รายงานการวิจัย

เรื่อง

ความสัมพันธ์ของจีนกับการสูบบุรี่และการติดเชื้อไวรัสต่อการเกิดมะเร็งปากมดลูกของสตรีไทย

Association between genes and cervical cancer susceptibility among smoking and viral infected Thai women

ชื่อผู้วิจัย

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โครงการวิจัย เรื่องความสัมพันธ์ของจีนกับการสูบบุรี่และการติดเชื้อไวรัสต่อการเกิดมะเร็งปากมดลูกของสตรีไทย ได้รับการสนับสนุนของทุนวิจัยประเภทอุดหนุนทั่วไป ประจำปีงบประมาณ พ.ศ. 2556 มหาวิทยาลัยขอนแก่น คณะผู้วิจัย ขอขอบพระคุณเป็นอย่างสูงมา ณ โอกาสนี้ด้วย

คำนำ

โครงการวิจัย เรื่องความสัมพันธ์ของจีนกับการสูบบุรี่และการติดเชื้อไวรัสต่อการเกิดมะเร็งปากมดลูกของสตรีไทย เป็นการศึกษาที่มีเป้าหมายในการหาแนวทางในการลดอุบัติการณ์การเกิดมะเร็งปากมดลูก โดยการสืบค้นสาเหตุ หรือปัจจัยเสี่ยง ที่จะนำไปสู่การเกิดมะเร็งปากมดลูกของสตรีที่อาศัยอยู่ในภาคตะวันออกเฉียงเหนือของไทย คณะผู้วิจัยหวังเป็นอย่างยิ่งว่าข้อมูลจากผลการวิจัยนี้จะเป็นประโยชน์ อันจะนำไปสู่การวางแผน ป้องกัน และให้คำแนะนำที่เหมาะสมต่อไป การวิจัยนี้สามารถดำเนินการได้ตามเป้าหมายจากการสนับสนุนของทุนวิจัยประเภทอุดหนุนทั่วไป ประจำปีงบประมาณ พ.ศ. 2556 มหาวิทยาลัยขอนแก่น คณะผู้วิจัย ขอขอบพระคุณเป็นอย่างสูงมา ณ โอกาสนี้ด้วย

คณะผู้วิจัย
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บทคัดย่อ

มะเร็งปากมดลูกเป็นโรคมะเร็งที่สำคัญในสตรี โดยมีไวรัสฮิวแมนแปปิโลมา(HPV) เป็นสาเหตุที่สำคัญ อย่างไรก็ตามภูมิหลังด้านพันธุกรรมของ พี 53 (*p53*) ซึ่งเป็นจีนต้านมะเร็งอาจจะมีบทบาทสำคัญต่อการพัฒนาของโรคมะเร็ง ในมนุษย์พบว่ามีความหลากหลายทางพันธุกรรมของจีน *p53* ที่ตำแหน่งโคนดอน 72 ซึ่งมีอัลลีลที่แตกต่างกัน 2 แบบ คือ arginine (Arg) อัลลีล และ proline (Pro) อัลลีล การศึกษานี้จึงต้องการศึกษาความสัมพันธ์ระหว่าง ความหลากหลายทางพันธุกรรมของ *p53* ตำแหน่งโคนดอน 72 กับความเสี่ยงของการเป็นมะเร็งปากมดลูกในสตรีภาคตะวันออกเฉียงเหนือของไทยที่ติดเชื้อ HPV และได้รับสูบบุหรี่ โดยทำการศึกษาในอาสาสมัครจำนวน 336 ราย แบ่งออกเป็นสองกลุ่มที่มีอายุเฉลี่ยใกล้เคียงกัน (age-matched study) คือ กลุ่มควบคุมซึ่งเป็นอาสาสมัครที่มีสุขภาพดีจำนวน 168 ราย และกลุ่มผู้ป่วยมะเร็งปากมดลูกจำนวน 168 ราย ผลการศึกษาพบว่าความหลากหลายทางพันธุกรรมของ *p53* ไม่มีผลเปลี่ยนแปลงความเสี่ยงของการเกิดมะเร็งปากมดลูกอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) แต่พบว่าการติดเชื้อ HPV สามารถเพิ่มความเสี่ยงต่อการเกิดมะเร็งปากมดลูกอย่างมีนัยสำคัญด้วยค่า OR เท่ากับ 41.84 ($p<0.0001$) และค่า adjusted OR เท่ากับ 44.48 ($p<0.0001$) อาสาสมัครที่มีสามีสูบบุหรี่จะส่งผลให้ความเสี่ยงต่อการเกิดมะเร็งปากมดลูกเพิ่มขึ้น ทั้งในอาสามัครที่มีสามีกำลังสูบบุหรี่ หรือเคยสูบบุหรี่ ด้วยค่า OR เท่ากับ 3.25 ($p<0.0001$) และ 2.15 ($p<0.01$) ตามลำดับ และมีค่า adjusted OR เท่ากับ 2.48 ($p<0.05$) และ 3.29 ($p<0.01$) ตามลำดับ อย่างไรก็ตาม ในสตรีที่ติดเชื้อ HPV ความหลากหลายทางพันธุกรรมของ *p53* ไม่มีผลเปลี่ยนแปลงความเสี่ยงของการเกิดมะเร็งปากมดลูก สำหรับความสัมพันธ์ระหว่างความหลากหลายทางพันธุกรรมของ *p53* กับการสูบบุหรี่ พบว่า ความเสี่ยงของการเป็นมะเร็งปากมดลูกจะเพิ่มขึ้น เมื่อ 1) สามีกำลังสูบบุหรี่ และ 2) อาสาสมัครที่ *p53* มี Arg อัลลีล อยู่ด้วย โดยถ้าเป็น Arg/Pro จีโนไทป์ จะเพิ่มความเสี่ยง 4.78 เท่า ($p<0.05$) และ ถ้าเป็น Arg/Arg + Arg/Pro จีโนไทป์ จะเพิ่มความเสี่ยง 4.18 เท่า ($p<0.05$) เมื่อปรับด้วยอายุและการติดเชื้อ HPV ดังนั้นการตรวจหาความหลากหลายทางพันธุกรรมของ *p53* ร่วมกับลดการสัมผัสกับสารก่อมะเร็งจากสิ่งแวดล้อมจะยังคงมีประโยชน์ในการป้องกันการเกิดมะเร็งปากมดลูกในกลุ่มที่มีความเสี่ยงสูง

Abstract

Cervical cancer is one of the most prevalent cancers in women worldwide. Human papillomavirus (HPV) is accepted the main causes of this cancer however, host genetic background, *p53* a tumor suppress gene, may play a crucial role in the cancer development. In humans, the *p53* is reported to be polymorphic at codon 72 to generate functionally different arginine (Arg) and proline (Pro) alleles. The purpose of this study was to investigate the association between *p53* polymorphism at codon 72 and the risk of cervical cancer by HPV and smoking status in northeast Thailand. A total of 336 age-matched study subjects were divided into two groups: 168 normal controls and 168 cervical cancer patients. Presence of *p53* polymorphism did not significantly alter the risk for cervical cancer ($p>0.05$). Among HPV carriers a significantly increased risk for cervical cancer with an OR of 41.84 ($p<0.0001$) and an adjusted OR of 44.48 ($p<0.0001$) was observed. A higher risk was observed among subjects whose partner had smoking habits, whether currently and formerly; with a respective OR of 3.25 ($p<0.0001$) and 2.15 ($p<0.01$); and a respective adjusted OR of 2.48 ($p<0.05$) and 3.29 ($p<0.01$). None of the genotypes showed significant difference in the risk for the cervical cancer susceptibility by the status of HPV infection. As for the associations between the *p53* genotypes and the smoking status, the risk was significantly increased among the subjects with the following conditions; 1) their partner had currently smoking status, and 2) the subjects had the Arg allele: 4.78-fold for the Arg/Pro genotype ($p<0.05$) and 4.18-fold for the Arg/Arg + Arg/Pro combined genotype ($p<0.05$) after adjusting by age and HPV infection. Detection of *p53* polymorphism and reducing exposure to environmental carcinogens remains useful preventive advice for Thai women in the high-risk group.

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หน้า

บทนำ (Introduction)

Cancer of the cervix is one of the most serious public health problems among Thai women. Although human papillomavirus (HPV) is a main cause of cervical cancer [1-3], few HPV-infected females develop cervical cancer [4, 5]. Other risk factors—such as certain carcinogens and genetic predisposition—of the host may have influence with the cervical carcinogenesis.

TP53 is a tumor suppressor protein with a highly conserved role as a 'guardian of the genome' via cellular anticancer mechanisms [6]. A base substitution at codon 72 of the exon 4 of the *p53* gene—resulting in either arginine (Arg; CGC) or proline (Pro; CCC)—has been identified as polymorphic in human populations [1, 7-9]. Association of the *p53* codon 72 polymorphism with an increased susceptibility to cancer development has been examined for many sites such as lung cancer [10], gastric cancer [11], and endometrial cancer [12]. HPV E6 oncogene protein binds to TP53 and promotes dysfunction of its activity. Storey *et al.* suggest that homozygous for the Arg allele was more effective in apoptosis persistent than homozygous for the *Pro* allele, leading to tumor development in HPV associated cancers [13].

Tobacco smoke contains more than 4,000 chemical substances; some of which are carcinogenic, such as polycyclic aromatic hydrocarbons and volatile N-nitrosamines [14-16]. Exposure to smoking is a leading cause of many types of cancer including lung, esophageal, gastric, bladder, liver and cervix [17-25].

As mentioned above, there should be several risk factors for cervical cancer including HPV infection, *p53* polymorphism and smoking; genotypes of *p53* may be related to tumorigenic potential [26-28]. There are insufficient experimental data, however, to prove consistent differences in biological activity between the two protein variants [1]. Therefore further epidemiologic investigation into the relationship between TP53 and cervical cancer is needed; particularly the polymorphism at codon 72 of the *p53* as a marker for risk of cervical cancer. The main aims of our study were to examine the independent effect of the polymorphism of codon 72 of the *p53* on cervical carcinogenesis, including the interaction of HPV infection and smoking on cervical cancer development.

วิธีดำเนินการวิจัย (Materials & Method)

Study subjects

Women between 27 and 81 were recruited between February 2009 and August 2011 at Khon Kaen Hospital and Srinagarind Hospital, Khon Kaen province, Thailand. The study comprised 168 cases and 168 controls. The cases had a confirmed diagnosis of squamous cell carcinoma of the cervix (SCCA), by pathological examination. Controls were recruited among healthy woman with normal cytology (Pap smear) and histology.

The controls and cases were matched within 5-year age groupings. The subjects were verbally informed and received documentation explaining the purposes and procedures involved in the study. All of the subjects signed an informed consent form prior to participation in the study. The protocol of this study was approved by the Ethics Committee of Khon Kaen University (No. HE 450333) and Khon Kaen Hospital (No.03/02/2554).

Detection of p53 codon 72 polymorphism

Genomic DNA was extracted from buffy coat with GF-1 Blood DNA Extraction Kits (Vivantis, USA). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for analysis of *p53* codon 72 polymorphism [29]. PCR was performed with the following primers to amplify the *p53* exon 4: 5'-CCCGGACGATATTGAACA-3' and 5'-AGAAGCCCAGACGGAAAC-3'.

The PCR products of 203 base pairs (bp) were loaded into a 2% agarose gel. After electrophoresis, the gel were stained with ethidium bromide and photographed under UV light. The PCR product was digested by *BstU*I (New England. USA). The *BstU*I recognizing the sequence CG/CG in the presence of the Arg allele generated a 125 bp and a 78 bp fragment for the Arg allele, whereas the CCC or Pro allele remained uncut.

Detection of HPV

The DNA from cervical cells was extracted using Genomic DNA (blood/cells) mini Kit (Genomic DNA mini kit, Geneaid, Taiwan). The samples were tested for the presence of HPV DNA by PCR amplification of the L1 region with the GP5+/GP6+ consensus primers (GP5+ 5'-

TTTGTTACTGTGGTAGATACTAC-3' and GP6+ 5'-GAAAAATAAACTGTAAATCATATTC-3' [30]) and using beta-globin as an internal control. The amplified product was verified by 2% agarose gel electrophoresis, ethidium bromide staining and photography under UV light.

Statistical analyses

The genotypic frequencies between the case and controls were compared using the $\chi 2$ test. To compare the risk for cervical cancer between the Pro/Pro genotype and the other genotypes and to evaluate the association among the variables, uni- and multi-variate logistic regression analyses (using 800-STATA on PC) were used to calculate the odds ratio (OR) at the 95% confidence interval (CI). Differences were considered statistically significant when the *p* value was < 0.05.

ผลการวิจัย (Results)

The allele frequencies and distribution of the *p53* codon 72 polymorphism are shown in Table 1. The genotype distribution was in Hardy-Weinberg equilibrium in the controls. Frequency of the Pro and Arg allele was not significantly different between the cases and controls ($p>0.05$). The genotype distribution of *p53* polymorphism did not significantly alter the risk for cervical cancer ($p>0.05$).

The relationships between HPV infection, smoking status and risk for SCCA are presented in Table 2. Among HPV carriers a significantly increased risk for SCCA with an OR of 41.84 ($p<0.0001$) and an adjusted OR of 44.48 ($p<0.0001$) was observed. Although the smoking status of the subject did not alter the risk for SCCA, a higher risk was observed among subjects whose partner had smoking habits—whether currently and formerly; with a respective OR of 3.25 ($p<0.001$) and 2.15 ($p<0.01$); and a respective adjusted OR of 2.48 ($p<0.05$) and 3.29 ($p<0.01$).

Interaction between the *p53* genotypes and the risk for SCCA by HPV infection status was determined (Table 3) and none of the genotypes showed any significant difference in the risk for the cervical cancer susceptibility.

Associations between the *p53* genotypes and the risk for SCCA by smoking status of the partner were examined (Table 4). Among the status 'currently smoking', the risk for cervical cancer was significantly increased in the presence of the Arg allele; 4.78-fold for the Arg/Pro genotype ($p<0.05$) and 4.18-fold for the Arg/Arg + Arg/Pro combined genotype ($p<0.05$) after adjusting by age and HPV infection. There was no association between the *p53* codon 72 polymorphism and the risk for cervical cancer among women who had been exposed to smoke by the partner ($p>0.05$).

Table1. Association of *p53* codon 72 genotype and risk for cervical cancer

Genotype of *p53*	Cases n	Controls n	OR [95% CI, *p*-value]	Adjusted OR[a] [95% CI, *p*-value]
Pro/Pro	37	49	1	1
Arg/Arg	52	46	1.50 [0.80-2.80, >0.05]	2.18 [0.89-5.35, >0.05]
Arg/Pro	79	73	1.43 [0.81-2.53, >0.05]	1.63 [0.72-3.68, >0.05]
Arg/Aro+Arg/Pro	131	119	1.46 [0.86-2.47, >0.05]	1.82 [0.85-3.91, >0.05]
Allele	Allele frequency			
	Cases	Controls		
Pro	0.46	0.51	1	
Arg	0.54	0.49	1.15 [0.80-1.68, >0.05]	

[a]adjusted with multiple logistic regression for age, smoking status of partner and HPV infection.

Table2. HPV infection, smoking status and risk for cervical cancer

Variable	Cases n (%)	Controls n (%)	OR [95%CI, *p*-value]	Adjusted OR[a] [95%CI, *p*-value]
HPV status				
negative	23 (13.69)	146 (86.90)	1	1
positive	145 (86.31)	22 (13.10)	41.84 [21.41-82.56, <0.0001]	44.48 [22.79-86.82, <0.0001]
Smoking status of				
non smoker	161 (95.8)	165 (98.2)	1	
smoker	7 (4.2)	3 (1.8)	2.39 [0.53-14.54, >0.05]	0.42 [0.08-2.11, >0.05]
Smoking status of				
non smoker	46 (27.38)	82 (48.81)	1	1
smoker	117 (71.78)	77 (48.43)	2.71 [1.66-4.42, <0.005]	2.78 [1.41-5.48, <0.005]
current smoker	71 (42.26)	39 (23.21)	3.25 [1.84-5.73, <0.0001]	2.48 [1.14-5.38, <0.05]
former smoker	46 (28.22)	38 (23.90)	2.15 [1.19-3.93, <0.01]	3.29 [1.41-7.66, <0.01]

[a]adjusted with logistic regression for age

Table 3 Association of *p53* codon 72 genotype, HPV status and risk for cervical cancer

HPV status	Genotype of *p53*[a]	Cases n	Controls n	OR [95%CI, *p*-value]	Adjusted OR[b] [95%CI, *p*-value]
HPV+	P/P	33	7	1	1
	A/A	43	5	1.82 [0.45-7.93, >0.05]	2.30 [0.62-8.47, >0.05]
	A/P	69	10	1.46 [0.43-4.69, >0.05]	1.77 [0.58-5.38, >0.05]
	A/A+A/P	112	15	1.58 [0.50-4.55, >0.05]	1.94 [0.68-5.51, >0.05]
HPV-	P/P	4	42	1	1
	A/A	9	41	2.30 [0.58-10.97, >0.05]	2.16 [0.61-7.65, >0.05]
	A/P	10	63	1.67 [0.44-7.73, >0.05]	1.55 [0.45-5.32, >0.05]
	A/A+A/P	19	104	1.92 [0.59-8.19, >0.05]	1.79 [0.57-5.63, >0.05]

[a]P: Pro; A: Arg [b]adjusted with multiple logistic regression for age and smoking status of partner

Table 4 Association of *p53* codon 72 genotype, smoking status of partner and risk for cervical cancer

Smoking status	Genotype of *p53*[a]	Cases n	Controls n	OR [95%CI, *p*-value]	Adjusted OR[b] [95%CI, *p*-value]
Current smoker	P/P	21	14	1	1
	A/A	17	10	1.13 [0.36-3.63, >0.05]	3.36 [0.62-18.05, >0.05]
	A/P	33	15	1.47 [0.53-4.02, >0.05]	4.78 [1.03-22.20, <0.05]
	A/A+A/P	50	25	1.33 [0.53-3.30, >0.05]	4.18 [1.02-17.12, <0.05]
Former smoker	P/P	7	9	1	1
	A/A	18	11	2.10 [0.51-8.75, >0.05]	1.33 [0.23-7.77, >0.05]
	A/P	21	18	1.50 [0.40-5.77, >0.05]	0.75 [0.13-4.22, >0.05]
	A/A+A/P	39	29	1.73 [0.50-6.13, >0.05]	0.96 [0.20-4.65, >0.05]

[a]P: Pro; A: Arg [b]adjusted with multiple logistic regression for age and HPV infection.

อภิปราย/วิจารณ์ (Discussion)

HPV infection persists as a main cause of cervical cancer development; it was identified in 86.31% of cases and increased the risk for development of SCCA by 44.5-fold. The current study, compared to our previous study with a 5-year interval that demonstrated 86.7% of cases to be infected with HPV with an increased risk for cancer by 43.5-fold [31]. Not all of the infected women developed cancer, as the healthy women were able to clear the infection within 1–2 years [1, 32]. Indeed, <1% of persons positive for oncogenic types of HPV develop cervical cancer [4, 5]; strongly suggesting that participation of other factors (*i.e.,* sexual behaviors, smoking, chemical substances and genetic backgrounds of the host) in higher incidence of the cervical cancer in this region.

The *p53* gene plays critical roles in apoptosis and cell cycle arrest; TP53 acts as a major regulator of DNA transcription to bind DNA strand breaks and interacts with proteins involved in DNA repair [33-35]. A polymorphism of the nucleotide at codon 72 on the exon 4 of the *p53* results in either Arg and Pro allele and these variants are suspected of having a different interaction pattern. The E6 oncoproteins from both the high- and low-risk HPV types are able to target the Arg allele more efficiently than the Pro allele, which results in inactivation of the function of the Arg allele through ubiquitin-mediated degradation [1, 36]. The ability to bind DNA may be lost so that the Arg allele is functionally inactive; leading to loss of the tumor suppressor function of *p53* and a subsequently higher risk of cancer development. Storey *et al.* [13] was the first to report that individuals homozygous for p53 72Arg have a 7-fold greater risk of HPV-associated cancers than those who are heterozygous. Several other studies have failed to confirm the role of this polymorphism and cervical cancer development—including in Thailand [22, 31], Japan [37], Netherlands [38], and southern England [39]. In the current study, subjects homozygous for the Arg did not show an increased OR whether they were positive or negative for HPV infection (Table 3), which supports the previous studies that were against Storey et al. (1998)[13]. The effect of the *p53* polymorphism on HPV-related cervical cancer may therefore be subtle.

It is our general understanding that smoking is a common risk for many cancers. Tobacco-related carcinogens in a smoking sex partner's seminal fluid are applied directly to the cervix mucus membrane during sexual intercourse [40, 41]. The current study confirmed our

previous work among Northeast Thai women that passive tobacco smoking contributed to an increased risk of SCCA development [22] and, moreover, found an association between genetic polymorphism of the *p53* codon 72 and the risk of cervical cancer among women whose partner was a current smoker (Table 4). Some studies have found a relationship between genotype for *p53* and carcinogenesis; that cells carrying the Arg allele require further *p53* mutations to increase their tumorigenic potential, while Pro/Pro cells could undergo this process with less damage [26]. Moreover, the *p53* mutant acted as a more potent inhibitor of TP53 activity when the *p53* has the Arg allele rather than the Pro allele [27, 28]. This higher risk for the development of cervical cancer in the Arg/Pro genotyped women with a current smoking partner would be attributed to the exposure to the tobacco carcinogens and less recovery of damages in the presence of the Arg allele. A more detailed survey for the smoking behavior of the partner is awaited.

In conclusion, although the A allele variant of codon 72 of the *p53* gene does not, by itself, increase the risk of cervical cancer, the *p53* polymorphism may act upon other co-factors namely tobacco smoking in development of cervical cancer. Detection of *p53* polymorphism and reduced exposure to carcinogenic environmental factors would be appropriate advice for women in the high-risk group.

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