RESEARCH COMMUNICATION

Selected Risk Factors, Human Papillomavirus Infection and the *P53* Codon 72 Polymorphism in Patients with Squamous Intraepithelial Lesions in Northeastern Thailand

Wannapa Settheetham-Ishida*[1], Pissamai Yuenyao[2], Wichittra Tassaneeyakul[3], Nipa Kanjanavirojkul[4], Atcharaporn Thawmor[1], Churairat Kularbkaew [4], Chariya Hahvajanawong[5], Dariwan Settheetham [6], Jintanaporn Wattanathorn[1], Taeko Kashima[7], Takafumi Ishida[7]

Abstract

Risk factors for cervical squamous intraepithelial lesions (SIL) including human papillomavirus (HPV) infection and the *p53* codon 72 polymorphism were investigated in a case-control study with 103 cases and 105 controls in Northeastern Thailand. Increased risk for SIL was observed for age at menarche (odds ratio (OR) = 2.2; $p<0.005$), age at the first sexual intercourse (OR=2.4; $p<0.05$), number of sexual partners (OR=2.7; $p<0.005$) and partners' smoking history (OR=2.3-3.2; $p<0.01$). Prevalence of malignant type of HPV infection in the control and SIL groups was 18.1% and 60.2%, respectively. HPV infection significantly increased risk for SIL 6.8-fold ($p<0.001$). HPV-16 infection was the commonest (31 out of 62 carriers) in SIL patients and highly associated with risk. The *p53* codon 72 polymorphism was not identified as a genetic risk for SIL in this study, as demonstrated in Thai cervical cancer. Therefore, to prevent cervical neoplasia or HPV infection, inclusion of knowledge on sexual behavior and effects of smoking into public health programs is important and, at the same time, a nation-wide screening scheme for cervical abnormalities including HPV-typing is a high priority in Thailand.

Key-Words: Risk factors - HPV - p53 polymorphism - SIL - Thailand

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Introduction

The prevalence of cervical cancer in Thailand is decreasing but remains high with an age-standardized incidence rate of 19.5 (Pengsaa et al., 2003). In the worldwide anti-cancer program, screening by Papanicolaou (Pap) smear test and early treatment of precancerous lesions have been employed to reduce incidence and mortality of cervical cancer. However, identification and early eradication of risk factors should also be emphasized to prevent cervical cancer in Thailand.

It has been suggested that infection with certain types of human papillomavirus (HPV), particularly malignant type, tightly correlates with development of cervical cancer (Schiffman and Castle, 2003; zur Hausen, 1991). Among HPV carriers, the majority remain asymptomatic and only a small fraction develop cervical preneoplastic lesions and/or an invasive cervical cancer as a result (Tommasino et al., 2003; Zehbe et al., 2001). This indicates that additional factors are also involved in determining the fate of HPV infection. Our previous studies on cervical cancer in Northeastern Thailand have confirmed that HPV infection and certain risk factors such as sexual behaviors and smoking contribute to the cancer development (Settheetham-Ishida et al., 2004a; Settheetham-Ishida et al., 2005). However, factors responsible for the progression of precursory lesion in the cervix into malignant neoplasia are still to be clarified.

In the search for genetic backgrounds responsible for

*[1] Department of Physiology, [2]Department of Obstetrics and Gynecology, [3]Department of Pharmacology, [4]Department of Pathology, [5]Department of Microbiology, Faculty of Medicine, [6]Department of Environmental Health, Faculty of Public Health, Khon Kaen University, [7]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan *Correspondence to: Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002 Thailand Tel/Fax [66]-43-348394 E-mail: wannapa@kku.ac.th*

cervical cancer development, the *p53* codon 72 polymorphism, arginine (*Arg*) and proline (*Pro*), was documented to be a genetic risk for HPV infected cervical carcinogenesis (Storey et al., 1998; zur Hausen and de Villiers, 1994). Biological and biochemical differences between p53 with Arg and Pro at amino acid position 72 indicate possible presence of different interactions between either p53 protein variant and the E6 protein of HPV (Storey et al., 1998; Thomas et al., 1999a; Thomas et al., 1999b). Arg type of p53 was more efficiently inactivated by the E6 oncoprotein than Pro type of p53 (Storey et al., 1998; Zehbe et al., 2001). Polymorphism of the *p53* in codon 72 was recorded in various human populations with varying allele frequencies (Helland et al., 1998; Yamada et al., 1997). Not all studies, however, did support the association between the *p53* polymorphism and cervical cancer (Comar et al., 2004; Nishikawa et al., 2000; Rezza et al., 2001; Yamashita et al., 1999) and the associations between the *p53* polymorphism and susceptibility to HPV infection as well as development of the cervical lesions were open to doubt.

In Northeastern Thailand, although high incidence of cervical cancer is present, few reports on the HPV infections and the *p53* polymorphism are available (Ekalaksananan et al., 2001; Lertworapreecha et al., 1998; Limpaiboon et al., 2000; Settheetham-Ishida et al., 2005). In this report, we studied relationships between cervical neoplasia, particularly, in squamous intraepithelial lesions and risk factors, namely HPV infection, the *p53* codon 72 polymorphism, history of reproduction, pattern of sexual behaviors, and history of smoking among Northeastern Thai women.

Materials and Methods

Study Subjects

Female volunteers aged 25-60 years were recruited during July 2002 and August 2004 at Srinagarind Hospital, Khon Kaen University, Thailand. A total 208 subjects were enrolled in this study, 103 cases were confirmed diagnosis of cervical squamous intraepithelial lesions (SIL) by pathological examination. _Controls were women with normal cytologic/ histopathologic appearance of cervix (n=105). The controls and cases were matched within 5-year age group. The same interviewer conducted a standardized questionnaire. All subjects were explained about purpose and procedures of this study. After informed consent was obtained, the participants were asked about interested risk factors, including age at menarche, age at the first sexual intercourse, number of sexual partners, number of vaginal deliveries, history of sexually transmitted diseases (STD: gonorrhea, syphilis, genital wart and herpes simplex type 2) and history of smoking. The Ethics Committee of Khon Kaen University has approved this study.

Detection of HPV DNA and Genotyping

All samples were tested for the presence and type of HPV by using polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) method. DNA was extracted from cells in the Pap test specimens with QIAamp DNA Mini Kit (Qiagen). The PCR for HPV was performed with a sets of primers (forward: 5'-TGTCAAAAACCGTTGTGTCC-3' and reverse: 5'-GAGCTGTCGCTTAATTGCTC-3') that amplify a fragment of E6 and E7 open reading frames (231-268 base pairs) in the malignant type of HPV strains -16, -31, -33, -35, -52b and -58 with Takara PCR Human Papillomavirus Typing Set (Takara), and a set of primers (forward: 5'-TGCCAGAAACCGTTGAATCC-3' and reverse: 5'-TCTGAGTCGCTTAATTGCTC-3') that amplify a corresponding region of HPV-18. For HPV genotypes, PCR products were digested by appropriate restriction enzymes as previously described (Settheetham-Ishida et al., 2005) and then electrophoresis on 4 % agarose gel (NuSeive, Takara).

Detection of p53 Codon 72 Polymorphism

DNAs from peripheral blood cells were used for analysis of the *p53* gene codon 72 polymorphism. The *p53* exon 4-specific PCR was performed with a set of primers (5' CCCGGACGATATTGAACA3' and 5' AGAAGCCCAGACGGAAAC3'). The PCR products, 203-base pair (bp), were electrophoresed on 2.5% agarose gel and visualized by ethidium bromide staining. The polymorphism at codon 72 was investigated by digesting the products with a restriction enzyme, *Bst*UI (New England bioLabs), which cleaves a CGCG sequence in the *Arg* allele (generating a 125 bp and 78 bp fragment).

Statistical Analyses

The c^2 test and Fisher's exact test were used to examine *p53* genotype frequencies between case and healthy subjects, and association between HPV infection and *p53* genotypes. Odds ratio (OR) at 95% confidence interval (CI) were calculated to compare risk for SIL and HPV infection between *Pro/Pro* genotype and other genotypes and to estimate the association of the variables in the questionnaire.

Results

ORs for the selected risk factors are summarized in Table 1. Significant difference was observed for age at menarche ($p<0.005$), age at the first sexual intercourse ($p<0.05$) and number of sexual partners ($p<0.005$). After adjusted by age, *p53* genotypes and HPV infection, a significant difference was still observed for age at menarche with *p*-value of 0.034. Smoking of partners also increased the risk to develop SIL. Increased ORs, 2.3 ($p<0.01$) and 3.2 ($p<0.01$), were observed when the partner had smoking history both at present and in the past, respectively; however, ORs turned to be not significant after adjusted for the confounding factors. Other factors including number of pregnancies, number of vaginal deliveries, age at the first delivery, history of STD both subjects and partners were not significantly different between the cases and controls ($p>0.05$).

Table 1. Selected Risk Factors for SIL

Variables	Subjects, n (%)		OR [95% CI]	
	SIL	Control	Crude OR	Adjusted OR[a]
Age at menarche				
>14 years old	46(44.7)	67(63.8)	1	1
≤ 14 years old	57(55.3)	38(36.2)	2.18[1.20-3.96]***	2.01[1.05-3.86]*
Age at the first sexual intercourse				
> 17 years old	72(69.9)	89(84.8)	1	1
≤ 17 years old	31(30.1)	16(15.2)	2.39[1.15-5.06]*	1.92[0.78-4.69]
Number of sexual partners				
≤ 1	71(68.9)	90(85.7)	1	1
> 1	32(31.1)	15(14.3)	2.70[1.29-5.79]***	1.62[0.71-3.68]
Number of pregnancies				
≤ 3	83(80.6)	79(75.2)	1	1
> 3	20(19.4)	26(24.8)	0.73[0.35-1.48]	0.57[0.21-1.56]
Number of vaginal deliveries				
≤ 3	96(93.2)	95(90.5)	1	1
> 3	7(6.8)	10(9.5)	0.69[0.21-2.11]	1.41[0.33-5.93]
Age at the first delivery				
> 20 years old	58(56.3)	63(60.0)	1	1
≤ 20 years old	45(43.7)	42(40.0)	1.16[0.64-2.09]	0.94[0.46-1.95]
History of STD				
Subjects				
No	92(89.3)	96(91.4)	1	1
Yes	11(10.7)	9(8.6)	1.27[0.45-3.65]	1.00[0.35-2.85]
Partners				
No	95(92.2)	96(91.4)	1	1
Yes	8(7.8)	9(8.6)	0.89[0.28-2.74]	0.63[0.20-1.96]
History of smoking				
Subjects				
Non smoker	100(97.1)	102(97.1)	1	1
Present smoker	2(1.9)	3(2.9)	0.68[0.05-6.07]	0.53[0.06-4.35]
Past smoker	1(1.0)	0(0.0)	not applicable	not applicable
Partners				
Non smoker	23(22.3)	44(41.0)	1	1
Present smoker	60(58.3)	49(46.7)	2.34[1.19-4.63]**	1.71[0.84-3.44]
Past smoker	20(19.4)	12(11.4)	3.18[1.22-8.44]**	2.15[0.75-6.13]

[a]Adjusted for age, p53 genotypes and HPV status

* p < 0.05 ** p < 0.01 *** p < 0.005

Prevalence of malignant type of HPV infection in the control and SIL group was 18.1% and 60.2%, respectively (Table 2). The HPV infection significantly increased the risk for SIL 6.8-fold (p<0.001) and moreover higher OR was observed as 8.6 (p<0.005) after adjusted by age, *p53* genotypes, age at menarche, age at the first sexual intercourse, number of sexual partners and smoking.

As for the distribution of HPV strains, infection of HPV-16, -18, -31, -33, -35, -52b, -58 and -67 was found with a variety of frequency (Table 2). A few HPV carriers were identified in the controls (n=19); they harbored namely HPV-18 (12/19), HPV-16 (4/19) and HPV-58 (4/19). On the other hand, in HPV carrying patients with SIL (n=62), HPV-16 infection was the commonest (31/62) followed by HPV-18 infection (13/62). Regarding to these prevalent strains, HPV-16 infection was significantly associated with risk for SIL

Table 2. Prevalence of Infection with Malignant Type of HPV

Subjects	HPV status, n (%)		OR [95% CI]	
	Negative	Positive	Crude OR	Adjusted OR[a]
Control	86(81.9)	19[b] (18.1)	1	1
SIL	41(39.8)	62[c] (60.2)	6.84[3.48-13.65]****	8.55[2.19-33.42]***

[a]Adjusted for age, p53 genotypes, age at menarche, age at the first sexual intercourse, number of sexual partners and smoking

[b]HPV-16 (4), -18 (12), -52b (1), -58 (4), not typed (2) and double infection of -16/-52b (1), -16/-58 (1), -16/not typed (1) and 58/not typed (1) were observed. Number of carrier is shown in each parenthesis.

[c]HPV-16 (31), -18 (13), -31 (6), -33 (5), -35 (2), -52b (2), -58 (7), -67 (1), not typed (6) double infection of -16/-35 (2), -16/-52b (1), -16/-58 (2), -16/not typed (1), -31/-33 (1), -33/not typed (1) and -58/not typed (3) were observed. Number of carrier is shown in each parenthesis.

*** p < 0.005; **** p < 0.001

Table 3. Prevalence of Infection with HPV-16 and HPV-18

HPV genotypes	Subjects	OR [95% CI]	
		Crude OR	Adjusted OR[a]
HPV-16	Control (n=4)	1	1
	SIL (n=31)	16.25[5.16-66.39] ****	5.03[0.45-55.75]
HPV-18	Control (n=12)	1	1
	SIL (n=13)	2.27[0.83-5.95]	14.26[0.90-224.75]

[a]Adjusted for age, *p53* genotypes, age at menarche, age at the first sexual intercourse, number of sexual partners and smoking
**** $p < 0.001$

(p<0.0001) even though the statistical difference was not observed after adjusted by age, *p53* genotypes, age at menarche, age at the first sexual intercourse, number of sexual partners and smoking (Table 3).

Allele frequencies and genotype distribution of *p53* in SIL patients are presented in Table 4. There was no significant difference in the allele distribution between the SIL and the controls (p>0.05). The genotype distribution of both groups was in Hardy-Weinberg equilibrium. There were no significant differences in the proportion of the *p53* codon 72 genotypes in the SIL and the control groups (p>0.05). There was no significant association between distribution of p53 codon 72 polymorphism and HPV infection (p>0.1) (Table 5).

As stated below, the number of sexual partners and smoking history increased risk for the high-risk HPV infection (data not shown in the tables). Women who had more than one sexual partner consistently showed significantly higher risks for HPV infection, more than 3-fold, both before and after adjusted by age and *p53* genotypes. Increased risk for high-risk HPV infection was also found for age at the first sexual intercourse with a marginally significant p-value of 0.052. Smoking history of the partners was significantly associated with risk for HPV infection; 3.2-fold at present smoking (p< 0.001) and 4.3-fold in past smoking (p<0.005). The increased risk for HPV infection was still observed for the smoking history of the partners both at present (p<0.005) and in the past (p<0.005) after adjusted for the confounding factors. This was also confirmed among the control subjects (p=0.035). Other factors, however, like sexual behavior and lifestyle, showed no relation with risk for HPV infection in this study (p> 0.05).

Discussion

The development of cervical cancer, progression of precursory lesion to malignant neoplasia, is probably multifactorial and risk factors such as genetic background, HPV infection and life styles may be synergically involved. Although the *p53* codon 72 polymorphism was suspected to be associated with cervical cancer (Storey et al., 1998), we have consistently failed to demonstrate the contribution of the polymorphic alleles to cervical cancer development (Settheetham-Ishida et al., 2004a) and to SIL development as well. It is thus concluded that the *p53* codon 72 polymorphism is not a genetic risk for cervical neoplasia as well as cervical cancer in Northeastern Thai women. We have also demonstrated lack of a significant difference in the distribution of the *p53* genotype between HPV positive and negative groups in this case-control study. Our previous and present results indicate no critical roles of the *p53* codon 72 polymorphism in the susceptibility to HPV infection as well as SIL and SCCA development in Thai women. On the other hand, infection with HPV is the major cause of cervical cancer (Munoz et al., 1992; zur Hausen, 1991) and this was also shown in this region (Settheetham-Ishida et al., 2005). In our study, we did not classify SIL into low and high SIL, although we had diagnosed the patients by pathological examinations. As the DNA samples used for HPV typing in this study were from scraped whole cervical cells but not from the legion itself, the origin of the DNA samples and the location of the tissue used for diagnosis did not correspond. To study the contribution of HPV strains to the progression of SIL, micro-dissection analysis is thus awaited.

Epidemiological evidence has suggested that sexual behaviors are the risk factors for cervical cancer in Thailand (Punyaratabunduh et al., 1982; Thomas et al., 1996). Sexual behaviors, particularly, having more than one sexual partners and younger age at the first sexual intercourse increased risk for malignant types of HPV infection and SIL development. These factors was also suggested in our previous report as the risks for HPV infection and cervical carcinoma with high ORs (Settheetham-Ishida et al., 2005; Settheetham-Ishida et al., 2004b). However, sexual history was found not to be a risk for HPV infection in women living in the capital of Thailand and in American Indians (Schiff et al., 2000;

Table 4. *p*53 Codon 72 Polymorphism: Allele and Genotype Frequencies

Subjects	Allele frequencies		Genotype distribution, n (%)		
	Pro	*Arg*	*Pro/Pro*	*Pro/Arg*	*Arg/Arg*
Control	0.53	0.47	25(23.8)	60(57.2)	20(19.1)
SIL	0.49	0.51	23(22.3)	55(53.4)	25(24.3)

Table 5. Genotype Distribution of the p*53* Codon 72 Polymorphism in HPV Positive Individuals

Subjects	Genotype distribution		
	Pro/Pro	*Pro/Arg*	*Arg/Arg*
Control	2	15	2
SIL	16	32	14

Sukvirach et al., 2003; Thomas et al., 2001a). Because of inadequate production of cervical mucus against infectious agents in very young women and of exposure to many sexual partners, HPV infection can readily occur and lead to higher risk for cervical abnormality as well as sexually transmitted diseases (Kahn et al., 2002; Thomas et al., 2001b) which themselves can facilitate HPV infection. Unfortunately we were not able to include husband's sexual behavior in the questionnaire; clearly extramarital sexual contact of a husband may cause HPV infection in his wife who is in a monogamous condition (Sukvirach et al., 2003; Thomas et al., 2001a).

Smoking experience has also been thought to be a risk factor for cervical lesions (Prokopczyk et al., 1997). Since the total number of smokers in our subjects was so small (6/208), we have then focused our attention on the mode of smoking, passive or secondary. We have confirmed the increased ORs ($p<0.01$) when the partner(s) had smoking habits either at present (OR=2.3) or in the past (OR=3.2). The harmful effects of smoking are well known and, moreover, tobacco specific carcinogens were detected from cervical mucosa of female smokers (Prokopczyk et al., 1997; Trimble et al 2005). Even though the women's smoking experience is passive or secondary, it is readily conceivable that tobacco specific carcinogens/mutagens may exist in the cervical mucus of such women. Here it should be noted the possible presence of alternative cervical exposure to tobacco specific carcinogens. Detection of tobacco specific carcinogens in semen of the smoking males (Pacifici et al., 1995; Vine et al., 1993; Zenzes et al., 1999) strongly suggests that cervical exposure to carcinogens through sexual contacts. Smoking causes a reduction in cervical immunity, which would enhance the persistence of HPV infection (Giuliano et al., 2002; Lazcano-Ponce et al., 2001; Poppe et al., 1995).

In conclusion, among a number of possible risk factors, a specific type of HPV infection, HPV-16, is a critical risk factor for the SIL progression. Some other factors such as sexual behaviors and smoking remain to be risk factors that may play to help and/or enhance the effects of HPV infection on the cervical hyperplasia and transformation, whereas the participation of the *p53* codon 72 polymorphism was not identified. Therefore, in line with prevention of HPV infection causing cervical abnormalities, incorporation of knowledge on the sexually transmitted diseases into the basic educational program and public health scheme is important. At the same time, a nation-wide screening scheme for the cervical abnormalities including HPV-typing should be a strong measure to lower the cervical cancer and recurrent practice of the test is thus awaited.

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