Correlation of HPV Types in Archived ASCUS Samples

Sharon Wah-Suet NG[1], *Stephen Tsui-Hoi Lo*[2], *Daniel Chuen-Chu TAM*[1]

[1] Genepath Technology Limited

[2] Molecular Pathology Laboratory, Department of Pathology, Caritas Medical Centre

Abstract

Objectives: To investigate the prevalence of HPV types in ASCUS samples in private sector in Hong Kong. *Materials and Methods:* One hundred and fifty two archived ThinPrep samples previously diagnosed as ASCUS were included. PCR with general primers GP 5+/6+ and SPF 1/2, and type specific primers for the detection of HPV 6, 11 (low-risk types) and 16, 18, 31 and 33 (high-risk types) were used. *Results:* A total of 140 ASCUS samples were HPV positive (92.1%). Individual HPV high-risk types 16, 33, 18, 31 were present in 15 (9.9%), 9 (5.9%), 8 (5.3%) and 4 (2.6%) cases respectively, contributing a HPV high risk type infection of 22.4% in ASCUS samples. Two cases had more than one type of HPV infection and all these cases harbored at least one high-risk type, while genotyping in 106 cases (69.7%) was unable to define. A high-risk aged-group of 41-50 year old was detected in the present study. *Conclusion:* A high incidence rate of HPV infection was associated with ASCUS samples. The statistical findings revealed that HPV positivity in ASCUS samples was relatively higher than previous studies in Hong Kong especially the presence of undefined types. Patients with normal and ASCUS cytology results should screen for HPV DNA, and consider for receiving vaccination for multiple protection of HPV infection. Analysis of HPV DNA detection in conjunction with cytology results is a useful tool for screening and early detection of cervical abnormalities, and especially to monitor the response of patients after HPV vaccination. An extended monitoring at old age should also be considered.

Key words: human papillomavirus (HPV), ThinPrep, cytology, vaccination

Introduction

Human papillomavirus (HPV) is a double-stranded circular DNA virus. The genome size of the virus is about 8000 bp. It consists of eight open reading frames (ORFs) which code for genes. Early (E) and late (L) regions are found in HPV. The early region is responsible for encoding E1, E2, E4, E5, E6 and E7 proteins while the late region encodes the L1 and L2 capsid proteins.[1] E1 contains DNA helicase for viral DNA replication and interacts with E2 to recruit cellular replication proteins to the viral replication origin. The function of E4 is for the binding of the cytokeratins and cyclin B during replication. E5, E6 and E7 are for down-regulating host immune surveillance.[2] There are over one hundred different types of HPV of which over forty types are pathogenic in human. According to the risk of causing cervical intraepithelial neoplasia (CIN) lesions and cervical cancer, HPV could be classified into two groups (high-risk and low-risk types). More than fifteen HPV high-risk types are currently known to be associated with cervical carcinoma. These high-risk types are 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. The low-risk types are 6, 11, 34, 40, 42, 44, 54, 61, 62, 70, 71, 72, 81, 83, 84, and 89.[3,4] Recent studies have indicated that HPV type 16 and type 18 were related with 50% of cervical intraepithelial neoplasia grade 3 (CIN3), and over 70% of cervical cancer. In addition, it was also found that 90% of genital warts were closely related with HPV type 6 and type 11.[1]

According to the statistical report of Hong Kong Cancer Registry in 2009, cervical cancer was the 7th most common female cancer.[5] Four hundred and fifty three new cervical cancer cases were registered in females that accounted for about 3.7 % of cancer occurrence in Hong Kong in that year. The median age of the females who had cervical cancer was about 53 year old. The age-standardized incidence rate of Hong Kong was about 8.4 per 100,000 women. It is of great interest to investigate the etiology, age relationship and the different stages of cervical abnormalities associated with HPV infection. However, previous studies were mainly focused on the interrelation between the HPV high-risk types with cervical carcinomas. It would therefore be informative to carry out typing of other HPVs and study their correlation with other cytological findings especially those conditions whereby pathogenesis of cervical carcinomas has not been developed. The aims of the present study are to find out the prevalence of HPV types and their correlation with cytology findings especially ASCUS samples in private sector. Hopefully, the data generated would provide information that can help in HPV prevention.

Materials and methods

One hundred and fifty two archived ThinPrep samples with cytology finding as atypical squamous cells of undermined significance (ASCUS) with patient age were provided by Biotechnology Limited previously collected between 2009 and 2010. The cytology grading of Pap smear results were reported according to WHO classification guidelines. This study was approved by the Ethics committee of the HKU SPACE and Edinburgh Napier University. DNA from archived sample was extracted by using NucliSens Magnetic extraction system (Biomerieux, France) as previously described.[6] Extracted HPV DNA was detected by using polymerase chain reaction (PCR). Three sets of general primers were used in this study. They were GP 5+/6+, SPF 1/2, and beta actin. The specific primers were for the detection of HPV types 6, 11, 16, 18, 31 and 33.[7-9] SPF 1/2 primer set targeted the 65bp fragment in the L1 region of the circular HPV DNA genome to detect the presence of 31 subtypes of Human HPV including high risk types (16, 18, 31, 33, 52, 53, 68, 70 and 82) and low risk types (6, 11, 13, 34, 44, 54, 61, 64, 71, 72, 81, and 84).[6] GP5+/6+ primers detected HPV types 6, 11, 16, 18, 31, 33, 35, 42, 45, 51, 55, 56, 58, 59, 66, 69, 70, 73.[7] The Beta actin primers acted as internal control. Ten microlitres extracted DNA template and individual primer sets were added into the master mix separately. The samples were placed to 35 cycles of amplification in a thermal cycler (Applied Biosystems GeneAmp PCR System 9700). All samples were heated to 94°C for 10 minutes in the thermal cycler before cycling in order to activate the hot-start AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, USA). Each cycle involved several steps: Denaturation, annealing and extension. The extra extension 72°C (10 minutes) and inactivation of PCR amplification (4°C) steps were performed after the last cycle. After completion of reaction, all PCR products (GP5+/6+, Beta-actin, T1 and T2) were subjected to gel electrophoresis through a 2% agarose gel while SPF PCR products were electrophoresed through a 2.5% agarose gel. The condition of electrophoresis was 100V, 75 minutes. After the electrophoresis was completed, all PCR products were stained with Ethidium Bromide (BioRad, Hercules, CA, USA; 1: 20000 dilutions in distilled water) for 30 minutes. The visualized bands of samples were observed under ultraviolet (UV) light. The image of DNA bands was captured with digital camera. DNA markers Φ174 DNA/Hae III fragments of 72-1353 bp and 100 bp DNA marker fragment of 100-1000 bp (Fermentas, USA) were used as reference markers for identification of PCR products.

The archived samples were divided into six age groups in this study: ≤20 years; 21-30 years; 31-40 years; 41-50 years; 51-60 years and ≥60 years for further analysis. The one-way ANOVA test was used to assess the distribution at different ages. All statistical testing was two-tailed. A P-value of <0.05 at 95% confidence level was regarded as statistically significant.

Results

A total of 152 archived ThinPrep ASCUS samples were analyzed in this study (Table 1). All samples indicated a sufficient amount of DNA concentration by Beta–actin PCR. Regardless of the breakdown of HPV types, 140 (92.1%) cases of ASCUS samples were associated with HPV. Individual HPV high-risk types 16, 33, 18, 31 were present in 15 (9.9%), 9 (5.9%), 8 (5.3%) and 4 (2.6%) cases respectively contributing a HPV high risk type infection of 22.4% in ASCUS samples. Two cases had more than one type of HPV infection and all these cases harbored at least one high-risk type, while genotyping in 106 cases (69.7%) was unable to define. After data analysis by one-way ANOVA (SPSS 11.0 Chicago, IL), a p value of 0.25 suggested that no significant difference with

Table 1. Prevalence of HPV in ASCUS samples (n=152).

Cytology results	Number of HPV positive types (%)		
ASCUS (n=152)	Any HPV types[a]	140/152 (92.1%)	
	High risk HPV types[b]	34/152 (22.4%)	
	HPV type 16	15/34 (44.1%)	15/152 (9.9%)
	HPV type 33	9/34 (26.5%)	9/152 (5.9%)
	HPV type 18	8/34 (23.5%)	8/152 (5.3%)
	HPV type 31	4/34 (11.8%)	4/152 (2.6%)
	Low risk HPV types[c]	1/152 (<1%)	
	Undefined HPV risk type[d]	106/152 (69.7%)	

Two archived ThinPrep samples with multiple infections were counted more than once.

[a] Indication of single or multiple HPV infection

[b] High risk types include 16,18,31,33

[c] Low risk types include 6, 11

[d] Undefined HPV risk types include 10, 13, 26, 30, 32, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62,64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 83, 84, 86, 86, 90 and CP8304

respect to age was observed.

Age-specific prevalence of HPV in ASCUS samples

The age range of archived samples included was from 20 to 92 year old (mean 43.2 years, median 45.0) (Table 2). One sample (<1%) at ≤20 years; 18 samples (11.8%) at 21-30 years; 34 samples (22.4%) at 31-40 years; 57 samples (37.5%) at 41-50 years; 25 samples (16.4%) at 51-60 years, and 5 samples (3.3%) were ≥60 years. The age-specific prevalence of HPV shows a bell shape pattern. The prevalence of any types of HPV infection (37.5%) peaked at the age group 41-50 years.

Discussion

Data regarding the prevalence of HPV genotyping in ASCUS samples have been few;

Table 2. Age-specific prevalence of HPV in ASCUS samples (n=152).

Age group	No. of HPV positive ThinPrep samples (n=152)		
	Any HPV types[a]	High risk types[b]	Undefined risk types[c]
≤ 20	1 (<1%)	0	1
21-30	18 (11.8%)	7	11
31-40	34 (22.4%)	10	24
41-50	57 (37.5%)	11	46
51-60	25 (16.4%)	3	22
≧60	5 (3.3%)	3	2

[a] Indication of single or multiple HPV infection.

[b] High risk types include 16,18,31,33

[c] Undefined HPV risk types include 10, 13, 26, 30, 32, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62,64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 83, 84, 86, 86, 90 and CP8304

results generated from the present study were in line with study previously reported in Hong Kong.[10] The overall HPV high risk types in ASCUS samples was 22.4% vs. 25.7%. The HPV high risk types found were in the order of HPV type 16 (9.9%) and HPV type 33 (5.9%), followed by HPV type 18 (5.3%) and HPV type 31 (2.6%). In contrast, the predominant HPV types in previous study were in the order of 18 (10.1%), 16 (8.3%), 31 (6.4%) and 33 (2.8%) in ASCUS samples. The discrepancy is not fully understood thus requires further investigation. More than two-third (69.7%) of the HPV positive cases in current study and near half (41.3%) of the HPV positive cases in previous study[10] were unidentified. This may account for the increase in HPV type positivity, 92.1% (140/152), in the present study vs. 77.1% (84/109) of previous study. The nature of the uncharacterized HPV types is not known, and may consider as undefined risk type for cervical oncogenesis.

In an epidemiological study performed by Chan *et al*. indicated that the downward orders of HPV types were 16 (18.3%), 52 (18.3%), 58 (17.7%) and 18 (9.1%).[11] Chan *et al*. further showed that the prevalence of HPV types in descending ordering was 16 (59.6%), 18 (23.9%), 52 (13.2%) and 58 (8.8%), and that the HPV types 16 and 18 were mostly associated with adenosquamous cell carcinoma cases.[12] The predominant HPV types were 52 and 58 after HPV types 16 and 18. In both studies, cervical scraped samples, and fresh frozen tumour tissues were included. No ASCUS samples were involved. The genotyping of HPV 52 and 58 was not included in the present study due to limitation of the method employed. It would be of interest to look at the presence of them and other genotypes among the unidentified risk types (69.7%) that were present in the high and low risk types. Recently, Li *et al*. indicated that the dominant HPV types were 16 (68.1%), 58 (8.3%) and 18 (6.3%) in the cervical cancer samples in Western China where microarray technique was applied.[13] In Macau, Yip *et al.* showed that HPV 52 (26.8%) was most dominant type, followed by HPV 16 (15.5%), HPV 68 (11.4%), HPV 18 and HPV 58 (8.9%) respectively in Thinprep samples consisting of 1256 normal cytology, 18 LSIL, 1 HSIL, 58 ASCUS and 267 inflammatory cases.[14] Since Yip *et al*. only focused on the overall prevalence and HPV genotype of all samples included, individual analysis of ASCUS samples only was not done.

Martin *et al*. investigated that the positive rate of HPV type in ASCUS samples was 84.5% (284/336) which is in line with our pervious study.[15] The most common genotypes detected were HPV 16 (31%), HPV 53 (16%), HPV 31 (11%), HPV 52 (11%) and HPV 6 (10%). The variation in epidemiological patterns of HPV types may due to difference in region of origin. In addition, the age distribution of HPV positive ASCUS samples only divided into 2 groups (below 33 yrs and above 33 yrs) and that the age-specific prevalence of HPV infection pattern was not observed in study of Martin *et al*. In the present study, 152 archived ThinPrep samples were mainly focused on ASCUS cytology without clinical follow-up. Sample size and variation in technology used may account for the discrepancy for the outcome.

The analysis of age-specific prevalence of HPV infection displayed uncommon pattern for low risk, high risk and undefined risk HPV infections in ASCUS samples. It showed a bell shape pattern peaked at 41-50 years and then declined when age decreased. This is in contrast to previous study of Chan *et al*. in Hong Kong which showed that HPV infection had two peaks: The first peak appeared in 26-30 years of women and the second peak was present in 46-55 years of women.[11] Xue *et al*. in China also found that the prevalence of HPV infection with age specific was a U shape pattern.[16] The first HPV infection peak occurred in younger and second peak occurred in women aged 61 or older. The author suggested that the number of sexual partners, smoking habits, education background, hormonal and immunity changes, cohort effect and environmental features might account for the age-prevalence of HPV types. However, the sample size at younger age was small in the present study which made it difficult to compare published data from previous studies. In the present study, the infected patient profile showed that the median is somewhere near 42 or 43 years of age, which is 10 years younger than the median age of cervical cancer in Hong Kong. This finding will be of significance for future vaccination study if progressions of cervical neoplastic diseases are preventable. Nonetheless local ASCUS population diagnosed with infection with HPV types should be monitored closely before they developed into cervical abnormalities and carcinomas.

Conclusion

A high incidence rate of HPV infection is associated with ASCUS samples. The statistical findings revealed that HPV positivity in ASCUS samples was relatively higher than previously studies in Hong Kong. Patients with normal and ASCUS cytology results should screen for HPV DNA, and consider for receiving vaccination for multiple protection of HPV infection. Analysis of HPV DNA detection in conjunction with cytology results is a useful tool for screening and early detection of cervical abnormalities, and especially to monitor the response of patients after HPV vaccination. An extended monitoring at old age should also be considered.

References

1. Schiffman M, Philip E, Jeronimo R, *et al*. Human papillomavirus and cervical cancer. Lancet 2007; 370: 890-907.
2. International Agency for Research on Cancer. Virus Taxonomy: The Classification and Nomenclature of Viruses. The Seventh Report of the International Committee on Taxonomy of Viruses. 2000.
3. Muñoz N, Bosch FX, de Sanjosé S, *et al*. International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic

classification of human papillomavirus types associated with cancer. N Engl J Med 2003; 348: 518–27.

4. Cogliano V, Baan R, Straif K, *et al.* WHO International Agency for Research on Cancer. Carcinogenicity of human papillomaviruses. Lancet Oncol 2005; 6: 204.
5. Hong Kong Cancer Registry (2009) Enquiry on cancer statistics. Available from http://www3.ha.org.hk/cancereg/eb2.asp [accessed 15 March, 2012].
6. Koidl C, Bozic M, Hadzisejdic I, *et al.* Comparison of molecular assays for detection and typing of human papillomavirus. Am J Obstet Gynecol 2008; 199(2): 144.e1-6.
7. Qu W, Jiang G, Cruz Y, *et al.* PCR Detection of Human Papillomavirus: Comparison Between MY09/MY11 and GP5+/GP6+ Primer Systems. J Clin Microbiol 1997; 35(6): 1304-10.
8. Kleter B, Van Doom LJ, Ter SJ, *et al.* Novel short-fragment PCR assay for highly sensitive broad- spectrum detection of anogenital human papillomavirus. Am J Pathol 1998; 153: 1731-9.
9. Lee CN, Cavanagh MA, Lo TH, *et al.* Human papillomavirus infection in non-neoplastic uterine cervical disease in Hong Kong. Br J Biomed Sci 2001; 58(2): 85-91.
10. Tam CC, Cheng PS, Ng WS, *et al.* High prevalence of Human Papilloma Virus (HPV) Infection in Women Tested on ThinPrep Samples in a Private Accredited Laboratory in Hong Kong. Abstract presented at 1st International ASM Conference in Beijing, 2008.
11. Chan PK, Ho WC, Wong MC, *et al.* Epidemiologic risk profile of infection with different groups of human papillomaviruses. J Med Virol 2009; 81(9): 1635-44.
12. Chan PK, Ho WC, Yu MY, *et al.* Distribution of human papillomavirus types in cervical cancers in Hong Kong: Current situation and changes over the last decades. Int J Cancer 2009; 125(7): 1671-7.
13. Li J, Zhang D, Zhang Y, *et al.* Prevalence and genotype distribution of human papilloma- virus in women with cervical cancer or high-grade precancerous lesions in Chengdu, western China. Int J Gynaecol Obstet 2011; 112: 131-4.
14. Yip YC, Ngai LK, Vong HT, *et al.* Prevalence and genotype distribution of cervical human papillomavirus infection in Macao. J Med Virol 2010; 82: 1724-9.
15. Martin P, Kilany L, GarcÍa D, *et al.* Human papillomavirus genotype distribution in Madrid and correlation with cytological data. BMC Infect Dis 2011; 15(316): 1-5.
16. Xue Y, Zhang W, Chen M, *et al.* “U” shape of age-specific prevalence of high-risk human papillomavirus infection in women attending hospitals in Shanghai, China. Eur J Obstet Gynecol Reprod Biol 2009; 145(2): 214-8.