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Genotype Frequency of Human Papillomavirus Determined by PCR and DNA Sequencing in Korean Women

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Human Papilloma viruses (HPVs) are etiological agents for cervical cancer and are classified into low- and high-risk categories. The aim of this study was to determine the frequency of the HPV genotype in the HPV screening test of Korean women using PCR-direct sequencing. Consensus primers of L1 legion were used for the amplification of HPV DNA and the PCR products (450 bps) obtained were analyzed by automatic sequencing. Sequences were compared with those in GenBank by using the BLAST program. Cervical swab samples of 3,978 women (20-73 years) were tested and the average age was 37.6 years. In this study, 1,174 samples were HPV positive out of 3,978 cervical swab samples screened (29.5%) and 136 samples (11.6%) showed a double infection. A total of 1,310 HPV genotypes were analyzed. The HPV positive rate was the lowest in the 20 years group (69.5%) and most of the samples of the > 60 years group were found HPV positive. Among thirty seven different HPV types identified by sequencing, 21 were HPV high risk types and 16 HPV low risk types were 69.8% (914/1,310) and 26.0% (340/1,310), respectively. In HPV high-risk types, 16 (13.21%), was the most frequently found. HPV 53 (9.62%) and 58 (9.24%) were also frequently found. This group was followed by HPV types 70 (5.50%), 33 (4.73%), 66 (4.20%), 18 (4.05%), 52 (4.05%), 31 (3.97%) and 56 (3.51%) in descending order of frequency. Among HPV low-risk types, 62 (4.20%), 6 (3.59%), 81 (3.59%), 84 (3.51%), and 11 (2.6%) were frequently found. In conclusion, PCR-direct sequencing could be used for quick and reliable typing of known and novel HPVs from clinical specimens. This data could be useful for epidemiological study of HPV and it also allows type-specific follow-up of women who have been treated for cervical intraepithelial neoplasia.

Key Words : HPV genotype frequency, PCR, DNA sequencing

I. INTRODUCTION

Cervical cancer is one of a most common malignancies and is a major cause of cancer mortality. Clinical and epidemiological studies have shown that the human papilloma virus (HPV) are the major infectious etiologic agents of genital precancerous lesions and cancers (Bosch et al, 1995). HPV are strictly epitheliotropic viruses infecting cutaneous or mucosal surfaces and display a very high selectivity for the specific epithelium infected (Zur Hausen, 2000). More than 100 different HPV genotypes have been described, of which at least 30 have been identified in the female genital tract and associated with epithelial neoplasm ranging from benign common warts to malignant carcinoma of the uterine cervix (McGlennen, 2000). HPVs are classified into low- and high-risk categories, based on their association with malignant lesions and phylogenetic relationships (Lorincz et al, 1992). Low-risk types of HPV may cause genital warts. High-risk types have been shown to cause more than 99 percent of all cases of cervical cancer. The identification of high-risk HPV genotypes may permit the

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selection of those patients who are at high risk for disease and may therefore provide additional clinical value (Walboomers et al, 1999). Knowledge of HPV status is becoming increasingly important as a triage screen after the detection of atypical cells of undetermined significance and as a primary screen for cervical cancer detection (Bollmann, et al, 2003a). An important requirement for this approach is that HPV testing and identification of high-risk HPV types should be highly sensitive and specific (Cuzick, 2000). Type specific HPV tests that are based on PCR and DNA sequencing are helpful and reliable tools in cervical cancer screening and diagnosis from cytological material, as well as from biopsies. In order to assess the epidemiological incidence and frequency of different HPV types, we applied the PCR and DNA sequencing approach in Korean women.

II. MATERIALS AND METHODS

Materials

Cervical swab samples of 3,978 women (median age, 37.6 years; range, 20-73 years) from Neodin Medical Institute, Korea, were screened for HPV in the absence of clinical signs of cervical dysplasia.

Methods

Cervical swab samples (10 mL) were centrifuged at 2000 g and the supernatant was removed. DNA was extracted from 200 μL of concentrated sample using PuregeneTM DNA purification kit (Gentra, USA). For the amplification of HPV DNA, PCR was performed using MY09/MY11 (Lin et al, 2005) of the HPV L1 gene. The PCR reaction mixture contained 4.5 μL of DNA template, 0.05 μL of PCR primers, 1 μL of dNTPs (2.5 mM/L), 0.2 μL of Taq polymerase (5 U/μL), 4 μL of 10x buffer and 10.2 μL of distilled water for a total volume of 20 μL. The amplification conditions included an initial denaturation for 3 min at 95℃, 35 cycles of amplification with denaturation at 94℃ for 45 sec, annealing at 55℃ for 45 sec, extension at 72℃ for 45 sec, followed by a final extension at 72℃ for 5 min. The PCR product of 450 bp fragment was detected by 2% agarose gel electrophoresis and was purified by using a High Pure PCR product purification kit (Roche Diagnostics, Swiss) according to the manufacturer's instructions. PCR products were subsequently sequenced with a Big-Dye Terminator sequencing kit (Applied Biosystems, USA). Sequencing reactions were also analyzed on the automatic DNA sequencer (Perkin Elmer, ABI 9600). Obtained sequences were compared with documented virus sequences that were available in GenBank by using the BLAST program (Altschul et al, 1997).

III. RESULTS

Among 3,978 cervical samples tested, the positive rate of HPV PCR was 29.5% (1,174), of which 11.6% (136/1,174) showed double infections. Age distribution and prevalence of high-risk HPV type of test samples are presented in Table 1. The number of test sample applied was the highest in the 30-year-old group. The prevalence of HPV high risk type was 100% in the >60-year-old group and 69.5% in the 20-year-old group (Table 1). In a total of 3,978 samples, 1,310 HPV sequences including double infections, were detected by PCR and DNA sequencing. In this study, 37 HPV genotypes were found, including 21 high-risk types and 16 low-risk types (Table 2). Fifty six samples of HPV 17, 71, 72 and 90 which were not classified as high or low-risk groups, were detected. The frequencies of HPV high-risk group and low-risk group were 69.8% (914/ 1,310) and 26.0% (340/1,310), respectively. The genotype frequencies of individual HPV are presented in Table 2. Among high-risk HPV group, HPV 16 was the most frequently found at a frequency of 13.21%, followed by HPV 53 and HPV 58 at 9.62% and 9.24%, respectively. These three types accounted for 32.07% of high-risk HPV sequences. This group was followed by HPV 70 (5.50%), 33 (4.73%), 66 (4.20%), 18 (4.05%), 52 (4.05%), 31 (3.97%), and 56 (3.51%). All other eleven HPV types

Table 1. Age distribution and frequencies of high or low risk group of HPV

Age	Sample distribution (%)	High risk HPV (%)
20 ~ 29	19.6%	69.5%
30 ~ 39	42.2%	80.1%
40 ~ 49	26.6%	79.3%
50 ~ 59	8.2%	73.5%
> 60	3.4%	100.0%

Table 2. Frequencies of HPV genotype by screening test in Korean women

High-risk group			Low-risk group		
HPV type	Number	% of total	HPV type	Number	% of total
16	173	13.21%	6	47	3.59%
18	53	4.05%	10	1	0.08%
26	1	0.08%	11	34	2.60%
30	1	0.08%	32	6	0.46%
31	52	3.97%	34	6	0.46%
33	62	4.73%	40	4	0.31%
35	8	0.61%	43	1	0.08%
39	22	1.68%	44	4	0.31%
45	3	0.23%	54	23	1.76%
51	8	0.61%	55	4	0.31%
52	53	4.05%	61	51	3.89%
53	126	9.62%	62	55	4.20%
56	46	3.51%	67	2	0.15%
58	121	9.24%	81	47	3.59%
59	9	0.69%	83	9	0.69%
66	55	4.20%	84	46	3.51%
68	18	1.37%			
69	12	0.92%			
70	72	5.50%			
73	1	0.08%			
82	18	1.37%			

were detected at frequencies lower than 2.5% and three HPV types, 26, 30 and 73 were detected only once. Among low-risk HPV group, HPV 62 (4.20%), 61 (3.89%), 6 (3.59%), 81 (3.59%), 84 (3.51%) and 11 (2.6%) were frequently found. All other ten types were detected at frequencies lower than 2.5% and two HPV types 10 and 43 were detected only once (Table 2). Other HPV low risk types, 13, 27, 28, 29, 42, 57 and 75 were not found in this study (Fig. 1).

IV. DISCUSSION

Specific anogenital type of human papilloma virus (HPV) is the main causal factor of cervical cancer. This virus is sexually transmitted and the male is the carrier. When HPV infection occurs, the viral DNA is present in the cell as an episomal plasmid and the infection is cured in the great majority of cases due to the host's immune response. Persistence of infection favors viral integration in the cell genome which, together with other factors, can progress to high grade squamous intra epithelial lesions (HSIL) and cancer (Lazo, 1997).

Pap smear screening has largely contributed in decreasing mortality by cervical cancer. Up to now, there is no serological test to detect the presence of HPV in cervical specimen (Weintraub, 1999). However, sensitive and specific methods, based on the detection of HPV DNA, including polymerase chain reaction (PCR), a variety of hybridization techniques; dot blots (Jacobs et al, 1995; Ylitalo et al, 1995), reverse hybridization line probe assays (Coutlee et al, 2002; van den Brule et al, 2002), DNA microarray (Kim et al, 2003; Klaassen et al, 2004; Oh et al, 2004; Choi et al, 2005; Kim et al, 2006) and PCR-direct sequencing (Speich et al, 2004) are available. A general drawback of hybridization techniques is that they may result in cross-hybridizations of closely related genotypes as well as non-specific hybridization (Coste-Burel et al, 1993; Jacobs et al, 1999). The commercially available Hybrid Capture II system (HCII, Digene Corp., USA) is used widely in routine analysis of cervical scrapings, but does not allow the typing of viruses. This test also permits the detection of only a limited number of genotypes that are included in the hybridization probe mixtures. Moreover, the HCII system has lower sensitivity than the PCR methods and it cannot detect relatively rare or novel HPV types (Cope et al, 1997; Smits et al, 1995).

It has been reported that the PCR-direct sequencing

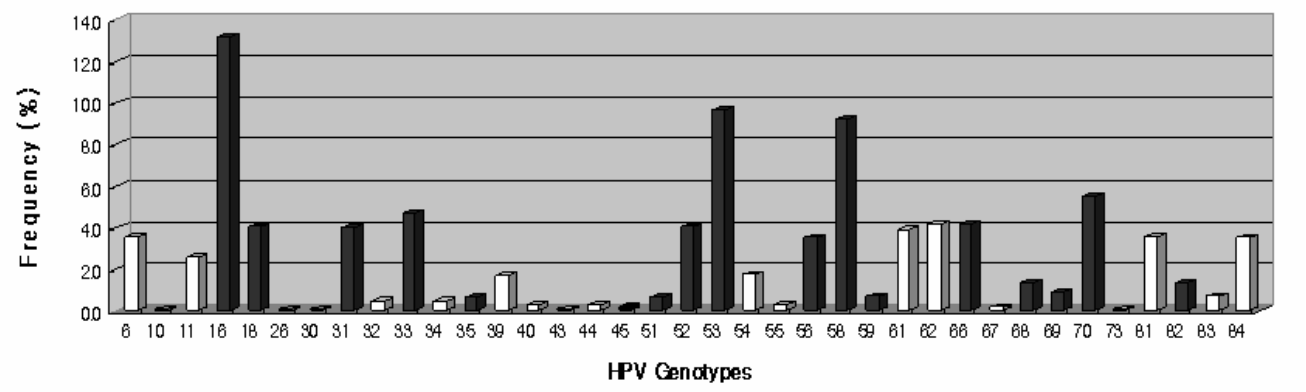

Fig. 1 Distribution of 1,310 HPV sequences detected by PCR-based DNA sequencing in 3,978 samples. Black bar represent HPV types classified as high-risk or probably high-risk, according to Munoz et al (2003).

method showed a 98.2% sensitivity rate in the identification of women with squamous intraepithelial lesions (SIL) and patients with high-grade intraepithelial lesions (HSIL) were all detected (Bollmann et al, 2003b). In this study, we analyzed HPV genotyping by PCR-direct sequencing method which is considered as the gold standard for genotyping study. Most of currently known high and low-risk genital HPV types were found in our samples. Our study showed a complex distribution of HPV types in the Korean population (Table 2).

Many of the sequences found in our samples were not included in the hybrid capture probe mixture. In our study, a significant rate of uncommon types were detected. Especially, HPV types, such as HPV 53, 66, 70 and 82 are important for early detection of cancer, as they are classified as high-risk or probably high-risk and are not included in the Hybrid Capture assay. In this study, a relatively high frequency of HPV 61 (3.89%), 62 (4.20%), 81 (3.59%) and 84 (3.51%) were observed in Korean women. These types are classified as low-risk by Munoz et al (2003). In contrast, the association of these types with aneuploidy in cases of squamous intraepithelial lesions (SIL) was reported (Bollmann et al, 2003b). Therefore, the clear identification of HPV genotype could be an important prognostic of therapeutic value, as it can distinguish between HPV types of high and low oncogenic risks.

Speich et al (2004) reported that 32.5% were HPV positive and mixed infections were 14% in German population by PCR–direct sequencing. It showed a little higher positive rate than our study in Korean women. They described that test samples were selected randomly, and samples screened for triage after detection of cytological signs of dysplasia were also included. Therefore, a relatively high rate of positive HPV test results were obtained. Kim et al (2006) reported that three high-risk types of HPV, including HPV 16 (42.9%), 58 (18.4%) and 31 (14.3%) made up the majority (75.5%) of the HPV found in Korean cervical cancer samples. Moreover frequent HPV genotypes were 16, 58, 31, 18, 35, 33 and 52 in descending order of frequency. On the other hand, in the screening samples of this study, frequencies of 16, 58 and 31 were 13.2%, 9.24% and 3.97%, respectively. And HPV genotypes 16, 53, 58, 70, 33, 66, 18, 52 and 31 were found in descending order of frequency in this study. Some differences of HPV genotype frequencies between the cervical cancer group reported by Kim et al. (2006) and the HPV screening sample of this study were shown.

HPV typing system with PCR-DNA sequencing is useful to provide clinicians and pathologists with relevant information about HPV infection in their patients. A positive HPV high-risk type has a higher sensitivity (90%) to detect recurrent carcinoma in situ (CIN) than in cytology alone. The persistence of HPV infection bears a high risk for recurrence of CIN or cancer, but only type-specific analysis can differentiate between true persistence of a specific type or a new HPV infection (Speich et al., 2004). This is especially important in cases of multiple HPV types (11.6% in our material). HPV sequencing studies in various populations will also help us acquire more knowledge of the epidemiological distribution of HPV types, as a necessary basis for vaccine development that is targeted at uncommon

genotypes. Moreover the sequencing method for HPV genotyping can be applied easily to the analysis of tissue samples and it therefore also allows type-specific follow-up of women who have been treated for cervical intraepithelial neoplasia.

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국문 초록

파필로마바이러스(Human papilloma virus; HPV)는 자궁경부암의 주요한 원인균으로 30종 이상의 여성성기감염과 관련된 유전자형이 보고되었으며 자궁경부암과 관련성이 높은 고위험군과 관련성이 낮은 저위험군으로 나뉘어 진다. 최근 HPV 유전자형의 임상적 활용이 높아짐에 따라 신속하고 정확하게 HPV 유전자형을 선별할 수 있는 방법이 요구되고 있다. 본 연구의 목적은 여러 가지 분자생물학적 방법 중에서 정확도가 높은 DNA 염기서열분석을 이용하여 한국인 여성에서 HPV의 유전자형분포와 빈도를 구하고자 하였다. 전국 각 지역의 3,978명으로부터 채취한 자궁경부 검체에서 DNA를 추출하고, HPV L1 유전자 영역에서 PCR을 실시하였다. PCR 양성이 나온 경우 DNA 염기서열분석을 실시하였으며 GenBank BLAST program을 이용하여 HPV 유전자형을 분석하였다. 검사대상의 평균 년령은 37.6세였으며 년령 범위는 20-73세였고, 30대 여성이 검사를 가장 많이 실시하였다(42.2%). 총 3.978명 중에서 1,174명(1,174/3,978, 29.5%)이 HPV 양성을 보였으며 136명(11.6%)이 중복감염을 보여, 총 1,310개의 HPV 유전자를 분석하였다. 본 연구에서는 21종의 고위험군, 16종의 저위험군을 포함하여 총 37종의 HPV 유전자형이 검출되었으며, HPV 고위험군의 빈도는 69.8%(914/1,310), 저위험군은 26.0% (340/1,310)로 나타났다. 년령은 20대에서 HPV 양성률이 가장 낮았으며(69.5%), 60대 이상의 검체에서 발견된 HPV는 대부분이 고위험군이었다. 고위험군에서는 HPV 16형이 13.21%로 가장 높게 나타났으며, HPV 53형이 9.62%, 58형이 9.24%로 높게 나타났다. 다음으로 HPV 70(5.50%), 33(4.73%), 66(4.20%), 18(4.05%), 52 (4.05%), 31(3.97%), 56(3.51%)의 순으로 나타났다. 저위험군에서는 HPV 62(4.20%), 61(3.89%), 6(3.59%), 81(3.59%), 84(3.51%), 11(2.6%)의 순으로 검출되었다. DNA 염기서열분석을 이용한 한국인 여성의 HPV 유전자형빈도 분석 결과는 HPV의 역학적 연구와 백신개발을 위한 자료로 유용할 것이며, 자궁경부암의 치료와 관련한 특이적 HPV 유전자형 관련 연구에 도움을 줄 것으로 사료된다.