Human papillomaviruses and cervical cancer

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Abstract

The central causal role of the human papillomaviruses (HPV) in cervical cancer has been well established. The association is present in almost all cases of cervical cancer in the world. The ability of HPV oncogenes to interact with host cell cycle and growth regulators, as well as to elude the immune response and protect infected cells from apoptosis, can promote the persistence of the infection and the development of cervical carcinoma. However, only a small number of HPV infections progress to cancer, while the majority are eliminated by the host, suggesting that the presence of other cofactors is important in carcinogenesis. Several diagnostic tools for HPV infection have been developed, from basic cervical cytology screening to signal-amplified hybridization and the most recent real-time polymerase chain reaction techniques. HPV testing has been recently introduced in clinical practice and plays an increasing role in the management of women with abnormal cervical cytological findings. This article provides review of the recent literature on HPV infection epidemiology, pathogenesis and diagnosis.

Keywords: *Human papillomavirus, cervical cancer, pathogenesis, diagnosis.*

Introduction

Genital infection with human papillomaviruses (HPV) is one of the most common sexually transmitted conditions. The Centers for Disease Control estimates that at least half of all sexually active individuals will acquire HPV at some point in their lives, whereas at least 80% of women will acquire an HPV infection by age 50[5].

Based on genomic differences detected by DNA sequencing, more than 200 HPV types have been recognized. Eighty-five HPV genotypes are well characterized. HPV can be grouped into high-risk (HR) types and low-risk (LR) types, based on their association with cervical cancer and precancerous lesions. High-risk, oncogenic HPV types (including HPV16 and HPV18) are associated with 99.7% of all cervical cancers, as well as low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and abnormal Papanicolaou (Pap) test results, which carry significant health care costs and psychosocial morbidity. Low-risk HPV types (HPV6 and HPV11) are responsible for additional abnormal Pap test results, as well as almost all cases of genital warts[5].

Today, it is well established that infection with HR-HPV is the central causal factor in cervical cancer[34].

Basic virology

Papillomaviruses are members of the *Papovaviridae* family. HPV is a small nonenveloped virus. Its capsid has an icosahedral structure composed of 72 capsomers which contain at least two capsid proteins, L1 and L2. The genome of the HPV (*Figure 1*) consists of a single closed double-stranded DNA molecule of approximately 8 kb in length and is functionally organized in three regions: the long control region (LCR), the early gene and the late gene regions.

The LCR, also named the noncoding region, or the upper regulatory region is a noncoding upstream regulatory region, and has the highest degree of variation in the viral genome[9]. It possesses numerous binding sites for many repressors and activators of transcription, suggesting that it may play a part in determining the range of hosts for specific HPV types[26].

The early gene region contains the open reading frames (ORFs) of the proteins E1, E2, E3, E4, E5, E6 and E7.

E1 and E2 encode proteins that are vital for extrachromosomal DNA replication and for the completion of the virus life cycle. E2 also encodes two proteins: one that increases and one that decreases the transcription of the early region[26]. The E4 protein has an important role for the maturation and replication of the virus. It is expressed when virions are assembled. The E5 protein interacts with various transmembrane proteins like the receptors of several growth factors. The E6 and E7 are oncoproteins that allow replication of the virus and the immortalization and transformation of the cell that hosts the HPV DNA[26].

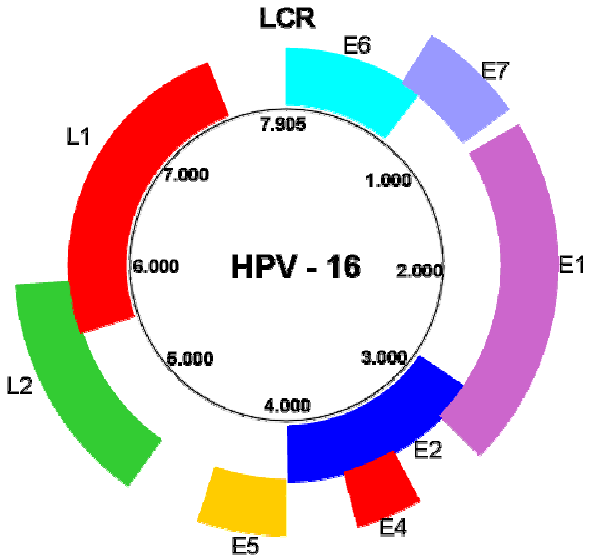

Figure 1. Schematic representation of the circular HPV genome[12]

The late region encodes for the L1 and L2 structural proteins for the viral capsid during the late stages of the virion assembly. The protein encoded by L1 is highly conserved among different papillomavirus species. The minor capsid protein encoded by L2 has more sequence variations than that of the L1 protein.

Epidemiology

Transmission of HPV occurs primarily by skin-to-skin contact. Epidemiologic studies clearly indicate that the risk of contracting genital HPV infection and cervical cancer is influenced by sexual activity[9].

Studies among virgin women who began their sexual life strongly confirm the sexually transmitted nature of HPV infection. Many cohort studies around the world among young women who were initially HPV negative have shown that the cumulative incidence of HPV infection exceeded 40% after 3 years, and the cumulative incidence is higher for high risk types than for low-risk types[5, 34]. It is noteworthy that the sensitivity of the methods used in assessing the viral load influences the incidence and prevalence rates of the infection. PCR has a higher sensitivity resulting in higher reported rates.

The probability of HPV transmission per sexual act is very high, several-fold higher than that for other viral sexually transmitted infections, such as human immunodeficiency virus or herpes simplex virus 2[34].

Condoms, although effective at preventing the spread of many other sexually transmitted infections, may not prevent all HPV infections since HPV can be transmitted by contact with infected labial, scrotal, or anal tissues that are not protected by a condom. A meta-analysis of more than 20 trials investigating the role of condoms in HPV transmission and the development of clinical complications concluded that, although condoms do not protect against cervical infection, they may offer some protection against HPV associated disease. Specifically, although there is conflicting evidence as to whether condom use protects against CIN 2/3, condoms may protect against cervical cancer. Condom use also seems to protect against some clinical sequelae of HPV infection and aids clearance of infection and clinical symptoms, even if it does not prevent primary infection[5].

In addition to the peno-vaginal intercourse route, HPV is also easily transmitted by other sexual practices, such as oral sex, peno-anal intercourse, digital vaginal sex, and use of insertive sex toys[34].

Epidemiological studies investigating risk factors for HPV have shown that the key risk factors for acquisition of HPV are the number of sexual partners, the age at which sexual intercourse was initiated, and the likelihood that each of her sexual partners was an HPV carrier. In the last years it has been proved that male circumcision protected men from being HPV carriers and their wives from developing cervical cancer[8].

Other risk factors for HPV infection include young age, other sexually transmitted infections, long-term oral contraceptive use, smoking, immunosuppression, multiparity and host genetics (HLA polymorphism) [23,26,34]. Women less than 30 years of age are significantly more likely to harbour HPV sequences, particularly HPV16[18]. *Herpes simplex* virus and *Chlamydia trachomatis* are each associated with 2-fold risk for the development of cervical cancer in HPV infected women[5].

An important emerging factor in the development of cervical neoplasia is the role of HPV variants. HPV variants differ in biological and chemical properties and pathogenicity[9].

Studies have shown that infections with multiple types of HPV can occur. The presence of multiple HPV genotypes tended to increase with the severity of cervical disease. The majority of multiple infections contain two HPV genotypes, but samples with three, four, or five genotypes were also seen[9].

In this context, recombination could be a significant issue and new recombinant types could be currently being generated. Recently, Angulo and Carvajal-Rodriguez[1] used a model-based population genetic approach to evidence that recombination exists in alpha papillomaviruses. They have detected significant recombination signal in the genes E6, E7, L2 and L1 at different groups, and importantly within the high-risk type HPV16. They found that the gene with recombination in most of the groups is L2 but the highest values were detected in L1 and E6. Gene E7 was recombinant only within the HPV16 type. Since E6 and E7 are involved in the oncogenic effect of HPV, variations in their sequences may have functional importance[9]. Recombination is an important evolutionary mechanism that could have high impact in both drug resistance due to genetic variation and in vaccine development[1].

Clinical manifestations of the infection

Sexually transmitted HPV infection can lead to three types of clinical condition[9]:

The first one is *condyloma acuminata* (genital warts) in both men and women. Anogenital warts are usually associated with low-risk HPV 6 and 11, do not lead to cancer and are generally asymptomatic. Their evolution can be thoward spontaneous resolution in 3 or 4 months, they can remain the same or increse in size and number. If warts are red-

brown in colour, a biopsy should be performed since they can be Bowenoid papulosis, which is due to HPV 16 or 18 and presents intraepithelial neoplasia[9].

The second condition is the asymptomatic status, when no or few clinical signs are present. The cytology of the infected areas remains normal. HPV DNA can be detected in 10% of citologically normal women. The HPV DNA detected is mainly of low-risk types.

The third condition is active infection with high-risk HPV types which can produce changes at cellular level and can lead to intraepithelial neoplasia. These infections can lead to cervical cancer.

Pathogenesis

Natural history of cervical cancer

The natural history of cervical cancer begins as a slow process of disruption of the normal maturation of the transformation zone epithelium of the uterine cervix near its squamo-columnar junction[34]. The abnormal changes initially limited to the cervical epithelium are referred to as dysplasia (or as cervical intraepithelial neoplasia, CIN, or as squamous intraepithelial lesion, SIL). This preinvasive condition is invariably asymptomatic and can be discovered only through cytological examination using the Papanicolaou (Pap) smear and confirmed by colposcopic examination and biopsy. If left untreated, the low grade lesions may eventually turn into cervical carcinoma in situ (CIS) and then become invasive. This process may take longer than a decade, but it occurs in an important proportion of patients with CIS. As an invasive cancer, the lesion will progress, reach lymph and blood vessels and become metastatic.

The major steps known to be necessary in cervical carcinogenesis include HR-HPV infection, persistence of that infection over a certain period of time, progression to precancerous lesions, and, eventually, invasion[34]. HPV infects the basal cells of stratified squamous epithelium and stimulates cellular proliferation. Other cell types appear to be relatively resistant[9]. Infected cells display a wide range of alterations. All precancerous lesions induced by HPV are reversible (*Figure 2*).

The vast majority of HPV infections are transient and only few of them become persistent[34].

Many studies have been performed in order to determine the risk for cervical precancer and cancer attributable to infection with HPV.

ALTS (ASCUS LSIL Triage Study)[11], a wide randomized, multicenter clinical trial determined the 2-year cumulative absolute risks for ≥ CIN3 attributable to baseline-detected HPV16 and other oncogenic HPV infection for more than 5000 women with equivocal or mild cytologic cervical abnormalities (ASCUS or LSIL Pap smear). HPV infection was detected using the Hibrid Capture 2 test (HC2; Digene Corporation). The baseline prevalences of HPV16 in women with ASCUS or LSIL cytology were 14.9% and 21.1%, respectively. Women with ASCUS cytology who were HPV16

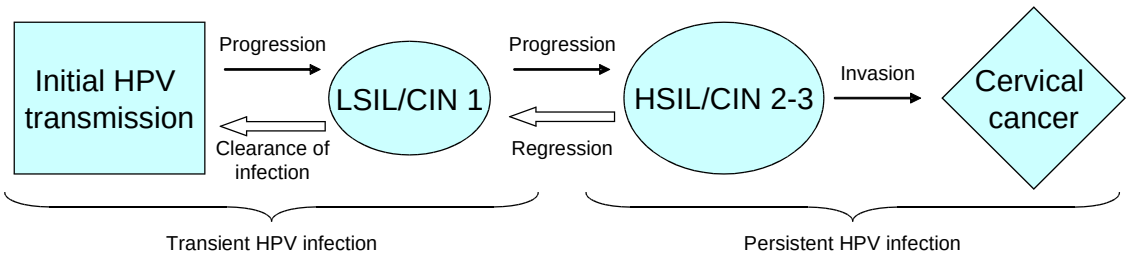

Figure 2. Cervical carcinogenesis, from HPV infection to cervical cancer[23]

DNA positive at baseline had a 2-year cumulative absolute risk for ≥ CIN3 of 32.5% and those with LSIL had a 2-year cumulative absolute risk of 39.1%. Women with ASCUS who were positive by HC2 for oncogenic HPV types other than HPV16 had an 8.4% risk for ≥ CIN3, which was similar to the risk posed by having ASCUS (risk = 8.8%) without knowledge of the oncogenic HPV DNA status. Women with LSIL who were positive by HC2 for other oncogenic HPV types combined had a 9.9% 2-year risk for ≥ CIN3, which was less than the risk posed by having LSIL (risk = 15.0%) without knowledge of the oncogenic HPV DNA status. Together, women with ASCUS or LSIL who were HPV16-positive had the highest 2-year risk for ≥ CIN3 compared with women who were HPV-negative (odds ratio [OR] = 38; p <0.001), fivefold greater than the increased risk in women who were positive for other oncogenic HPV types (OR = 7.2, p <0.001).

The prospective Kaiser Permanente cohort study[21] of over 20000 women found that the 10-year cumulative incidence rates of ≥ CIN3 were 17.2% among HPV16-positive women and 13.6% among HPV18-positive (HPV16-negative) women, but only 3.0% among HC2-positive women negative for HPV16 or HPV18. The 10-year cumulative incidence among HC2-negative women was 0.8%. In women aged 30 years and older, the 10-year cumulative incidences of ≥ CIN3 among HPV16- and 18-positive women were 20% and 15%, respectively. Stratification by age (<30 years versus ≥ 30 years) demonstrated the high risks associated with HPV16 and HPV18 in both younger and older women. Because of the study limitations due to aggressive treatment and censoring, it is possible that its findings underestimate the true cumulative incidence rates.

HPV types and cervical neoplasia

Human papillomaviruses are generally classified into high-risk and low-risk HPV according to their potential to induce malignant transformation. LR-HPV include types 6, 11, 42, 43, and 44. HR-HPV include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70[9]. HPV 31, 33, 35, 51 and 52 are sometimes regarded as “intermediate risks” because they are more common in mild or severe dysplastic lesions than in carcinomas[26]. The most common HR types are HPV 16, 18, 31, 33, and 45. The most commonly found LR types are HPV 6 and 11[33].

Infection with LR-HPV types, such as HPV 6 and 11, can cause benign lesions of the anogenital areas, as well as a large proportion of lowgrade squamous intraepithelial lesions of the cervix[34].

The association between HR-HPV and cervical cancer is supported by strong epidemiological evidence. About 15 HPV types are involved in over 95% of the cervical cancer cases[8]. HPV-16 is the most prevalent HR-HPV, and is present in approximately 54% of cervical tumor specimens worldwide, whereas HPV-18 is associated with approximately 17% of cervical cancers[34]. All types of high-risk HPV can induce malignancy even when they are present in low levels[9].

However, HPV has also been detected in a wide range (3-30%) of asymptomatic controls, indicating that other events are required for development of neoplasia, such as viral persistence and/or altered expression of viral genes. It is clear that only a small minority of persistent HPV infections progress to cancer[4]. The central causal role in cervical carcinogenesis of the high-risk oncogenic (HR-) HPV genotypes, such as HPV16, has been established as a likely but not sufficient cause of virtually all cases of cervical cancer worldwide[34].

Bosch et al[8] have recently reviewed the epidemiological data on the association of HPV and cervical cancer. They discussed the causality criteria proposed by Hill in human cancer research. Briefly, these are: (*) strength, (*) consistency, (*) specificity, (*) temporality, (*) bi-

ological gradient, (*) plausibility, (*) coherence, (*) experimental evidence, and (*) analogy.

The **strength** of association is discussed by examining the magnitude of the relative risk (RR) or the odds ratio (OR) in case-control studies. Results of the IARC (International Agency for Research on Cancer) multicentre case-control study on invasive cervical cancer have reported ORs for cervical cancer in the range of 50 to 100 fold for HPV DNA. ORs for specific associations (such as HPV-16 and squamous cell cancer and HPV-18 and cervical adenocarcinomas) range between 100 and 900.

The association between HPV and cervical cancer is **consistent** among over 50 studies conducted in different countries with high risk and low risk for cervical cancer.

The association of type specific HPV DNA and cervical cancer has been found to be significantly different from random. Systematic patterns of HPV type and cervical cancer histology suggest a fair degree of **specificity**.

The **temporality** criterion is also satisfied: HPV infections precede cervical precancerous lesions and cervical cancer by a substantial number of years.

The **biological gradient** criterion refers to the presence of a dose–response curve indicating that the magnitude of the exposure is related to the risk of disease. It has been thought that HPV viral load correlates with the severity of the disease. Studies based on type-specific real time PCR for HR and LR HPV have demonstrated that HPV16 can reach higher viral loads than other HPV types. Only HPV16 increased viral load seems to correlate with increased severity of cervical disease[9]. In one study, high viral loads could be detected up to 13 years before the diagnosis of cervical cancer. Women with high viral loads for HPV16 had a 30 fold greater risk of developing cervical cancer than did HPV negative women. A related paper using the same population showed that the 20% of the population with the highest viral loads for HPV16 had a 60 fold higher risk of developing carcinoma in situ when compared with HPV negative women. Of importance for clinical and screening purposes, another study confirmed that high viral loads predicted cervical lesions and, more interestingly, that the reduction of viral load or clearance of viral DNA in repeated visits predicted regression of CIN lesions to normalcy[8,35]. On the other hand, a large prospective study using quantitative hybrid capture did not find viral load to be a determinant of risk of future CIN 3. More research is needed to validate these methods and the results need to be extended and confirmed in clinical studies[8].

The association of HPV DNA in cervical specimens and cervical cancer is **biologically plausible** and **coherent** with laboratory evidence of the oncogenic potential of high risk type HPV genes (E6 and E7 viral oncogenes)[4,8].

Experimental evidence shows that species specific papillomaviruses (PV) induce papillomas and cancers in the susceptible host. There are animal models of PV infections that induce warts and carcinoma in the skin, mucosa, and the digestive tract.

The HPV and cervical cancer model is **analogous** to many other examples of PV-induced papillomas and carcinomas and cancers caused by other viruses[8].

HPV genome integration and viral oncogene expression

It is assumed that the HPV replication cycle begins with entry of the virus into the cells of the *stratum germinativum* (basal layer) of the epithelium[9]. Establishment is tied to the tissue proliferative activity of epithelial cells and, in the case of extensive tissue repair, the viral infection can become widely disseminated[8].

HPV employs host cell factors to regulate viral transcription and replication. Once inside the host cell, HPV DNA replicates as the basal cells differentiate and progress to the sur-

face of the epithelium. In the basal layers, viral replication is considered to be nonproductive and the virus establishes itself as a low-copy-number episome. In the differentiated keratinocytes of the suprabasal layers of the epithelium, the virus amplifies its DNA to high copy number, synthesizes capsid proteins, and causes viral assembly to occur[9].

Normally, cell growth is regulated by two cellular proteins, the tumor suppressor protein, p53, and the retinoblastoma gene product, pRB[9].

p53 protein is activated upon phosphorylation via DNA damage sensing proteins. Activated p53 stops the cell cycle in the G phase as a result of direct stimulation of p21cip1 by this molecule[8]. p53 also participates in DNA repair[9]. Alternatively, in the case of major DNA damage or high amounts of viral replication, p53 may activate an apoptotic pathway[8].

The HPV E6 gene product binds to p53 and leads to its rapid degradation via a cellular ubiquitin ligase[9]. Thus, HPV mediated loss of p53 function decreases the cell's susceptibility to apoptosis, and promotes cellular survival after DNA damage or development of genomic instability, allowing accumulation of genetic changes that may drive further progression to malignancy[4]. Unlike in other cancers, in most of the cervical cancer cases, p53 is inhibited and not mutated[9].

Low-risk HPV E6 proteins do not bind p53 at detectable levels and have no effect on p53 stability in vitro[9].

A number of proteins other than p53 are targeted by E6. These include proteins involved in the regulation of transcription and DNA replication; proteins involved in apoptosis and immune evasion; proteins involved in epithelial organization and differentiation; proteins involved in cell-cell adhesion, polarity, and proliferation control that contain a PDZ-binding motif; and proteins involved in DNA repair. So, E6 helps HPV interfere apoptotic pathways by both p53-dependent and -independent mechanisms[15].

Under normal conditions, pRB forms a complex with histone deacetylase (HDAC) and binds to the E2F transcription factor in the G1 phase of the cell cycle. This prevents E2F from transactivating genes that are necessary for proliferation until the cell enters the S phase. However, when E7 is expressed in cells, it binds to pRB and HDAC and relieves their repression of E2F, resulting in the constitutive activation of E2F-responsive genes. The actions of E7 cause the cell to reenter the S phase where cellular replication factors that are necessary of viral replication are activated.[15]. E7 also binds and activates cyclins, such as cyclin E, and cyclin complexes, such as p33–cyclin dependent kinase 2, which control progression through the cell cycle[8,9]. The outcome is stimulation of cellular DNA synthesis and cell proliferation[9]. Additionally, E7 can induce abnormal centrosome duplication and chromatin condensation possibly leading to chromosome instability[29].

HPV E7 proteins of both LR and HR types have an ability to promote unscheduled DNA replication in spinous cells[8], but the E7 protein from LR-HPV types binds pRB with decreased affinity[9].

Temporary functional abrogation of p53 and pRB by HPV E6 and E7 proteins is important for viral DNA replication in senescent or terminally differentiated (non-cycling) squamous epithelial cells[4]. Expression of E6 and E7 proteins of HR-HPV types can induce immortalization of cells through their inhibitory effects on the tumor suppressor proteins[6].

The E5 gene product induces an increase in mitogen-activated protein kinase activity, thereby enhancing cellular responses to growth and differentiation factors[9]. E5 protein appears to alter signaling from growth factor receptors for epidermal growth factor and platelet derived growth factor[4]. These effects result in continuous proliferation and delayed differentiation of the host cell[9]. E5 modulates signal transduction triggered by apoptotic stimuli from

immune effector cells and also interferes with MHC class I protein expression. It is possible that E5 interferes with the ability of the immune system to eliminate infected cells by impairing death receptor signaling[15]. This way, HPV is able to regulate the survival of infected cells in order to facilitate its replication cycle and thus ensure the production and spread of progeny[15]. E5 is highly expressed in basal cells of premalignant cervical lesions. This expression declines as cells differentiate and move toward the apical face of the epithelium whereas E6 and E7 expression increases. E5 is detected throughout all epithelium layers in high grade lesions such as CIN 3. In contrast, expression is restricted to layers closest to the basal cells in low grade lesions, implying that E5 expression may be limited to undifferentiated basal cells[30]. In the first study that identified viral tags in human SAGE (serial analysis of gene expression) libraries, Ashleen Shadeo et al[30] found a high expression of HPV16 E5 gene transcripts in CIN 3 specimens.

The E1 and E2 gene products are synthesized after the E5, E6 and E7. The E2 gene product is a DNA binding protein which blocks transcription of the E6 and E7 genes and permits the E1 gene product to bind to the LCR. This binding initiates replication of the viral genome as extrachromosomal elements in the S phase of the cell cycle[9]. HPV replicates in these cells to reach a low number of about 25–50 genomes/cell[8]. The down-regulation of E6 and E7 by E2 leads to the release of p53 and pRB proteins and the normal differentiation process of the host cell is allowed to continue[9].

Then, L1 and L2 genes are activated and viral particles are assembled into the nucleus. Maturation and release of papillomavirus particles are aided by the E4 gene product. The process does not seem to be cytolytic[9].

In benign lesions caused by HPV, viral DNA is located extrachromosomally in the nucleus. In high-grade intraepithelial neoplasias and cancers, HPV DNA is generally integrated into the host genome[9]. It is believed that a combination of persistent infection by HR strains along with the inability of the immune system to adequately clear the virus from infected cells are the main factors contributing to the integration of HPV genomes into the DNA of the host[15].

There is a consistent pattern of disruption of the circular viral DNA upon integration. The recombination event frequently occurs within the viral genes E1 or E2, sometimes causing focal deletions, allowing the overexpression of E6 and E7 genes, which leads to profound loss of function of p53 and pRB proteins[4]. As a consequence, the host cell accumulates more and more damaged DNA that cannot be repaired. Eventually, mutations accumulate that lead to fully transformed cancerous cells.[9]. The genetic analysis of cervical cancer progression has revealed that the acquisition of specific chromosomal aneuploidies appears to be a mandatory event. It has been shown that more than 85% of invasive cervical carcinomas carry specific genomic imbalances that result in copy number increases of chromosome arm 3q[19].

Wang-Johanning et al reported that copy number of both RNA and DNA of HPV E6 and E7 increased with increasing SIL grade[29]. Increased expression of E6 and E7 has been observed to lead to the malignant transformation of the host cells and to tumor formation[26]. HPV viral integration into the host genomic DNA is associated with progression from polyclonal to monoclonal status in CIN, and these events play a fundamental role in the progression from low-grade to high-grade cervical neoplasia (9.) Progression to cancer generally takes place over a period of 10 to 20 years. Some of the lesions may progress even more rapidly[9].

A recent study performed by Bahnassy et al[6] on the interaction between HPV oncogenic proteins and cell cycle regulatory genes concluded that aberrations involving $p27^{KIP1}$, cyclin E, CDK4, $p16^{INK4A}$ are early events in

HPV 16- and 18-associated cervical carcinoma, whereas cyclin D1 and p53 pathway abnormalities are late events. They investigated a number of samples ranging from CIN 1 to invasive squamous cell carcinoma, together with normal samples that served as controls. They found an increased expression of cyclin E, CDK4 and $p16^{INK4A}$ and a significant decrease of the expression of $p27^{KIP1}$ from normal cervical tissue to invasive squamous cervical carcinoma. They also found that, among these markers, p53, cyclin D1 and $p27^{KIP1}$ are independent prognostic factors that might help in predicting outcome of cervical cancer patients.

In addition to the effects of activated oncogenes and chromosome instability, potential mechanisms contributing to transformation include methylation of viral and cellular DNA, telomerase activation, and hormonal and immunogenetic factors[9].

An important step in immortalization of the cervical cells is related to telomeres. Normally, telomeres shorten every cell generation and once they reach a critical length the cells die. Telomere length is maintained by telomerase, which can stabilize and even lengthen telomeres, allowing cells to continue dividing. E6 can activate telomerase and additional cell mutations can then stabilise the telomeres and allow cells to continue the process of immortalization[8]. A recent study[19] provided evidence that the acquisition of specific chromosomal aneuploidies that result in a gain of the human telomerase gene (TERC) is associated with progression of premalignant dysplastic lesions of the uterine cervix.

HPV infection also induces alterations in cellular adhesion molecules.

Integrins are transmembrane glycoproteins important in interactions between basal epithelial cells and basement membranes. In neoplastic cervical epithelium, dysplastic cells express integrins throughout the full thickness of the epithelium in CIN 3[4].

Epithelial cadherin (E cadherin) is a cell-cell adhesion molecule that connects epithelial cells via homotypic, calcium dependent interactions. In normal cervix, E cadherin is expressed on the cell membrane of basal and parabasal cells. Cytoplasmic expression of E cadherin is present only in rare basal cells. In CIN, there is increased cytoplasmic expression, which is strongly correlated with the grade of the CIN lesion. Reduced membranous and increased cytoplasmic expression is seen in squamous carcinomas[4].

The intercellular adhesion molecule 1 (ICAM-1) plays an important role in many aspects of leukocyte function, including the adhesion of lymphocytes and monocytes to target cells, MHC-restricted antigen presentation and T cell-mediated cytotoxicity. In a study, Coleman et al investigated the expression of ICAM-1 in squamous neoplasia of the cervix and noted a significant induction of the molecule in high-grade intra-epithelial lesions. They suggested that expression of ICAM-1 by cervical keratinocytes is induced by the process of HPV-related neoplastic transformation, rather than HPV infection alone[13].

Diagnosis

Cytology

The Papanicolaou-stained (Pap) smear is a screening tool that looks for changes in cells of the transformation zone of the cervix. These changes are often caused by HPV.

The current Pap smear reporting system is The Bethesda System (TBS). It was introduced in 1988 and updated in 1991, 1999 and 2001. It contains a standardized approach for reporting if an individual specimen is adequate for evaluation.

TBS 2001 reports the adequacy of cervical cytology preparations in two categories: “satisfactory” and “unsatisfactory” for evaluation. The unsatisfactory category includes specimens that do not contain sufficient cells for re-

liable interpretation. However, any specimen with abnormal cells will be described as satisfactory for evaluation regardless of the number of cells present[2].

In TBS 2001, cervical cytologic specimens that contain no epithelial abnormalities are listed under the category "negative for intraepithelial lesion or malignancy." The presence of organisms such as *Trichomonas vaginalis* or "fungal organisms morphologically consistent with Candida species" must be included as a comment in this "negative" category.

The Bethesda System 2001 classifies squamous cell abnormalities into four categories:

1. ASC (atypical squamous cells),
2. LSIL (low-grade squamous intraepithelial lesions),
3. HSIL (high-grade squamous intraepithelial lesions),
4. squamous cell carcinoma.

The ASC category contains two subcategories:

5. ASC-US (atypical squamous cells of undetermined significance);
6. ASC-H (atypical squamous cells, cannot exclude HSIL).

The ASC-US subcategory includes lesions that have cellular abnormalities suggestive of SIL. The use of the qualifier "undetermined significance" emphasizes that a specific diagnosis cannot be made and that further triage may be appropriate. ASC-US excludes cytology suggestive of HSIL. ASC-H is interpreted as cytologic changes that are suggestive of HSIL but lack criteria for definitive interpretation. Low-grade lesions (LSIL) include mild dysplasia, cervical intraepithelial neoplasia (CIN) 1 and other lesions generally considered to be due to transient HPV infection[2]. Specific to mild dysplasia is koilocytic atypia, which is manifest in mature superficial or intermediate cells by enlarged, irregular nuclei with hyperchromasia and perinuclear halo, a slightly convoluted nuclear membrane, occasional binucleation, a moderately increased nuclear/cytoplasmic ratio, and sometimes, a densely eosinophilic staining of the cytoplasm around the halo[34].

High grade lesions (HSIL) encompass moderate and severe dysplasia, carcinoma in situ, CIN 2, and CIN 3[2]. HSIL contains malignant basal/parabasal cells of different numbers (on Pap test) or level of epithelial involvement in histology[34].

In TBS 2001, results of HPV DNA testing can be added if appropriate and, preferably, reported with the cytology results. If automated computer systems are used to scan slides, the type of system used and the result should be reported.

The Pap smear procedure has its limitations. Up to 8% from the samples are inadequate and false negative rates rise to 20-30%[9]. Some of the reasons for the false negative results may be: (*) other contents of the cervical specimen such as blood or bacteria that can superimpose on abnormal cells preventing their detection; (*) the non-uniform spreading of the cells on the slide; (*) a low number of abnormal cells that may be "missed" in a crowded background with normal cells (the number of cells on a slide can reach several hundred thousands)[9].

In order to reduce the high number of false-negative results, other methods of sample collection and processing have been developed. In these methods, the specimen is collected in a preservative solution rather than being spread directly on the microscope slide by hand. Cellular structure is better preserved because the cells are immediately fixed. The uniform monolayer created by these methods is easier for a technician to read, and the process prevents drying artifacts and removes most contaminating mucus, protein, red blood cells, bacteria, and yeast[9].

There are many studies that compare monolayer cytology to conventional Pap smear cytology. Their results show that monolayer cytology has a significantly higher diagnostic sen-

sitivity and provides fewer inadequate samples than the standard Pap smear procedure[9].

Computer-assisted systems that scan microscope slides and recognize abnormal cells have been developed and some are approved to be used in routine practice.

Histopathology

Cytology is a screening tool, not a diagnostic test[34]. Pacients with abnormal cervical cytological results are evaluated by colposcopy and colposcopy-directed biopsy[9].

Colposcopy can detect low-grade and high-grade dysplasia but does not detect microinvasive disease.

Biopsy can be used to confirm most diagnoses by observing characteristic pathologic features of HPV infection such as acanthosis (epithelial hyperplasia) and koilocytosis in terminally differentiated keratinocytes with atypical nuclei. In addition, stains can be used which detect HPV antigens or HPV nucleic acids. Monoclonal and polyclonal antibodies that can detect HPV common antigen are available. Bound antibody is detected by peroxidase-antiperoxidase immunocytochemical staining[9]. The advantage of *in situ* nucleic acid detection techniques is that it retains the morphology and the histological context of the lesion. *In situ* methods can be nonamplified, signal amplified or target amplified by PCR.

HPV nucleic acid detection

Nucleic acid hybridization methods used for HPV detection can be classified into three categories: direct nucleic acid probe methods, hybridization signal amplification, and target amplification methods[20].

Direct probe methods

The Southern blot technique has been used in the early studies on HPV. Another direct probe method is *in situ* hybridization (ISH), which can detect HPV DNA or RNA inside cells in biopsy tissues, within the context of histopathology. ISH uses probes marked with either radioactive or non-radioactive ligands. The former contain radioactive isotopes and can be detected by autoradiography. The non-radioactive ligands are chemically reactive and can be detected by fluorescence or after a colour reaction. The preferred methods for HPV nucleic acids detection in clinical samples are the enzymatic methods[9]. The staining pattern can differentiate between integrated and episomal HPV DNA and the intensity of the signal reflects the viral load.

The disadvantages of the direct probe methods include low sensitivity and specificity[33]. Southern blot hybridization cannot be performed on fixed tissues, because of the DNA degradation caused by formalin-catalyzed DNA cross-linking[20]. These are also time-consuming techniques.

Signal amplification

Signal amplification methods include the branched DNA (bDNA) assay and liquid hybridization. These are proprietary technologies. The former is owned by Bayer Corporation and the latter by Digene.

Digene's Hybrid Capture II (HC2) has been widely used in studies. It uses specific RNA probes that are directed toward individual DNA sequences comprising the HPV genotypes to be detected. Digene owns a proprietary antibody that is directed toward DNA-RNA hybrids. The antibody is used both for the capture step and the detection step. The qualitative detection of the presence of the HPV uses chemiluminescence[9,20]. In essence, the HC2 assay is an immunoassay[20].

HC2 uses two RNA probe pools, which can be used together or separately. One can detect LR-HPV 6, 11, 42, 43, and 44 and the other one can detect HR-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The assay does not distinguish among the HPV types within these groups. The assay has a microwell plate format, so it can be automated.

This test has some limitations. False positive results can appear due to cross-reactivi-

ty. Cross-reactivity may occur in the presence of HPV13 since both probes cross-react with HPV-13[9]. The documented cross-reactivity between HPV6 and HPV42 could lead to the mistaken categorization of a specimen as positive for a HR type in situations of high concentration of the LR genotype HPV[20]. Cross-reactivity between both HPV probes and high levels of bacterial plasmid pBR322 which can be found in cervical samples is also possible[9]. Semiquantitation provided by the HC2 assay relates to the concentration of viral DNA per milliliter of specimen transport medium but does not control for variability in lesion size, specimen adequacy, or viral copy per infected cell[24].

HC2 can be used to screen patients with ASC-US Pap smear results so as to determine the need for referral for colposcopy[9]. In a meta-analysis, Arbyn et al[3] concluded that HC2 has improved accuracy (higher sensitivity, similar specificity) than the repeat Pap smear using the threshold of ASCUS for an outcome of CIN2+ among women with equivocal cytologic results.

Signal amplification can be also used to increase the sensitivity of *in situ* detection techniques. With the use of chromogenic tyramide-signal-amplified ISH it is even possible to detect and localize single or very few HPV copies within infected nuclei[7].

Target amplification

Target amplification is the most flexible and sensitive of all DNA analysis techniques. This technology can be used for detection, viral load quantitation, DNA sequencing, and mutation analysis[20]. The most widely used target amplification method for HPV detection is based on the polymerase chain reaction (PCR) using either type-specific or general primers[33].

Type-specific PCR assays are based on the sequence variations present in the E6 and E7 genes of HPV subtypes. Internal control primers are included to detect inhibitory substances[9].

Most PCR-based studies have used consensus primers to amplify a wide range of HPV types in a single PCR reaction[9]. These primers target sequences in the conserved regions of the L1 HPV gene. For example, the MY09/MY11 primer pair amplifies a 450-bp fragment in the L1 ORF, and the GP5/6 primer pair amplifies a 152-bp fragment in the L1 ORF. HPV-consensus CPIIG/CPI primer pair amplifies a 188-bp fragment in the highly conserved E1 ORF region[33].

PCR-based tests that can be used in screening for HR-HPV infection in the presence of cervical lesions have been developed and are commercially available. In mid-2004, the PCR-based AMPLICOR HPV test (Roche Molecular Systems) was launched on the European market. The AMPLICOR test is designed to detect the same 13 HR-HPV genotypes as the HC2 high-risk probe cocktail and, in principle, has been developed for HR-HPV screening. Like HC2, the AMPLICOR HPV test is sensitive, specific, feasible, and easy to handle in routine but does not provide specific genotype information[16]. These tests show good agreement for samples with high-grade cytology and similar sensitivity in detecting CIN2+ lesions[10]. Both tests are Conformité Européenne (CE) marked[36].

After the general primer PCR amplification step, a number of methods can be used for classification based on sequence differences. These are based on type-specific oligonucleotide hybridizations including the reverse line blot detection method, restriction fragment length polymorphism (RFLP), or, as the gold standard, sequencing[33].

A PCR-based detection system, known as the line probe assay (LiPA), has recently been developed. Line probe assays detect amplified genotype-specific DNA by selective hybridization with oligonucleotides immobilized on nylon membranes. Using line probe assay techniques, individual HPV genotypes can be classified with a very high level of sensitivity, useful in resolving individual genotypes in

mixed infections[20].

SPF_{10}-INNO LiPA (Innogenetics) assay uses a general primer set, SPF_{10} that amplifies a 65-bp segment of the L1 ORF region of the HPV genome. Amplicons are then detected using a DNA enzyme immunoassay (DEIA) using mixture of general HPV probes recognizing a broad range of HR, LR, and possible high-risk HPV genotypes in a microtiter plate format. In the positive samples, HPV genotype is determined using a line blot assay in which oligonucleotide probes are immobilized as parallel lines on membrane strips. The LiPA strips are then manually interpreted. The SPF_{10}-INNO LiPA assay is capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously[36]. The short- fragment-PCR used by SPF_{10}-INNO LiPA can be successfully used to assess HPV genotypes in formalin-fixed paraffin-embedded tissue samples which often yield poorly amplifiable DNA[9].

The Roche Linear Array (LA) HPV (Roche Molecular Systems) genotyping test is a recently launched new HPV genotyping assay able to genotype 37 HPV types, concurrently assessing human β-globin as an indicator for sample quality. The LA test uses biotinylated PGMY primers to amplify a 450-bp fragment within the polymorphic L1 region of the HPV genome.

Comparing the two tests, van Hamont et al found them highly comparable. Apparently, if an infection encompasses multiple genotypes, the SPF_{10}-INNO LiPA assay is less sensitive than the LA. The LA seems to be less sensitive than the LiPA if a sample has a single infection with some specific HPV genotypes that are poorly amplified by PGMY. The LA assay is unable to distinguish HR-HPV 52 from other HR genotypes[36].

Wallace et al developed a high-throughput, fast, single-tube-typing assay capable of simultaneously typing 45 HPV by blending multiplex PCR and multiplex hybridization using spectrally addressable liquid bead microarrays[37].

As an inconvenient, PCR assays are subject to environmental contamination with previously amplified material (amplicons) that can potentially contaminate negative specimens such that false-positive results could be obtained. In almost all laboratories, this possibility is obviated by the use of stringent amplicon-containment procedures coupled with enzymatic amplicon elimination systems involving the uracil-N-glycosylase (AMPerase) enzyme[20].

Another disadvantage of the above-mentioned methods is that they are not suited for quantitative determination of viral load and require extensive processing after PCR. The introduction of real-time reporting of PCR amplification with fluorogenic reporter probes such as TaqMan and molecular beacon probes provides quick and convenient tools for sequence variation detection (genotyping) and quantification[33].

All real-time PCR systems rely on the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. Quantitation of target DNA, such as a viral pathogen, using real-time PCR has the advantage of being reproducible, rapid, and applicable in a clinical setting. It provides a real quantification by determination of standard curves, which then can be used to extrapolate the amount of starting DNA target in a patient's specimen. Reactions can be run in multiplex with the use of different fluorochromes, such that the starting concentrations of several target DNAs can be analyzed at once[20].

Rapid real-time PCR using consensus primers and SYBR Green I as a reporter has been used to detect the HPV infection, quantify viral load and identify the two most frequent HR-HPV (HPV16 and 18) using melting curve analysis[14].

The temperature at which a DNA

strand dissociates or melts when heated is dependent on sequence, length, and GC content. Melting temperatures can vary between strands with the same length but different GC /AT contents, but also between strands with similar length and GC /AT contents but with different GC/AT sequence.

SYBR Green I is a fluorescent dye which binds to the minor groove of double stranded DNA and emits light on excitation. Thus, as the PCR product accumulates, fluorescence increases. Melting curve profile is analyzed by slowly increasing the temperature of the amplified samples and monitoring the fluorescence. On denaturation of the product, SYBR Green I is released and fluorescence rapidly decreases. The melting curve chart shows the sample fluorescence versus temperature. The melting temperature (T_m) of a sample is defined as the temperature at which half the probes (or dye) have melted off the DNA. T_m of the sample can be seen as a peak on the first negative derivative (-dF/dT) of the sample melting curve (*Figure 3*).

In mixed samples, two separate peaks of distinct T_m are seen, even when there is a considerable difference in the copy number of each type. The determination of melting curves can be carried out on each sample after amplification without opening the reaction vessels. HPV16 and HPV18 products were clearly separated by T_m analysis in mixtures varying from equivalence to 1/1000. This approach has been confirmed with type specific primers[14].

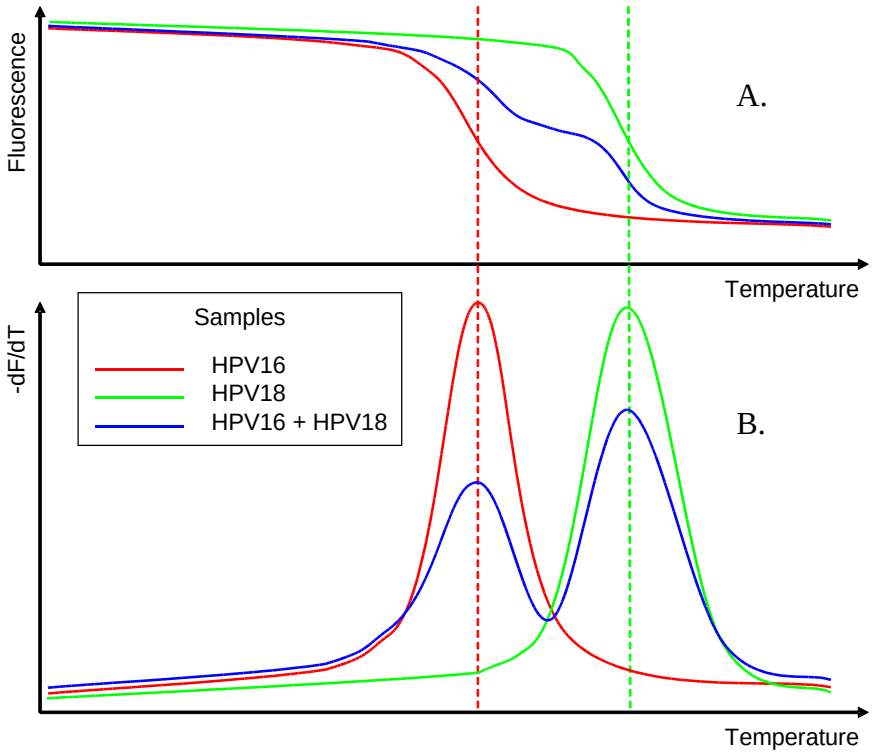

Figure 3. Melting temperature analysis of three samples: respectively, HPV16 positive, HPV 18 positive and positive for both HPV16 and HPV18. (A) Melting curve chart. (B) Melting peak chart. Y axis: negative differential of fluorescence over temperature (-dF/dT). The analysis of the sample positive for both HPV16 and HPV18 (blue) shows two melting peaks, corresponding to different melting temperatures of sequences amplified for the two viruses.

Both real-time PCR and conventional PCR have greater sensitivity for the detection of HPV16 and 18 in formalin-fixed, paraffin-embedded cervical CIS and cancer specimens compared to signal amplified *in situ* hybridization. Though, there is an inhibitory effect of formalin fixation and paraffin embedding on the evaluation by real-time PCR quantification, resulting in underestimation of the viral copy number[7].

Another approach to HPV detection is the detection of mRNA of the HPV E6 and E7 oncogenes in clinical samples. These tests determine if HPV genes that have transforming potential are present and active instead of simply detecting the presence of the virus. Lamarcq et al performed a study that aimed to detect and measure gene expression in cells scraped from the cervix using real time quantitative reverse transcription-PCR (qRT-PCR) (TaqMan). Their report was the first demonstration that TaqMan RT-PCR can reliably measure gene expression in RNA extracted from samples collected for cervical cancer screening. They demonstrated that presence of detectable HPV E7 transcripts can distinguish between normal and abnormal samples[22].

Scheurer et al were the first to use a less expansive SYBR Green I - based real-time PCR for the measurement of HPV E7 expression in cervical cytobrush specimens[29].

Invirion developed the In-Cell, a viral load test for HPV that detects mRNA of the E6 and E7 genes. The assay can be automated on any analytic instrument that detects fluorescence. Liquid-based cytology specimens can be analyzed using this assay on flow cytometry instruments. The assay can also be done directly on Pap smear slides and visualized using a fluorescence microscope[9].

Quantitative measures of gene expression between samples require some form of normalization to a reference that provides a common basis for the comparison. Most methods of qRT-PCR use one or more so called "housekeeping genes" whose expression is considered to be stable. Steinau et al selected the appropriate references for normalizing qRT-PCR assays of gene expression in exfoliated cervical cells. ACTB (β-actin) was the most stable single gene. The addition of PGK1 (phosphoglycerate kinase 1) and RPLP0 (large ribosomal protein P0) increased the robustness in qRT-PCR applications not stratified by disease. If special attention to intraepithelial lesions is appropriate, RPL4 (ribosomal protein L4) and PGK1 are recommended as the best combination of two genes[32].

The World Health Organization is involved in the assessment of the performance of various HPV DNA detection assays and examines the feasibility of generating HPV DNA standard reagents. The "Results of the First World Health Organization International Collaborative Study of Detection of Human Papillomavirus DNA" have been recently published and showed that a proposed material derived from cloned plasmid DNA representing double-stranded full genomicHPV DNA sequences fulfilled the requirements for international standards[28].

As an inconvenient, most PCR methods, while generally available for research use only testing, often involve the use of patented HPV sequences that limits their applicability as in vitro diagnostic tests due to legal or proprietary restrictions[20].

Utility of the HPV DNA testing in clinical practice

Based on the results of the ALTS study, The American Society for Colposcopy and Cervical Pathology (ASCCP) 2001 guidelines recommend HR-HPV DNA testing for colposcopy triage of ASC-US. They further recommend that patients found harboring HR-HPV types undergo colposcopy and biopsy to rule out CIN or cancer, while those negative for HPV or carrying LR-HPV types are spared from such pro-

cedures and followed with yearly conventional cervical cytology studies. If those HPV positive are not found having CIN/cancer, then Pap smear should be repeated at 6 and 12 months or the HPV DNA testing should be repeated at 12 months, with referral back to colposcopy if HR-HPV is found[37].

According to ASCCP 2001 guidelines, pacients with ASC-H are referred for immediate colposcopy. However, in their analysis, Srodon et al[31] concluded that, in routine practice, HPV testing for the triage of ASC-H would have the potential to reduce the number of pacients referred to colposcopy. They used the "routine practice" approach, meaning that the liquid-based cervical cytology specimens were interpreted by a single pathologist rather than being subjected to a consensus review process. They compared their results with those of the ALTS study which were generated through rigorous expert consensus diagnosis. Like in the ALTS study, HPV DNA testing was done using the HC2 assay. The authors found that the frequency of HPV positivity for routinely diagnosed ASC-H was high enough to distinguish these patients from ASC-US patients yet low enough, compared with the ALTS data for ASC-H, to potentially reduce the number of women who are referred for colposcopy by one-third compared with immediate colposcopy approach recommended by ASCCP for all women with ASC-H. HPV testing would have a colposcopy triage role for patients with ASC-H, similar with its triage role for ASC-US. HPV negative patients with either ASC-US or ASC-H have a similar low risk of underlying HSIL, so this approach would not lead to an increased risk of failure to detect HSIL in HPV negative women[31].

Most current HPV screening protocols identify the presence of one of a pool of HR HPV types but do not identify the individual genotype or determine whether repeated positive tests are due to the persistence of one particular type; women who are infected with any HR HPV type are treated in the same way[35].

Given the finding that some HR-HPV genotypes have been shown to differ in oncogenic potential, it is reasonable to assume that detection of individual HPV genotypes in cervical specimens could assist in more precise risk stratification[20]. HPV screening that distinguishes HPV16 and HPV18 from other oncogenic HPV types may identify women at the greatest risk of $\geq$ CIN3 and may permit less aggressive management of women with other oncogenic HPV infections[21]. Type-specific HPV testing is a potentially stronger long-term predictor of cervical disease than cytology in women aged 30 years and older[21]. Future management of patients with neoplasia will probably include not only HPV detection but HPV genotyping as well[35].

Testing for HR-HPV on self-sampled vaginal material gave results comparable to those of the cervical smear. The prevalence of HR-HPV on the basis of self-sampled vaginal material is consistently about 5% to 10% lower than for cervical smears, which would decrease the sensitivity in detecting CIN. However, for women who decline to participate in screening programs that use cervical smears, vaginal self-sampling may be a good alternative and could largely reduce the risk of cervical cancer associated with not participating in a screening program[25].

The "Guidelines for the management of asymptomatic women with screen detected abnormalities" introduced in Australia, the country with the second lowest incidence and the lowest mortality for cervical canver in the world, claim that HPV testing can be used as a 'test of cure' after the treatment of an HSIL. The woman should return to her gynecologist for a repeat colposcopy and Pap test 4–6 months after treatment of an HSIL. If these are satisfactory, she can see her usual practitioner 12 months post-treatment for both a Pap and HPV test. These two tests should be done annually until the woman has tested negative on

both tests on two consecutive occasions. When all four tests are negative the woman can return to the usual 2 yearly screening interval[17].

In patients with definitive therapies such as conization of uterine cervix lesions, a negative HPV test at 6- or 12-month follow up is highly predictive of eradication of the lesion whereas a positive result is associated with persistence of cervical lesion[37].

In patients with cervical cancer, the plasma HPV DNA is associated with metastasis and could be used as a marker representing the circulating free cervical cancer DNA[27].

Future direction

Screening for cervical cancer remains an important concern throughout the world. In the recent years, the screening programs available have helped reducing the incidence of this disease and the mortality associated with it.

Large studies have helped the development and improvement of guidelines for the management of patients with abnormal cervical cytology. These studies indicate the utility of HPV testing in patient management. High sensitivity and specificity assays for HPV detection are commercially available, FDA and CE marked, and the development of new ones is on the way. New guidelines, acknowledging our increased understanding of the role HPV plays in genital disease are being formulated. The new 2006 ASCCP Consensus Guidelines for the Management of Women with Abnormal Cervical Cancer Screening Tests will be published in October 2007 (http://www.asccp.org).

A new direction in cervical cancer prophylaxis is the development of efficient vaccines against HPV. Recent research on the safety and efficacy of 2 candidate prophylactic vaccines has shown nearly 100% efficacy in preventing persistent infections and development of cervical precancerous lesions. These 2 vaccines (quadrivalent Gardasil®, already available commercially, and bivalent Cervarix™, in final stages of clinical development) protect against the 2 main HPV types (HPV 16 and 18) that together cause about 75% of all cervical cancers[34].

There is ongoing development of new technologies for diagnosis and research. The possibility of performing fast and small-volume nucleic acid amplification and analysis on a single chip has attracted great interest. Devices based on this idea, the "Lab-on-a-chip" systems, have witnessed steady advances over the last years. Viral DNA, including HPV DNA, is a potential application for them[38]. Given their tremendous analytical power, it may someday be possible that, in a single specimen, concurrent testing is performed for all HPV genotypes and all known HPV mutations within the context of pacient genetic factors, such as HLA haplotype or mutations within tumor suppressor genes[20].

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