RESEARCH ARTICLE

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Study of the Role of Circulating Cell-Free DNA in the Diagnosis of Cholangiocarcinoma

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Abstract

Background: Cholangiocarcinoma (CCA) is an aggressive biliary malignancy usually diagnosed at advanced stages because early symptoms are vague. Conventional serum markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), have limited sensitivity and specificity. Circulating cell-free DNA (cfDNA), particularly ALU-115, ALU-247 and DNA integrity index, may provide improved diagnostic accuracy by reflecting tumor-derived DNA fragmentation. **Methods:** A case-control study enrolled 160 subjects divided into four groups, each comprising 40 individuals: CCA (Group I), other hepatobiliary malignancies including hepatocellular carcinoma and pancreatic head cancer (Group II), benign biliary disorders (Group III), and healthy controls (Group IV). Clinical data, biochemical parameters, tumor markers, and cfDNA fragments were assessed. cfDNA was quantified by real-time PCR using ALU-115 (short fragments) and ALU-247 (long fragments). The DNA integrity index was calculated. Receiver operating characteristic (ROC) curves were used to determine diagnostic performance. **Results:** Serum CEA and CA19-9 were significantly higher in Groups I and II compared with Groups III and IV ($p < 0.001$), but they showed limited discrimination between CCA and other malignancies. cfDNA markers demonstrated significantly higher values in CCA than in other groups ($p < 0.001$). Against Group II, ALU-247 >800 copies/ml yielded 80% sensitivity and 75% specificity, while DNA integrity >0.89 achieved 70% sensitivity and 90% specificity. Against Group III, ALU-115 >700 copies/ml showed 85% sensitivity and 90% specificity. Versus controls, an ALU-115 >580 copies/ml reached 95% sensitivity and 95% specificity. **Conclusion:** cfDNA concentrations and integrity indices outperform CEA and CA19-9 for distinguishing CCA from other malignancies, benign biliary disease, and healthy subjects. These findings support the incorporation of cfDNA-based assays as minimally invasive diagnostic adjuncts for CCA pending larger validation studies.

Keywords: Cholangiocarcinoma- Cell-Free DNA- ALU 115- ALU 247- DNA Integrity- CEA- CA 19-9

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Introduction

Cholangiocarcinoma (CCA) arises from the biliary epithelium and represents the second most frequent primary hepatic malignancy, with increasing incidence worldwide [1]. Major risk factors include chronic liver disease, biliary and gallbladder inflammation, smoking, advanced age, and liver fluke infection particularly *Opisthorchis viverrini* [1]. CCA carries poor outcomes, with 5-year overall survival between 7–20%, largely because early symptoms are absent and diagnosis is usually delayed [2, 3]. Early detection may improve survival [4].

Histological biopsy remains the diagnostic standard, but anatomical complexity, small tumor size, and procedural risks limit sampling, making ERCP suboptimal for routine diagnosis or monitoring [5]. Molecular studies have identified genetic alterations including *PIK3CA, ERBB2, IDH1*, and *FGFR*, offering targeted therapy options in selected cases [5]. Serum CA19-9 and CEA have low sensitivity and specificity, particularly in early disease, and are often elevated in benign cholestasis, reducing their diagnostic value for CCA [6]. Thus, minimally invasive markers with higher accuracy are needed. Circulating cell-free DNA (cfDNA) from apoptotic and necrotic cells has emerged as a potential "liquid biopsy," providing prognostic utility in various cancers [7].

Quantification of fragment size, particularly short (ALU-115) and long repeats (ALU-247), allows calculation of DNA integrity index, which may help

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discriminate malignant from non-malignant conditions [8]. This study evaluates the diagnostic performance of cfDNA in CCA versus malignant, benign biliary disorders, and healthy subjects.

Materials and Methods

This case-control study included 160 participants (95 males, 59.4% and 65 females, 40.6%) classified into four equal groups (n=40 each): Group I, cholangiocarcinoma (CCA); Group II, other hepatobiliary malignancies including hepatocellular carcinoma (n=24) and pancreatic head cancer (n=16); Group III, benign biliary disorders (choledocholithiasis); and Group IV, healthy controls free from clinically confirmed neoplasms, autoimmune diseases, or active inflammatory conditions at the time of sampling. Participants were recruited from Menoufia Liver Institute between September 2022 and December 2023. Written informed consent was obtained from all subjects. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Menoufia University (IRB 5/2022TROP11) and conducted in accordance with the Declaration of Helsinki.

All participants underwent detailed history taking (including DM, HTN, malignancy history, and medications) and thorough clinical examination. Laboratory investigations included complete blood count (CBC), liver and renal function tests (bilirubin, AST, ALT, albumin, urea, creatinine), coagulation profile (PT, PTT, INR), tumor markers (CA 19-9 and CEA), and circulating cfDNA quantification (ALU-115 and ALU-247). Radiological evaluation included abdominal ultrasonography, CT abdomen, and magnetic resonance cholangiopancreatography (MRCP). Esophagogastroduodenoscopy (EGD) and endoscopic retrograde cholangiopancreatography (ERCP) were performed when indicated. Histopathological examination confirmed the diagnoses.

Blood (8 mL) was collected from each participant; 3 mL in EDTA tubes for DNA extraction and plasma storage at −20°C, and 5 mL allowed to clot for biochemical and tumor marker analysis. CEA and CA 19-9 were measured by electrochemiluminescence (Cobas 6000 e601), and liver and kidney function by Cobas 6000 c501 (Hitachi, Tokyo, Japan).

cfDNA was quantified by real-time PCR of ALU115 and ALU247 fragments, with total cfDNA from ALU115 and DNA integrity index as ALU247/ALU115. Primers: ALU115 (F: 5′-CCTGAGGTCAGGAGTTCGAG-3′, R: 5′-CCCGAGTAGCTGGGATTACA-3′) and ALU247 (F: 5′-GTGGCTCACGCCTGTAATC-3′, R: 5′-CAGGCTGGAGTGCAGTGG-3′). PCR was in 20 µL reactions with SYBR Green on QuantStudio 5 (Applied Biosystems) using 95°C for 10 min, then 35 cycles of 95°C for 10 s and 64°C for 1 min. Quantification used a standard curve from serial dilutions of TaqMan® Control Human Genomic DNA Standard. Negative controls were included; cfDNA was expressed as copies/ml [9].

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA, 2018). Qualitative variables are presented as number and percentage, quantitative variables as mean ± SD. Group comparisons used one-way ANOVA with Tukey's post-hoc test and chi-square for categorical data. Diagnostic performance was assessed by ROC curves, and correlations by Spearman's rank test. P-values < 0.05 were considered significant, < 0.001 highly significant.

Results

No significant differences were observed for age or sex (P > 0.05). Diabetes was more frequent in Group II than Groups I and III (65% vs. 40% and 35%, P = 0.016), while hypertension was similar (Table 1). Anorexia and weight loss occurred in 90–95% of Groups I and II but were absent in Group III (P < 0.001). Jaundice was seen in 100% of Group I, 55% of Group II, and 85% of Group III (P < 0.001). Right hypochondrial pain, fatigue, fever, itching, dark urine, pale stool, and lower limb edema differed significantly, whereas nausea and vomiting did not (Table 2).

Groups I–III showed marked biochemical abnormalities. ALT and AST were highest in Group II (115.5 ± 71.4; 104.7 ± 61.2 U/L) (Table 2). ALP was elevated in Groups I–III (370.8 ± 138.7, 198.4 ± 134.8, 296.4 ± 115.4 U/L) vs. controls (66.3 ± 12.1 U/L). Total bilirubin peaked in Group I (8.52 ± 5.49 mg/dl), albumin was lowest in Groups I (3.06 ± 0.64) and II (2.93 ± 0.93 g/dl) vs. Groups III–IV (4.28 ± 0.46, 4.22 ± 0.49 g/dl). Serum creatinine was higher in Group II (1.53 ± 0.58 mg/dl). HBsAg positivity: 35%, 20%, 5% in Groups I–III (P = 0.004); HCV antibodies most frequent in Groups I (50%)

Table 1. Basic Demographic Data among the Studied Groups

		Groups				Test	
		Group I	Group II	Group III	Group IV	F	P value
Age (Years)	Range	45 - 75	49 - 79	49 - 72	44 - 69	2.066	0.107
	Mean ±SD	62.35 ± 8.79	64.9 ± 9.907	62.05 ± 7.257	60.125 ± 8.358		
Chi-Square		N %	N %	N %	N %	X^2	P-value
Sex	Male	28 70	22 55	19 47.5	26 65	5.053	0.168
	Female	12 30	18 45	21 52.5	14 35		
Comorbidities	DM	16 40	26 65	14 35	--- ---	8.304	0.016*
	HTN	6 15	2 4	6 15	--- ---	2.588	0.274

Data was presented as mean ±SD or range or frequency (%); SD, standard deviation; DM, Diabetes Mellitus; HTN, hypertension

Table 2. Symptomatology among the Studied Groups

	Groups						Chi-Square	
	Group I		Group II		Group III			
	N	%	N	%	N	%	X^2	P-value
Fever	24	60	12	30	2	5	28.036	<0.001*
Fatigue	28	70	36	90	2	5	63.838	<0.001*
Pain in the right hypochondria	34	85	20	50	40	100	31.031	<0.001*
Anorexia	36	90	38	95	0	0	96.733	<0.001*
Weight loss	32	80	38	95	0	0	85.851	<0.001*
Nausea	30	75	24	60	30	75	2.857	0.240
Vomiting	26	65	24	60	30	75	2.1	0.350
Itching	34	85	22	55	4	10	45.6	<0.001*
Dark urine	34	85	22	55	34	85	12.8	0.002*
Pale stool	34	85	22	55	34	85	12.8	0.002*
Jaundice	40	100	22	55	34	85	26.25	<0.001*
LL edema	18	45	20	50	0	0	28.036	<0.001*

Data was presented as number and frequency (%). *, statistically significant P value ≤ 0.05

and II (55%), rare in Group III (10%, P < 0.001) (Table 3).

Biliary dilatation in all Group I (CCA) and Group III (choledocholithiasis) patients, while Group II presented mainly with hepatic or extrahepatic masses (24 HCC, 16 pancreatic head cancers). MRCP and ERCP in Group I revealed irregular long-segment strictures, proximal duct dilatation, shouldering, and ductal cut-off; choledocholithiasis showed ductal stones without strictures (Table 4).

Conventional tumor markers, CEA and CA 19-9, were significantly elevated in Groups I and II (CEA: 6.26 ± 3.28 and 5.25 ± 3.00 ng/mL; CA 19-9: 51.5 ± 21.8 and 45.0 ± 22.6 U/mL) compared with lower levels in Groups III and IV (CEA: 3.33 ± 2.13 and 2.78 ± 1.94 ng/mL; CA 19-9: 27.2 ± 15.9 and 28.6 ± 10.9 U/mL, P < 0.001) (Table 5).

Cell-free DNA markers demonstrated highly significant differences among groups (P < 0.001). ALU 247 was highest in Group I (1315.4 ± 527.8 copies/mL) followed by Group II (745.2 ± 462.2), Group III (413.4 ± 253.6), and controls (217.4 ± 191.9). ALU 115 and DNA integrity followed similar trends (ALU 115: 1425.4 ± 487.5 to 374.3 ± 191.6; DNA integrity: 0.876 ± 0.161 to 0.539 ± 0.124) (Supplementary Table 1). In Group I, CEA correlated positively with ALU 247 (r = 0.374, P = 0.017), ALU 115 (r = 0.345, P = 0.029), and DNA integrity (r = 0.454, P = 0.003), whereas CA 19-9 showed no significant correlations. ALP and total bilirubin were positively correlated with cfDNA markers, while albumin showed negative correlations (Supplementary Table 2).

ROC curve analysis demonstrated superior diagnostic performance of cfDNA markers compared to conventional tumor markers. Between Groups I and II, ALU 247 >800 copies/mL showed 80% sensitivity and 75% specificity (accuracy 79.4%), ALU 115 >1000 copies/mL had 80% sensitivity and 70% specificity (accuracy 77.9%), and DNA integrity >0.89 reached 70% sensitivity and 90% specificity (accuracy 81.7%). CEA and CA 19-9 showed lower sensitivity or specificity. Between Groups I and III,

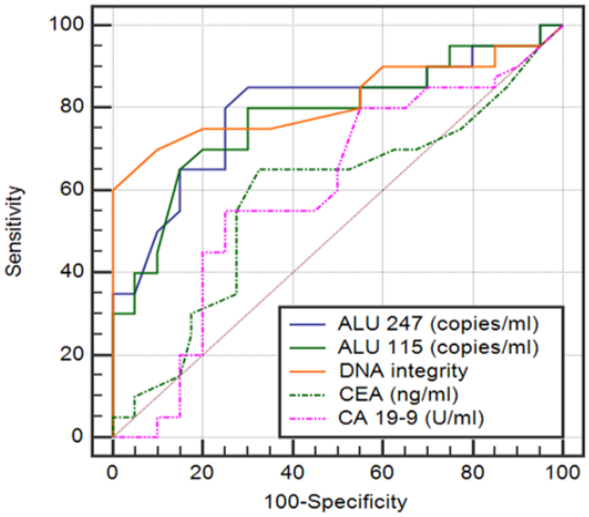

Figure 1. ROC Analysis of Tumor and cfDNA Markers between Group I and Group II

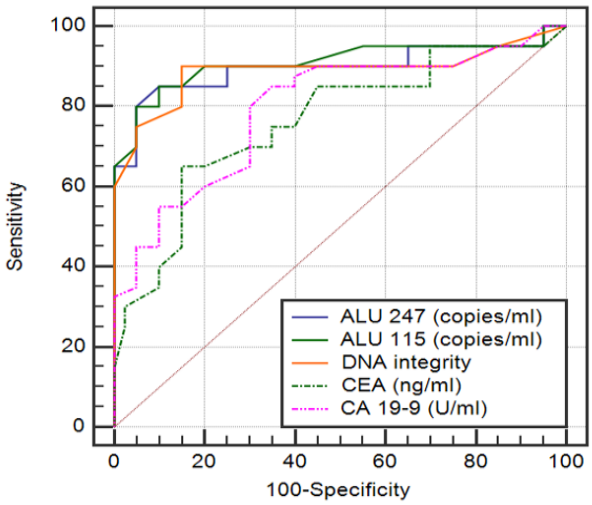

Figure 2. ROC Analysis of Tumor and cfDNA Markers between Group I and Group III

Table 3. Biochemical Parameters among Studied Groups

		Groups				ANOVA	
		Group I	Group II	Group III	Group IV	F	P-value
ALT (IU/L)	Range	43- 270	33- 300	50- 132	18 - 40	27.068	<0.001*
(10-40IU/L)	Mean ±SD	89.450 ± 46.932	115.450 ± 71.382	82.4 ± 19.952	29.250 ± 6.025		
AST (IU/L)	Range	40 - 210	32 -270	60 - 165	18- 40	30.964	<0.001*
(10-40IU/L)	Mean ±SD	83.800 ± 35.613	104.650 ± 61.234	81.350 ± 22.142	27.850 ± 5.985		
ALP (IU/L)	Range	200 - 800	45 - 560	130 -500	44 - 90	54.504	<0.001*
(44-147IU/L)	Mean ±SD	370.750 ± 138.678	198.350 ± 134.767	296.400 ± 115.444	66.300 ± 12.098		
T. Bilirubin (mg/dl)	Range	2-25	0.9 - 17	2.5 - 12	0.4 - 1.2	26.645	<0.001*
(0.2-1.2mg/dl)	Mean ±SD	8.515 ± 5.488	6.415 ± 5.576	6.905 ± 2.580	0.795 ± 0.261		
Albumin (g/dl)	Range	1.5 - 4	1.5- 4.2	3.5 - 5.2	3.5 - 5	49.319	<0.001*
(3.5-5g/dl)	Mean ±SD	3.065 ± 0.639	2.930 ± 0.925	4.280 ± 0.458	4.225 ± 0.49		
Creatinine (mg/dl)	Range	0.9 - 2.3	0.9 - 3	0.8 - 1.5	0.7 -1.2	17.801	<0.001*
(0.6-1.1mgdl)	Mean ±SD	1.348 ± 0.350	1.534 ± 0.580	1.088 ± 0.212	1.005 ± 0.155		

	TUKEY'S Test					
	I&II	I&III	I&IV	II&III	II&IV	III&IV
ALT (IU/L)	0.044*	0.89	<0.001*	0.005*	<0.001*	<0.001*
AST (IU/L)	0.063	0.991	<0.001*	0.029*	<0.001*	<0.001*
ALP (IU/L)	<0.001*	0.019*	<0.001*	0.001*	<0.001*	<0.001*
T. Bilirubin (mg/dl)	0.107	0.303	<0.001*	0.951	<0.001*	<0.001*
Albumin (g/dl)	0.793	<0.001*	<0.001*	<0.001*	<0.001*	0.982
Creatinine (mg/dl)	0.105	0.009*	<0.001*	<0.001*	<0.001*	0.74

	Groups						Chi-Square	
	Group I		Group II		Group III			
	N	%	N	%	N	%	X^2	P-value
HBs-Ag	14	35	8	20	2	5	11.25	0.004*
HCV AB	20	50	22	55	4	10	20.588	<0.001*

Data was presented as mean ±SD or range or frequency (%). ALP, Alkaline phosphatase; ALT, alanine alanine aminotransferase; AST, Aspartate aminotransferase

Table 4. Diagnostic Modalities and ERCP Findings in the Studied Groups

		Groups					
		Group I		Group II		Group III	
		N	%	N	%	N	%
US	IHBD	40	100	0	0	40	100
	Hepatic Focal lesion	0	0	24	60	0	0
	Extraheptic mass	0	0	16	40	0	0
CT	IHBD	-	-	20	50	-	-
	Ductal thickening and periductal dilatation	-	-	0	0	-	-
	Hepatic mass	-	-	24	60	-	-
	Extrahepatic mass	-	-	16	40	-	-
MRCP	Irregular asymmetric stricture with shouldering	40	100	-	-	0	0
	Proximal duct dilatation	40	100	-	-	40	100
	stones	0	0	-	-	40	100
	Ductal cut off	40	100	-	-	0	0
ERCP	Irregular asymmetric stricture	40	100	-	-	0	0
	Long segment affection	40	100	-	-	-	-
	Proximal duct dilatation	40	100	-	-	40	100
	Shouldering	40	100	-	-	-	-
	stones	0	0	-	-	40	100

Data was presented as frequency (%). US, Ultrasonography; CT, Computed tomography; MRCP, Magnetic Resonance Cholangiopancreatography; ERCP, Endoscopic Retrograde Cholangiopancreatography.

Table 5. Comparison of Serum CEA and CA 19-9 Levels among the Studied Group

		Groups												ANOVA	
		Group I			Group II			Group III			Group IV			F	P-value
CEA (ng/ml)	Range	1.5	-	13	1.5	-	12	1.5	-	9	1	-	8.5	15.094	<0.001*
(<3ng/ml)	Mean ±SD	6.26	±	3.283	5.25	±	3	3.33	±	2.133	2.78	±	1.941		
CA 19-9 (U/ml)	Range	10	-	86	10	-	90	5	-	65	14	-	55	17.142	<0.001*
(<37U/ml)	Mean ±SD	51.5	±	21.83	45	±	22.596	27.2	±	15.915	28.55	±	10.943		

	TUKEY'S Test					
	I&II	I&III	I&IV	II&III	II&IV	III&IV
CEA (ng/ml)	0.325	<0.001*	<0.001*	0.008*	<0.001*	0.79
CA 19-9 (U/ml)	0.395	<0.001*	<0.001*	<0.001*	0.001*	0.988

Data was presented as mean ±SD or range. *, statistically significant P value ≤ 0.05; CEA, Carcinoembryonic Ag

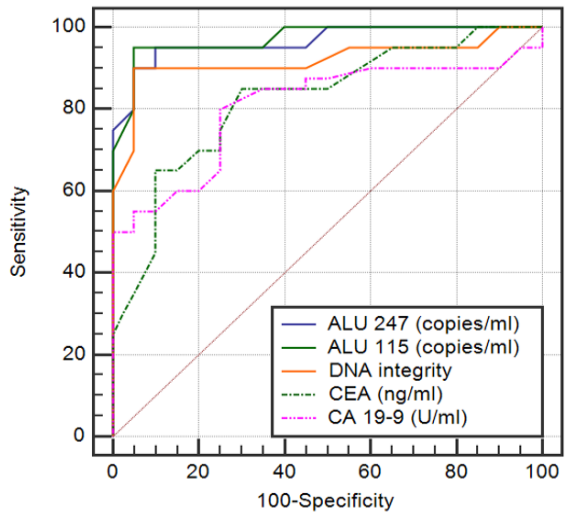

Figure 3. ROC Analysis of Tumor and cfDNA Markers between Group I and Group IV

ALU 247 >552 copies/mL and ALU 115 >700 copies/mL achieved 85% sensitivity and 90% specificity (accuracies 89.6% and 90.9%), while DNA integrity >0.7 reached 90% sensitivity, 85% specificity, and 88.9% accuracy. Between Groups I and IV, ALU 247 >325 copies/mL showed 95% sensitivity, 90% specificity (accuracy 96.5%), ALU 115 >580 copies/mL had 95% sensitivity and 95% specificity (accuracy 97.1%), and DNA integrity >0.67 yielded 90% sensitivity, 95% specificity (accuracy 91.9%) (Supplementary Table 3-5) (Figures 1–3).

Discussion

This study assessed demographic, clinical, biochemical, tumor marker, and cfDNA profiles in cholangiocarcinoma patients versus other groups, highlighting the enhanced diagnostic and prognostic value of cfDNA.

Age and sex did not differ significantly among groups ($p > 0.05$). Mean ages were 62.3 ± 8.8 years in CCA, 64.9 ± 9.9 in Group II, 62.0 ± 7.3 in Group III, and 60.1 ± 8.4 in controls, consistent with CCA predominating in older adults without sex bias [10]. Diabetes was more frequent in Group II (65%) than in CCA (40%) and other groups ($p = 0.016$), supporting reported links between diabetes and hepatobiliary malignancies, especially HCC [11], as our findings reinforce the role of metabolic comorbidities in liver-related cancers, while confirming that CCA does not consistently cluster with metabolic risk factors.

Analysis of symptomatology revealed marked variation between groups. Anorexia and weight loss were present in 90% and 80% of CCA patients, respectively, but absent in choledocholithiasis ($p < 0.001$). Fatigue (70%) and pruritus (85%) were also significantly more frequent in CCA compared to Group III (5% and 10%, respectively; $p < 0.001$). Jaundice was universal in CCA (100%), compared to 55% in Group II and 85% in Group III ($p < 0.001$). These findings agree with prior reports identifying obstructive jaundice, weight loss, and pruritus as hallmark symptoms of CCA [1]. Interestingly, nausea and vomiting showed no significant differences between groups, reflecting their nonspecific nature. Such overlapping gastrointestinal symptoms highlight the diagnostic challenge in differentiating CCA from benign biliary disease.

The biochemical profile demonstrated striking abnormalities in CCA patients compared to controls. ALT and AST were significantly elevated in CCA (89.5 ± 46.9 IU/L and 83.8 ± 35.6 IU/L, respectively) relative to controls (29.3 ± 6.0 and 27.9 ± 6.0; $p < 0.001$). ALP was also markedly increased in CCA (370.8 ± 138.7) compared to 66.3 ± 12.1 in controls ($p < 0.001$). Total bilirubin reached the highest levels in CCA (8.5 ± 5.5 mg/dl), while albumin was significantly reduced (3.1 ± 0.6 g/dl). These abnormalities reflect cholestasis and impaired hepatic synthetic function, consistent with previous observations in biliary malignancies [12]. Notably, creatinine was significantly higher in Group II (1.53 ± 0.58 mg/dl), reflecting the impact of systemic illness or renal impairment in hepatocellular carcinoma, rather than in CCA. Viral markers revealed HBsAg positivity in 35% and HCV antibodies in 50% of CCA patients, consistent with regional epidemiology linking chronic viral hepatitis to biliary tract cancers [13].

Radiological and endoscopic findings further supported the diagnosis. All CCA patients demonstrated intrahepatic biliary dilatation on ultrasound, and MRCP/ERCP consistently revealed irregular asymmetric strictures, proximal duct dilatation, shouldering, and long-segment involvement. By contrast, choledocholithiasis

was characterized by proximal duct dilatation with intraductal stones, without strictures. These imaging patterns are well documented as classic features of CCA and remain essential for initial diagnostic suspicion [14].

When evaluating tumor markers, both CEA and CA 19-9 were significantly elevated in patients with CCA. The mean CEA level was 6.26 ± 3.28 ng/ml compared with 2.78 ± 1.94 ng/ml in controls ($p < 0.001$), while CA 19-9 reached 51.5 ± 21.8 U/ml versus 28.6 ± 10.9 U/ml in controls ($p < 0.001$). These findings align with earlier and more recent studies demonstrating elevation of both markers in CCA, though specificity remains limited due to overlap with benign biliary disorders and other gastrointestinal malignancies [15]. In our cohort, both markers achieved moderate diagnostic performance, but failed to differentiate CCA from other malignancies ($p > 0.05$ for Group I vs. Group II).

In contrast, cfDNA parameters demonstrated superior discriminatory power. ALU 247 levels were highest in CCA (1315.4 ± 527.8 copies/ml) compared to Group II (745.2 ± 462.2), Group III (413.4 ± 253.6), and controls (217.4 ± 191.9; $p < 0.001$). Similarly, ALU 115 was markedly elevated in CCA (1425.4 ± 487.5) versus controls (374.3 ± 191.6). DNA integrity index was also significantly higher in CCA (0.876 ± 0.161) compared to controls (0.539 ± 0.124; $p < 0.001$). These findings are consistent with reports by Umetani et al. [16], and Yu et al. [17], who demonstrated that longer cfDNA fragments and increased DNA integrity indices are associated with malignancy, reflecting tumor necrosis and apoptosis. Importantly, our results extend these observations by confirming their diagnostic utility specifically in CCA.

Correlation analysis revealed strong associations between cfDNA and biochemical parameters. In CCA patients, ALU 247 and ALU 115 correlated positively with CEA ($r = 0.374$, $p = 0.017$; $r = 0.345$, $p = 0.029$) and ALP ($r \approx 0.44$, $p < 0.01$), while negatively correlating with albumin ($r \approx –0.40$, $p < 0.05$). This suggests that cfDNA reflects tumor activity and cholestatic injury. Similar correlations between cfDNA and disease severity markers have been observed in hepatobiliary malignancies Xu et al. [18], supporting its role as a surrogate marker of tumor burden.

ROC curve analyses confirmed the superior diagnostic accuracy of cfDNA markers. Against Group II, ALU 247 at a cutoff >800 copies/ml achieved sensitivity of 80% and specificity of 75% (accuracy 79.4%), while DNA integrity >0.89 provided specificity of 90% with overall accuracy of 81.7%. Against Group III, ALU 115 at a cutoff >700 copies/ml achieved 85% sensitivity and 90% specificity (accuracy 90.9%), surpassing both CEA (accuracy 76.2%) and CA 19-9 (79.5%). Most strikingly, against controls, ALU 115 >580 copies/ml achieved 95% sensitivity and 95% specificity, with the highest accuracy of 97.1%. These values far exceed those of conventional markers, confirming the diagnostic superiority of cfDNA. Comparable performance was reported in meta-analyses of cfDNA across multiple cancers, where AUC values ranged between 0.89–0.92 [19].

Taken together, our findings demonstrate that cfDNA quantification and integrity indices outperform conventional tumor markers for CCA detection. This agrees with emerging literature establishing cfDNA as a powerful liquid biopsy tool [20]. However, discrepancies exist; some studies reported lower sensitivity for cfDNA compared to CA 19-9, particularly in early-stage disease [21]. These differences may be attributable to methodological variations, patient populations, or disease stages. Nonetheless, the high sensitivity and specificity observed in our cohort underscore cfDNA's potential for routine clinical application in CCA diagnosis.

Despite these promising results, limitations must be acknowledged. The relatively small sample size may limit generalizability, and the cross-sectional design precludes assessment of cfDNA dynamics over time. Furthermore, pre-analytical variability such as sample processing may affect cfDNA quantification. Standardization of cfDNA methodologies remains essential before clinical translation. Future studies should validate these findings in larger multicenter cohorts, incorporate longitudinal monitoring, and explore cfDNA methylation or mutational profiles for enhanced diagnostic accuracy.

In conclusion, our study confirms that cfDNA markers (ALU 247, ALU 115, and DNA integrity) are significantly elevated in CCA and provide superior diagnostic accuracy compared to CEA and CA 19-9. These results support the integration of cfDNA analysis into the diagnostic algorithm of CCA, while highlighting the need for methodological standardization and larger validation studies.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

Ethical approval

The research protocol was authorized by the Ethical Committee of Menoufia University's Faculty of Medicine with IRB (5/2022TROP11).

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

cfDNA: Cell- free DNA
cfDI: cfDNA integrity
CCA: Cholangiocarcinoma
BBD: Benign biliary disorders
CEA: Carcinoembryonic antigen
CA19-9: Carbohydrate antigen 19-9
qRT-PCR: Quantitative real time PCR

References

1. Banales JM, Marin JJG, Lamarca A, Rodrigues PM, Khan SA, Roberts LR, et al. Cholangiocarcinoma 2020: The next horizon in mechanisms and management. Nat Rev Gastroenterol Hepatol. 2020;17(9):557-88. https://doi.org/10.1038/s41575-020-0310-z.
2. Cambridge WA, Fairfield C, Powell JJ, Harrison EM, Søreide

K, Wigmore SJ, et al. Meta-analysis and meta-regression of survival after liver transplantation for unresectable perihilar cholangiocarcinoma. Ann Surg. 2021;273(2):240-50. https://doi.org/10.1097/sla.0000000000003801.
3. Nada A, Alkhatib A, Abdelmalik F, El-Abd M, Elabd NS, Abdel-Latif HE-D. Bile level of cytokeratin 7 as a diagnostic marker for cholangiocarcinoma: A case-control study in egyptian patients. Egyptian Liver Journal. 2024;14(1):46. https://doi.org/10.1186/s43066-024-00353-4.
4. Khuntikeo N, Chamadol N, Yongvanit P, Loilome W, Namwat N, Sithithaworn P, et al. Cohort profile: Cholangiocarcinoma screening and care program (cascap). BMC Cancer. 2015;15:459. https://doi.org/10.1186/s12885-015-1475-7.
5. Brandi G, Venturi M, Pantaleo MA, Ercolani G. Cholangiocarcinoma: Current opinion on clinical practice diagnostic and therapeutic algorithms: A review of the literature and a long-standing experience of a referral center. Dig Liver Dis. 2016;48(3):231-41. https://doi.org/10.1016/j.dld.2015.11.017.
6. Loosen SH, Roderburg C, Kauertz KL, Koch A, Vucur M, Schneider AT, et al. Cea but not ca19-9 is an independent prognostic factor in patients undergoing resection of cholangiocarcinoma. Sci Rep. 2017;7(1):16975. https://doi.org/10.1038/s41598-017-17175-7.
7. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6(224):224ra24. https://doi.org/10.1126/scitranslmed.3007094.
8. Grabuschnig S, Bronkhorst AJ, Holdenrieder S, Rosales Rodriguez I, Schliep KP, Schwendenwein D, et al. Putative origins of cell-free DNA in humans: A review of active and passive nucleic acid release mechanisms. Int J Mol Sci. 2020;21(21). https://doi.org/10.3390/ijms21218062.
9. Soliman SE, Alhanafy AM, Habib MSE, Hagag M, Ibrahem RAL. Serum circulating cell free DNA as potential diagnostic and prognostic biomarker in non small cell lung cancer. Biochem Biophys Rep. 2018;15:45-51. https://doi.org/10.1016/j.bbrep.2018.06.002.
10. Khan SA, Tavolari S, Brandi G. Cholangiocarcinoma: Epidemiology and risk factors. Liver Int. 2019;39 Suppl 1:19-31. https://doi.org/10.1111/liv.14095.
11. Wang P, Kang D, Cao W, Wang Y, Liu Z. Diabetes mellitus and risk of hepatocellular carcinoma: A systematic review and meta-analysis. Diabetes Metab Res Rev. 2012;28(2):109-22. https://doi.org/10.1002/dmrr.1291.
12. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. Semin Liver Dis. 2004;24(2):115-25. https://doi.org/10.1055/s-2004-828889.
13. Zhou Y, Zhao Y, Li B, Huang J, Wu L, Xu D, et al. Hepatitis viruses infection and risk of intrahepatic cholangiocarcinoma: Evidence from a meta-analysis. BMC Cancer. 2012;12:289. https://doi.org/10.1186/1471-2407-12-289.
14. Razumilava N, Gores GJ. Cholangiocarcinoma. Lancet. 2014;383(9935):2168-79. https://doi.org/10.1016/s0140-6736(13)61903-0.
15. Liang B, Zhong L, He Q, Wang S, Pan Z, Wang T, et al. Diagnostic accuracy of serum ca19-9 in patients with cholangiocarcinoma: A systematic review and meta-analysis. Med Sci Monit. 2015;21:3555-63. https://doi.org/10.12659/msm.895040.
16. Umetani N, Kim J, Hiramatsu S, Reber HA, Hines OJ, Bilchik AJ, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: Direct quantitative pcr for alu repeats. Clin Chem. 2006;52(6):1062-9. https://doi.org/10.1373/clinchem.2006.068577.
17. Yu SC, Lee SW, Jiang P, Leung TY, Chan KC, Chiu RW, et al. High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. Clin Chem. 2013;59(8):1228-37. https://doi.org/10.1373/clinchem.2013.203679.
18. Xu RH, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat Mater. 2017;16(11):1155-61. https://doi.org/10.1038/nmat4997.
19. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer. 2017;17(4):223-38. https://doi.org/10.1038/nrc.2017.7.
20. Shen N, Zhang D, Yin L, Qiu Y, Liu J, Yu W, et al. Bile cell-free DNA as a novel and powerful liquid biopsy for detecting somatic variants in biliary tract cancer. Oncol Rep. 2019;42(2):549-60. https://doi.org/10.3892/or.2019.7177.
21. Prasopdee S, Pholhelm M, Yusuk S, Tangphatsornruang S, Butthongkomvong K, Kunjantarachot A, et al. Investigation of plasma cell-free DNA and mirna in cholangiocarcinoma and opisthorchiasis viverrini patients. Asian Pac J Cancer Prev. 2024;25(3):739-46. https://doi.org/10.31557/apjcp.2024.25.3.739.