RESEARCH ARTICLE

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miR-1294 Upregulation as a Diagnostic Biomarker for Cholangiocarcinoma

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Abstract

Objective: To investigate plasma miR-1294 levels in cholangiocarcinoma (CCA) patients compared with healthy individuals and *Opisthorchis viverrini*-infected subjects. **Methods:** To assess its potential as a plasma-based diagnostic biomarker, plasma samples were collected and total RNA was extracted from 15 healthy controls (HC), 12 *O. viverrini* (OV)-infected individuals, and 33 patients with CCA. miR-1294 expression was quantified using RT-qPCR (MiRXES). **Results:** Relative expression analysis demonstrated a significant upregulation of plasma miR-1294 in patients with CCA compared with both HC and OV groups ($P < 0.0001$). Receiver operating characteristic (ROC) curve analysis showed that miR-1294 discriminated CCA from non-CCA subjects, yielding an area under the curve (AUC) of 0.8676, with a specificity of 96.30% and a sensitivity of 63.64%. **Conclusion:** The upregulation of plasma miR-1294 was investigated in this study. Despite these promising findings, the moderate sensitivity suggests that miR-1294 alone may not be sufficient as a standalone diagnostic marker. Nevertheless, its high specificity and non-invasive detectability highlight its potential utility as a complementary biomarker, particularly in combination with other diagnostic indicators for improved detection of CCA.

Keywords: miR-1294- diagnosis- cholangiocarcinoma- *Opisthorchis viverrini*

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Introduction

Cholangiocarcinoma (CCA), or bile duct cancer, is a highly lethal malignancy characterized by poor prognosis and limited therapeutic options [1–3]. In Southeast Asia, particularly Northeastern Thailand, chronic infection with the liver fluke *Opisthorchis viverrini* (OV) in combination with dietary exposure to nitrosamine compounds, represents a major risk factor for CCA development [4]. The typical asymptomatic clinical course delays diagnosis and consequently reduces treatment efficacy. Currently, diagnosis relies on imaging modalities followed by histological confirmation, underscoring the urgent need for reliable, minimally invasive biomarkers for early detection [5]. Although ultrasonography has been explored as a screening tool, its use imposes a medical burden and remains largely confined to research settings rather than routine clinical practice. MicroRNAs (miRNAs), a class of small noncoding RNAs that regulate gene expression at the post-transcriptional level, have emerged as promising circulating biomarkers across multiple cancer types [6–10]. Among these, miR-1294 has been reported to be aberrantly expressed in several malignancies, and implicated in the regulation of the PI3K/AKT/mTOR signaling pathway [11–13]. This pathway, together with Wnt/β-catenin signaling, plays a central role in CCA progression [14–21]. Despite these associations, data regarding the expression patterns and clinical relevance of miR-1294 in CCA remain limited.

Previous studies initially proposed plasma miR-1294

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as a candidate housekeeping miRNA based on comparable expression levels across healthy controls, *O. viverrini*–infected individuals, and CCA patients, as assessed by next-generation sequencing (NGS) [9] and the nCounter® SPRINT Profiler (NanoString) [8]. However, miR-1294 exhibited low abundance and inadequate stability for normalization purposes, and was therefore excluded as a reference miRNA [8]. Notably, subsequent analyses unexpectedly revealed an upregulation of miR-1294 in CCA patients, suggesting its potential as a noninvasive diagnostic biomarker. In the present study, reverse transcription quantitative PCR (RT-qPCR) was employed to further evaluate the diagnostic utility of miR-1294

Materials and Methods

Chemicals and reagents

EDTA blood collection tubes were purchased from Becton Dickinson. The circulating miRNA was extracted from plasma using miRNAeasy Serum/Plasma Kit (Qiagen, Gaithersburg, MD, USA). The concentration of isolated circulating RNA was measured using a microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, DE, USA). Quantitation of miR-1294 was performed using the ID3EAL miRNA qPCR Assay (MiRXES Pte Ltd, Singapore).

Subjects

This study was approved by the Human Ethics Committee of Udon Thani Cancer Hospital, Udon Thani, Ministry of Public Health, Thailand (UCH-CT 11/2563). A total of 60 subjects were recruited and categorized according to health statuses into three groups: 15 healthy control subjects (HC), 12 *Opisthorchis viverrini*-infected individuals (OV), and 33 patients with cholangiocarcinoma (CCA).

Subjects were stratified based on predefined clinical criteria. The HC group comprised individuals with normal physical examination findings, no evidence of hepatomegaly or jaundice, and negative fecal examination for *O. viverrini* eggs. The OV group included participants with positive fecal examinations for *O. viverrini* eggs but normal liver and bile ducts on ultrasonography. The CCA group consisted of individuals with abnormal liver findings on physical examination, ultrasonographic abnormalities of the liver and bile ducts, and histopathological confirmation of CCA. A summary of the demographic and clinical characteristics of the study population is presented in Table 1.

Plasma circulating RNA isolation and RT-qPCR analysis

Plasma circulating RNAs were isolated from 200 µL of plasma obtained from 60 participants, including 15 healthy controls (HC), 12 *O. viverrini*–infected subjects (OV), and 33 patients with cholangiocarcinoma (CCA). RNA extraction was performed using the miRNeasy Serum/Plasma Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, 100 ng of RNA was reverse-transcribed into cDNA using the ID3EAL cDNA Synthesis System with ID3EAL RT primers (MiRXES, Singapore) under the following conditions: 42°C for 30 min and 95°C for 5 min.

miRNA expression was quantified by RT-qPCR using the ID3EAL miRNA qPCR Assay (MiRXES, Singapore) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Each 20 µL reaction contained 5 µL of 1:10 diluted cDNA, 10 µL of ID3EAL miRNA qPCR Master Mix, and 2 µL of ID3EAL miRNA qPCR assay. Cycling conditions were as follows: denaturation at 95°C for 10 min, 40°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s.

Spike-in controls from the previous study [8], Iso 1 (RNA extraction) and Iso 2 (RT-qPCR), were measured concurrently, and their average Ct values were used to normalize miR-1294 expression in this study. Relative expression levels for each sample were calculated using the ΔCt and $2^{-\Delta Ct}$ methods. This approach ensured methodological consistency and facilitated evaluation of the diagnostic potential of miR-1294.

Statistical analysis

The IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA), was used for statistical

Table 1. The Demographic and Clinical Statuses of Participants

	Group		
	HC	OV	CCA
Sample size	15	12	33
Sex (Male/Female)	2/13	10/2	19/14
Age (year)			
Min/Max	25/60	19/64	48/87
Mean ± SD	37.6 ± 9.4	49.1 ± 11.4	66.5 ± 11.6
Alcohol consumption			
No	8 (53.3%)	1 (8.3%)	12 (36.4%)
Yes	7 (46.7%)	11 (91.7%)	21 (63.6%)
Smoking			
No	15 (100.0%)	5 (41.7%)	16 (48.5%)
Yes	0 (0%)	7 (58.3%)	17 (51.5%)
Raw fish-eating habit (Source of O. viverrini infection)			
No	11 (73.3%)	2 (16.7%)	4 (12.1%)
Yes	3 (20.0%)	10 (83.3%)	28 (84.8%)
Uncertain	1 (6.7%)	0 (0.0%)	1 (3.0%)
History of O. viverrini infection			
No	14 (93.3%)	10 (83.3%)	22 (66.7%)
Yes	0 (0%)	2 (16.7%)	10 (30.3%)
Uncertain	1 (6.7%)	0 (0.0%)	1 (3.0%)
Fermented food-eating habit (Source of nitrosamine)			
No	0 (0%)	0 (0%)	0 (0%)
Yes	15 (100.0%)	12 (100.0%)	32 (97.0%)
Uncertain	0 (0%)	0 (0%)	1 (3.0%)
Cancer stage			
Stage 1 (%)	0 (0%)	0 (0%)	1 (3.0%)
Stage 2 (%)	0 (0%)	0 (0%)	0 (0.0%)
Stage 3 (%)	0 (0%)	0 (0%)	1 (3.0%)
Stage 4 (%)	0 (0%)	0 (0%)	31 (93.9%)

analysis. The mean $2^{-\Delta Ct}$ values of miR-1294, along with their corresponding standard deviations (SD), were reported. Prior to performing one-way analysis of variance (ANOVA), homogeneity of variance was assessed using Levene's test. If the P-value was greater than 0.05, a standard ANOVA F-test followed by a Tukey Tukey's honestly significant difference (HSD), post hoc test was applied. Conversely, if the P-value was ≤ 0.05, Welch's ANOVA was performed, followed by Dunnett's T3 post hoc test.

For diagnostic evaluation, box plots and receiver operating characteristic (ROC) curves along with the corresponding area under the curve (AUC) were generated using GraphPad Prism version 10.6.0 for macOS (GraphPad Software, Boston, MA, USA; www.graphpad.com). The optimal cut-off value was determined based on the maximum likelihood ratio. Diagnostic performance parameters, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, were calculated using the MedCalc Diagnostic Test Evaluation Calculator (Version 23.3.7; MedCalc Software Ltd., Ostend, Belgium; https://www.medcalc.org/calc/diagnostic_test.php; accessed August 27, 2025).

Results

Investigation of miRNA expression by real-time RT-qPCR

The Ct and $2^{-\Delta Ct}$ values for each group were presented in Figures 1 and 2, respectively. Levene's test indicated unequal variances in $2^{-\Delta Ct}$ levels of miR-1294 among the groups ($F[2, 57] = 11.947$, $P < 0.05$). Accordingly, Welch's ANOVA was performed and revealed a significant overall difference among the groups (Welch's $F [2, 18.192] = 19.849$, $P < 0.001$). Post hoc analysis using Dunnett's T3 test demonstrated significant differences between the CCA and the HC groups ($P < 0.05$, 95% CI = 0.438607-1.348708), and between the CCA group and the OV group ($P < 0.05$, 95% CI = 0.847772-1.747513). In contrast, no significant difference was observed between the HC and OV groups ($P > 0.05$).

Diagnostic potential of miRNA

ROC curve analysis demonstrated that miR-1294 effectively discriminated CCA from non-CCA subjects (HC&OV) with an AUC of 0.8676 ($P < 0.0001$, 95% CI: 0.7721–0.9630). The biomarker showed the highest diagnostic performance when comparing the OV and CCA groups, yielding an AUC of 0.9192 ($P < 0.0001$, 95% CI: 0.8390–0.9994). miR-1294 also successfully differentiated

Table 2. A Receiver Operating Characteristic (ROC) Curve Analysis of miR-1294

	HC vs. OV	HC vs. CCA	OV vs. CCA	HC&OV vs. CCA
Area under curve (AUC)	0.7722	0.8263	0.9192	0.8676
Standard. Error	0.09406	0.0601	0.04091	0.04869
95% confidence interval (CI)	0.5879-0.9566	0.7085-0.9441	0.8390-0.9994	0.7721-0.9630
P-value	0.0168	0.0003	<0.0001	<0.0001

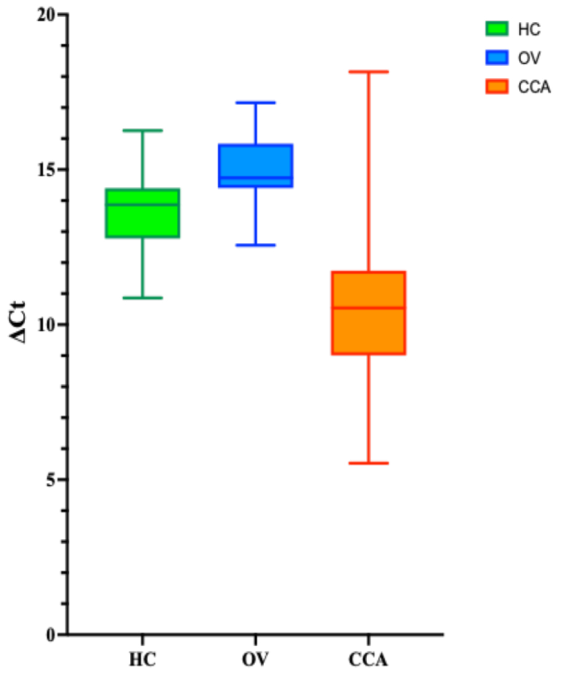

Figure 1. The Box-Plot of Ct of RT-qPCR of miR-1294. The Ct (y-axis) is plotted against each group (x-axis). The green, blue, and orange colors are used to indicate the HC, OV, and CCA group, respectively.

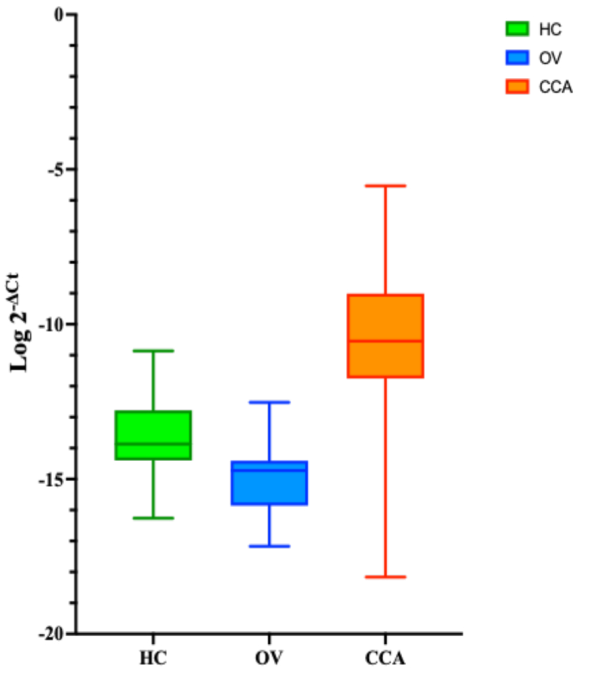

Figure 2 The box plot of Log $2^{-\Delta Ct}$ Levels of RT-qPCR of miR-1294. The green, blue, and orange colors are used to indicate the HC, OV, and CCA group, respectively. The y-axis shows the Log$2^{-\Delta Ct}$ levels and groups in the x-axis.

Table 3. The Interpretation of miR-1294 of HC, OV and CCA. The cut-off Ct of <37.68 are Used for Calculation

	CCA					
	Present	N	Absent	N	Total	
Positive	True Positive (TP)	21	False Positive (FP)	1	TP+ FP	22
Negative	False Negative (FN)	12	True Negative (TN)	26	FN+ TN	38
Total	TP+FN	33	FP+TN	27		

Table 4. Diagnostic Parameters of miR-1294 of HC&OV vs. CCA Using Cut-off <37.68.

	Value	95% CI
Cut-off (Ct)	<37.68	
AUC of ROC	0.8676	0.7721-0.9630
Sensitivity	63.64%	45.12% to 79.60%
Specificity	96.30%	81.03% to 99.91%
Positive Likelihood Ratio	17.18	2.47 to 119.63
Negative Likelihood Ratio	0.38	0.24 to 0.60
Positive Predictive Value	95.45%	75.10% to 99.32%
Negative Predictive Value	68.42%	57.83% to 77.39%
Accuracy	78.33%	65.80% to 87.93%

HC from CCA (AUC = 0.8263, P = 0.0003, 95% CI: 0.7085-0.9441) and HC from OV (AUC = 0.7722, P = 0.0168, 95% CI: 0.5879-0.9566,) (Table 2 and Figure 3). Using a cut-off value of <37.68, 21 true positives, 26 true negatives, 1 false positive, and 12 false negatives were identified and used to calculate diagnostic performance (Table 3). At this threshold, miR-1294 demonstrated a sensitivity of 63.64%, specificity of 96.30%, positive predictive value (PPV) of 95.45%, negative predictive value (NPV) of 68.42%, and an overall accuracy of 78.33% in distinguishing CCA from HC and OV groups (Table 4).

Discussion

Currently, no effective diagnostic method exists for the detection of cholangiocarcinoma (CCA), despite its increasing global incidence. Numerous efforts have been undertaken to identify reliable biomarkers, including cell-free DNA, cell-free RNA, cell-free miRNA, exosomal miRNA, and circulating tumor cells [6–9, 22–24]. However, none of these approaches have yet been translated to a diagnostic tool ready for routine

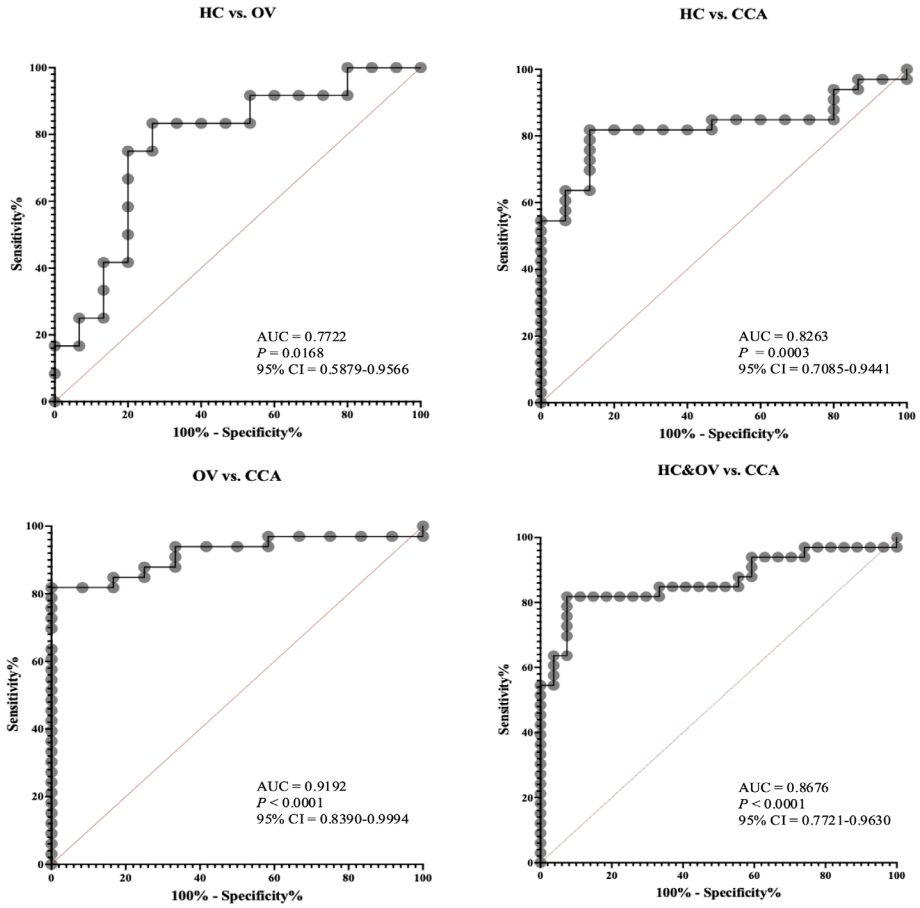

Figure 3. A Receiver Operating Characteristic (ROC) Curve Analysis of miR-1294 of HC vs. OV (upper left), HC vs. CCA (upper right), OV vs. CCA (lower left), and HC&OV vs. CCA (lower right). The AUC, 95% confidence interval (CI), and P values are indicated.

clinical use, and several biomarkers remain under rigorous investigation.

In the present study, we investigated the potential of circulating or cell-free miR-1294 as a diagnostic biomarker for CCA. Interestingly, the diagnostic potential of miR-1294 was an unexpected finding. The differential expression of miR-1294 observed in this study may appear inconsistent with our previous work that suggested miR-1294 as a candidate housekeeping miRNA. In earlier discovery-phase profiling studies using NGS and NanoString platforms that are excellent for broad profiling from pooled plasma, miR-1294 showed relatively comparable expression levels across all cohorts [9]. However, miR-1294 was detected at very low abundance in those datasets, leading to its exclusion as a reference.

In the present study, individual plasma samples from 60 subjects were analyzed using RT-qPCR following an optimized RNA isolation protocol. Unlike the previous discovery-phase studies that used pooled plasma samples, this approach eliminated the pooling effect and allowed assessment of biological variation among individuals. The RT-qPCR method also provided higher analytical sensitivity for detecting low-abundance miRNAs.

Although miR-1294 has been proposed as a prognostic indicator in esophageal squamous cell carcinoma [25], gastric cancer [26], and ovarian cancer [27], its diagnostic utility has not yet been established in any cancer type. Furthermore, data regarding circulating or cell-free miR-1294 levels in plasma or serum remain limited and inconclusive [28].

Dysregulation of miR-1294 has also been implicated in several other malignancies, with different molecular targets across signaling pathways, including ERK in breast cancer [29], TGFBR1 in hepatocellular carcinoma [30], c-Myc [31], and EGFR [15]. Notably, miR-1294 is involved in the PI3K/AKT/mTOR and Wnt/β-catenin signaling pathways, both of which are commonly upregulated and contribute to CCA cell proliferation [12, 15-16, 18–21].

In many solid tumors, miR-1294 acts as a tumor suppressor; its downregulation leads to the overactivation of targets like AKT1, IGF1R, and c-Myc, often through sponging by long non-coding RNAs or circular RNAs [13, 17, 27]. Conversely, miR-1294 has previously been shown to be significantly upregulated in acute lymphocytic leukemia (ALL), where it promotes cell proliferation via the Wnt/β-catenin signaling pathway by repressing inhibitors like SOX15, thereby activating Wnt/β-catenin signaling [32]. Supporting this, human umbilical cord mesenchymal stem cell therapy has been shown to affect CCA cell proliferation through the Wnt/β-catenin pathway [33, 34]. Additionally, a proteomic study of the CCA plasma proteome identified the PI3K/AKT pathway as a key regulator, with PIK3CB highlighted as a potential biomarker [35]. Together, these findings underscore the potential role of miR-1294 as a diagnostic biomarker.

Interestingly, our study revealed that plasma levels of miR-1294 were significantly elevated in CCA patients compared with healthy controls (HC) and patients with *O. viverrini* (OV) infection, a result that contrasts with its reported low expression in several tumor tissues and cancer cell lines [11]. This discrepancy remains unexplained, as the behavior of miR-1294 in body fluids such as plasma and serum is poorly characterized. Moreover, the role of miR-1294 in CCA pathogenesis has not yet been elucidated. We propose future mechanistic studies of miR-1294 in CCA. These include gain- and loss-of-function experiments using miR-1294 mimics or inhibitors in CCA cell lines, evaluation of downstream target genes, and functional assays such as proliferation, migration, invasion, and apoptosis analyses. One proposed mechanism, demonstrated in ALL, involves SOX15 downregulation and Wnt3a/β-catenin upregulation, which enhances proliferation and decreases apoptosis [32].

While the sensitivity of miR-1294 as a diagnostic marker was moderate (63.64%), its specificity was remarkably high (96.30%). These results suggest that miR-1294 may serve as a valuable diagnostic biomarker for CCA, particularly as a confirmatory rather than a screening marker. Given these limitations, we recommend quantification of total circulating miRNA or cell-free DNA concentration for initial CCA screening [6], followed by confirmatory testing with specific miRNA species.

The present study has a relatively moderate sample size (n=60) and recruitment from a single geographic area where *O. viverrini* is endemic. Therefore, we emphasize the need for future validation involving larger cohorts and more diverse populations, particularly non-liver fluke-associated CCA to confirm broader clinical applicability.

In summary, this study provides the first evidence supporting a diagnostic role for miR-1294 in CCA, potentially contributing to the development of more accurate and non-invasive diagnostic approaches for this challenging malignancy.

The present study aimed to assess circulating miR-1294 as a diagnostic biomarker for cholangiocarcinoma (CCA) in plasma. Circulating miR-1294 was upregulated in the plasma of CCA patients and was able to distinguish CCA cases from healthy individuals and those infected with *O. viverrini*, with 96.30% specificity and 63.64% sensitivity. These findings suggest that miR-1294 holds promise as a diagnostic biomarker for CCA. To our knowledge, this is the first study to report a diagnostic role for miR-1294 in CCA.

Author Contribution Statement

Conceptualization: Thitapakorn V. Data Curation: Supradit K., Phanaksri T., Prasopdee S., Tangphatsornruang S., Sangsrakru D., Wongprasert K., Pholhelm M., Butthongkomvong K., Kulsantiwong J., Kunjantarachot A., Thitapakorn V. Formal Analysis: Supradit K., Phanaksri T., Prasopdee S., Tangphatsornruang S., Sangsrakru D., Wongprasert K., Pholhelm M., Butthongkomvong K., Kulsantiwong J., Kunjantarachot A., Thitapakorn V. Funding Acquisition: Supradit K., Prasopdee S., Thitapakorn V. Investigation: Supradit K., Phanaksri T., Prasopdee S., Tangphatsornruang S., Sangsrakru D., Wongprasert K., Pholhelm M., Butthongkomvong K., Kulsantiwong J., Kunjantarachot A., Thitapakorn V. Methodology: Supradit K., Phanaksri T., Prasopdee S., Tangphatsornruang S., Wongprasert K., Pholhelm M.,

Butthongkomvong K., Kulsantiwong J., Kunjantarachot A., Thitapakorn V. Validation: Supradit K., Phanaksri T., Prasopdee S., Thitapakorn V. Writing: Original Draft: Supradit K., Phanaksri T., Prasopdee S., Pholhelm M., Thitapakorn V. Writing, Review and Editing: Supradit K., Phanaksri T., Prasopdee S., Pholhelm M., Thitapakorn V.

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Ethical Declaration

This study was approved by the Human Ethics Committee of Udon Thani Cancer Hospital, Udon Thani, Ministry of Public Health, Thailand (UCH-CT 11/2563).

Data Availability

All data is included in this publication

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT to check for typographical and grammatical errors with the prompt "Typo and grammar check". After using this tool, the author(s) reviewed and edited the content for the final content of the publication.

Conflict of interest

The authors declare no competing interests for this study.

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