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ORIGINAL ARTICLE

Cell-free DNA as a prognostic and predictive biomarker in resectable distal common bile duct cancer

Bor-Shiuan Shyr, Shih-Chin Chen, Yi-Ming Shyr, Shin-E Wang, Bor-Uei Shyr*

Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, and National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC

Abstract

Background: Cell-free DNA (cfDNA) as an oncological biomarker has drawn much attention in recent years, but very limited effort has been made to investigate the prognostic values of cfDNA in distal common bile duct (CBD) cancer.

Methods: Plasma cfDNA was measured in 67 patients with resectable distal CBD cancer. Survival outcomes and the correlation of cfDNA with other conventional prognostic factors were determined.

Results: cfDNA levels were significantly higher in female patients, and those with poor tumor differentiation, abnormal serum carcinoembryonic antigen (CEA) level, and stage III cancer. The significant prognostic factors included a high cfDNA level (>8955 copies/mL), abnormal serum CEA level, stage III cancer, and positive resection margins. Compared with patients with high cfDNA level, those with lower cfDNA level (≤8955 copies/mL) had significantly better overall survival outcomes (74.4% vs 100% and 19.2% vs 52.6%, for 1- and 5-year survival rates, respectively, $p = 0.001$). The cfDNA level, perineural invasion, CEA level, and radicality were identified as independent prognostic factors for distal CBD cancer after multivariate analysis.

Conclusion: Circulating cfDNA levels play a significant role in predicting the prognosis and survival outcome for resectable distal CBD cancer. Furthermore, acting as a promising liquid biopsy, cfDNA could serve as a prognostic and predictive biomarker in combination with current conventional markers to improve diagnostic and prognostic efficacy.

Keywords: Biomarker; Cell-free DNA; Distal common bile duct; Prognosis

1. INTRODUCTION

As a biomarker, circulating cell-free DNA (cfDNA) has been applied in oncological diagnosis and prognostic prediction in recent years. cfDNA is an extracellular DNA[1,2] and some studies[1,3] have reported that plasma levels of cfDNA could be higher in patients with cancer than those in healthy individuals. Cancer cell turnover rate is thought to be increasing during tumorigenesis and, therefore, resulting in more cell apoptosis and necrosis, which would be released and circulating in the bloodstream as an accumulation of cfDNA.[1,2] This finding implies that plasma levels of cfDNA would be higher in patients with cancer than in those without cancer. Given molecular characteristics, circulating cfDNA might provide some valuable information in early detection and accurate prediction of the oncological outcome for some cancers. Therefore, cfDNA could be considered as a promising prognostic and predictive biomarker in various cancers.[1,3–8]

Bile duct cancer is relatively rare compared to other periampullary cancers, such as pancreatic head and ampullary cancers. Patients with bile duct cancer tend to experience poor outcome. Therefore, it is crucial to identify potential biomarkers for early detection, prognostic prediction, and novel treatment strategies in these patients. So far, data regarding diagnosis and prognosis for bile duct cancer are limited, partly because of the rarity of the disease. Although cfDNA has been studied extensively in other cancers,[4,9–11] there is little evidence on the diagnostic or prognostic values of cfDNA in resectable distal common bile duct (CBD) cancer, especially in comparison to traditional serum protein tumor markers, such as carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA).

This study was conducted to measure plasma cfDNA in patients with resectable distal CBD cancer undergoing pancreaticoduodenectomy. The aim was to clarify the role of circulating cfDNA as a prognostic and predictive biomarker and correlation between cfDNA and clinicopathological factors in resectable distal CBD cancer. The prognostic factors for resectable distal CBD cancer were determined, and the impact of cfDNA levels on survival outcomes was further evaluated using univariate and multivariate analyses.

2. METHODS

2.1. Patient selection

Patients with pathologically confirmed distal CBD adenocarcinoma at our hospital from March of 2003 to July of 2021 were included in this study. Data were collected prospectively and kept in a computer database. Only patients undergoing resection with pancreaticoduodenectomy were included, and those receiving biopsy or bypass surgery were excluded. All surgical procedures were performed with the same technique by the same team led by an experienced surgeon. The surgical technique was previously described in

* *Address correspondence. Dr Bor-Uei Shyr, Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC. E-mail address: punkid830@gmail.com (B.-U. Shyr).*

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detail.[12–14] Standard lymph node dissection was performed around the common hepatic artery, head of the pancreas, superior mesenteric vein, and hepatoduodenal ligament. Chemotherapy was routinely considered, except for stage 1 with well-differentiated CBD cancer; radiotherapy was performed in those with palliative resections. The study was approved by the Institutional Review Board (IRB) of the Taipei Veterans General Hospital (IRB-TPEVGH NO.: 2021-08-008CC). This study was conducted in accordance with the guidelines and regulations of the IRB. Informed consent was waived for this retrospective cohort study with data anonymity.

The primary study aim was to clarify the role of plasma cfDNA as a prognostic and predictive biomarker in resectable distal CBD cancer by determining survival outcomes. The secondary study aim was to evaluate the correlation between plasma cfDNA and other conventional prognostic factors, including tumor size, lymph node involvement, tumor cell differentiation, lymphovascular invasion, perineural invasion, serum CEA level, serum CA 19-9, tumor stage, and radicality of the resection.

2.2. cfDNA Quantification

After obtaining written informed consent, patient blood samples were collected before surgical resection and stored in the anonymous biobank at our hospital. Details of the measurement of serum cfDNA levels were described previously.[1,3] Briefly, according to the manufacturer's instructions, the serum cfDNA copy number was quantified by a quantitative polymerase chain reaction (qPCR) assay. The cfDNA from each biobank plasma sample was isolated and purified using a commercial QIAamp DNA Tissue Kit and MinElute Virus Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quality and quantity of plasma DNA were evaluated using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A TaqMan qPCR assay (Thermo Fisher Scientific, Waltham, MA, USA) of the housekeeping gene cyclophilin, which is known to be correlated with cancer and commercially available at our institute, was used to quantify the cfDNA copy numbers in the plasma samples. qPCR was performed using TaKaRa Ex Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The sequence of cyclophilin primers were as follows: forward ACATGGGTACTAAGCAACAAAATAAG and reverse CACAATTGGAACATCTTTGTTAAAC. The probe primer was Fam-TTGCAGACAAGGTCCCAAAGACAGCA-Tamra. To reduce the batch effect, small aliquots of the pooled samples in standard tubes (1 mL) and a large volume tube of pre-mixed plasma samples were prepared and stored at −80 °C. Blood samples stored in the biobank from patients and the pre-mix standard tubes were used when measuring cfDNA. The cfDNA copy numbers from each patient were measured based on the threshold cycle (Ct) value and the standard curve.

As the control group, serum cfDNA levels of 90 healthy volunteers were also measured, including 65 males and 25 females, with a mean age 53.1 ± 15.2 years. These healthy volunteers were recruited from individuals being seen for a regular health check-up at least 2 years before this study and followed up for at least 2 years. These healthy individuals had no previous and subsequent malignant diseases for at least two years after blood sampling. The cfDNA assays of these healthy volunteers and patients with cancer were not performed simultaneously because it was impossible to collect their blood samples at the same time but used the same standardized methods.

2.3. Statistical analysis

Statistical analyses were carried out using Statistical Product and Service Solutions (SPSS) version 26.0 software (SPSS Inc., IBM, Armonk, NY, USA). Categorical variables were compared by χ^2 or Fisher's exact test. Continuous variables were compared by two-tailed Student's *t*-test. The Wilcoxon rank-sum test was used for continuous variables without a normal distribution. All continuous data are presented as mean (standard deviation), median and range. The cutoff value to define low and high cfDNA was based on the median cfDNA to avoid a too small sample size in one arm for analysis. Cumulative survival rates were estimated by the Kaplan-Meier method. The log-rank test was used to compare differences between the survival curves. Variables with *p* values <0.2 by univariate analysis were included for multivariate analysis with a Cox proportional hazards regression model. A *p* value <0.05 was considered statistically significant.

3. RESULTS

A total of 67 patients with resectable distal CBD cancer undergoing pancreaticoduodenectomy were included in this study. The median follow-up time was 51 months. Plasma cfDNA levels were measured in all patients as the study group, with a median of 8955 copies/mL, ranging from 2109 to 28 489 copies/mL, and a mean of 10 019 ± 4581 copies/mL. The median value, 8955 copies/mL, was used as the cutoff level for statistical analysis in this study (≤8955 copies/mL was considered the low-level group and >8955 copies/mL the high-level group). Plasma cfDNA levels were measured in 90 healthy individuals as the control group, with a median of 547 copies/mL, ranging from 0 to 2438 copies/mL, and a mean of 819 ± 693 copies/mL (Fig. 1).

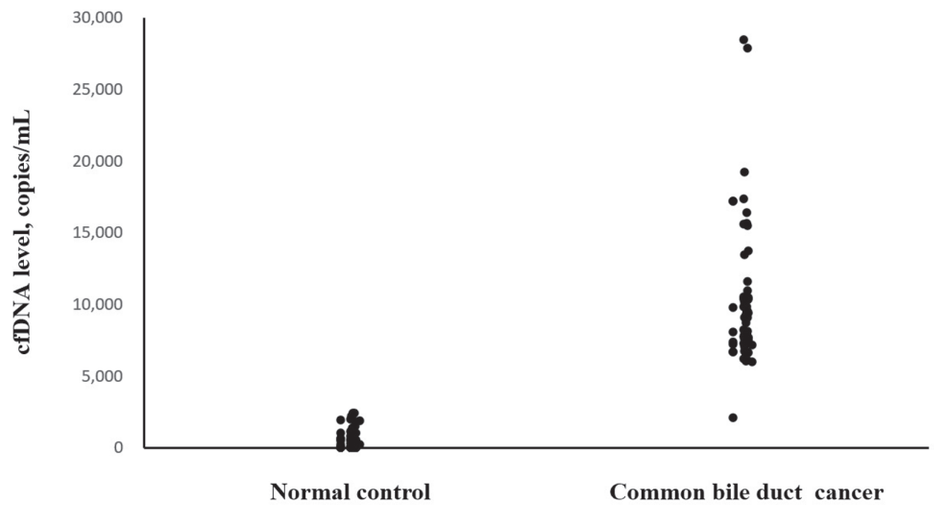

Fig. 1 The scatterplot for healthy volunteers and common bile duct cancer.

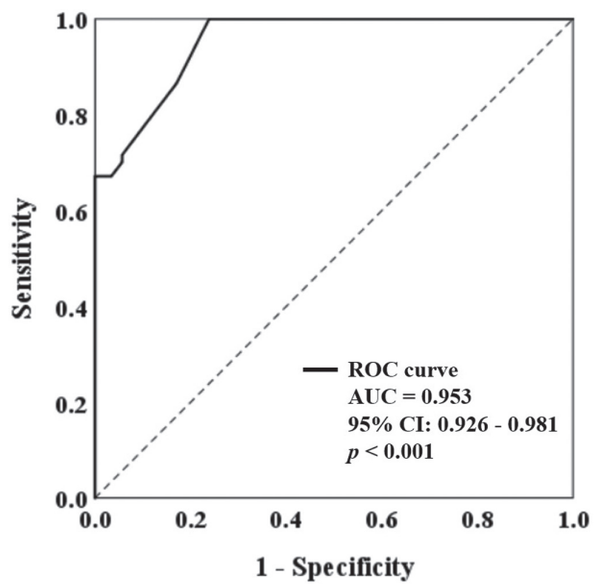

Fig. 2 ROC curve analysis for discriminating distal common bile duct cancer from healthy volunteers using plasma cfDNA levels. Plasma cfDNA level yielded an AUC of 0.953, with a 95% CI of 0.926-0.981 in discriminating the distal common bile duct cancer from healthy controls. AUC = area under the curve; cfDNA = cell-free deoxyribonucleic acid; CI = confidence interval; ROC = receiver operating characteristics.

Plasma cfDNA levels were significantly different between study and control groups ($p < 0.001$). The receiver operating characteristic (ROC) curve is shown in Fig. 2. The ROC curve is a widely used method to determine a diagnostic test's accuracy and a cutoff point for the disease in question. The cutoff point of the ROC curve is 1278 copies/mL, meaning this value can classify whether the patient has the disease with the highest sensitivity and specificity.

The circulating plasma cfDNA levels were significantly higher in female patients (median: 9515 vs 8154 copies/mL in male patients, $p = 0.040$), poor tumor cell differentiation (median: 13 605 vs 8732 copies/mL for well and moderate tumor cell differentiation, $p < 0.001$), abnormal serum CEA level (median: 8489 vs 17 182 copies/mL for normal CEA levels, $p = 0.011$), and stage III cancer (median: 10 552 vs 8489 copies/mL for stage I and II, $p = 0.014$) (Table 1). Otherwise, the cfDNA level had no correlation with age, tumor size, lymph node involvement, lymphovascular invasion, perineural invasion, serum CA 19-9, and radicality of resection.

Table 2 shows the prognostic analysis for distal CBD cancer after pancreaticoduodenectomy. The univariate analysis revealed that significant poor prognostic factors included: (1) cfDNA level > 8955 copied/mL, $p = 0.001$; (2) Abnormal CEA level > 5 ng/mL, $p = 0.013$; (3) Stage III cancer, $p = 0.007$; and (4) Positive resection margins, including R1 and R2 resection, $p = 0.026$.

The Cox proportional hazards regression model for multivariate analysis identified cfDNA level, perineural invasion, CEA level, and radicality of resection as independent prognostic

Table 1

Circulating cell-free DNA level in patients with distal common bile duct cancer undergoing pancreaticoduodenectomy

Variable	Mean ± SD	Median	Range	*p*
Total, n = 67	10019 ± 4581	8955	2109–28 489	
Age, y/o				0.667
≤65, n = 25	9703 ± 4894	8006	2109–27 867	
>65, n = 42	10207 ± 4435	9160	6060–28 489	
Sex				0.040
Male, n = 52	9403 ± 3931	8154	2109–27 867	
Female, n = 15	12 153 ± 6030	9515	6703–28 489	
Tumor size, cm				0.210
≤2, n = 40	9439 ± 2772	8909	6060–17 182	
>2, n = 27	10 877 ± 6361	8955	2109–28 489	
Lymph node involvement				0.610
Negative, n = 42	9796 ± 4384	8489	6003–27 867	
Positive, n = 25	10 392 ± 4966	9509	2109–28 489	
Tumor cell differentiation				<0.001
Well and moderate, n = 57	9153 ± 2937	8732	2109–17 376	
Poor, n = 10	14 955 ± 8271	13605	6060–28 489	
Lymphovascular invasion (n = 66)				0.320
Negative, n = 46	9686 ± 4406	8489	2109–27 867	
Positive, n = 20	10 921 ± 5040	10104	6677–28 489	
Perineural invasion (n=66)				0.714
Negative, n = 19	10 391 ± 5333	8955	6003–27 867	
Positive, n = 47	9926 ± 4331	9096	2109–28 489	
CEA, ng/mL (n=61)				0.011
Normal ≤5, n = 58	9698 ± 4534	8489	2109–28 489	
Abnormal >5, n = 3	16 707 ± 2769	17182	13 731–19 207	
CA 19-9, U/mL (n = 65)				0.174
Normal ≤37, n = 26	8956 ± 3493	7957	2109–17 376	
Abnormal >37, n = 39	10 544 ± 5147	9255	6060–28 489	
Stage				0.014
I and II, n = 64	9724 ± 4103	8489	2109–28 489	
III, n = 3	16 303 ± 10 014	10552	10 491–27 867	
Radicality of resection				0.174
R0, n = 59	10 300 ± 4795	9085	2109–28 489	
R1 and R2, n = 8	7946 ± 1334	7448	6677–10 366	

CA 19-9 = carbohydrate antigen 19-9; CEA = carcinoembryonic antigen.

Table 2

Prognostic factors by univariate analysis for the patients with resectable distal common bile duct cancer undergoing pancreaticoduodenectomy

	Survival time, mo					
Prognostic factors	**Mean ± SD**	**Median**	**Range**	**1-y survival, %**	**5-y survival, %**	***p***
Total, n = 67	70.4 ± 8.7	45.2	1.8–148.7	87.6	37.8	0.001
cfDNA level, copies/mL						0.001
Low (≤ 8955), n = 34	89.0 ± 11.8	70.5	8.4–148.7	100.0	52.6	
High (> 8955), n = 33	46.4 ± 11.2	22.3	1.8–145.9	74.4	19.2	
Age, y/o						0.796
≤65, n = 25	39.1 ± 36.7	29.8	8.4–148.7	100.0	52.6	
>65, n = 42	37.2 ± 30.5	30.5	1.8–145.9	74.4	19.2	
Sex						0.962
Male, n = 52	38.2 ± 32.5	28.4	1.8–148.7	86.7	45.5	
Female, n = 15	37.1 ± 34.4	31.3	4.9–110.5	87.9	33.9	
Tumor size						0.898
≤2, n = 40	67.7 ± 10.5	45.2	1.8–148.7	89.8	35.7	
>2, n = 27	57.7 ± 9.3	45.8	4.9–101.9	84.2	41.7	
Lymph node involvement						0.111
Negative, n = 42	76.7 ± 11.0	49.3	4.9–148.7	90.1	42.1	
Positive, n = 25	51.6 ± 9.8	26.7	1.8–110.3	83.5	34.2	
Tumor cell differentiation						0.068
Well and moderate, n = 57	74.2 ± 9.6	47.4	1.8–148.7	89.2	40.9	
Poor, n = 10	35.9 ± 13.1	23.0	4.9–112.9	77.1	15.4	
Lymphovascular invasion						0.297
Negative, n = 46	75.7 ± 10.2	49.3	1.8–148.7	84.5	42.4	
Positive, n = 20	37.7 ± 5.8	36.6	5.9–67.0	94.1	27.2	
Perineural invasion						0.084
Negative, n = 19	94.6 ± 15.2	44.9	8.6–148.7	94.4	53.0	
Positive, n = 47	51.5 ± 7.2	38.1	1.8–112.9	84.5	32.4	
CEA, ng/mL						0.013
Normal ≤5, n = 58	79.5 ± 9.7	45.8	1.8–148.7	89.2	42.5	
Abnormal >5, n = 3	20.0 ± 12.6	10.2	4.9–44.9	33.3	0.0	
CA 19-9, U/mL						0.184
Normal ≤37, n = 26	82.5 ± 15.1	70.5	8.6–148.7	95.8	51.3	
Abnormal >37, n = 39	65.8 ± 10.7	44.9	1.8–145.9	81.3	32.6	
Stage						0.007
I and II, n = 64	72.2 ± 8.9	30.6	1.8–148.7	90.4	39.0	
III, n = 3	12.4 ± 1.6	11.1	9.8–16.2	33.3	0.0	
Radicality of resection						0.026
R0, n = 59	77.5 ± 9.8	29.9	1.8–148.7	87.6	44.6	
R1 and R2, n = 8	30.6 ± 4.6	28.2	8.6–45.8	87.5	0.0	

CA 19-9 = carbohydrate antigen 19-9; CEA = carcinoembryonic antigen; cfDNA = cell-free deoxyribonucleic acid.

factors for distal CBD cancer (Fig. 3). Patients with lower cfDNA level (≤8955 copies/mL) had significantly better overall survival outcomes (100% and 52.6% for 1- and 5-year survival rates, respectively), as compared with those with high cfDNA levels (>8955 copies/mL) (74.4% and 19.2% for 1- and 5-year survival rates, respectively), $p = 0.001$ (Fig. 4).

4. DISCUSSION

Traditionally, the most reliable prognostic predictors for distal CBD cancer are perineural invasion, tumor cell differentiation, lymph node involvement, cancer staging, and radicality of resection.[15,16] Furthermore, CEA and CA19-9 are the most well-known tumor markers for pancreaticobiliary malignancies. However, application of these two serum tumor markers in clinical practice is limited due to a lack of sensitivity and specificity. Recently, cfDNA has been used as a liquid biopsy in several malignancies and has drawn much attention because of its potential application in early diagnosis, prediction of survival outcomes, and monitoring of disease progression.[4,17] Thus, the potential for cfDNA as a liquid biopsy in distal CBD cancer is of interest.

Plasma cfDNA, first reported in 1948,[17] is thought to be released into the bloodstream through cell necrosis or apoptosis, and is usually detected as double-stranded DNA fragments of 150 to 200 base pairs.[4,17] Circulating cfDNA levels can be detected in patients with malignancy, and cfDNA levels are quantitatively correlated with prognosis and tumor burden.[17] As a part of metabolism, cfDNA could also be actively released from normal cells; nevertheless, 4–40 times greater levels can often be measured in patients with cancer.[18–21] Therefore, measurement of circulating cfDNA can potentially provide a less invasive approach in making a diagnosis of malignancy, monitoring tumor progression, and predicting prognosis of malignancies.[17] There are various techniques for quantification of the cfDNA level, with their advantages and disadvantages. Of these, real-time PCR is most widely used for cfDNA quantification, and the cyclophilin gene is one of the most suitable housekeeping genes analyzed for expression studies.[22,23] Therefore, the qPCR assay of the housekeeping gene cyclophilin was used to measure cfDNA in this study. The only available kits at our institute are commercial QIAamp DNA Tissue Kit and MinElute Virus Kit (Qiagen), although these are not the usual method for extracting cfDNA.

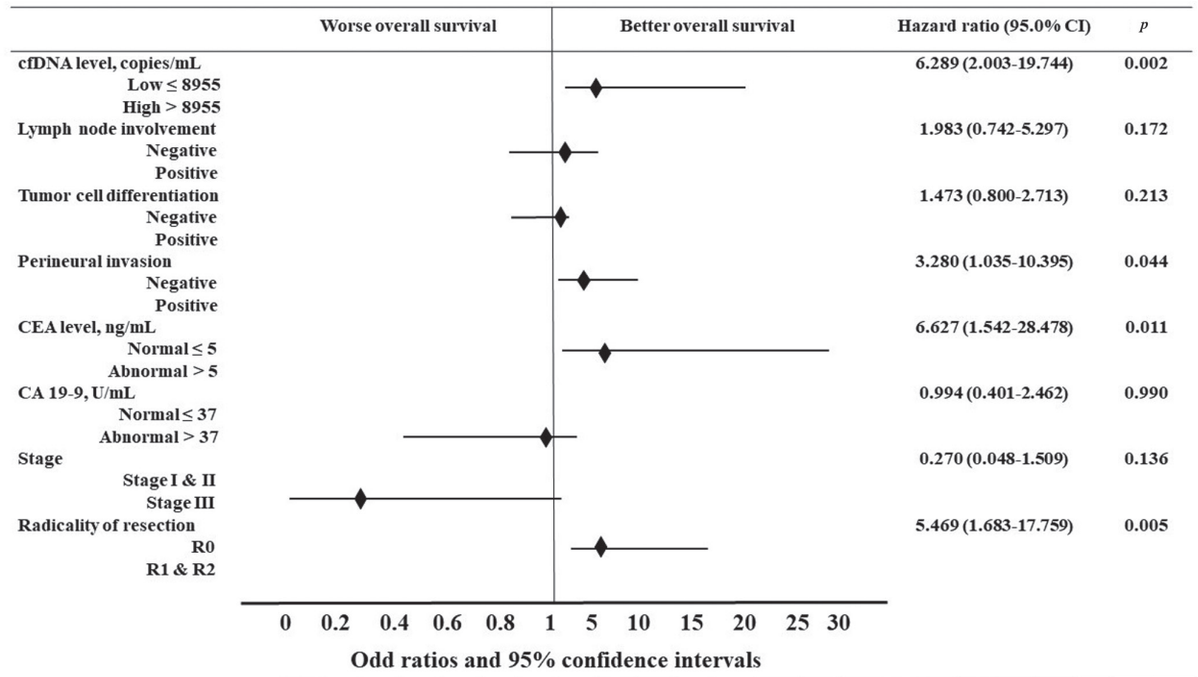

Fig. 3 Forrest plot for multivariate analysis of the independent prognostic factors for resectable distal common bile duct cancer undergoing pancreaticoduodenectomy. Variables with *p* value <0.2 in the univariate analysis were included for multivariate analysis. The independent prognostic factors were identified by multivariate analysis with Cox proportional hazards regression model.

Besides cfDNA, circulating tumor DNA (ctDNA), circulating tumor cell (CTC), and microRNA have also been suggested as potential liquid biopsies.[17] Ideally, either ctDNA or CTC would be more specific as a biomarker for tumor detection. Theoretically, ctDNA would provide better detection of the specific cancer under investigation because ctDNA could only be released from the genomes of cancer cells and not from normal cells. Although the fractions of ctDNA tend to parallel tumor burden, detection of ctDNA could be very challenging because plasma levels of ctDNA from tumor-specific mutations might be too low to be detected accurately.[4,17] CTCs have the same limitation of low plasma concentrations, typically less than 10 CTCs/mL.[17,24] Therefore, the low concentrations of ctDNA and CTCs would lower the sensitivity in detecting cancer and limit their clinical application.[25,26] In contrast, measurement of circulating cfDNA could be clinically feasible and practical as a liquid biopsy for distal CBD cancer.

Although cfDNA has been studied extensively in other cancers,[4,9–11] to our knowledge, so far there is no study investigating the diagnostic or prognostic value of cfDNA in resectable CBD cancer. In a study of the influence of fetal sex and maternal characteristics on fetal cfDNA in maternal plasma, Zhao et al[27] found that concentration of female fetal cfDNA was higher than male fetal cfDNA (mean fetal cfDNA, 13.07% vs 8.37%, $p < 0.0001$). Our study also showed that cfDNA levels were significantly higher in female patients. Given that there are no similar reports regarding sex discrepancies in plasma cfDNA for patients with cancer, adequate sample sizes for reliable clinical investigation are of utmost importance.

In this study, advanced stage III was associated with higher levels of cfDNA, as compared with early stage I and II. This association implied that serum levels of cfDNA may reflect tumor burden. Moreover, levels of cfDNA were also higher in those with poor differentiation of tumor cells and abnormal serum CEA levels. These findings suggest that serum cfDNA could be a surrogate marker of biological behavior for CBD cancer. In addition to stage and radicality of resection, cfDNA level remained as an independent prognostic predictor after multivariate analysis. Therefore, serum cfDNA could act as a prognostic and predictive biomarker for distal CBD cancer.

There are some limitations to this retrospective and single-center study with a limited sample size. Several variables exist in quantifying cfDNA, including blood sampling, time to processing, sample quality, duration, and storage temperature. Since distal CBD cancer is rare, it would be impossible to complete this study within a short period of time without using archived blood samples from our biobank. Inevitably, the time of collection and the sensitivity and specificity of the cyclophilin gene could affect the results. Measurement of cfDNA is sensitive to the presence of cfDNA degradation, genomic DNA contamination from lysed cells in poorly manipulated samples, and even enzymatic inhibitors. Since it was impossible to collect blood samples from our healthy volunteers and patients with cancer at the same time, and thus measurements of the plasma cfDNA levels were not performed simultaneously, technical error are inevitable. Measurement of cfDNA might not be specific as a cancer biomarker because it could also increase with inflammation and other conditions. Thus, correlation between cfDNA and prognosis with a limited number of patients may not have direct implications for patients. Since the presence of cfDNA could elicit an immune response, the levels of cfDNA should be taken in tandem with other such conditions in patients with CBD cancer. These limitations should be taken into consideration when interpreting plasma cfDNA. Due to the limitations of this retrospective study, the timing of tumor recurrence could not be exactly noted for all patients, and therefore, the predicting probability of cfDNA in tumor recurrence and recurrence-free survival is not available. The postoperative level of plasma cfDNA are also not available to assess their impact on prognosis.

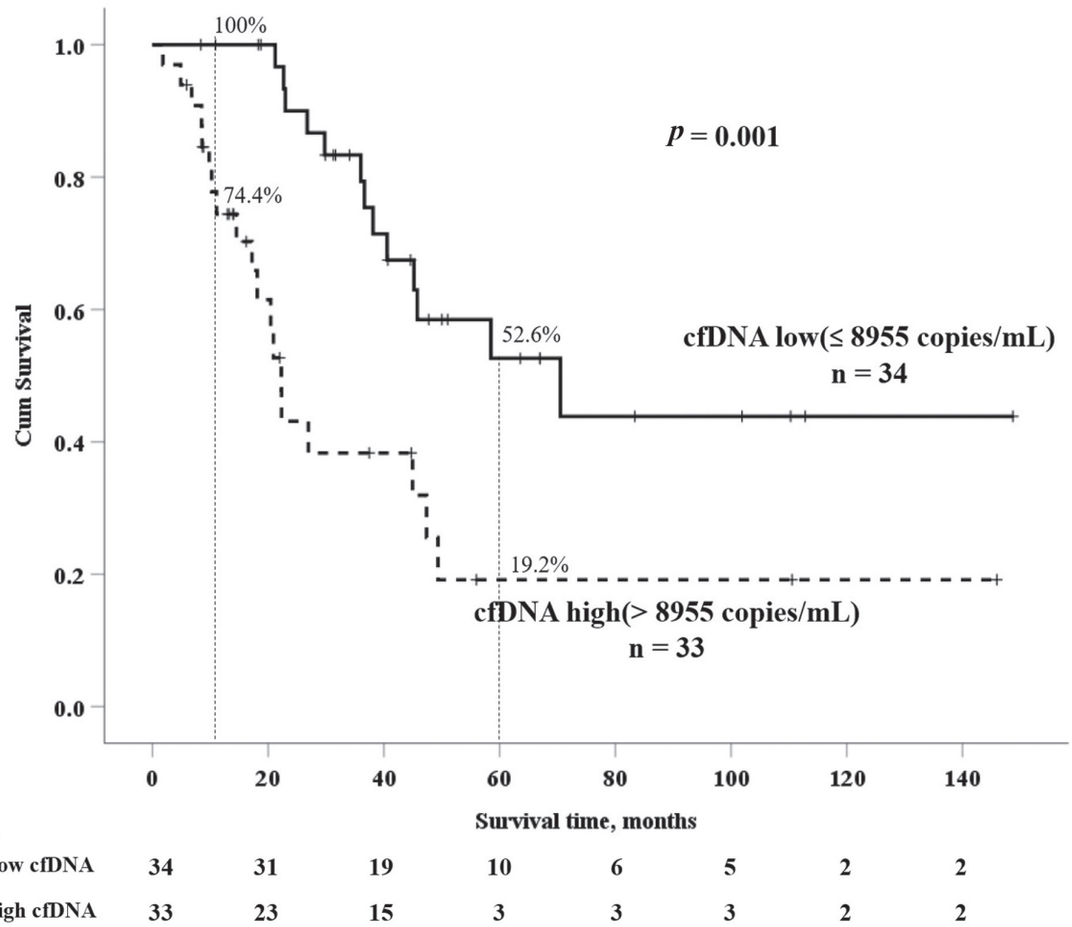

Fig. 4 Overall survival rates comparing patients with low cfDNA levels (≤8955 copies/mL) and those with high cfDNA (>8955 copies/mL) levels. cfDNA = cell-free deoxyribonucleic acid.

In conclusion, circulating cfDNA levels could predict tumor burden and biological behavior in resectable distal CBD cancer and play a significant role in predicting the prognosis and survival outcomes of this disease. Furthermore, acting as a promising liquid biopsy, cfDNA could serve as a prognostic and predictive biomarker to improve diagnostic and prognostic efficacy. A comparative analysis between the amounts of cfDNA and ctDNA or CTCs would substantiate the use of cfDNA in the future.

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