Original Article

Cyclin D1 expression as a potential prognostic factor in advanced *KRAS*-mutant non-small cell lung cancer

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Background: East Asian, including Thailand, lung cancer population may have a relatively lower prevalence of *KRAS* mutations than Caucasians. We investigated the prevalence and clinical characteristics of *KRAS*-driven non-small cell lung cancer (NSCLC) patients and the expression of cyclin D1, one of the KRAS downstream targets.

Methods: Lung cancer patients who received treatment at the King Chulalongkorn Memorial Hospital between January 2015 and July 2017 were enrolled. We identified *KRAS* mutations using allele specific PCR *KRAS* mutation testing. Cyclin D1 expression was determined using immunohistochemistry.

Results: After excluding 376 *EGFR* mutations and inadequate samples, we enrolled 95 patients eligible for *KRAS* mutation testing. *KRAS* mutations were identified in 28 out of 95 patients. There were 26 patients with *KRAS* codon 12/13 and 2 patients with *KRAS* codon 61 mutations. The prevalence of *KRAS* mutations among informative samples was 28 out of 357 (7.8%) which was relatively lower than that reported in Caucasian population. Smoking and male were significantly associated with *KRAS* mutations. The prognosis of *KRAS*-mutant NSCLC patients in particular codon 61 mutations was worse than that found in *KRAS*- and *EGFR*-wild-type (*KRAS* WT/*EGFR* WT) NSCLC patients (P=0.048). The levels of cyclin D1 expression in *KRAS*-mutant NSCLC were significantly higher than those in *KRAS* WT/*EGFR* WT NSCLC (P=0.02). A better prognosis of *KRAS*-mutant NSCLC patients with low cyclin D1 expression was observed when compared with those with high cyclin D1 expression (median overall survival 41.7 *vs.* 3.5 months, P=0.037).

Conclusions: We found a moderate prevalence of *KRAS* mutations in lung cancer in Thailand. Clinical characteristics were similar to those of Caucasian population. Most *KRAS*-mutant NSCLC had high cyclin D1 expression. Cyclin D1 expression may serve as a useful prognostic biomarker in *KRAS*-mutant lung cancer. Validation of this finding in larger cohort is required.

Keywords: Non-small cell lung cancer (NSCLC); East Asia; Kirsten rat sarcoma (*KRAS*); cyclin D1

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Introduction

Mutations of *Kirsten rat sarcoma* (*KRAS*) in non-small cell lung cancer (NSCLC) are frequently found approximately 20–30% of lung adenocarcinomas in Western countries including Europe and America (1,2) and are associated with smoking (3) and poor prognosis (4). Though commonly found, *KRAS* mutation remains an un-targetable oncogene. In East Asian countries, lung cancers have been reported to have lower prevalence of *KRAS* mutations than those in western countries. Part of the reasons includes the higher prevalence (approximately 40–55%) of epidermal growth factor receptor (*EGFR*) mutations which is associated with non-smoking (5,6). Mutations of *KRAS* in East Asian regions reported from Japan, China and Taiwan were at 8–10% (5,6). Similarly, in Thailand, *EGFR* mutations are the most common driver mutations in NSCLC (7); however, the frequency of *KRAS* mutations is less described.

Cyclin D1, encoded by *CCND1*, is a CDK4/6-dependent regulator of the G1-S checkpoint of cell cycle. Cyclin D1 overexpression has been reported in many types of human cancers including lung cancer (8). In NSCLC, the reported frequencies of cyclin D1 overexpression were 18–76% while the frequencies of *CCND1* amplifications were lower at 5–20% (9). These findings suggest additional mechanisms of cyclin D1 overexpression beyond *CCND1* amplifications, particularly an activation of mitogenic signaling pathways including RAS-MEK-ERK pathways. Therefore, we hypothesized that *KRAS*-mutant NSCLC would drive cyclin D1 overexpression. To investigate this possibility, we determined the levels of cyclin D1 expression in correlation with *KRAS* mutation status in NSCLC tissues. Prognostic roles of cyclin D1 expression and *KRAS* mutations in NSCLC were also investigated.

Methods

DNA specimens

Eligible patients (aged ≥18 years) were those diagnosed with NSCLC at the King Chulalongkorn Memorial Hospital between January 2015 and July 2017. Based on prior report of mutually exclusive between *EGFR* and *KRAS* mutation, we excluded tissue samples with known *EGFR* mutations or inadequate amount (less than 100 ng) or quality of DNA specimens. Clinicopathological characteristics including demographic data, smoking status and TNM staging according to the 7th edition AJCC which were retrospectively reviewed from medical records. The study was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No. 560/59).

EGFR and KRAS mutation testing

Tumor specimens for all patients were obtained either from diagnostic biopsy or surgical procedures. DNAs were extracted from formalin-fixed, paraffin-embedded (FFPE) tissues using QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. *EGFR* mutation testing platform was Cobas® *EGFR* mutation test v2 (Roche Diagnostics GmbH, Berlin, Germany) which was used to detect *EGFR* mutations in exons 18, 19, 20 and 21.

KRAS mutations at codons 12, 13 and 61 were examined using Cobas® *KRAS* mutation kit according to the manufacturer's instructions (CE-IVD, Roche Diagnostics, Pleasanton, CA, USA). PCR amplifications and automated real-time mutation detections were performed using a Cobas z 480 analyzer (Roche Diagnostics, USA).

Immunohistochemistry for cyclin D1

Two-micron FFPE sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using Dako PT link (Dako, Glostrup, Denmark). Immunostaining was performed using the automated staining systems, Dako Autostainer Link48 (Dako, Glostrup, Denmark). Primary antibody was FLEX monoclonal rabbit anti-human cyclin D1 clone EP12 ready-to-use (Dako, Glostrup, Denmark). The cyclin D1 immunostaining was evaluated by an experienced lung pathologist who was blinded from *KRAS* status. Cyclin D1 was evaluated for both its intensity and percentage of positivity. The intensity was classified into 0, 1+, 2+, 3+ where 0 was for no staining, 1+ for noticeable nuclear staining in 400× magnification, 2+ for distinct nuclear staining in 200× magnification, and 3+ for distinct nuclear staining in 100× magnification (*Figure 1*). The cyclin D1 immunoreactivity was previously reported as percentage of distinct nuclear staining (10) (defined as 2+ and 3+ in this study). Percentage of cyclin D1 expression was calculated using the following formula, % of cyclinD1 expression = (% cells with staining 2+) + (% cells with staining 3+).

Statistical analysis

The relationship between *KRAS* mutations and other

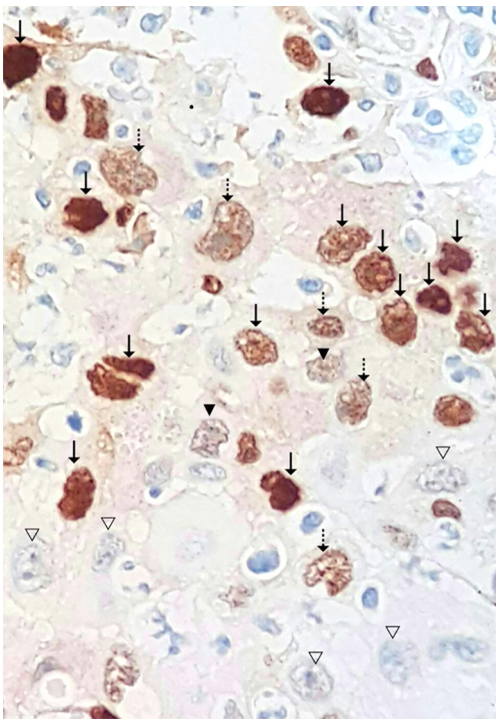


Figure 1 Immunohistochemical staining of cyclin D1 in lung cancer (∇, ▼, ⇣, ↓ represented negative, 1+, 2+, 3+, respectively).

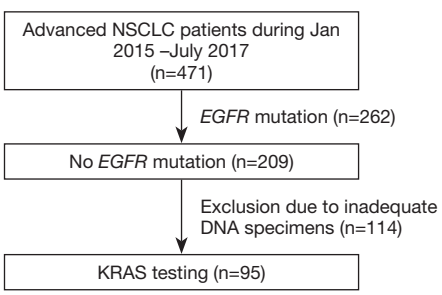


Figure 2 Patient flow diagram. From January 2015 to July 2017, 471 patients with NSCLC were evaluated for *EGFR* mutation test. There were 262 (55.6%) patients with *EGFR* mutations and 209 (44.4%) patients without *EGFR* mutations. After excluding 114 inadequate DNA specimens, 95 patients were evaluable for *KRAS* mutation testing. NSCLC, non-small cell lung cancer.

clinicopathologic characteristics was analyzed using Chi-squared test or Fisher's exact test (when the minimum expected count was less than 5). Binary logistic regression was performed to calculate odds ratio. Overall survival (OS) rate was measured from the date of diagnosis until the date of death from any causes and was analyzed by using the Kaplan-Meier method. Comparisons were done by using the log-rank test. The Cox proportional hazard model for survival was used for univariate and multivariate analyses. Median follow-up time was calculated using the reverse Kaplan-Meier method with the cut-off date on 28 September 2018. SPSS Statistical software version 21 (IBM Corp., Armonk, USA) was used to analyze the data. All tests were two-tailed and a P value <0.05 was considered statistically significance.

Results

Prevalence, clinical characteristics and prognosis of KRAS mutations

A total of 471 NSCLC patients received treatment at the King Chulalongkorn Memorial Hospital from January 2015 to July 2017. Of these patients, 262 (55.6%) had *EGFR*-mutant NSCLC. In NSCLC, *EGFR* and *KRAS* mutations are mutually exclusive (5), we thus identified *KRAS* mutations in NSCLC patients with *EGFR* wild-type (*EGFR* WT). After excluding 114 inadequate DNA specimens, there were 95 patients included for KRAS mutation testing (*Figure 2*). The median age was 67, and men accounted for 66.3% of the study population. Fifty-four point seven percent of the patients had history of smoking (*Table 1*). The majority of their histology (90.5%) was adenocarcinoma and 73.7% of the patients presented as stage IV at diagnosis. *KRAS* mutations were identified in 28 out of 95 patients with *EGFR* WT. The finding represented *KRAS* mutation rate of 7.8% of informative DNA status of the total population (28 out of 357, in which 262 were *EGFR*-mutant and 95 were *EGFR* WT NSCLC patients). Of 28 *KRAS*-mutant NSCLC patients, there were 26 (93%) patients with codon 12/13 mutations and 2 (7%) with codon 61 mutations.

The clinical parameters that were significantly associated with *KRAS* mutations were sex (P<0.001) and smoking history (P=0.001) (*Table 1*). *KRAS* mutations were more frequently found in men [odds ratio 23.25; 95% confidence interval (CI): 2.98–181.13; P=0.003] and former/current smoker patients (odds ratio 5.71; 95% CI: 1.93–16.88; P=0.002). The median follow-up time of this study was 38.8 months and 80.0% (76 out of 95) of the patients died. The median OS of *KRAS*-mutant *vs.* *KRAS* WT/*EGFR* WT NSCLC patients were 5.2 *vs.* 13.2 months, respectively (*Figure 3*). *KRAS*-mutant NSCLC patients had statistically significant shorter OS (log rank P=0.05). Of note, patients

Table 1 Relationship between *KRAS* mutation and clinicopathological characteristics of 95 NSCLC patients with *EGFR* wild type

Clinical characteristic	All (%)	*KRAS* mutation (%)	*KRAS* WT/*EGFR* WT (%)	P value
Total	95 (100.0)	28 (29.5)	67 (70.5)	–
Age at diagnosis				
Median [range]	67 [28–89]	73.5 [38–89]	66 [28–88]	–
≤65	42 (44.2)	10 (35.7)	32 (47.8)	0.281
>65	53 (55.8)	18 (64.3)	35 (52.2)	
Sex				<0.001
Men	63 (66.3)	27 (96.4)	36 (53.7)	
Women	32 (33.7)	1 (3.6)	31 (46.3)	
ECOG PS				0.395
0–1	67 (70.5)	18 (64.3)	49 (73.1)	
2–3	22 (23.2)	8 (28.6)	14 (20.9)	
Unknown	6 (6.3)	2 (7.1)	4 (6.0)	
BW loss				0.827
≤5%	53 (55.8)	17 (60.7)	36 (53.7)	
>5%	32 (33.7)	11 (39.3)	21 (31.3)	
Unknown	10 (10.5)	0 (0)	10 (14.9)	
Smoking				0.001
Never	41 (43.2)	5 (17.9)	36 (53.7)	
Former and current	52 (54.7)	23 (82.1)	29 (43.3)	
Unknown	2 (2.1)	0 (0)	2 (3.0)	
Histology				0.600
Adenocarcinoma	86 (90.5)	25 (89.3)	61 (91.0)	
NSCLC-NOS	7 (7.4)	2 (7.1)	5 (7.5)	
Squamous cell carcinoma	1 (1.1)	1 (3.6)	0 (0)	
Lymphoepithelioma carcinoma	1 (1.1)	0 (0)	1 (1.5)	
Stage (7^{th} AJCC)				0.930
IA	2 (2.1)	0 (0)	2 (3.0)	
IB	1 (1.1)	0 (0)	1 (1.5)	
IIA	3 (3.2)	1 (3.6)	2 (3.0)	
IIB	2 (2.1)	1 (3.6)	1 (1.5)	
IIIA	8 (8.4)	2 (7.1)	6 (9.0)	
IIIB	9 (9.5)	3 (10.7)	6 (9.0)	
IV	70 (73.7)	21 (75.0)	49 (73.1)	

NSCLC, non-small cell lung cancer; WT, wild type; ECOG PS, the Eastern Cooperative Oncology Group performance status; BW, body weight; NOS, not otherwise specified; AJCC, the American Joint Committee on Cancer.

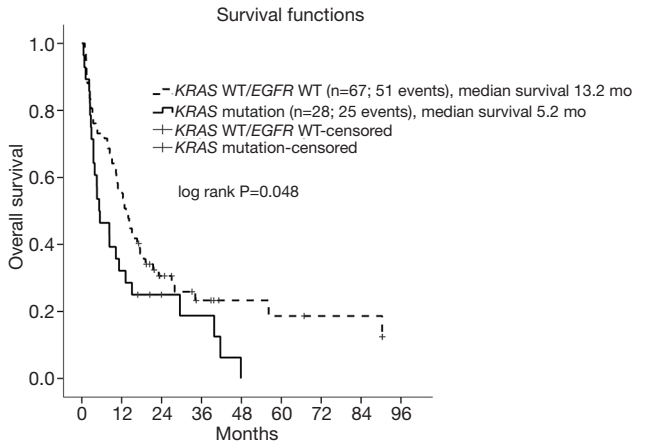


Figure 3 Overall survival of NSCLC patients with *KRAS* mutation *vs. KRAS* WT/*EGFR* WT. NSCLC, non-small cell lung cancer; WT, wild type.

with *KRAS* codon 61 mutations had limited survival comparing to those with *KRAS* codon 12/13 mutations (OS of 2.4 *vs.* 5.4 months).

Levels of cyclin D1 in KRAS mutant vs. KRAS WT/EGFR WT lung cancer

To test the hypothesis that *KRAS*-mutant NSCLC could drive cyclin D1 overexpression, immunohistochemistry of cyclin D1 was performed in 24 patients with *KRAS* mutant lung cancer and 26 patients with *KRAS* WT/*EGFR* WT lung cancer. The results demonstrated that cyclin D1 was mainly expressed in nucleus. The mean percentages of cyclin D1 expression were 68.83 (SD 27.11) and 50.50 (SD 27.5) in *KRAS*-mutant and *KRAS* WT/*EGFR* WT lung cancer, respectively. Cyclin D1 expression in *KRAS*-mutant lung cancer was significantly higher than that in *KRAS* WT/*EGFR* WT lung cancer (P=0.02) (*Figure 4A*).

Prognosis role of cyclin D1 in KRAS mutant lung cancer

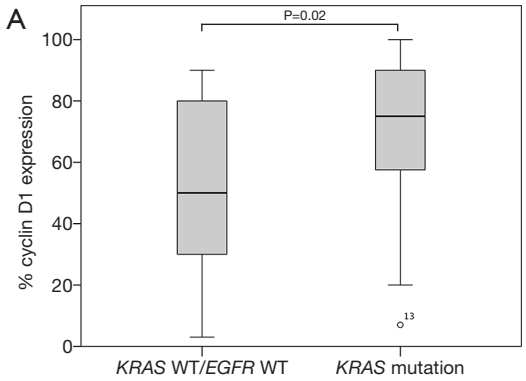
To investigate the prognostic role of cyclin D1, the expression levels of cyclin D1 were divided into two groups: high cyclin D1 and low cyclin D1 levels using the mean value of 60% as a cut point. High cyclin D1 levels were defined as the percentage of cyclin D1 expression of more than or equal 60% whereas low cyclin D1 levels were defined as cyclin D1 expression of less than 60%. Kaplan-Meier survival analyses of stage IV patients were performed to determine the prognostic role of cyclin D1 in *KRAS*-mutant and *KRAS* WT/*EGFR* WT NSCLC, separately.

In the *KRAS*-mutant NSCLC (n=18), patients with low cyclin D1 levels (n=4) had significantly better prognosis when compared with those with high cyclin D1 levels (n=14) (OS 41.7 *vs.* 3.5 months, P=0.037) (*Figure 4B*). In contrast, in the *KRAS* WT/*EGFR* WT NSCLC (n=19), there was no survival difference between patients with low (n=13) and high cyclin D1 (n=6) levels (OS 10.7 *vs.* 15.1 months, P=0.645) (*Figure 4C*).

Discussion

In the present study, we found a moderate prevalence of *KRAS* mutation, 7.8%, in Thai lung cancer. Similar to those of East Asian countries (11-13), where share a higher number of *EGFR* mutation especially in never or light smoking, *KRAS* mutation seems to be lower than those in Caucasian, 8–10% *vs.* 26.1% (5). However, we found that the prevalence of the underlying etiology for this group of *KRAS* mutation, smoking, was similar in both ethnicities. These results are consistent with the idea of a strong correlation between *KRAS*-mutant lung cancers to smoking. Consistent with previous studies (11,14,15), the prognosis of *KRAS*-mutant NSCLC was worse than that of *KRAS* WT/*EGFR* WT NSCLC (median survival of 5.2 *vs.* 13.2 months, P=0.048). NSCLC patients with *KRAS* codon 61 mutations had worse prognosis than those with *KRAS* codon 12/13 mutations. Different *KRAS* mutations subtypes have previously demonstrated different prognosis outcomes (16). In agreement with our results, *KRAS* codon 61 mutant lung cancers carried worse disease-free survival compared with those with *KRAS* codon 12 mutation (16). The worse outcome of *KRAS* codon 61 mutations could be explained by the more severely deficient in GTPase activity and relatively increased KRAS activity when compared with *KRAS* codon 12 or 13 mutations from an *in-vitro* study (17). The prevalence of *KRAS* mutations at codon 61 seems to be under-recognized as most studies selected only exon 2 (not exon 3) for determination of *KRAS* mutations. However, due to the small number of patients in this study, this needs validation in a larger patient population.

To our knowledge, this is the first study comparing the expression of cyclin D1 between NSCLC patients with *KRAS* mutation and those with *KRAS* WT/*EGFR* WT. Cyclin D1 expression in *KRAS*-mutant NSCLC was significantly higher than that found in *KRAS* WT/*EGFR* WT NSCLC (P=0.02). This could imply that *KRAS*-



% cyclin D1 expression	Mean	SD
All (n=50)	59.30	28.57
KRAS mutation (n=24)	68.83	27.11
KRAS WT/*EGFR* WT (n=26)	50.50	27.5

		All (%)	*KRAS* WT/*EGFR* WT (%)	*KRAS* mutation (%)	P value
Cyclin D1 expression	low (<60%)	20 (40.0)	14 (53.8)	6 (25.0)	0.038
	high (>60%)	30 (60.0)	12 (46.2)	18 (75.0)	

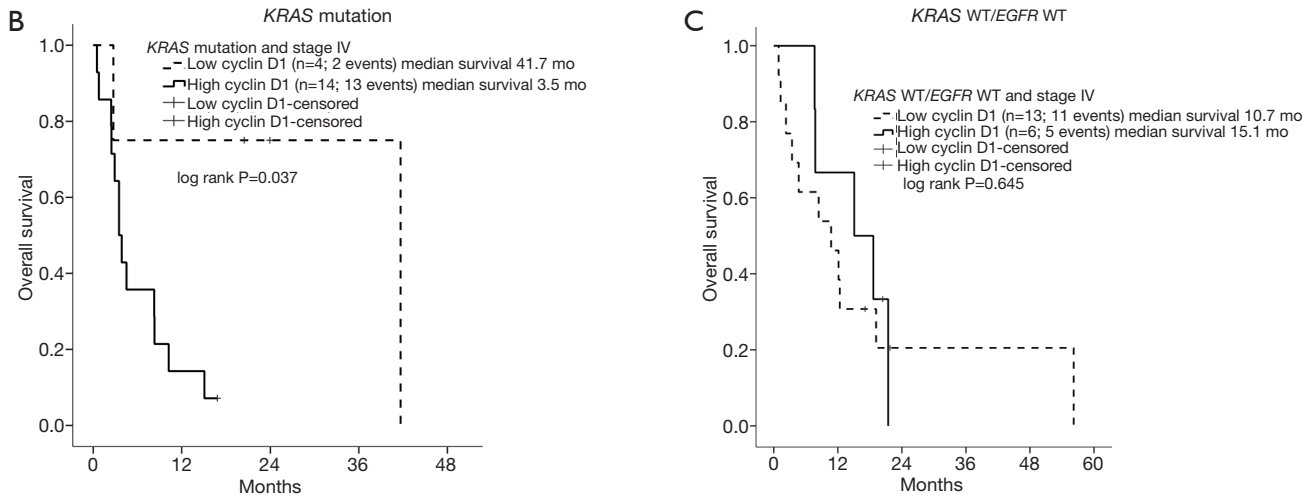


Figure 4 XXXXXXX. (A) Cyclin D1 expression in *KRAS*-mutant lung cancer (n=24) was significantly higher than those in *KRAS* WT/*EGFR* WT lung cancer (n=26); (B,C) overall survival of stage IV NSCLC patients with low cyclin D1 *vs*. high cyclin D1 in *KRAS* mutation (n=18) (B) and *KRAS* WT/*EGFR* WT (n=19) (C) (log-rank; P=0.037 for *KRAS* mutation and P=0.645 for *KRAS* WT/*EGFR* WT). NSCLC, non-small cell lung cancer; WT, wild type.

mutant NSCLC could drive cyclin D1 overexpression beyond *CCND1* amplification via an activation of mitogenic signaling pathways including RAS-MEK-ERK pathways (17). In line with our findings, there was a small study exploring *KRAS* mutation and cyclin D1 mRNA expression in 30 patients with early stage (I–III) NSCLC comprising 9 adenocarcinomas and 21 squamous cell carcinomas. It was found that *KRAS* mutations was associated with cyclin D1 mRNA expression (18). In addition, *KRAS* mutations and CCND1 amplifications were found to be mutually exclusive according to Pan-Lung cancer database (TCGA, Nature Genetics 2016) (19-21).

Interestingly, among NSCLC patients with *KRAS* mutations, those with low cyclin D1 levels had much better prognosis than those with high cyclin D1 levels (OS 41.7 *vs*. 3.5 months) and numerically better than those with *KRAS* WT/*EGFR* WT (OS 13.2 months). We speculated that defective cyclin D1 expression downstream of MAPK pathway could render *KRAS* mutant tumor cells less aggressive. This finding should be interpreted with caution because of the low number of patients with *KRAS*-mutant NSCLC in this retrospective, single-center study. Despite the limitations of this study, this is the first study demonstrated the potential prognosis role of cyclin D1 in *KRAS*-mutant NSCLC patients that could be worth further exploring in larger size prospective studies.

Conclusions

In Thailand, there was a moderate prevalence of *KRAS* mutation in lung cancers at about 7.8%. Smoking was the strong clinical parameter corelated with *KRAS* mutations.

Most *KRAS*-mutant NSCLC had high cyclin D1 expression and conferred poor prognosis. In contrast, *KRAS*-mutant NSCLC with low cyclin D1 level had much better prognosis.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (No. 560/59).

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