RESEARCH COMMUNICATION

Prevalence of Human Papillomavirus in Abnormal Cervical Smears in Malaysian Patients

Noor Akmal Sharifah[1], A Seeni [1], MI Nurismah [1], CH Clarence-Ko [1], AZ Hatta[2], Ng-Paul Ho[2], T Rafaee[2], N Adeeb[2], AR Jamal[3]

Abstract

Cervical cancer is the second most common female malignancy in Malaysia. Despite advances in treatment, the overall survival for this disease has not changed in the last decade. Infection by certain types of HPV is recognized as a causal and necessary factor for its development. This study was carried out to determine the prevalence of HPV infection in abnormal cervical smears in Malaysian patients using archival cervical smears retrieved from the Cytopathology Unit, Universiti Kebangsaan Malaysia Medical Centre (UKMMC) between the years 1992-1995. DNA was extracted from 38 abnormal smears comprising 25 intraepithelial lesions and 13 cervical carcinomas and 10 normal smears. Amplification of HPV genes was carried out using the polymerase chain reaction (PCR) technique. HPV genotypes were determined using direct sequencing and the results were compared to the database from Genebank. DNA was successfully extracted from all 48 cervical smears. High-risk HPV (HR-HPV) genotypes were detected in 95% of the abnormal smears. Eight high-risk oncogenic types were identified: 16, 18, 31, 51, 52, 56, 58 and 66. All (100%) cervical cancer smears showed presence of HR-HPV compared to 92% of the cervical intraepithelial lesions. Among the eight HR-HPV genotypes identified, HPV 16 and 52 were the commonest (23.7% each) HPV genotypes encountered and among the CIN lesions, HPV 16 (28%) was the most frequent. We conclude that HPV 16 is the most prevalent HPV genotype present in abnormal cervical smears in Malaysian patients, and that the use of archival material to assess the presence of HPV is potentially worthwhile, and can be utilized for longitudinal studies of HPV presence and persistence.

Key Words: Cervical smears - HR-HPV - HPV genotypes - PCR, direct sequencing

Introduction

Traditionally, cervical carcinogenesis was thought to evolve through a series of increasingly abnormal intraepithelial patterns, i.e. mild to moderate to severe dysplasia, followed, in a minority of cases, by acquisition of the ability to invade and metastasize. Harald zur Hausen went against the dogma and postulated that oncogenic human papilloma virus (HPV) caused cervical cancer, the second most common cancer among women. He documented numerous epidemiological and experimental studies on HPV types known to contribute to the development of cervical cancer (Zur Hausen, 1994).

More than 40 of 100 distinct HPV types identified infect the genital tract. High-risk (HR)-HPV include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. LR-HPV include 6, 11, 26, 40, 42, 53, 54, 55, 57, 61, 62, 64, 67, 69-73, 81, 82, 82v, 83, 84, 89 (Wheeler et al., 2006). Over the years, overwhelming evidence has accumulated that infection with HR-HPV is necessary for the development of cervical cancer. Studies have shown that women who have persistent HR-HPV in their smears show progressive CIN disease, whereas women without HR-HPV do not show clinical progression to high grade CIN (Ho et al., 1995; Remmink et al., 1995).

In Malaysia, cancer of the cervix was shown to be the second most common cancer in all the major ethnic groups (Lim et al., 2002). Screening for cervical cancer is based on the presence of cytomorphologically abnormal epithelial cells. Recent data showed that cervical cancer is strongly associated with the presence of high risk oncogenic HPV types of up to 100% (Van den Brule et al., 1991; Parkin et al., 1993). In addition, a number of clinical follow-up studies of women with CIN indicate that the presence of HPV-DNA is a predictive value in progressive CIN disease (Katja et al., 1992; Gaarenstroom et al., 1994). Shibata et al (1988) studied the role of HPV in cervical carcinogenesis using DNA extracted from formalin-fixed paraffin-embedded tissue (PET). This method has proven to be useful for retrospective characterization of molecular defects leading to malignancy. In the present study, we aimed to determine

[1]*Department of Pathology,* [2]*Department of Obstetrics and Gynaecology, Universiti Kebangsaan Malaysia Medical Centre,* [3]*UKM Medical Molecular Biology Institute, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia* *For Correspondence: sharifah@ppukm.ukm.my*

the prevalence of HPV present in abnormal cervical smears and to identify the most common HPV genotype encountered in Malaysian patients.

Materials and Methods

Selection of Study Material

Ten normal smears and 38 routinely processed abnormal cervical smears between 1992-1995 were selected from the Cytopathology archives, UKMMC for this study. Ethics approval was granted by the UKM Research & Ethics Committee (F/03/00). All 48 smears were previously fixed in 95% alcohol and stained with Papanicolaou stain. The 38 abnormal smears include 25 cervical intraepithelial neoplasia (CIN), 11 squamous cell carcinoma (SCC) and 2 adenocarcinoma smears taken from the cervix. The storage times of the cervical smears vary from 16 days to seven years.

Removal of Cellular Material from Slides

The slides were soaked in xylene for two to seven days until the coverslips were removed. This is followed by washes in absolute ethanol and 70% ethanol (2 mins each) respectively. The slides were then air-dried. The washes were done gently so as not to detach the cells from the slide. The material was scraped from each slide using a sterile scalpel blade and transferred to a vial containing 0.5-1.5ml TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

Extraction of DNA from Cellular Material

The tubes were centrifuged for 5 mins at 13,000 rpm. The supernatant was removed, the pellet was resuspended in 1ml TE buffer and kept at 4°C for one hour to dissolve excess stain. The sample was again centrifuged at 13,000 rpm for 5 mins, and the supernatant was removed. The pellet was then suspended in 50 to 100 microlitre of lysis buffer containing 50mM KCl; 10mM Tris HCL at pH 8.3; 2.5 mM $MgCl_2$; 0.5% Tween and 100 microgram/ml proteinase K. The sample was then incubated at 55°C for 60 mins and boiled for 10 mins to inactivate the proteinase K. The tubes were finally centrifuged for 2 mins at 13,000 rpm. The samples were stored at -20°C until used for PCR.

PCR Procedure for Amplification of HPV Genes

SPF1/SPF2 (short PCR fragment) concensus primers were used to amplify 65bp fragments of the HPV genes. These primers allow detection of at least 43 different HPV genotypes at the target region of L1.

Each sample (2µl) was added to PCR mix containing 200µM of each dNTP, 2.0mM $MgCl_2$, 1.5U Taq polymerase (Perkin Elmer) and 100nM of each primer, 5µl reaction buffer, to a final volume of 50µl. Forty cycles of amplification were performed using a thermal cycler (30 seconds at 94°C, 45 secs at 52°C, 45 secs at 72°C). The first cycle was preceded by a 9 minute denaturation at 94°C and the last cycle was extended by a 5 minute elongation at 72°C.

The amplified products were analysed by electrophoresis in a 3% agarose gel containing ethidium bromide and photographed (Kodak Digital Science IDTM). PCR positive controls consisted of DNA from the cell line SiHa, which contains HPV 16 (donated by Professor Bernard Hans-Ulrich, from the National University of Singapore). Distilled water was used to replace the DNA in the negative control tube.

DNA Sequencing

PCR product was purified using the QIAquick PCR purification kit (QIAGEN). HPV were typed by direct sequencing (ABI prism 377 DNA sequencer) and comparison to known HPV sequence database using the bioinformatics computer programme FASTA 3 (www.ebi.ac-uk/fasta3/) and BLAST algorithm (www.ncbi.nlm.nih.gov/blast/).

Results

DNA was successfully extracted from all 10 normal smears and 38 abnormal cervical smears (25 CINs, 11 squamous cell carcinoma, 2 adenocarcinoma). HPV were detected in all 38 abnormal pap smears, 36 (94.7%) showed HR HPV genotype and 2 (5.3%) showed LR HPV genotype (Table 1). HPV was not detected in all 10 normal smears. The range of HPV genotypes identified were 6, 11, 16, 18, 31, 51, 52, 56, 58 and 66. HPV 16 and 52 were each identified in 9 of 38 (23.7%) abnormal smears, followed by HPV 51 (6 of 38; 15.8%) and HPV 56 (3 of 38; 7.9%) (Table 1). HR-HPVs were detected in all (100%) cervical cancer smears, while CIN lesions showed 92% (23 of 25) HR HPV genotypes. The three commonest HPV genotype identified from the CIN lesions (n=25) were HPV 16 (7 of 25; 28%), followed by HPV 51 (6 of 25; 24%) and HPV 52 (5 of 25; 20%). The three commonest HR-HPV genotypes in invasive cancers were HPV 31 and 52 (4 of 13 each; 30.8%) and HPV 16 (2 of 13; 15.4%).

Discussion

In women worldwide, cervical cancer is the second most common type of cancer after breast cancer; in many

Table 1. Distribution of HPV Genotypes in the 38 Abnormal Cervical Smears

Lesions	HPV Genotypes										Total
	6	11	16	18	31	51	52	56	58	66	
LGSIL	1	1	6	1	1	6	4	2	-	1	23
HGSIL	-	-	1	-	-	-	1	-	-	-	2
SCC	-	-	2	-	4	-	3	1	1	-	11
Adenocarcinoma	-	-	-	1	-	-	1	-	-	-	2
Total	1	1	9	2	5	6	9	3	1	1	38

developing countries, it is the leading cause of cancer death among women (Ferlay et al., 2004). Likewise, in Malaysia, cervical cancer has been the second most common cancer among women with an ASR of 21.5 per 100,000 population, comprising 12% of total female cancers. In comparison with Western countries and other Asian countries, Malaysia has a higher incidence of cancer of the cervix (Lim et al., 2002). Intensive screening programmes, especially the widespread use of Pap smear testing that detects preinvasive lesions, have dramatically decreased the incidence of cervical cancer in the developed countries of the world (National Cancer Institute). In the US, approximately 60 million women receive a Pap test each year (Sirovich and Welch, 2004).

HPV, a common sexually transmitted infection, is the primary and necessary aetiological agent in the development of cervical neoplasia. The lifetime of sexually active women becoming infected with HPV is estimated to be up to 70% (Bosch and de Sanjos, 2003). In the US, approximately 20 million people are infected with HPV with 5.5 million acquiring new HPV infections annually (The National Centre for HIV, STD, and TB Prevention). Most of these infections are benign. However, persistent HPV infections are associated with increased risk of CIN and cancer.

Several large meta-analysis have tabulated the HPV types associated with cervical cancers (Munoz et al., 2003; Bosch et al., 1995; Clifford et al., 2003). A recent conference sponsored by the IARC (Cogliano et al., 2005) concluded that 13 HPV types are carcinogenic to humans, including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66. Establishment of HPV infection status and identification of the HPV genotype in clinical samples are gaining ground as important prognostic indicators in the clinical screening of women and management of those found to be at risk for the development of cervical cancer (Cox, 1996).

Cervical cancer is largely preventable through screening programmes designed to diagnose and treat cervical lesions that may progress to invasive cancer. Conventional screening for cervical cancer is based on the presence of cytomorphologically abnormal epithelial cell smears. In spite of the success of this examination, major drawbacks exist. In many developing countries, to establish and maintain effective screening programmes have been very challenging.

Diagnosis of HPV infection is dependant on molecular techniques. Various types of HPV can be detected using this technique. HPV DNA has been shown to be present in over 99% of cervical cancers (Walboomers et al., 1999) and it is now clear that persistent infection with HR HPV increases the risk of developing cervical carcinoma (Ho et al., 1998).

We have managed to successfully extract DNA from 38 archival pap smears and apply molecular techniques to determine the presence of HPV genotypes using this material. Our results showed a strong association between HR-HPV type infection with cervical cancer and that HPV 16 is the commonest HPV genotype present among abnormal smears and CIN lesions. This result is in keeping with other studies worldwide. This type of retrospective

studies using archival smears can allow the natural history of HPV infection to be studied over a considerable length of time and the relationship of HPV detection to normal and abnormal cytology to be evaluated. Walboomers et al. (1995) reported the detection of HPV DNA in smears taken up to six years before the diagnosis of cervical cancer. This same group also detected HPV DNA of high-risk types in cervical smears up to nine years old (Roda Husman de et al., 1995). Poljak et al. (1995) and Puranen et al. (1996) also reported successful HPV DNA detection using polymerase chain reaction (PCR)-based techniques.

Worldwide, HPV 16 is the most common type identified (Clifford et al., 2003). HPV 16 is clearly a much stronger viral carcinogen than any other type (Khan et al., 2005; Castle et al., 2005), persisting longer than most other types (Schiffman et al., 2005) and therefore with a higher prevalence. When it persist for more than one or two years, HPV 16 is most likely to cause cervical precancer and cancer than the other potentially carcinogenic types (Schiffman et al., 2005). Results from our study showed that HPV 16 was one of the most prevalent among abnormal smears and that HPV 16 was also the most common among CIN lesions. In a recent study, persistence of HPV infection was shown to have an effect on risk of disease progression, and that the higher risk was observed for women who showed HPV 16 infection (Wheeler et al., 2006).

The high rates of infection with HPV genotypes in sexually active Malaysian women make molecular investigation for HPV 16 essential for clinical approach in patients with proven dysplasia in their screening tests and also to those patients with borderline reports. Since vaccines are available and found to be effective in preventing cervical neoplasia, their utilization will be soon justified. Further studies with larger samples are essential to choose which are compatible with prevalent genotypes.

Acknowledgement

References

Bosch FX, de Sanjos S (2003). Chapter 1: Human Papillomavirus and cervical cancer-burden and assessment of causality. *J Natl Cancer Inst Monogr*, **31**, 3-13.

Bosch FX, Manos MM, Muñoz N, et al (1995). Prevalence of Human Papillomavirus in cervical smear: a worldwide perspective. *J Natl Cancer Inst*, **87**, 796-802.

Castle PE, Solomon D, Schiffman M, et al (2005). Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. *J Natl Cancer Inst*, **97**, 1066-71.

Clifford GM, Smith JS, Aguado T, et al (2003). Comparison of human papillomavirus type distribution in high grade cervical lesions and cervical cancer: a meta-analysis. *Br J Cancer*, **89**, 101-5.

Clifford GM, Smith JS, Plummer M, et al (2003). Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer*, **88**, 63-73.

Cogliano V, Baan R, Straif K, et al (2005). WHO International Agency for Research on Cancer. Carcinogenecity of human papillomavirus. *Lancet Oncol*, **6**, 204.

Cox JT (1996). Clinical utility of HPV testing. In: Obstetrics and Gynaecology Clinics of North America (Editor, Lorincz AT) 23(4) pp. 811-852. WB Saunders, Publication.

Ferlay J, Bray F, Pisani P, et al (2004). GLOBOCAN 2002: Cancer Incidence, mortality and prevalence. Worldwide IARC Cancer Base no.5. Version 2.0 Lyon: IARC Press.

Gaarenstroom KN, Melkert P, Walboomers JM, et al (1994). Human papillomavirus DNA and genotypes; prognostic factors for progression of cervical intraepithelial neoplasia. *Int J Gynecol Cancer*, **4**, 73-8.

Ho GY, Bierman R, Beardsley L, et al (1998). Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med*, **338**, 423-8.

Ho GY, Burk RD, Klein S, et al (1995). Persistent genital HPV infection is a risk factor for persistent cervical dysplasia. *J Natl Cancer Inst*, **87**, 1365-71.

Kataja V, Syrjänen S, Mäntyjärvi R, et al (1992). Prognostic factors for cervical human papillomavirus infections. *Sex Transm Dis*, **19**, 54-60.

Khan MJ, Castle PE, Lorincz AT, et al (2005). The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst*, **97**, 1072-9.

Lim GCC, Halimah Y, Lim TO (2002). The First Report of the National Cancer Registry: Cancer Incidence in Malaysia 2002 [online]. Available from URL: http://www.crc.gov.my/ncr.

Muñoz N, Bosch FX, de Sanjosé S, et al (2003). International Agency for Research on Cancer Multicenter Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med*, **348**, 518-27.

National Cancer Institute. Cervical Cancer Physician data query (PDQ): Screening (health professional version) [online]. Available from URL: http://cancer.gov/.

Parkin DM, Pisani P, Ferlay J (1993). Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int J Cancer*, **54**, 606.

Poljak M, Barlik J, Seme K, et al (1995). Isolation of DNA from archival Papanicolaou-stained cytological smears using a simple salting-out procedure. *J Clin Pathol*, **48**, M55-6.

Puranen M, Saarikoski S, Syrjänen K, et al (1996). Polymerase chain reaction amplification of human papillomavirus DNA from archival, Papanicolaou-stained cervical smears. *Acta Cytol*, **40**, 391-5.

Remmink AJ, Walboomers JM, Helmerhorst TJ, et al (1995). The presence of persistent high risk human papillomavirus genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. *Int J Cancer*, **61**, 306-11.

de Roda Husman AM, Snijders PJ, Stel HV, et al (1995). Processing of long-stored archival cervical smears for human papillomavirus detection by polymerase chain reaction. *Br J Cancer*, **72**, 412-7.

Schiffman M, Herrero R, Desalle R, et al (2005). The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology*, **337**, 76-84.

Shibata D, Fu YS, Gupta JW, et al (1988). Detection of human papillomavirus in normal and dysplastic tissue by polymerase chain reaction. *Lab Invest*, **59**, 555-9.

Sirovich BE, Welch HG (2004). The frequency of Pap smear screening in the United States. *J Gen Intern Med*, **19**, 243-50.

The National Centre for HIV, STD, and TB Prevention. Genital HPV Infection [online]. Available from URL: http:www.cdc.gov/.

Van Den Brule AJ, Walboomers JM, Du Maine M, et al (1991). Difference in prevalence of human papillomavirus genotypes in cytomorphologically normal cervical smears is associated with a history of cervical intraepithelial neoplasia. *Int J Cancer*, **48**, 404-8.

Walboomers JM, Jacobs MV, Manos MM, et al (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol*, **189**, 12-9.

Walboomers JM, de Roda Husman AM, Snijders PJ, et al (1995). Human papillomavirus in false negative archival smears: implications for screening for cervical cancer. *J Clin Pathol*, **48**, 1-5.

Wheeler CM, Hunt WC, Schiffman M, et al (2006). Human papillomavirus genotypes and the cumulative 2-year risk of cervical precancer. *J Infect Dis*, **194**, 1291-9.

Zur Hausen H (1994). Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. In: Human Pathogenic Papillomavirus (Editor, Zur Hausen H) pp 131-56. Springer Verlag, Berlin.