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TRAN HAI YEN

**STUDY ON THE METHYLATION STATUS OF *SEPTIN9*
GENE IN PLASMA AND TUMOR TISSUE SAMPLES OF
PATIENTS WITH HEPATOCELLULAR CARCINOMA**

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Scientific Instructor:

- 1. PhD. Ho Huu Tho**
- 2. PhD. Duong Quang Huy**

Critique 1: Associate Professor, PhD. Nguyen Canh Binh

Critique 2: Associate Professor, PhD. Nguyen Thi Xuan

Critique 3: Associate Professor, PhD. Vu Anh Hai

The thesis is defended at the University-level Thesis Grading Council
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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common types of cancer globally and in Vietnam, particularly in men, and is associated with a relatively high mortality rate. Early detection of HCC is a critical factor for improving treatment outcomes and prognosis. The development of novel biomarkers is expected to replace traditional markers such as alpha-fetoprotein (AFP) in the diagnosis and prognosis of HCC in the future. One promising research direction involves the investigation of epigenetic alterations, particularly abnormal DNA methylation at promoter regions of tumor suppressor genes, including methylation of the *SEPTIN9* gene (m*SEPT9*).

The *SEPTIN9* gene is a key regulator of cell division and tumor suppression. Hypermethylation of this gene promotes cellular proliferation, including proliferation of hepatocytes. While *SEPTIN9* expression is commonly observed in normal tissues, it is often reduced or silenced in HCC patients due to aberrant hypermethylation. Parallel analysis of m*SEPT9* in both plasma and tumor tissue samples from HCC patients not only helps elucidate the relationship between tumor-specific genetic alterations and the presence of circulating cell-free DNA, but also provides insights into the accuracy and biological relevance of m*SEPT9*. This, in turn, supports the potential application of plasma m*SEPT9* as a biomarker for diagnosis, prognosis, and therapeutic monitoring in HCC.

1. OBJECTIVES

1.1. To assess the degree of SEPTIN9 gene methylation in plasma and compare it with tumor tissue samples, while analyzing its correlation with selected clinical and paraclinical features in patients diagnosed with hepatocellular carcinoma.

1.2. To evaluate the diagnostic utility of plasma SEPTIN9 gene methylation levels in identifying hepatocellular carcinoma.

2. CONTRIBUTIONS

This is the first study in Vietnam to quantify plasma m*SEPT9* levels using the Semi-nested quantitative RT-PCR (sqRT-PCR) technique with TaqMan probes combined with Extension Blocker Probe (ExBP) technology. The study was conducted on plasma samples from 133 patients with hepatocellular carcinoma (HCC), 70 patients with chronic liver disease (CLD), 40 healthy controls (HD), and 58 tumor tissue samples.

The results showed a moderate positive correlation between mSEPT9 levels in plasma and tumor tissue among the 58 HCC patients ($r = 0.33$; $p < 0.01$). The correlation was stronger in subgroups with typical histological features, large tumors (>50 mm), bilobar lesions, and advanced disease stages (BCLC B/C/D). Additionally, plasma mSEPT9 levels were significantly associated with tumor progression indicators such as portal vein thrombosis, tumor diameter >50 mm ($p = 0.01$), bilobar liver involvement ($p = 0.004$), elevated AST and ALT levels ($p = 0.002$ and 0.001 , respectively), and plasma AFP levels >20 ng/mL ($p = 0.04$).

Plasma mSEPT9 alone demonstrated good diagnostic performance for HCC compared to the overall control group, with an AUC of 0.827. At the threshold of 26.94 cycles, it achieved a sensitivity of 82.71% and specificity of 72.07%. Against the CLD group, the AUC was 0.824, with a sensitivity of 84.21% and specificity of 72.86% at a threshold of 28.25 cycles. Notably, mSEPT9 also showed good diagnostic efficacy in HCC patients with $\text{AFP} \leq 20$ ng/mL (AUC = 0.817; $p < 0.001$) and in early-stage HCC (BCLC 0/A) (AUC = 0.771; $p < 0.001$).

The combination of plasma mSEPT9 and AFP yielded excellent diagnostic accuracy for differentiating HCC from CLD, with an AUC of 0.90, sensitivity of 87.22%, and specificity of 77.14%—significantly higher than either marker alone ($p < 0.001$).

3. LAYOUT

The dissertation comprises a total of 138 pages, including: 2 pages for the introduction, 33 pages for the literature review, 28 pages outlining the research subjects and methodology, 39 pages presenting the research findings, 32 pages dedicated to discussion, 1 page addressing the study's limitations, 2 pages for the conclusion, and 1 page for recommendations. The dissertation contains 45 tables, 17 charts, 9 figures, and 3 diagrams. A total of 145 references were cited, of which 16 are in Vietnamese and 129 in English.

Chapter 1. OVERVIEW

1.1. *SEPTIN9* Gene

1.1.1. *Structure*

SEPTIN9 gene is located on the long arm of chromosome 17, at position 17q25.3, and spans approximately 240 kilobases. Through the process of alternative splicing, various *SEPTIN9* transcripts are generated. This diversity results in multiple isoforms of the septin-9 protein, each with a distinct N-terminal region, while all retain a conserved central GTP-binding domain (GTPase).

1.1.2. *Function*

SEPTIN9 encodes septin-9, a member of the septin family of proteins. Septin-9 plays a critical role in maintaining cellular architecture and function. It is essential in processes such as cell division, establishment of cell polarity, cell migration, and cytoskeletal stabilization. During mitosis, septin-9 contributes to the formation of the contractile ring at the division site, ensuring effective cytokinesis. Additionally, by interacting with microtubules and actin filaments, septin-9 helps maintain cell shape and guide directional movement within tissues. It is also involved in stress response, apoptosis regulation, and cell growth control. Notably, aberrant methylation of the *SEPTIN9* promoter region can lead to suppressed gene expression, thereby compromising its regulatory role in cell proliferation.

1.2. Methylation of the *SEPTIN9* Gene and Carcinogenic Mechanisms

1.2.1. *SEPTIN9* Gene Methylation

Methylation of the *SEPTIN9* gene is an epigenetic modification in which methyl groups are added to cytosine residues within CpG islands located in the gene's promoter region. Under physiological conditions, this region remains unmethylated, permitting the binding of RNA polymerase and initiation of transcription, leading to the synthesis of septin-9 protein. Septin-9 is a key regulatory molecule in cell division, cytoskeletal integrity, and proliferation homeostasis.

1.2.2. *Oncogenic Mechanism of mSEPT9*

Under normal physiological conditions, the promoter region of *SEPT9* is unmethylated. However, when hypermethylation occurs at the CpG islands within this region, gene transcription is silenced, resulting in a significant reduction in septin-9 protein expression. This leads to loss of cell cycle control, unregulated proliferation, and increased risk of malignant transformation. Furthermore, reduced septin-9 expression has been associated with Enhanced HIF-1 transcriptional activity, Sustained JNK pathway activation (via inhibition of JNK degradation), Upregulated RhoA signaling.

These changes promote not only tumor development but also invasiveness and metastatic potential. Due to the high specificity of this epigenetic alteration, m*SEPT9* has emerged as a valuable biomarker in cancer diagnosis and prognosis.

1.2.3. mSEPT9 in Hepatocellular Carcinoma

Villanueva et al. (2015) demonstrated that the *SEPTIN9* gene exhibits high levels of methylation in HCC. In their analysis of 331 HCC tumor samples collected from hepatic resections, 61% showed promoter hypermethylation, particularly within CpG islands near the transcription start site of *SEPTIN9*.

In a study investigating the immune microenvironment of liver tumors, Park et al. (2025) found that *SEPTIN9* interacts with PAI-1, a pro-tumorigenic protein, contributing to a specialized immune context within the tumor microenvironment. This interaction was associated with higher histological grades, poorer overall prognosis, and potential implications for immunotherapy responsiveness.

Several international studies have highlighted the diagnostic and prognostic potential of plasma m*SEPT9* in HCC, including works by Oussalah et al. (2018), Kotoh et al. (2020), and Saeki et al. (2023). However, no study in Vietnam has yet examined this issue in depth.

Chapter 2. OBJECTS AND METHODS

2.1. Subjects of study

Including 244 patients divided into 3 groups: HCC group, CHD group and HD group, all were examined and/or treated at Military Hospital 103.

Study group: 133 HCC patients

Control group: 70 patients with cerebral palsy (cirrhosis, chronic hepatitis) and 41 patients with chronic hepatitis.

Study period: from 1/2021 to 6/2024.

2.1.1. Selection criteria

- HCC group: diagnosis according to the standards of the Ministry of Health of Vietnam in 2020.

- CHD group: Cirrhosis patients are clinically and subclinically diagnosed with all 2 syndromes of hepatic insufficiency and portal hypertension. Diagnosis of chronic hepatitis B and C according to the guidance of the Ministry of Health of Vietnam.

- HD group: no chronic liver disease, periodic health check-ups at Military Hospital 103.

2.1.2. Exclusion criteria

- HCC group: HCC patients who have been treated. Co-morbid with other types of cancer. Patients did not agree to participate in the study or did not have sufficient data according to the research protocol.

- CHD group: Patients are having serious complications. Patients did not agree to participate in the study or did not have sufficient data according to the research protocol.

2.2. Research methods

2.2.1. Research design

A comparative cross-sectional descriptive study with control groups

2.2.2. Sample size and sample selection method:

Sample size for correlation analysis between mSEPT9 levels in tumor tissue and plasma samples in patients with HCC. The sample

size was calculated using the paired t-test formula, based on the findings of He N. et al. (2019). The minimum required sample size was 45 paired samples. In this study, 58 pairs of tumor tissue and plasma samples were actually analyzed.

Sample size for evaluating the diagnostic value of plasma mSEPT9 in HCC. The sample size was determined based on sensitivity and specificity estimation formulas, using reference data from Oussalah A. et al. (2018). The minimum required sample size was 88 patients. The actual sample size in this study included 133 patients with HCC and 111 individuals in the reference group.

Sampling methods: A purposive sampling approach was applied. All patients diagnosed with HCC, CLD, and HD who met the inclusion and exclusion criteria were consecutively enrolled until the required sample size was fulfilled.

2.2.3. Means and chemicals used in research

Equipment and reagents were used for blood testing, diagnostic imaging, and quantification of mSEPT9 levels in the analyzed samples.

Table 2.1. Primer Sequences for Semi-Nested Real-Time PCR

Môì/Probe	Sequence	Amplification Round
<i>SEPT9_Fo</i>	<u>CGACGTAAAACGACGGCCAGT-</u> CAACCCAACACCCACCT	Round 1
<i>SEPT9_Ro</i>	<u>CACACAGGAAACAGCTATGACCATGGATTC-</u> GTTGTTTATTAGTTATTATGT	
<i>SEPT9_Fi</i> Forward Primer	TGGATTTTGTGGTTAATGT- AAATAATCCCATCCAACTA	Round 2
TaqMan probe (<i>SEPT9_P</i>)	FAM-TTAACCGCGAAATCCGAC-BHQ1	
<i>SEPT9_Ri</i>	CACACAGGAAACAGCTATGACCATG	

Reverse Primer		
Forward Primer -Ft	AATCCGAAATAATCCCATCCAACTA	Total mSEPT9 Quantification
<i>SEPT9_Ri</i> Reverse Primer	CACACAGGAAACAGCTATGACCATG	

2.2.4. Steps to proceed

Collect clinical and paraclinical data of patients with HCC through appropriate examinations and tests. Assess liver function using the Child-Pugh and ALBI scores. Classify disease stage according to the 2018 BCLC staging system.

Collect 244 blood samples (plasma separated) from patients with HCC, benign liver tumors, and healthy individuals; and 58 tumor tissue samples from 58 patients with HCC.

Determine mSEPT9 levels in plasma and tissue samples at the Military Institute of Medical and Pharmaceutical Research, Military Medical Academy, following these steps:

- + Extract DNA from plasma and tissue samples using the corresponding kits, following the manufacturer's instructions (Qiagen, Germany).

- + Perform bisulfite conversion of the extracted DNA using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, USA).

Quantify mSEPT9 expression using sqRT-PCR with TaqMan probes and extendable blocking probe (ExBP) technology. A two-round amplification process is employed to achieve high specificity and sensitivity, especially in cases with low tumor DNA concentration. The ΔC_t value reflects the level of mSEPT9 expression (the higher the ΔC_t value, the lower the mSEPT9 expression). ΔC_t is treated as a continuous quantitative variable.

2.2.5. Research criteria

Clinical and paraclinical indicators, tumor burden, histopathology, liver function, and disease stage.

mSEPT9 levels in tumor tissue and plasma samples in the HCC group. Plasma mSEPT9 levels in the HCC group, the subgroup of HCC patients with AFP < 20 ng/mL, and early-stage HCC (BCLC stage 0/A), compared with the benign liver tumor group and the healthy control group.

Diagnostic value: calculate the AUC of plasma mSEPT9 levels in diagnosing HCC, HCC with AFP < 20 ng/mL, using benign liver tumors, healthy controls, and the combined non-cancer group (benign + healthy controls) as reference groups. Evaluate the early diagnostic value in BCLC stage 0/A and compare the performance of combined markers (mSEPT9 + AFP) versus each marker alone.

Determine the association between plasma mSEPT9 levels (as indicated by Δ Ct values) and age, sex, risk factors (HBV infection, HCV infection, alcohol abuse), clinical characteristics, blood test results, AFP levels, liver dysfunction (Child-Pugh score, ALBI grade), tumor burden, tumor cell differentiation in histopathology, and disease stage according to BCLC classification.

2.3. Data analysis: Data are processed by medical statistical methods, using SPSS 25.0 and Medcal 22.0 software.

2.4. Research ethics

The research outline was approved by the Ethics Council of the Military Medical Academy, Decision No. 15/2021/CNChT-HDDD.

All patients voluntarily participate in the research, and the implementation process is strictly in accordance with the regulations of the Ministry of Health. Mutation tests and gene expression do not affect the treatment of the disease or the patient's financial burden.

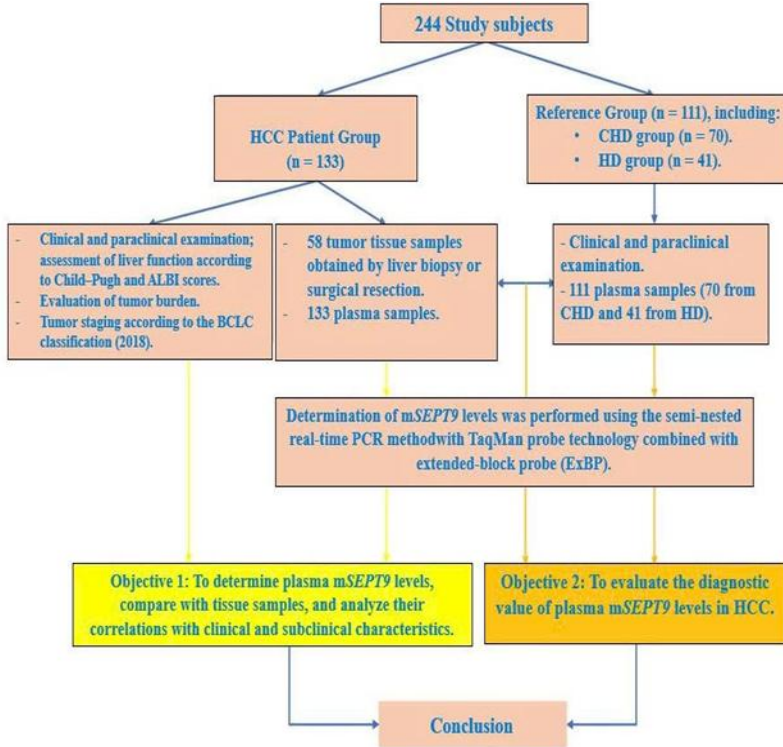


Figure 2.1. The research process flowchart

3.1. Characteristics of the research group

+ The average age of patients with HCC was 61.82 ± 11.18 years, with males accounting for 94.74%. Fatigue and right hypochondriac pain were reported in 48.12% and 43.61% of cases, respectively.

+ Median values of several liver function tests were within normal limits. The proportion of patients with $\text{AFP} \leq 20 \text{ ng/mL}$ was 42.11%.

+ Most HCC patients had a single tumor (61.65%), with tumor size > 5 cm in 43.61% of cases. Portal vein thrombosis was present in 26.32%, and distant metastasis in 6.77%.

+ Tumor tissue samples predominantly showed trabecular architecture (60.3%) and typical cellular morphology (72.4%). Moderate cellular differentiation was found in 84.5% of cases according to WHO grading, and grade II differentiation in 63.8% according to the Edmondson-Steiner (ES) classification.

+ Liver function was classified as Child-Pugh A in 84.96% of patients, with 65.41% in ALBI grade 2. Tumors were in early stages (BCLC stage 0/A) in 53.39% of patients, according to the 2018 BCLC classification.

3.2. Plasma mSEPT9 level in relation to tumor tissue and selected Clinical and Paraclinical characteristics

3.2.1. Plasma mSEPT9 Levels Compared with Tumor Tissue

Analysis of 58 paired samples of tumor tissue and plasma collected from the same patients with HCC showed that 57 samples (98.3%) exhibited specific amplification signals for mSEPT9.

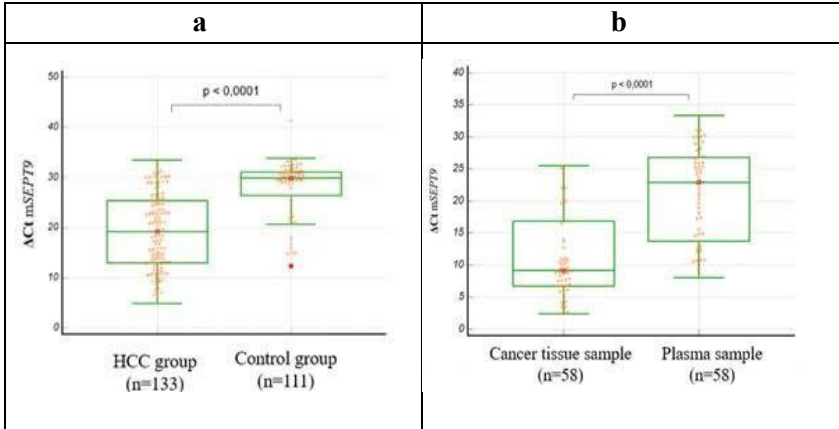


Figure 3.1. ΔC_t Values of mSEPT9 in Study Samples
a) HCC group & non HCC group b) Tumor tissue & plasma samples

The median ΔC_t in the HCC group was significantly lower than that in the reference group, and markedly lower in tumor tissue compared to plasma samples ($p < 0.001$).

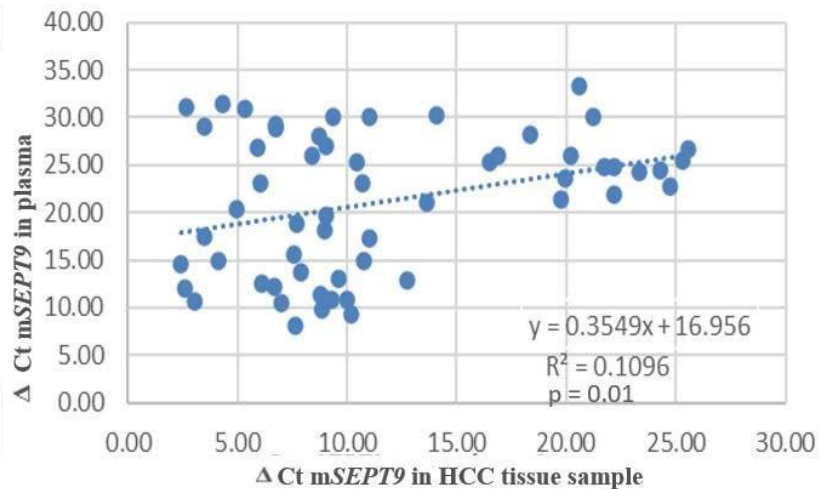


Figure 3.2. The correlation between mSEPT9 in plasma and tumor tissue ($n = 58$)

A weak positive correlation was observed between the ΔCt values of plasma and tumor tissue mSEPT9 (correlation coefficient $r = 0.33$; $p = 0.01$).

3.2.2. The association between plasma mSEPT9 levels and certain clinical and paraclinical characteristics

Table 3.1. The correlation between ΔCt values of plasma mSEPT9 and selected laboratory parameters and liver function assessment scores

Chỉ số $\Delta\text{Ct mSEPT9}$	Hệ số tương quan (rho)	p
Platelets (T/L)	0,08	0,33
Prothrombin(%)	0,13	0,11
AST (U/L)	-0,19	0,02
ALT (U/L)	-0,13	0,14
Bilirubin TP($\mu\text{mol/L}$)	-0,16	0,07
Albumin (g/L)	0,23	0,01
AFP (ng/ml)	-0,25	0,005
Child-Pugh point	-0,18	0,03
ALBI point	-0,25	0,004

The mSEPT9 level showed a weak negative correlation with AST activity, plasma AFP concentration, Child–Pugh score, and ALBI score, and a positive correlation with albumin concentration.

Table 3.2. The association between plasma mSEPT9 and tumor burden (n = 133)

Tumor burden			mSEPT9 in plasma (Δ Ct)		
			Median (Quartile)	p	
Indices		HCC ^a (n = 133)	CHD ^b (n = 70)	HD ^c (n = 41)	p
mSEPT9 (Δ Ct)	Median	19,20	29,93	29,29	p _{a-b} < 0,001
	Quartile	12,97 - 25,37	26,43 - 31,10	25,96 - 30,39	p _{a-c} < 0,001
	Min - Max	4,88 - 33,50	12,37 - 41,34	20,75 - 33,70	p _{b-c} > 0,05
Tumor diameter (mm)		< 50 (n=75)	21,46 (14,54 - 26,66)		0,003
		rho	-0,4		< 0,001**
Inferior vena cava thrombosis		No (n=98)	21,43 (14,29 - 26,49)		< 0,001*
		Yes (n=35)	13,00 (9,53 – 20,64)		
Extrahepatic metastasis		No (n=124)	19,29 (13,00 - 25,40)		0,62*
		Yes (n=9)	15,59 (9,61 - 26,14)		

The patients with tumors involving both hepatic lobes, ≥ 2 tumor nodules, tumor size ≥ 50 mm, or portal vein thrombosis had significantly lower Δ Ct values of mSEPT9.

3.3. Diagnostic value of plasma mSEPT9 levels in hepatocellular carcinoma

3.3.1. Diagnostic value

Table 3.3. The comparison of plasma mSEPT9 levels among study subjects

Indices		HCC ^a (n = 133)	CHD ^b (n = 70)	HD ^c (n = 41)	p
mSEPT9 (ΔCt)	Median	19,20	29,93	29,29	p_{a-b} < 0,001
	Quartile	12,97 - 25,37	26,43 - 31,10	25,96 - 30,39	p_{a-c} < 0,001
	Min - Max	4,88 - 33,50	12,37 - 41,34	20,75 - 33,70	p_{b-c} > 0,05

The median plasma mSEPT9 level in the HCC group was significantly higher than that in CHD group and HD groups ($p < 0.001$).

Table 3.4. The diagnostic value of plasma mSEPT9 in hepatocellular carcinoma compared with reference groups

Index	AUC	Cut-off value	95% CI	Se (%)	Sp (%)	OR	p
Non-cancer control group (CHD và HD) (n=111)							
mSEPT9 (ΔCt)	0,827	26,94	0,764 - 0,874	82,71	72,07	11,72	< 0,001
CHD control group (n=70)							
mSEPT9 (ΔCt)	0,824	28,25	0,764 - 0,874	84,21	72,86	14,32	< 0,001
HD control group (n=41)							
mSEPT9 (ΔCt)	0,833	24,75	0,769 - 0,885	71,43	87,80	17,35	< 0,001

- Plasma mSEPT9 demonstrated good diagnostic performance for HCC compared with the non-cancer reference group, with an AUC of 0.827 ($p < 0.001$).

- Plasma mSEPT9 showed good diagnostic value for differentiating HCC from benign liver disease, with an AUC of 0.824 ($p < 0.001$).

- Plasma mSEPT9 also exhibited good diagnostic performance for distinguishing HCC from normal controls, with an AUC of 0.833 ($p < 0.001$).

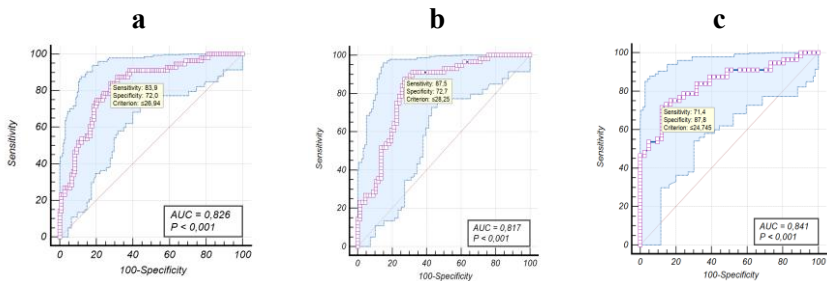


Figure 3.3. Diagnostic value of mSEPT9 for hepatocellular carcinoma (HCC)

- (a) Compared with the non-cancer group;
(b) Compared with the benign liver disease CHD group; (c) Compared with HD group.

3.3.2. Diagnostic value in cases with AFP < 20 ng/mL

Table 3.5. The plasma mSEPT9 levels in the HCC group with AFP < 20 ng/mL compared with the CHD and HD groups

Index		AFP < 20ng/mL ^a in HCC (n = 56)	CHD ^b (n = 70)	HD ^c (n = 41)	p
mSEPT9 (ΔCt)	Median	20,97	29,93	29,29	p_{a-b} < 0,05
	Quartile	14,89 - 25,39	26,43 - 31,10	25,96 - 30,39	p_{a-c} < 0,05
	Min - Max	6,03 - 31,06	12,37 - 41,34	20,75 - 33,70	p_{b-c} > 0,05

The plasma mSEPT9 ΔCt value in HCC patients with AFP < 20 ng/mL was significantly lower than that in the two reference groups ($p < 0.05$).

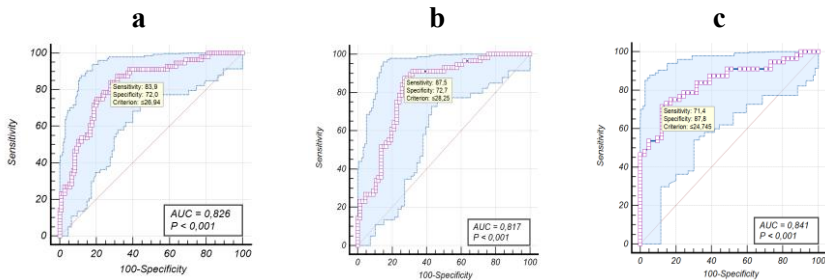


Figure 3.4. The diagnostic value of mSEPT9 for HCC with AFP < 20 ng/mL

- Compared with the non-cancer group, plasma mSEPT9 showed good diagnostic performance for HCC patients with AFP < 20 ng/mL, with an AUC of 0.826.

- Compared with the benign liver disease (BGL) group, plasma mSEPT9 also demonstrated good diagnostic value for HCC, with an AUC of 0.817, $p < 0.001$.

3.3.3. Diagnostic value by stage

Table 3.6. The comparison of plasma mSEPT9 levels in very early and early-stage HCC patients versus the CHD and HD groups

Index		HCC at very early and early stages ^a (n = 71)	CHD ^b (n = 70)	HD ^c (n = 41)	p
mSEPT9 (ΔCt)	Median	22,71	29,93	29,29	p_{a-b} < 0,001
	Quartile	15,05 - 27,71	26,43 - 31,10	25,96 - 30,39	p_{a-c} < 0,001
	Min - Max	6,02 - 33,50	12,37 - 41,34	20,75 - 33,70	p_{b-c} = 0,091

The plasma mSEPT9 ΔCt value in very early and early-stage HCC patients (BCLC 0/A) was significantly lower than that in the CHD and HD groups ($p < 0.001$).

Table 3.7. The mSEPT9 in plasma for the diagnosis of very early and early-stage HCC compared with reference groups

Index	AUC	Cut-off value	95%CI	Se (%)	Sp (%)	OR	p
Non-cancer control group (CHD và HD) (n=111)							
mSEPT9 (ΔCt)	0,769	26,94	0,70 - 0,83	74,65	72,07	7,46	< 0,001
CHD control group (n=70)							
mSEPT9 (ΔCt)	0,771	28,25	0,69 - 0,84	77,46	72,86	9,23	< 0,001
HD control group (n=41)							
mSEPT9 (ΔCt)	0,766	24,72	0,68 - 0,84	57,75	87,80	9,84	< 0,001

- Compared with the non-cancer group, plasma mSEPT9 demonstrated moderate diagnostic value for very early and early-stage HCC, with an AUC of 0.769.

- Compared with the benign liver disease (BGL) group, plasma mSEPT9 showed diagnostic value for very early and early-stage HCC, with an AUC of 0.771.

- Plasma mSEPT9 also exhibited moderate diagnostic performance for very early and early-stage HCC compared with the HD group, with an AUC of 0.766.

3.3.4. Diagnostic value when combined with AFP

Bảng 3.8. Diagnostic value of combining plasma mSEPT9 with AFP for HCC (the CHD reference group)

Index	AUC	Cut-off value	95% CI	Se (%)	Sp (%)	OR	p
mSEPT9 (Δ Ct)	0,824	28,25	0,76 - 0,87	84,21	72,86	9,22	< 0,001
AFP (ng/mL)	0,827	8,59	0,77 - 0,88	72,18	85,71	11,04	< 0,001
mSEPT9 + AFP	0,90	-	0,85 - 0,94	87,22	77,14	-	< 0,001

The combination of plasma mSEPT9 and AFP markedly improved the diagnostic performance for HCC, achieving an AUC of 0.90.

Table 3.9. The diagnostic value of combining plasma mSEPT9 with AFP for very early and early-stage HCC (reference group: CHD)

Index	AUC	Cut-off value	95% CI	Se (%)	Sp (%)	OR	p
mSEPT9 (Δ Ct)	0,771	28,25	0,69 - 0,84	77,46	72,86	9,23	< 0,001
AFP (ng/mL)	0,798	8,59	0,72 - 0,86	64,79	85,71	11,04	< 0,001
mSEPT9 + AFP	0,867	0,36	0,80 - 0,92	90,14	70,00	-	< 0,001

The combination of plasma mSEPT9 and AFP improved the diagnostic performance for very early and early-stage HCC, achieving a good level with an AUC of 0.867.

Table 3.10. Logistic regression analysis was performed to evaluate the diagnostic value of several factors for HCC (reference group:CHD).

Variables	Univariate analysis			Multivariate analysis		
	OR	95%CI	p	OR	95%CI	p
Gender	1,07	1,04 - 1,10	< 0,001	1,06	1,02 - 1,10	0,0007
Giới tính	0,50	0,17 - 1,49	0,21			
HBsAg (Positive and Negative)	2,86	1,04 - 7,87	0,04	5,58	1,68 - 18,50	0,005
AST (> 40 và ≤ 40 U/L)	1,49	0,83 - 2,69	0,18			
ALT (> 40 và ≤ 40 U/L)	1,19	0,66 - 2,13	0,56			
AFP (ng/mL)	1,02	1,01 - 1,03	0,003	1,01	1,003 – 1,03	0,01
Cirrhosis (Yes and No)	0,79	0,44 - 1,41	0,42			
Δ Ct-mSEPT9	0,83	0,79 - 0,88	< 0,001	0,86	0,81 - 0,92	< 0,001

Multivariate regression analysis showed that the plasma mSEPT9 Δ Ct value was an independent factor associated with HCC (OR = 0.86; 95% CI: 0.81–0.92; $p < 0.0001$), along with other factors such as age, AFP, and HBsAg positivity.

Chapter 4. DISCUSSION

4.1. Plasma mSEPT9 Levels in Relation to Tissue Samples and Association with Clinical and Paraclinical Characteristics

4.1.1. Plasma mSEPT9 Levels in Relation to Tissue Samples

4.1.1.1. Levels of mSEPT9 in Tissue and Plasma

Analysis of 58 HCC patients with both tumor tissue and plasma samples revealed that the ΔCt value of mSEPT9 was significantly higher in tumor tissue than in plasma. This supports the hypothesis that tumor tissue is the primary source of circulating mSEPT9. The median ΔCt difference of approximately 13 cycles indicates that the ratio of mSEPT9 to total SEPT9 in plasma is roughly 1,000 times lower than in tumor tissue (corresponding to 2^{13} or about 13,600-fold difference), reinforcing the understanding that plasma mSEPT9 represents only a minor fraction of the actual methylation level in tumor tissue. According to Huang F. et al. (2022), mSEPT9 levels in HCC tissue are significantly higher than in normal tissue. Similarly, Kotoh Y. et al. (2020) reported that methylated SEPT9 copy numbers in serum were significantly lower than in tumor tissue ($p < 0.001$).

4.1.1.2. Correlation Between SEPTIN9 Gene Methylation Levels in Tissue and Plasma

There was a moderate linear correlation between the ΔCt values of mSEPT9 in plasma and tumor tissue ($r = 0.33$; $p < 0.01$). This suggests that higher mSEPT9 levels in tumor tissue tend to be accompanied by increased methylation levels in plasma. However, the relationship is not strictly proportional. This implies that while plasma mSEPT9 may reflect epigenetic alterations in the tumor, it is also influenced by multiple factors. Tissue analysis is based on a small biopsy sample and may not fully represent tumor heterogeneity, whereas plasma mSEPT9 levels can be affected by systemic biological factors such as tumor size, number, invasiveness, necrosis, and the tumor's capacity to release cfDNA into circulation. Thus, a tumor with a high methylation rate at the biopsy site might still exhibit low plasma mSEPT9 levels if it is small, releases little DNA, or if the DNA is rapidly degraded by enzymes. Conversely, a large tumor with central necrosis may release substantial cfDNA, increasing circulating mSEPT9 levels even if the biopsy shows low methylation.

4.1.2. Association of Plasma mSEPT9 Levels with Clinical and Paraclinical Parameters

4.1.2.1. Association with Clinical Presentation, Biochemical Markers, and Liver Function

Lower ΔCt values of plasma mSEPT9 were significantly associated with elevated AST and ALT enzyme levels. Increases in AST and ALT reflect hepatocellular injury and necrosis, which may lead to higher release of cfDNA into plasma, including methylated mSEPT9 fragments. Analysis of the relationship between mSEPT9 and AFP revealed a trend toward higher plasma mSEPT9 levels in patients with markedly elevated AFP levels (> 400 ng/mL), indicating a greater tumor burden. According to the Child-Pugh and ALBI grading systems—used to assess liver functional reserve— ΔCt values of mSEPT9 decreased progressively across Child-Pugh and ALBI grades. Statistically significant differences were observed between Child-Pugh A vs. C ($p = 0.037$) and ALBI grade 1 vs. 3 ($p = 0.05$).

4.1.2.2. Association with Tumor Burden

Plasma mSEPT9 ΔCt values were significantly associated with tumor characteristics in HCC patients. Specifically, lower plasma mSEPT9 ΔCt values were found in patients with bilobar liver tumors compared to those with tumors confined to either the right or left lobe ($p < 0.05$), suggesting that widespread tumors may release more methylated SEPT9 DNA into circulation. Additionally, patients with multiple liver tumors had lower plasma mSEPT9 ΔCt values than those with a solitary tumor. This aligns with findings by Huang F. et al., who reported a positive mSEPT9 detection rate of 31.6% in patients with multifocal HCC, significantly higher than the 9.3% in those with a single lesion ($p = 0.004$). Regarding tumor size, lower ΔCt values were observed in patients with larger tumors (≥ 50 mm) compared to those with smaller ones (< 50 mm), $p = 0.003$, consistent with findings from Huang F. et al. (2022).

4.2. Diagnostic Value of Plasma mSEPT9 in Hepatocellular Carcinoma

4.2.1. Diagnostic Accuracy in Comparison with Reference Groups

ROC curve analysis comparing HCC patients to the reference group (70 patients with liver cirrhosis and 41 healthy controls) showed that the ΔCt mSEPT9 assay had good diagnostic performance, with an AUC of 0.827 ($p < 0.001$), sensitivity of 82.71%, and specificity of 71.07%. When compared specifically to the cirrhosis group, the AUC remained high (0.824), with sensitivity of 84.21% and specificity of

72.86%. Compared with international studies, our AUC was similar to those reported by Kotoh Y. et al. (2020, AUC = 0.81), He N. et al. (2020, AUC = 0.85), and Bannaga A.S. et al. (2021, AUC = 0.854). However, specificity was somewhat lower in our study, possibly due to differences in study population characteristics. Notably, our reference group included both patients with cirrhosis of uncertain progression and healthy controls, whereas some studies limited comparisons to either healthy individuals or patients with clearly established cirrhosis.

The cumulative findings of this study demonstrate that *mSEPT9* is a highly specific epigenetic biomarker capable of effectively distinguishing HCC from reference groups, including patients with chronic liver disease, despite variations in analytical methods. Our team developed a semi-quantitative semi-nested PCR assay incorporating an ExBP probe—offering high sensitivity and practical optimization—which represents a significant advancement toward domestic technology ownership. This assay positions *mSEPT9* as a feasible tool for early detection and screening of HCC in Vietnam.

4.2.2. Diagnostic Value of mSEPT9 in HCC Patients with AFP < 20 ng/mL

This study also explored the diagnostic utility of plasma *mSEPT9* in cases where alpha-fetoprotein (AFP) levels were below 20 ng/mL. Results showed that plasma *mSEPT9* Δ Ct values were significantly lower in HCC patients with AFP < 20 ng/mL compared to those in the cirrhotic and healthy control groups (median 21.0 vs. 29.9 and 29.3, respectively; $p < 0.05$). Compared to non-HCC individuals, plasma *mSEPT9* demonstrated strong diagnostic performance for AFP-low HCC, with an AUC of 0.826. At a cutoff of 26.94, the test achieved 83.9% sensitivity and 72.07% specificity. Against the cirrhosis reference group, the diagnostic efficacy remained high (AUC = 0.817), with 87.5% sensitivity and 72.7% specificity at a cutoff of 28.25 cycles. Notably, patients with Δ Ct $\leq$ 28.25 had an 18.67-fold higher risk of HCC compared to those with Δ Ct > 28.25 ($p < 0.001$). Consistent with our findings, Li B. et al. (2020) reported similar positivity rates for *mSEPT9* in HCC patients with AFP $\leq$ 20 ng/mL and > 20 ng/mL (79.5% vs. 85.0%, $p = 0.468$).

4.2.3. Diagnostic Value of mSEPT9 in Very Early and Early-Stage HCC

Our data show that plasma mSEPT9 Δ Ct values were significantly lower in BCLC stage 0/A HCC patients (median = 22.71 cycles) than in cirrhotic patients (median = 29.93 cycles; $p < 0.0001$) and healthy individuals (median = 29.29; $p < 0.001$). When comparing BCLC 0/A HCC to cirrhotic patients, mSEPT9 Δ Ct values yielded a diagnostic AUC of 0.771 ($p < 0.001$), indicating good diagnostic performance. Kotoh Y. et al. (2020) reported a comparable AUC of 0.86 in distinguishing early-stage HCC from cirrhosis. These findings highlight the potential of plasma mSEPT9 as a practical clinical screening tool for early detection of HCC in high-risk populations such as those with chronic liver disease.

4.2.4. Diagnostic Value of Combining Plasma mSEPT9 with AFP

The diagnostic performance of mSEPT9 was significantly enhanced when combined with plasma AFP levels. The combined biomarker model achieved an AUC of 0.900, markedly higher than AFP alone (AUC = 0.824) or mSEPT9 alone (AUC = 0.827), with the difference being statistically significant ($p < 0.001$). At the optimal cutoff, the combination yielded a sensitivity of 87.22% and specificity of 77.14%, offering strong discriminatory power between HCC and reference groups. Li B. et al. (2020) also demonstrated that combining mSEPT9 with AFP raised HCC detection sensitivity to over 91%, compared to 82.7% for mSEPT9 alone, with an AUC of 0.986, indicating superior diagnostic capacity. These results underline the value of mSEPT9 not only as a stand-alone marker for HCC but also as a valuable adjunct to AFP, especially in cases where AFP levels are not elevated. This combination enhances the clinical utility of mSEPT9 in early detection and risk stratification strategies for HCC.

CONCLUSION

1. Plasma mSEPT9 level in comparison with tissue samples and their association with clinical and paraclinical features

1.1. Plasma mSEPT9 Levels Compared with Tissue Samples

+ The median Δ Ct value of plasma mSEPT9 in the HCC group was 19.22 cycles, significantly lower than in the non-cancer group (29.55 cycles, $p < 0.001$), indicating higher plasma mSEPT9 levels in HCC patients.

+ Specific amplification signals of mSEPT9 were detected in 98.3% (57/58) of HCC tumor tissue samples, with a median Δ Ct of

9.20 cycles (IQR: 6.74–16.88). The corresponding plasma ΔCt values were significantly higher (median 22.93; $p < 0.001$), reflecting that plasma mSEPT9 levels are approximately 13,000 times lower than those in tumor tissue.

+ A moderate positive correlation was found between mSEPT9 levels in plasma and tumor tissue ($r = 0.33$; $p < 0.01$). This correlation was more evident in patients with typical histological features (trabecular architecture, grade II differentiation), large tumors (> 50 mm), bilobar involvement, advanced stage (BCLC B/C/D), absence of cirrhosis, and $\text{AFP} \leq 400$ ng/mL.

1.2. Association Between Plasma mSEPT9 Levels and Clinical/Paraclinical Characteristics

+ Lower ΔCt values (indicating higher mSEPT9 levels) were observed in patients with hepatocellular injury and those with severe liver dysfunction (Child-Pugh C, ALBI 3), compared to those with mild dysfunction (Child-Pugh A, ALBI 1), $p < 0.05$.

+ Plasma mSEPT9 levels were significantly associated with tumor progression features such as portal vein thrombosis, tumor diameter > 50 mm ($p = 0.01$), bilobar liver involvement ($p = 0.004$), and AFP levels > 20 ng/mL ($p = 0.04$).

+ No significant associations were found between plasma mSEPT9 levels and patient age, sex, or common clinical symptoms.

2. Diagnostic Value of Plasma SEPTIN9 Methylation in Hepatocellular Carcinoma

+ Plasma mSEPT9 alone showed good diagnostic performance for HCC compared to the combined reference group (cirrhotic and healthy individuals), with an AUC of 0.827. At the cutoff of 26.94 cycles, sensitivity was 82.71% and specificity 72.07%; compared with the cirrhosis group alone, the AUC was 0.824 with a cutoff of 28.25 cycles, yielding 84.21% sensitivity and 72.86% specificity.

mSEPT9 retained good diagnostic value in HCC cases with $\text{AFP} \leq 20$ ng/mL (AUC = 0.817; OR = 18.67; $p < 0.001$) and fair performance in early-stage HCC (BCLC 0/A, AUC = 0.771; OR = 9.23; $p < 0.001$).

Combining plasma mSEPT9 with AFP significantly improved diagnostic accuracy (AUC = 0.900), notably higher than using either biomarker alone ($p < 0.001$).

Plasma mSEPT9 Δ Ct was identified as an independent factor associated with HCC (OR = 0.86; 95% CI: 0.81–0.92; $p < 0.0001$), alongside other factors such as age, AFP levels, and HBsAg positivity.

RECOMMENDATIONS

Plasma SEPT9 methylation is a promising epigenetic biomarker for HCC diagnosis, especially in early-stage disease and AFP-negative cases. Therefore, large-scale, multicenter studies are needed to validate its clinical accuracy and applicability. Additionally, technical protocols should be standardized, and cost-effectiveness analyses should be conducted to support its integration into routine diagnostics.

To enhance diagnostic accuracy and enable better risk stratification, further studies should investigate the combination of mSEPT9 with other molecular markers and aim to develop multi-marker diagnostic models using modern molecular biology platforms such as quantitative PCR or sequencing technologies.

LIST OF WORKS ANNOUNCING THE RESEARCH RESULTS OF THE THESIS TOPIC

1. Yen Hai Tran, Trang Thuy Dao, Ung Dinh Nguyen, Thien Ba Tran, Loi Phuc Luu, Huy Quang Duong, Tho Huu Ho (2025). Sensitive detection of circulating methylated *SEPT9* in hepatocellular carcinoma patients using a novel quantitative PCR assay. *Anal Methods*, 17(9), 2181 - 2190.
2. Tran Hai Yen, Ho Huu Tho, Duong Quang Huy (2025). Association between plasma *SEPTIN9* gene methylation status and liver tumor characteristics in patients with hepatocellular carcinoma. *Vietnam Journal of Medicine*, **555**(1), 316–321.
3. Tran Hai Yen, Ho Huu Tho, Duong Quang Huy (2025). Correlation between plasma and tumor tissue SEPTIN9 gene methylation in patients with hepatocellular carcinoma. *Vietnam Journal of Medicine*, **555**(2), 157-161.