DOI:10.31557/APJCP.2022.23.4.1279

RESEARCH ARTICLE

Editorial Process: Submission:10/19/2021 Acceptance:04/05/2022

Lack of Association between *IFN-γ*, *CXCL10* and *TGF-β1* Gene Polymorphisms and Liver Complication in HIV-infected Thais

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Abstract

Objective: Chronic liver disease has become a leading cause of illness and death in people living with HIV and the production of the cytokines IFN-γ and TGF-β1, and chemokine CXCL10 during chronic inflammation contributes to liver disease progression in HIV patients under long-term anti-retroviral therapy. This study aimed to examine association of IFN-γ +874T/A, CXCL10 G-201A and C-1596T, and TGF-β1 -509C/T single nucleotide polymorphisms (SNPs) with liver complications in the HIV-infected Thais. **Methods:** A cross-sectional study was conducted in 200 Thai HIV patients who were evaluated for transaminitis and significant liver fibrosis by fibrosis-4 score (FIB-4), and genotypes for IFN-γ +874T/A, CXCL10 G-201A and C-1596T, and TGF-β1 -509C/T SNPs using PCR-based methods. **Result:** There were high rates of transaminitis (30.1%) and significant liver fibrosis assessed by FIB-4 score > 1.45 (18.8%) in this group of patients, mostly under anti-retroviral therapy (73.0%). The genotypes and alleles of IFN-γ +874T/A, CXCL10 G-201A and C-1596T, and TGF-β1 -509C/T SNPs were not associated with either transaminitis or FIB-4 score > 1.45 (p > 005). Logistic regression analysis identified age and gender as risk factors, and $CD4^+$ cell count higher than 350 cells/ul as a protective factor of liver fibrosis in this study group. **Conclusion:** The IFN-γ +874T/A, CXCL10 G-201A and C-1596T, and TGF-β11 -509C/T SNPs were not significantly associated with liver complication in HIV-infected Thais, mostly under ART.

Keywords: IFN-γ +874T/A- CXCL10 G-201A- CXCL10 C-1596T- TGF-β1 -509C/T- Liver fibrosis

Asian Pac J Cancer Prev, **23 (4)**, 1279-1284

Introduction

Human immunodeficiency virus (HIV) infection remains a major health issue globally. The advances in anti-retroviral therapy (ART) for people living with HIV (PLWH) has led to a decrease of acquired immunodeficiency syndrome (AIDs)-related morbidity and mortality with an increased evidence of liver disease. Chronic liver disease has become a leading cause of illness and death in PLWH and there is increasing prevalence of hepatocellular carcinoma (HCC) with longer lifespan of patients with HIV (Ceccarelli et al., 2020; Chamroonkul and Bansal, 2019). Major etiologies of liver-related diseases in HIV patients include HIV replication in the liver, coinfection with hepatitis B (HBV) and C (HCV) viruses and some anti-retroviral drug regimens. Previous studies have indicated multiple risk factors for development and severity of liver disease in HIV patients including age, sex, $CD4^+$ cell count, $CD4^+/CD8^+$ ratio, HIV RNA levels, HCV coinfection, active alcohol use and diabetes mellitus (Akekawatchai et al., 2015; Androutsakos et al., 2020; Chiraunyanann et al., 2019; DallaPiazza et al., 2010). Due to the variability of liver disease progression in PLWH having similar risk factors, host genetic background is suggested to be a contributor, and some immune gene polymorphisms involving liver disease progression particularly in HIV/HCV coinfection have been reported (Medrano et al., 2017).

Accumulating studies indicate contribution of chronic immune activation and persistent inflammation to liver-related diseases in HIV-infected patients under long-term ART (Zicari et al, 2019). Various cytokines and chemokines released during chronic inflammation participate in progression of liver fibrosis. Interferon-γ (IFN-γ), known as an anti-fibrotic cytokine, is found to be underexpressed during viral hepatitis and HIV infection,

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leading to a decrease in IFN-γ-mediated apoptosis of activated hepatic stellate cells (HSCs) and potentiate a profibrotic state in the liver (Kaspar and Sterling, 2017). C-X-C motif chemokine 10 (CXCL10) or interferon-γ-inducible protein 10 (IP-10) is secreted from various cell types in response to IFN-γ, and known to play roles in recruitment and immune response in the liver. The CXCL10 chemokine and its respective receptors, CXCR3, participate in pathogenesis of liver diseases especially caused by HBV and HCV infection (Marra and Tacke, 2014). Transforming growth factor-β1 (TGF-β1) is well established as a master profibrogenic cytokine. The TGF-β1 signaling pathway drives HSCs activation and induces extracellular matrix production, leading to hepatic fibrosis (Dewidar et al., 2019). Many previous studies support the significant roles of IFN-γ, CXCL10 and TGF-β1 in different types of chronic liver diseases (Kaspar and Sterling, 2017; Marra and Tacke, 2014).

Several lines of evidence also indicated potential roles of the polymorphisms in IFN-γ, CXCL10 and TGF-β1 genes in different types of liver diseases. The single nucleotide polymorphisms (SNPs) in the first intron of IFN-γ gene, +874T/A, in the promotor region of CXCL10 gene, G-201A and C-1596T, and in the promotor region of TGF-β1 gene, -509C/T, demonstrated to affect the expression and secretion levels of IFN-γ, CXCL10 and TGF-β1, respectively, *in vitro* and *in vivo* (Pravica et al., 1999; Xu et al., 2013; Deng et al., 2008; Rathod and Tripathy, 2015), and accumulating studies demonstrated the contribution of these SNPs in development and severity of liver diseases of different etiologies including chronic hepatitis C, chronic hepatitis B, and HCC (Dai et al., 2006; Deng et al., 2008; Ma et al., 2015). While cytokine production during chronic immune activation and persistent inflammation in HIV-infected patients under long-term ART has been suggested to be one of the key mechanisms for chronic liver disease in HIV patients, relationships of polymorphisms in these cytokine and chemokine genes with liver complication in HIV patients are still unclear.

Our previous study has demonstrated the relatively high rates of HBV and HCV coinfection, and liver abnormalities in HIV-infected Thais mostly under long-term ART, suggesting high potential of progression to chronic liver diseases and HCC (Akekawatchai et al., 2015; Chiraunyanann et al., 2019). This study aimed to investigate association of genetic variation in cytokine and chemokine genes, IFN-γ +874T/A, CXCL10 G-201A and C-1596T and TGF-β1 -509C/T SNPs, with liver complications in the HIV-infected group. The data obtained from this study provide understanding in an impact of genetic determinants in development of liver disease in PLWH under long-term ART.

Materials and Methods

Study population, clinical data, and laboratory investigation

A cross-sectional study was conducted in 200 HIV patients attending the ART clinic in Nakhon Nayok hospital between October 2011 and June 2013. Inclusion criteria were being older than 15 years, documented HIV infection, and availability of blood samples and clinical data. Patients who regularly consume alcohol, herbal and steroid medication, opportunistic infection including tuberculosis were excluded. All subjects provided written informed consent. The study protocol was reviewed and approved by the Human Ethics Committees No. 2, Thammasat University, Thailand (project no. 141/2559) and Certificated Biological Safety by Biological safety Committee, Thammasat University, Thailand (certificate no. 136/2561).

Clinical and laboratory data were obtained as described in the previous study (Akekawatchai et al., 2015). Ethylene-diamine-tetra-acetic acid (EDTA) blood samples left over from routine testing were subjected to plasma separation within 8 hours after the collection and stored at -80^{O}C before use. In this study, liver complications in HIV patients were determined by transaminitis defined as an increase of either aspartate aminotransferase (AST), alanine aminotransferase (ALT) from the normal upper limit (ULNs) (> 40 U/L), and liver fibrosis assessed by fibrosis-4 (FIB-4) score, classified into class I (< 1.45), class 2 (1.46 to 3.25) and class 3 (> 3.25) (Foca et al., 2016; Sterling et al., 2006), and by AST to platelet ratio index (APRI), classified into class I (< 0.5), class 2 (0.5 to 1.5) and class 3 (> 1.5) (Wai et al., 2003).

Genotyping of IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs

Genomic DNA was isolated from blood samples according to the manufacturer's instruction using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). DNA samples were genotyped for IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs using different PCR-based assays. The genotyping of +874 T/A SNP was performed by a specific sequence primer polymerase chain reaction (SSP-PCR) (Ghasemian and Shahbazi, 2016; Akekawatchai et al., 2022), while that of G-201A and C-1596/T SNPs were analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP) as described (Limothai et al., 2016; Yang et al., 2013; Tunkor et al., 2018). The TGF-β1 -509C/T SNP was genotyped using amplification refractory mutation system-PCR (ARMS-PCR) assay (Heidari et al., 2013; Akekawatchai et al., 2022). At least 10% of DNA samples were subjected to direct DNA sequencing and analyzed by Unipro UGENE v.1.24.2. There was 100% agreement between the results obtained by the PCR-based methods and by direct sequencing.

Statistical analysis

Descriptive statistics, mean, median and percentages were used to describe characteristics of study population. Genotype and allele frequencies were calculated by direct counting. Hardy-Weinberg equilibrium was assessed by chi-square test with one degree of freedom from online analysis tool, considering equilibrium when $p > 0.05$ (Rodriguez et al., 2009). Chi-square test was used to determine an association between categorical variables, while a Mann-Whitney U test was used to analyze the differences between continuous variables. Odd ratio (OR)

with 95% confidence interval (CI) were calculated by chi-square test and binary logistic regression. p values < 0.05 were considered as statistically significant. The PASW statistic 18 software (SPSS Inc.) and the online calculator VassarStats: Website for Statistical Computation (Lowry R, 2021) were used for statistical analysis.

Results

Characteristics of the study population

Table 1 presents the characteristics and clinical features of 200 HIV patients recruited in this study. Prevalence of patients with liver abnormality evaluated by transaminitis was 30.1% (53/176) and those with significant liver fibrosis assessed by FIB-4 score > 1.45 and APRI > 0.5 were 18.8% (33/176) and 13.6% (24/176) respectively. Subgroup analysis on characteristics of HIV patients with liver abnormality assessed by transaminitis and FIB-4 score compared with those who were not was demonstrated in Table 2. The characteristics of patients with and without transaminitis were similar (p > 0.05), except for gender (p = 0.013). Binary logistic regression also indicated that male had a higher risk of transaminitis than female patients (OR = 2.4, 95% CI 1.2-4.7, p = 0.014). Most clinical features of patients with significant fibrosis, age, gender, $CD4^+$ cell count, and duration of ART, were statistically different from those without fibrosis (p =0.001, p = 0.005, p =0.001, p = 0.034). Univariate logistic regression analysis indicated that the ages older 40 years and being male were risk factors of FIB-4 score > 1.45 (OR = 6.8, 95% CI 2.6-17.5, p = 0.001 and OR = 3.7, 95% CI 3.7 (1.5-8.8), p = 0.005). Patients with CD4+ cell count ≥ 350 cells/ul and under ART longer than 6 months had lower risks than those with $CD4^+$ cell count < 350 cells/ul and naïve to ART (OR = 0.2, 95% CI 0.1-0.6, P = 0.001 and OR = 0.3, 95% CI 0.1-0.7, P = 0.012). In multivariate analysis, male patients, and the ages older than 40 years were risk factors of significant liver fibrosis (OR = 3.5, 95% CI 1.3- 10.0, P = 0.017 and OR = 7.0, 95% 2.4-20.1, P = 0.001), and maintaining levels of $CD4^+$ cell count ≥ 350 cells/ul was protective factors of significant fibrosis (OR = 0.3, 95% CI 0.1-0.9, P = 0.031).

Genotypic and allelic frequencies and association of IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs with liver complication in HIV patients

Genotypic and allelic frequencies of IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs were previously reported (Table 3) (Akekawatchai et al., 2022; Tunkor et al., 2018). The distribution of all SNP alleles and genotypes were consistent with the Hardy-Weinberg equilibrium (p > 0.05). To examine the effect of genetic factors involving immune function on liver complication in HIV patients, association of the SNPs with transaminitis and FIB-4 score > 1.45 was analyzed. The analysis by chi-square test and binary logistic regression shown in Table 3 demonstrated no statistically significant relationship of genotypes and alleles of all SNPs with transaminitis or significant fibrosis assessed by FIB-4 score (p > 0.05). Additionally, the analysis of genotypes under dominant and recessive models of all SNPs also indicated no statistically significant association (p > 0.05) (Data not shown).

Table 1. General and Clinical Characteristics of HIV Patients (n = 200)

Characteristics	N (%)
Age[(a)] (Years)	40.2 (±11.4)
Gender	
Female	91 (45.5)
Male	109 (54.5)
$CD4^+$ cell count[(b)] (cells/ul) (n=174)	364.5 (1-1,601)
HBV and HCV coinfection (n=167)	
HIV monoinfection	136 (81.4)
HIV-HBV coinfection	15 (9.0)
HIV-HCV coinfection	15 (9.0)
HIV-HBV-HCV coinfection	1 (0.6)
ARV drugs	
Naïve to ARV treatment	54 (27)
Lamivudine/Stavudine/Nevirapine	13 (6.5)
Lamivudine/Zidovudine/Nevirapine	72 (36)
Lopinavir/Ritonavir combination	15 (7.5)
Others	46 (23.0)
Nevirapine experience	
Naive to ARV treatment	54 (27.0)
Nevirapine-based regimens	92 (46.0)
Others	54 (27.0)
Duration of ARV treatment	
0 months (naïve to ARV treatment)	54 (27.0)
< 3 months	10 (5.0)
3-6 months	6 (3.0)
> 6 months	130 (65.0)
Transaminitis (n=176)	
AST and/or ALT ≤ ULN (40 U/L)	123 (69.9)
AST and/or ALT > ULN (40 U/L)	53 (30.1)
FIB-4 score (n=176)	
≤ 1.45	143 (81.3)
> 1.45	33 (18.8)
APRI (n=176)	
≤ 0.5	152 (86.4)
> 0.5	24 (13.6)

[(a)] and [(b)], data shown as mean ± S.D. and median (range) respectively. Some variables had missing data and n is given in parentheses. AST, aspartate aminotransferase; ALT, alanine aminotransferase; FIB-4, fibrosis-4 score; APRI, AST to platelet ratio index

Discussion

This cross-sectional study demonstrated the relatively high prevalence of transaminitis (30.1%) and significant liver fibrosis, assessed by FIB-4 > 1.45 (18.8%) and APRI > 0.5 (13.6%), in Thai HIV-infected patients with relatively high rates of HBV (9.0%) and HCV (9.0%) coinfection and longer than 6-month suppressive ART (65.0%). The subgroup analyses indicated that

Table 2. Clinical Characteristics of Patients with and without Liver Abnormalities Assessed by Transaminitis and FIB-4 Score in HIV Patients

Characteristics	Transamainitis			Liver fibrosis by FIB-4 score				
	Without	With	P	≤ 1.45	> 1.45	P	Crude OR	Adjusted OR
	N (%)	N (%)		N (%)	N (%)		(95% CI)	(95% CI)
Age (years) (n=176)								
≤ 40	67 (54.5)	25 (47.2)	0.374	86 (60.1)	6 (18.2)	0.001*	1	1
> 40	56 (45.5)	28 (52.8)		57 (39.9)	27 (81.8)		6.8 (2.6-17.5)	7.0 (2.4-20.1)
Gender (n=176)								
Female	62 (50.4)	16 (30.2)	0.013*	71 (49.7)	7 (21.2)	0.003*	1	1
Male	61 (49.6)	37 (69.8)		72 (50.3)	26 (78.8)		3.7 (1.5-8.8)	3.5 (1.3-10.0)
$CD4^+$ cell count (cells/ul) (n = 170)								
< 350	52 (44.1)	30 (57.7)	0.101	58 (41.7)	24 (77.4)	0.001*	1	1
≥ 350	66 (55.9)	22 (42.3)		81 (58.3)	7 (22.6)		0.2 (0.1-0.6)	0.3 (0.1-0.9)
Hepatitis virus coinfection (n= 146)								
HIV monoinfection	84 (84.8)	34 (72.3)	0.073	99 (83.9)	19 (67.9)	0.053	1	
HBV and/or HCV coinfection	15 (15.2)	13 (27.7)		19 (16.1)	9 (32.1)		2.5 (1.0-6.3)	-
Anti-retroviral therapy (n = 176)								
Naïve to ART	28 (22.8)	8 (15.1)	0.488	24 (16.8)	12 (36.4)	0.034*	1	1
≤ 6 months	9 (7.3)	5 (9.4)		11 (7.7)	3 (9.1)		2.1 (0.4-9.9)	0.9 (0.2-4.7)
> 6 months	86 (69.9)	40 (75.5)		108 (75.5)	18 (54.5)		0.3 (0.1-0.7)	0.4 (0.1-1.3)

P was calculated by Chi-square test. Crude OR and Adjusted OR were analyzed using univariate and multivariate logistic regression models respectively. Some variables had missing data and n is given in parentheses. *Data shown as P values < 0.05, OR, Odd ratio; CI, Confidence interval; FIB-4, fibrosis-4 score

characteristics of the patient group stratified by having transaminitis were similar, except for gender, while those stratified by having significant fibrosis, FIB-4 > 1.45, were significantly different including age, gender, $CD4^+$ cell count and duration of ART. In uni- and multivariate logistic regression analysis, being male and age older than

Table 3. Frequencies and Association of IFN-γ +874 T/A, CXCL10 G-201A/C- 1596T and TGF-β1 -509C/T SNPs with Transaminitis and Liver Fibrosis in HIV-Infected Thais

SNPs		Frequencies	Transaminitis				Liver fibrosis by FIB-4 score			
			Without	With	*P*	OR	≤ 1.45	> 1.45	*P*	OR
			N (%)	N (%)		(95% CI)	N (%)	N (%)		(95% CI)
IFN-γ +874T/A		-	100 (70.4)	42 (29.6)	-	-	114 (80.3)	28 (19.7)	-	-
Genotypes	AA	0.527	10 (76.9)	3 (23.1)	0.438	1	9 (69.2)	4 (30.8)	0.505	1
	AT	0.383	34 (64.2)	19 (35.8)		1.9 (0.5-7.6)[a]	42 (79.2)	11 (20.8)		0.6 (0.2-2.3)[a]
	TT	0.090	56 (73.7)	20 (26.3)		1.2 (0.3-4.8)[a]	63 (82.9)	13 (17.1)		0.5 (0.1-1.8)[a]
Allelles	A	0.719	54 (68.4)	25 (31.6)	0.639	1	60 (76.0)	19 (24.0)	0.254	1
	T	0.281	146 (71.2)	59 (28.8)		0.9 (0.5-1.5)[b]	168 (82.0)	37 (18.0)		0.7 (0.4-1.3)[b]
CXCL10 G-201A/-1596C/T (n=199)		-	122 (69.7)	53 (30.3)	-	-	142 (81.1)	33 (18.9)	-	-
Genotypes	GG or CC	0.764	97 (70.3)	41 (29.7)	1.000	1	113 (81.9)	25 (18.1)	0.504	1
	GA or CT	0.226	24 (68.6)	11 (31.4)		1.3 (0.6-2.8)[a]	28 (80.0)	7 (20.0)		1.1 (0.4-2.9)[a]
	AA or TT	0.010	1 (50.0)	1 (50.0)		2.5 (0.2-40.1)[a]	1 (50.0)	1 (50.0)		4.5 (0.3-74.7)[a]
Allelles	G or C	0.122	218 (65.1)	117 (34.9)	0.841	1	254 (81.7)	57 (18.3)	0.475	1
	A or T	0.878	26 (66.7)	13 (33.3)		0.9 (0.4-1.9)[b]	30 (76.9)	9 (23.1)		1.3 (0.6-3.0)[b]
TGF-β1 -509C/T (n=191)		-	117 (69.6)	51 (30.4)	-	-	137 (81.5)	31 (18.5)	-	-
Genotypes	CC	0.157	21 (77.8)	6 (22.2)	0.601	1	22 (81.5)	5 (18.5)	0.973	1
	CT	0.440	50 (68.5)	23 (31.5)		1.7 (0.6-4.8)[a]	59 (80.8)	14 (19.2)		1.0 (0.3-3.2)[a]
	TT	0.403	46 (67.6)	22 (32.4)		1.6 (0.6-4.4)[a]	56 (82.4)	12 (17.6)		0.9 (0.3-3.0)[a]
Allelles	C	0.377	92 (72.4)	35 (27.6)	0.383	1	103 (81.1)	24 (18.9)	0.862	1
	T	0.623	142 (67.9)	67 (32.1)		1.2 (0.8-2.0)[b]	171 (81.8)	38 (18.2)		1.0 (0.5-1.7)[b]

p, was calculated by chi-square test. [a]ORs and 95% CIs were calculated by binary logistic regression. [b]ORs were analyzed by Chi-square test. Some variables had missing data and n is given in parentheses. * Data shown as *p* values < 0.05, OR, Odd ratio; CI, Confidence interval, FIB-4, fibrosis-4

40 years were identified as risk factors, while maintaining $CD4^+$ cell count higher than 350 cells/ul and ART longer than 6 months were protective factors of significant fibrosis. The data indicated that there were multi-factors contributing to development of liver fibrosis in this patient group, consistent with several previous reports suggesting the high rates of liver fibrosis and multiple associated risk factors in HIV patients (Androutsakos et al., 2020; Chiraunyanann et al., 2019; DallaPiazza et al., 2010).

The genotypes of all SNPs tested in this study were in Hardy-Weinberg equilibrium ($p > 0.05$), suggesting that genetic background of the study group remains constant. Although previous studies suggested that the IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs had a significant effect on production levels of IFN-γ, CXCL10 and TGF-β1 (Pravica et al., 1999; Xu et al., 2013; Deng et al., 2008; Rathod and Tripathy, 2015) and were demonstrated to influence the susceptibility to hepatitis B progression, cirrhosis in chronic hepatitis C and HCC (Dai et al., 2006; Deng et al., 2008; Ma et al., 2015), our data indicated no statistically significant association of the genotypes and alleles of all SNPs, with transaminitis and significant liver fibrosis in the HIV patient group. This is probably because liver fibrosis can be influenced by confounding factors as shown in the subgroup analyses including ARV drug treatment. Most HIV patients in this study had protective factors, under ART (73%) and maintained $CD4^+$ cell count higher than 350 cells/uL. These factors possibly lessened an influence of the genetic determinants in liver fibrosis as shown in our previous study indicating strong association of CXCL12 G801A SNP with significant fibrosis in the same HIV patient group especially in the subgroup with ART (Chiraunyanann et al., 2019).

This study provides evidence that the genetic variation, IFN-γ +874 T/A, CXCL10 G-201A/C-1596T and TGF-β1 -509C/T SNPs, were not significantly associated with liver fibrosis in HIV-infected Thais, mostly under long-term ART. Limitations of this study should be noted. Firstly, this a cross-sectional study with relatively small number of subjects may limit statistical significance for variables tested. The study design is unable to control confounding factors. Secondly, there was no functional assessment of the genotypes of all SNPs tested. Further examination of IFN-γ, CXCL10 and TGF-β1 levels in circulation and liver tissues is suggested. Lastly, the findings in Thai population would be interesting to extend the study to different ethnic patients.

Author Contribution Statement

C. A. contributed to funding acquisition, resources, supervision, study design, data analysis, manuscript preparation, review and editing. K. C. is responsible for supervision, data analysis and manuscript preparation. A. T. and C. P. performed experimental work, data collection and analysis. T. S. and M. F. contributed to experimental work and data collection. T. C. and W. S. participated in blood sample and data collection, and data analysis. All authors have read and approved the manuscript.

Acknowledgements

General

Special thanks are due to all patients, staff in Nakorn Nayok hospital and undergraduate students in Medial Technology Program, Faculty of Allied Health Sciences, Thammasat University, Thitipan Jongchotchutchawan and Piyapat Boonsri, who contributed in this study.

Funding Statement

This study was supported by the fiscal year budget of the office of the National Research Council of Thailand to Thammasat University (Contract no. 30/2562), the Program Management Unit for Human Resources & Institutional Development, Research and Innovation, NXPO (Grant number B05F630043), and Thammasat University Research Unit in Diagnostic Molecular Biology of Chronic Diseases related to Cancer (DMB-CDC). Apikhun Tunkor is a recipient of the Research Grant for Graduate Studies from the National Research Council of Thailand (Fiscal year 2018, contract no. 9/2561).

Approval

This study has been approved by Graduate Program in Medical Technology, Faculty of Allied Health Sciences, Thammasat University as parts of master theses of Apikhun Tunkor and Chada Phuegsilp.

Ethical Declaration

The study protocol has been approved by the Human Ethics Committees No. 2, Thammasat University, Thailand (project no. 141/2559).

Data Availability

The data supporting this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

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