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Molecular Diagnosis of Human Papillomavirus Infections

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1. Introduction

Human Papillomavirus (HPV) is arguably the most common sexually transmitted agent worldwide, either in its clinical (genital warts) or subclinical presentation in men and women. The main interest in HPV relates to its recognized as a causal and necessary factor for cervical cancer one of the most common cancers in women (80% of cases in most developing countries, with an annual incidence of almost half a million and a mortality rate of approximately 50%) [1-5], and other types of cancer, such as penis, anal or oral cancer [6].

The overall prevalence of HPV in cervix in women in the general population is 10%. This prevalence is higher in the less developed world than in more developed regions [7, 8]. A review of studies has also shown prevalence of HPV in men as usually 20% or greater, depending on population tested and the type and number of anatomic sites evaluated [9].

HPV infection is most common in sexually active young women 25 years of age or younger but cervical cancer is common in older woman, suggesting infection at younger age and slow progression to cancer [10].

The most significant predictor for acquiring HPV infection in men or women appears to be the life time number of sexual partners [11,12,13]. For women, the sexual activity of their partner(s) is also important, with increased risk of acquiring HPV if their partner had, or currently has, other partners [12].

Not all women infected with high-risk HPV develop cervical cancer, other factors are necessary: genotype, persistent infection, viral variants, viral load, integration, coinfection, age of 30 years old, immunosupresión, smoking, condom use, coinfections, long-term use of oral contraceptives, parity and circumcision. [10, 12, 14-24]

About 189 HPV genotypes have been sequence and classified according to their biological niche, oncogenic potential and phylogenetic position [25]. From them, about 40 can infect the genital tract [26]. HPV types are classified based on their association with cervical cancer and precursor lesion into low-risk types (**LR-HPV**), which are found mainly in genital warts, high-risk types (**HR-HPV**), which are frequently associated with invasive cervical cancer and undetermined risk types (table 1) [27, 28, 29].

Risk category	HPV types
High-risk	16,18,31,33,35,39,45,51,52,56,58,59,68,73,82
Low-risk	6,11,40,42,43,44,54, 61,70,72, 81, 83, 89
Undetermined risk	26,53,66

Table 1. HPV types classification according their oncogenic potential

Worldwide, HPV-16 is the most common HPV type across the spectrum of HPV related cervical lesions. In women with ICC (invasive cervical cancer), the most common HPV types are HPV-16,18,33,45,31 and 58 [30, 31], but among these genotypes, certain variants have linked to different clinical outcomes. It is now generally accepted that HPV has co-existed with its human host over a very long period of time and has evolved into multiple evolutionary lineages [25, 32]. Intratypic variants of HPV16 have been identified from different geographic locations and are classified according to their host ethnic groups as European (including prototypes and Asian types), Asian American, African and North American [33]. Through epidemiological and in-vitro experimental studies, natural variants of HPV16 have shown substantial differences in pathogenicity, immunogenicity and tumorigenicity. IARC Study [34] and IARC Meta-analysis [31] are very robust in identifying that HPV-16 and 18 contribute approximately 70% of all ICC. HPV-16,18 and 45 are the three most relevant types in cervical adenocarcinoma [30]. The geographical variation in type distribution is of minor significance variation.

Among men and women, cancers of the ano-genital tract and their precursor lesions have been strongly linked to infection with sexually transmitted human papillomavirus. In men, HPV infection has been strongly associated with anal cancer and is associated with approximately 85% of the anal squamous cell cancers that occur annually worldwide. Likewise, approximately 50% of cancers of penis have been associated to HPV infection [35]. Genital warts are a common sexually transmitted condition with an estimated prevalence of 1-2% of young adults [36]. Although having genital warts is not associated with mortality, represent a significant public health problem (clinical symptoms and psychosocial problems) and healthcare costs for society [37-39]. More than 90% of genital warts are related to HPV-6 and 11 (low risk genotypes) in general these types are not associated with malignant lesions, however 20-50% of these also contained coinfection with oncogenic HPV types [39-41].

On the other hand, between 33-72% of oropharyngeal cancers, and 10% of cancer of the larynx may be attributed to HPV infection [42-44].

2. Etiopathogenesis of HPV

The HPV virion has a double-stranded, circular DNA genome of approximately 7900bp, with eight overlapping open reading frames, comprising early (E), and late (L) genes and an untranslated long control region, within an icosahedral capsid. The L1 and L2 genes encode the mayor and minor capsid proteins. The capsid contains 72 pentamers of L1, and a pproximately 12 molecules of L2. The early genes regulate viral replication and some have transfor-mation potential. Late genes L6 and L7 code for structural capsid proteins which encapsidate the viral genome. (Figure 1).

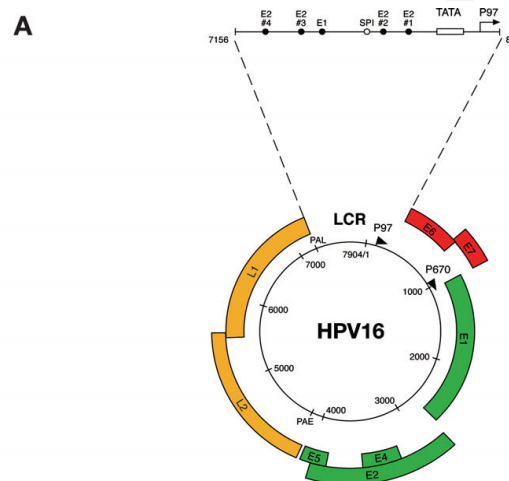


Figure 1. Organization of the HPV genome. Adapted from Doorbar J. [45]

Infection by papillomaviruses requires that virus particles gain access to the epithelial basal layer and enter the dividing basal cells. Having entered the epithelial tissues, the HPV virus enters the nucleus of a basal epithelial cell, where early genes E1 and E2 are expressed, replicating the viral genome and transcribing messenger RNA needed for viral replication; in addition to its role in replication and genome segregation, E2 can also act as a transcription factor and can regulate the viral early promoter and control expression of the viral oncogenes (E6 and E7). At low levels, E2 acts as a transcriptional activator, whereas at high levels E2 represses oncogene expression [45]. As the host cells differentiate, genes E4 and E5 assist in the production of the viral genome by controlling epidermal growth factor. E6 and E7 are viral oncogenes which now become important. E6 causes degradation of the tumour suppressor gene p53, while E7 completes for retinoblastoma protein (pRb), allowing the transcription factor E2F to drive cell proliferation processes. The p16 protein, encoded by the suppressor gene CDKN2A (MTS1, INK4A) at chromosome 9p21, is an inhibitor of cyclin dependent kinases (cdk) which slows cell cycle by inactivating the function of the complex-cdk4 and cdk6-cyclin D. These complexes regulate the control point of the G1 phase of the cell cycle with subsequent phosphorylation and inactivation of retinoblastoma (pRb), which E2F released and which allows cells to enter S phase. It has been demonstrated existence of a correlation between pRb and p16 reciprocal, which is why there a strong overexpression of p16 both in carcinomas

as in lesions premalignant cervix. In cervical cancer, pRb is functionally inactivated from the initial stages of cervical carcinogenesis as a consequence of expression of HPV E7 gene. Genes E6 and E7 therefore act to remove two principle mechanisms of cell defence, and drive the cell replication machinery towards production of new virus particles. E6 and E7 are also known to promote oncogenesis. [45]

On the other hand, integration of HPV-DNA into the host DNA is a well known topic in cervical cancer. Integration of HPV 16 DNA correlates with dysfunction of HPV E1 or E2 ORF, which are active during HPV replication. E2 loss of function allows up-regulation of E6 and E7 oncoproteins, because E2 is a repressor of E6 and E7. (Figure 2).

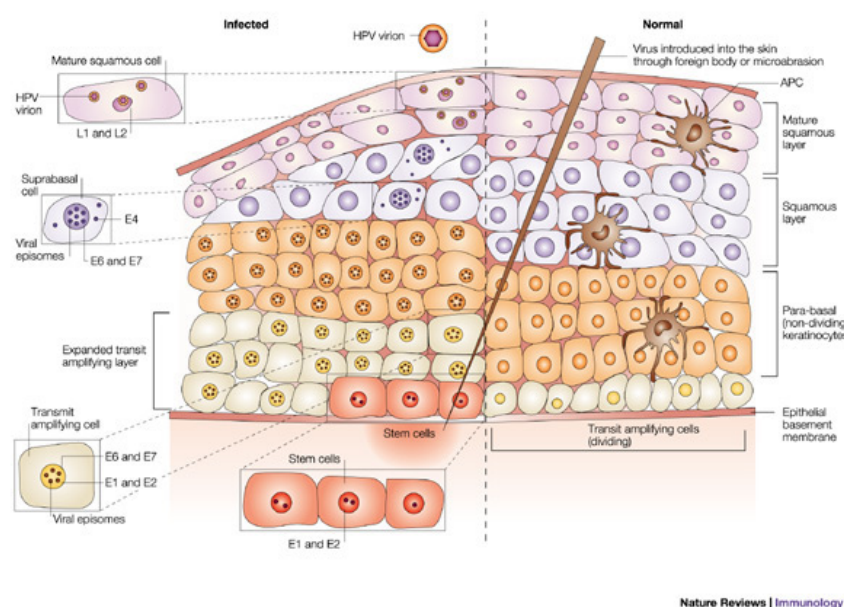


Figure 2. The location in squamous epithelium of the main stages of the papillomavirus life cycle. [46]

3. Diagnosis of HPV infections

Despite the promising outcomes, vaccination does not exempt from performing periodic control visits, because the effects of the vaccine at 15-20 years and the role other genotypes with oncogenic capacity not included in the vaccine may play are still unknown. Furthermore, there is still a large population of women which has had no access to it. Then, secondary prevention by screening and treatment will continue to be crucially important in cervical cancer prevention programs. Moreover, the fact that infection by HPV provokes long-term symptomatology demands a close follow-up (screening) of those individuals susceptible to infection in order to avoid related problems.

Currently, cervical cancer screening is acknowledged as the most effective approach for cervical cancer control. The primary screening and diagnostic methods have been cytology

and histology, but two limitations of the Pap smear exist: low specificity leading to the need for repeat screening at relatively short intervals and cervical cancer screening, based on Pap smear, remains beyond the economic resources of nation in developing world. This economic disparity has meant that cervical cancer incidence and mortality rates in the developing world have remained high, with large reductions in these rates being limited primarily to the industrialized world. Thus, the reduction of cervical cancer in developing nations remains an unmet need of high priority. Since the link between HPV and cervical cancer is known and numerous large scale studies have been done, molecular methods to detect HPV DNA in clinical specimens (vaginal, urethral, paraurethral, anal or pharyngeal exudates, biopsies, and, especially, endocervical exudates) have been introduced into screening algorithms.

Increased sensitivity has important clinical outcomes because reduce mortality and an elongation of screening, and implies better compliance with screening and lower cost [47]. An Italian study showed that HPV-based screening is more effective than cytology in preventing invasive cervical cancer, by detecting persistent high-grade lesions earlier and providing longer low-risk period [48].

HPV serves as paradigm for the use of NAATs for its diagnosis and typification due to how difficult it is to obtain the virus via cell cultures or to develop indirect diagnosis techniques [49].

The first protocols for detect HPV were described about 20 years ago, using L1 consensus primers PCR systems, particularly MY09/11 and GP5+/6+ [50-52]. These primer systems have been widely used to study the natural history of HPV and their role in the development of genital cancer [53-55]. Nowadays, several kits are commercially available which allow for the detection of the virus or the detection and typification of the most relevant HPVs: Amplicor HPV test and Linear array HPV Genotyping test (Roche Diagnostics, Switzerland), Innolipa HPV Genotyping Extra (Innogenetics, Belgium), Biopat kit (Biotools, Spain) or Clart Papillomavirus 2 (Genómica, Spain). The latter uses microarray technology to increase the number of hybridizations in a reduced space. Besides genome amplification, direct hybridization protocols on the sample (hybrid capture) approved by the FDA for diagnosing HPV in women (Hybrid Capture II, Digene, USA) is also used. These protocols identify high and low-risk genotypes without specifying the infecting genotype.

The sensitivity of such methods has left out cytological methods (Papanicolau), which are less sensitive and specific. This high degree of sensitivity allows to extending the period between control visits of women to 5 or 6 years [56, 57].

3.1. Signal amplification systems

The Hybrid Capture II system (HCII, Digene, USA) is a non radioactive signal amplification method based on the hybridization of the target HPV-DNA to labeled RNA probes in solution. The resulting RNA-DNA hybrids are captured onto microtiter wells and are detected by specific monoclonal antibody and chemiluminescence substrate, providing a semi-quantitative measurement of HPV-DNA. Two different probe cocktails are used, one containing probes for five low-risk genotypes: HPV 6, 11, 42,43 and 44 and the other containing probes for 13 high-risk genotypes: HPV 16,18,31,33,35,39,45,51,52,56,58,59 and 68.

However, HCII has some limitations. It distinguishes between the high-risk and low-risk groups but does not permit identification of specific HPV genotypes. Hybrid Capture II (HCII) has been shown to have similar analytic sensitivity to some PCR methods for HPV DNA detection [58], but present cross-reactivity of the two probe cocktails can reduce the clinical relevance of a positive result [59, 60].

The Hybrid Capture III (HCIII, Digene, USA) is being evaluated as the next generation of hybrid capture clinical assays. A primary technical distinction between HCIII and HCII is that HCIII employs a biotinylated DNA oligonucleotide specific for selected HPV DNA sequences (HPV16 and HPV18) for the capture of the DNA-RNA complexes on streptavidin-coated wells, to reduce false positivity [59].

3.2. Target amplification systems (PCR)

Type specific primers designed to amplify exclusively a single HPV genotype can be used but multiple type-specific PCR reactions must be performed separately to detect the presence of HPV in a sample. This method is labor-intensive, a little bit expensive and the type –specificity of each PCR primer set should be validated. Alternatively, consensus or general PCR primers can be used to amplify a broad-spectrum of HPV types: genome amplification protocols (PCR) with degenerate primers targeted towards the L1 gene fragment (MY09/MY11) allow for the detection of a wide range of viral subtypes, which are then identified with specific probes [50, 61]. Other consensual primers (PGMY, GP5+/GP6+ or SF10) used on the same target enhance diagnostic sensitivity [52, 62, 63]. Thanks to these protocols, the low and high cancer progression risk genotypes were identified [25].

Amplification protocols have also experimented great advancements with the application of real-time PCR, which reduces reaction times (e.g. HPV RealTime test, Abbot, USA; GenoID, Hungary). In fact, it is now possible to automate the whole process (Cobas® 4800 HPV Test with 16/18 Genotyping, Roche Diagnostics, Switzerland).

Type-specific PCR primers can be combined with fluorescent probes to real-time detection [64-66] although multiplexing several type specific primers within one reaction can be technically difficult. Broad-spectrum PCR primers have also been used in real-time PCR [67, 68].

The HCII method and consensus PCR assays are currently the most frequently applied. In last years, RT-PCR is being introduced in clinical microbiology laboratories.

3.3. Full spectrum genotyping

About 40 different HPV types (involved in human genital infections) have been identified based on DNA sequence analysis so far, with a subset of these being classified as high risk. DNA of these types is found in almost all cervical cancers, however, regional variation in the distribution of certain HPV types should be taken into account in the composition of screening “cocktails” for high-risk HPV types from different populations [29]. The diversity of virus types and the incidence of multiple infections have made it necessary to develop reliable methods to identify the different genotypes, for epidemiological studies as well as for the

patient follow up [69]. Over the last few years, virus genotyping has become an important way to approach cervical cancer. Then HPV genotype detection could increase specificity in a routine screening program or in post –treatment follow-up (i.e. test of cure) by differentiating transient and sequential infection from persistent infection [70-72].

Population-based genotyping characterizations pre- and post-vaccination will be important to determinate vaccine effectiveness and potential unmasking of niche replacements by non-vaccines HPV types in cytologically normal women and women with low and high grade lesions.

Genotyping assays have been developed, like GP5+/6+ reverse line blot, or MY90/11 dot-blot. Based in these technologies, specific kits have been comercialized: PGMY09/11 linear array (Linear Array® HPV genotyping test; Roche Molecular Systems, Switzeland) and SPF10 LiPA 25 (Inno-LiPA® HPV test, Innogenetics, Belgium). The assays are based on consensus broad spectrum PCR which are subsequently differentiated by type-specific oligonucleotide probe hybrydizacion. These assays have the ability to identify multiple several viruses in cases of multiple infections. In the last years, others assays for HPV genotyping has been commercialised and introduced in clinical and research laboratories with full or partial automation (PapilloCheck HPV-Screening Test, Greiner Bio-One; Clart HPV2, Genomica, Infiniti HPV Genotyping assay, Autogenomics; Cobas 4800 HPV Test, Roche diagnostics; Real Time High Risk HPV test, Abbott Molecular) [73]

As already reported and in spite of its limitations, sequencing could be considered the gold standard for HPV genotyping, due to the possibility of identifying virtually all virus types without mistaken classifications through cross-reactions among similar types, which can occur using tests based on hybriditation [74, 75]. Nevertheless, it was disadvantaged at identifying genotypes in samples with multiple infections, in which viral sequences overlap and it is not possible to distinguish the various types [74, 76].

In any case, genotyping is a technology that has to be incorporated in the HPV surveillance. Waiting for massive sequencing, now the most promising field is automated methods, because simplifies the testing procedure, increases the sample processing capability, minimizes the human errors, facilitates the quality assurance, reduces the cost and can be developed in multiples laboratories.

4. Screening and progression prognostic biomarkers technologies

Because molecular testing for HR-HPV DNA may detect infection too early in the process, with only a small subset of women developing disease that progresses to cancer, there is interest in defining secondary markers that have potential application in identification of women who need to be followed more closely because they are at higher risk of developing high-grade lesions [77]; especially, when the positive predictive value of current screening strategies will be diminished in a vaccinated population [78]. Then, the impetus for new screenig or progession technologies in the developed world is thus predominately driven by the need to increase

positive predictive value and reduce over-management of low-grade and often transient abnormalities.

In these situations, several surrogate markers are in research.

4.1. HPV viral load

Several studies have suggested that a high HPV-DNA viral load may be a candidate marker that could help identify women at greater risk of CIN progression [64, 65, 79, 80]. It has been reported that average HPV DNA copy number increases significantly with the grade of CIN mainly for HPV 16, but not for other HR-HPV types [81-83]. Some studies have pointed out that high viral load in cytological normal epithelium could also be a risk factor for neoplastic progression but other studies suggested an important limitation to the utility in screening algorithms for the substantial overlap of HPV load values between women without and with CIN and the common presence of more than one carcinogenic HPV type [64, 84].

Real-time PCR techniques have been developed to quantify HPV in clinical samples. Moreover, the HCII provides semiquantitative measurement of HPV-DNA, and some studies have demonstrated that the estimated HCII load correlated well with the precise load generated by RT-PCR [85-86]. However, real-time PCR assays more accurately measure HPV 16 viral load by adjusting the signal obtained for HPV 16 DNA with the amount of cellular DNA calculated for amplification of a human gene, therefore providing a more accurate viral load [64, 65, 87, 88]. However, due to low multiplicity for different HR-HPV types, real-time PCR methods are not suitable as a high-throughput screening tool.

4.2. HPV mRNA

Although HR-HPV genotypes are associated with any grade of dysplasia, these types can be detected in a significant proportion of women with normal cytology. It is known that HPV E6 and E7 genes are overexpressed throughout the thickness of epithelial cells in high-grade lesions and cancer. Then, mRNA could be more efficient than cytology for the triage of HPV DNA-positive women, and provides high specificity for high grade cervical intraepithelial neoplasia identification [69, 89-93].

Some authors have developed a real time reverse transcriptase amplification (RT-PCR) for HPV detection strategies and suggested that it may be more specific for the detection of symptomatic infections and quantitative increased coordinately with severity of the lesion [94, 95].

These assays incorporate NASBA amplification of E6/7 mRNA transcripts prior to type specific detection via molecular beacons for HPVs 16,18,31,33, and 45. Initial data, on the prognostic value and specificity for underlying disease, is promising, but the value of this method compared with DNA based assays remains to be determined in large-scale prospective studies [96,97].

Detection of human papillomavirus (HPV) E6/E7 oncogene expression may be more predictive of cervical cancer risk than test HPV-DNA. Commercial test targeting HPV mRNA has been

developed: NucliSENS-EasyQ® HPV E6/E7 mRNA assay (Biomerieux, USA) and Aptima HPV test (Gen-Probe, USA) both are a type-specific E6/E7 mRNA test for HR-HPV types performed in one NASBA reaction. NucliSENS-EasyQ® HPV E6/E7 mRNA assay detected HPV 16,18,31,33 and 45 with detection and genotyping and Aptima HPV test detects E6/E7 mRNA of 14 oncogenic types HPV16,18,31,33,35,39,45,51,52,56,58,59,66, and 68.

4.3. HPV integration (E2/E6-7 ratio)

Most HR-HPV infections are either latent or permissive. Latent infections are not very well defined, but it is assumed that the viral genome is maintained as an episome in the basal and parabasal cells of the epithelium without inducing obvious phenotypic alterations in the host cell.

The transformation process is characterized by the deregulation of viral oncogenes E6 and E7 in cycling cells which ultimately results in chromosomal instability and the accumulation of mutations. The underlying mechanisms for deregulation are manifold. Integration of the HPV genome is a characteristic step in cervical carcinogenesis and its appearance correlates with the progression of precancerous lesions (CIN2/3) to invasive carcinoma [98-100].

However, integration is not mandatory in this process and was shown to be HPV-type dependent. Vinokurova and colleagues observed that HPV16, 18 and 45 were substantially more often present in an integrated state compared with HPV types 31 and 33 [101].

The loss of the viral E2 gene is a common consequence of HPV integration. This event may lead to an elevated expression of the oncogenes E6 and E7 due to the fact that E2 is no longer able to repress the expression of the viral oncogenes in trans [102, 103]. However, in a recent analysis of biopsy material no correlation between the expression levels of viral oncogene transcripts and the physical state of the viral genome was found [104].

Several investigators have also focussed on the impact integration may have on the host genome. Methods for detection of integrated HPV have been described [87, 105]. However, they are affected by similar limitations described for HPV viral load. On the other hand, cervical epithelial cells for women with CIN may simultaneously contain episomal and integrated HPV DNA. Recent data suggest that integration frequency in CIN3 and ICC is variable by HPV genotype, further reducing the desired gains in specificity [101].

4.4. E6-T350G HPV 16 variant

A variety of HPV types have been characterized on the basis of differences greater than 10% in L1 gene sequence [25]. Isolates of the same type are referred to as “variants” when the nucleotide sequences of their coding genes differ by less than 2%, or when the non-coding region (LCR) differs by as much as 5% [106]. HPV 16 is one of the most important HPV genotypes which cause serious cervical disorders, but among these genotypes, certain variants have been linked to different clinical outcomes. HPV 16 variants have been grouped into six distinct phylogenetic branches: E (European), AA (Asian-American), Af1 (African 1), Af2 (African 2), NA (North American), As (Asian) with different geographic distributions. Most

HPV16 variants from European and North American samples were classified as European prototype (EP) [107]. Several studies have shown that the infection by the European L83V HPV16 variant, harbouring a nucleotide substitution at position 350 in the E6 gene (E6-T350G), is a risk factor for advanced cervical disease although some discrepant results have also been found [21, 104, 108, 109].

Detection of HPV variant has been performed mainly by Sanger sequencing, pyrosequencing or high resolution melting analysis [110, 111]. A new one-step allelic discrimination real time PCR assay to detect the E6-T350G HPV 16 variant was evaluated in clinical samples, this novel allelic discrimination assay is a fast sensitive and specific method [24].

4.5. p16 enzyme linked immunosorbent assay

Protein p16 is a cell cycle regulation protein which accumulates in abnormal epithelial cells infected with HR-HPVs as a result of a loss of negative regulation by the retinoblastoma protein induced by E7 expresion [112]. In immunostaining studies, p16 (INK4a) has shown potential as a marker of high grade cervical intraepithelial neoplasia (CIN) and invasive cervical cancer [113, 114]. A recent literature report demonstrates different p16 accuracy according to different anatomical sub-sites. In this complex scenario the p16-IHC test alone or in association to CDKN2a promoter methylation could be used only as screening methods but need to be associated with molecular tests in order to detect HPV-DNA and to assess its integration status. Furthermore, non-dysplastic cells, particularly methaplastic, atrophic and endocervical cells, may display p16 immunoreactivity, thereby reducing specificity [115].

4.6. Methylation profile

Methylation of CpG islands within gene promoter regions can lead to silencing of gene expression. Methylation of tumor-relevant genes has been identified in many cancers: p16 methylation is the paradigm for epigenetic inactivation of a tumor suppressor gene, leading to abrogation of cell cycle control, escape from senescence, and induction of proliferation.

Methylation has been detected already at precancerous stages, suggesting that methylation markers may have value in cervical cancer screening [116]. Furthermore, methylated DNA is a stable target and allows for flexibility of assay development. The detection of methylated genes from cervical specimens is technically feasible and represents a source for detecting potential biomarkers of relevance to cervical carcinogenesis. In particular, there is the ultimate hope of finding methylation markers that, among HPV-infected women, would indicate the presence of CIN2+ and risk of cancer.

A clear role of methylation in carcinogenesis has been demonstrated only for 6 genes (DAPK1, RASSF1, CDKN2A, RARB, MLH1, and GSTP1 [117].

During the last years, several new platforms have been developed that allow for accurate high-throughput genome-wide DNA methylation profiling [118]. Markers or marker panels identified in these approaches could be translated to smaller scaled assays such as MethyLight to be used in cervical cancer screening, but their use is in research.

4.7. Human telomerase RNA component (hTERC)-gain

It has been generally accepted that carcinogenesis involves the progressive accumulation of genetic abnormalities. Gain at 3q is a common feature of squamous-cell carcinoma (SCC), with an overlapping area of gain at 3q26 having been reported in SCC at different anatomic sites [119], including cervix of the uterus [120, 121].

The human telomerase RNA component (hTERC) gene, localized on chromosome 3q26, encodes the RNA component of human telomerase, and acts as a template for the addition of the repeat sequence [122]. Genetic studies have shown that amplification of *hTERC* gene might be an early event commonly involved in the progression of CIN to cervical cancer [123-127].

Amplification of hTERC gene has been identified in many tumor samples and immortalized cell lines using techniques such as fluorescence in situ hybridization (FISH) and Southern blot analysis, suggesting that transcription is upregulated during tumorigenesis [128]. Lan YL et al. confirm that measuring hTERC gene gain could be a useful biomarker to predict the progression of CIN-I or -II to CIN-III and cervical cancer [129]. The present limitation to this assay is the technical complexity and requirement of highly trained individuals to interpret the FISH staining, however automated methods for reading TERCH FISH slides are under development.

4.8. Other proliferation/cell cycle markers

HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins (E6 and E7) and is manifested by changes in the expression of host cell cycle regulatory proteins [130]. Such differentially expressed host proteins and nucleic acids may have a role as “biomarkers” of dysplastic cells.

To date, a wide array of molecular markers has been evaluated. Three markers that have shown the greatest potential are the cyclin dependant kinase inhibitor p16^{INK4} [131, 132] and the DNA replication licensing proteins CDC6 (cell division cycle protein 6) and MCM5 (mini chromosome maintenance 5) [133]. Some authors found that three markers showed a linear correlation between their presence or absence and the grade of dysplasia [132].

5. Summary

In summary, the relevance of HPV infections requires a close monitoring, especially in certain groups of individuals (e. i. Women older than 30 years old). The accuracy of methods using NAATs has emerged as election in the control of HPV infection. But the search is ongoing for safer: more precise markers which may allow for a better control of the infection [134]. These markers include genome quantification via real-time PCR, viral integration into the human genome via E2-E1/E6-E7 genes ratio or the search of viral variants by sequencing, pyrosequencing or allelic discrimination techniques [24, 109, 135].

Addition of new technologies into existing, highly effective screening programs are considered according to the ability to increase the efficiency of the program (high sensitivity with reduction in unnecessary follow-up of minor, transient infection) [136].

The table 2 presents a summary of the technologies relative to their intended or perceived benefit and limitations compared to existing screening and progression prognostic biomarkers methods [136].

Technology	Benefits	Limitations
HCII	Non radioactive signal amplification method	Not identification of specific HPV genotypes
	Distinguishes between the high-risk and low-risk HPV	Cross-reactivity between high-risk and low-risk HPV
	Similar analytic sensitivity to some PCR methods for HPV DNA detection	
PCR	Non radioactive signal amplification method	Contamination
	Low cost	
	Amenable to use with many-samples	
HPV genotyping	Discrimination of HPV-18/ 18 from other high-risk types may have greater positive predictive value.	Moderate to high complexity even with standardized commercial reagents.
	May differentiate sequential infection with different types from persistent infection with the same type.	Very difficult to establish consensus primer-based genotyping de novo with adequate quality control
	Useful for test of cure.	Algorithms may be too complicated to be readily translated into clinical practice.
	Amenable to use with self-sampling.	High cost
	Compatible with many collection buffers.	
	Objective output.	
HPV mRNA	Potential to increase specificity	Moderate to high complexity
	Objective output.	RNA less stable, not compatible with some common collection buffers
		Compatibility with self-sampling unknown
		High cost
HPV viral load	Potential to increase specificity	High complexity
	Objective and quantitative output.	Not pronostic (except for HPV 16)
		Requires type-specific quantitation
		High cost

Technology	Benefits	Limitations
HPV integration	Potential to increase specificity	Moderate complexity for DNA methods
	Objective output.	Very high complexity to detect integrated transcripts
		Integrated DNA may not be transcriptionally active
		Requires type-specific assay
		Common occurrence of mixed episomal and integrated HPV in cervical intraepithelial neoplasia
		High cost
p16 enzyme linked immunosorbent assay	Single analyte (p16protein) to detect infection with any high-risk HPV	Moderate complexity
	May increase specificity by detecting active infection	Compatibility with self-sampling unknown
	Subjective output	Not compatible with all collection buffers
	Cost may be lower than DNA/RNA test	Order of sampling may affect performance
		Low specificity
Methylation profile	As a marker of disease and not infection, may increase specificity	High complexity
	Compatible with urine sampling	Sensitivity limited; questionable reproducibility
	Objective output.	High cost
TERC-gain	As a marker of disease and not infection, may increase specificity	Very high complexity
	Subjective output	High cost
	May be useful as a prognostic marker	
Other proliferation/ cell cycle markers	As a marker of disease and not infection, may increase specificity	High complexity
	Subjective output	Questionable reproducibility
		High cost

TERC: telomerase RNA component. Adapted from Gravitt et al [135]

Table 2. Screening and progression prognostic biomarkers technologies.

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References

- [1] Ferlay J, Bray F, Pisani P, Parkin DM. Globocan 2000: cancer incidence, mortality and prevalence world wide. IARC CancerBase no.5. Lyons, France: IARC press 2001.
- [2] Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; 94:153-6.
- [3] Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;(2):342-50.
- [4] Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV. The casual relation between human papillomavirus and cervical cancer. *J Clin Pathol* 2002;55:244-65.
- [5] Steenbergen RDM, de Wilde J, Wilting SM, Brink AATP, Snijders PJF, Meier CJLM. HPV-mediated transformation of the anogenital tract. *J Clin Virol* 2005; 32(suppl):43-51.
- [6] Georgieva S, Iordanov V, Sergieva S. Nature of cervical cancer and other HPV-associated cancers. *J Buon* 2009(14): 391-8.
- [7] Castellsague X, de San José S, Aguado T, Louie KS, Bruni L, Muñoz et al., editors. HPV and Cervical cancer in the world. 2007 Report. WHO/ICO Information Centre on HPV and Cervical cancer (HPV Information Centre). *Vaccine* 2007; 25(suppl 3).
- [8] De San José S, Diaz M, Castellsague X, Clifford G, Bruni L, Muñoz N et al. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. *Lancet Infect Dis* 2007;7(7):453-9.
- [9] Dunne EF, Nielson CM, Stone KM, Markowitz LE, Giuliano AR. Prevalence of HPV infection among men: a systemic review of the literature. *J Infect Dis* 2006; 194:1044-57.
- [10] Molano M, van den Brule A, Plummer M, Weiderpass E, Poso H, Arslan A, por el HPV Study group. Determinants of clearance of human papillomavirus infections in Colombian womens with normal cytology: a population –based, 5 years follow-up study. *Am J Epidemiol.* 2003;158:486-94.
- [11] Partridge JM, Koutsky LA. Genital human papillomavirus infection in men. *Lancet Infect Dis* 2006; 6:21-31.
- [12] Vaccarella S, Franceschi S, Herrero R, Muñoz N, Snijders PJ, Clifford GM, et al. Sexual behaviour, condom use, and human papillomavirus: pooled analysis of the IARC

human papillomavirus prevalence surveys. *Cancer Epidemiol Biomarkers Prevention* 2006;15: 326-33.

- [13] Wiley D, Masongsong E. Human Papillomavirus: the burden of infection. *Obstet Gynecol Surv.*2006;61(suppl)3-14.
- [14] Smith JS, Herrero R, Bosetti C, Muñoz N, Bosch FX, Eluf-Neto J, Castellsagué X, Meijer C, van den Brule A, Franceschi S, Ashley R, por el International Agency for Research on Cancer (IARC) Multicentric Cervical Cancer Study Group. Herpes simplex virus-2 as a human papillomavirus cofactor in the etiology of invasive cervical cancer. *J Nat Cancer Inst.* 2002;94:1604-13.
- [15] Castellsagué X, Muñoz N. Cofactors in human papillomavirus carcinogenesis-role of parity, oral contraceptives, and tobacco smoking. *J Natl Cancer Inst Monogr.* 2003;31:20-28.
- [16] Palefsky JM, Holly EA. Immunosuppression and co-infection with HIV. *J Natl Cancer Inst Monogr.* 2003;31:41-6.
- [17] Hopman AH, Kamps MA, Smedts F, Speed EJ, Herrington CS, Ramaekers FC. HPV in situ hybridization: impact of different protocols on the detection of integrated HPV. *Int J Cancer.*2005;115:419-28.
- [18] Khan M, Castle P, Lorincz A, Wacholder S, Sherman M, Scott D, et al. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst.* 2005;97:1072-9.
- [19] Trimble C, Genkinger J, Burke A, Hoffman S, Helzlsouer K, Diener-West M, et al. Active and passive cigarette smoking and the risk of cervical neoplasia. *Obstet Gynecol.* 2005;105:174-81.
- [20] Tseng HF, Morgenstern H, Mark T, Peters RK. Risk factors for penile cancer: results of a population-based case-control study in Los Angeles County (United States). *Cancer Causes Control* 2005;2:298.
- [21] Grodzki M, Besson G, Clavel C, Arslan A, Franceschi S, Birembaut P,et al. Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. *Cancer Epidemiol Blomarkers Prev.* 2006;15:820-2.
- [22] Peter M, Rosty C, Couturier C, Radvanyi F, Teshima H, Sastre-Garau X. MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors. *Oncogene.* 2006;25:5985-93.
- [23] Winer R, Hughes J, Feng Q, O'Reilly S, Kiviat N, Colmes K, et al. Condom use and the risk of genital human papillomavirus infection in young woman. *N Engl J Med .* 2006; 354:2645-54.

- [24] Perez S, Cid A, Araujo A, Lamas MJ, Saran MT, Alvarez MJ, et al. A novel real-time genotyping assay for detection of the E6-350G HPV 16 variant. *J Virol Methods* 2011; 173(2):357-63.
- [25] Bernard HU, Burk RD, Chen Z, van Doorslaer K, Hausen Hz, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology* 2010; 401(1):70-79.
- [26] De Villiers EM. Taxonomic classification of papillomaviruses. *Papillomavirus Rep* 2001;12:57-63.
- [27] Davies P, Kornegay J, Iftner T. Current methods of testing for human papillomavirus. *Best Pract Res Clin Obstet Gynecol* 2001; 15:677-700.
- [28] Van der Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJLM, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. *J Clin Microbiol* 2002; 40:779-87.
- [29] Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, et al. Epidemiological classification of human papillomavirus types associated with cervical cancer. *N. Engl.J.Med.* 2003;348:518-27.
- [30] Castellsague X, Diaz M, de San José S, Muñoz N, Herrero H, Franceschi S. et al. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its factors: implications for screening and prevention. *J Nat Cancer Inst* 2006; 985:303-15.
- [31] Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, et al. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer* 2007; 121(3):621-32.
- [32] Bernard HU, Calleja-Macias IE, Dunn ST. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer.* 2006;118:1071–6.
- [33] Picconi MA, Alonio LV, Sichero L, Mbayed V, Villa LL, Gronda J, et al. Human papillomavirus type-16 variants in Quechua aboriginals from Argentina. *J Med Virol.* 2003;69:546–52.
- [34] Muñoz N, Bosch FX, Castellsague X, Diaz M, de San José S, Hammouda D et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004; 111(2):278-85.
- [35] Lont AP, Kroon BK, Horenblas S, Gallee MP, Berkhof J, Meijer CJ et al . Presence of high-risk human papillomavirus DNA in penile carcinoma predicts favorable outcome in survival. *Int J Cancer* 2006; 119(5):21078-81.
- [36] Fairley CK, Donovan B. What can surveillance of genital warts tell us? *Sex Health.* 2010;7(3):325-7.
- [37] Insigna RP, Dasbach EJ, Elbasha EH. Assessing the annual economic burden of preventing and treating anogenital human papillomavirus related disease in the US:

- analytic framework and review of the literature. *Pharmacoeconomics* 2005; 23(11): 1107-22.
- [38] Brown RE, Breugelmans JG, Theodoratou D, Bernard S. Cost of detection and treatment of cervical cancer, cervical dysplasia and genital warts in the UK. *Curr Med Res Opin* 2006;22(4):663-70.
 - [39] Lacey CJ, Lowndes CM, Shah KV. Chapter 4: Burden and management of non-cancerous HPV-related conditions: HPV 6/11 disease. *Vaccine* 2006;Aug 21;24 (suppl 3):35-41.
 - [40] Potocnik M, Kocjan BJ, Seme K, Poljak M. Distribution of human papillomavirus (HPV) genotypes in genital warts from males in Slovenia. *Acta Dermatoven APA* 2007;16(3): 91-6.
 - [41] Cremin S, Menton JF, Canier L, Horgan M, Fanning LJ. The prevalence and genotype of human papillomavirus on cervical samples from an Irish female population with external genital warts. *Hum Vaccin Immunother* 2012; 8 (7): 916-920.
 - [42] IARC .Cancer incidence in five continents, Vol IX. IARC Scientific Publications No. 160.Lyon:IARC, 2007.
 - [43] IARC. Human papillomavirus.IARC Monographs on the Evaluation of carcinomagenic risk to humans.2007, Vol.90.Lyon.
 - [44] D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med* 2007; 356(19): 1944-56.
 - [45] Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clinical Science* 2006; 110: 525-41.
 - [46] Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nature Reviews Immunology* 2004; 4:46-55
 - [47] Origoni M, Cristoforoni P, Costa S, Mariani L, Scirpa P, Lorincz A, et al. HPV-DNA testing for cervical cancer precursors: from evidence to clinical practice. *e-cancer* 2012; 6:258.
 - [48] Ronco G, Giorgi-Ross P, Caorzzi F et al for the NewTechnologies for Cervical Cancer screening (NTCC) Working Group. Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial. *Lancet Oncol*, 2012; 11:259-57.
 - [49] Dillner J. The serological response to papillomavirus. *Semin Cancer Biol* 1999; 9:423-30.
 - [50] Manos MM, Ting Y, Wright DK, Lewis JA, Broker TR, Wolinsky SM. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* 1989; 7:209-14.
 - [51] Bauer HM, Greer CE, Mannos MM. Determination of genital human papillomavirus infection using consensus PCR. C.s. Herrington and J.O.D Mc Gee (ed.). *Diagnostic*

molecular pathology: a practical approach. Oxford University Press, Oxford, United Kingdom. 1992; p.132-152.

- [52] Jacobs MV, Snijders PJ, van den Brule AJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A general primer GP5+/GP6+-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in genital scrapings. *J Clin Microb.*1997;35:791-5.
- [53] Schiffman M, Bauer H, Hoover R, Glass AG, Cadell DM, Rush BB, et al. Epidemiologic evidence showing the HPV infection causes most cervical intraepithelial neoplasia. *J Natl Cancer Inst.* 1993; 85:958–64.
- [54] Kjaer SK, van den Brule AJ, Bock JE, Poll PA, Engholm G, Sherman ME, et al. Human papillomavirus—the most significant risk determinant of cervical intraepithelial neoplasia. *Int J Cancer* 1996; 65:601–06.
- [55] Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N. Engl. Med.* 1998; 338:423-8.
- [56] Dillner J, Rebolj M, Birembaut P, Petry KU, Szarewski A, Munk C, et al. Long term predictive values of cytology and human papillomavirus testing in cervical cancer screening: joint European cohort study. *BMJ* 2008; 337:a1754.
- [57] De Oña M, Álvarez-Argüelles ME, Torrents M, Villa L, Rodríguez-Feijoo A, Palacio A, et al. Prevalence, evolution, and features of infection with human papillomavirus: a 15-year longitudinal study of routine screening of a women population in the north of Spain. *J Med Virol* 2010; 82:597-604.
- [58] Bozzetti M, Nonnenmacher B, Mielzinska I, Villa L, Lorincz A, Breitenbach V et al. Comparison between Hybrid Capture 2 and polymerase chain reaction results among women at low-risk for cervical cancer. *Ann Epidemiol* 2000; 10:466.
- [59] Castle PE, Schiffman M, Burk RD, Hildesheim A, Herrero R, Bratti MC, et al. Restricted cross-reactivity of hybrid capture 2 Test with low-risk human papillomavirus types. *Cancer Epidemiol. Biomark. Prev.* 2002; 11:1394-9.
- [60] Poljak M, Marin IJ, Seme K, Vince A. Hybrid Capture II HPV test detects at least 15 human papillomavirus genotypes not included in its current high-risk probe cocktail. *J Clin Virol* 2002; 25(suppl 3):89-97.
- [61] Ting Y, Manos MM. Detection and typing of genital human papillomaviruses, p. 356-367. In: M. Innis, D. Gelfand, J. Sninsky, and T. White (ed.), *PCR Protocols: A Guide to Methods and Applications*. 1990 Academic Press, Inc., San Diego, Calif.
- [62] Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, et al. Development and clinical evaluation of highly sensitive PCR-reverse hybridization line probe assay of detection and identification of anogenital human papillomavirus. *J Clin Microbiol* 1999; 37:2508-17.

- [63] Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlée F, Hildesheim A, et al. Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000; 38: 357-61.
- [64] Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qvar-forth-Tubbin P, Andersen PK, et al. Viral load of human papillomavirus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. *Lancet*, 2000.355:2189-93.
- [65] Ylitalo N, Josefsson A, Melbye M, Sorensen P, Frisch M, Andersen PK et al. A prospective study showing long-term infection with human papillomavirus 16 before the development of cervical carcinoma in situ. *Cancer Res* 2000; 60:6027-32.
- [66] Tucker RA, Unger ER, Holloway BP, Swan DC. Real-Time PCR-based fluorescent assay for quantitation of human papillomavirus types 6,11,16 and 18. *Mol Diagn* 2001;6:39-47.
- [67] Strauss S, Desselberger U, Gray JJ. Detection of genital and cutaneous human papillomavirus types: differences in the sensitivity of generic PCRs, and consequences for clinical virological diagnosis. *Br J Biomed Sci* 2000; 57:221-5.
- [68] Cubie HA, Seagar AL, McGoogan E, Whitehead J, Brass A, Arends MJ, et al. Rapid real-time PCR to distinguish between human papillomavirus types 16 and 18. *Mol Pathol* 2001;54:24-9.
- [69] Sotlar K, Stubner A, Diemer D, Menton S, Menton M, Dietz K, Wallwiener D, Kandolf R, Bültmann B. Detection of high-risk human papillomavirus E6 and E7 oncogene transcripts in cervical scrapes by nested RT-polymerase chain reaction. *J Med Virol*. 2004;74(1):107-16.
- [70] Schiffman M. Integration of human papillomavirus vaccination, cytology and human papillomavirus testing. *Cancer* 2007;111(3):145:53.
- [71] Wright Jr TC, Massad LS, Dunton CJ, Spitzer M, Wilkinson EJ, Solomon D. 2006 consensus guidelines for the management of women with abnormal cervical screening test. *J low Genit Tract Dis* 2007;11(4):201-22.
- [72] Rodriguez AC, Schiffman M, Herrero R, Wacholder S, Hildesheim A, Castle PE, et al. Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections. *J Natl Cancer Inst* 2008;100(7):513-7.
- [73] Torres M, Fraile L, Echevarría JM, Hernández-Novoa B, Ortiz M. Humana Papillomavirus (HPV) Genotyping: automation and application in routine laboratory testing. *Open Virol J*, 2012; 6:144-150.
- [74] Serrano ML, Correa M, Medina O, Melgarejo D, Bravo MM. Tipificación de virus del papiloma humano mediante secuencia directa en mujeres con citología normal. *Rev Colomb Cancer*. 2003;7: 18-24.
- [75] Fontaine V, Mascaux C, Weyn C, Bernis A, Celio N, Lefevre P, et al. Evaluation of combined general primer-mediated PCR sequencing and type-specific PCR strategies

for determination of human papillomavirus genotypes in cervical cell specimens. *J Clin Microbiol* 2007;45: 928-34.

- [76] Choi YD, Jung WW, Nam JH, Choi HS, Park CS. Detection of HPV genotypes in cervical lesions by the HPV DNA chip and sequencing. *Gynecol Oncol* 2005;98: 369-75.
- [77] Schiffman M, Kjaer SK. Natural history of anogenital human papillomavirus infection and neoplasia. *J Natl Cancer Inst Monogr.* 2004 ;31:14-9.
- [78] Clifford GM, Gallus S, Herrero R, Muñoz N, Snijders PJ, Vaccarella S, et al. Worldwide distribution of human papillomavirus types in cytologically normal women in the IARC HPV prevalence surveys: a pooled analysis. *Lancet* 2005;366(9490):991-8.
- [79] Van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbels MA, et al. Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. *Int J Cancer* 2002; 98:590-5.
- [80] Carcopini X, Henry M, Olive D, Boubli L, Tamalet C. Detection and quantification of human papillomavirus genital infections: virological, epidemiological, and clinical applications. *Med Mal Infect* 2011;41 (29):68-79.
- [81] Gravitt PE, Peyton CL, Wheeler C, Apple R, Higuchi R, Shah KV. Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. *J Virol Methods* 2003; 112: 23–33.
- [82] Weissenborn SJ, Funke AM, Hellmich M, Mallmann P, Fuchs PG, Pfister HJ, et al. Oncogenic human papillomavirus DNA loads in human immunodeficiency virus-positive women with high-grade cervical lesions are strongly elevated. *J Clin Microbiol* 2003;41: 2763–67.
- [83] Tábor N, Ferrera A, Bakkers J, Massuger L, Melchers WJG. High HPV 16 Viral Load is Associated with Increased Cervical Dysplasia in Honduran Women. *Am J Trop Med Hyg* 2008; 78(5):843-6.
- [84] Fontaine J, Hankins C, Mayrand MH, Lebre J, Money D, Gagnon S et al. High levels of HPV-16 DNA are associated with high-grade cervical lesions in women at risk or infected with HIV. *AIDS* 2005;19(8):785-94.
- [85] Sun CA, Liu JF, Wu DM, Nieh S, Yu CP, Chu TY. Viral load of high-risk human papillomavirus in cervical squamous intraepithelial lesions. *Int J Gynaecol Obstet* 2002; 76: 41–7.
- [86] Dalstein V, Riethmuller D, Pretet JL, Le Bail Carval K, Sautiere JL, Carbillet JP, et al. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int J Cancer* 2003; 106: 396–403.
- [87] Nagao S, Yoshinouchi M, Miyagi Y, Hongo A, Kodama J, Itoh S, et al. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. *J Clin Microbiol* 2002;40: 863–7.

- [88] Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* 2002; 40: 886–91.
- [89] Castle PE, Dockter J, Giachetti C, Garcia FA, Mc Cormick MK, Mitchell AI, et al. A cross-sectional study of a prototype carcinogenic human papillomavirus E6/E7 messenger RNA assay for detection of cervical precancer and cancer. *Clin Cancer Res* 2007; 13(9): 2599-605.
- [90] Arbyn M, Ronco G, Cuzick J, Wentzensen N, Castle PE. How to evaluate emerging technologies in cervical cancer screening? *Int J Cancer*. 2009;125(11):2489-96.
- [91] Halfon P, Benmoura D, Agostini A, Khiri H, Martineau A, Penaranda G, et al. Relevance of HPV mRNA detection in a population of ASCUS plus women using the NucliSENS EasyQ HPV assay. *J Clin Virol*. 2010;47(2):177-81.
- [92] Benevolo M, Vocaturo A, Caraceni D, French D, Rosini S, Zappacosta R, et al. Sensitivity, specificity, and clinical value of human papillomavirus (HPV) E6/E7 mRNA assay as a triage test for cervical cytology and HPV DNA test. *J Clin Microbiol*. 2011;49(7):2643-5.
- [93] Perez S, Iñarrea A, Lamas MJ, Saran MT, Cid A, Alvarez MJ, et al. Human Papillomavirus (HPV) E6/E7 mRNA assay as a triage test after HPV16 and 18 DNA detection. *J Med Virol*, 2012; in press.
- [94] Lamarcq L, Deeds J, Ginzinger D, Perry J, Padmanabha S, Smith Mc Cune K. Measurements of human papillomavirus transcripts by real time quantitative reverse transcription polymerase chain reaction in samples collected for cervical cancer screening. *J Mol Diagn* 2002;4(2):97-102.
- [95] Wang-Johanning F, Lu DW, Wang Y, Johnson MR, Johanning GL. Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep papanicolau test using realtime polymerase chain reactions analysis. *Cancer* 2002; 94(8):2199-210.
- [96] Kraus I, Molden T, Erno LE, Skomedal H, Karlsen F, Hagmar B. Human papillomavirus oncogenic expression in dysplastic portio; an investigation of biopsies from 190 cervical cones. *Br J Cancer* 2004;90:1407-13.
- [97] Lie AK, Risberg B, Delabie J, Begum S, Rimalla R, Hagen B. DNA versus RNA based methods for HPV testing in screening evaluation of Hybrid Capture II and pre-tect HPV proofer in Norway 2004. In: *Proceedings of the 21st International Papillomavirus Conference*; 2004.p.55 [abstract].
- [98] Kalantari M, Calleja-Macias IE, Tewari D, Hagmar B, Lie K, Barrera-Saldana HA, et al. Conserved methylation patterns of human papillomavirus type 16 DNA in asymptomatic infection and cervical neoplasia. *J. Virol*. 78: 12762-2.

- [99] Wentzensen N, Vinokurova S, von Knebel Doeberitz M. Systematic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. *Cancer Res* 2004;64: 3878–84.
- [100] Pett M, Coleman N. Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? *J Pathol* 2007;212: 356–67.
- [101] Vinokurova S, Wentzensen N, Kraus I, Klaes R, Driesch C, Melsheimer P, et al. Type dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer Res* 2008;68(1):307-13.
- [102] Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, et al. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *Journal of virology* 1987;61: 962–971.
- [103] Romanczuk H, Howley PM. Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proceedings of the National Academy of Sciences of the United States of America* 1992;89: 3159–63.
- [104] Hafner N, Driesch C, Gajda M, Jansen L, Kirchmayr R, Runnebaum IB et al. Integration of the HPV16 genome does not invariably result in high levels of viral oncogene transcripts. *Oncogene* 2008;27: 1610–7.
- [105] Luft F, Klaes R, Nees M, Duerst M, Heilmann V, Melsheimer P, et al. Detection of integrated papillomavirus sequences by ligation-mediated PCR (DIPS-PCR) and molecular characterization in cervical cancer cells. *Int J Cancer* 2001; 92(1):9-17.
- [106] Bernard HU, Calleja-Macias IE, Dunn ST. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int J Cancer*. 2006; 118:1071–6.
- [107] Yamada T, Mannos MM, Peto J, Greer CE, Muñoz N, Bosch FX, et al. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective *J Virol* 1997;71 :2463-72.
- [108] Xi LF, Kiviat NB, Hildesheim A, Galloway DA, Wheeler CM, Ho J, et al. Human papillomavirus type 16 and 18 variants: race-related distribution and persistence. *J Natl Cancer Inst* 2006; 98: 1045-52.
- [109] Zuna RE, Moore WE, Shanesmith RP, Dunn ST, Wang SS, Schiffman M, et al. Association of HPV16 E6 variants with diagnostic severity in cervical cytology samples of 354 women in a US population. *Int J Cancer* 2009;125: 2609-13.
- [110] Sabol I, Cretnik M, Hadzisejdić I, Si-Mohamed A, Matovina M, Grahovac B, Levanat S, Grce M. A new approach for the evaluation of the human papillomavirus type 16 variability with high resolution melting analysis. *J Virol Methods*. 2009;162(1-2):142-7. Epub 2009 Aug 5.
- [111] Swan DC, Limor JR, Duncan KL, Rajeevan MS, Unger ER. Human papillomavirus type 16 variant assignment by pyrosequencing. *J Virol Methods*. 2006;136(1-2):166-70.

- [112] Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U et al. Overexpression of p16 as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001;92:276–84.
- [113] O'Neill CJ, McCluggage WG. p16 expression in the female genital tract and its value in diagnosis. *Adv Anat Pathol*. 2006;13(1):8-15.
- [114] Mao C, Balasubramanian A, Yu M, Kiviat N, Ridder R, Reichert A, et al. Evaluation of a new p16(INK4A) ELISA test and a high-risk HPV DNA test for cervical cancer screening: results from proof-of-concept study. *Int J Cancer*. 2007 1;120(11):2435-8.
- [115] Wentzensen N, Bergeron C, Cas F, Eschenbach D, Vinokurova S, von Knebel Doeberitz M. Evaluation of a nuclear score for p16INK4a-stained cervical squamous cells in liquid-based cytology samples. *Cancer Cytopathology* 2005; 105(6):461–7.
- [116] Esteller M. Epigenetics in cancer. *N Engl J Med*. 2008 Mar 13;358(11):1148–59.
- [117] Wentzensen N, Sherman ME, Schiffman M, Wang SS. Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecol Oncol*. 2009;112(2):293-9.
- [118] Gargiulo G, Minucci S. Epigenomic profiling of cancer cells. *Int J Biochem Cell Biol*. 2009;41(1):127-35.
- [119] Sugita M, Tanaka N, Davidson S, Sekiya S, Varella-Garcia M, West J, et al. Molecular definition of a small amplification domain within 3q26 in tumors of cervix, ovary, and lung. *Cancer Genet Cytogenet*. 2000;117:9–18.
- [120] Hopman AH, Theelen W, Hommelberg PP, Kamps MA, Herrington CS, Morrison LE, et al. Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC at 3q26 are strongly associated events in the progression of uterine cervical dysplasia to invasive cancer. *J Pathol*. 2006;210:412–9.
- [121] Caraway NP, Khanna A, Dawlett M, Guo M, Guo N, Lin E, et al. Gain of the 3q26 region in cervicovaginal liquid-based pap preparations is associated with squamous intraepithelial lesions and squamous cell carcinoma. *Gynecol Oncol*. 2008;110:37–42.
- [122] Meyerson M. Role of telomerase in normal and cancer cells. *J Clin Oncol*. 2000;18:2626–34.
- [123] Ramsaroop R, Oei P, Ng D, Kumar N, Cotter PD. Cervical intraepithelial neoplasia and aneusomy of TERC: assessment of liquid-based cytological preparations. *Diagn Cytopathol* 2009; 37: 411-5.
- [124] Sui W, Ou M, Dai Y, Chen J, Lan H, Yan Q, et al. Gain of the human telomerase RNA gene TERC at 3q26 is strongly associated with cervical intraepithelial neoplasia and carcinoma. *Int J Gynecol Cancer* 2009; 19: 1303-1306.
- [125] Zhang Y, Wang X, Ma L, Wang Z, Hu L. Clinical significance of hTERC gene amplification detection by FISH in the screening of cervical lesions. *J Huazhong Univ Sci Technolog Med Sci* 2009; 29: 368-71.

- [126] Jalali GR, Herzog TJ, Dziura B, Walat R, Kilpatrick MW. Amplification of the chromosome 3q26 region shows high negative predictive value for nonmalignant transformation of LSIL cytologic finding. *Am J Obstet Gynecol* 2010; 202: 581.e1-e5.
- [127] Jiang J, Wei LH, Li YL, Wu RF, Xie X, Feng YJ, et al. Detection of TERC amplification in cervical epithelial cells for the diagnosis of high-grade cervical lesions and invasive cancer: a multicenter study in China. *J Mol Diagn* 2010; 12: 808-17.
- [128] Guilleret I, Yan P, Guillou L, Braunschweig R, Coindre JM, Benhattar J. The human telomerase RNA gene (hTERC) is regulated during carcinogenesis but is not dependent on DNA methylation. *Carcinogenesis*. 2002;23:2025–2030.
- [129] Lan YL, Yu L, Jia CW, Wu YM, Wang SY. Gain of human telomerase RNA gene is associated with progression of cervical intraepithelial neoplasia grade I or II. *Chin Med J (Engl)*. 2012;125(9):1599-602.
- [130] Giannoudis A, Herrington SC. Human papillomavirus variants and squamous neoplasia of the cervix. *J Pathol* 2001;193:295–302.
- [131] Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, et al. p16INK4A immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol*, 2002;26:1389–99.
- [132] Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, et al. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep™ smears. *J Clin Pathol* 2003;56:53–63.
- [133] Bonds L, Baker P, Gup C, Shroyer KR. Immunohistochemical localization of CDC6 in squamous and glandular neoplasia of the uterine cervix. *Arch Pathol Lab Med* 2002;126:1162–8.
- [134] Grce M, Matovina M, Milutin-Gasperov N, Sabol I. Advances in cervical cancer control and future perspectives. *Coll Antropol* 2010;34:731-6.
- [135] Tamalet C, Richet H, Carcopino X, Henry M, Leretraite L, Heid P, et al. Testing for human papillomavirus and measurement of viral load of HPV 16 and 18 in self-collected vaginal swabs of women who do not undergo cervical cytological screening in Southern France. *J Med Virol* 2010;82:1431-7.
- [136] Gravitt PE, Coutlée F, Iftner T, Sellors JW, Quint WGV, Wheeler CM. New technologies in cervical cancer screening. *Vaccine* 2008, 26(suppl) 42-52.