Review

Human papilloma virus (HPV) molecular diagnostics

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Abstract

Human Papilloma Virus (HPV) is becoming a menace world-wide, especially to the developing world, due to its involvement in a variety of malignancies, with cervical cancer being the most important and prevalent. There are many HPV types; HPV 16/18 are the most carcinogenic but few others are also characterized as high-risk (HR). They can cause a variety of low- or high-grade cellular abnormalities, most frequently detected in a routine Pap test. Most infections clear within 2 years, however, a minority persists and potentially could progress to cervical cancer. Molecular tests detecting HPV DNA, RNA or proteins are now being available either commercially or in-house developed. DNA detection is nowadays an established tool for diagnosis and monitoring of HPV-related disease, however, there is lack of a reference method and standardization with reference materials. The various available test formats create confusion on which molecular test to choose and what are its limitations. Therefore, the need for lab accreditation and participation in proficiency testing has to be stressed. Novel HPV biomarkers (RNA, protein etc.) are now intensively examined for their inclusion as adjunct tools. Recently, developed prophylactic vaccines for HPV 16/18 have already proven safe and efficient and raise high expectations for the complete eradication of these types in the future.

Keywords: cervical cancer; DNA/RNA biomarkers; HPV; molecular diagnostics; viral persistence.

Introduction

The evidenced discovery of HPV (human papilloma virus) correlation with condylomata and uterine cervical cancer some 30 years ago has made an impressive trip in the world scientific community that started with many doubts initially but ended with a final recognition and a 2008 Nobel laureate in Medicine and Physiology for Professor Harald zur Hausen (1, 2).

With its continually increasing global prevalence HPV is nowadays a serious environmental threat for cancer, second only to cigarette smoke. According to updated data, HPV is responsible for 10% of total cancer cases in women (5% of total cancer cases in both sexes) (1). The number of HPV infections is significantly increasing in the developing world and correspondingly the number of HPV-related cancer cases is expected to double by 2050 due also to population and life expectancy increases. Hopefully the arrival of the prophylactic HPV vaccines will reverse the above situation and eventually the course of this type of infection might mimic that of hepatitis B where a substantial decrease of related cancers was observed after introduction of successful HBV vaccination programs.

HPV prevalence and related diseases

HPV is an epitheliotropic species-specific virus containing a small circular double-stranded DNA (approx. 8 Kb) within an icosahedral coat or capsid comprised by 72 pentameric capsomers. Its footprint is small (55 nm) resembling a golf ball under the electronic microscope. HPV comprises a family by itself (*Papillomaviridae*) and its types are classified in 16 genera (named after the Greek alphabet letters α-π). The genera are classified in many species and within species so far, at least 128 different genotypes have been identified (106 of them infect humans) that along with their subtypes (differ 2%–10% in DNA sequence) and variants (differ <2%) they have surpassed 200 (1, 3).

HPV types are also divided according to their target epithelial site to: a) cutaneous when they infect foot and hand epidermis and cause most likely warts (but also rarely epidermodysplasia bullosa and squamous skin cancer and melanoma) and b) mucosal when they infect mostly transformation zones between squamous and glandular (columnar) epithelia e.g., of the genital areas of both sexes almost exclusively through sexual contact. In this review, we will examine properties and diagnostics of these mucosal types.

It has been estimated that mucosal HPV types could infect as many as 370 million individuals annually; however, they do not cause any symptoms in the majority of them (300 million). In 30 million people, they can cause disturbing but still benign warts or condylomata (or papillomas, hence the name of the virus) and in another about 40 million people, potentially dangerous precancerous lesions. These lesions and especially the so-called high-grade precancerous lesions must

be either confronted successfully by the human organism itself or medically treated, since they can lead to female cervical uterine cancer in a very small percentage of the totally infected (0.14%); therefore in about 0.5 million women (X. Bosch, 25th International Papillomavirus conference 2009, Malmo, Sweden).

In 2002 there were records of about 560,000 tumors attributed to sexually transmitted HPV in both sexes with the vast majority being cervical uterine cancer in women (493,000 cases, 85% of them squamous carcinoma but also 15% adenocarcinoma). For this particular disease, HPV is responsible for at least 95%–98% of cases. However, HPV also causes 90% of vaginal cancer and 30%–35% of vulvar cancer in women (totally attributed 16,000 cases/year) and 50% of penile cancer in men (10,000 cases/year). Additionally, in both sexes, 80% of anal cancers (27,000 cases/year) and possibly a significant percentage (ranging from 25%–35%) of oral cavity and pharynx/larynx/esophageal tumors (total 14,000 cases/year) could be attributed to HPV (4, 5). Its involvement has been also proven in recurrent respiratory papillomatosis (mostly in neonatal babies by their passage in the birth canal of HPV infected mothers) and has been postulated in other carcinomas like breast, lung etc (6–8).

HPV genome structure

Depending on the type, HPV genome structure could contain as many as eight to 10 open reading frames (ORFs) (1). However, for practical reasons, genomes from all HPV types are divided in three regions (Figure 1):

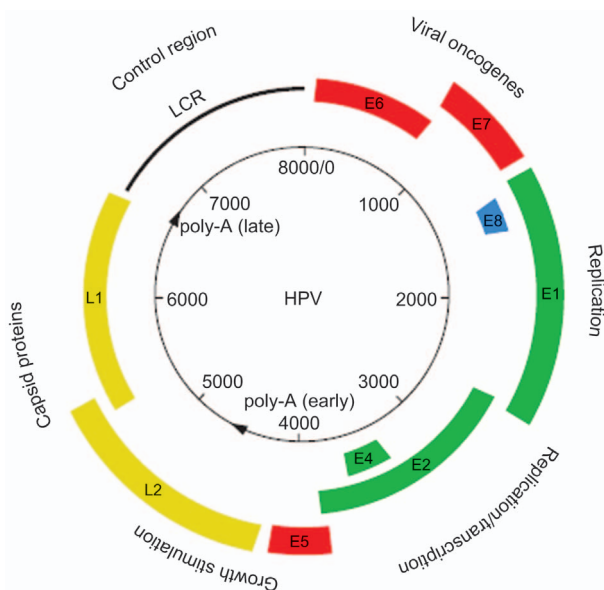a. A non-coding upstream regulatory region (URR) or else long control region (LCR) that contains many elements, such as an origin of replication (ori), a promoter for early genes (p_E), four DNA binding regions for E2 HPV protein and at least 20 other binding areas for host transcription factors specific for epithelial cells.

b. An early region, that encodes for viral E1, E2, E4, E5, E6 and E7 (in some types also E3 and E8) proteins. E1 protein possesses helicase function and binds to ori as an hexamer and initiates viral DNA replication. E2 is the viral transcription factor and also a regulator of E6/E7 expression. E5 is a putative oncogene and its product interacts with EGFR and also suppresses host *MHC I/II* gene expression. E6 and E7 are proven oncogenes (see below).

c. Finally, a late region that encodes for structural proteins of the capsid: L1 (360 molecules/capsid) and L2 (12 molecules/capsid). Erroneously-named E4 is a late protein interacting with host cytoskeleton in order to facilitate the release of viral particles (virions) (9).

Mechanism of HPV infection in the cervix

Mucosal HPV types can cause papillomas and/or precancerous lesions in the cervix in the following way (10): through an abrasive lesion during sexual contact the virus can invade, most likely through L2 interaction with α_6-integrin and heparin proteoglycans, the membranes of the lower basal keratinocytes and especially those of the transformation zone of the cervix. This is an area of immature epithelial cells that are especially hormone-sensitive in women of reproductive age and therefore are susceptible to infections and mutations. The area comprises mainly of soft and vulnerable glandular epithelium that is more exposed during adolescence and that is slowly transformed through metaplasia to the hard and durable squamous epithelium later in life. With this argument, one could easily explain the significantly higher HPV prevalence in younger ages. Among these infected basal cells, there could be stem cells where the virus could potentially hide in a latent state even after infection clearance (not a widely accepted hypothesis). The cells of the basal layer have the potential to divide and it is their proliferative potential along with their transcription factors that the virus exploits in order to express its E proteins and drives its replication as a circular episome at least at a minimal level (50 copies per cell). The daughter cells migrate to the suprabasal layer. In an ideal scenario for humans, infection remains asymptomatic due to an effective control from host cellular immunity factors, such as the local area antigen-presenting cells (Langherhans cells) and T-cells. This encounter could be very well influenced by an interplay between HPV genotype and host genetic polymorphisms in immunity or cancer susceptibility genes e.g., HLA, γIFN, IL6, p53 (11–13).

In another scenario, supra basal cells are trying to differentiate but are also forced by the viral E proteins to a G1 to S-cell cycle phase transition and therefore to proliferate with two side consequences: i) dramatic increase in viral replication (1000 copies per cell) and ii) cellular hyperplasia (along with koilocytic atypia, dyskeratosis). The most benign HPV types cause either simple papillomas (warts) or sub-

Figure 1 HPV genome organization [reprinted from (2), with permission from Taylor and Francis, London].

clinical flat or explantar acuminata condylomata. These phenotypic changes of the keratinocytes can be observed either visually or under the microscope. If the scenario has a happy end - this is the scenario of a productive infection (Figure 2, upper part)-HPV infected cells divide and eventually differentiate in the next layer (granular, stratum spinosum layer) where production of E4, L1 and L2 viral proteins occurs, the virus is assembled and released through apoptosis of the superficial cells and possibly can infect another individual through sexual contact (14, 15).

However, there is another not so happy scenario where malignant transformation can occur during an infection with certain HPV types under certain environmental conditions and/or host genetic background, especially when increased E6 and E7 oncogene expression occurs. There are therefore intermediate scenarios where cellular gene deregulation, hyperplasia, potentially precancerous lesions (low or high grade) exist along with a productive infection (middle part Figure 2).

In the worst of all scenarios, circular episomal DNA becomes linearized, usually with a breakage of the E2/E4 area and integrates into human genomic DNA (Figure 2, bottom part). The transcription check through suppressor gene E2 is lost and uncontrollable transcription of E6 and E7 occurs with the concomitant overproduction of their oncoproteins (16). In this scenario, no productive infection occurs but instead the HPV infected malignant keratinocytes fill all the layers and at a certain time point they break the basement membrane and invasion occurs (17).

E6 and E7 genes of the high-risk HPV types are by themselves true oncogenes since when they transfect normal keratinocytes in vitro, they can induce carcinogenesis: their products interact and inactivate tumor suppressor proteins, such as p53 (through E6-associated protein and ubiquitination) and retinoblastoma protein (Rb) with concomitant release of E2F transcription factor. As a consequence of the above, *p16INK4A* and telomerase expression are up regulated, cyclin inhibitors are inactivated and cyclins are activated and chromosomal instability, aneuploidy and apoptosis evasion occur (10).

Clinical course and evaluation of an HPV infection in the cervix

The most important preventive measure for cervical carcinoma so far has been the Pap test (from George Papanicolaou, Lusker Award 1950) where cervicovaginal smear is obtained from a woman, layered and fixed in a glass slide and then cytochemically stained and evaluated under the microscope in the cytology lab. In this way dysplastic cellular abnormalities compatible with HPV infection like koilocytic atypia, dyskaryosis and dyskeratosis can be detected (Figure 3A). Significant improvement for the conventional Pap test came with liquid-based cytology (LBC) technology where the collected cells are solubilized in a small container with suitable buffer along with preservatives. With the use of special equipment, cells are prepared as a monolayer in a circular area of a slide. In this way, overlays between cells are avoided, cells are gathered in a smaller area and the sensitivity of the test in the hands of an experienced cytologist can increase up to 80% for the detection of precancerous lesions (more could also be found in AACC-sponsored and EFCC/EDMA-backed website www.labtestsonline.org when searching for parameters, such as Pap test or cervical cancer).

HPV infection is suspected in a routine Pap test when in the report there is a positive finding of atypical squamous cells of undetermined significance (ASC-US, Bethesda criteria). In this case, the best strategy to verify the presence or not of the virus is its molecular detection (18). If indeed there was a true finding of an HPV infection, then depending on the HPV genotype, age of the woman ($<$ or $>$30 years old), her immune status and absence/ presence of other numerous cofactors with either a proven ugly role [smoking, oral contraceptive (OC) use, HIV co-infection, number of sexual partners] or an hypothetical one (unhealthy way of living and nutrition, high number of births, co-infection with chlamydia or HSV2) (19, 20) the virus could be effectively contained and the lesion could regress (transient infection) (Figure 4). However, if follow-up tests within a 6- or 12-month period show that the infection is persistent (with the same type or types), it is possible that the cellular abnormalities increase in intensity and quantity and then the most likely positive finding in the Pap test would be LG-SIL (low grade squamous intraepithelial lesion) (21). At this point, many gynecologists refer infected women to colposcopy, especially of the transformation zone (Figure 3B), assisted by the differential stain of cells with either acetic acid or Lugol's iodine. If lesions are observed, then cervical biopsies are excised and sent to the Pathology lab where they are staged according to the CIN (Cervical Intraepithelial Neoplasia) nomenclature.

According to the judgment of the treating physician, at this stage or even more imperatively if the histological diagnosis or a new Pap diagnosis shows advances to high grade squamous intraepithelial lesion (HG-SIL) or CIN stages 2 and 3, treatment options are offered. It has to be stressed that even at the advanced CIN3 stage, a significantly high percentage of lesions regress (up to 70%) however, it is rather unethical not to select for treatment at this point (22), (also Patrick Walker and Mark Schiffman, 25th International Papillomavirus conference 2009, Malmo, Sweden).

Treatment could be pharmaceutical with podophyllin/podophyllotoxin or 5-fluouracil or immune enhancers, such as imiquimod but also invasive (either destructive with electrocauterization/cryocoagulation/laser vaporization or subtractive: cone excision with cold-knife, laser or a loop procedure like LEEP/LLETZ) (23). The goal of any therapy would be initially to completely remove both the precancerous lesion and the area of the virus spread and then to avoid long-term complications, such as carcinoma in situ initially and invasive cervical carcinoma in the future. The treatment effectiveness can be judged by the negative results of follow-up tests and especially of the molecular detection of the virus in cervicovaginal specimen.

The various HPV types possess different oncogenic potential most likely due to differences in E6 and E7 protein
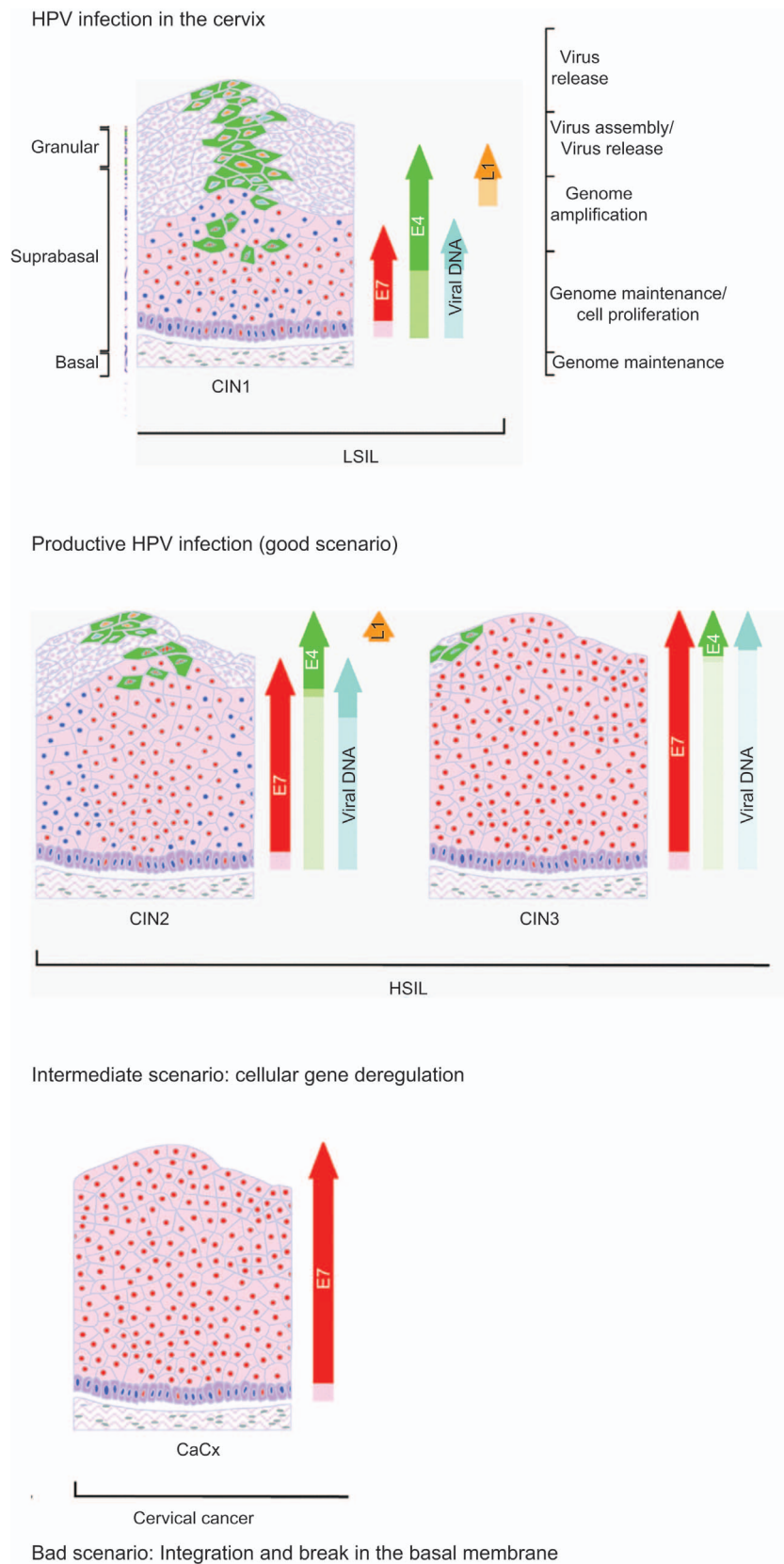
Figure 2   Differences in viral HPV gene expression in various infectious scenarios in the uterine cervix [adapted from (14) with permission from The Biochemical Society, London].
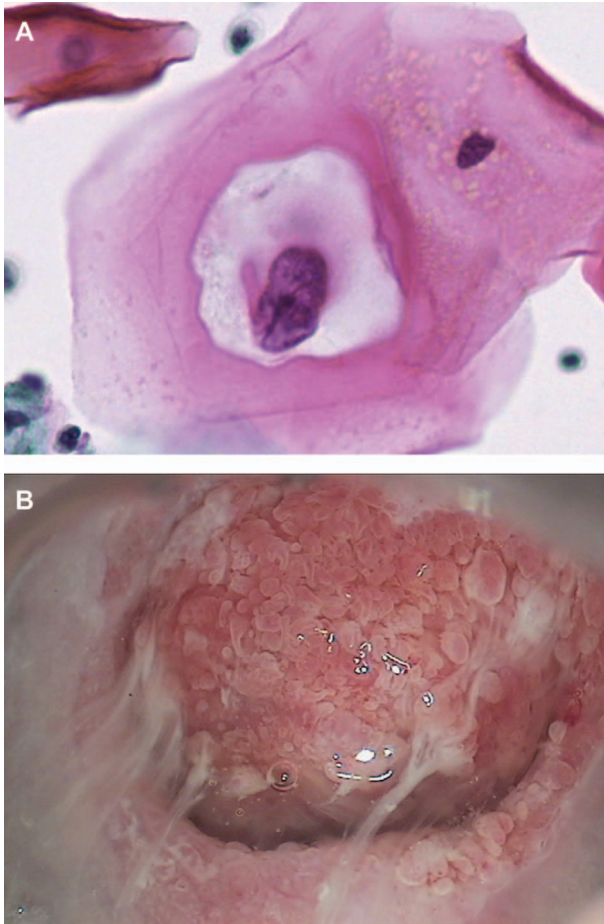
Figure 3 (A) Thin prep cytology image of a typical koilocyte with dyskaryosis, perinuclear halo and dense cytoplasm and (B) a colposcopy image of a cervical transformation zone indicative of LG-SIL HPV infection (both from Mitera Center archives).

sequence. Some researchers claim also that the presence of an E5 protein is important as well (24). Their tumorigenicity is classified to various risk groups (low, high etc.) according to either phylogenetic or epidemiological observations (19, 25). Due to existing confusion regarding risk of some types, we provide a somewhat modified table (Table 1) evidenced according to arguments described elsewhere (26, 27) and in the next paragraphs.

Low risk HPV types are usually found in condylomata and low grade dysplasias (ASC-US, LG-SIL, CIN 1) of the uterine cervix or even in negative for dysplasia Pap tests while intermediate and high-risk HPV types are detected in the whole spectrum of findings. In high grade dysplasias (HG-SIL, CIN3) and in carcinomas, only high-risk types are detected almost exclusively. The large majority of cervical carcinomas (about 73%) are due to HPV16 and HPV 18, the two highest risk types both in squamous (SCC) and also in the rarer adenocarcinomas (ADC). This observation is very important. Their prevalence rise continuously from normal tests (28) to LG-SIL (29), then to HG-SIL (30) and cervical Ca (27), as illustrated in Figure 5. The total HPV16/18 percentage in cervical tumors varies slightly worldwide and is increased in Western/Central Asia (82%) while decreased in Eastern Asia (68%). Another six types HPV 58, 33, 45, 31, 52 and 35 fill the gap till about 92% for squamous cell carcinomas (31, 32). HPV 58 is frequent in Eastern Asia. Five other types HPV 59, 39, 51, 56 and 68 provide another 3%–4% of tumor burden. HPV 16 is not only a majority in both the aforementioned cervical carcinomas but also in those tumors mentioned in the beginning of this review (vaginal, vulvar, penile, anal, oropharyngeal). As noticed in Figure 5, HPV 18 has increased role relatively to HPV 16 in adenocarcinomas (36.8% in ADC vs. 13.2% in SCC) which are tumors that more easily evade diagnosis. HPV typing therefore has prognostic value. Incubation period till carcinogenicity varies depending on HPV type and host background and it could typically be over 10 years (33).
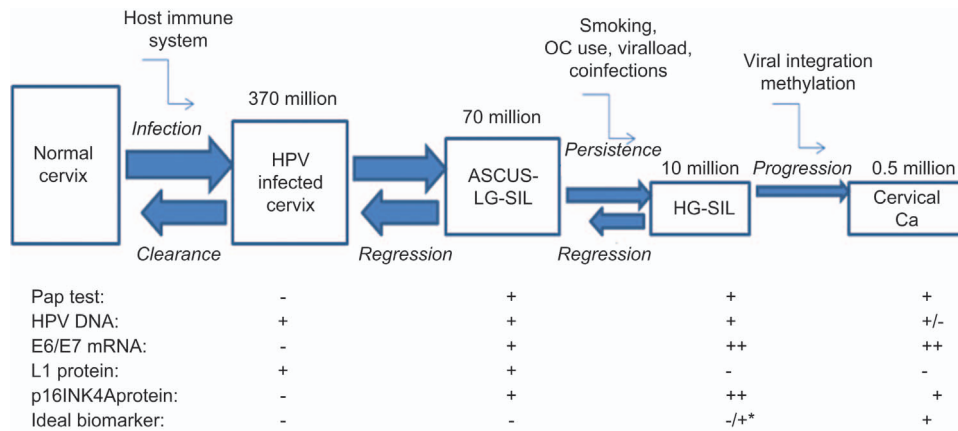

	Normal cervix → HPV infected cervix	ASCUS-LG-SIL	HG-SIL	Cervical Ca
Pap test:	-	+	+	+
HPV DNA:	+	+	+	+/-
E6/E7 mRNA:	-	+	++	++
L1 protein:	+	+	-	-
p16INK4Aprotein:	-	+	++	+
Ideal biomarker:	-	-	-/+*	+

Figure 4 Typical clinical course of an HPV infection in the uterine cervix (numbers indicate cases/year). Time between mild cytological abnormalities to cancer can vary (typically between 7 and 10 years). Factors affecting the progression are shown above and various test results are shown underneath the line of the time course. For the ideal biomarker, -/+* denotes the situation where (−) could provide the HG-SILs that regress and (+) the HG-SILs that progress to cancer.

Table 1   HPV type classification according to risk (highest frequencies in types printed bold, types found more rarely in parentheses).

Low risk:	**6**, **11**, (40, 42, 43, 44, 54, 61, 62, 72, 81, 89)
Intermediate to low risk:	26[a], **53**[a], **66**[a], **70**, **73**, **82**, **83**, **84**
High to intermediate risk:	**31**, **33**, **35**, **45**, **52**, **58**, (39, 51, 56, 59, 68)
Highest risk:	**16**, **18**

[a]Debated risk in literature (many classify them as low risk).

HPV types 16 or 18 are far more important risk factors for cervical carcinogenesis in relation to the rest of the HPV types (either high or intermediate risk) as shown elegantly in a recent study that monitored 20,000 women for a big time frame (Figure 6). It is impressive that even a single negative molecular HPV test attributes a very low cancer risk for the same big time frame (34).

Besides the prognostic value of the HPV type, the presence of persistent infection with the same type is very important. In the vast majority, HPV types regress and the cervical area is ''cleared'' in 70% of the cases within 2 years and in 90% of cases within 7 years (Figure 7) (33).

Certain HPV types ''clear'' fast (e.g., the 80% of HPV 6 infections that clear within 6 months) and they never cause cancer while others, like HPV16, are both more persistent and potentially more cancer prone. Other types are persistent but they do not cause cancer. It must be stressed that even 80% of infections with the most carcinogenic type, HPV 16, clear within 2 years without any consequences. Maybe significant roles for both persistent and carcinogenic infection might be played by the presence or not of the E5 gene (24) and by variations in the DNA sequence detected so far for types HPV16/18 (35, 36) e.g., Q14H/H78Y/L83V variants in E6 gene of HPV 16 are associated with increased susceptibility for infected keratinocytes to undergo cell-detachment-induced apoptosis (anoikis) in relation to wild type and therefore could lead to increased viral persistence (37).

The above observations have certainly their importance for selection of both the proper therapeutic treatment and the proper molecular test of detection and its accurate evaluation.

Maybe the easier clearance observed in some women might have to do with their previous exposure to HPV. In a subpopulation of HPV infected women, low titers of antibodies are being detected. The reason for the low level of antibody-mediated immunity might be due to the following: the virus acts in a non-lytic and non-viremic fashion, is suppressing local immunity and is assembled fully with its main antigenic L1/L2 epitopes only in the upper layers of keratinocytes where no significant numbers of APC (antigen presenting cells) reside. This is not the same situation with vaccination where in both approved vaccines (tetravalent 16/18/6/11 Gardasil or bivalent 16/18 Cervarix) the L1 antigen has been prepared biotechnologically, has been packaged in high quantities of virus-like particles (VLPs) and administered intramuscularly. The human immune system in this case, confronts efficiently the epitopes with dendritic cells and B lymphocytes in the local lymph nodes and therefore, high titers of neutralizing anti-L1 IgG antibodies are secreted in peripheral blood (38). These levels are apparently fully protecting from infection from the HPV types included in the vaccines and from HPV-related cancer development, at least for 7 years, as it has been shown with the clinical studies conducted so far. These vaccines also offer some cross protection against other HPV types phylogenetically related
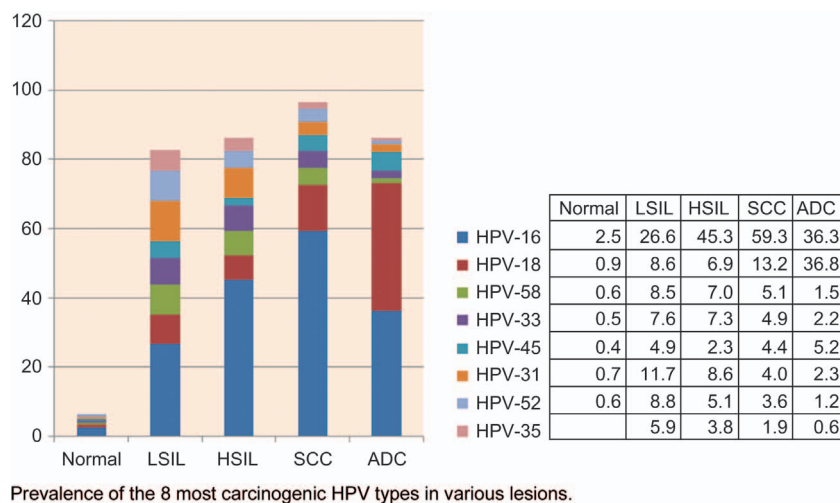

	Normal	LSIL	HSIL	SCC	ADC
HPV-16	2.5	26.6	45.3	59.3	36.3
HPV-18	0.9	8.6	6.9	13.2	36.8
HPV-58	0.6	8.5	7.0	5.1	1.5
HPV-33	0.5	7.6	7.3	4.9	2.2
HPV-45	0.4	4.9	2.3	4.4	5.2
HPV-31	0.7	11.7	8.6	4.0	2.3
HPV-52	0.6	8.8	5.1	3.6	1.2
HPV-35		5.9	3.8	1.9	0.6

Prevalence of the 8 most carcinogenic HPV types in various lesions.

Figure 5   Distribution of the 8 most carcinogenic HPV types in normal samples, LG-SIL, HG-SIL and SCC/ADC cervical carcinomas [compiled with data from (27–30)].
Some of the tested samples contain multiple HPV types.
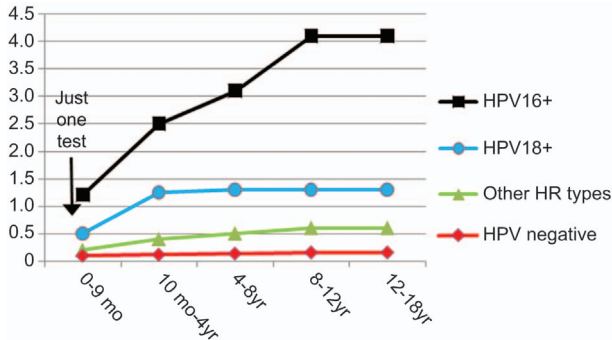
Figure 6 Significant cumulative risk differences for cervical cancer for a time period over 16 years between HPV 16- or HPV18-infected women (typed at baseline), women infected by other HR types or HPV negative women (n = 20,810, adapted from M. Schiffman, 25th International Papillomavirus conference 2009, Malmo, Sweden).

(38–40). The vaccines have proven to be safe and wherever introduced successfully in school-based programs, prior to any exposure to the virus (e.g., in Australia with coverage of about 80%), they have already not only dramatically reduced genital warts (41) but also cervical, vaginal and vulvar precancerous lesions (42). However, in other areas, such as in European countries with different legislations and public perceptions, coverage differs substantially between them and efforts of public health systems must be intensified (43, 44). In some European countries, female-adult catch-up vaccinations are offered free of charge. In the third world, there is a debate on whether to vaccinate males as well in order to obtain herd immunity (40).

Value of molecular detection of HPV DNA/RNA/ proteins

As aforementioned, the molecular detection for HPV virus is already used:

a. As a tool for proper triage of patients with a suspicious or positive finding in the Pap test (referral to colposcopy and biopsy). It must be noted that in the evidence-based Medicine era, there is no place for an argument for the presence of HPV infection without any real proof. And the only objective proof is through the molecular test since all findings in the other related tests (Pap test, colposcopy image and biopsy) are only indications for the virus presence. It is known that the microscopic examination is prone to subjective errors and is as good as the examiners' experience. The value of the HPV molecular detection is more noticeable when there are contradicting results between the other three tests; a common situation. In this case, the molecular result clears the confusion. If the result is negative, the tested woman is relieved since the molecular test possesses high negative predictive value (NPV). If the result is positive and the typing result shows the presence of high-risk HPV type(s), the woman and the treating physician are proportionally alerted while if the type is low risk, they could reasonably expect that the infection is transient and no immediate and useless invasive therapeutic procedure is needed especially in LG-SILs (45).

b. As a test of cure to assess complete eradication of the virus 12 months after therapy (45) and for monitoring of potential recurrence in the following years.
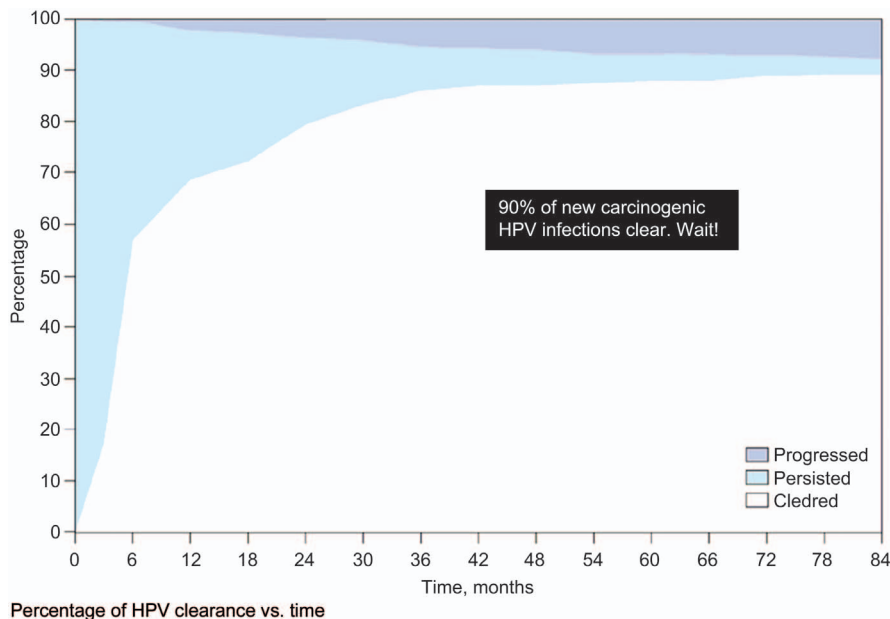   However, studies have shown the value of molecular HPV detection even in this third application:

Figure 7 Monitoring of HPV clearance percentage (or persistent infection or progression) over time in 10,000 women from Guanacaste, Costa Rica: even after for 7 years there are persistent infections with the same type that have not progressed (M. Schiffman, 25th International Papillomavirus conference 2009, Malmo, Sweden).

c. As a screening test instead of Pap test (46). In a recent study 10,000 women were randomized for screening with either Pap test or HPV DNA test (HC II). Not only the estimated clinical sensitivity for CIN2 detection was greater for the molecular test than the Pap test, as expected (95% vs. 55%), but also clinical specificity was not lagging significantly (94% vs. 97%). [If used in combination the sensitivity reached 100%] (47). Therefore, it has been proposed to use the molecular test as a first-line screening test and then in the case of positivity to triage according to the result of the Pap test for cellular abnormalities.

However, it has become evident that in developed countries, that have in place organized national screening programs for prevention of cervical cancer (this can only mean one thing: invitations for Pap tests in schedule every 3–5 years through a free public health system), the target of dramatic reduction of this disease has been achieved in the most efficient and cost-effective way. In this setup of efficient organized screening programs, the molecular test has only place after a positive or suspicious Pap test result. On the other hand, in the developing countries, there is a completely different situation: the proposed action is screening with the combination of either a self-testing device (48) or a visual colposcopic observation (aided with acetic acid or Lugol's iodine) and a fast molecular point of care (POC) test (such as either a DNA test with FastPath by Qiagen or a protein E6 latex test by Arbor Vita) and in case of positive findings, direct treatment by cryocoagulation (49). It is believed that such approaches combined with mass vaccination programs could eradicate HPV-related disease in the third world.

Evaluation of HPV molecular methods

In contrast with the beginning of the HPV molecular detection era some 20 years ago where only cumbersome methods, like Southern Blot and in situ hybridization, existed, nowadays there is a plethora of methods (for HPV DNA/RNA/protein) easier to perform and amenable also to automation: they either detect a certain number of HPV types in aggregate or they directly genotype. Some of the in-house methodologies initially developed in research laboratories have been evolved to standardized commercial kits with enhanced characteristics e.g., better analytical sensitivity and increased coverage for a wider range of HPV types. However, so far there is no established reference molecular method.

Their analytical evaluation is performed with: a) precision studies (repeatability-reproducibility), b) trueness and limit of detection (LOD) with the use of certified reference materials or commercial controls or plasmids with either cloned *HPV* genes or full genome sequences from WHO reference labs (HPV Labnet), c) comparison with other molecular methods, and most likely with the only FDA-approved method available till 2009; Hybrid capture (by Digene initially,

now Qiagen) and finally e) analytical specificity is examined in the presence of other viruses.

Their clinical evaluation is performed in women with carcinomas or in women with a Pap test result or even better with a colposcopy-guided biopsy result since this is considered the gold standard (however there have been even small in size CIN3s that have evaded this kind of diagnosis). Most of the existing molecular tests have excellent clinical sensitivities and negative predictive values however they suffer in clinical specificity and positive predictive value (PPV).

Possible reasons for the above observations and for the difficulties for objective comparison between various tests formats are the following:

a. So far, clinical studies have used different end points for clinical evaluation: e.g., either CIN2 or ≥CIN2 or CIN3 or cancer, therefore are not comparable.

b. Regarding CIN2, if used as an end point, there are tremendous differences between observers in the pathology report (borders are not always distinct between CIN1 and CIN2) and as a result many CIN2 results are not ''true'' (33, 50, 51).

c. HPV infections as aforementioned easily regress: more easily the CIN2 lesions e.g., a molecular test might have detected the presence of the most dangerous type HPV 16 correctly, however, this might not have being correlated at the time of the molecular result with a ''clinical phenotype CIN2 or 3'' due to favorable modifying genetic and/or environmental factors that assisted in the regression of the virus (or in the opposite direction: at this time period not being correlated but finally correlated after many-many years).

d. Finally, various molecular tests detect or genotype different panels of HPV types: others rather limited (e.g., the two dangerous HPV16/18), others five types (e.g., the most oncogenic 16/18/45/31/33) or 14 types [the most frequent high-risk types (HR)] or even many more and among them low risk types (LR) that could never cause CIN3, however, they can explain observed condylomata or low grade lesions detected e.g., in the Pap test. As the number of the detected types included in the panel of the test decreases, clinical sensitivity decreases also however clinical specificity and PPV are increased. Methods with novel biomarkers, such as HPV mRNA and proteins are working towards this goal in order to pinpoint a more advanced stage of infection with cellular deregulation and therefore, of lesser chance for regression and of higher clinical utility and specificity.

For the aforementioned reasons, we will not provide a direct head-to-head comparison of the methods regarding their analytical and clinical sensitivities and specificities but rather we will make an effort to classify them, provide information about the principles involved along with some characteristics and references in the next sections and in Table 2. Most widely-used commercial or in-house methods are mentioned in an historical perspective; however, some might have been missed accidentally.

Table 2   Molecular detection of HPV DNA, RNA or proteins [HR denotes high-risk HPV types and LR low risk; several control (ctrl) genes used for internal QC are mentioned].

Test (company)	Method	Targeted types	References
A. HPV DNA detection			
I. Screening for some HPV types in aggregate			
a) Hybrid capture II (ex Digene, now QIAGEN) FDA approved	RNA probes + chemiluminescent detection of bound RNA/DNA antibodies	-13 HR types and/or -5 LR types (No IC)	(52–55)
b) Cervista HPV test (Hologic) FDA approved	Fluorimetric invader third wave technology	-14 HR types (in 3 separate tubes) -histone2 ctrl	(56–58)
c) Amplicor HPV test (Roche) CE-IVD	Biotinylated MY-PCR (L1 gene) and colorimetric ELISA	13 HR types b-globin ctrl	(52, 59)
d) Full spectrum amplification and detection (GenoID) CE-IVD	Biotinylated L1F/L1R-PCR (L1 gene) and colorimetric ELISA	46 HR+LR types b-globin ctrl	(60)
e) In-house primer sets: -MY, GP, GP+, SPF10 -CP, pU	Consensus PCR L1 gene E1 gene, E6/7 gene	>35 HR+LR types	(61–63) (7, 64, 65)
II. HPV Typing (better genotyping)			
IIA. Full genotyping			
i) In-house MY-PCR (L1 gene) + RFLP (66, 67)			
ii) Line blots			
a) In-house	GP+ PCR + reverse line blot or EIA		(68)
b) Linear array genotyping test (Roche) CE-IVD	PGMY- PCR (L1 gene) + reverse line blot	37 HR+LR types + ctrl	(69–73)
c) INNO-LiPa HPV Genotyping extra (DDL-Innogenetics)	SPF10 PCR (L1 gene)+reverse line blot	28 HR+LR types + ctrl	(74)
d) Ampliquality HPV type (AB Analitica) CE-IVD	L1-PCR+reverse line blot	29 HR+LR types	(75)
iii) Microarrays			
a) Clart2 clinical arrays HPV2 (Genomica) CE-IVD	MY-PCR (L1 gene)+array hybridization	35 HR+LR types + b-globin ctrl	(76)
b) Papillocheck HPV (Greiner bio-one)	PCR (E1 gene) +array hybridization	24 HR+LR types +ADAT1 ctrl	(77)
c) Gene square HPV (Kurabo)	Multiplex SSP-PCR +array hybridization	23 HR+LR types +G3PDH ctrl	(78)
d) Easy chip HPV (King car)	MY11/GP6+ PCR +array hybridization	39 types	(79)
e) PANArray (Panagene)	array hybridization (with PNA probes)	19 HR+ 13 LR types+2 b-globin ctrl	(80)
f) APEX array (Genorama)	E7-multiplex PCR, hybridization in an array, primer extension	19 HR types+2 b-globin ctrl	(81)
iv) Liquid Arrays: –Multiplex HPV genotyping Kit (Multimetrix) –Genosearch (MBL)	GP+ PCR or type-specific PCR and hybridization in luminex bead array	24 HR+LR types + b-globin ctrl 31 types	(82, 83)
v) -fHPV typing (Molgentix-genomed) –Seeplex HPV 18 plex (Seegene)	QF-PCR (E6 and E7 genes) and Fragment analysis in capillary electrophoresis	15 HR+LR types Human STR IC 13 HR+5LR types + IC	
vi) MassArray HPV (sequenom)	Multiplex PCR in L1 gene-extension and detection in MALDI-TOF mass spectroscopy	14 HR HPV types	(84, 85)
IIB. Partial genotyping			
a) Cobas 4800 HPV (Roche)	Real-time PCR 4 channels	-12 HR types -HPV 16 -HPV18 -b-globin ctrl	(86)

(Table 2 continued)

Test (company)	Method	Targeted types	References
b) Abbott m2000 Real time high-risk HPV test	Real-time PCR 4 channels	-12 HR types -HPV 16 -HPV18 -b-globin ctrl	(87)
c) Cervista HPV 16/18 (Hologic) FDA approved	Fluorimetric invader third wave technology	-HPV 16 -HPV18 -histone2 ctrl	(56, 58)
d) Real time PCR HPV assay -GenoID -SACACE	Real time PCR with molecular beacons 4 channels (for ABI)	-HPV16/18 -HPV 6/11 -rest HR types -IC	(88)
B. HPV E6/E7 mRNA detection			
a) Nuclisens HPV (Biomerieux) previously pre-tect proofer (Norchip) CE-IVD	NASBA	Full transcript five types in three tubes: -HPV 16/U1 ctrl -HPV 33/45 -HPV18/31	(89–91)
b) APTIMA (GenProbe)	TMA	14 HR types (aggregate) Full transcript	(76, 92–94)
c) HPV onco tect (incellDx)	Flow cytometry-FISH CK(-)/CD16(-)/E6/7 mRNA(+) cells	All HR types Full transcript	(95)
d) In house nested	RT-PCR and gel electrophoresis	Full and spliced transcript	(96)
C. HPV protein detection			
I. Viral HPV proteins			
a) Cytoactiv (Cytoimmun)	L1 protein IHC	HR types	(97)
b) In house	E4 protein IHC	All types	(9)
II. Host human proteins			
a) CINtec histology CINtec cytology CINtec ELISA (mtm laboratories)	i) p16INK4A IHC ii) p16INK4A ICC iii) p16INK4A ELISA		(15, 92, 93, 98–100)
b) ProEx C (BD)	TopoII2a/MCM2 IHC		(101)
D. HPV novel biomarkers (Research in house tests)			
a) Viral integration	PCR, MLPA		(102–104)
b) Viral load	Real time-QPCR		(102, 103, 105)
c) Epigenetic markers (HPV and host)	Methylation-specific PCR, bisulfite Sequencing etc		(106–110)
d) HPV16/18 variants	DNA sequencing		(35)

HPV DNA detection methods

In-house PCR methods were the first that have been designed with degenerate or consensus primers like MY09/11 (61) GP 5/6, GP5+/6+ (62), SPF10 (63) targeting the highly conserved HPV DNA sequences in L1 gene or CP I/IIG (64, 111) in E1 gene and pU (7, 65) in E6/7 genes. Their goal was to detect in aggregate the majority of HPV types known up to that period under less stringent amplification conditions. Strengths and weaknesses of the above systems related with the detection of particular HPV types in the setup of single or multiple-type HPV infection have been described in the literature (64, 111, 112). The use of biotinylated and optimized MY primers allowed the detection of the corresponding amplicon for HR HPV types in a colorimetric ELISA format and led to the introduction of the successful

CE-IVD Amplicor by Roche, amenable to automation in Cobas equipment (52, 59). An analogous effort was followed also by Genoid with a properly optimized primer set in the L1 most conserved area to detect the majority of HPV types (60) (Table 2. A.I.c,d,e).

Primer sets MY, GP+ and SPF10 proved to work very well and were the basis for HPV full genotyping in post-PCR procedures either simple ones like restriction fragment length polymorphism (RFLP) (66, 67) or more complicated like those based on hybridization of the amplicon with specific oligonucleotides either placed in a line blot (paper strip) or spotted in a microarray format. Reverse line blots were developed either in-house with the GP+ primer set (68) or commercially: after replacing the degenerate MY set with a pool of specific upstream and downstream primers called PGMY (69) the system was evaluated (70, 71) and intro-

duced successfully by Roche as Linear Array, also a CE-IVD reagent (72, 73). The same occurred with the SPF10 short amplicon that was later on incorporated in the widely used INNO-LiPa assay by Innogenetics (74). The range of the detected HPV types of the above systems along with another recently introduced reagent kit from Analitica (75) are described in Table 2. A.IIA.ii. The introduction of the commercial microarray systems soon followed: companies used also additional oligos as hybridization controls and for orientation purposes. Popular systems include Clart2 from Genomica (76), Papillocheck from Greiner (77) and Easy chip from King Car (78) that are based on the hybridization of the product of a single PCR (MY or other) but also others exist that use multiplex PCR (79–81) as described in Table 2. A.IIA.iii.

In the meantime, Hybrid capture -and especially its latest version HC II- has become the most wide spread method in labs and in clinical studies worldwide since it is simple, less technically demanding, less prone to contamination but also because it is FDA approved. It is a non-PCR isothermal method that uses RNA probes for hybridization to denatured HPV DNA and then is followed by detection of RNA/DNA hybrids with antibodies coupled with an enzyme reacting to a chemiluminescent substrate. The method has been used in numerous method comparisons (as the only FDA approved till 2009) and performs rather well in detecting, in aggregate, 13 HR types with its HR probe cocktail, despite the facts that it cross reacts also with some LR types and that it does not control for DNA quantity and quality (52–55, 78). Lately, another FDA approved method was introduced from Hologic (Cervista) that works also with a non-PCR, isothermal, well proven in the literature, fluorometric Invader Third wave technology (Table 2. A.I.a,b). It detects 14 HR types in three separate tubes and cares for a control of DNA quality (56, 57), however, reports have already appeared claiming analytical problems with this method (58). At this point, it has to be stressed that the reverse line blot and microarray systems described in the previous paragraph are also plunged with analytical issues and especially with false-positive results in the multiple-type HPV infection setup as various method comparison manuscripts or interlaboratory WHO HPV Labnet comparisons have shown (72, 76, 78, 113).

In the section describing the clinical course of an HPV infection, it was made clear that HPV types 16 and 18 stand above all other types in terms of carcinogenic potential and persistence. This is the reason that both companies with FDA approved reagents have or are developing kits for their special detection (QIAGEN 16/18/45 and Cervista 16/18). Also big manufacturers like Roche and Abbott, but others also (e.g., Genoid, Sacace), have started to market kits performing partial HPV genotyping (Table 2. IIB) with the use of the robust real-time PCR technique. They usually exploit all four or five channels of the real-time equipment and genotype with specific probes: with a probe separately for HPV16 and 18, with a general probe for the aggregate of the other HR types and finally with a probe for a human housekeeping (reference) control gene (86–88). These partial HPV genotyping methods are also amenable to automation and high-throughput analysis and are expected to expand significantly.

Other efforts that deserve further attention also belong to the area of HPV full genotyping (Table 2. IIA.iv,v,vi): a) application of the Luminex technology looks very promising because the availability of the spectrally-resolved beads that could be coupled with HPV type-specific oligos and hybridize to e.g., a biotinylated PCR product exceeds the number of types in detection (82, 83), b) multiplex PCR with fluorescently-labeled primers and fragment analysis in capillary electrophoresis (e.g., in a Sequencing DNA analyzer) and finally c) an even more impressive but also expensive technology: multiplex PCRs fragmented and resolved in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy like in Sequenom's Mass Array HPV test (84, 85).

HPV E6/7 mRNA and protein detection methods

Molecular methods detecting HPV E6/7 mRNA and/or human p16INK4A protein are aiming at more advanced infections; those producing cellular deregulation although boundaries are not clear and infections showing high levels of these biomarkers could very well regress in the immediate next time period. So far, IARC has not issued a guideline for their routine application (25), so at the moment they should be only used as additional tools for clinical decisions. Their sole application would also create confusion in relation with Pap test or colposcopy results. The most widely used commercial method is Nuclisens HPV that by using isothermal NASBA, detects the E6/7 transcript of the five most dangerous types in three separate tubes and checks also for RNA quality (89–91). Another recently launched kit uses isothermal TMA technique (APTIMA) to detect in aggregate the E6/7 transcript in 14 HR HPV types (76, 92–94). In this format, results would agree more with those of the Pap test; however, the clinical specificity for progression would diminish in comparison to NucliSens HPV. Table 2B is supplemented also with an in-house nested RT-PCR method for detecting both the full and spliced E6/7 transcript (96) and with an elegant flow-cytometric way of detecting E6/7 HPV mRNA in cervical cells with a consensus probe labeled with fluorescein, after gate exclusion of cytokeratin and CD16-expressing endocervical cells and leukocytes (95).

Host p16INK4A protein is a surrogate marker of HPV E7 expression and as it can be seen in Table 2C, the available monoclonal antibody is used in three different formats depending on the testing matrix: tissue slides, cells on a cytospin or cell extracts. It is a method already extensively tested in the same context for higher clinical specificity as above for E6/7 mRNA detection (15, 92, 93, 98–100). Maybe the future of this method lies with its coupling with immunohistochemistry of a proliferation marker, such as ki-67 and with assistance from an automated image analysis system (von Knebel Doeberitz, personal communication). Other efforts include detection of host topoisomerase II2a/MCM2 IHC (101) or, in the opposite direction of the aforemen-

tioned, looking at good prognostic markers: HPV proteins indicative of a productive infection like L1 (97) or E4 (9).

HPV novel biomarkers

Novel biomarkers are urgently needed to pinpoint the low- or high-grade lesions that will progress beyond any doubt. These biomarkers should be detected early enough so that appropriate treatment in a timely fashion could prevent cancer. As seen in Table 2D, research groups have developed methods for: a) detecting HPV viral integration with real-time PCR or elegant MLPA techniques (102–104) and for b) quantification of viral loads especially for the carcinogenic HPV types 16/18 (102, 103, 105). Epigenetics is another hot area of research with groups detecting promoter CpG island hypermethylation either in *L1* or *E2 HPV* genes (106–108) or in human host genes like *DAPK, TIMP3, ER, PTEN, RASSF1A, FHIT* etc (109, 110) by various techniques, such as methylation-specific PCR, pyrosequencing of bisulfite-treated DNA etc. Finally, looking at HPV 16/18 variants with DNA sequencing could yield interesting conclusions (35, 37).

Points to remember

Furthermore, one should not forget that the two key points to improve our effort to battle HPV-related cancer are the following:

a. The treating physician should understand fully what the molecular test detects and which are its limitations and
b. The clinical laboratory responsible for the molecular detection should: i) verify or validate its methods with the use of available certified reference materials (like NIBSC HPV 16/18) and/or commercially available controls (Optiqual Acrometrix, Accurun BBI, Advanced Biotechnologies etc.), ii) perform regularly internal QC and iii) participate successfully in EQAs (external quality assessments) [available from Instand www.instandev. de, EHEQAS www.mendelcenter.org/eheqas and CAP www.cap.org] and/or in WHO HPV LabNet proficiency panels (useful observations have already being published (113, 114) regarding different HPV type detection efficiencies). The above are also requirements for the ISO15189 accreditation standard which is the suitable standard with which these laboratories should comply.

Expert opinion

As the importance of HPV 16/18 genotyping is widely now recognized, methods detecting HPV DNA in aggregate will become less common. So far methods detecting or genotyping these two most dangerous types are performing well even at low viral loads as the interlaboratory comparisons have shown. However, methods providing partial or full genotyping for the other HPV types have to better standardize with proper reference materials in order to avoid false-positives. Selection of the type panel for detection or genotyping has to be better refined: the usual 14-HPV type setup followed by many methods is including types, such as HPV 39 and HPV 66 with either less frequency or debatable high-risk; therefore clinical specificity is reduced. Correspondingly for HPV RNA detection the five-type format should be rather expanded to include all types providing clinical significant lesions (e.g., all eight types being responsible for the majority of cervical cancer). Methods detecting RNA/protein biomarkers will be increasingly used, at least initially as adjunct tools. Finally, a novel ideal biomarker or a combination of protein markers of cellular deregulation is desperately needed that would clearly differentiate between the HG-SIL that regresses and the HG-SIL that progresses to cancer.

Outlook

With the advent of the two effective and safe HPV vaccines, it is expected that the burden of disease will reduce and apparently HPV types 16/18 will become substantially less frequent worldwide. So far, there is no indication for type replacement by other HPV types due to the low mutation rate of the virus throughout the ages. Maybe methods partially genotyping for HPV 16/18 will be not needed in the future. However, proper monitoring with the combination of Pap test and molecular detection will still have to be in place, most probably at a reduced frequency. Novel vaccination approaches either by including more types (the big eight) in the L1 VLP cocktail or by including the L2 antigen in the VLPs, which is less polymorphic, will probably eradicate the majority of the HPV types. Till then, much needed E6/E7 therapeutic vaccines or si-RNA therapy arising from research will clearly benefit those suffering from any HPV-related malignancy.

Highlights

- HPV is responsible for the majority of cervical cancers but also for a significant proportion of other anogenital cancers plus cancers in other remote sites.
- Diagnostic algorithms include Pap test (the best cost-effective screening tool available) and also nowadays molecular HPV tests as the best combination for prevention of cervical cancer.
- Most HPV infections clear within 2 years.
- Among the plethora of HPV types, types 16/18 stand above all the other types for their capacity to both transform and persist.
- Safe and effective preventive vaccination against HPV 16/18 exists and has to be implemented forcefully in order to increase coverage especially in schoolgirl populations where prior exposure to the virus is not likely.

- Molecular detection of HPV DNA or RNA or protein even with its limitation is a powerful tool when evaluated properly in terms of clinical sensitivity and specificity.
- Method validation with proper reference materials and lab accreditation are necessary, they can guarantee for the analytical value of molecular HPV detection tests.

Conflict of interest statement

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