p53 STATUS AND HUMAN PAPILLOMAVIRUS INFECTION IN THAI WOMEN WITH CERVICAL CARCINOMA

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Abstract. Loss of p53 function has been implicated in a wide variety of human malignacies. Many studies suggest that in cervical carcinoma p53 function is inactivated either by gene mutation or by complex formation with E6 oncoprotein product of high-risk human papillomavirus (HPV). The aim of this study was to determine the status of HPV infection and p53 gene mutation as well as their correlation in cervical carcinomas. Formalin-fixed paraffin-embedded tissues of 12 cervicitis, 21 cervical intraepithelial neoplasia grade 3 (CIN 3) and 17 squamous cell carcinomas were determined for the presence of HPV using polymerase chain reaction (PCR) amplification and dot blot hybridization. The status of p53 mutations in exons 5-8 was evaluated by polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) and confirmed by direct nucleotide sequencing. HPV infections were detected in all CIN 3 and squamous cell carcinomas (100%). Mutations of p53 were present in 3 of 38 HPV-positive samples: one with an ATG→TTG transversion (Met→Leu) in codon 237 of exon 7; and the others with a TGC→TGG transversion (Cys→Trp) in codon 242 of exon 7, and a CGT→CCT transversion (Arg→Pro) in codon 273 of exon 8, respectively. Our findings show that the frequency of p53 mutation is low in primary cervical carcinoma and that the p53 gene mutation and HPV infection are not mutually exclusive events in the development of cervical cancer. Thus, other genetic events independent of p53 inactivation may also significantly contribute to the carcinogenesis of the uterine cervix.

INTRODUCTION

Clinical and epidemiologic evidence have implied that human papillomavirus (HPV) is frequently associated with the development of cervical carcinoma (Munoz and Bosch, 1989). The high-risk HPV types (notably HPV 16 and 18) are associated with high-grade squamous intraepithelial lesions and invasive cervical carcinomas, whereas the low-risk types (HPV 6 and 11) are found mainly in low-grade lesions (Lorincz *et al*, 1992; Munoz and Bosch,1989). Although infection with high-risk HPV types may be quite common, only a small percentage of infected women develop invasive cervical cancer (Young *et al*, 1989). Thus, HPV infection alone may not be sufficient for the process of malignant transformation, suggesting the requirement of additional cellular events that facilitating the accumulation of genetic lesions.

Tumor suppressor gene p53 located on chromosome 17p13.1 encodes a 53 kDa nuclear phosphoprotein with 393 amino acid residues. The wild type p53 functions as a negative regulator by controlling cell cycle at the G1-S transition (Kastan *et al*, 1991; Levine *et al*, 1991). Generally, one allele of the p53 gene is lost through a chromosomal deletion and the second allele undergoes mutation within the gene. Chromosomal deletions or point mutations in the region of the p53 gene have been demonstrated in various human cancers, including colon (Vogelstein *et al*, 1988), lung (Mori *et al*, 1989), breast (Mazars *et al*, 1992), bladder (Presti *et al*, 1990), and ovarian cancers (Eccles *et al*, 1992). The mutations identified in the p53 gene are clustered in 4 hotspot regions which coincide with the evolutionarily most highly conserved regions of the gene (Nigro *et al*, 1989).

In addition, abnormalities in p53 could be mediated through protein inactivations. Many viral oncoproteins such as simian virus (SV) 40 large T antigen and adenovirus E1B protein bind to and

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cause p53 protein dysfunction (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al*, 1982). Previous studies have shown that E6 protein of high risk HPV formed a complex with p53 protein resulting in degradation of p53 through the ubiquitin-dependent proteolysis pathway (Scheffner *et al*, 1990; Werness *et al*, 1990). Moreover, HPV-negative cervical cancer-derived cell lines have shown to contain mutations in the p53 gene, whereas cervical cancer cells associated with HPV infections express the wild type p53 gene (Crook *et al*, 1991; Scheffner *et al*, 1991). Taken together, the inactivation of p53 is considered as an important role in the development of cervical carcinoma and loss of p53 activity can be achieved by two different mechanisms; either by mutation of the p53 gene itself or by complex formation with HPV encoded E6 protein.

In this study, we examined cervical carcinomas for the presence of p53 mutation by polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) and confirmed by direct sequencing. HPV infection was determined by PCR amplification and dot blot hybridization. The status of p53 and HPV infection was also analyzed.

MATERIALS AND METHODS

Cervical tissue samples and DNA extraction

A total of 50 formalin-fixed paraffin-embedded tissues with pathological diagnosis as 12 cervicitis, 21 cervical intraepithelial neoplasia grade 3 (CIN 3), and 17 squamous cell carcinomas were used in this study. Genomic DNA from sections of paraffin-embedded tissues was isolated by the method of Wu *et al* (1990) and used for detection of HPV DNA and p53 mutations.

Detection and typing of HPV

The presence of HPV DNA was detected by PCR amplification of HPV L1 region using consensus primers MY09 and MY11 (Manos *et al*, 1989) as described by Bauer *et al* (1991). The consensus primers detect approximately 25 different HPV types by a 450 base pair product. Human β-globin gene was simultaneously amplified as a control. Amplification was performed in 50 μl reaction mixture containing 1 μl template DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.5, 4mM $MgCl_2$, 200 μM each of deoxyribonucleoside triphosphate (dNTP), 25 pmole of each HPV L1 consensus primer, 2.5 pmole of each β-globin primer (GH20 and PC04), and 1.25 units of Taq DNA polymerase (Amplitaq, Perkin-Elmer Cetus, USA). The samples were subjected to 40 cycles of denaturation at 95ºC for 1 minute, annealing at 50ºC for 1 minute and extension at 72ºC for 2 minutes, with an additional 10 minutes at 72ºC during the last cycle.

HPV typing was done by dot blot hybridization of HPV PCR products as described by Lertworapreecha *et al* (1998) using type specific HPV probes MY12, MY13, MY14, WD74, and MY16 which are specific to HPV 6, 11, 16, 18, and 33, respectively. Oligonucleotide sequences of HPV L1 consensus primers, β-globin gene and type specific HPV probes are shown in Table 1.

Detection of p53 mutations

Mutations of p53 gene were firstly screened by an improved nonisotopic PCR-SSCP as previously described by Pooart *et al* (1999). The intronic primers specific for exons 5-8 of the p53 gene were used for amplification (Koga *et al*, 1994) (Table 1). Each reaction mixture (50 μl) contained 1 μl of DNA sample, 200 μM each of dNTP, 50 mM KCl, 10 μM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$ for exons 6-8 (2 mM $MgCl_2$ for exon 5), 26 μM of each primer and 1.25 units *Taq* DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). Amplification was carried out by 35 cycles of 30 seconds at 94ºC, 30 seconds at 60ºC and 30 seconds at 72ºC with an additional 5 minutes at 94ºC before the first cycle and 7 minutes at 72ºC after the last cycle. Two microliters of PCR products were added to 10 μl of formamide loading dye (90% formamide, 20 mM EDTA, 0.025% bromphenol blue and 0.025% xylene cyanol). Samples were heated to 95ºC for 5 minutes and immediately put into an icebath. Ten microliters of each sample were loaded onto a 82x80x0.75 mm 15% polyacrylamide (69:1 acrylamide to N,N´-methylene-bis-acrylamide), 90 mM Tris-borate pH 8.0, 2 mM EDTA (TBE) gel. The electrophoresis was carried out on a horizontal electrophoresis apparatus (Mighty Small II SE 250, Hoefer Scientific Instruments, San Francisco, USA) for 1 hour at 400 V at 15ºC using 1xTBE as a running buffer.

The SSCP bands were detected using a silver staining method as described by Bassem *et al* (1991) with slight modifications. Briefly, the gels were fixed in 10% ethanol for 3 minutes and in 1% nitric acid for 3 minutes. The gels then were soaked in impregnation solution (0.1 g $AgNO_3$, 150 μl formaldehyde in 100 ml deionized water) for 10

Table 1
Oligonucleotide sequences of primers and probes for HPV, β-globin and p53.

	Oligonucleotide sequences (5′ → 3′)	
	Consensus primers for HPV L1 region	
MY 09	CGT CCM ARR GGA WAC TGA TC	
MY11	GCM CAG GGW CAT AAY AAT GG	
	Type specific HPV probes	
MY12	CAT CCG TAA CTA CAT CTT CCA	(HPV type 6)
MY13	TCT GTG TCT AAA TCT GCT ACA	(HPV type 11)
MY14	CAT ACA CCT CCA GCA CCT AA	(HPV type 16)
WD74	GGA TGC TGC ACC GGC TGA	(HPV type 18)
MY16	CAC ACA AGT AAC GAG TGA CAG	(HPV type 33)
	β-globin gene primers	
GH20	GAA GAG CCA AGG ACA GGT AC	
PC04	CAA CTT CAT CCA CGT TCA CC	
	Primers for exons 5-8 of p53 gene	
Exon 5 sense	TTC AAC TCT GTC TCC TTC CT	
antisense	CAG CCC TGT CGT CTC TCC AG	
Exon 6 sense	GCC TCT GAT TCC TCA CTG AT	
antisense	TTA ACC CCT CCT CCC AGA GA	
Exon 7 sense	AGG CGC ACT GGC CTC ATC TT	
antisense	TGT GCA GGG TGG CAA GTG GC	
Exon 8 sense	TTC CTT ACT GCC TCT TGC TT	
antisense	AGG CAT AAC TGC ACC CTT GG	

M = A+C, R = A+G, W = A+T, Y = C+T

Table 2
The distribution of HPV types in samples tested.

Histological diagnosis	Cases	Types of HPV						Total	
		11	16	18	16/18	33	untype	HPV (+)	HPV (-)
Cervicitis	12	-	-	-	-	-	-	-	12
CIN 3	21	1	6	2	1	1	10	21	-
Squamous CA*	17	-	8	5	2	2	-	17	-
Total	50	1	14	7	3	3	10	38	12

CA*: carcinoma

minutes and subsequently in developing solution (3 g Na_2CO_3, 150 µl formaldehyde, 100 µl of 2 mg/ml $Na_2S_2O_3$ in 100 ml deionized water) until SSCP bands were discerned, then immersed in 5% acetic acid for 5 minutes to stop reaction.

Samples which showed a band shift by SSCP were subsequently sequenced by Sequenase PCR product sequencing kit (Sequenase® version 2.0; United States Biochemical, Cleveland, OH, USA) according to the manufacturer's instruction.

RESULTS

HPV analysis

HPV DNA was detected in 21 of 21 (100%) CIN 3 and in 17 of 17(100%) squamous cell carcinomas. No HPV DNA was detected in 12 cervicitis subjects. The high-risk HPV types were mostly found in both CIN 3 and squamous cell carcinomas and the predominant types appeared to be HPV 16 and 18. The distribution of HPV types in all

subjects is shown in Table 2.

P53 mutations

All samples were subjected to PCR-SSCP analysis to screen for mutations in the p53 coding sequences of exons 5-8. The PCR products of exons 5-8 were 248, 181, 177 and 231 base pairs, respectively. No p53 mutation was found in 12 cervicitis. Of 38 HPV-positive samples detected, three showed mobility shifts of DNA (Fig 1). One was in exon 7 of CIN 3 with untyped HPV (Fig 1A) and the others were in exon 7 with HPV 16 and in exon 8 with HPV 16/18 of squamous cell carcinomas (Fig 1B and 1C).

Dideoxynucleotide sequencing confirmed the presence of p53 point mutations in these samples. CIN 3 sample had an ATG to TTG transversion at codon 237, resulting in substitution of methionine for leucine (Fig 2A). Each squamous cell carcinoma showed a missense point mutation in codons 242 and 273 changing TGC to TGG and CGT to CCT transversion and causing substitution of cysteine for tryptophane and arginine for proline in encoded protein, respectively (Fig 2B and 2C). In these samples, a normal allele and mutated allele were found at these sites.

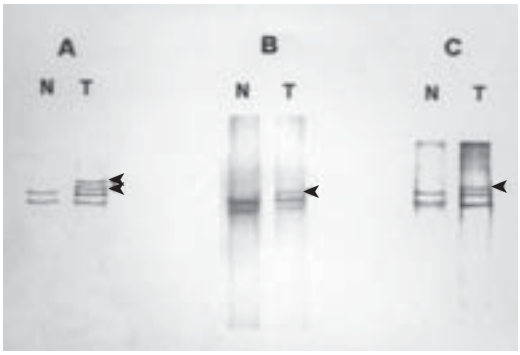

Fig 1–Screening of p53 mutations by PCR-SSCP. N, normal DNA; T, tumor DNA. (A) CIN 3 exon 7. (B) Squamous cell carcinoma exon 7. (C) Squamous cell carcinoma exon 8. Mutations are indicated by arrowheads.

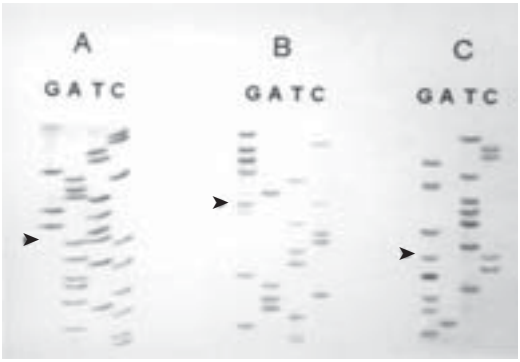

Fig 2–Nucleotide sequence analysis of the cases that showed mobility shift on SSCP. (A) CIN 3 exon 7 (ATG→TTG). (B) Squamous cell carcinoma exon 7 (TGC→TGG). (C) Squamous cell carcinoma exon 8 (CGT→CCT). Arrowheads indicate positions of mutated bases.

DISCUSSION

More than 70 different HPV types have been characterized and approximately 30 types are known to be associated with male and female anogenital tract diseases (Bergeron *et al,* 1992). Among these, HPV 16, 18, 31, 33, and 45 are the most common types associated with high grade CIN and invasive cervical cancers (zur Hausen, 1991). Several investigations show that the development of cervical carcinoma correlates closely with the presence of certain HPV types, such as HPV 16 and 18 (Campion *et al,* 1986; Schneider *et al,* 1987), our findings are consistent with those previous reports.

Inverse correlation between infection of oncogenic HPV and mutation of p53 gene has been observed in both cervical cancer-derived cell lines and primary cervix tumors (Crook *et al,* 1991, 1992; Iwasaka *et al,* 1993; Scheffner *et al,* 1991*;* Srivastava *et al,* 1992). This correlation raises the hypothesis that p53 inactivation can be achieved either by mutation in HPV-negative cases or by complex formation with HPV E6 oncoprotein in HPV-positive cases. Contrary to previous reports, this study showed that mutations of p53 gene could occur in HPV-positive cervical cancers. Moreover, we also studied p53 status in 12 selected HPV-negative cases and found no p53 mutation in these cases (data not shown). Furthermore, many recent studies have demonstrated that p53 mutation may not be a functionally important feature in some HPV-negative cervical carcinoma cell lines and primary tumors (Busby-Earle *et al,* 1994; Choo and Chong, 1993; Kessis *et al,* 1993; Kurvinen *et al,* 1994; Pao *et al,* 1994). They also suggested that some cervical cancers could develop without involvement of either HPV infection or p53 gene mutation. The HPV-negative carcinomas must have achieved their malignant phenotype by a different pathway including genetic aberration other than inactivation of p53. Although previous studies

have revealed that a vast majority of mutational hot spots exist in the exons 5-8 of the p53 gene (Nigro *et al*, 1989), the possibility that mutations locate outside these regions cannot be excluded.

The presence of p53 mutations in HPV-positive cases does not support the hypothesis. The coexistence of p53 mutation and HPV genome in cervical carcinomas has been demonstrated by several investigators (Helland *et al*, 1993; Kim *et al*, 1997; Mittal *et al*, 1995; Munirajan *et al*, 1998) and is consistent with our observations. It should be noted that the presence of p53 mutation in a HPV-positive CIN 3 sample may help in the prognosis of cervical cancer since p53 mutation has been shown to occur in a late stage of this disease (Bremer *et al*, 1995). Three point mutations at codons 237, 242, and 273 were found in this study, whereas only a point mutation at codon 273 has been reported by others in cervical carcinomas (Crook *et al*, 1991,1992; Park *et al*, 1994).

In conclusion, our findings indicate that mutations in the highly conserved regions of the gene are relatively infrequent in cervical carcinoma. No correlation has been found between p53 mutational status and the presence of HPV in this cancer. In contrast to other solid tumors, alterations of p53 gene does not appear to be involved in cervical carcinogenesis. The results also suggest that an alternative pathway independent of p53 inactivation may play an important role in contributing the subsequent accumulation of genetic alterations associated with cervical carcinogenesis.

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