Short Communications

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Investigation of Plasma Cell-Free DNA and MiRNA in Cholangiocarcinoma and Opisthorchiasis Viverrini Patients

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Abstract

Objectives: This study aimed to assess the diagnostic potential of cell-free DNA (cfDNA) and cell-free miRNA (cf-miRNA) for distinguishing between Healthy, asymptomatic opisthorchiasis viverrini and cholangiocarcinoma in a preliminary manner. **Methods:** In this study, 36 participants were enrolled into three health status groups: a healthy control group (HC), *Opisthorchis viverrini*-infected group (OV), and a cholangiocarcinoma group (CCA), each comprising 12 participants. Concentration measurements of cfDNA and cf-miRNA from plasma were conducted. Additionally, ultra-low-pass whole-genome sequencing (ULP-WGS) was employed to investigate DNA alterations. **Results:** The study revealed a significant elevation in plasma cfDNA concentration in the cholangiocarcinoma (CCA) group compared to healthy controls (HC) and *Opisthorchis viverrini*-infected (OV) groups ($P < 0.001$). The cfDNA concentration demonstrated a sensitivity of 75.00% and specificity of 95.83% for differentiating cholangiocarcinoma, with a cut-off of > 30.50 ng/ml plasma. Likewise, the concentration of cf-miRNA in the CCA group significantly differed from that in the HC and OV groups, demonstrating a sensitivity of 83.33% and specificity of 95.83% with a cut-off set at > 70.50 ng/ml plasma. Furthermore, a positive correlation between plasma concentrations of cfDNA and cf-miRNA suggests a potential relationship between these two biomarkers. These findings indicated the diagnostic potential of cfDNA and cf-miRNA in distinguishing cholangiocarcinoma, emphasizing their role as promising biomarkers for further investigation and clinical applications. **Conclusion:** Elevated plasma concentrations of cfDNA and cf-miRNA could serve as potential diagnostic tools for distinguishing cholangiocarcinoma from other conditions. cf-miRNA was superior to cfDNA in terms of sensitivity.

Keywords: Cell-free DNA- cell-free miRNA- diagnosis- cholangiocarcinoma- *Opisthorchis viverrini*

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Introduction

Cholangiocarcinoma (CCA), a rare cancer affecting the bile ducts, exhibits a high prevalence in Southeast Asian countries, particularly in regions endemic to the carcinogenic liver fluke, *Opisthorchis viverrini* (OV) [1]. An asymptomatic nature of OV infection allows for chronic inflammation and, consequently, contributes to the development of cholangiocarcinoma, posing challenges to effective diagnoses for both conditions [2]. The aggressive nature of CCA, coupled with its poor survival rates, indicates the critical need for early detection and accurate diagnosis to enhance treatment efficacy and outcomes. Numerous studies have explored biomarkers involving proteins, carbohydrates, and nucleic acids, but these attempts have been limited by sensitivity, specificity, and accuracy, leaving effective diagnostic methods currently unavailable [3-5].

In recent developments, cell-free DNA (cfDNA) and cell-free miRNA (cf-miRNA) have shown promise in diagnosing various diseases, including heart disease, kidney disease, infections, and several cancers [6-9].

The cfDNA and cf-miRNA, found in biological fluids like blood or plasma, are released by death or damaged cells [10]. Although fragmented and degraded, cfDNA in the bloodstream has been utilized as a diagnostic tool,

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providing insights into genetic mutations and changes in affected cells. Current limitations in cancer diagnosis, particularly in early-stage detection, highlight the ongoing need for research and development in this area [11-13]. The use of liquid biopsy, which involves minimally invasive sampling, has been extensively investigated for this purpose [14]. Numerous attempts have been made to identify effective cancer biomarkers using liquid biopsy-based samples, with recent focus turning to cfDNA, cf-miRNA, exosomal miRNA, and circulating tumor cells (CTCs). [10, 15-21].

However, the use of cfDNA as a diagnostic tool is still in the early stages of development, and further research is essential to fully understand its potential and limitations. The microRNAs (miRNA), small non-coding RNA molecules regulating gene expression and influencing cellular processes, have also been explored for diagnostic purposes, including cancer. Similar to cfDNA, the utilization of cf-miRNA as a diagnostic tool is still in the research stage. Therefore, the aim of this study is to conduct a preliminary investigation into the feasibility and efficacy of using cfDNA and cf-miRNA levels in plasma for the differential diagnosis of opisthorchiasis and CCA.

Materials and Methods

Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA) blood tubes were purchased from Becton Dickinson. The extraction of plasma cfDNA and cf-miRNA was performed using the QIAamp® Circulating Nucleic Acid kit (QIAGEN). Measurement of cfDNA and cf-miRNA concentration utilized the Qubit™ dsDNA HS Assay Kit (Invitrogen) and Qubit™ miRNA Assay Kit (Invitrogen), respectively."

Subjects

The study was conducted in accordance with the ethical guidelines in the Declaration of Helsinki and was approved by the Human Ethics Committee of Udon Thani Cancer Hospital, Udon Thani, Ministry of Public Health, Thailand (UCH-CT 11/2563). Regarding OV, a liver fluke that poses a risk factor for CCA, OV-infected patients were included in this study. The subjects were categorized into three groups, each comprising 12 participants, based on their health statuses: the healthy control (HC) group, consisting of subjects with a normal physical examination, no liver enlargement, no jaundice, and who tested negative for OV eggs in fecal examination; the group with *O. viverrini* (OV)-infected subjects, including those who tested positive for OV eggs in fecal examination with a normal liver and bile duct as confirmed by ultrasonography; and the group with cholangiocarcinoma (CCA) subjects, comprising individuals with hepatomegaly and/or jaundice upon physical examination, abnormal liver and bile duct ultrasonography, and confirmed CCA through tissue histopathology. All subjects in the OV group were asymptomatic. Demographic details and a summary of the clinical statuses of the subjects are presented in Table 1.

Blood collection and plasma preparation

Ten milliliters of venous blood samples were collected in an EDTA blood tube. The tube was immediately centrifuged at 3,000 x g for 10 minutes. The supernatant was then transferred to a new tube and spun again at 15,000 x g for another 10 minutes. The resulting 700 µl of plasma (supernatant) was aliquoted into a fresh tube to prevent the freeze-thaw cycle and stored at -80ºC until use. Turbid, hemolyzed, or clotted plasmas were excluded from this study.

Isolation of circulating cfDNA and cf-miRNA

The QIAamp® Circulating Nucleic Acid Kit (QIAGEN) was employed to isolate circulating cfDNA and cf-miRNA from the plasma of each subject, following the manufacturer's instructions. In brief, the plasma underwent a brief centrifugation at 16,000 x g for 5 minutes at 4ºC, followed by transfer to a new tube and mixing with 300 µl of phosphate-buffered saline. Subsequently, 800 µl of buffer ACL with carrier RNA (0.9 ml ACL buffer mixed with 5.6 µl carrier RNA) and 100 µl proteinase K were added and mixed for 30 seconds by vortexing. The mixture underwent incubation at 60°C for 30 minutes. Next, 1.8 ml of ACB buffer was added, mixed for 15 seconds by vortexing, and incubated on ice for 5 minutes. Upon completion, 600 µl of ACW1 buffer was added, and the entire solution was loaded onto the column.

The subsequent steps included the addition and suctioning through the column of the following solutions: 750 µl AWC2 buffer and 750 µl ethanol. The column was then centrifuged at 16,000 x g for 3 minutes and heated at 56°C to thoroughly dry the column membrane. To elute the circulating nucleic acid, encompassing both cfDNA and cf-miRNA, 30 µl of AVE buffer was added to the center of the membrane. After incubating for 3 minutes at room temperature, the solution was centrifuged at 16,000 x g for 1 minute, and the eluate was utilized for further experiments.

Measurement of cell-free DNA (cfDNA) and cell-free microRNA (cf-miRNA) concentration

To quantify the concentration of cfDNA and cf-miRNA, two microliters of each eluate were employed in duplicate, utilizing the Qubit™ dsDNA HS Assay Kit (Invitrogen) and Qubit™ miRNA Assay Kit (Invitrogen), respectively. In brief, 1 µl of Qubit reagent was combined with 199 µl of Qubit buffer to prepare Qubit mixture, and the 2 µl sample was mixed with 198 µl of the Qubit mixture before being measured using the Qubit 4 Fluorometer. The concentrations of cfDNA and cf-miRNA were determined in ng/ml plasma and subsequently utilized for statistical analysis.

Investigation of cell-free DNA by ultra-low-pass-whole-genome sequencing (ULP-WGS)

The investigation of cfDNA using ultra-low-pass-whole-genome sequencing (ULP-WGS) unveiled quality changes in DNA sequences. An equal volume of 10 plasmas from each group was combined to create pooled HC, pooled OV, and pooled CCA samples. Subsequently, these three pools underwent further analysis through ultra-low-pass-whole-genome sequencing (ULP-WGS).

The MagMAX™ Cell-Free DNA Isolation Kit

(Thermo Fisher Scientific) was employed to isolate cfDNA. The isolated cfDNA was then used to construct a cfDNA library following the provided protocol (Ion Plus Fragment Library Kit, Applied Biosystems™, Thermo Fisher Scientific). The library constructs underwent sequencing using the MGISEQ-2000 sequencing system (MGI Tech Co. Ltd., China).

The analysis involved determining the percentage of tumor fraction and identifying somatic copy number alterations (SCNAs), encompassing deletion, gain, amplification, and average ploidy number. ULP-WGS was conducted with 0.1-0.3x genome-wide coverage using the ichorCNA software (available for download at https://doi.org/10.1038/s41467-017-00965-y) [22].

Statistical and diagnostic analyses

The statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp.). The normality of the data was assessed through a normal Q-Q plot, with a diagonal line indicating normal distribution. For normally distributed data, a Student's t-test was applied for two-sample comparisons, and a one-way analysis of variance (ANOVA) followed by post-hoc tests for multiple comparisons were utilized to compare plasma cfDNA and cf-miRNA concentrations across multiple groups.

In cases where the data did not follow a normal distribution, the Mann-Whitney U test was employed for two-sample comparisons, and the Kruskal-Wallis test, followed by the Dunn-Bonferroni post-hoc test, was used for multiple group comparisons to identify significant differences.

GraphPad Prism 9 was employed for dot plot analysis and calculating the area under the receiver operating characteristic curve (AUC). For diagnostic calculations, the cut-off at the maximum likelihood ratio was used. MedCalc, a diagnostic test evaluation calculator available at https://www.medcalc.org/calc/diagnostic_test.php, was utilized to calculate diagnostic parameters, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy.

Results

Plasma level of Cell-free DNA (cfDNA) and cell-free miRNA (cf-miRNA)

The measurement of cfDNA and cf-miRNA concentrations using Qubit Fluorescence revealed average (maximum/minimum) cfDNA levels in the HC, OV, and CCA groups to be 12.55 ng/ml plasma (range: 4.46-26.70 ng/ml plasma), 20.369 ng/ml plasma (range: 7.37-39.13 ng/ml plasma), and 100.97 ng/ml plasma (range: 17.66-501.43 ng/ml plasma), respectively (Table 2). Correspondingly, cf-miRNA levels in the HC, OV, and CCA groups showed averages of 36.87 ng/ml plasma (range: 17.14-62.57 ng/ml plasma), 34.91 ng/ml plasma (range: 2.23-73.71 ng/ml plasma), and 376.69 ng/ml plasma (range: 20.14-1,924.29 ng/ml plasma), respectively (Table 2).

The highest concentrations of both plasma cfDNA (Figure 1A) and cf-miRNA (Figure 1B) were observed in the CCA group. Additionally, Pearson correlation analysis demonstrated a significant correlation between plasma cfDNA and cf-miRNA concentrations ($r = 0.9874$, 95% CI = 0.9751-0.9936, $R^2 = 0.9749$, $P < 0.0001$) (Figure 2). As the data for cfDNA and cf-miRNA concentrations did not meet the assumptions of parametric tests, the non-parametric Kruskal-Wallis test was employed to determine significant differences between groups. Results indicated significant differences in cfDNA concentration (χ^2 (^{2}) = 17.944, $P < 0.001$) and cf-miRNA concentration (χ^2 (^{2}) = 13.319, $P = 0.001$) among groups, with the CCA group showing higher levels compared to the HC and OV groups. The difference in concentration levels of cfDNA between the CCA group and the HC+OV group was further tested using the Mann-Whitney U test, revealing a significantly higher cfDNA concentration level in the CCA group ($U = 27.00$, $P < 0.001$). A similar trend was observed for cf-miRNA, with the CCA group having a higher concentration level than the HC+OV group ($U = 26.00$, $P < 0.001$).

Assessment of the diagnostic potential of cell-free DNA (cfDNA) and cell-free miRNA (cf-miRNA) in plasma

The results demonstrated a significantly higher concentration of cfDNA in the plasma of the CCA group compared to the HC and OV groups ($P<0.001$). Using a cut-off of >30.50 ng/ml plasma, the cfDNA test exhibited a sensitivity of 75.00% and specificity of 95.83% for the differential diagnosis of CCA (Table 3). The AUCs of the ROC curves for plasma cfDNA, comparing the

Table 1. Summary of Subject Demography

	Group		
	HC	OV	CCA
Sample size	12	12	12
Sex (Male/Female)	3/9	9/3	9/3
Age (year)			
Min/Max	25/82	19/60	52/87
Mean ± SD	36.7 ± 7.8	48.1± 10.7	66.5 ± 10.2
Alcohol consumption			
No	5 (42%)	2 (17%)	1 (8%)
Yes	7 (58%)	10 (83%)	11 (92%)
Smoking			
No	10 (83%)	5 (42 %)	3 (25%)
Yes	2 (17%)	7 (58 %)	9 (75%)
Raw fish eating-habit			
(Source of *O. viverrini* infection)			
No	9 (75%)	2 (17%)	1 (8%)
Yes	2 (17%)	10 (83%)	11 (92%)
Uncertain	1 (8%)	0 (0%)	0 (0%)
History of *O. viverrini* infection			
No	11 (92%)	11 (92%)	9 (75%)
Yes	0 (0%)	1 (8%)	3 (25%)
Uncertain	1 (8%)	0 (0%)	0 (0%)
Fermented food eating-habit (Source of nitrosamine)			
No	0 (0%)	0 (0%)	0 (0%)
Yes	12 (100%)	12 (100%)	12 (100%)

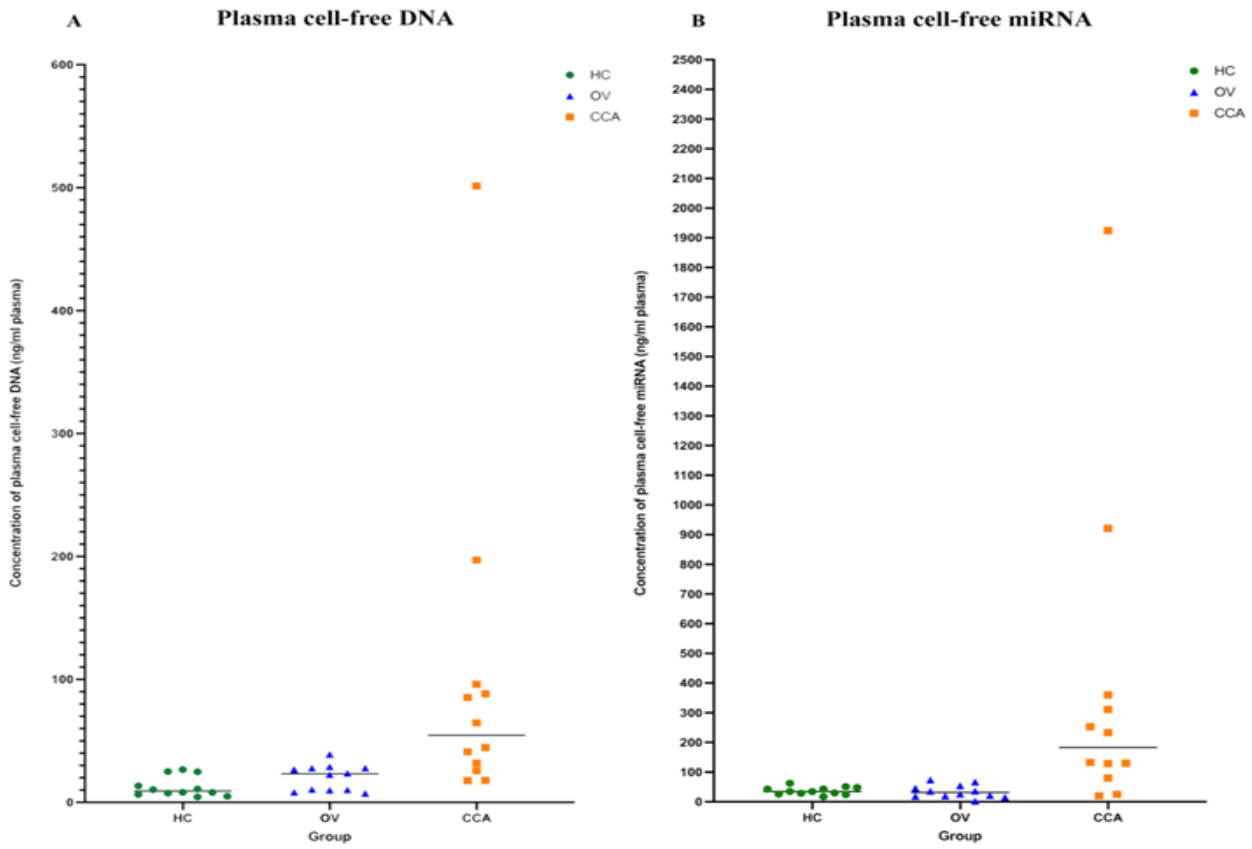

Figure 1. Scatter Plots of Plasma cfDNA (A) and cf-miRNA (B) Concentrations for Healthy Controls (HC) Subjects, *O. viverrini*-infected (OV) Subjects, and Cholangiocarcinoma (CCA) Subjects. A: concentration of plasma cfDNA in ng/ml plasma is on y-axis. B: concentration of plasma cf-miRNA in ng/ml plasma is on y-axis. Group is on x-axis. The green circle, blue triangle, and orange square indicate HC, OV, and CCA.

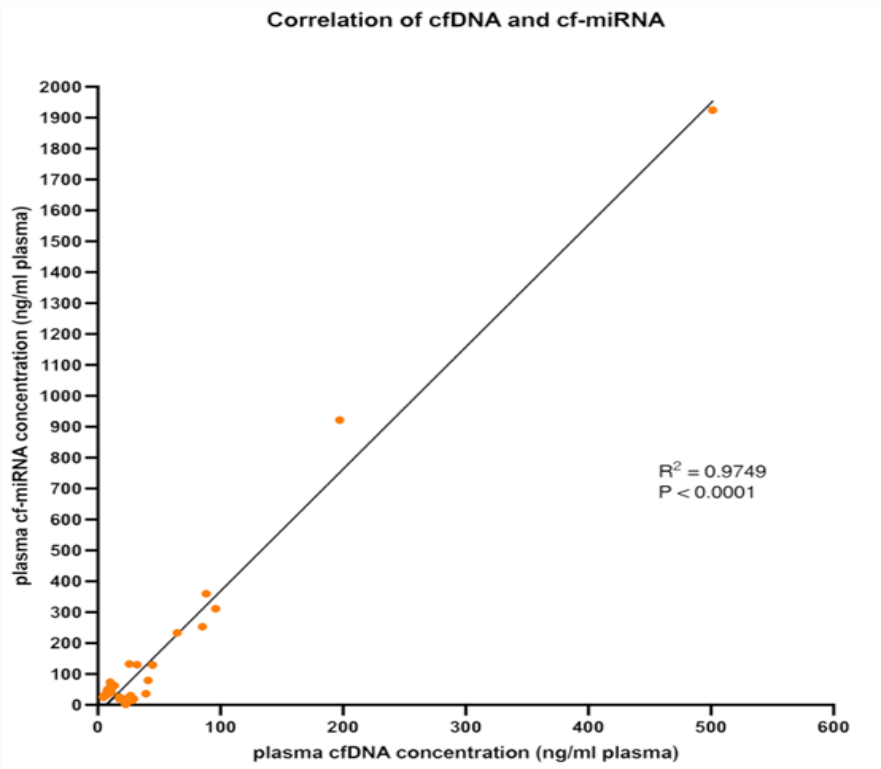

Figure 2. The Correlation of Plasma cfDNA and cf-miRNA. Positive correlation between plasma cfDNA and cf-miRNA concentration shown by Pearson correlation coefficient ($R^2 = 0.9740$, $P < 0.0001$). The concentration of plasma cf-miRNA in ng/ml plasma is on y-axis and the plasma cfDNA is on x-axis. The orange circle indicates the cfDNA and cf-miRNA of each subject.

Table 2. Concentration of Plasma cfDNA and cf-miRNA

	HC		OV		CCA	
	cfDNA	cf-miRNA	cfDNA	cf-miRNA	cfDNA	cf-miRNA
1	26.7	30.04	23.79	18.43	64.71	233.14
2	7.46	34.54	22.71	2.23	44.57	129
3	6.43	34.84	26.96	26.44	88.29	360
4	25.07	24.21	27.99	21.99	17.83	20.14
5	4.46	25.97	8.40	36.3	17.66	25.37
6	24.86	17.14	29.14	19.71	501.43	1924.29
7	4.97	28.46	7.37	45.86	25.63	132.86
8	8.14	51.00	27.99	15.43	31.97	130.29
9	10.29	48.00	10.29	54.86	96	311.14
10	13.37	62.57	10.63	67.29	85.29	252.86
11	10.8	42.86	10.03	73.71	197.14	921.43
12	8.06	42.86	39.13	36.64	41.06	79.71
Min	4.46	17.14	7.37	2.23	17.66	20.14
Max	22.7	62.57	39.13	73.71	501.43	1924
Average	12.55	36.87	20.37	34.91	100.97	376.69

CCA group to the HC and OV groups, were 0.9514 and 0.8611, respectively (Table 4). Similarly, the concentration of cf-miRNA in the CCA group differed significantly from that in the HC and OV groups. With a cut-off of >70.50 ng/ml plasma, the cf-miRNA test demonstrated a sensitivity of 83.33% and specificity of 95.83% for the differential diagnosis of CCA (Table 3). The AUCs of the ROC curves for plasma cf-miRNA, comparing the CCA group to the HC and OV groups, were 0.8542 and 0.8958, respectively (Table 4).

Furthermore, the AUCs of the ROC curves for plasma cfDNA and cf-miRNA, comparing the CCA group to the HC+OV groups, were 0.9063 and 0.8750, respectively (Figure 3 and Table 4). The combination of plasma cfDNA and cf-miRNA concentrations resulted in 83.33% sensitivity and 100% specificity, indicating a high sensitivity and specificity of the test (Table 3).

Analysis of cell-free DNA alteration by ultra-low-pass-whole-genome sequencing (ULP-WGS)

Ultra-Low-Pass Whole Genome Sequencing (ULP-WGS) was employed to detect changes in DNA sequences, revealing gains, amplifications, and deletions of cfDNA (Figure 4). These alterations were observed from an unspecified time point after OV infection. Intriguingly, these changes were predominantly evident in the CCA

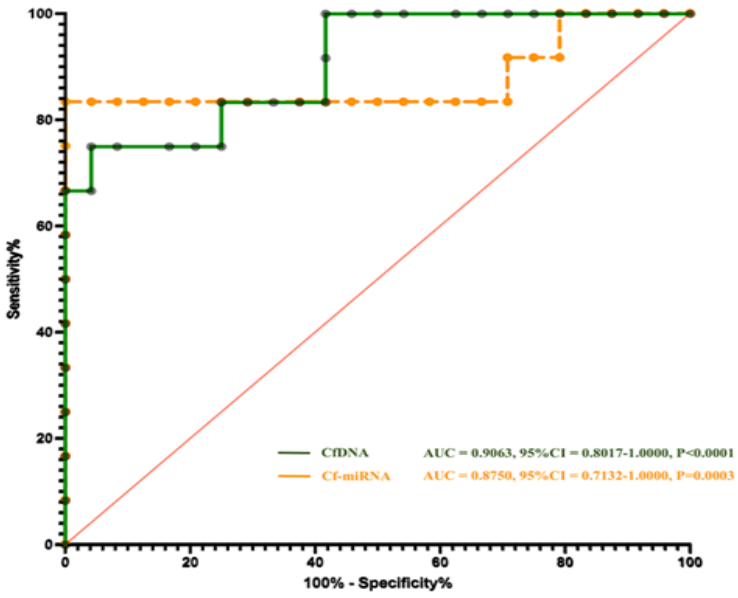

Figure 3. Area under the Curve (AUC) of the Receiver Operating Characteristic (ROC) Curve for Plasma cfDNA (Green Line) and cf-miRNA (Orange Line). The % sensitivity is plotted against 100% - % specificity on the x-axis and y-axis, respectively. The AUC of ROC curves and 95% CI are calculated and indicated in each curve. The highest AUC of ROC curve is observed in plasma cfDNA concentration.

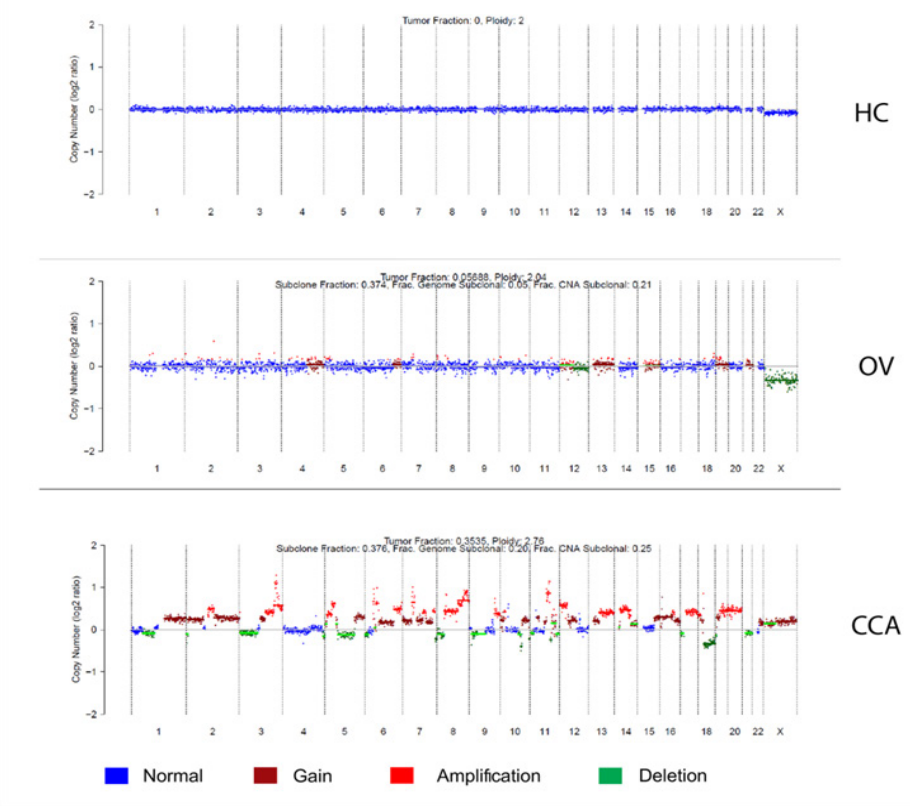

Figure 4. The Somatic Copy Number Alteration (SCNA) Analysis of Plasma Cell-Free DNA (cfDNA) of Healthy Control (HC) Subjects, *O. viverrini* infected (OV) subjects, and cholangiocarcinoma (CCA) subjects. The log2 ratio of copy number (x-axis) indicates the degree of chromosomal gain or loss, with blue representing normal, brown representing gain, red representing amplification, and green representing deletion. The number of chromosomes is on the x-axis (Chromosome number 1-22 and X chromosome).

group, where the ploidy of CCA increased to 2.76. The tumor fraction for OV and CCA was 5.7% and 35.4%, respectively. Notably, no significant DNA alterations were observed in the HC group.

Table 3. The Diagnostic Parameters of Plasma cfDNA and cf-miRNA for Cholangiocarcinoma

	cfDNA		cf-miRNA		cfDNA + cf-miRNA	
	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	75.00%	42.81% to 94.51%	83.33%	51.59% to 97.91%	83.33%	51.59% to 97.91%
Specificity	95.83%	78.88% to 99.89%	95.83%	78.88% to 99.89%	100.00%	85.75% to 100.00%
Positive Likelihood Ratio	18	2.57 to 126.05	20	2.89 to 138.53	-	-
Negative Likelihood Ratio	0.26	0.10 to 0.70	0.17	0.05 to 0.62	0.17	0.05 to 0.59
Positive Predictive Value	90.00%	56.24% to 98.44%	90.91%	59.08% to 98.58%	100.00%	-
Negative Predictive Value	88.46%	74.14% to 95.35%	92.00%	76.39% to 97.61%	92.31%	77.20% to 97.70%
Accuracy	88.89%	73.94% to 96.89%	91.67%	77.53% to 98.25%	94.44%	81.34% to 99.32%

Table 4. The Area under the ROC Curve (AUC) of Plasma cfDNA and cf-miRNA

		AUC	Standard error	95% CI	P-value
cfDNA	HC vs OV	0.7396	0.1018	0.5400-0.9392	0.0464
	HC vs CCA	0.9514	0.03939	0.8742-1.0000	0.0002
	OV vs CCA	0.8611	0.07757	0.7091-1.0000	0.0027
	HC+OV vs CCA	0.9063	0.05335	0.8017-1.0000	<0.0001
cf-miRNA	HC vs OV	0.7396	0.1018	0.5400-0.9392	0.0464
	HC vs CCA	0.9514	0.03939	0.8742-1.0000	0.0002
	OV vs CCA	0.8611	0.07757	0.7091-1.0000	0.0027
	HC+OV vs CCA	0.9063	0.05335	0.8017-1.0000	<0.0001

Discussion

Extensive research has been conducted on cfDNA and cf-miRNA as potential biomarkers for predicting, prognosing, and treating cancer [23, 24]. cfDNA and cf-miRNA are released from dead cells through processes such as apoptosis or necrosis [17, 25]. The increased circulation of these biomarkers in cancer patients has been demonstrated across various cancer types, attributed to the higher rate of cell death.

cfDNA has been suggested to have the capability to detect leukemias at least ten years earlier [10], underscoring its significance in early cancer diagnosis. Nevertheless, the potential of cfDNA or miRNA in CCA has not been firmly established. Thus, this study aims to explore their potential as biomarkers for this specific disease.

In the diagnostic approach for CCA, both cfDNA concentration and cf-miRNA exhibited superior results with high sensitivity, specificity, and accuracy when compared among CCA, HC, and OV. Nevertheless, for practicality, cost-effectiveness, and ease of use, the application of plasma cfDNA or cf-miRNA concentration for CCA diagnosis using a fluorescence dye-based method appears more suitable. The increase in cfDNA and cf-miRNA concentration was positively correlated and demonstrated 100% specificity when combined. However, it is noteworthy that the elevated concentration of cfDNA and cf-miRNA may also occur in other cancers and diseases [26], suggesting the need for further investigation. Therefore, to screen for CCA, this method should be implemented in high-incidence areas of CCA, particularly in endemic regions for liver fluke infections such as *O. viverrini*, *Clonorchis sinensis*, and *Opisthorchis felineus*. Notably, employing low-coverage sequencing, such as ULP-WGS, can predominantly detect changes in cfDNA, providing a cost-effective advantage, as deep sequencing may not be necessary [22]. ULP-WGS proves effective in differentiating CCA from OV and HC by utilizing the percentage of tumor fraction. The application of ULP-WGS for CCA diagnosis is suggested and warrants further analysis for its efficacy in large populations.

In conclusion, measuring cfDNA or cf-miRNA concentration through a fluorescence dye-based method offers advantages in terms of cost, ease, and efficacy. However, further research in large populations is essential.

Author Contribution Statement

Sattrachai Prasopdee: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, software, validation, writing - review and editing. Montinee Pholhelm: data curation, formal analysis, investigation, methodology. Siraphatsorn Yusuk: data curation, formal analysis, investigation, methodology. Sithichoke Tangphatsornruang: data curation, formal analysis, methodology, software. Kritiya Butthongkomvong: data curation, formal analysis, investigation, methodology. Teva Phanaksri: data curation, formal analysis, investigation, methodology. Anthicha Kunjantarachot: data curation, formal analysis, investigation, methodology. Jutharat Kulsantiwong: data curation, formal analysis, investigation, methodology. Smarn Tesana: conceptualization, formal analysis, methodology, supervision. Veerachai Thitapakorn: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, software, supervision, validation, writing - review and editing.

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General

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3. Chulabhorn International College of Medicine, Thammasat University, Fund Contract No. T3/2562 to Dr. Veerachai Thitapakorn and Dr. Sattrachai Prasopdee.

Ethical Declaration

This study was conducted in adherence to the ethical guidelines established by the Declaration of Helsinki and received approval from the Human Ethics Committee of Udon Thani Cancer Hospital, Udon Thani, Ministry of Public Health, Thailand (UCH-CT 11/2563). Prior to the commencement of blood collection, all participants provided signed informed consent.

Data Availability

All data has been included in this manuscript.

Approval

It was not approved by any scientific body or part of an approved student thesis.

Conflict of interest

The authors declared no conflict of interest for this study.

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