ORIGINAL ARTICLE

Human Papillomavirus DNA and Virus-encoded Antigens in Cervical Carcinoma

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Summary

The present study was undertaken to evaluate the prevalence of HPV in formalin-fixed, paraffin-embedded cervical carcinoma tissues using PCR followed by non-radioactive Southern hybridization with type-specific oligonucleotides for HPV 16 and 18. In addition, the tissue sections were immunohistochemically screened with two monoclonal antibodies, for expression of HPV 16 L1 and HPV 18 E6 proteins. A total of 57 of 60 cervical carcinomas (95.0%) were found with HPV using both techniques. HPV 16 and HPV 18 were present in equal proportions. Results of both DNA hybridization and immunohistochemistry were in agreement for the majority of the cases. HPV 16 and 18 DNA and virus-encoded antigens, L1 and E6 were found highly prevalent in these cervical carcinomas. Due to the high prevalence of HPV with cervical carcinoma in Malaysia, the implementation of routine diagnosis for the virus in cervical biopsies would be clinically useful.

Key Words: Human papillomavirus, PCR, Southern hybridization, Immunohistochemistry, Cervical carcinoma

Introduction

About a quarter of all malignancies in Malaysian females occur in the uterine cervix and in general a high percentage of the diseased tissue carry human papillomavirus (HPV) types 16 and 18. More than 70 different HPV types have been molecularly identified[1-3]. Several of the HPVs have been aetiologically linked with the development of cervical carcinoma and are collectively referred to as high-risk anogenital types. In contrast to the extrachromosal episomal state in normally infected tissues, the high-risk HPVs particularly HPV 16 and HPV 18 DNA, are often found to be integrated into the human genome lesions[4]. The integration event leads to deletion of some viral genes resulting in the enhanced dysregulated expression of virus-encoded proteins specifically E6 and E7. The coordinated expressions of E6 and E7 provides a selective growth advantage and are thought to play an important role in cervical carcinogenesis because they possess oncogenic properties when transfected in human keratinocytes[5,6] and also when tested in transgenic mice[7,8].

Intraepithelial HPV-related lesions of the genital tract may be divided into two histologic groups. The first group, low-grade lesions, has as its distinctive features; perinuclear halos or koilocytotic atypia and nuclear atypia evident in the middle and superficial areas of the epithelium. These morphological features have been associated with HPV infection. The second group, high-grade lesions, often demonstrate these features, but is distinguished by an increased mitotic index, a typical mitotic figures, and increased cellular and nuclear atypia toward the basal zone[9]. If the association between certain types of HPV and pre-invasive and

invasive cancer of the uterine cervix appears be causal rather than casual then, rapid and accurate diagnosis of the type of HPV infection may be important in the clinical management of patients. Currently, diagnosis of the specific type of HPV relies heavily upon DNA hybridization techniques, which are time-consuming, expensive and not feasible for screening large numbers of samples. However, in contrast immunohistochemical assays for the detection of HPV encoded-antigens in tissues may provide a simpler and a routine-adaptable method for the identification of 'high risk' virus infection in cervical tissues.

Until recently, it has not been possible to produce virus type-specific antibodies because of the lack of techniques for the replication of HPV in tissue culture. Moreover, the presence of low amounts of viral proteins in genital lesions makes it difficult to use them for preparation of virus-specific antibodies. These difficulties have now been overcome by cloning and expression in bacterial vectors of selected sequences for the production of fusion proteins[10]. The major capsid protein, L1 of HPVs is the most abundant virus-encoded protein and is highly conserved between different HPV types with as high as 80% homology in their amino acids[11]. The availability of type specific monoclonal antibodies (MAb) against the L1 protein[12] and E6[13] has encouraged us to compare DNA detection techniques with immunochemical localization of viral antigens in cervical carcinomas.

The objective of the present investigations was to analyse the usefulness of detection for virus-encoded antigens HPV 16 L1 and HPV 18 E6, in archival tissues with two monoclonal antibodies. The data obtained was compared to that by the PCR technique followed by Southern hybridization. HPV 16 and HPV 18 are the two predominant HPV infection previously noted in Malaysian cervical carcinoma samples[14,15].

Materials and Methods

Source of clinical material

Sixty formalin-fixed, paraffin-embedded cervical carcinoma tissue samples were obtained from patients with carcinoma *in situ,* squamous cell carcinoma and adenocarcinoma. Ten biopsies of cytologically normal women were also available as controls. The histological diagnoses were defined using conventional morphological criteria[16]. These cases were diagnosed during regular screening of surgical biopsies for cervical carcinoma at the General Hospital of Seremban, Negeri Sembilan from 1986 to 1988 by one of us (A.R.A.G.) and the cytologically normal tissues from 1994 to 1995 by (S.K.). The tissues were fixed in 10% buffered formaldehyde solution for less than 48 hours but usually not more than 24 hours.

In addition, three ribbons of four micron thick sections from the block were placed in an 1.5ml Eppendorf tube and stored for PCR. A new sterile, disposable scalpel blade was used for each tissue biopsy paraffin block to avoid contamination between specimens.

Four micron thick sections were cut from the same blocks onto two-welled silanized slides for immunohistochemical analysis. Plain wax blocks empty of tissues were cut in-between the sample blocks and these sections were also extracted for DNA and run routinely as negative controls. A separate section was cut before and after the sample sections were taken, for hematoxylin and eosin staining to confirm the original histopathology. All sections were evaluated by a pathologist (A.R.A.G. or S.K.).

Polymerase chain reaction

DNA extraction from the paraffin-embedded tissues was performed according to the protocol previously described[17]. Amplification was performed in a 50µl reaction mixture which contained approximately 100ng of genomic DNA. The PCR amplified products were identified by agarose gel pattern size determination and Southern hybridization. PCR amplification, Southern transfer and hybridization procedures have been described previously[15]. In brief, PCR amplification was carried out using HPV L1 consensus primers, MY09 (5'CGTCCMMARRGGAW ACTGATC3') and MY11 (5'GCMCAGGG WCATAAYA ATGG3'); (M: A+C, R: A+G, W: A+T, Y: C+T) (Perkin-Elmer Cetus, USA). Maximal sensitivity in the detection of HPV DNA sequences was achieved by optimization of PCR reagent concentration and reaction conditions. The target DNA was amplified using a PCR thermal cycler (Perkin-Elmer

Cetus, USA) with the following profiles : 40 cycles of amplification each consisting of 1 minute denaturation at 94°C, 1 minute of annealing at 55°C and 2 minutes of primer extension at 72°C. The last cycle was followed by a final step prolongation at 72°C for 5 minutes. A separate amplification reaction for a 268 basepair β-globin fragment was performed as a measure of DNA sufficiency for subsequent HPV sequence amplification. Positive controls consisting 100ng DNA extracted from CaSki and HeLa cell lines were amplified during each run, which gave consistent positive results. Negative controls included reaction mixtures lacking DNA. All PCR products were electrophoresed through a 2% agarose gel, stained with ethidium bromide and viewed through ultraviolet transillumination.

Southern hybridization

The DNA was denatured, neutralized and transferred to a nylon membrane (Hybond N+, Amersham). Hybridization was performed with type-specific digoxigenin-11-dUTP 3'-tailed oligonucleotide probes, namely, MY14 (5'-CATACACCTCCAGCACCTAA-3') for HPV 16 and WD74 (5'-GGATGCTGCA CCGGCTGA-3') for HPV 18. Filters were prehybridized in 5X SSC-0.5% blocking reagent (Boehringer Mannheim)-0.1% N-lauroylsarcosine, sodium salt-0.02% SDS for a minimum of 1 hour and were then hybridized overnight at 42°C. Filters were washed at low stringency in 2X SSC, 0.1% SDS at room temperature followed by high stringency washing with 0.1X SSC, 0.1% SDS at 42°C. Incubation with anti-digoxigenin antibody conjugated to alkaline phosphatase, subsequent washes and overnight incubation in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in an alkaline buffer were performed[18].

HPV-positive cell lines; (CaSki, SiHa and HeLa) served as positive controls which did not cross-react with each other. All PCR product analyses were interpreted without knowledge of cytologic or pathologic findings.

Immunohistochemistry

Immunohistochemical analysis was performed using two monoclonal antibodies; one directed to the L1 gene product of HPV 16, designated as CAMVIR-1 (courtesy of Dr. M. Stanley) and the other to the E6 gene product of HPV 18, MAB 879 (Chemicon International Inc., USA). The tissue culture supernatant used as a monoclonal antibody for CAMVIR-1 identifies an epitope of HPV 16 L1 that reacts strongly and consistently with specimens containing HPV 16 or HPV 33[12] however, the MAb for HPV 18 E6 was highly specific[13]. The tissue sections were dewaxed by heating at 56°C for 20 minutes followed by immersion in xylene for 3 minutes and then hydrated in decreasing concentrations of alcohol. To block endogenous peroxidase activity, the tissues were incubated with 3% (v/v) H_2O_2 for 10 minutes at 37°C. Subsequently, the sections were incubated with appropriate diluted antibody in phosphate buffered saline pH 7.6 at room temperature for 1 hour. The sections were then incubated successively with biotinylated antibody link and streptavidin (LSAB Kit, Dakopatts) at 37°C. A substrate-chromogen solution, 3-amino-9-ethylcarbazole (AEC, Dakopatts) was applied to the sections and incubated until the desired colour intensity developed. Slides were counterstained with Mayer's hematoxylin (Fluka, USA) and mounted in aqueous glycergel (Dakopatts, Denmark).

For each run, a negative control was included in which the primary monoclonal antibody was replaced by phosphate buffered saline. A positive control consisting of the cervical carcinoma cell lines SiHa and CaSki for HPV 16 and HeLa for HPV 18 grown on teflon coated slides was also run parallel to these samples. Type specificity of the MAbs was tested by incubating SiHa and CaSki cell lines with the MAb for HPV 18 E6 and the HeLa cell line with the MAb for HPV 16 L1. The lack of cross reactivity between these MAbs and the cell lines further proved the specificity of the MAbs.

Statistical analysis

The χ^2 test (contingency tables) was performed to assess the significant correlation between HPV DNA prevalence and HPV protein expression. 'Statistics Graphics System Version 4.0' was used to conduct the correlation tests (contingency tables). The level of significance used throughout the statistical test was 0.05 (5%).

Results

DNA hybridization of PCR amplicons

The DNA extracted from the cervical tissues were subjected to PCR specific for HPV gene L1 sequences.

Ethidium bromide stained gels showed that 46/60 (76.7%) of the carcinomas were positive for HPV (Figure 1A). Initial amplification of these samples for the human beta-globin gene were all positive, thus indicating that the DNA was adequate for PCR studies. The PCR amplified product was further subjected to DNA hybridization using digoxigenin-labelled probes specific for HPV 16 and HPV 18 (Figure 1B). A total of forty nine (81.6%) specimens were positive by Southern hybridization (Table I). Three cases (1 keratinizing squamous cell carcinoma and 2 carcinoma *in situ*) were positive only upon Southern hybridization but negative on agarose gel ultraviolet visualization.

Dual infection for HPV 16 and HPV 18 was found in 24 (40.0%) biopsies. In comparison, 21 (35.0%) patients were positive for only HPV 16 and 3 (5.0%) for only HPV 18. HPV 16 was present in 45 (75.0%) samples and HPV 18 in 24 (40.0%) cases. The cervical carcinomas analyzed in this study showed high dual infection of HPV 16 and 18. Only 2 cases of the 10 analyzed with normal cytology were found positive for HPV infection and both cases were single infections of HPV 16.

Immunohistochemical analysis

The concentration of the MAbs used were optimized to minimize weak signals in tissues that led to equivocal results. Among the limitations observed before optimization were weak nuclear signals, a signal limited to very few cells and/or increased background that diminished the signal to noise ratio. Hence, best optimal signal to noise ratio was seen with a 1:40 dilution for CAMVIR-1, for HPV 16 L1 and a 1:80 dilution for the MAb HPV 18 E6.

Table I summarizes the results of the immunohistochemical analysis. Of the 60 samples tested, 48 (80.0%) were positive for HPV 16 L1 (Figure 2) or HPV 18 E6 (Figure 3) or both viral antigens. In 8 (13.3%) cases for HPV 16 and 9 (15%) cases for HPV 18 the viral protein was found as a single infection but in 31 (51.7%) cases both types were detected. The cervical carcinomas showed a common high rate of dual infection for HPV 16 and 18 as compared to single HPV infection. In the ten

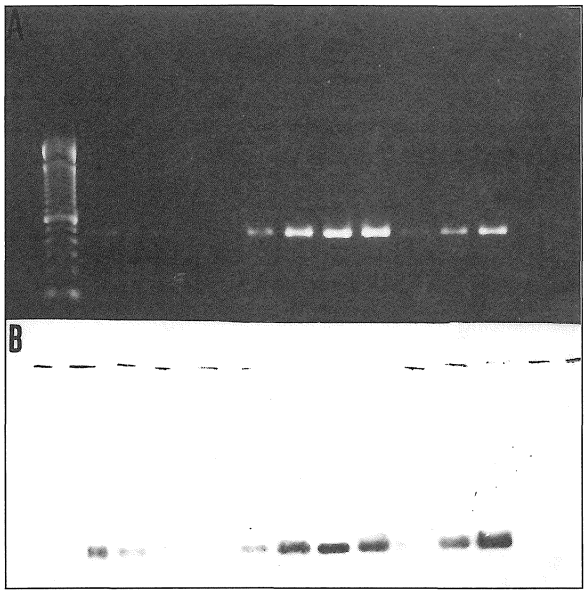

Fig. 1: Detection by PCR of HPV sequence in genomic DNA extracted from cervical carcinoma tissues.

A) Lane 1 : 100 base pair (bp) ladder, molecular weight marker. Lane 2-11 : PCR amplified HPV DNA sequence derived from cervical cancer tissue samples. The 450 bp fragment is visible in lanes 2-3 and 6-11. Lane 12 : Positive control DNA derived from the cervical carcinoma cell line, CaSki. Lane 13 : Negative control.

B) DNA from the gel was transferred to a nylon membrane then hybridised to digoxigenin-labelled oligonucleotide HPV 16 probe. The 450 bp PCR products are noted in lanes 2-3 and 6-11 and these amplicons and the positive control in lane 12 hybridized to the probe.

cases with normal histology of the cervix, two were found positive; with one positive for HPV 16 and the other for HPV 18.

The HPV 16 L1 MAb showed both cytoplasmic and

Table I
Prevalence of Human Papillomavirus 16 and 18 identified by Southern hybridization (SH) of PCR amplicons and immunohistochemistry (IHC) in cervical carcinomas

Carcinoma types	No. cases analyzed	HPV positive		HPV negative	
		SH	IHC	SH	IHC
Squamous cell carcinoma					
Keratinizing	13	10	9	3	4
Nonkeratinizing	26	21	23	5	3
Carcinoma *in situ*	21	18	16	3	5
Total	60	49	48	11	12
Controls	10	2	2	8	8

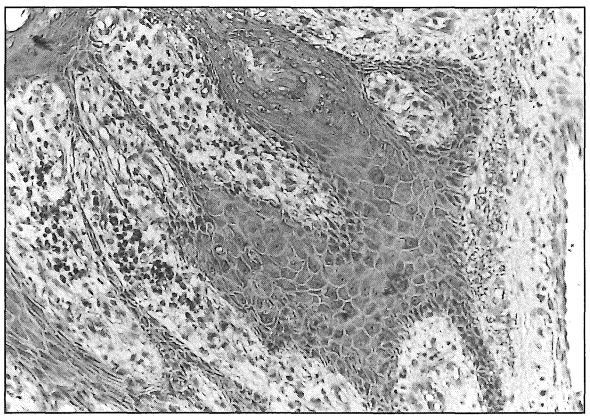

Fig. 2: Positive staining of the HPV 16 major capsid protein in the cytoplasm of a transformed region of squamous cell carcinoma by immunohistochemistry (200X).

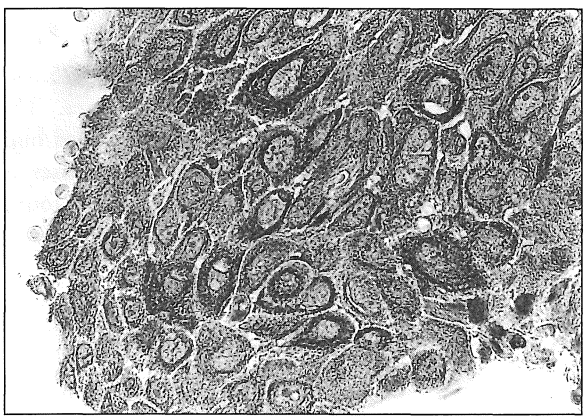

Fig. 3: Immunohistochemical detection of HPV-encoded proteins in formalin-fixed and paraffin-embedded sections of cervical carcinoma. Section of a large cell nonkeratinizing squamous carcinoma shows intense reactivity (reddish brown) of the cytoplasm with the MAb for HPV 18 E6. Nonreactive cells show only blue hematoxylin counterstain.

intense nuclear staining in the superficial layers, also extending over the basal and parabasal regions of the epithelial layer. Similarly, a high dual nuclear and cytoplasmic immunolocalization was found using the MAb against HPV 18 E6. In addition, the MAbs also showed staining in cells with koilocytotic atypia. Immunolocalization of both MAbs was also prominent in the transformed cell regions of the tissue. This was a common feature particularly in the cases of invasive carcinomas. Since the E6 oncoprotein is believed to have transforming properties, it is not surprising that this type of staining was encountered frequently with use of the MAb for HPV 18 E6.

In the cases of keratinizing SCCs, staining of the keratin pearls were not a distinctive feature as histologically expected. In fact, in only 50% of these cases, were the keratin pearls positively stained.

All negative controls run with omission of the primary antibody (MAb for HPV 16 or 18) gave consistent negative staining (Figure 4).

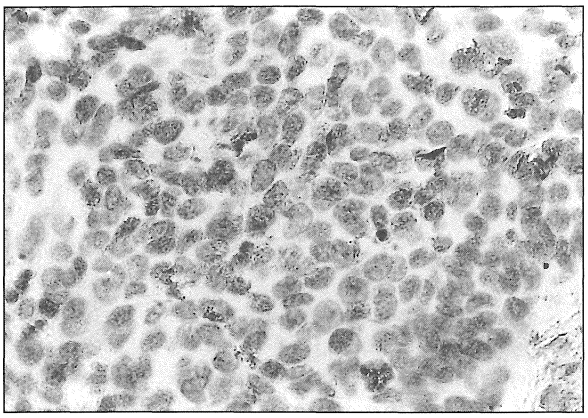

Fig. 4: Negative controls achieved by omitting the primary antibody (MAb for HPV 16 L1 or 18 E6).

Statistical association between DNA prevalence and protein expression

Table II shows that in general the data on the presence of HPV DNA in the carcinomas did not correlate significantly ($p = 0.55$) with the HPV protein expressed in the tissues as tested by the Chi-square test (contingency tables). A significant association was observed between HPV 16 DNA and HPV 16 L1 expression ($p = 0.03$) but not between HPV 18 DNA and HPV 18 E6 expression ($p = 0.82$).

Discussion

The prevalence of HPV DNA found in this series of cervical carcinoma is similar to that of an earlier study[15]. The incidence of dual infection by HPV 16 and 18 in a single genital lesion varies from 2% to 71% with reporting rates of 10% to 20%[19] which is slightly lower than observed here. The studies just cited employed the Southern blot hybridization. The immunohistochemical detection lends support to this high rate of dual infection. It is not possible to distinguish with Southern hybridization between contamination of a HPV type from an adjacent site and actual multiple infection of the tissue[20].

Detection of viral DNA only informs us of the presence of the genome without revealing much on the virus replication and pathogenesis.. The changes of viral protein expression may reflect neoplastic changes of viral phenotype and enlighten us on the progression and prognosis[21]. Perhaps, the use of monoclonals in immunohistochemistry may contribute significantly in this context.

One of the most promising aspects of this study, is the potential of using MAbs as reagents to diagnose genital HPV infections by immunohistochemical staining. The development of monoclonal antibodies

Table II
HPV DNA prevalence associated with HPV protein expression

Parameters	Southern hybridization		χ^2 test (with Yates correction)
HPV Immunohistochemistry	+	−	
+	42	8	P = 0.55
−	7	3	(Not significant)
HPV 16 Immunohistochemistry			
+	35	7	P = 0.03
−	9	8	(Significant)
HPV 18 Immunohistochemistry			
+	18	24	P = 0.82
−	9	9	(Not significant)

to the major capsid protein L1 of HPV 16 encouraged us to compare the immunohistochemical detection of this protein to the DNA detection as the consensus primers for PCR and the oligonucleotide probes used during DNA hybridization were also derived from the L1 region. However, as a MAb for HPV 18 L1 was not available, a type-specific MAb for the E6 protein of HPV 18 was utilized instead. The MAb for HPV 16 L1 designated as CAMVIR-1 reacts strongly and consistently against HPV 16 and 33. However, as HPV 16 had been established as the predominant type in these cervical carcinoma cases by DNA hybridization, its ability to react with HPV 33 did not pose as a serious deterrent for its use.

Although a high degree of agreement was observed between both methods, dissimilarities were seen between the rates of DNA detection and protein expression of both HPV 16 and 18 within the different types of cervical carcinomas. The total number of patients with HPV infection detected using both methods were highly similar with 81.6% for the PCR-Southern hybridization and 80.0% for immunohistochemistry.

However, there were 7 cases which were HPV DNA positive but were negative for HPV proteins by immunohistochemistry. It is possible that in these cases the HPV DNA was not transcriptionally active or prolonged formalin-fixation may have masked the virus protein. The discrepancy in 11.7% of the cases suggests that the immunohistochemical method may not be useful as routine procedure for detection of HPV-encoded proteins particularly when histopathology indicates presence of virus infection. There were 8 (13.3%) cases which were positive for immunohistochemistry but negative for HPV DNA presence. These discrepancies may be due to the loss of viral DNA copies during the rigorous process of formalin-fixation and paraffin-embedding and Southern blot analysis where the tissue analyzed may not have been representative of the section analyzed histologically. Also viral DNA sequences which are not intact are not amplifiable using PCR[22] whereas the use of immunohistochemistry targets the viral antigen *in situ* within the tissue which is fixed directly onto a slide.

The HPV 16 DNA prevalence was significantly correlated with HPV 16 L1 protein expression in the carcinoma cases analyzed. However, no significant correlation was observed between DNA prevalence and HPV protein expression of HPV infection in general and HPV 18 infection. Hence, it may not be possible to confirm all carcinoma cases that were positive for HPV by DNA studies using the immunohistochemical analysis.

The use of a monoclonal antibody against the viral oncoprotein E6 for HPV 18 saw an increased prevalence of this particular HPV type. This may be due to the fact that this protein is believed to play an important part in transformation of epithelial cells which may lead to malignancy and is thus found more frequently in the carcinomas studied. It has been found that a continuous expression of the HPV E6 oncoprotein may contribute to the accumulation of DNA damage associated with the progression of cervical cancer[23]. The L1 viral protein however, was abundantly expressed in the nuclei of the targeted transformed cells and cells showing nuclear atypia.

During this study, 3 cases (5.0%) were persistently HPV-negative by both techniques used. While it has been reported that a low percentage of cervical carcinoma are not HPV associated[24], another possibility may be that the HPV types were not detectable by the primers and probes used here. It is possible that these HPV-negative carcinomas are truly HPV-independent and other factors might be involved in the initiation of the tumours[24]. Recent studies have demonstrated that HPV negative cervical carcinomas posses mutated p53 gene resulting in dysregulated p53 function[25].

In comparison to the cervical carcinoma cases, HPV presence was also analyzed in patients with normal cytology. HPV has been demonstrated in 19.5-26%[26,27] of women with normal cytology and we noted a similar frequency of 20.0%. The rates of HPV infection however, vary greatly in women of abnormal and normal cytology.

It is not known whether these women positive for HPV would develop abnormalities in the future. The progression of HPV positive women from normal cytology to CIN or carcinoma occurs at an annual

frequency of 0.082%. Assumimg an estimated, infected lifespan of 45 years, then the lifetime risk of acquiring the disease is 3.7%[28].

This study demonstrates that PCR followed by Southern hybridization remains the most sensitive and reliable technique for analysis of HPV infection in archival material. Although employing immunohistochemistry techniques was not conducive for routine diagnostic screening for HPV infection, it may however, be performed to further relate the infection with cell morphology and to determine whether histo-morphological cellular changes suggestive of HPV infection are actually HPV-associated.

The HPV infection rates reported here by each technique may vary due to methodological differences. It can be resolved however, that Malaysian patients are infected with HPV types highly related to those in many other European and Asian countries around the world and with similar type-specific prevalence. HPV 16 and 18 are predominantly associated with cervical carcinomas worldwide as well as in the Malaysian population screened. In view of this, detection and typing of high risk HPV genotypes is recommended as a diagnostic exercise to identify women most at risk of developing genital neoplasia.

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