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ORIGINAL ARTICLE

การกลายพันธุ์ของยีน *PIK3CA* ตำแหน่งใหม่ในผู้ป่วยมะเร็งปากมดลูก

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บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาการกลายพันธุ์ของยีน *PIK3CA* จากตัวอย่าง Pap smear ในผู้ป่วยมะเร็งปากมดลูก ด้วยเทคนิค SYBR green real-time PCR (SYBR green RT-PCR) และเพื่อประเมินความถี่การกลายพันธุ์ของยีน *PIK3CA* ในผู้ป่วยมะเร็งปากมดลูกที่มารับการตรวจที่โรงพยาบาลกาฬสินธุ์ วิธีการศึกษา: ทำการสกัด DNA จากผู้ป่วยมะเร็งปากมดลูก 73 ราย ตรวจหาการกลายพันธุ์ของยีน *PIK3CA* exons 9 และ 20 ด้วยเทคนิค SYBR green RT-PCR จากนั้นวิเคราะห์ผลโดยใช้ melting curve และทำการตรวจยืนยันการกลายพันธุ์ ด้วยวิธี DNA sequencing ผลการศึกษา: มีผู้ป่วย 3 ราย (16.6%) ที่พบการกลายพันธุ์ของยีน *PIK3CA* ใน exon 9 โดยมี 2 รายที่มีการกลายพันธุ์ที่ตำแหน่ง S555T (serine to threonine c. 1668 G> C และ c.1669T del) อีกตัวหนึ่งพบกับการกลายพันธุ์ที่ตำแหน่ง S555T (c. 1668 G> C และ c.1669T del) ร่วมกับ E545A (glutamic acid to alanine; c.134 A> C) ทั้งหมดมีค่า Tm ต่ำกว่าค่า Tm ของ negative control ส่วนใน exon 20 ไม่พบการกลายพันธุ์ สรุปผลการศึกษา: ตัวอย่าง Pap smear สามารถใช้สำหรับการตรวจคัดกรองการกลายพันธุ์ของยีน *PIK3CA* ใน exons 9 และ 20 โดยใช้เทคนิค SYBR green RT-PCR ก่อนการส่งตรวจ DNA sequencing ซึ่งอาจช่วยประหยัดค่าใช้จ่าย และสามารถทำได้ในโรงพยาบาลทั่วไป

คำสำคัญ: Phosphatidylinositol kinase catalytic subunit alpha, *PIK3CA*, Real-time PCR, Pap smear, มะเร็งปากมดลูก

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ORIGINAL ARTICLE

New *PIK3CA* gene mutation in cervical cancer patients

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Abstract

Objectives: This study aimed to detect *PIK3CA* gene mutations in Pap smears from cervical cancer patients using SYBR green real time-PCR (SYBR green RT-PCR) and to estimate the frequency of *PIK3CA* gene mutations in cervical cancer patients from Kalasin Hospital, Kalasin Province, Thailand. **Method:** The DNA from Pap smears of 73 cervical cancer patients was extracted. Mutations of the *PIK3CA* gene on exons 9 and 20 were detected using SYBR green PCR. Then the melting curve was analyzed. The mutations in the samples were confirmed by DNA sequencing. **Result:** There were three (16.6%) cases that had a mutation of the *PIK3CA* gene on exon 9. Two of them were found to have the mutation at S555T (serine to threonine; c. 1668 G>C and c.1669T del). Another one was found with the mutation at S555T (c. 1668 G>C and c.1669T del) plus E545A (glutamic acid to alanine; c.1634A>C). All of them had Tm values lower than the Tm values of the negative control. There were no mutations on exon 20. **Conclusion:** Pap smear samples could be used for screening of the mutation of *PIK3CA* on exons 9 and 20 using SYBR green PCR before DNA sequencing. This could save costs and could be performed in general hospitals.

Keywords: Phosphatidylinositol kinase catalytic subunit alpha, *PIK3CA*; Real-time PCR, Pap smear, Cervical cancer

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Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is one of the most commonly activated signal pathways in several cancer types. This pathway controls cell proliferation, growth, differentiation, protein synthesis, glucose metabolism, migration, and apoptosis. Activation of this pathway is initiated by the binding of the corresponding ligands to tyrosine kinase receptors[1]. A regulatory subunit of PI3K is phosphorylated and results in the activation of a 110-kDa catalytic subunit[2]. Aberrant activation of this pathway is involved in cell metabolism and survival, cell cycle progression, regulation of apoptosis, protein synthesis, and genomic instability while it also promotes carcinogenesis and tumor angiogenesis[3-4]. There are several reports that showed the overexpression and mutation of the phosphatidylinositol 3-kinase catalytic subunit alpha (*PIK3CA*) was associated with several cancer types, for example, breast cancer[5], bladder cancer[6], and cervical cancer[7], etc. In cervical cancer, there were several reports showing that *PIK3CA* mutations were predictive of a poor response to standard radiochemotherapy ± cetuximab[8] and cisplatin-based concurrent chemoradiotherapy[9]. Whereas other reports indicated that cervical cancer patients with a *PIK3CA* mutation had higher responses to target therapy, PI3K/Akt pathway inhibitors, than patients with no *PIK3CA* mutations. Activation is frequently mediated by mutations in the p110a subunit of PI3K, *PIK3CA*, with most mutations occurring either in exon 9, which codes for the helical domain, or exon 20, which codes for the kinase domain[10-11].

Therefore, detection of *PIK3CA* gene mutations in cervical cancer patients can be used as a guide for treatment planning and selection of proper medicine for the highest response and benefits for the patients. However, the detection of *PIK3CA* gene mutations in cervical cancer patients can be complicated by the use of formalin-fixed, paraffin-embedded tissue. Therefore, the objectives of this study were to detect *PIK3CA* gene mutations in Pap smears from cervical cancer patients by SYBR green real time-PCR (SYBR green RT-PCR) and to estimate the frequency of *PIK3CA* gene mutations in cervical cancer patients from Kalasin Hospital, Kalasin Province, Thailand.

Materials and Methods

Pap smear samples

Pap smear samples were from 73 cervical cancer patients attending Kalasin Hospital, Kalasin Province, Thailand, during 2010 to 2014. Their pathological results were confirmed by the Institute of Pathology, Department of Medical Service Ministry of Public Health, Bangkok, Thailand.

DNA extraction

The coverglass was removed from the Pap smear slide by soaking in xylene overnight. The Pap tissue was scratched and transferred to an

Eppendorf tube, 1 mL of TE buffer was added, mixed well, and centrifuged at 14,000 rpm for 10 min at room temperature. The supernatant was discarded. Then the pellet was washed two times using 1 mL of absolute ethanol, mixed well, and centrifuged (14,000 rpm) at room temperature for 10 min. The washed tissue pellet was extracted

using a Genomic DNA Mini Kit (Geneaid Company, Taiwan) according to the manufacturer's protocol. The extracted DNA concentration was estimated by a NanoVue™ spectrophotometer (Fisher Scientific, UK) at the absorbance of 260 nm. The extracted DNA was kept at -70°C until use.

SYBR green RT-PCR

The primers for the *PIK3CA* gene on exon 9 for SYBR green RT-PCR were ex9*PIK3CA*-1F, 5′-AATCATCTGTGAATCAGAGG-3′, ex9*PIK3CA*-1R, 5′-TGAGATCAGCCAAATTCAGTT-3′ and the primers for exon 20 were ex20*PIK3CA*-1F, 5′- CTCAATGATGCTTGGCTCTG -3′, ex20*PIK3CA*-1R, 5′- TGGAATCCAGAGTGAGCTTTC -3′[12]. The SYBR green RT-PCR reaction was conducted using an ExiCycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, South Korea). The 50 µl of the PCR mixture were composed of 2 µl of 10 pmole of each forward and reverse primer, 25 µl of 2X Greenstar Master Mix (AccuPower Greenstar qPCR Master Mix Bioneer, South Korea), 1 µl of 50X ROX dye, 5 µl of DNA, and DW. The negative control was Pap smear samples that were negative for malignancy without the *PIK3CA* mutation on exons 9 and 20, which were confirmed by DNA sequencing. The reaction conditions were initial denaturation (at 95°C, for 5 min), 40 cycles of DNA denaturation (at 95°C, for 30 sec), annealing (57°C, for 30 sec), and extension (72°C, for 30 sec). The melting temperature (Tm) from the SYBR Green signal was analyzed. A positive result was a Tm value lower than the Tm value of the negative control. Then the positive sample was confirmed as having the *PIK3CA* mutation on exons 9 and 20 by DNA sequencing.

DNA sequencing

The *PIK3CA* genes were amplified using the conventional PCR technique. PCR products, forward primer, and reverse primer were sent to First Base Laboratories SDN BHD (Selangor, Malaysia) for sequencing. Each DNA sequence was analyzed by BioEdit version 7.2.6.1.

Ethical clearance

This study was reviewed and approved by the International Ethics Board, Khon Kaen University, Thailand (Reference No. HE582029).

Results

Demographic and clinical characteristics

Based on WHO age group classification, their median age was 52 years (range from 31-71 years). Pap smears were diagnosed as 82% squamous cell carcinomas and 18% adenocarcinomas (**Table 1**).

Table 1 Demographic and clinical characteristics of 73 cervical cancer patients.

Characteristics	Patients	
	N(73)	%
Age (years)		
Median	52	
Range	31-71	
<30	0	0
30-34	3	4.1
35-39	4	5.5
40-44	4	5.5
45-49	11	15.1
50-54	27	37
55-59	11	15.1
60-64	10	13.7
65-69	2	2.7
>69	1	1.4
Histology		
Squamous cell carcinoma	60	82
Adenocarcinoma	13	18

SYBR green RT- PCR

Melting curve analysis of the *PIK3CA* exon 9 and exon 20 by SYBR green RT-PCR is shown in **Figure 1.** The melting curves of *PIK3CA* on exon 9 were divided into two patterns via the Tm values **(Table 2)**. The first patterns had Tm values of 82°C, which were equal to the wild type *PIK3CA* (control), and the second patterns had Tm values of 80ºC **(Figure 1)**. For *PIK3CA* exon 20, there were two patterns of the Tm values similar to those from exon 9.

***PIK3CA* exon 9 and exon 20 sequencing analysis**

Due to different Tm values from the *PIK3CA* exon 9 amplification, 20 conventional PCR products were selected for DNA sequencing. The DNA sequences of *PIK3CA* exon 9 were analyzed by BioEdit version 7.2.6.1 and it was found that three cases had mutations. The mutated *PIK3CA* on exon 9 were S555T (serine to threonine; c. 1668 G>C and c.1669T del), and S555T (c.1668G>C and c.1669T del) plus E545A (glutamic acid to alanine; c.1634A>C) (**Table 2 and Figure 2**). All mutations were found from the Tm values of 80ºC, and no mutations were found from the Tm values of 82ºC. There were on mutated *PIK3CA* from exon 20 found in this study.

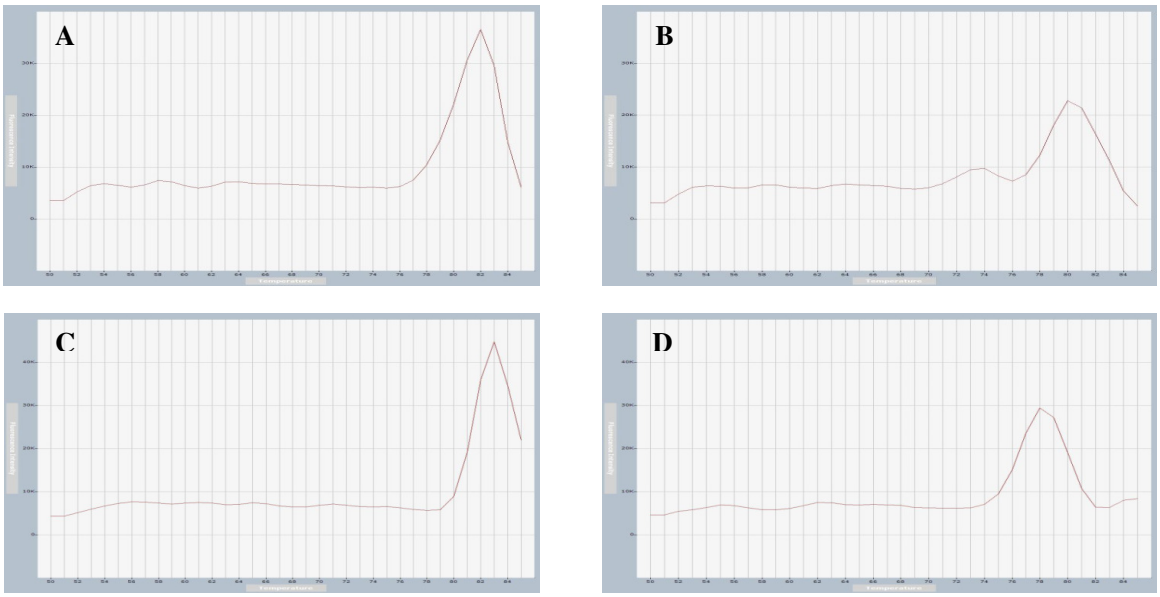

Figure 1 SYBR green real-time PCR melting temperature (Tm) patterns of amplified PIK3CA exon 9 and 20 in Pap smear tissues from cervical cancer patients. (A) Examples of PIK3CA gene exon 9 melting peaks from some samples and wild type (control), (B) negative control (Tm = 82ºC), (C) sample 16 (Tm = 80ºC), and (D) sample 19 (Tm = 80ºC).

Table 2 SYBR green real-time PCR melting temperature (Tm) patterns of amplified PIK3CA exon 9 and exon 20 in Pap smear tissues from cervical cancer patients.

[a]Tm compared with negative control	*PIK3CA* exon9		*PIK3CA* exon 20	
	N(73)	%	N(73)	%
Normal	34	46.6	65	89
Abnormal	39	53.4	8	11

[a]Tm; melting temperature

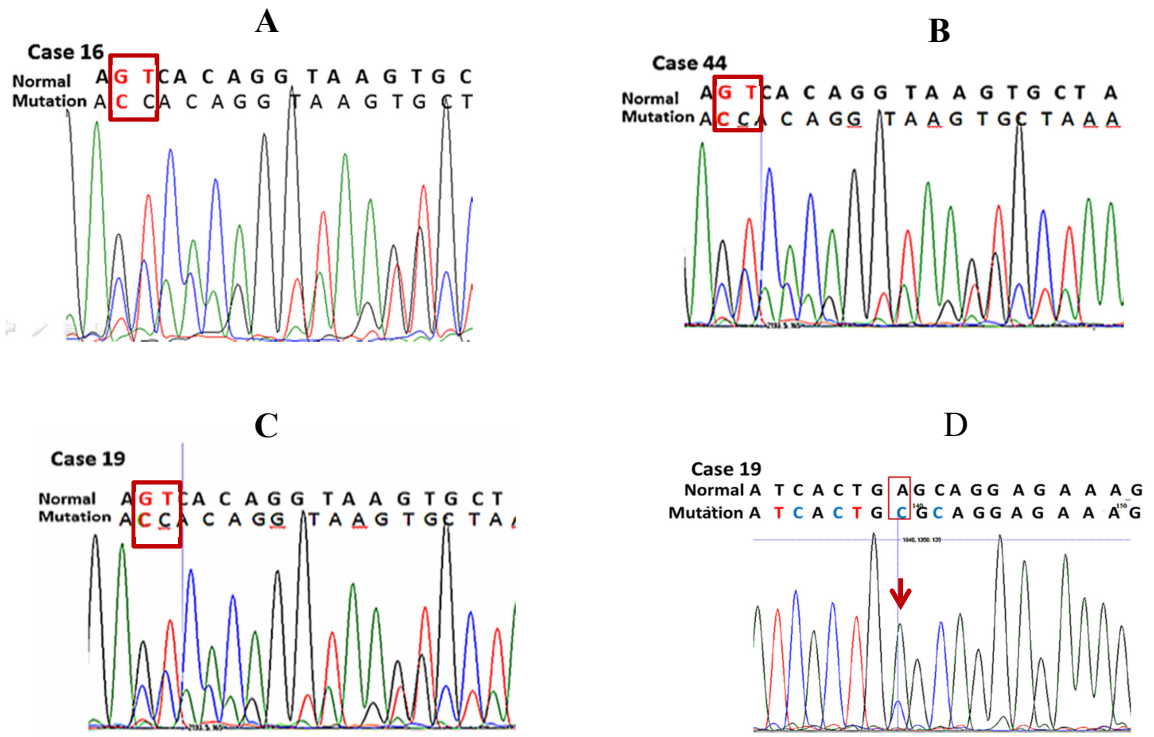

Figure 2 Mutation of *PIK3CA* on exon 9 from DNA sequencing results analyzed by Bioedit. (A) Sample 16, *PIK3CA* exon 9 mutations S555T (serine to threonine; c. 1668 G>C and c.1669T del), (B) sample 44, *PIK3CA* exon 9 mutations S555T (c. 1668 G>C and c.1669T del), (C) sample 19, PIK3CA exon 9 mutations S555T (c. 1668 G>C and c.1669T del), and (D) sample 19, *PIK3CA* exon 9 mutation in E545A (glutamic acid to alanine; c.1634A>C).

Discussion

Previous reports showed that the most common *PIK3CA* exon 9 mutation was PIK-3CA-E545K, which accounted for approximately 60% of all cases with mutations and most of *PIK3CA*-E545K (c.1633G>A) were heterozygous[13]. Other mutations of *PIK3CA* exon 9 in squamous cell carcinoma and adenocarcinoma of the cervix were E542K, E545A, D527N, E547K, S541Y, and Q546Q[14-15]. These mutations were considered to be about 11% and 5% in adenocarcinoma and squamous cell carcinoma, respectively[15]. Our study showed that there was no common *PI3KCA* exon 9 mutation as E545K or E542K were not found, but the uncommon mutation E545A (c. 1634 A>C) and new mutation S555T (c. 1668G>C, c.1669T del) were found. The S55T mutation occurred due to a single base substitution at 1668 in *PIK3CA* exon 9 from G to C, and then 1669T deletion with a frame shift mutation occurred. In the wild type PIK3CA exon 9, there was a stop codon (TAA) at the codon position 558. Due to the frame shift mutation 1669T del, the stop codon at codon 558 disappeared, so that the mutant protein might be longer than in the wild type. This mutation was not previously reported in the Catalogue of Somatic Mutations in Cancer (COMIC). Overall, the prevalence of *PIK3CA* mutations in this study was 16.6% (3 cases/18),

two were new mutations and one was E545A. S55T mutations were found in three cases, and two cases were squamous cell carcinoma FIGO stage IB and IIA, whereas another was adenocarcinoma FIGO stage IB. E545A was found together with S55T mutated squamous cell carcinoma stage IIA. Most reports showed that *PIK3CA* mutations were found in squamous cell carcinoma more than adenocarcinoma or non-squamous cell tumors*(14, 16-17)*. Moreover, these mutations were correlated with worse disease-free survival when treated with standard radiochemotherapy[8-9, 16].

On *PIK3CA* exon 20, there was no mutation detected in this study. Previous reports showed that exon 20 had fewer mutations than exon 9. The PIK3CA exon 20 mutations previously reported were H1047R, H1047L, G1049R, M1043V, and M1043I[10].

The melting temperature of *PIK3CA* exon 9 with SYBR green RT-PCR may be useful for screening mutations because mutations were found in the cases of lower Tm patterns (< 82ºC) than Tm pattern of 82ºC, which was equal to the wild type *PIK3CA* (control). However, it must be confirmed by sequencing or other specific methods.

From the results of this study, we can conclude that Pap smear samples could be used for screening the mutations of *PIK3CA* on exons 9 and 20 using SYBR green RT- PCR before DNA sequencing. The method could save costs and could be performed in general hospitals. Patients will receive procedures more rapidly. Moreover, the frequency of *PIK3CA* exons 9 and 20 mutations in cervical cancer patients derived from Kalasin Hospital was 16.6%.

However, this study is a primary report that had only 20 samples for DNA sequencing. A further study should be conducted to investigate a greater number of samples. Moreover, other techniques should be studied to develop accurate and rapid testing for this mutation.

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