RESEARCH ARTICLE

Polymorphisms in TP53 (rs1042522), p16 (rs11515 and rs3088440) and NQO1 (rs1800566) Genes in Thai Cervical Cancer Patients with HPV 16 Infection

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Abstract

    The risk of cervical cancer development in women infected with HPV varies in relation to the individual host's genetic makeup. Many studies on polymorphisms as genetic factors have been aimed at analyzing associations with cervical cancer. In this study, single nucleotide polymorphisms (SNPs) in 3 genes were investigated in relation to cervical cancer progression in HPV16 infected women with lesions. Two thousand cervical specimens were typed by PCR sequencing methods for TP53 (rs1042522), p16 (rs11515 and rs3088440) and NQO1 (rs1800566). Ninety two HPV16 positive cases and thirty two normal cases were randomly selected. Analysis of TP53 (rs1042522) showed a significantly higher frequency in cancer samples (OR=1.22, 95%CI=1.004-1.481, p-value=0.016) while differences in frequency were not significant within each group (p-value=0.070). The genotype distributions of p16 (rs11515 and rs3088440) and NQO1 (rs1800566) did not show any significantly higher frequency in cancer samples (p-value=0.106, 0.675 and 0.132, respectively) or within each group (p-value=0.347, 0.939 and 0.111, respectively). The results indicated that the polymorphism in TP53 (rs1042522) might be associated with risk of cervical cancer development in HPV16 infected women. Further studies of possible mechanisms of influence on cervical cancer development would be useful to manage HPV infected patients.

Keywords: Single nucleotide polymorphisms - TP53 - p16 - NQO1 - human papillomavirus

Introduction

    Human papillomavirus (HPV) is a double-stranded DNA virus infecting cutaneous and mucosal epithelial cells of the human body. HPV genomes consist of early, late, upstream regulatory and non-coding regions. HPV is a major risk factor for invasive cervical cancer (ICC) especially HPV16 and 18. In developing countries, cervical cancer is the most common gynecological cancer. These viruses usually clear in 70-90% of HPV infected individuals. However, a few per cent of infected women develop low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) or cervical cancer (Walboomers et al., 1999). Epidemiological studies have strongly supported a genetic link to cervical cancer based on the observation that biological relatives of women with cervical tumor have a two-fold higher risk of cervical tumor development than non- biological relatives. The genetic susceptibility for cervical cancer has been defined as 27% (Hemminki et al., 1999; Magnusson et al., 1999 Chattopadhyay, 2011).

    The host's genetic makeup such as single nucleotide polymorphisms (SNPs), hormonal, nutritional and environmental factors contribute to the risk of cancer development (Kohaar et al., 2007). Therefore, studies on cancer risk focus to a large extent on human genetic factors. Genetic polymorphisms in a variety of genes have been mainly studied to establish a correlation between specific allele variants and cancer progression.

    The tumor suppressor gene p53 (TP53) is responsible for regulating DNA repair, cell cycle control and apoptosis. The TP53 gene encodes the tumor protein 53 (p53) which controls the p53 pathway. The p53 protein comprises 393 amino acid residues which consist of an N-terminal transactivation domain (TAD), a proline-rich domain (PRD), a DNA binding domain (DBD), tetramerization domain (4D) and C-terminal domain (CTD). In unstressed cells the p53 protein is present at low levels while the p53 pathway is up-regulated via p21, BAX and p53 up-regulated modulator of apoptosis (PUMA) in response to cellular stress inducing apoptosis and DNA repair or cell cycle and growth arrest (Brooks et al., 2006; Rogler

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et al., 2011). All human tumors harbor mutated p53 with 50-90% of those mutations leading to a decreased ability to bind DNA targets thus inducing proliferation, cell growth and incomplete DNA repair and eventually, progression to malignancy (Meek, 2009). HPV targets p53 and other proteins to promote viral replication (Narisawa-Saito et al., 2007) while disruption of cell cycle control may induce HPV persistence and cervical carcinogenesis (Koshiol et al., 2008). Polymorphisms in the TP53 gene change the conformation of the p53 protein which leads to degradation of p53 and HPV binding. The most widely studied of the non-synonymous SNPs on exon 4 at codon 72 of the TP53 gene (rs1042522), Arginine/Arginine (Arg/Arg) has been associated with a higher risk of cancer development. Both variants have the same binding affinity to DNA while their capacity to bind components of the transcription factor is different (Thomas et al., 1999). Hence, it appeared prudent to investigate the association of SNPs with development of different neoplasias. The previous study has shown that homozygous TP53Arg might interfere with protein stability and is seven times more susceptible to cervical squamous cell carcinoma than the heterozygous variant. The p53 variant containing arginine (G) was seven times more susceptible to E6-mediated degradation than that containing proline (C). Thus, the Arg/Arg genotype was degraded more efficiently which increased the risk for cervical cancer development (Storey et al., 1998). In some populations, the arginine variant is more common. For example, the arginine variant is more common in Caucasian than in African and Asian populations. Therefore, several studies have not been successful in detecting this association. The ethnic variations of this SNP might be a genetic marker for cancer susceptibility and balance natural selection (Sousa et al., 2007; 2011; Oliveira et al., 2008).

p16, known as CDKN2/MTS-1/INK4a, is a tumor suppressor gene on chromosome 9p21 consisting of three exons encoding 156 amino acids. CDK4 and CDK6 bind with the p16 gene product to inhibit interaction with cyclin D1 which prevents retinoblastoma protein (pRb) phosphorylation and release of E2F thus leading to cell cycle inhibition in the G1/S transition (Yan et al., 2008). The over-expression of p16 due to inactivation of Rb by the HPV E7 protein may act as a specific biomarker for cervical cancers. The p16 gene has two polymorphisms (C540G, rs11515 and C580T, rs3088440) at the 3'-untranslated region of exon 3 (Sauroja et al., 2000). A previous study has shown that polymorphisms are associated with progression of various cancers such as ovarian and upper gastrointestinal tract but their association with the risk of cervical cancer progression has so far remained uncertain (Zheng et al., 2002; Geddert et al., 2005; Thakur et al., 2012).

NQO1, known as NAD(P)H quinine oxidoreductase 1 is located on chromosome 16q22.1 consisting of 6 exons and 5 introns (Rosvold et al., 1995). NQO1 has two protein subunits whose expression is transcriptionally controlled. It catalyzes two electron reductions of quinones and nitrogen-oxides to defend the cell against oxidative stress and mutagenicity by preventing the formation of semiquinones and highly reactive oxygen species (ROS).

It is considered essential for protection against cancer (Tsvetkov et al., 2010). The SNP of NQO1 at nucleotide position 609C>T in exon 6 (rs1800566) has been found in nearly 50% of the human population with the proline to serine amino acid substitution at codon 187 as the most extensively studied. However, this polymorphism is variable in different ethnic groups with 20% in Asians and 2-5% in Caucasians and Africans (Kelsey et al., 1997). The variant T allele has been associated with reduction of NQO1 enzymatic activity with homozygotes (TT) devoid of NQO1 activity while the wild-type homozygotes (CC) display the highest enzymatic activity (Siegel et al., 1999). The T allele has been associated with increased risk of several solid tumors (Chao et al., 2006) such as colorectal cancer (Schulz et al., 1997) and urological malignancies (Lafuente et al., 2000). The wild-type NQO1 partially inhibits p53 degradation mediated by the HPV E6 protein while this does not occur with the C609T mutant. Hence, the inactive NQO1 gene in high-risk HPV-infected individuals may be more prone to p53 degradation and confer a higher risk of cervical carcinoma development (Niwa et al., 2005).

The aim of this study has been to analyze the distribution of the polymorphisms of the TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440) and NQO1 gene (rs1800566) in women infected with HPV16 compared with a healthy control group. We conducted this research on low-grade, high-grade, cervical squamous cell carcinoma and normal cervical squamous cells to analyze the association between the risk of cervical cancer development and these polymorphisms in Thai women.

Materials and Methods

The study was conducted on specimens collected from previous studies and stored as anonymous. Permission had been granted by the Director of King Chulalongkorn Memorial hospital. Patient identifiers including personal information (name, address) and hospitalization number were removed from these samples to protect patient confidentiality and neither did they appear in any part of document in this study. The research protocol was approved by the Institutional Review Board (IRB number 48/55), Faculty of Medicine, Chulalongkorn University. IRB waived the need for consent because the samples were anonymous.

Study population and sample collection

The specimens were kept frozen (–20°C) at the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University. Polymorphism analysis was performed on samples positive for HPV16. Healthy cervical squamous cells were used as controls for comparison with the 2 groups of HPV16 infection. The first group consisted of non-cancerous samples such as normal cytology, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, while the second group comprised samples of cervical squamous cell carcinoma. Two thousand stored cervical samples were obtained during the patients' routine checkup and treatment from the National Cancer Institute (NCI),

Bangkok 9 International and Samitivej Srinakharin hospital between January 2011 and January 2012. These samples were consisting of 1,500 of normal cytology, 300 of abnormal cytology and 200 of cervical cancer. These samples were screened for HPV16 infection by polymerase chain reaction with consensus primers and further analyzed by direct sequencing to detect HPV DNA. We randomly selected abnormal and normal lesions of HPV 16 positive specimens for this study. Ninety two cervical samples with HPV16 infection and 32 normal cytological specimens were examined for polymorphisms in the TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440) and NQO1 gene (rs1800566).

Laboratory methods

DNA extraction: Human genomic and HPV DNA were extracted from 100 μl of cervical swab samples. The standard organic method (phenol-chloroform) and alcohol precipitation of the specimens as described by Chansaenroj et al. (2012) was applied for DNA extraction. The purified substance was re-suspended in a final volume of 30 μl of deionized water.

HPV detection and typing: HPV DNA was detected by using consensus polymerase chain reaction (PCR) of the E1 and L1 regions as described by Chansaenroj et al. (2010). HPV DNA positive samples were identified by electrophoresis in 2% agarose gel (FMC Bioproducts Rockland, ME) and purified with the agarose gel extraction mini kit (5PRIME, Hamburg, Germany) according to the manufacturer's specifications. The purified DNA was sequenced by FirstBASE Laboratories SDNBHD (Selangor Darul Ehsan, Malaysia). The nucleotide sequences were analyzed by Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Single nucleotide polymorphism determination: The HPV16 specimens were randomly selected. The TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440) and NQO1 gene (rs1800566) were analyzed using specific sets of primer pairs for direct sequencing. The polymerase chain reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: Initial denaturation at 94ºC for 5 min, followed by 40 cycles at 94ºC for 30 s for denaturation, 30s at 50ºC for annealing and 1 min at 72ºC for extension, and final extension at 72ºC for 7 min. The PCR reaction mixture contained 100 ng/ml of genomic DNA, 0.5 μl of 10 μM primers (Table 1), 12.5 μl of 2X Mastermix (Eppendorf, Hamburg, Germany) and distilled water to a final volume of 25 μl. The PCR products were subjected to direct sequencing to analyze sequence polymorphisms.

Statistical analysis

Genotype frequencies were determined by direct counting from the sequences. The Statistical Package for the Social Sciences (SPSS) software for Windows, version 13.0 (Chicago, IL) was used for statistical analysis of the association between the TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440), NQO1 gene (rs1800566) and control group. The association between all genotypes, odds ratios (ORs) and 95% confidence intervals (CIs)

for cervical cancer risk were calculated by χ^2 exact test analysis (2-tailed exact significance). Differences were considered statistically significant at p-values<0.05.

Results

Two thousands cervical specimens were screened by PCR then the typing was performed by direct sequencing with consensus primers in the E1 and L1 regions. After typing, 87 specimens with cervical cancer, 131 specimens with abnormal cytology and 22 specimens with normal cytology were found positive for HPV16 infection. Of those, samples which were found positive for HPV16 were selected randomly to be the case group and the sample which were HPV negative with a normal cytology were selected randomly to be the negative control group. This study employed PCR and a sequencing-based assay to analyze SNPs. Ninety-two HPV 16 infected samples (56 invasive cervical cancers and 36 cervical lesions) and 32 normal control samples were subjected to polymorphism analysis of the TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440) and NQO1 gene (rs1800566). The average age of cases and controls was 44.6±12.5 years and 42.7±11.2 years, respectively as shown in Table 2. As shown in Table 3, the polymorphism analysis of all genes is represented as genotype frequencies.

In this study, we found that the polymorphism in genotype frequency of the TP53 gene (rs1042522) was significantly higher in patients with cancer (OR=1.22, 95%CI=1.004-1.481, p-value=0.016) with a link between the G allele and non-cancer lesion while this difference was not significant within the HPV16 infection of HSIL, LSIL and normal groups (p-value=0.07). Yet, the frequency in polymorphisms in the p16 gene (rs11515 and rs3088440) were not statistically higher in patients with G and T alleles in cancer and non-cancer and were not significantly in the HPV16 infection of HSIL, LSIL and normal groups (p-value=0.106, 0.675 and p-value=0.347,

Table 1. Conserved Primers for Amplification and Sequencing of TP53 Gene (rs1042522), p16 Gene (rs11515 and rs3088440) and NQO1 Gene (rs1800566)

Gene	Primer name	Sequence (5' - 3')	Product (bp)
TP53	TP53_F	TTC ACC CAT CTA CAG TCC C	252
	TP53_R	CCA GAC GGA AAC CGT AGC	
p16	p16_F	GCC ACA CAT CTT TGA CCT C	257
	p16_R	GGA CAT TTA CGG TAG TGG G	
NQO1	NQO1_F	GCC TCC TTA TCA GAG TGT C	253
	NQO1_R	ACA GTG GTG TCT CAT CCC A	

Table 2. Demographic Data of All Samples

		Control	Case Non-cancer	Case Cancer
Number		32	36	56
Age:	Range	30-79	19-57	28-83
	Average	42.7	36.1	50.1
	SD	11.2	9	11.4
Cytology:	Invasive carcinoma			56
	HSIL		17	
	LSIL		9	
	Normal	32	10	100.0

6.

75.0

Table 3. Genotype Frequencies of TP53 Gene (rs1042522), p16 Gene (rs11515 and rs3088440) and NQO1 Gene (rs1800566) Polymorphism in HPV16 and Controls (n=124); CI, Confidence Interval

Gene (SNP)	Genotype	HPV16 CA	HPV 16 HSIL	HPV 16 LSIL	HPV 16 Normal	Normal	p-value*	p-value**	Odd ratio***
			Non-cancer (n=36)			Controls group			
		(%) (n=56)	(%) (n=17)	(%) (n=9)	(%) (n=10)	(%) (n=32)			
TP53 (rs1042522)	CC	23 (41.1)	5 (29.4)	1 (11.1)	4 (40.0)	6 (18.8)	0.016*	0.07	1.22 (1.004-1.481)
	GC	16 (28.6)	6 (35.3)	2 (22.2)	2 (20.0)	18 (56.2)			
	GG	17 (30.3)	6 (35.3)	6 (66.7)	4 (40.0)	8 (25.0)			
p16 (rs11515)	CC	52 (92.9)	17 (100.0)	9 (100.0)	10 (100.0)	28 (87.5)	0.106	0.347	1.52 (0.753-3.057)
	GC	4 (7.1)	0	0	0	4 (12.5)			
	GG	0	0	0	0	0			
p16 (rs3088440)	CC	46 (82.1)	13 (76.5)	8 (88.9)	8 (80.0)	25 (78.1)	0.675	0.939	1.06 (0.800-1.402)
	CT	10 (17.9)	4 (23.5)	1 (11.1)	2 (20.0)	7 (21.9)			
	TT	0	0	0	0	0			
NQO1 (rs1800566)	CC	26 (46.4)	7 (41.2)	1 (11.1)	4 (40.0)	10 (31.2)	0.132	0.111	1.11 (0.909-1.366)
	CT	18 (32.1)	9 (52.9)	7 (77.8)	4 (40.0)	11 (34.4)			
	TT	12 (21.5)	1 (5.9)	1 (11.1)	2 (20.0)	11 (34.4)			

*p-value between Cancer and non-Cancer groups; **p-value of each cytology group (Cancer, HSIL, LSIL and Normal); ***95% Confidence interval
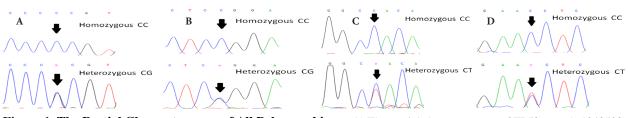

Figure 1. The Partial Chromatograms of All Polymorphisms. A) The partial chromatograms of TP53 gene (rs1042522) polymorphism, B) The partial chromatograms of p16 gene (rs11515) polymorphism, C) The partial chromatograms of p16 gene (rs3088440) polymorphism and D) The partial chromatograms of NQO1 gene (rs1800566) polymorphism

0.939, respectively). The polymorphism frequency in the NQO1 gene (rs1800566) was not found significantly higher in patients with T allele in cancer and non-cancer and were not significantly in the HPV16 infection of HSIL, LSIL and normal groups (p-value=0.132 and p-value=0.111, respectively). The partial chromatograms of each SNP are shown in Figure1a-d.

Discussion

Cervical cancer is the most common gynecological cancer among women especially in developing countries. The high-risk HPV genotypes play a major role for lesion development especially HPV16 which commonly found in the cervical cancer and abnormal lesion. This study found the HPV16 infection in cervical cancer and abnormal lesion are nearly 45% of all cases while the normal cytology was found very low percentage of less than 2%. The result showed that viral factor is the main strategy for the risk identification of HPV infection. Novel strategies for early identification of populations at high risk due to environmental factors and host genetics are required to prevent cervical cancer. One of the main oncogenic mechanisms depends on host genetics which may increase the risk of disease development. Tumor development is largely attributed to genetic variations in the host's cell cycle. Many studies have focused on deregulation of the cell cycle during tumor development. The relationships between four polymorphisms (TP53 gene (rs1042522), p16 gene (rs11515 and rs3088440) and NQO1 gene (rs1800566)) and the risk of cervical cancer progression have been investigated in this study. The association

between the TP53 gene (rs1042522) polymorphism and cervical cancer in various populations has been explored and various factors such as ethnicity and HPV infection status may have to be taken into consideration upon analyzing the results. The frequency of Arg variants in a population is important to identify the variant's effects. The Arg variant of the p53 protein is more readily degraded by the E6 oncoprotein than the Pro variant. This pathway causes increasing vulnerability. Based on previous studies, the Arg polymorphism depends on the population group (Jee et al., 2004; Koushik et al., 2004; Piña-Sánchez et al., 2011). Moreover, a meta-analysis conducted between 1998 and 2002 suggested that any effect is a slightly increased risk associated with arginine at codon 72 (Koshiol et al., 2009). The homozygous Arg are associated with high risk of invasive cervical cancer with OR nearly 1.1-1.2 while studies conducted in most European countries have not observed any significant association (Jee et al., 2004; Koushik et al., 2004; Sousa et al., 2007; Piña-Sánchez et al., 2011; Koshiol et al., 2009). However, other biological factors in conjunction with p53 also have an effect on the population. DNA extracted from formalin-fixed tissues increased the OR but its quality is poor and thus, can inhibit PCR amplification (Jee et al., 2004). Both variants have non conservative change (Thurow et al., 2011). The proportions of the Arg/Arg, Arg/Pro and Pro/Pro genotypes showed varying Kappa coefficients ranging from 0.49-0.63. Moreover, the OR for the Arg/Arg genotype can increase from 1.5-8.0 when the discordant genotypes in cervical cancer are excluded (Makni et al., 2000). For example, the homozygous Arg is 31% in South Africa, 50% in India and 76% in Finland

(Ojeda et al., 2003). According to the study by Eltahie et al. (2012) this polymorphism may contribute to the risk for cervical cancer, but on its own, does not cause carcinogenesis (Eltahir et al., 2012). In this study we found that the TP53 gene (rs1042522) polymorphisms were present at significantly higher frequency in abnormal cervical lesions. The results imply this polymorphism may be associated with abnormal cervical lesions and increased risk for progression to cancer.

The genetic variations in cell cycle regulatory genes p16 (rs11515 and rs3088440) in the 3' Un-translated region of exon 3 can contribute to cancer development in various ways. These polymorphisms may induce the development of cervical cancer. A previous study reported that both polymorphisms were significantly associated with faster progression of primary melanoma to metastatic disease and increased risk of familial melanoma (Sauroja et al., 2000). In contrast, Geddert H et al. suggested that these polymorphisms were not associated with the risk of upper gastrointestinal and esophageal adenocarcinomas (Geddert et al., 2005). In this study, these adjacent polymorphisms were not present at significantly higher frequency in abnormal cervical lesions. Therefore, these polymorphisms may not be associated with the risk of lesion development.

The NQO1 gene (rs1800566) TT genotype is associated with a null anticancer enzyme activity and may affect cancer development through the reduction cytotoxic agents containing the quinone moiety into hydroquinone individuals with lung, bladder and colorectal cancer (Siegel et al., 1999). The allele frequency of the T allele is depending on specific ethnic groups such as 0.217 in Caucasians and 0.398 in Japanese. A study of head and neck squamous cell carcinoma has reported no association with NQO1 SNP609. In contrast, Niwa Y et al. suggested that the effect of NQO1 genotypes on cervical carcinogenesis varies in relation to smoking behaviors and showed that the TT genotype was a risk factor only for cervical squamous cell carcinoma while the C allele was over-expressed in invasive cervical cancer and cervical intraepithelial neoplasia 3 (Niwa et al., 2005; Hu et al., 2010). In this study we found that the NQO1 gene (rs1800566) polymorphisms were not present at significantly higher frequency in abnormal cervical lesions. Hence, this polymorphism may not be associated with abnormal cervical lesions and increased risk for progression to cancer.

The important environment factors or host factors are necessary cause which can clarify the complications of the diseases. Genetic variations allow characterization of the genetic profile in population with epidemiological parameters that can support to identify the molecular markers. However, the results of this study need to be confirmed in larger population and clarified of the association between these polymorphisms and cervical cancer development. Detection and prevention of cancer development are urgently required. The method presented here applied the useful and easy technique of conventional PCR to detect the SNPs. The host genetic factors are considered important for early determining susceptibility to HPV infection, lesion progression and treatment prognosis. In conclusion, the risk of cervical cancer was showed significant association between TP53 gene (rs1042522) polymorphisms and strongly associated with HPV type16. The further studies on correlation between the p53 pathway, Human papillomavirus and the other factors are considered.

Acknowledgements

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