Special Article

Overview: Detection of Human Papillomavirus in Clinical Samples

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Human papillomavirus (HPV) is the most common sexually-transmitted virus and it is known that persistent infection by high-risk HPV is a necessary factor for cervical carcinogenesis. Although cytological screening has decreased the incidence of cervical cancer, the sensitivity and specificity of testing is limited. To date, HPV-driven molecular techniques have provided a number of potential biomarkers for both diagnostic and prognostic use in clinical management. In addition, they can provide insights into the biology of HPV-induced cancers leading to non-surgical therapy. This review summarizes current knowledge of detection methods for HPV and related biomarkers that can be used to discriminate lesions with a high risk of progression of cervical cancer.

Keywords: *Human papillomavirus, Detection methods, Cervical cancer*

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Human papillomavirus (HPV) is the most common sexually-transmitted virus[1,2]. There are around 100 types of HPV with different variations in their genetic and oncogenic potential[3]. There is international consensus that persistent infection of "high-risk" genotypes (hr-HPV), including genotypes-16, -18, -31, -33, -35, -39, -45, -52 and -58, can lead to cervical cancer. Infection with other genotypes, termed "low-risk" (lr-HPV), can cause benign or low-grade cervical tissue changes and genital warts (condyloma acuminata).

Based on strong epidemiological data and laboratory studies, there is no doubt that persistent hr-HPV infection is a necessary but not sufficient cause of cervical cancer, which develops over a long period of time through pre-cancerous lesions[4,5]. The majority of these lesions regress spontaneously without treatment. In Thailand, a total of 20 HPV genotypes were identified and the four most common high-risk HPV genotypes in Thai women are HPV-16 (83.2%), -18 (59.3%), -58 (9.3%), and -45 (3.8%)[6]. The challenge of cervical screening is to detect the lesions that have a high risk of progression[7].

Although cytological screening has decreased the incidence of cervical cancer, some problems, such as low sensitivity and specificity as well as significant variability in the diagnosis of cervical dysplastic lesions, are still major drawbacks. To date, the molecular mechanisms underlying the development of cervical cancer have provided a number of potential biomarkers for both diagnostic and prognostic use in clinical management.

Considering the importance of HPV detection and related biomarkers, several methods are being developed. This review summarizes current knowledge about detection methods for HPV and related biomarkers that can be used to discriminate lesions with a high risk of progression of cervical cancer.

Molecular methods for HPV detection

Since HPV cannot be cultured, its accurate identification relies on molecular biology techniques. All HPV genotypes contain a double-stranded, circular DNA genome approximately 8,000 base pairs (bp) in size that can be generally divided into three major regions, early (E), late (L) and long control region (LCR) (Fig. 1). The tests of choice for detecting HPV in clinical specimens are commonly based on nucleic probe technology[8].

The main clinical applications of HPV DNA testing are: (i) triage of women with equivocal or low-grade cytological abnormalities; (ii) follow-up of women with abnormal screening results who are negative at colposcopy/biopsy; (iii) prediction of the therapeutic outcome after treatment of cervical intraepithelial

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neoplasia (CIN); (iv) primary screening of HPV DNA testing, alone or in combination with Pap smear, to detect cervical cancer precursors[9]; (v) gaining valuable information about the persistence of certain HPV types[10] and (vi) investigations of regional and country-based prevalence of type-specific HPV to provide baseline values against the global impact of HPV vaccination in the future[11].

Nowadays, two types of HPV vaccine are commercially available, a bivalent (type -16/18) and quadrivalent (type -6/11/16/18)[12]. Vaccination against HPV -16/18 in particular can potentially prevent more than two-thirds of cervical cancer worldwide. Promising broad-spectrum HPV vaccines are in development. In addition, novel strategies based on the use of HPV DNA assays for primary cervical screening are increasingly recommended[12]. For detecting and typing of HPV, nucleic acid hybridization assays, signal amplification assays and nucleic acid amplification are available (Fig. 2 and Table 1).

Nucleic acid hybridization assays

Initially, techniques based on hybridization, such as Southern blotting, in situ hybridization and dot-blot hybridization, used radio-labeled probes to detect HPV infection in cervical samples. Although these techniques generated high quality information, disadvantages included low sensitivity, they need for relatively large amounts of purified DNA, and time-consuming procedures.

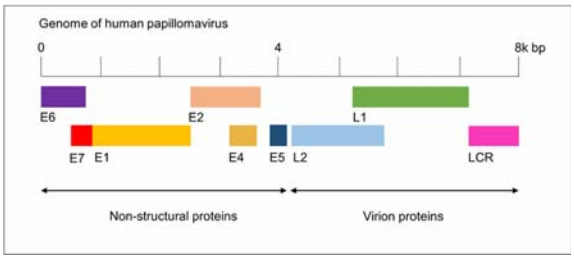

Fig. 1 Simplified genome organization of HPV.

Signal amplification assays

The Digene® HPV test using Hybrid Capture® 2 (hc2) technology and the Cervista® HPV HR assay are the only methods that currently have FDA approval for diagnostic testing in the United States[13].

The Hybrid Capture® 2 system is a non-radioactive signal amplification method based on hybridization of the target HPV-DNA to labeled RNA probes in solution[14]. This test detects 13 hr-HPV genotypes (-16, -18, -31, -33, -35, -39, -45, 51, -52, -56,

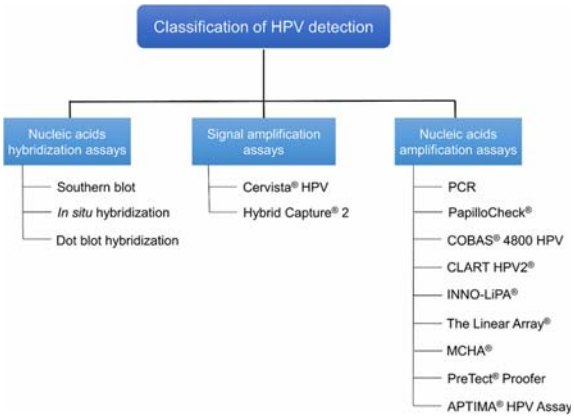

Fig. 2 Schematic diagram of classification for HPV detection.

Table 1. Benefits and weaknesses of molecular methods for HPV detection

Method	Benefits	Weaknesses
Nucleic acids hybridization assays	Southern blot is gold standard for HPV genomic analysis Presence of HPV in association with morphology	Low sensitivity, time-consuming, relatively large amounts of purified DNA
Signal amplification assays	Quantitative FDA approved (hc2) Lower false-positive rate High sensitivity to genotyping	Licensed and patented technologies Was not designed for individual genotyping
Nucleic acids amplification assays	Flexible technology (viral load and genotype) Very high sensitivity Multiplex analysis	Lower amplification signals of some genotypes Contamination with previously amplified material can lead to false-positive

-58, -59 and -68) or 5 lr-HPV genotypes (-6, -11, -42, -43 and -44)[13].

This assay distinguishes between high-risk and low-risk groups, but was not designed for genotyping single HPV genotype. This is a significant finding since, with persistent infection, the risk of a pre-cancerous lesion is between 10 and 15% with HPV genotype 16 and 18, and below 3% for all other high-risk types combined. Therefore, HPV genotyping is very important to identify single oncogenic HPV types and to provide more information regarding risk-stratification as well as persistence of infection[15-17].

The Cervista® HPV detects the presence of all HPV types by Hybrid Capture® 2 assay plus HPV-66[13]. This assay also utilizes a signal amplification method for the detection of specific nucleic acids sequences. In comparison with Hybrid Capture® 2, the Cervista® assay demonstrated 100% sensitivity in the detection of CIN III and 98% sensitivity in the detection of CIN II[18]. Additionally, this assay also showed a lower false positive rate and high sensitivity and specificity to genotyping HPV-16/18[19, 20].

Nucleic acid amplification methods

Polymerase chain reaction

Polymerase chain reaction (PCR)-based techniques are highly sensitive, specific and widely used. In a conventional PCR, the thermostable DNA polymerase recognizes and extends a pair of primers that flank the region of interest. In the final process, the PCR can generate a billion copies from a single double-stranded DNA molecule.

The PCR protocols for HPV detection use consensus primers such as PGMY09/PGMY1 and GP5+/GP6+, which allow amplification of a large number of HPV genotypes in a single reaction. The primers target conserved regions of the HPV genome, such as L1 capsid gene[21]. After amplification, the HPV genotypes can be determined separately using techniques such as restriction-fragment length polymorphism (RFLP), linear probe assay, direct sequencing, or genotype-specific primers[22]. Some researchers have used a type-specific PCR with primers that amplify the long region L1 and E6/E7[23].

These PCR techniques also have some drawbacks, mainly in competition for reagents that lead to false negative results for multiple type detection, which contained low copy numbers. Due to this problem, the PCR method may not detect all the HPV genotypes that are present in a sample. Amplification of samples containing DNA more than one genotype can lead to a much stronger amplification than other genotypes.

This assay detects and genotypes 25 HPV types in a single reaction (HPV-6, -11, -16, -18,-31, -33, -35, -39, -40, -42, -43, 44, -45, -51, -52, -53, -55, -56, -58, -59, -66, -68, -70, -73 and -82). The assay uses a multiplex PCR with fluorescent primers to amplify 350 bp fragment of the E1 gene of HPV, comprising 28 probes, each in 5 replicate spots fixed on a DNA chip. Co-amplification of human ADAT1 gene is used as an internal control. The hybridization is performed on a microarray chip which is automatically scanned and analyzed using the CheckScanner™ at both 532 and 635 nm[24].

The main advantage of the PapilloCheck® (Greiner Bio-One GmbH, Frickenhausen, Germany) is high-risk and low-risk HPV identification, which is multiple identification compatible. However, the cost is still relatively high since it requires specific apparatus.

Abbott® real-time PCR

The Abbott® Real-Time HR-HPV test is a novel assay based on concurrent individual genotyping for HPV-16/18 and pooled detection of 12 HPV genotypes: -31, -33-, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68[25]. This assay is a reliable, sensitive and specific diagnostic tool for detection and genotyping of tissue specimens.

This test features automated sample preparation combined with real-time PCR technology to detect 14 hr-HPV. The amplification and detection occur in a single tube, (i) HPV-16, (ii) HPV-18, (iii) 12 hr-HPV (-31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68) as a pooled detection, (iv) β-globin as the control for extraction and amplification adequacy[26].

The agreement between COBAS® 4800 (Roche Molecular Systems, CA) and real-time PCR was strong in a study that determined the reproducibility involving a sequence of several consecutive steps, both intra- and inter-laboratory[26]. The results can be obtained in approximately 4 hours. COBAS® fulfills all requirements as defined in the international guidelines to consider it clinically validated for screening[27]. This test has been clinically validated for ASC-US triage[28].

CLART® Human Papillomavirus 2

The CLART® Human Papillomavirus 2 (Genomica, Madrid, Spain) assay uses biotinylated primers that amplify a 450 bp fragment within HPV L1 region. Co-amplification of an 892 bp region of the FTR gene and 1,202 bp fragment of a transformed plasmid provides a control to ensure DNA extraction adequacy

and PCR efficiency. Amplicons are detected by hybridization in low-density microarray containing triplicate DNA probes specific for 35 HPV genotypes (-6, -11, -16, -18, -26, -31, -33, -35, -39, -40, -42, -43, -44, -45, -51, -52, -53, -54, -56, -58, -59, -61, -62, -66, -68, -70, -71, -72, -73, -81, -82 ,-83, -84, -85 and -89). Semi-quantitative results can be obtained in an automatic reader with highly comparable outcomes showing excellent sensitivity, specificity and reproducibility[29].

This assay genotypes all 14 HPV covered by real-time PCR assay[25]. INNO-LiPA® (Innogenetics NV, Ghent, Belguim) is based on the co-amplification of the 65 bp region of the HPV L1 gene and the 270 bp of the human HLA-DP1 gene using SPF10 biotinylated primers followed by genotyping[30,31]. Some carcinogenic genotypes such as HPV-35, -39, -52, -56 and -66 are not included in this method[32]. This kit can also be used on specimens taken with swabs, brushes, tampons and lavage[33,34].

The Linear Array®

The Linear Array® HPV genotyping (Roche Molecular Diagnostics, CA) is a PCR-based assay coupled with a reverse line blot hybridization. This assay allows the discrimination of 36 HPV genotypes, including 15 hr-HPV (-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73 and -82), 3-pooled hr-HPV (-26, -53 and -66), 10 lr-HPV (-6, -11, -42, -54, -61, -70, -72, -81 and -CP6108) and 9 genotypes for which the risk is still undetermined (-55, -62, -64, -67, -69, -71, -83, -84 and -IS39)[35].

This test uses biotinylated PGMY09/11 primers to amplify a 450 bp fragment within the polymorphic L1 region of the HPV genome. Co-amplification of the 268 bp region of the human β-globin gene provides a control to ensure DNA extraction adequacy and PCR efficiency. The hybridization and detection of the amplified product are performed with the Auto-LIPA™ instrument (Innogenetics, Ghent, Belgium) which can process up to 30 strips simultaneously in a perfectly standardized fashion. Colored signals on the strips are read by the naked eye and interpreted according to the Linear Array® reference guide. Equivocal results can be obtained for HPV-52 when -33, -35 or -58 are also present, because it is detected through a cross-hybridization probe for these 4 HPV types. In addition, a specific probe is present on the strip to confirm the detection of HPV -33, -35 and -58 but not HPV-52[36,37].

This kit (Genomica SAU, Madrid, Spain) allows the detection and genotyping of HPV. The DNA extraction method is a modified procedure using absorption columns. The kit employs biotinylated primers to define a sequence of 451 nucleotides within the polymorphic L1 region of the HPV genome. A human cystic-fibrosis transmembrane conductance regulator (CFTR) gene and control plasmids are used to check both the PCR procedure and the integrity of the DNA[17].

This also allows the detection of the 35 genotypes that are individually associated with hr-HPV (-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -70, -73, -82 and -85) or lr-HPV (-6, -11, -40, -42, -43, -44, -54, -61, -62, -71, -72, -81, -83, -84 and -89). It is possible to identify simple infections or co-infections[17,38].

Microplate colorimetric hybridization assay (MCHA)

The MCHA (Boehringer Mannheim, Germany) is a method for identifying 6 hr-HPV (-16, -18, -31, -33, -39 and -45) and is based on the amplification by PCR of the 150 bp fragment within the L1 region by consensus primers GP5+/6+ followed by colorimetric hybridization to six type-specific probes on microwell plates (Immobilizer™ Amino Surface, Roskilde, Denmark)[39].

The MCHA showed very good agreement with PapilloCheck® for HPV-31, -33, and -45, and higher sensitivity in identifying HPV -16/18 but poor agreement for -39. To improve MCHA for detection of other genotypes, probes for HPV-35, -52, -56 and -58 should be included[39].

HPV mRNA detection

E6 and E7 are the main genes responsible for cell transformation mediated by hr-HPV and they modulate the activities of cellular proteins that regulate the cell cycle. Thus, the presence of E6 and E7 can be a specific marker for diagnosing pre-cancerous lesions by HPV[40]. For this reason, the search for transcripts of E6/E7 could increase the specificity and sensitivity of the tests in screening for cervical lesions that have a great chance of progressing, compared with a simple detection of HPV-DNA[8,13,41].

The main techniques used to detect mRNA for E6/E7 oncogenes are two commercial assays: PreTect® HPV-Proofer and APTIMA® HPV assay[42]. The chemistry is based on transcription-mediated amplification of full-length E6/E7 transcripts pre-empted by target capture.

The PreTect® HPV-Proofer assay (NorChip AS, Norway) detects HPV E6/E7 mRNA of the 5 hr-HPV (-16, -18, -31, -33 and -45). Clinical studies have

shown high sensitivity. This assay is based on real-time multiplex PCR and is more specific than conventional PCR for the detection of underlying high-grade squamous intraepithelial lesions (HSIL)[13].

The APTIMA® HPV assay (Gen-Probe, Santiago, CA) detects HPV E6/E7 mRNA of the 14 hr-HPV (-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68) which provide better sensitivity than the Pretect® which detects only 5 hr-HPV[41]. This assay has several advantages over other HPV tests, including: (i) detects HPV E6/E7 mRNA, which may be a better marker of advanced disease than hc2; (ii) the limit of detection is lower than other tests; (iii) it does not cross-react with lr-HPV types tested in the current study and (iv) it is compatible with a fully automated system.

HPV viral load quantification and integration HPV-DNA viral load

The association between the HPV viral load and cervical lesions with malignant potential remains unclear. Many studies of HPV viral load address the utility of predicting the progression or severity of disease[43-46]. Lowe et al demonstrated differences in viral load between CIN I and CIN II and between normal and all CIN, but not between CIN II and CIN III[46]. Thus, the results from viral load history and cytology are in agreement. This research group also reported that the viral load declines in response to therapy and provides an acceptable alternative for decisions to pursue further clinical trials[46]. However, other studies have shown that viral load assessment had no added value over cytology and that testing for high viral load level may not be clinically useful, except in the case of HPV-16[44,45,47].

HPV viral load can be determined by real-time PCR techniques. These techniques have been used for semi-quantification in clinical samples that can also be determined by hc2[48].

HPV-DNA integration

HPV-DNA is usually present in extra-chromosomal or episomal form in beginning cervical precursor lesions. Integration of viral DNA frequently occurs in HSIL and carcinoma. These lesions may often contain episomal and integrated HPV-DNA at the same time[49]. During HPV-DNA integration into the host cell, the viral genome usually breaks at E1 and/or E2 open reading frames (ORFs), whereas the E6/E7 ORFs and LCR region remain intact[50,51]. Loss of the E2 gene function results in uncontrolled and increased expression of the oncogenic proteins E6 and E7. A high copy number of HPV-DNA directly contributes to HPV-DNA integration and increased expression of E6 and E7[49,52], and is related to persistent HPV infection[48].

The viral integration is a very early event, occurring before than the onset of morphological changes. Molecular events precede morphological features leading to malignancy, and that integration does not always temporally coincide with a high-grade lesion. It is also possible that viral integration is not necessarily always followed by immediate viral E1 and/or E2 expression[53,54].

The main methods used for detecting HPV integration are PCR, in situ hybridization and real-time PCR. The latter allows calculation of the ratio between E2 and E6/E7 genes. When there is HPV integration, the viral genome shows a 1:1 ratio between E2 and E6/E7 genes[48].

Conclusion

Cervical cancer develops over a long period through pre-cancerous lesions that may regress spontaneously without treatment. The challenge of cytological screening is to detect the lesions that have a risk of disease progression. Consequently, various markers associated with the risk of progression have been investigated and most of them are associated with hr-HPV. Molecular techniques are most commonly used for HPV testing which is the gold standard for HPV infection.

Cell infection by HPV is shown by changes in function or in host gene expression that play a major role in the screening and follow-up of patients. HPV-DNA viral load quantification and integration are promising biomarkers that can predict the disease progression. To date, there is no one ideal biomarker. That said, a combination of biomarkers could contribute to early determination of cervical cancer, which increases the positive predictive value of screening. In spite of their value, molecular techniques still must become more rapid, automated and low-cost to be of practical use in developing countries.

What is already known on this topic?

This review highlights the current knowledge of detection methods for HPV used to evaluate the risk of cervical cancer progression.

What this study adds?

The power of molecular techniques in clinical investigation of HPV detection is summarized and

updated here.

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Potential conflicts of interest

None.

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การตรวจหาเชื้อไวรัสฮิวแมนแปปิโลมาในสิ่งส่งตรวจทางคลินิก

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เชื้อไวรัสฮิวแมนแปปิโลมาเป็นเชื้อไวรัสที่มีการติดต่อทางเพศสัมพันธ์ ซึ่งเป็นที่ทราบกันดีว่าการติดเชื้อไวรัสชนิดนี้เป็นสาเหตุที่สำคัญในการก่อให้เกิดมะเร็งปากมดลูก อย่างไรก็ตามแม้ว่าการตรวจคัดกรองด้วยวิธีทางเซลล์วิทยาจะช่วยลดอุบัติการณ์ของโรคได้ แต่เทคนิคนี้ยังมีข้อจำกัด เนื่องจากเป็นเทคนิคที่มีความไวและความจำเพาะต่ำ ด้วยเหตุนี้จึงมีการนำเทคนิคทางอณูชีวโมเลกุลมาช่วยในการวินิจฉัยและติดตามผลในทางคลินิกมากขึ้น นอกจากนี้ยังมีส่วนช่วยให้เข้าใจกระบวนการก่อโรคของเชื้อไวรัสมากยิ่งขึ้น บทความนี้จึงเป็นการสรุปกระบวนการในการตรวจหาเชื้อไวรัสฮิวแมนแปปิโลมาในสิ่งส่งตรวจทางคลินิกซึ่งจะช่วยในการคัดกรองผู้มีความเสี่ยงในการเกิดมะเร็งปากมดลูกต่อไป