1A promoter methylation has exhibited high co-specificity in pan-cancer diagnosis and a certain degree of screening ability[21]. Additionally, the combined detection of SEPTIN9 and SHOX2 methylation was associated with tumor-node-metastasis (TNM) staging, histological grading, and lymphatic infiltration in pre-treatment tumors[22,23]. Furthermore, HOXA9 methylation dynamics predicted the therapeutic efficacy of novel ovarian cancer therapies, bridging the gap in efficacious predictive biomarkers[24].

In particular, recent advancements have indicated the potential of ctDNA methylation markers for improving diagnostic accuracy in CCA. A study by Hu *et al*[13] demonstrated the potential of RASSF1A promoter methylation as a biomarker for colorectal cancer, emphasizing its potential applicability in other cancers, including CCA. Liang *et al*[23] recently introduced a new set of DNA methylation biomarkers for identifying malignant pleural effusion, underscoring the versatility of methylation markers across different cancer types. These recent reports highlight the evolving landscape of epigenetic biomarkers in cancer diagnosis and prognosis.

In this study, we aimed to evaluate the clinical utility of combined plasma methylation of SHOX2, HOXA9, SEPTIN9, and RASSF1A, alongside CTC enumeration, in CCA diagnosis and monitoring. We used these epigenetic and cellular biomarkers to develop a non-invasive detection platform that could facilitate early CCA diagnosis, prognosis assessment, and disease monitoring, ultimately guiding personalized therapeutic strategies and improving patient outcomes.

MATERIALS AND METHODS

Genome data
The genomic data used in this study were obtained from the Gene Expression Omnibus database (http://gepia2.cancer-pku.cn/) and The Cancer Genome Atlas (TCGA) database (http://www.ualcan.path.uab.edu/).

Patients and healthy controls
The study cohort comprised 156 patients diagnosed with CCA at Hubei Cancer Hospital, China, between June 2020 and December 2022. The patients were included based on pathological evidence in accordance with the World Health Organization criteria and TNM classification. All patients were treatment-naïve at the time of enrollment. The cohort included 11 patients at stage I-II disease and 145 patients at stage III-IV disease. As a control group, 70 healthy individuals were enrolled from Wuhan's TCWM Hospital health check-up clinic. The disease controls were 28 patients with benign bile duct disease (17 with bile duct or gallbladder polyps, 11 with biliary calculus). The Ethics Committee of Hubei Cancer Hospital approved this study (approval number: LLHBCH2023YN-002).

Blood samples
Peripheral blood samples (10 mL per case) were collected from each patient and control by venipuncture into EDTA-coated tubes at the time of diagnosis and prior to any therapeutic intervention. Blood samples for CTC detection were also collected before and after clinical therapy. The samples were immediately processed and stored at 4 °C until further analysis.

DNA was extracted from the plasma using a commercially available kit according to the manufacturer's instructions (Tellgen Corporation, Shanghai, China). Before the methylation was detected, the DNA was treated with bisulfite to convert unmethylated cytosines into uracils while preserving the methylated cytosines. The treated DNA was referred to as sDNA (*i.e.*, DNA after bisulfite conversion). This conversion is a crucial step in methylation detection, as it enables the specific detection of methylated DNA sequences through subsequent PCR techniques. Methylation in the genes of interest was detected by subjecting DNA to bisulfite conversion, which converts unmethylated cytosines to uracil while leaving methylated cytosines unchanged. The methylation status of the *SHOX2*, *HOXA9*, *SEPTIN9*, and *RASSF1A* genes was determined using quantitative real-time PCR with TaqMan probes specific for the CpG islands in the promoters of these genes. The methylation levels were expressed as comparative threshold cycle (ΔCt) values, where ΔCt = Ct (gene of interest) - Ct (internal control, β-actin). β-Actin was selected as the internal reference gene given its relatively stable expression in most tissues and cells, which aids in standardizing and comparing gene expression levels across different samples, and it is a widely used as an internal reference gene. A sample was deemed methylation-positive when satisfying these quantitative thresholds: Ct value for SHOX2 < 32 with DCt ≤ 9; RASSF1A < 35 with DCt ≤ 12; SEPTIN9 <

35 with DCt ≤ 9; HOXA9 < 32 with DCt ≤ 8[22]. The methylation reagents for the four genes were from LungMe Assay (Tellgen Corporation, Shanghai, China).

DNA Extraction and Purification: High-purity DNA was extracted using commercial kits (Tellgen Corporation). The manufacturer's instructions were strictly followed to ensure the quality and purity of the extracted DNA. Methylation detection quality control consists of three parts. First, whole blood is processed to separate the plasma within 2 hours after collection. The separated plasma is stored at -20 °C and tested within 1 month to ensure its quality. Second, an internal control (β-actin) is used as a whole-process quality control to monitor from extraction to bisulfite conversion to PCR detection. Third, PCR positive and negative controls are used to individually assess the PCR system and reactions. These three parts ensure the quality control of methylation detection and provide indications for identifying the causes of detection failure.

RNA in situ hybridization assay for CTC identification

CTCs were enriched from peripheral blood samples using the CanPatrol™ CTC enrichment technique (SurExam, Guangzhou, China). Following enrichment, the CTCs were identified by RNA *in situ* hybridization using a panel of molecular markers. These markers included EpCAM and cytokeratins 8/18/19 as epithelial cell biomarkers, CD45 as a leukocyte biomarker, and vimentin and Twist as mesenchymal cell biomarkers. Before hybridization, probe accessibility was enhanced by permeabilizing blood cells and treating them with a protease. Hybridization was performed using capture probes targeting the selected molecular markers. Fluorescence signals were detected using an automated imaging fluorescence microscope (Zeiss, Oberkochen, Germany). Red and green fluorescent signals represented epithelial and mesenchymal marker expression, respectively, while bright white fluorescent signals identified leukocytes.

Carbohydrate antigen 19-9 detection

Carbohydrate antigen 19-9 (CA199) concentrations in serum were determined by employing a chemiluminescence-based immunoassay kit, supplied by Snibe Diagnostic (Shenzhen, China), adhering to the provided instructions. The normal reference range for CA199 was defined as ≤ 41 U/mL, and values exceeding this threshold were considered abnormal, indicating potential malignancy.

Statistical analysis

SPSS 25 (IBM) was utilized for statistical analyses. Normality of continuous variables was checked with Shapiro-Wilk. Depending on data distribution, we applied either non-parametric tests (Mann-Whitney *U*, Kruskal-Wallis *H*) or parametric ones (ANOVA, independent *t*-test) for group comparisons. Categorical variables were analyzed using the χ^2 test. Spearman's correlation was used to evaluate patient variable relationships. An assessment of the diagnostic precision of single methylation markers, their various combinations, along with CA199, was conducted through the receiver operating characteristic (ROC) curve analysis. We quantified diagnostic efficacy by computing the area under the curve (AUC) of the ROC curve. Independent prognostic factors for CCA survival were identified using univariate and multivariate Cox regression analyses. Statistical significance was set at $P < 0.05$.

RESULTS

Study cohort

The study cohort comprised 156 patients diagnosed with CCA at Hubei Cancer Hospital between June 2020 and December 2022. Our study cohort comprised 87 male participants (mean age: 58 years, ranging from 40 to 74) and 69 female participants (mean age: 57 years, ranging from 40 to 73). The disease-specific features of this cohort are detailed in Table 1.

Methylation frequency and association with clinicopathologic features in plasma samples

TCGA data analysis revealed that the CCA tissue had significantly higher *HOXA9*, *RASSF1A*, *SEPTIN9*, and *SHOX2* expression than normal tissue. Beta values, which span from 0 indicating no methylation to 1 signifying complete methylation, were utilized to represent the DNA methylation status of *HOXA9*, *RASSF1A*, *SEPTIN9*, and *SHOX2* gene promoters in both normal tissues (*n* = 9) and CCA tissues (*n* = 36). Hypermethylation was signified by beta values between 0.7 and 0.5, whereas hypomethylation was indicated by values from 0.3 to 0.25 (Figure 1).

Figure 1 depicts the DNA methylation of the *SHOX2*, *RASSF1A*, *SEPTIN9*, and *HOXA9* genes. Utilizing the optimal cutoff for gene-specific methylation, the sensitivity and specificity for CCA detection ranged from 25% to 64.1% and from 93.88% to 95.92%, respectively, as illustrated in Figure 2. The ROC curves for DNA methylation-based CCA diagnosis are shown in Figure 3. For the diagnosis of CCA, the methylation markers SHOX2, HOXA9, SEPTIN9, and RASSF1A exhibited sensitivities of 23.72%, 51.92%, 28.21%, and 29.49%, and specificities of 93.88%, 95.92%, 95.92%, and 93.88%, respectively. The sensitivity and specificity of the four methylations combined for diagnosing CCA were 64.74% and 93.88%, respectively. The AUC for the complex evaluation to diagnose CCA was 0.828 (range: 0.764-0.891) (Figure 4A). In the context of CCA diagnosis, the AUC values for methylation markers SHOX2, HOXA9, SEPTIN9, and RASSF1A were found to be 0.799 (range: 0.724-0.874), 0.777 (range: 0.705-0.850), 0.713 (range: 0.632-0.795), and 0.765 (range: 0.689-0.841), correspondingly. The critical values for SHOX2, HOXA9, SEPTIN9, and RASSF1A methylation for diagnosing CCA were DCtSHOX2 ≤ 9; DCtRASSF1A ≤ 12; DCtSEPTIN9 ≤ 9; and DCtHOXA9 ≤ 8, respectively, which was the same as that in a previous study[23].

Table 1 The clinical characteristics of the 156 patients with cholangiocarcinoma and 98 controls

Clinicopathological data	n	SHOX2 positive	SEPT9 positive	HOXA9 positive	RASSF1A positive	Comprehensive methylation Positive	CTC counts/ 5 mL
Tumor location							
iCCA	33	24.24%	24.24%	42.42%	27.27%	57.58%	10
pCCA	83	21.69%	27.71%	56.63%	27.71%	66.27%	12
dCCA	40	27.5%	32.5%	50%	35%	67.5%	14
iCCA *vs* dCCA		0.795	0.604	0.638	0.614	0.467	0.342
Gender							
Male	87	22.99%	34.48%	47.13%	32.18%	68.97%	13
Female	69	24.64%	20.29%	57.97%	26.09%	59.42%	10
Male *vs* female		0.851	0.702	0.199	0.481	0.24	0.760
Age at diagnosis							
≤ 60 years	91	21.98%	26.37%	41.76%	26.37%	59.34%	11
> 60 years	65	26.15%	30.77%	66.15%	33.85%	72.31%	14
Median age (years)	57						
Mean age (years)	59						
≤ 60 years *vs* > 60 years		0.571	0.591	0.195	0.374	0.126	0.276
Tumor stage							
I-II	11	9.09%	9.09%	18.18%	9.09%	36.36%	3
III-IV	145	24.83%	29.66%	54.48%	31.03%	66.21%	13
I-II *vs* III-IV		0.237	0.144	0.020	0.176	0.046	0.001
Lymphatic invasion							
L0	105	25.71%	27.62%	48.57%	32.38%	62.86%	4
L1	51	19.61%	29.41%	58.82%	23.53%	68.63%	16
L0 *vs* L1		0.400	0.815	0.331	0.255	0.479	0.001
Follow-up							
Follow up available	43						
Median follow-up months	14						
Mean follow-up months	17						
Range (months)	0-58						
Deceased	23						
Censored	20						
Healthy donors							
Male	42	3/42	2	2	3	3	1
Female	28	1/28	1	1	1	1	0
≤ 60 years	51	1	1	0	1	1	0
> 60 years	19	2	2	2	2	2	1
Benign bile duct patients							
Male	21	2/21	1	1	1	1	0
Female	7	0	0	0	1	1	0

≤ 60 years	23	2	0	1	2	2	0
> 60 years	5	1	1	1	1	1	0

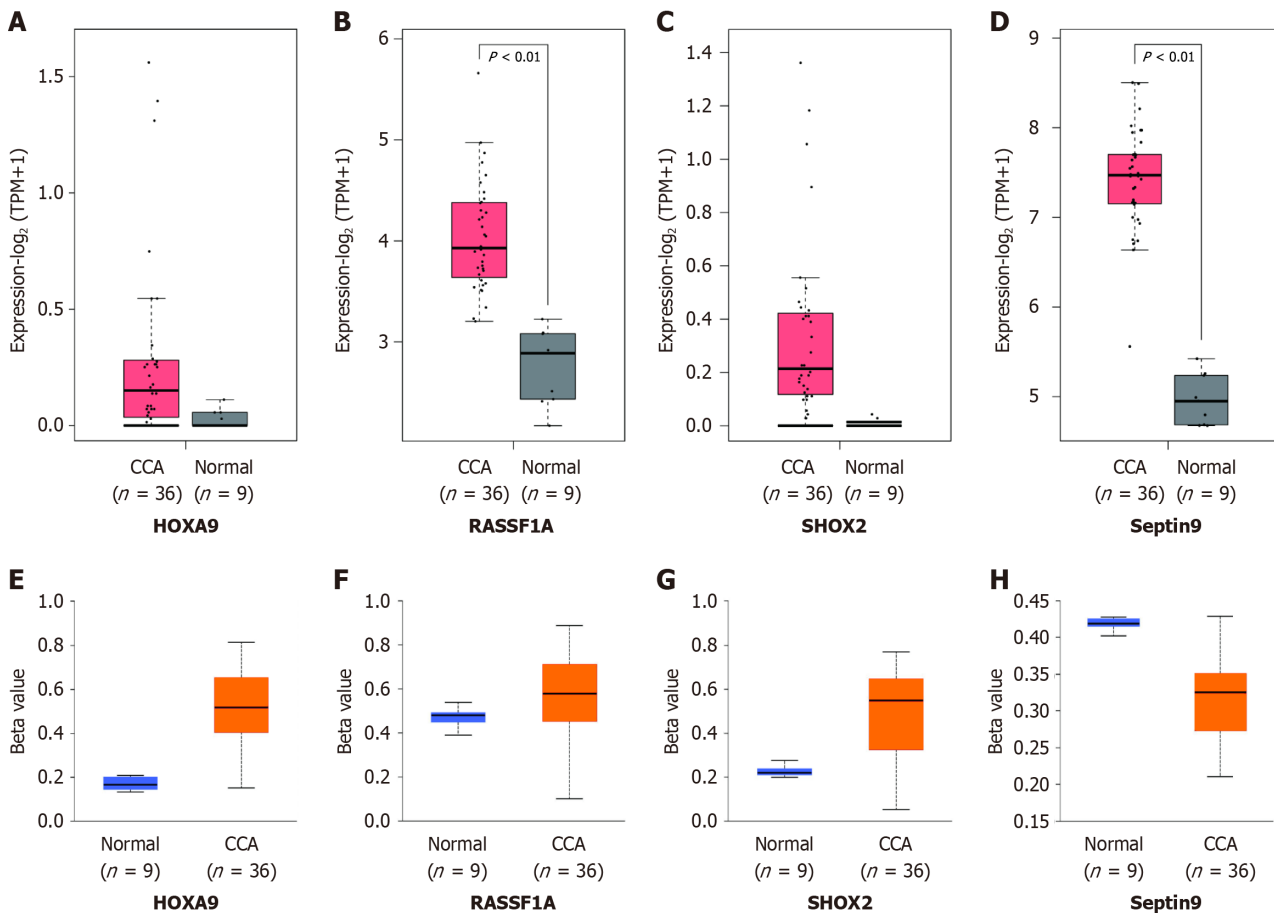CTC: Circulating tumor cell; CCA: Cholangiocarcinoma.

Figure 1 Analysis of *SHOX2*, *RASSF1A*, *SEPTIN9*, and *HOXA9* gene expression in normal tissue (*n* = 9) and cholangiocarcinoma tissue (*n* = 36) from previously reported genome data. A-D: *SHOX2*, *RASSF1A*, *SEPTIN9* and *HOXA9* gene expression was upregulated in cholangiocarcinoma (CCA) tissue (*n* = 36); E-H: Beta values ranging from 0 (unmethylated) to 1 (fully methylated) indicate the DNA methylation levels of the *SHOX2*, *RASSF1A*, *SEPTIN9* and *HOXA9* promoters in normal tissue (*n* = 9) and CCA tissue (*n* = 36). CCA: Cholangiocarcinoma.

Correlations between sDNA methylation and CA199 for detecting CCA

CA199 demonstrated a sensitivity of 51.28% and a specificity of 87.76% for detecting CCA, which was significantly lower than the sensitivity of the combined methylation evaluation. The AUC for CA199 was 0.746 ± 0.04, whereas the AUC for the combined methylation evaluation was 0.828 ± 0.032, indicating superior diagnostic performance.

Combining CA199 with the methylation panel improved the sensitivity and specificity to 68.59% and 85.76%, respectively (Table 2).

Among 156 CCA patients, 11 were diagnosed with stage I-II, while 145 presented with stage III-IV disease, as detailed in Table 1. For the diagnosis of stage I-II and III-IV CCA, the complex evaluation exhibited sensitivities of 36.36% and 75.17%, and specificities of 93.83% and 93.58%, correspondingly. The AUC of the complex evaluation and CA199 to diagnose early CCA was 0.682 ± 0.014 (Figure 4B) and 0.542 ± 0.092 (Figure 4C). Table 2 presents the sensitivity (68.59%) and specificity (85.76%) of the complex evaluation of CA199 combined with methylation.

The ROC curves of CTCs for the diagnosing CCA progression

The ROC curves for CTCs had an AUC of 0.653 (95%CI: 0.533-0.774) for diagnosing tumor progression, with a cut-off value of 10 CTCs/5 mL (Figure 3E). Among the 156 patients, 51 underwent surgery and chemotherapy. Another 105 patients underwent chemotherapy, of which 67 also received immunotherapy as first-line treatment. The remaining 45 patients were treated with targeted therapy. Postoperative CTC counts were monitored in 43 patients, revealing significantly decreased counts after surgical resection. Notably, the CTC counts of 10 out of 22 patients surpassed the threshold 3 months before imaging-confirmed recurrence or metastatic lesions.

Table 2 Performance of biomarkers and their combinations for diagnosing cholangiocarcinoma

Biomarker or combination	Positive test, CCA No.		Negative test, CCA No.		Sensitivity (%)	Early-stage CCA (%)	Specificity (%)	PPV (%)
SEPTIN9	44	3	112	95	28.21	9.09	96.94	54.72
HOXA9	81	3	75	95	51.92	18.18	96.94	69.29
SHOX2	37	2	119	96	23.72	9.09	97.96	52.36
RASSF1A	46	2	110	96	29.49	9.09	97.96	55.91
CA199	80	12	47	86	51.28	9.09	87.76	65.35
SEPTIN9 + HOXA9	81	3	75	95	51.92	18.18	96.94	69.29
SEP + HOXA9 + SHO	88	3	68	95	56.41	27.27	96.94	72.05
SEP + HOXA9 + SHO + RASS	101	6	55	92	64.74	36.36	93.88	75.98
Sep + HOXA9 + SHO + RASS + CA199	107	12	49	86	68.59	45.45	87.76	75.98

CA199: Carbohydrate antigen 19-9; CCA: Cholangiocarcinoma; PPV: Positive predictive value.
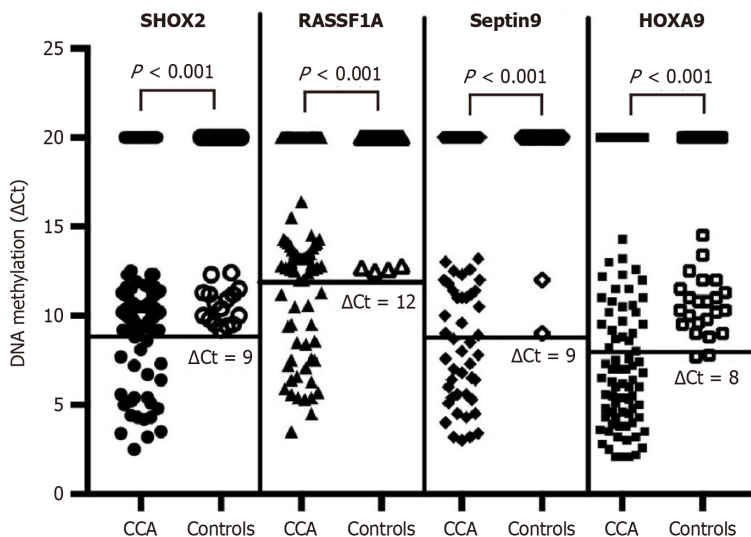
Figure 2 Quantitative analysis of SHOX2, RASSF1A, SEPTIN9, and HOXA9 DNA methylation in cholangiocarcinoma (*n* = 156) and control (*n* = 98) specimens. CCA: Cholangiocarcinoma.

Figure 5 presents the relapse-free survival (RFS) and OS of patients with CCA with varying CTC counts at admission. Patients with > 10 CTCs/5 mL had faster disease progression and a poorer outcome.

The evaluation of sDNA methylation for monitoring CCA

The utility of plasma sDNA methylations and CTCs in monitoring CCA progression was assessed using postoperative evaluation of these biomarkers in 22 patients 1 month following resection. Four patients exhibited a notable decrease in plasma DNA methylation levels. Kaplan-Meier survival analysis demonstrated that patients with high methylation levels (defined as DCtSHOX2 ≤ 9; DCtRASSF1A ≤ 12; DCtSEPTIN9 ≤ 9; DCtHOXA9 ≤ 8) had shorter OS than those with low methylation levels (Figure 6). Notably, patients with advanced CCA exhibiting RASSF1A methylation had a significantly worse RFS ($P = 0.002$), as illustrated in Figure 5C. Nevertheless, there was no notable link found between CCA metastasis and the methylation status of SEPTIN9, HOXA9, or SHOX2.

The prognostic nomogram for OS of patients with CCA constructed using methylation and CTCs

A prognostic nomogram for predicting the OS of patients with CCA was constructed based on multivariate analysis. The nomogram incorporated age, tumor stage, CTC count, methylation status, and CA199 levels. Each predictor in the nomogram was assigned a score, and the sum of these scores indicated the probability of the 1- and 2-year OS (Figure 7). The C-index of this nomogram was 0.705 (95%CI: 0.605-0.805), demonstrating its discriminative ability. Internal cross-validation revealed close approximation between the calibration plots and observed estimates for the 1-year and 2-year OS (Figure 8).
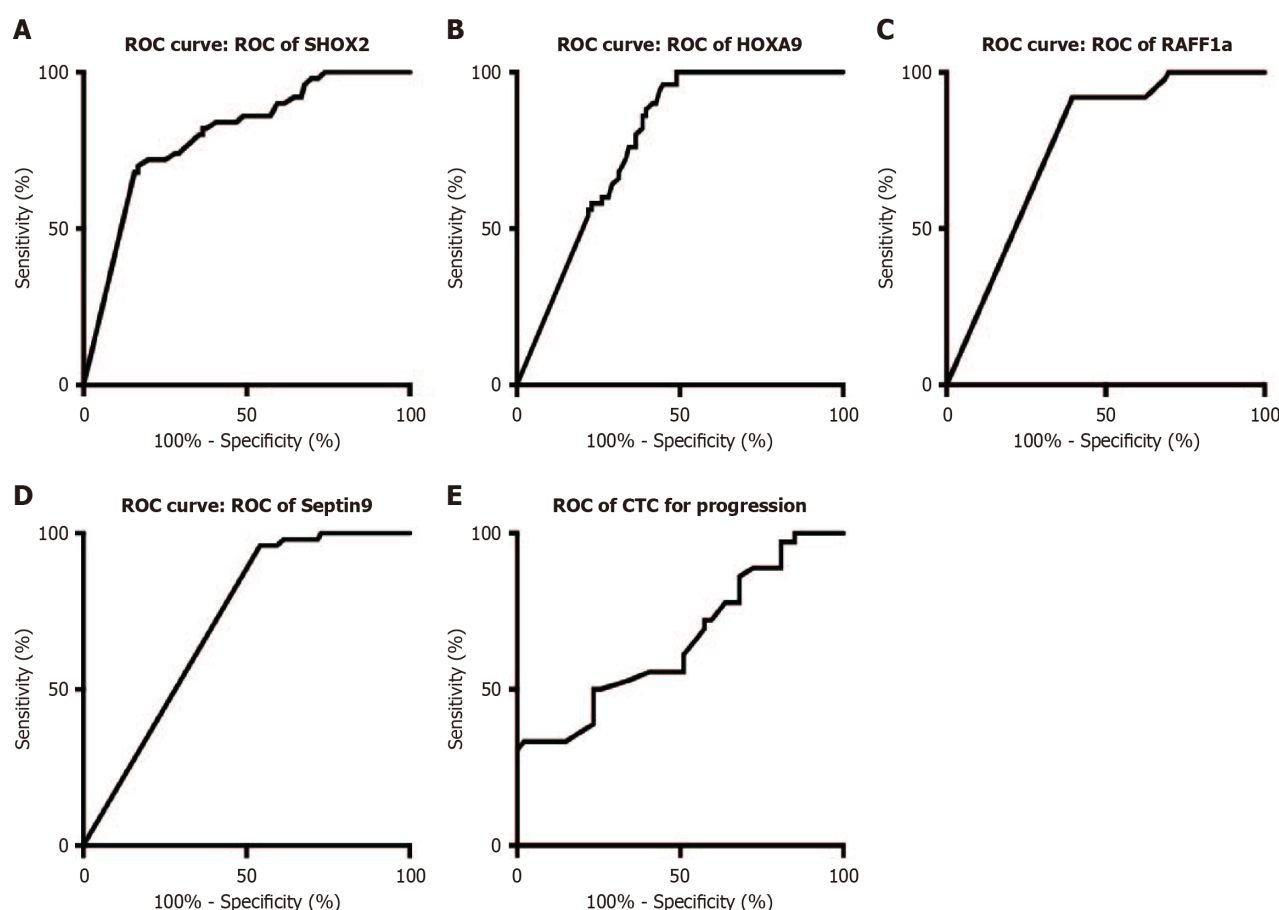
Figure 3 The receiver operating characteristic curves, calculated area under the curves, and sensitivity and specificity values of the four DNA methylation biomarkers in the plasma of patients with cholangiocarcinoma and the receiver operating characteristic curves for circulating tumor cells to assess cholangiocarcinoma progression. A: The receiver operating characteristic (ROC) of SHOX2; B: ROC of HOXA9; C: ROC of RASSF1A; D: ROC of SEPTIN9; E: ROC of circulating tumor cell for progression. The ROC curves and resulting area under the curve (AUC) for 96 patients with cholangiocarcinoma are depicted. AUC-SHOX2 = 0.799 (95%CI: 0.724-0.874), AUC-SEPTIN9 = 0.713 (95%CI: 0.632-0.795), AUC-RASSF1A = 0.765 (95%CI: 0.689-0.841), AUC-HOXA9 = 0.777 (95%CI: 0.705-0.850). AUC-circulating tumor cells = 0.653 (95%CI: 0.533-0.774). ROC: The receiver operating characteristic; CTC: Circulating tumor cell.
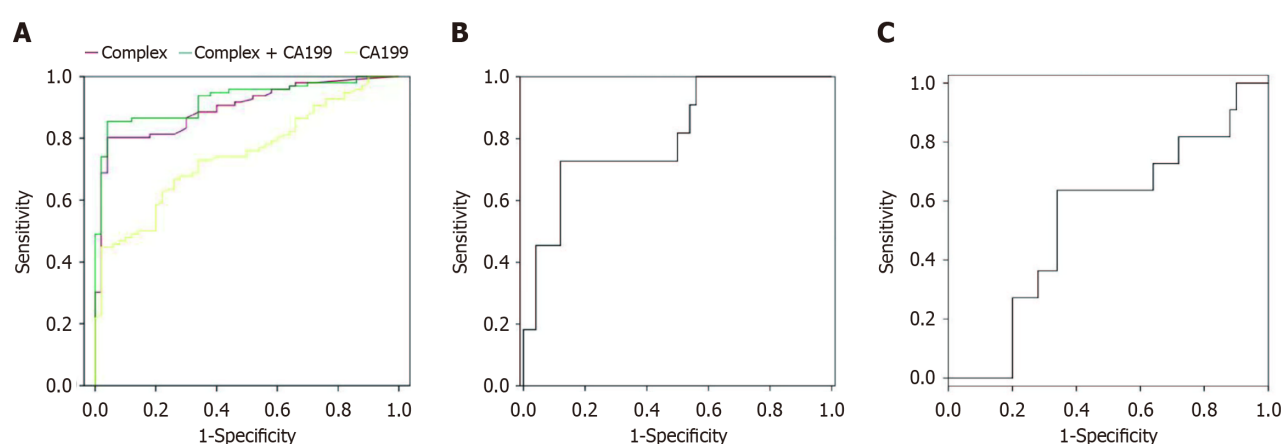
Figure 4 The receiver operating characteristic curves and resulting area under the curve values for patients with cholangiocarcinoma (*n* = 156) and early cholangiocarcinoma (*n* = 11). A: The receiver operating characteristic curves of complex methylation and carbohydrate antigen 19-9 (CA199) of the patients. area under the curve (AUC)-complex methylation = 0.828 (95%CI: 0.764-0.891), AUC-CA199 = 0.746 (95%CI: 0.666-0.826), AUC-complex methylation + CA199 = 0.851 (95%CI: 0.792-0.910); B and C: The AUC of the complex evaluation and CA199 for diagnosing early cholangiocarcinoma. AUC-complex methylation = 0.682 (95%CI: 0.654-0.710), AUC-CA199 = 0.542 (95%CI: 0.358-0.726). CA199: Carbohydrate antigen 19-9.
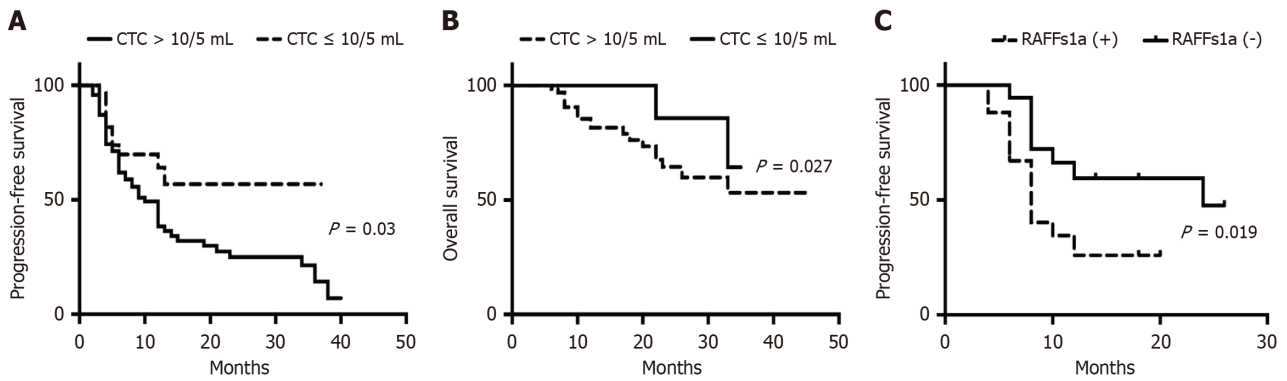
Figure 5 The relapse-free survival and overall survival curves for the initial circulating tumor cell counts of the patients with cholangio-carcinoma, and the relapse-free survival curve for RASSF1A methylation. A and B: The progression free survival and overall survival curves for the initial circulating tumor cell counts before therapy in the patients with cholangiocarcinoma; C: The progression free survival curves for the RASSF1A methylation in the patients with cholangiocarcinoma. CTC: Circulating tumor cell.
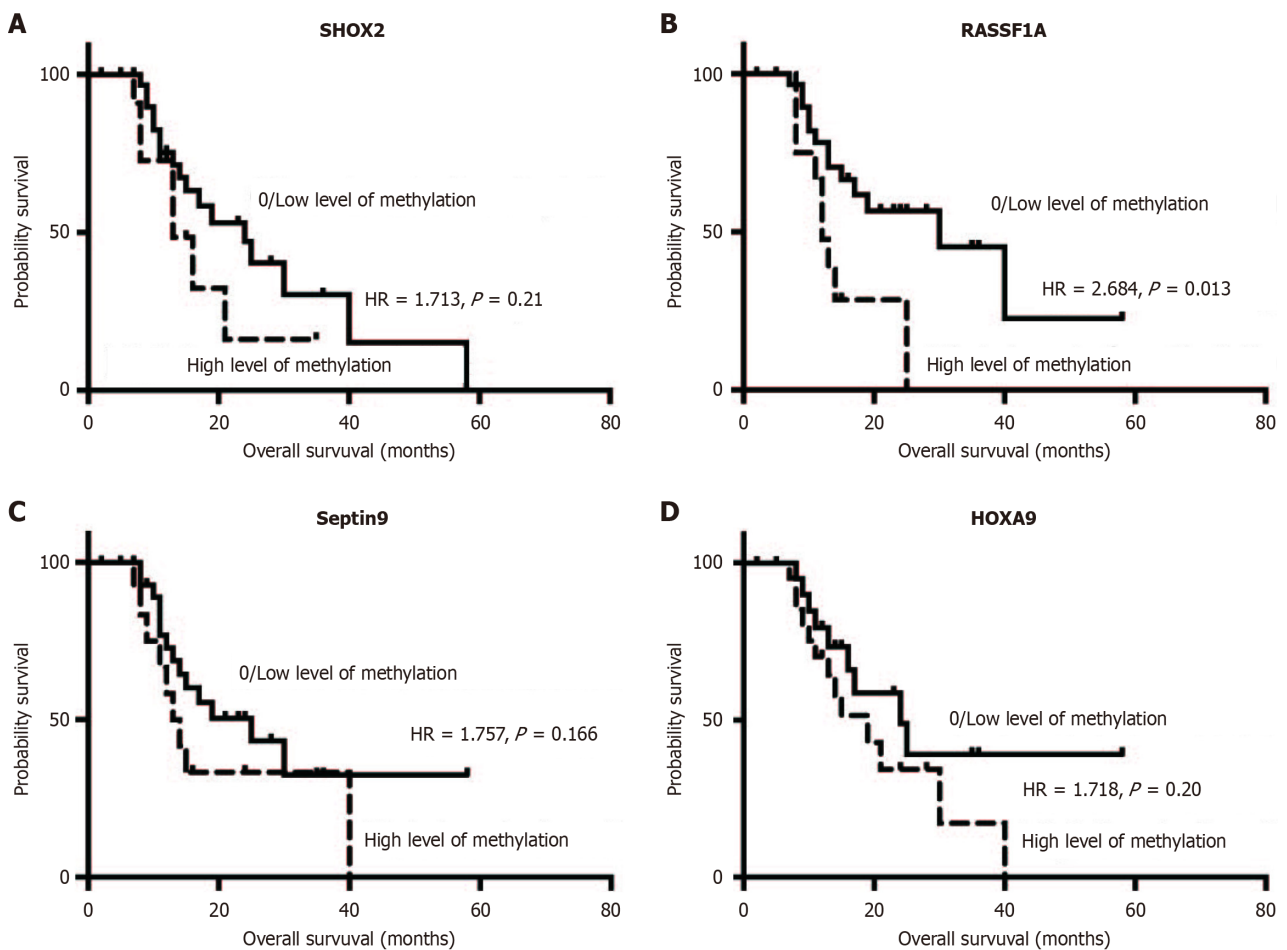
Figure 6 Kaplan-Meier curves modeling the effect of methylation on overall survival in cholangiocarcinoma. Overall survival was calculated from plasma sampling time until death from any cause, and cases were censored at last follow-up. The effect of high and low methylation levels of SHOX2, RASSF1A, SEPTIN9, and HOXA9 were evaluated using a univariate approach with the log rank method. A: SHOX2; B: RASSF1A; C: SEPTIN9; D: HOXA9. Survival curves were estimated with the Kaplan-Meier method. Group definition: The cholangiocarcinoma samples were divided into high (DCtSHOX2 ≤ 9; DCtRASSF1A ≤ 12; DCtSEPTIN9 ≤ 9; DCtHOXA9 ≤ 8) and no/low methylation levels (DCtSHOX2 > 9; DCtRASSF1A > 12; DCtSEPTIN9 > 9; DCtHOXA9 > 8). HR: Hazard ratio.

Univariate and multivariate Cox regression analyses identified tumor stage, lymphatic invasion, CTC count > 10/5 mL, and RASSF1A methylation as independent prognostic factors for CCA (Table 3). In detail, the risk estimates, expressed as hazard ratios, for tumor stage, lymphatic invasion, CA199 levels, CTC counts, and RASSF1A methylation status were 3.13 (with a 95%CI: 1.06-9.242, P value 0.039), 2.902 (95%CI: 1.069-7.874, P = 0.037), 2.41 (95%CI: 1.079-5.394, P = 0.028), 3.542 (95%CI: 1.354-9.266, P = 0.027), and 2.684 (95%CI: 1.0-7.557, P = 0.013). These results highlight the importance of

Table 3 Results of univariate and multivariate survival analyses (Cox proportional hazard models)

Variables		Number of patients	Univariate		Multivariate	
			HR (95%CI)	*P* value	HR (95%CI)	*P* value
Tumor location	(iCCA *vs* dCCA)	33	1.246 (0.495-3.138)	0.624		
Gender	(male *vs* female)	43	1.536 (0.673-3.861)	0.284		
Age at diagnosis	(≤ 60 *vs* > 60 years)	43	1.527 (0.673-3.467)	0.273		
Tumor stage	(I-II *vs* III-IV)	43	3.13 (1.06-9.242)	0.039	2.871 (1.056-7.653)	0.03
Lymphatic invasion	(V0 *vs* V1)	38	2.902 (1.069-7.874)	0.037	3.425 (1.081-8.523)	0.035
CA199	≤ 41 U/mL *vs* > 41 U/mL)	43	2.41 (1.079-5.384)	0.028		
SHOX2 methylation	(SHOX2- *vs* SHOX2+)	43	1.713 (0.623-4.713)	0.210		
SEPT9 methylation	(SEPT9- *vs* SEPT9+)	43	1.757 (0.698-4.422)	0.166		
HOXA9 methylation	(HOXA9- *vs* HOXA9+)	43	1.718 (0.743-3.975)	0.200		
RASSF1A methylation	(RASSF1A- *vs* RASSF1A+)	43	2.684 (1.0-7.557)	0.013		
CTC count > 10/5 mL	(> 10/5 mL *vs* ≤ 10/5 mL)	43	3.542 (1.354-9.266)	0.027	3.24 (1.465-7.562)	0.021

CTC: Circulating tumor cells; CA199: Carbohydrate antigen 19-9; CCA: Cholangiocarcinoma; HR: Hazard ratio.
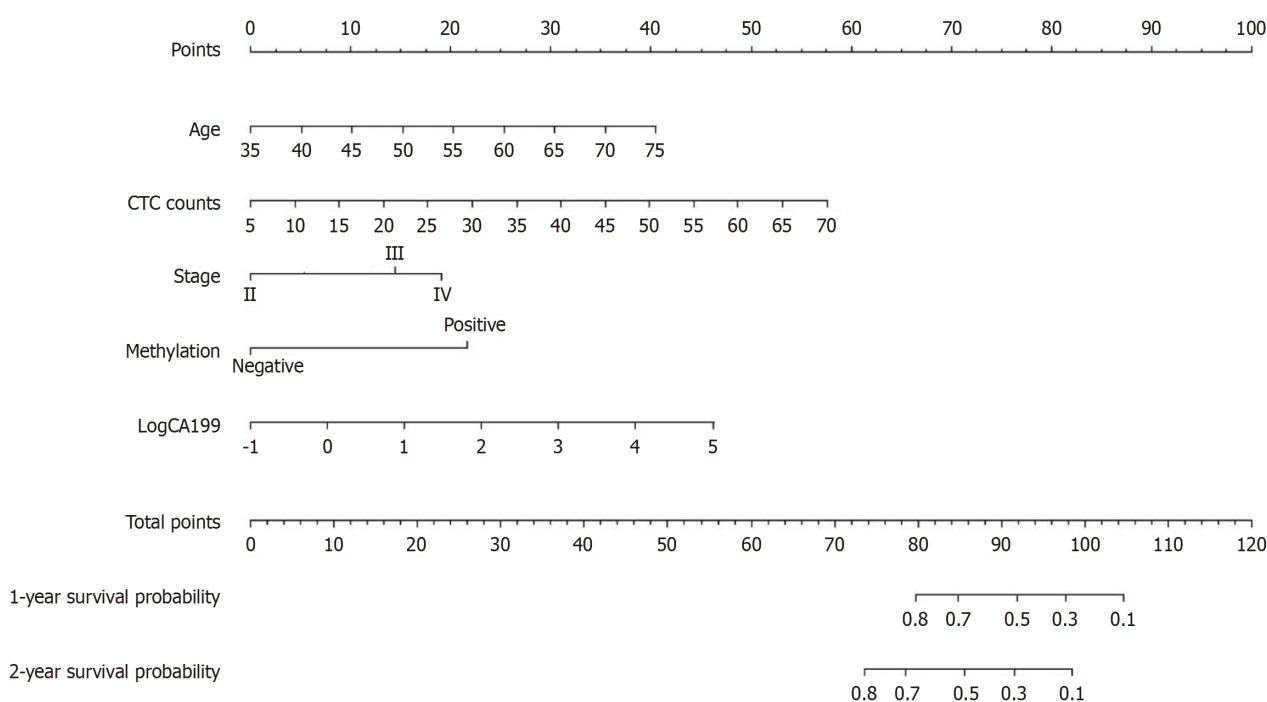
Figure 7 Nomograms to predict the diagnosis of patients with cholangiocarcinoma. The nomograms comprise the levels of carbohydrate antigen 19-9, methylation, and other significant indicators. The points total is located on the total point axis, and a vertical line is traced down to the diagnostic axes to predict the likelihood of a risk. CTC: Circulating tumor cell; CA199: Carbohydrate antigen 19-9.

combining multiple biomarkers, including plasma DNA methylations and CTCs, for comprehensive CCA management.

DISCUSSION

The significance of ctDNA methylation in clinical oncology has been increasingly recognized due to its potential to detect early-stage cancers with high sensitivity and specificity[21]. The present study demonstrates the value of plasma SHOX2, HOXA9, SEPTIN9, and RASSF1A methylation in CCA diagnosis and monitoring. These markers, together with CTCs, present a promising non-invasive approach to improve early detection and management of CCA.
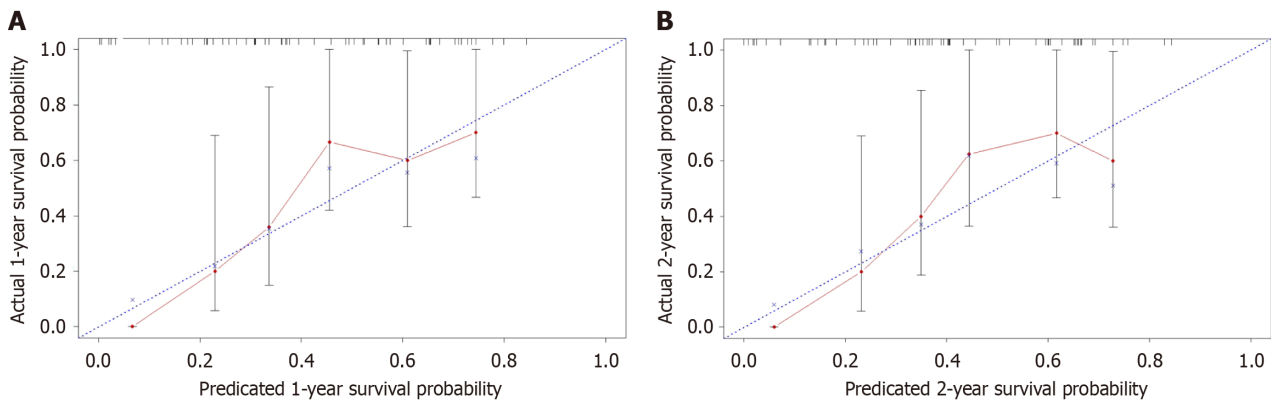
Figure 8 Internal cross-validation calibration curves at 1 and 2 years.

The high sensitivity (64.74%) and specificity (93.88%) of the combined methylation panel for detecting CCA underscore its potential as a diagnostic tool. Importantly, a detailed comparison with previous studies that used similar markers or techniques for CCA diagnosis revealed that our study achieved comparable or even superior performance. For example, Branchi *et al*[17] reported 55% sensitivity and 90% specificity using a combination of SHOX2 and SEPT9 methylation for diagnosing biliary tract cancer . With the addition of HOXA9 and RASSF1A methylation, our study reports higher sensitivity (64.74%) while maintaining a high specificity (93.88%). Moreover, the AUC of our complex evaluation (0.828) was higher than that reported by Liang *et al*[23] (0.801) for a panel of novel methylated DNA markers in pleural effusion. These comparisons highlight the advantages of our multi-marker panel in identifying early disease.

In addition to its diagnostic utility, plasma methylation potentially informs prognosis and treatment monitoring. Our results indicate that RASSF1A methylation is significantly associated with the prognosis of patients with CCA, with hypermethylated patients exhibiting poorer OS. This result aligns with previous studies demonstrating the prognostic significance of RASSF1A methylation in various cancers[13]. Furthermore, our study is unique compared to other studies that explored the prognostic value of individual methylation markers in CCA, given its comprehensive approach, where multiple markers and clinical factors were incorporated into a prognostic nomogram. For example, Peng *et al*[18] found that RASSF1A hypermethylation was associated with increased risk of hepatocellular carcinoma, but did not construct a prognostic model. Contrastingly, our nomogram, which includes age, tumor stage, CTC count, methylation status, and CA199 levels, achieved a C-index of 0.705, demonstrating its discriminative ability in predicting OS.

The clinical implications of this study extend beyond diagnosis. Due to its brief half-life, ctDNA enables immediate assessment of therapy effectiveness and tumor load, aiding precise tumor classification and early recurrence identification. The decrease in CTC counts post-surgery and their subsequent increase prior to imaging recurrence highlights the potential of CTCs as a complementary tool for monitoring disease progression. In clinical practice, integrating these biomarkers into routine diagnostics or monitoring for patients with CCA could significantly improve patient outcomes. For example, in the early diagnosis of CCA, the combined use of our methylation panel with imaging techniques, such as computed tomography or magnetic resonance imaging, could increase the detection rate of early-stage tumors. Similarly, in the prognosis assessment and monitoring of patients with advanced CCA, the combined use of our prognostic nomogram with biopsy results could provide a more comprehensive evaluation of the patient's condition and guide personalized treatment strategies.

It is also crucial to acknowledge the limitations of our statistical models, particularly regarding the potential for overfitting, which could affect the robustness and real-world applicability of our results. We mitigated this risk by using internal cross-validation and assessed the calibration of our nomogram, and identified a close approximation between the calibration plots and observed estimates for the 1-year and 2-year OS (Figure 8). Additionally, using bootstrap methods could further validate the stability and reliability of our model. Despite these efforts, future studies with larger patient cohorts and longer follow-up durations are necessary to fully evaluate the generalizability of our results. The inclusion of additional biomarkers and clinical factors, such as imaging findings and biopsy results, in our model may also enhance its diagnostic and prognostic accuracy.

The non-invasive nature of liquid biopsy techniques offers several advantages over traditional methods. First, it enhances patient compliance by eliminating the need for invasive procedures, such as biopsy. Second, the high sensitivity and specificity of the methylation panel allow earlier disease detection, which is crucial for improving outcomes in CCA, where late-stage diagnosis is common. Third, the ability to monitor disease progression and treatment response non-invasively could inform timely adjustments to treatment plans, ultimately improving patient survival.

Our study also clarifies the potential applications of these markers in specific patient populations. For example, plasma methylation markers may complement existing detection methods for high-risk groups, such as patients with primary sclerosing cholangitis, who are at an increased risk of developing CCA. Furthermore, the ability to monitor disease progression and treatment response non-invasively could inform personalized treatment strategies, particularly in patients with unresectable disease.

Despite the promising results, the present study was subject to limitations. The relatively small sample size and limited follow-up duration may have affected the robustness of our conclusions. Therefore, our results should be validated through studies with larger patient cohorts and longer follow-up periods. Additionally, a more detailed exploration of

the relationship between methylation status and OS, and the potential for overfitting in the nomogram construction, would enhance the clinical applicability of our results.

In conclusion, ctDNA contains abundant cancer cell genetic information, among which methylation variation partly reflects the presence and malignancy of cancer *in situ*. Here, we constructed a nomogram for predicting the OS of patients with CCA (model: Age + stage + CTC + methylation + CA199)**,** attaining a C-index score of 0.705 with a 95%CI: 0.605-0.805. The model could assess the clinical risk factors to predict the OS of patients with CCA. Clinicians could utilize these data to determine the most effective and tailored treatment approach for CCA patients. The non-invasive procedure and high-quality cancer information render ctDNA methylation and CTCs promising and clinically valuable liquid biopsy projects.

CONCLUSION

ctDNA contains abundant cancer cell genetic information, among which methylation variation partly reflects the presence and malignancy of cancer *in situ*. Here, we constructed a nomogram for predicting the OS of patients with CCA (model: Age + stage + CTC + methylation + CA199) with a C-index score of 0.705 with a 95%CI: 0.605-0.805. The model has the capability to evaluate clinical risk factors for forecasting the OS in CCA patients. These data could aid clinicians in selecting an optimal and customized management strategy for treating patients with CCA. The high sensitivity (64.74%) and specificity (93.88%) of the combined methylation panel for detecting CCA underscored its potential as a diagnostic tool. The methylation panel exhibited superior diagnostic performance compared to the tumor marker CA199 (AUC: 0.828 *vs* 0.746). This result highlighted the advantages of ctDNA methylation-based assays in identifying early disease, as demonstrated by the ability of the panel to detect early-stage CCA with 36.36% sensitivity and 93.83% specificity. Furthermore, RASSF1A plasma methylation and CTCs were valuable predictors for assessing CCA prognosis and recurrence. Despite the promising results presented herein, it is important to recognize that the present study has several limitations, notably a comparatively modest sample size and a restricted period of follow-up. Our results should be validated and refined through studies that involve larger patient cohorts and longer follow-up durations. Additionally, exploring the combination of more epigenetic markers and integrating them with other clinical parameters, such as imaging findings and biopsy results, may enhance the diagnostic and prognostic accuracy for CCA. Furthermore, the development of more sensitive and specific detection methods for ctDNA methylation and CTC enumeration is also crucial for improving the clinical utility of these biomarkers. Investigating the potential mechanisms underlying the methylation alterations of these genes in CCA may also provide deeper insights into its pathogenesis and progression. Ultimately, translating these results into clinical practice, such as integrating plasma methylation and CTC analysis into routine diagnostic algorithms for CCA, could significantly improve patient outcomes through earlier diagnosis, personalized treatment strategies, and more effective disease monitoring.

FOOTNOTES

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