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Cervical Cancer Screening: From Molecular Basis to Diagnostic Practice, Going Through New Technologies

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In the era of Human Papillomavirus (HPV) vaccination, a lot of misunderstanding still exists among healthcare professionals and patients regarding HPV infection. The purpose of this review is to synthesize the clinical molecular mechanisms that contribute to HPV-mediated cervical carcinogenesis, as well as to appraise the current status of new biomarkers and technologies in terms of available data on clinical applications and future promises.

Key words: HPV review; E6/E7 mRNA; Cervical cancer; New technologies.

The Real Dimension of the Problem

The global burden of HPV-associated disease is very high. Cervicocarcinoma is the second most common cancer among women worldwide, but its rank among women's cancers varies between countries depending on the effectiveness of screening programs (1). American Cancer Society estimated that approximately 10,000 American women would have been identified as having cervical cancer in 2006 and that 3700 would be died because of this disease (2). In addition, in 2002, worldwide assess of new cases of cervical cancer was 500,000, with approximately 280,000 deaths from cervical cancer recorded (1).

The vast majority of cervicocarcinoma cases (83%) were diagnosed in developing countries that have access to less than 5% of global cancer resources and where cytological screening programs have not been successfully implemented (3).

On the contrary, a decline of the incidence and mortality of cervical cancer has been observed in most of the Western Countries since the second half of the past century, with the introduction of the Pap test. Cytology-based screening has reduced the incidence of cervical cancer by up to 75% in countries that have been able to realize quality-controlled screening programs (4, 5).

These data impressively demonstrated how successful early detection and prevention programs for cervical cancer might work.

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Abbreviations: ASC-H, Atypical squamous cells suspicious but not diagnostic of high-grade squamous intraepithelial lesion; ASC-US, Atypical squamous cells of undetermined significance; FISH, *In Situ* Hybridation with Fluorescent probe; CIN, Cervical Intraepithelial Neoplasia; HC2, Hybrid Capture 2; HPV, Human Papillomavirus; HR, High Risk; HSIL, High-grade squamous intraepithelial lesion; ICC, Invasive Squamous Carcinoma; ISH, *In Situ* hybridation; LBC, Liquid-Based Cytology; LCR, Long Control Region; LR, Low Risk; LSIL, Low-grade squamous intraepithelial lesion; NASBA, Nucleic Acid Sequence Amplification; ORF, Open Reading Frame; PC, PreservCyt fluid; PCR, Polymerase Chain Reaction; Sb, Southern blot; SIL, Squamous intraepithelial lesion; URR, Upper Regulatory Region; VLP, Virus-Like Particle.

Cytological Testing Limitations

There is no doubt that well-organized screening programs that realize high compliance and good quality control are effectual in saving lives. However, a program based solely on conventional cervical cytology has important limitations; among these weak points, there is the problem of inadequate samples. The prevalence of unsatisfactory specimens varies widely around the world and depends on the method of sampling and on the criteria used for specimen adequacy. Actually, inadequate samples constitute about 8% of cervical cytological slides; so the estimated true specificity and sensitivity of conventional cervical cytology is only in order of 80-85% and 76%, respectively (6).

A review of the UK program found that 47% of women under 70 years old who developed invasive cervical cancer have had an apparently adequate screening history within 5 years of detection (7). Some patients failed to follow-up on an abnormal smear result, while many had a history of negative smear results. Most cases of cervical cancer occur because of false-negative results of Pap test.

Another problem concerns the high proportion of cervical smears diagnosed as "borderline/low-grade cervical lesions". Each year in the United States, almost 50 million cervical cytological examinations are performed with more than 3 million diagnoses of borderline/low-grade lesions; these comprise atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells suspicious but not diagnostic of high-grade squamous intraepithelial lesion (ASC-H) and low-grade squamous intraepithelial lesion (LSIL). Although most cases with borderline/low-grade cytological results do not reflect clinically significant lesion, about 5%-10% of these cases underlying high-grade lesion of the cervical mucosa by colposcopic biopsy (8).

For the reasons listed above and because of the subjective interpretations of scoring criteria that are used to classify ASCUS/ASC-H and LSIL cytological samples, such lesions are placed by cytopathologist in the grey diagnostic zone ranging from normal to abnormal smears (9-14).

In consideration of Pap test limitation, even in well-organized/quality-assured programs, the mortality rate of cervical cancer in screened population is not been completely removed. This consideration suggests that the frontier of effectiveness of conventional Pap smear have been reached and that is imperative to improve the diagnostic assessment of cytological reporting.

Monolayer Cytology

In order to realize further reductions in the incidence and mortality of cervical cancer, attention has focused on the screening test itself. The low sensitivity of a single cervical smear is due to a variety of factors including: incorrect or inadequate sampling of cervix; poor transfer of cells to the glass slide; non-representative sample placed on the slide; poor fixation. Sampling and preparation together are guilty for about two-third of false-negative tests (15). New methods of collection and processing Pap smear have recently developed in order to go over these deficiencies and to improve the diagnostic accuracy of cervical cytology.

Liquid-based cytology (LBC) technique is a new method that involves the transfer of all the material collected on the sampling device into a preservative solution, to produce a cell suspension. Then, this cell suspension is sent to the laboratory in place of glass slide smeared with cellular material by hands. By this innovative tool, the cells are immediately fixed and well preserved for several weeks at room temperature (16). Furthermore, the device used to acquire the specimen (cervical brush) provides more numerous epithelial cells than other collection instrument.

Slides preparation from LBC specimens takes place by an automated process that prevents drying artifacts (common in conventional Pap slides) and removes most contaminating mucus, red blood cells, and most saprophytic bacteria. Background material such as inflammatory exudates, cytolysis, microorganisms, and tumor diathesis can still be identified but it does not obscure the epithelial cells.

The small, randomized aliquot of epithelial cells deposited in a thin layer on a glass side, contains a proportional representation of all the cells removed from the cervix. Cytological evaluation and interpretation of these slides is facilitated by this thin layer distribution of the cells (17).

To date, there are three currently FDA-approved LBC methods: SurePath™ System (TriPath Imaging Inc., Burlington, NC, USA), ThinPrep® System (Cytyc Corp., Boxborough, MA, USA), MonoPrep System (MPPT; MonoGen, Lincolnshire, IL) (15); the last is the only disposable thin-layer preparation system that requires no instrumentation. ThinPrep and SurePath methods are the most widely studied technologies, in literature; their underlying principles are similar. The only difference is that ThinPrep collects samples into methanol-based preservative solution, SurePath disperses cells into ethanol-based fluid.

More than forty publications promote the use of these preparation methods. In particular, all the authors show statistically significant improvement (about 10% or more) of the diagnostic sensitivity of conventional cytology in all categories of disease (18-21). In particular, in the US, unsatisfactory rates with liquid cytology are generally less than 1% to slightly over 2% (19). This enhancement is primar-

ily achieved by a better cellular preservation and by a fairly uniform thickness of cellular deposit that allows for optimal viewing of cellular morphology and gives the opportunity of computer-assisted screening (22).

Currently, LBC constitutes over 80% of cervical screening tests in the USA. In 2003, the UK National Institute for Clinical Excellence (NICE) recommended the introduction of LBC as the primary way of processing samples in Cervical Cancer Screening Program (23). Another undoubted advantage of the LBC approach is that, since only an aliquot of cell suspension is removed from the vial to prepare cytological glass slide, the residual cell suspension provides a specimen for additional investigations such as immunocytochemical or molecular procedures. These ancillary tools, more closely related to cervical carcinogenesis, would further improve the sensitivity and the specificity of the screening test and would offer a better diagnostic value in identifying the correct management of women whose cytological lesions remain controversial.

The Health Risks of HPV Infection

Recently, a significant increase in the understanding of the natural history and the molecular pathogenesis of cervical cancer has been achieved. At present, Human Papillomavirus (HPV) infections are among the most common sexually transmitted disease (24). In the United States alone, it was estimated that 6.2 million of new infections occur annually in individuals, with an approximately prevalence of 20 million (25). The typical age of cervical HPV infection is similar to other sexually transmitted diseases, the highest peak of prevalence being present at the third decade (24-30%). Cumulative prevalence of HPV infection in sexually active asymptomatic women of reproductive age is on the order of 5-40%. High risk HPV (HR-HPV) prevalence is between 4 and 20% (26); it is age-dependent and gradually decreases with age (27). In most cases, genital HR-HPV infection is transient or intermittent: in particular, only 10% of infected women remain HPV-positive within 5 years. Centers for Disease Control (CDC) calculated that at least half of all sexually active individuals will acquire HPV at some points of their lives, whereas at least 80% of women will acquire an HPV infection by age 50. Rates of infection appear to continue to be rapidly increasing.

Cervical cancer is characterized by a well-defined pre-malignant phases that can be highlighted by cytological examination of exfoliated cervical cells. These pre-malignant changes represent a spectrum of abnormalities ranging from LSIL to high-grade squamous intraepithelial lesion (HSIL). The identification of viral and host factors that modulate the risk of developing cervicocarcinoma in HPV infected women necessarily requires a complete understanding of viral biology.

HPV is a small, ubiquitous DNA-virus with an approximately 7900 base-pairs genome enclosed in icosahedral capsid consisting of two late proteins: L1 and L2. HPV genome consists of a single molecule of double-stranded circular DNA. The Open Reading Frame (ORF) protein-coding sequences are restricted to one strand. The genome is functionally divided into three regions. The first is a non-coding one referred as Long Control Region (LCR) or Upper Regulatory Region (URR). This section of HPV's genome includes enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. The second is the Early Region (E), consisting of the ORFs E1, E2, E4, E5, E6, and E7, which are implicated in viral replication and oncogenesis. The third is the Late Region (L) that encodes the aforesaid capsidic structural proteins L1 and L2.

More than 100 genotypes of HPV have been isolated and branded molecularly. They can infect basal epithelial cells of the skin or inner lining of tissues and are categorized as cutaneous or mucosal types. Cutaneous varieties are epidermotrophic and recognize as elective center the skin, in particular that of hands and feet; mucosal types show tropism towards respiratory, intestinal, and lower genital tract epithelium. Each type of HPV is associated to a preferential site and to an own natural history of infection. Extremely important in ano-genital pathology field are the forty so-called genital type HPVs; they preferentially infect stratified squamous epithelium of cervix, urethra, and anus, and skin of vagina, vulva, penis, and perianal areas.

Based on their association with cervical cancer and precursor lesions, genital HPV types can also be grouped: High-risk (HR-HPV) or oncogenic types (16, 18, 26, 31, 33-35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73, 82), associated with a high relative risk of cancer; Low-risk (LR-HPV) or non-oncogenic viruses (6, 11, 40, 42-44, 54, 61, 70, 72, 81, 89), associated with benign epithelial proliferation within ano-genital area but not with cancer (28, 29). Among the nineteen HR-HPV types, five are most often found associated with cervical cancers: virus 16 is accountable for about half of cervicocarcinoma cases discovered in Northern America, Europe, and Australia; viruses 18, 31, 33, and 45 are responsible for additional 30% of cases (30-32).

The second significant milestone in cervical cancer prevention, being Pap test introduction the first, came in the 1980s, with the finding of the link between cervical cancer and HPV (33). During the following 20 years, epidemiological studies clearly confirmed that cervical infection with specific HR-HPV types represents the initial event of most cervical cancer (34).

HR-HPVs establish a dramatically elevated risk of developing squamous intraepithelial lesion (SIL) and cervical cancer, in the order of 50-300-fold higher than the risk attended for

HR-HPV-negative women. A meta-analysis of J. S. Smith *et al*. shows that the overall prevalence of HR-HPVs in ICC is on the order of 87%, ranging from 86% to 94% by region; overall HR-HPVs prevalence in HSIL ranges from 78% in Asia to 88% in Europe. HPV-16 appears to be the most common type (ranging from 52% in Asia to 58% in Europe), being HPV-18 the second (it ranges from 13% in South-Central America to 22% in North America) (35).

Molecular Diagnosis of HPV-related Disease

The consciousness that cervical cancer is a multistep process and that it only occurs in women who are infected with HR-HPVs, led to development of molecular techniques capable of identifying carcinogenic HPV in cervical sample and able to improve the quality and the accuracy of cervical screening (26).

Due to the difficulties to induce HPV growth in conventional tissue and cell culture and to the unreliability of serological tests in determining whether an HPV infection is present or past (36), an accurate diagnosis of HPV infection relies on the detection of viral nucleic acid. So, direct detection of HPV genome in cervical specimens may offer an alternative or a complement to population-based cytological screening.

Wide ranges of methods are available for HPV-DNA detection in cytological specimens; these molecular technologies can be broadly divided into those that operate without amplification (such as nucleic acid probe tests), and those that utilize amplification (such as Polimerase Chain Reaction). Amplification techniques can be further divided into two separate categories: (i) target amplification (*i.e*., PCR), in which the assay amplifies a target nucleic acid; (ii) signal amplification, in which the signal generated from each probe is increased by a compound-probe or branched-probe technology. To date, all the above techniques, in addition to non-amplified ones, have been applied to HPV detection field.

Southern Blot

Initial methods applied for HPV recognition were direct probe hybridization such as Southern blot (Sb). Sb utilizes enzymes to break HPV-DNA chain extracted from the specimen. The product, integrated into a gel, is consequently subjected to an electrophoretic process that separates viral DNA basing on the size of each fragment. The separated DNA fragments are next transferred to a nitrocellulose membrane and hybridized with HPV genomic probes, which are labeled with radioisotopes. In addition to poor labor applicability (reliance on radiolabeled probes) and high time-consuming, Sb procedure shows low sensitivity, due to the request of large amounts of DNA in clinical samples. In consequence of these disadvantages, Sb technique has now largely been superseded by amplification technologies, which allow detection of low-level HPV-DNA copy number.

Direct Hybridization

In situ hybridization (ISH) is another direct probe method to assess the presence of a target nucleic acid or gene expression within either paraffin-embedded tissue or cervical smear. The nucleic acid probes used in ISH are derivatized, typically with biotin, in multiple sites. Detection is frequently achieved employing a sandwich approach involving streptavidin-chromogen complexes.

Improvements in sensitivity of ISH have been reached with fluorescent probe (FISH) utilization, in order to add a further amplification of the signal.

The major advantage of ISH/FISH techniques is that HPV-DNA can be identified inside specific cells (normal, koilocytes, neoplastic) and that viral physical status may also be determined (integration versus episomal) (37). Low sensitivity, low specificity (30-72%), nucleic acid degradation during sample processing and high time-consuming (due to multiple assay that must be carried out for HPV typing), are the main factors that make these techniques troublesome in its performance (38, 39).

Amplification

DNA amplification is a laboratory-based procedure that duplicates specific target sequences of DNA present in biological specimens. Polymerase Chain Reaction (PCR) is the mostly common employed amplification technologies in HPV detection. The method exponentially increases HPV-DNA sequences present in cervical samples, theoretically producing, after 30 cycles of amplification, one billion HPV-DNA copies from a single double-stranded molecule.

There are two main approaches to detect HPV-DNA by PCR: type-specific PCR and consensus PCR.

Type specific procedure is designed to amplify a single HPV genotype; so, multiple PCRs must be done to detect the presence of viral DNA in one sample. Instead, consensus assay enables the immediate detection of a broad range of HPV types (40).

The most extensively used PCR assay utilizes consensus primers that target a highly conserved region of HPV L1 genome, thus amplifying a vast spectrum of HPV types in one reaction. Initially, most laboratories used PCR assay with degenerated primers pair MY09/11. The use of these containing degeneracies oligonucleotides resulted in a lack of reproducibility and in high variation between PCR runs. These prim-

ers are now been replaced by a new set of oligonucleotides pool: GP5/6 and modified GP5+/GP6+; PGMY09/11 (modified MY09/11); SPF1/2, especially appropriated for formalin-fixed paraffin-embedded tissue samples that often offer a small amount of amplifiable DNA (41, 42). The employ of these primers produces a broad-spectrum size of amplification fragment and different levels of sensitivity for detection of certain HPV genotypes. Nevertheless, it's commonly accepted that consensus PCR frequently misses clinically important HPV infections such as those induced by multiple HPV types.

A recent study evaluated cervical specimens of 120 women with HPV infection; of those, 11 had multiple infections. PGMY09/11-based method detected most of this multiple infection (9/11); MY09/11 identified 2/11 whereas the GP5+/GP6+ method detect none (43).

Subsequently to the amplification of the target DNA by consensus PCR, the specific HPV type can be determined by using nucleic acid hybridation, restriction fragment length polymorphism, or sequencing techniques. Actually, nucleic acid hybridation (dot blot, SB, microtiter ELISA plate, reverse line blot strip assays, and microchip format assays) is probably the most common employed procedures for HPV genotyping; obviously, the analytic sensitivity and reproducibility of results are different, depending on the employed method.

PCR assay can be also greatly affected by various unrelated substances that can inhibit the amplification reaction. Most laboratories includes in PCR protocol an internal control of reaction, such as beta-globin gene, present at one copy/human cell.

To try to by-pass these disadvantages, a number of commercial HPV detection PCR-based assays have newly been released. Roche diagnostics (Amplicor, Indianapolis, IN, USA) have recently brought a new PCR standardized test that, similarly to HC2, is able to detect 13 HR-HPV types. However, this assay is not yet FDA approved, is still relatively expensive, and provides evidence of cost-effectiveness only in high-throughput laboratories (44, 45); in addition, Roche PCR test is not able to discriminate genotypes specifically.

Recent literature reports clinical sensitivity of PCR varying from 75% to 95% (46), with a median of 82%. Of interest is the PCR versus HC2 data obtained from ALTS study: on 278 cases of CIN3/cancer, PCR test employing the PGMY09/11 primers achieved clinical sensitivity and specificity of 87.4% and 55.6% respectively, while the corresponding value for HC2 test were 92.5% and 51.1% (47).

Hybrid Capture 2

HC2 (Digene, Gaithersburg, MD, USA) assay is the only kit currently approved by the US Food and Drug Administration (FDA) for the detection of HPV-DNA in cervical samples. It is widely used in the majority of clinical diagnostic laboratories.

HC2 is a relatively simple, high-throughput, semi-automated, solution-phase hybridation test for detecting 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 5 LR-HPV types (6, 11, 42-44). This is the mostly commonly applied and clinically validated assay on the market today. Because the detection of LR-HPV types has no oncologic significance, testing is usually done only with HR-HPV probe set. There are two preferred methods to collect cervical cytology smear for HC2 testing: Specimen Transport Medium (STM) (Digene Diagnostics Corporation, Gaithersburg, MD, USA) and PreservCyt fluid (PC) (Cytic Corporation, Marlborough, MA, USA).

HC2 technology operates on the principle of signal amplification; the method utilizes long single-stranded RNA probes to facilitate both capture and detection of target molecules within the specimens. RNA probes are complementary to genomic sequences of HPV DNA that this test is able to detect. In this assay, DNA is first denatured and subsequently mixed with RNA probe pool in a buffered solution. Two RNA probe pool are used. The assay can be performed using both probe pool together or separately. Probe A recognizes LR-HPV types; pool B identifies HR-HPVs. DNA-RNA complexes are immobilized onto microplates which has been coated with antibody that recognize specific DNA-RNA hybrids at room temperature. The immobilized hybrids are then identified by a second anti-DNA/RNA antibody, conjugated to alkaline phosphatase and bounded to the chemiluminescent substrate CDP Star (Tropix PE, Bedford, MA, USA). Several molecules of alkaline phosphatase are conjugated to each antibody and multiple conjugated antibodies bind to each captured hybrids, in order to amplify the signal. Excess of antibodies and non-hybridized probes are removed by washing. Microplates are then transferred into a software program where results are analyzed; in particular, CDP Star light is measured by a luminometer.

The intensity of emitted light is expressed as RLUs, which are proportional to the amount of target HPV-DNA present in the specimen. RLU value represents a semi-quantitative measure of the viral load. The assay works at the recommended cut-off value for a positive result of 1.0 Relative Light Units (RLUs), equivalent to 1 pg HPV-DNA/mL of sampling buffer and corresponding to 5,900 HPV genome/test well. The test is relatively easy to perform; it has in-built positive and negative controls, and exhibits robust performance (48, 49). Reproducibility is better from STM than from PC specimens. For PC samples, FDA has approved a retest zone for initial RLU/cut-off value of 1.0-2.5. In this case, one or two supplementary tests need to be executed to

confirm or contradict the previous result. HC2 assay also permits high reproducibility. The main advantages of this technology are the high sensitivity and the abundant clinical data obtained from its application. Conversely, the test is rather restrictive in the era of HPV vaccination; the reason is its inability to give information about the specific implicated HPV types. This also limits the recognition of persistent infection by the same viral type, which is the most important risk factor for progression to cancer (50).

DNA Test: Alternative or Complement to Cytology?

The knowledge that infection by HR-HPV types is the necessary cause of cervical neoplasia, has led to improved interest in the use of HPV-DNA test.

Actually, the two potential uses for HPV-DNA include population screening and triage. In population screening, as a primary test or as an adjunct to Papanicolaou test, the purpose of HPV-DNA assay is the detection of latent or subclinical infection among symptom-free women. J. Belinson *et al*. (51) performed a large cross-sectional study (1997 women aged 35-45) to compare the sensitivity of LBC and HC2 test for detection of CIN2+ lesions. Removing of 100% of verification bias, they showed no difference between LBC and HC2 (94% and 95%, respectively). Essentially, the rationale of the use of adjunctive HPV-DNA testing in screening applications is based on the accepted concept of necessary causality of HPV in determining cervical cancer and on the basis of the very high negative predictive value-NPV (typically 90-100%) of the combination HPV-DNA test/Pap test (52).

In triage, the goal is to guide the management of patients with borderline or mildly diskaryotic smears (ASCUS and LSIL lesions, respectively) (53, 54).

ASC-US/LSIL Triage Study – ALTS (55, 56), is a multicenter randomized clinical trial, sponsored by National Cancer Institute (NCI), built in order to evaluate the three management strategies of women with ASC-US and LSIL cytological results: (i) immediate colposcopy for all patients; (ii) repeated cytology with referral to colposcopy if cytological findings showed High-grade Squamous Intraepithelial Lesion (HSIL); (iii) HPV triage (with referral to colposcopy in case of HPV-DNA test positivity) (57, 58).

ALTS establish that: (a) HPV-DNA triage is as sensitive as immediate colposcopy triage in HSIL+ detection; then, the primary use of the test would spare HPV-negative women from emotional and financial weight of colposcopy. (b) Repeating cytology, a strategy that would offer at least 90% sensitivity for HSIL+ lesion, is failure in ASC-US/LSIL triage; the reason is that this approach would refer more than two-third of the above abnormalities to colposcopy. Vice versa, HPV testing has substantially greater sensitivity than repeated cytology with similar specificity (57).

In summary, ALTS study attests that the HPV method represents the best triage, particularly for ASC-US/LSIL cervical lesions (55). It's generally agreed that combining HPV-DNA testing with the Pap test improves the performance of Pap smear alone and is useful when cervical cytology is ambiguous (*i.e*., ASCUS and LSIL lesions) (57). However, the main problem with DNA testing is the high prevalence of HPV-DNA positivity among women with normal, ASC-US or LSIL Pap smear (about 90%), compared with the number of women really developing HSIL+ lesions (less than 10%). The evidence that the majority of Papillomavirus infections are transient or non-transforming creates a high false positive rate and a low clinical specificity for HPV-DNA testing methods. In a follow-up study of 240 ASC-US and LSIL cases, it was observed that 73% of cases that were histologically normal in loop electrosurgical excision biopsies, were HC2 positive (Bjerre *et al*. data not shown). These results should evoke in medical community the indication to emphasize persistent papillomavirus infection rather than single-time HPV-DNA detection, in management strategies and health messages (58).

Molecular Basis of Transformation

Within population, the prevalence of HPV infection in asymptomatic women is estimated to range from 2% to 44%. The U.S. National Health and Nutrition Examination Survey (NHANES) determined the overall prevalence of HPV infection in a representative sample of 1921 woman, aged 14-59 years to be 26.8% (54). The highest prevalence was reported in women younger than 25 years of age (44.8%) compared with 24.5% for women aged 14-19 years and 27.4% among women aged 25-29 years. Then, HPV infection gradually declined to 4-5% around age 45. It's actually unclear whether incidence begins to increase again in older age (59).

The vast majority of HPV infection is usually transient and resolves within 2 years, without necessarily led to clinically significant cervical lesions. Given the high incidence of HR-HPV DNA positivity compared with the low prevalence of cervical cancer, it's then reasonable to deduce the following conclusions: infection with HR-HPV represents only the necessary but far from sufficient condition for the development of H-SIL and ICC (60); the single best predictor of risk of cervical cancer is viral persistence (50).

HPV infection is established by penetration of virions in the basal and parabasal cells layers (stratum germinativum) through micro-abrasions located in the transformation zone of the cervical epithelium. During an acute infection, to replicate its genomes and successfully produce new infectious virions, HPV requires a certain degree of terminal differentiation.

Then, once inside the host cell, HPV replicates as the basal cells differentiate and progress to the surface on the epithelium. In the basal layer, viral replication stay as non-productive and HPV establish itself as a low copy number episome by using the host DNA replication machinery to synthesize its nucleic acid on usual once/cell cycle (61). Instead, in the differentiated keratinocytes of the suprabasal layers of epithelium, the virus switches to amplify its DNA to high copy number, synthesises capsid protein and causes viral assembly. This strategy to avoid viral gene expression and replication in epithelial stem cells but to permit it in differentiated cells (committed to die because of their physiological differentiation processes) is a very meticulous mechanism that consents maximal production of virions causing almost no injure to infected host.

In the basal and parabasal cells, only very little, if any, gene expression activity of the virus can be observed (61). Conversely, expression of early viral genes, E1, E2, E4, E5, E6, and E7, is evident in differentiated epithelial cells of upper layers of epithelium, which have lost the capacity to replicate their genome and are at no further risk of acquiring functional mutations. Here, viral genome is further replicated, with the additional expression of late L1/L2 genes, and of early E4 gene. L1/L2 "encapsidate" the viral genome to form HPV progeny in the nucleus; E4 expression is necessary for the production of L2 protein. E4 also interrupts cytoplasmic cytokeratin network, causing condensation of tonofilaments at the cell periphery and perinuclear cytoplasmic halo: the result is the koilocyte. At that time, the virus can initiate a new infection. Mild cervical abnormalities support productive viral replication. The situation changes radically if the regulatory intracellular mechanisms that drive the fine-tuned expression control of the viral gene along with the differentiation processes of the epithelium are disturbed and deregulated, and uncontrolled expression of genes involved in the replication of viral genome suddenly occur in epithelial stem cells. In these circumstances, interference of viral genes with cellular pathways that control replication and life cycle of epithelial cell may result in chromosomal instability.

One of the key consequences of HPV-induced chromosomal instability is the integration of HPV genome into the host-cell genome and the continuous and deregulated expression of viral oncogenes E6/E7 in replicating epithelial stem cells (basal and parabasal cells). There are no data for particular specific integration loci within host cell genome, although fragile sites are apparently favored (62). HPV genome integration results in the loss of E2 regulatory gene. E2 gene product usually blocks transcription of E6/E7 genes. E2-mediated down-regulation of E6/E7 transcription results in the release of pRb and p53 proteins; the normal differentiation process of the host cell is legitimate to carry on. Disruption or deletion of E2 gene occurring during HPV-DNA integration interferes with down-regulation of E6/E7 transcription genes and leads to an increased expression of these oncoproteins. As result of the above event, E6/E7 products bind to cell cycle regulators and stimulate cell-cycle progression, inducing instability of infected basal cells (61). In particular, E6 gene product binds to p53, a protein that causes cell-cycle arrest in G1 phase in response to DNA damage and induces apoptosis. E6 supports premature degradation of p53 tumor suppressor gene, *via* cellular ubiquitin ligase. As a consequence, the normal activities of p53, which govern G1 arrest and DNA repair, are abrogated (63, 64). LR-HPV E6 protein does not bind p53 at detectable levels and has no effect on p53 stability, *in vivo*. E7 gene product binds the hypophosphorilated form of the retinoblastoma protein (pRb) complex. This binding disrupts pRb-cellular transcription factor E2F-1 bond and lead to E2F-1 release; this one permits the transcription of genes whose products are required to enter in S phase of cellular cell cycle. LR-HPV E7 protein binds pRb with decreased affinity (65). Both HR-E6/E7gene products deregulate the key cell cycle checkpoints (G1/S, G2/M). HR-HPV infected cells with E6/E7 expression may acquire defects in differentiation, immortal phenotype, chromosomal instability, and an increased probability of mutations allowing invasion. Such described are obviously extraordinary molecular accidents occurring during HPV life cycle and, in view of many HPV-infected cells, are extremely rare events. On the other hand, the continuous, deregulated and persistent viral oncogenic E6/E7 activity in cervical stem-cells compartment enhances the neoplastic progression of the respective cell clones. This persistence represents the essential and indispensable requisite to develop cervical cancer.

Numerous studies concerning HPV infection in immunocompromised individuals reported that E6/E7 would also play an important role in the inhibition of the host cell immune response (31): in particular, evasion of immune response would contribute to survival and propagation of HPV-infected cells. E6/E7 genes would have a negative impact on immune response by inhibiting the production of immune mediators (*i.e.*, Interleukin-18); E6/E7 oncoproteins would also have the ability to down-regulate Interleukin-8 (IL-8) expression (IL-8 is a T-cell chemoattractant) (66), and to suppress the expression of Chemochine Monocyte Chemoattractant Protein 1 (MCP-1) in epithelial cells of female genital tract (67). In addition E6/E7 would favor the escape from the antiviral and antiproliferative properties of Tumor Necrosis Factor Alpha (TNF-α) (68).

Consequently, detection of E6/E7 transcripts is considered the best biological marker of neoplastic risk, the more precise indicator of progression towards malignancy (70).

The numerous assays we examined until this time have different characteristics (cost, applicability on various biologi-

cal material, and level of automation) but all have a common factor: identification of the mere HPV-DNA in cervical smear.

Previous reported studies indicate that these tests have good diagnostic accuracy due to their high negative predictive value (52). Nonetheless, HPV-DNA tests are only indicative of a generic possibility of developing cervical carcinoma; approximately two-thirds of the women with ASCUS/LSIL abnormalities, after additional analysis, result as not being oncogenetically active (70). Then, despite the advantages offered by DNA test, it is not capable of distinguishing a clinically transient infection, which is common in sexually active women, from a clinically active infection with an elevated risk of carcinogenic transformation (71, 72).

Based on this conceptual consideration, and given the high number of HR-HPV infected individuals, the best and presumably also most cost-effective target for cervical cancer early detection assays appears to be cells population that just acquired chromosomal instability and that was, thus, initiated to transformation.

Novel Candidate Biomarkers to Identify Cervical Cells with Deregulated Viral Oncogenes Expression

Ideally, the candidate marker defining the different stages (clearance, persistence, progression) of cellular change associated with HPV infection, should give a high positive predictive value in predicting progression to cervical cancer. Many reflex tests to use on HPV-DNA positive samples are under investigation; we will restrict the discussion to the markers that have reached some level of clinical applicability and have been evaluated in large clinical trials.

Viral Load

Among women with HR-HPV positive test, cytological abnormalities are more common in those having high viral load (73). Then, some authors have proposed viral load as the principal predictor of HSIL+ lesions (21). However, it is now clear that the liaison between viral load and cervical disease is more complex than it was previously thought. Whereas many cross-sectional studies reported an increased viral load with growing disease severity, other found either no association; in some cases viral load was quite higher in patients with LSIL than in those with HSIL. Longitudinal studies were also unsuccessful in finding a statistically significant association between viral load and duration of infection, clearance or progression of disease (75). There are some probable reasons for these contradictions: HPV integrated status increases with enhancing of disease severity; integration status is characterized by a declined viral replication. In addition, the relationship between viral load and cervical disease varies among HPV types. Studies using quantitative type-specific PCR for HR-HPV-16, -18, -31, -33, and -45 and LR-HPV-6 and –11 showed that HPV-16 can reach a much higher viral load than the other types listed above and that only HPV-16 viral load might correlate with increased severity of cervical disease (76, 77). Then, all HR-HPV types but 16 are able to provoke cervical cancer even when they are present at low levels (41).

DNA Methylation

More recently, epigenetic alterations of HPV-infected cells genome have been considered as diagnostic marker for cervical cancer. Aberrant presence of CpG-rich DNA sequences (so-called CpG islands) in the promoter regions of tumor suppressor genes represents one of the several epigenetic changes that contribute to carcinogenesis (78). DNA methylation involves the covalent addition of a methyl ($-CH_3$) group at the carbon-5-position of a cytosine that precedes a guanosine. Usually, DNA methylation plays a role in maintaining genome stability and in regulating gene expression (79). However, global hypermethylation of CpG clusters present in the promoter region of multiple genes have been associated with malignancy (80). Numerous clinical studies demonstrated that these epigenetic methylation changes are often present in a variety of cancer. In this framework, silencing of the Tumor Suppressor Lung Cancer 1 (TSLC1) gene by promoter hypermethylation may be a valuable biomarker to detect cervical lesions with high malignancy potential. TSLC1 was found to be silenced in 91% of cervical cancer cell lines, primarily resulting from promoter hypermethylation (81). Moreover, such hypermethylation was detected in 58% of cervical carcinomas and in 35% of High-grade CIN lesions, but not in low-grade CIN or in normal cervix (82). The high frequency of TSLC1 methylationin cervical cancer was confirmed by studies of Li *et al*. (83) and Gustafson *et al*. (84). These data suggest that the analysis of methylation patterns of TSLC1 gene may be a valuable tool in forthcoming screening programs: however, they appear more likely to play a role in detecting cervical cancer cell clones rather than cells in early initiating events of cervical carcinogenesis.

Proliferation-associated Gene Products

Cervical cancer cells present in a cervical smear usually predominantly include differentiated epithelial cells that have undergone cell cycle arrest and are not likely to express high level of proteins involved in active DNA metabolism; otherwise, proliferating dysplastic cells arrive to upper epithelium surface from where they are taken to collect cervical specimen. This suggests that a group of proliferation-associated gene products may provide a potential source of novel candidate predictive markers.

Accordingly, Ki67 or PCNA have extensively been used in attempt to identify dysplastic cells. Deeper analysis could not confirm the initially hopeful results and showed that these markers did not have adequate sensitivity and specificity to supply the request of additive prognostic markers in cytological screening (85).

p16^{INK4a}

Cellular protein markers are currently available as indirect indicator of HPV integration into the host genome and of aberrant cell cycle control. Expression of gene related to specific interference of HR-HPV E6/E7 oncogenes with host cells, may represent a supplementary resource for these potential useful biomarkers. One of these candidates is the gene coding for the cyclin-dependent kinase inhibitor p16^{INK4a} (85).

p16^{INK4a} is expressed at very low levels in normal cells, while it is strongly over-expressed in cervical cancer cell lines in which pRb has been inactivated by HR-HPV E7. Physiologically, pRb may act as negative regulator of p16^{INK4a} expression (86). If this mechanism is lost (disruption of the pRb-dependent p16^{INK4a} regulation *via* HR-HPV E7 protein), the respective cell starts to over-express p16^{INK4a} at very high levels.

p16^{INK4a} overexpression has been found to be associated with pre-neoplastic cervical lesions and ICC (87); on the other hand, normal epithelium does not show overexpression of this protein. These data suggest that p16^{INK4a} realizes most of the criteria required to identify cells or lesions with deregulated viral oncogenes expression pattern. Despite the high correlation between p16^{INK4a} immunodetection and morphological cervical lesion classification, there are special conditions in those also nondysplastic cells stain for p16^{INK4a} in histological as well as cytological specimens: metaplastic, atrophic, and endocervical cells may display p16 immunoreactivity. The expression of p16^{INK4a} in these cells may be referred to irregularities in normal differentiation pattern. So in cytological specimens, in which histological context is lost, the evaluation of p16^{INK4a} positive cells is more difficult and requires a depth analysis of cellular and nuclear morphology (88).

In practical terms, there are several criteria that may help to correctly interpret p16^{INK4a} staining patterns in cervical cytology samples; the most important method utilized contemplates the score of nuclear abnormalities (nucleocytoplasmatic ratio, hypercromasia, nuclear border abnormalities, anisokaryosis, nuclear chromatin structural aspects). Nuclear Score (NS) is then actually proposed to facilitate the assessment of p16^{INK4a}-stained cells (89).

Despite NS classification, there are still disagreement in literature regarding the significance of p16-positive cell numbers and stain location, especially due to high intra- and inter-observer discrepancy in stained cells evaluation, and to poor reproducibility of p16^{INK4a} immunocytochemical assay. Therefore, larger numbers studies are needed to answer all question about the clinical value of p16^{INK4a} immunocytostaining as a potential biomarker in cervical cancer screening.

Novel Approach with Prognostic Significance

Although several biomarkers have been proposed for the detection of neoplastic changes in cervical cells, these biomarkers must not be considered a replacement of the detection of specific oncogenic HPV genes.

Persistent expression of viral oncogenes E6/E7 is a necessary step for HPV-induced carcinogenesis. Consequently, detection of HR-HPV E6/E7 is considered to be the best biological indicator of risk of progression towards cervical cancer.

HPV E6 expression is regulated at transcriptional or post-transcriptional level. HPV-16 E6 ORF encodes for three different variants of E6 protein, which may have dissimilar roles in the viral cell cycle. These transcripts are either unspliced (full length-FL- E6/E7 transcripts) or spliced. Interestingly, only FL E6 protein is found to be powerfully bound to p53 and promotes its degradation. Moreover, only the unspliced E6 form was found to be more strongly associated with tumorigenicity. Studies carried out on cervical cancer samples show that FL transcripts are always present (90). These studies indicates FL transcripts as being the most important biomarkers for the carcinogenic process (91).

PreTect HPV-Proofer™ assay (NorChip AS Klokkarstua, Norway) is a commercially available kit based on a multiplex nucleic acid sequence amplification (NASBA) technique, which amplifies E6/E7 mRNA (FL transcripts) in a DNA background, and utilizes molecular beacon probes for real-time detection of HPV types 16, 18, 31, 33, and 45 (41).

Molecular beacons are oligonucleotide probes consisting of stem-loop structure and of two regions: loop region is a single-stranded 18-30 base pair sequence that is complementary to the target sequence; stem region typically consists of 5-7 base pair long double-stranded sequences that lie on both arms of loop region; 5′ sequences are labeled with a fluorescent dye (fluorophore) while 3′ sequences are covalently labeled with a non-fluorescent quencher. In absence of a complementary target sequence, molecular beacon remains closed and in a non-hybridized state; in this situation, the quencher captures the fluorescent signal. When beacon unfolds the presence of the complementary target (E6/E7 mRNA), loop region hybridizes with this sequence, fluorophore separates itself from the quencher, and the fluorescent signal is transmitted.

In PreTect HPV-Proofer™ procedure, two different labeled

molecular beacon probes for each multiplex reaction are used. Fluorescein (FAM) is used as fluorophore for the detection of HPV-16, 31, and 33; Texas Red (TxR) as fluorophore for the detection of U1A gene, HPV-18, and HPV-45.

U1A is a small nuclear specific ribonucleoprotein A included in HPV-Proofer kit to avoid false-negative results, and to monitor sample mRNA integrity.

Isolation of mRNA is carried out by extracting aliquots of freshly collected LBC samples not over a time course of 14 days (91), and performed by using a silica-based method (*i.e.*, Qiagen RNeasy Mini protocol).

NASBA amplification is achieved through coordinated activities of three enzymes (Avian Myeloblastosis Virus Reverse Transcriptase, *E.Coli* Rnase H and T7 RNA polymerase) and two DNA oligonucleotide primers that are specific for the target sequence of interest. RNA amplification is performed for one hundred and fifty minutes at isothermal temperature of 41 ºC. In presence of the target sequences, a fluorescent signal is observed. A fluorescent analyzer measures, in real-time, the emitted fluorescence from molecular beacon hybridized with amplified mRNA (92).

PreTect HPV-Proofer assay makes available artificial and standardized oligo's, designed to monitor the integrity of primers, probes, and reagents, and used as positive controls for HPV types 16, 18, 31, 33, and 45. The reproducibility of a positive result by PreTect HPV-Proofer™ assay was in the range of 96-100% (93).

The high clinical accuracy of E6/E7 mRNA test has been confirmed by a large cross-sectional study carried out on 4,136 women older than 30 years, assessed longitudinally for a minimum of 2 years, and based on detection of HPV-DNA *via* GP5+/6+ consensus PCR and on detection of E6/E7 mRNA by PreTect HPV-Proofer assay. Cytological and histological data related to follow-up diagnosis of women with ASCUS/LSIL Pap smear were also included (71). DNA and RNA test showed identical sensitivity to detect CIN2+ lesion. On the other hand, specificity for RNA and DNA test was 85% and 50%, respectively.

Comparative studies between HC2 test and RNA test by using PreTect HPV-Proofer assay, showed that the specificity for detecting HSIL+ lesion, in population below 30 years of age, was 20% for HC2 and 70% for PreTect HPV-Proofer, while was 40% and 81%, respectively, for women above 30 years of age (95). All of the 20 biopsies, confirming invasive cervical cancer and included in these last studies, were E6/E7 mRNA positive; only 90% of those were HC2 positive. The main reason of this discrepancy is that PreTect HPV-Proofer has high analytical sensitivity and that its capacity to locate target sequences is not affected by viral integration. Vice versa almost all DNA assays detect the L1 region, which is deleted by integration of the viral genome into the host cell. Then, DNA tests fail in identifying 4-25% of persistent infections and cervical cancer cases.

A cross-sectional outpatients population-based follow-up study carried out by our group demonstrated that among women with ASC-US cytology, 39% were positive for HR-HPV DNA, while E6/E7 transcripts were detected in only 15% of cases. Among patients with LSIL cytology, the corresponding proportions were 76% and 26%, respectively (70). These data, combined with others 15 extensive studies carried out in many countries, show that RNA test may reduce the number of ASC-US and LSIL cases that have to be followed by colposcopy-directed biopsies by more than 70% (95).

At present, only five (16, 18, 31, 33, and 45) HR-HPV types are detectable by the above-described RNA methodology; at the first sight this may be considered a disadvantage in comparison with the available DNA genotyping strategies. However, studies carried out to find E6/E7 mRNA in cervical smears of women with cervical cancer demonstrated a 100% coverage by the listed above HPV genotypes (31, 95, 96).

PreTect HPV-Proofer assay is a reproducible tool in order to monitor HPV infections with transforming potential. Therapeutic HPV vaccination strategies, now in early developing, are based on the induction of a cell-based immune response to E6/E7; the result is a destruction of HPV-associated precancerous and cancerous cells that express these viral oncoproteins. In this background, RNA test may also represent a useful tool in order to individuate cervical lesion in which E6/E7 transcripts are expressed (97).

In conclusion, the introduction of mRNA test in the clinical work-up of DNA positive women, particularly of those with ASC-US/LSIL cervical abnormalities, would certainly increase the diagnostic accuracy of Pap smear and DNA test, through a better identification of the infections, which is more likely to persist and induce HSIL+ lesions in future, and by reducing psychological distress and cost for women who have only a transient infection.

The Future

Significant clinical and commercial development of prophylactic vaccines began only with the discovery of L1 intrinsic capability to self-assemble into virus-like particles (VLPs). VLPs are morphologically indistinguishable from naïve virions and are able to induce high titers of neutralizing antibodies (98); they are created by cloning the major capsid L1 genes, acquired from different HPV types, and by inserting them into yeast or baculovirus vectors. Large amounts of L1

protein for each HPV types separately are then obtained in eukaryotic tissue culture system (99).

HPV VLP-based vaccines are produced and commercialized by two pharmaceutical companies: GlaxoSmithKline (GlaxoSmithKline, Rixensart, Belgium) and Merck (Merck & Co., Whitehouse Station, NJ, USA).

GlaxoSmithKline vaccine (Cervarix) is bivalent; it's composed by HPV16 and 18 VLPs; Merk vaccine (Gardasil) is tetravalet and contains HPV-6/11/16/18 VLPs. The last is designed to combat two different diseases: cervical cancer and genital warts. To date, results from VLP vaccine trials are encouraging with regard to safety and efficacy. Several randomized placebo-controlled trial in human volunteers reported that these prophylactic vaccines significantly reduced the incidence of persistent HPV-16 and HPV-18 infections.

Currently, most national vaccination program are primarily aimed to preadolescents and adolescents (9-23 years of age).

It is an extremely positive fact to hope in use of a vaccine capable to prevent a neoplasia with so strong social impact such as cervicocarcinoma. However, despite the initial promises, there are several issues that still need to be addressed before the fully appreciation of HPV vaccination in matter of overall potential and impact for public health (100-101).

First, the duration of protection is unknown. Second, the vaccines will only protect against HR-HPV types targeted vaccine. Third, prophylactic vaccines are likely to provide limited benefits to women previously infected with HR-HPV. Fourth, VLP vaccines are relatively expensive and vaccine delivery in developing world is more difficult. Fifth, the effects of vaccination on the female psychology is dangerous: if vaccinated women will believe to be at no further risk of developing HPV-induced cancer and will leave screening programs, the last impact of vaccination on the incidence of cervicocarcinoma will be invalided (98). Therefore, it is important that both, women and healthcare professionals, do not perceive HPV vaccination as an immediate alternative to cervical cancer screening. Only integrating HPV vaccination into screening programs will maximize the benefits offered by vaccine and will lead to a greater reduction of cervical cancer prevalence, incidence, and mortality.

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