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The prevalence of HPV-18 and variants of *E6* gene isolated from cervical cancer patients in Taiwan

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Abstract

Background : Cervical cancer is the second most common cancer in women worldwide. It has been considered that human papillomavirus (HPV) is associated with cervical cancer. Currently, more than 80 different serotypes of HPV have been characterized and they are divided into low- and high-risk groups. The most common types that lead to cervical cancer are HPV-16 and -18. The viral oncogenes *E6* and *E7* are associated with the development of cervical cancer. In previous study, the variants of HPV-16 *E6* gene have been reported. It suggests that variants may influence the morbidity of carcinogenesis, but the variant study on HPV-18 remains unknown.
Objectives : To identify the variants of integrated HPV-18 *E6* gene in the prevalent infection of HPV-18 of cervical cancer patients.
Study design : 25 cervical cancer patients were clinically identified and the biopsies were obtained. The infectious HPV types were identified by PCR and Southern blotting analysis. The DNA fragments of the integrated HPV-18 *E6* were amplified by PCR and cloned. The nucleotide sequences were obtained by sequencing.
Results : The prevalence of HPV infection in our 25 cases was HPV-18 (100%) and 7 out of these 25 cases (28%) were co-infected with HPV-16. The most dominant mutation among 25 tested patients was a silence mutation C183G of the *E6* coding region.
Conclusions : The prevalent HPV infectious serotype is HPV-18, which differs from the worldwide prevalent type. The identified HPV-18 *E6* variants had a unique silence mutation located on C183G in E6 coding region.
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Keywords: Human papillomavirus type 18 (HPV-18); Cervical cancer; Variants

1. Introduction

Human papillomavirus (HPV) is a risk factor of cervical cancer, the second most common cancer in women worldwide. Studies on epidemiology showed that cervical cancer still remained a major death reason of malignant tumor patients among women.

There are more than 80 different serotypes identified and subdivided into "high-risk" and "low-risk" groups for their potential of tumorigenesis (Mcmurray et al., 2001). The members of high-risk group, HPV-16 and -18, represent 58% and 12% in prevalence, respectively (Bosch et al., 2002). The genome of HPV consists of about 8000 nucleotides, with a circular DNA form. The genome can be separated into three parts including early genes, late genes and the upstream regulatory region (Corden et al., 1999; Wolf and Ramirez, 2001).

The viral early oncogenes play important roles in cancer development. After persistent infection of high-risk HPV, the viral genome can integrate into the host chromosome. The viral genome integration interrupts its regulatory functions of viral oncogenes, *E6* and *E7* (Corden et al., 1999; Jeon and Lambert, 1995). E6 protein reacted with E6-AP and formed an ubiquitin-protein ligase, it functioned following the ubiquitination pathway to degrade cellular tumor suppressor p53

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(Scheffner et al., 1993; Talis et al., 1998). On the other hand, E7 protein interacted with another cellular tumor suppressor, pRb, and disrupted its normal functions. Interaction with E7 protein leads to the disassociation of the pRb–E2F complex, and stimulates the transcription of cellular genes involved in S-phase entry (Chan et al., 2001; Dyson, 1998; Wiman, 1993). The synergism of viral oncogenes leads to abnormal proliferation and abolishes apoptosis induced by the p53-dependent pathway (Huibregtse and Scheffner, 1994; Song et al., 2000).

Studies on viral variants revealed that variants may rise the opportunity for reinfection or evasion from host immune system, like influenza virus and hepatitis B virus (Ferguson et al., 2003 and references there in; Hwang et al., 2003). The most frequent variants of HBV *x* gene isolated from hepatocellular carcinoma patients were located in the B- or T-cell epitopes (Hwang et al., 2003). On the other hand, the studies on HPV-16 *E6* variants showed the most common variation isolated from cervical cancer patients was T to G at nucleotide position 350 (Brady et al., 1999; Hu et al., 1999). This variant resulted in a change from a leucine to a valine at amino acid position 83, which is located in the potential epitope region (Villada et al., 2000). These results revealed that the viral variants may contribute to carcinogenesis or evasion from host immune system (Brady et al., 1999; Zehbe et al., 1998).

The researches of HPV-16 *E6* variants were discovered progressively, but the study on HPV-18 *E6* was unclear. Since the possible epitopes of HPV-18 E6 oncoprotein have been described (Bleul et al., 1991), we would like to know the biological significance of the variants in human papillomavirus. In this study, the infectious serotypes of cervical cancer patients were firstly identified, and the variants of HPV-18 *E6* were also shown. The prevalent type of HPV was HPV-18, and the unique variant was also found in all tested cervical cancer patients. These results implicate that the epidemiological survey was important in designing vaccine for a particular population.

2. Materials and methods

2.1. Specimens

The 25 tissues of the clinically confirmed cervical cancer patients, which were used after the informed consent were obtained from Department of Obstetrics and Gynecology of Changhua Christian Hospital, Taiwan. The information of these patients was collected, including ages, geographic location, and the stages of disease. These tissues were kept in liquid nitrogen or −80 °C freezer before tested.

2.2. Genomic DNA extraction

Each 5 mm^3 of cervical tissues were minced and digested in 1 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA) with 100 μg/ml protease K (Boehringer Mannheim) and 1% SDS at 37 °C overnight. The mixture was extracted with phenol twice and chloroform once by centrifugation 5 min, 14,000 rpm. Genomic DNA was precipitated by 10% v/v sodium acetate (pH 4.6, 3 M) and 2.5 times of absolute alcohol. After washing by 70% alcohol, pellets were re-suspended in DNase-free double distilled water and stored in −80 °C.

2.3. PCR amplification of the HPV-16 and -18 E6 genes

Amplification of the integrated HPV genes were performed by using type-specific primers that designed according to HPV-16 (accession number NC_001526) and HPV-18 (accession number NC_001357) genomes. These type-specific primers were HPV16E6F (position 83–102), HPV16E6R (position 559–540), HPV18E6F (position 105–119), HPV18E6R (position 581–567), HPV18E7F (position 590–604), HPV18E7R (position 907–893), HPV18L16672 (position 6672–6689), HPV18L1R (position 7136–7122). The thermal program was started with pre-heat at 94 °C for 5 min, 30–40 cycles of suitable annealing temperature and extensive time which depended on the primers, and the final extension was at 72 °C for 5 min.

2.4. Southern blotting

The PCR products were separated on 1.5% agarose gel. After depurination (0.25 M HCl) for 15 min, denaturalization (0.5 M NaOH, 1.5M NaCl) for 30 min twice, and neutralization (1 M Tris–HCl, pH 8.0, 1.5 M NaCl) for 30 min twice, the DNA fragments were transferred to positive-charged nitrocellulose membrane with 10× SSC, overnight. After prehybridization with hybridization buffer (50% formamide, 5× SSC, 5× Denhard's, 10 ug/ml ssDNA, 1% SDS) at 42 °C for 1 h, the membranes were probed with Klenow fragment (New England Biolabs) amplified ^{32}P-labeled full-length HPV-16 *E6* probe or HPV-18 *E6* probe, which was amplified from the HPV-16 or -18 genome, at 42 °C overnight. Membranes were washed with washing buffer I (2× SSC, 0.05% SDS) at room temperature for 30 min, and then washed with washing buffer II (0.1× SSC, 0.1% SDS) for another 30 min at 52 °C. The radiation signal was exposed to film at −80 °C in a freezer and then developed.

2.5. Cloning the amplified PCR products and DNA sequencing

Amplified HPV-18 *E6* gene fragments were ligated with pCR II-TOPO TA cloning vector (Invitrogen) and screened as the description from the manufacturer. The identified clones were sequenced and aligned with reference sequence (accession number NC_001357).

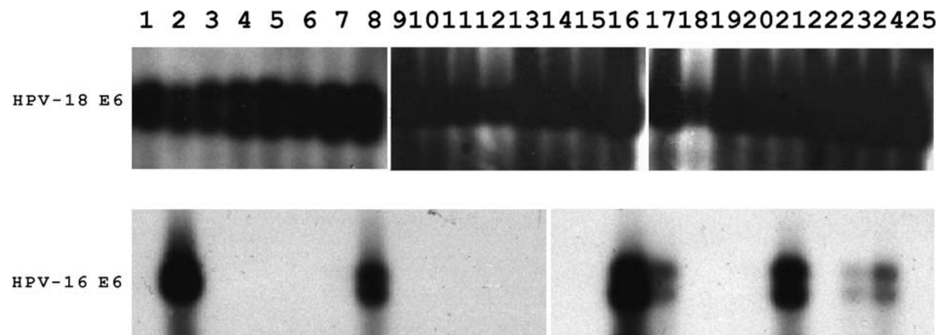

Fig. 1. The serotypes of infected HPV in the cervical cancer patients were identified by Southern blotting. The amplified HPV *E6* fragment from cervical cancer patients were probed with ^{32}P-labeled full-length HPV-18 *E6* (upper panel) or HPV-16 *E6* (lower panel) probe.

Fig. 2. The integrations of HPV-18 *E7* and *L1* genes. The PCR products of full-length HPV-18 *E7* (upper panel) and partial *L1* (lower panel) gene fragments were amplified in all tested samples.

3. Results

3.1. Serotype identification

The amplified *E6* fragments were screened by Southern blotting with two kinds of *E6* probes. The results showed that all the 25 patients were infected with HPV-18 (100%) (Fig. 1, upper panel), while seven patients were co-infected with HPV-16 (28%) (Fig. 1, lower panel). In order to eliminate the possibility of cross-reaction between HPV-16 and -18, the hybridizations of amplified fragments of HPV-16 *E6* and -18 *E6* were performed. The results showed no cross-reaction in these two viral serotypes (data not shown).

To further proof the typing of HPV, we also used the other type-specific primers to amplify the integrated viral genes, including HPV-18 *E7* and partial *L1* genes. The HPV-18 *E7* gene fragment was amplified by semi-nest PCR, which the first reaction reacted with HPV18E6F/HPV18E7R primers, and the second reaction reacted with HPV18E7F/HPV18E7R primers. The result revealed that the HPV-18 *E7* gene fragments were amplified in all tested samples (Fig. 2, upper panel). The full-length of HPV-18 *E6* and *E7* genes were usually integrated into host chromosome after long-term infection, but the *L1* gene presented as a partial-integration form in the host chromosome (Corden et al., 1999). Thus, we used the shorter *L1* primers near the 3′-end of *L1* gene to prevent the false-negative amplification. After amplification, we found the partial HPV-18 *L1* gene fragments were also amplified in all tested cases (Fig. 2, lower panel).

Our results showed the predominant type among patients was HPV-18, and it was quite different from the worldwide distribution and previous studies. In Bosch et al.'s study, HPV-16 was the major infectious type in worldwide (Bosch et al., 2002). The similar prevalence was also observed in previous studies in Taiwan (Shyu et al., 2001; Wu et al., 1994; Yang et al., 1997). Our results imply the unique epidemiology of the HPV-18 infection in our cases.

3.2. Sequences of the variants of HPV-18 E6 among patients

The integrated HPV-18 *E6* fragments were cloned, sequenced, and aligned with the prototype HPV-18 *E6* gene. Several variants were found in the integrated HPV-18 *E6* among patients and the changes in amino acid were also shown (Table 1). The most frequently variant was at

Table 1
The variants of HPV-18 *E6* genes in cervical cancer patients

Variant	Numbers[b] of patients	Results[c]
C183G only[a]	16	Prototype
With A36G and A171G	1	Prototype
With A64G and A95G	1	N22D C32Y
With A95G	1	C32Y
With A107G	1	K36R
With A200G	1	K67R
With A276G	1	Prototype
With A334G	1	Prototype
With A350G	1	Prototype
With A356G	1	R11K
Total	25	

[a] This variant was based on the reference sequence NC_001357.
[b] All cases presented the variant at C183G, and the number indicated the amount of C183G only or with additional variants in the sequences.
[c] The number of patients with the variant and the changes in amino acids were also shown.

nucleotide position 183 C to G of the *E6* coding region. Although this frequent variant was a silence mutation without amino acid change, it may contribute as a tracer to study viral transmission.

4. Discussion

In this study, we found two interesting results, the prevalence of HPV-18 among patients and the predominant variant of integrated HPV-18 *E6* gene. Previous etiological studies on cervical cancer focus on HPV-16, based on its high prevalence in worldwide (Bosch et al., 2002; Munoz, 2000). In Taiwan, similar results were also shown via different approaches (Shyu et al., 2001; Wu et al., 1994; Yang et al., 1997). However, our typing results of the PCR-base hybridization and sequencing data showed the prevalent type was HPV-18. We found that all the 25 patients were infected with HPV-18, while seven patients were co-infected with HPV-16 (Fig. 1). We further used other type-specific primers of HPV-18 *E7* and *L1* to verify our data, and the PCR results also supported the prevalence of HPV-18 (Fig. 2). We also obtained the same result from the western blot analysis on protein level (data not shown). These results also confirmed that HPV-18 was a predominant serotype in severe clinical cancer stages based on in situ PCR approach (Shyu et al., 2001). As we tested all obtained samples without selected on purpose, the results revealed the dominant prevalent serotype of HPV-18 in Taiwan. Hence, the prevalence of different serotypes were reported in several recent studies, like HPV-58 in Mexico (Gonzalez-Losa et al., 2004) and HPV-16 and -18 in Indonesia (Schellekens et al., 2004). On the other hand, the multiple HPV infections were also reported, the range was from 13% to 40% in cervical cancer patients (Cuschieri et al., 2004; Sasagawa et al., 2001; Schellekens et al., 2004). It implied that the multiple infections of HPV was associated in adenosquamous carcinoma (Huang et al., 2004; Schellekens et al., 2004), but was not observed in our studies.

These results may be due to the restriction of the habitation among patients who were almost from the same county (22/25). However, there was no other relationship among these patients, like age, clinical stage and so on. Since we could not collect samples broadly in whole Taiwan area, these results could not be defined as a general or a local event.

On the other hand, the histological analysis showed the relationship between infected cell types and HPV types. Co-infected HPV-16 was only represented in squamous cell carcinoma (SCC), but absent in adenocarcinoma (ADC) (Table 2 and data not shown). Similar results were also reported in other studies (Sasagawa et al., 2001; Schellekens et al., 2004; Tseng et al., 1996; Yang et al., 1997), but the correlation between virus types and cell types remains unclear.

The other aim in our study was to identify the variants of HPV-18 *E6* among patients. In a previous study, it showed that the variants of HPV-16 *E6* were more prevalent in carcinoma than the prototype (Zehbe et al., 1998), which may be due to its ability in evasion from the host immune system. In our study, the most frequent variation at nucleotide position C183G of the *E6* coding region was found in all tested patients (Table 1). This variant was a silence mutation without any change in amino acid. Our study suggests that the limited HPV-18 *E6* variants have the lower mutation rate in DNA virus than in RNA virus. The variant was based on comparing with the reference sequence (accession number NC_001357) of GenBank, but the guanine at *E6* coding region nucleotide position 183 is represented as the prototype of another HPV-18 genome (accession number AY262282). Thus, the variant C183G may be a common mutation in worldwide.

In conclusion, the prevalence of the HPV-18 infection was quite unique from the worldwide based on the results from PCR, hybridization, and sequencing. The dominant silence mutation, C183G, was also identified in the variants of HPV-18 *E6* coding region.

Table 2
Histological diagnosis of cervical cancer patients

Diagnosis[a]	Numbers of patients	HPV-16 co-infection
SCC	13/25	4/13 (30.7%)
ADC	9/25	0/9 (0%)

[a] The squamous cell carcinoma (SCC) and adenocarcinoma (ADC) were identified by histology, but the remaining three samples we obtained were poor for pathological diagnosis. The ratios of different carcinoma among 25 patients and HPV-16 co-infection are also shown.

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