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Human Papillomavirus Laboratory Testing: the Changing Paradigm

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SUMMARY

High-risk human papillomaviruses (HPVs) cause essentially all cervical cancers, most anal and oropharyngeal cancers, and some vaginal, vulvar, and penile cancers. Improved understanding of the pathogenesis of infection and the availability of newer tests are changing the approach to screening and diagnosis. Molecular tests to detect DNA from the most common high-risk HPVs are FDA approved for use in conjunction with cytology in cervical cancer screening programs. More-specific tests that detect RNA from high-risk HPV types are now also available. The use of molecular tests as the primary screening tests is being adopted in some areas. Genotyping to identify HPV16 and -18 has a recommended role in triaging patients for colposcopy who are high-risk HPV positive but have normal cytology. There are currently no recommended screening methods for anal, vulvar, vaginal, penile, or oropharyngeal HPV infections. HPV testing has limited utility in patients at high risk for anal cancer, but p16 immunohistochemistry is recommended to clarify lesions in tissue biopsy specimens that show moderate dysplasia or precancer mimics. HPV testing is recommended for oropharyngeal squamous cell tumors as a prognostic indicator. Ongoing research will help to improve the content of future guidelines for screening and diagnostic testing.

INTRODUCTION

Papillomaviruses are common worldwide and are known to infect birds and most mammals, including humans. Because of similar structure, papillomaviruses and polyomaviruses were initially taxonomically positioned in the same family, *Papoviridae*. Both are small (40- to 60-nm) viruses with nonenveloped icosahedral capsids and carry their genetic material as circular double-stranded DNA associated with histones. The size of the genome is about 8 kb for papillomaviruses and 5 kb for polyomaviruses, and, with the exception of a small homologous segment in the polyomavirus T-antigen and papillomavirus E1 genes that correspond to a helicase, they do not share any substantial sequence similarity. Further, although both viruses replicate in the host cell nucleus, the replication strategies are very different, with papillomavirus DNA transcription occurring on only one DNA strand in one direction while polyomavirus DNA replication is bidirectional. Because of these differences, papillomaviruses have been separated into their own family, *Papillomaviridae*. Papillomaviruses contain stable DNA genomes that are replicated with high fidelity by the host cell machinery. Sequence changes by mutation or recombination are rare events and appear to occur at frequencies similar to those in the DNA genomes of the infected host. Papillomaviruses are generally considered to be species specific; however, there are rare reports of cross-species infection, such as human papillomavirus DNA associated with cutaneous squamous lesions of the cat (1, 2).

Human papillomavirus (HPV) is the broad term referring to the group of related papillomavirus strains that infect humans. HPV genomes are similarly organized and are divided into three major regions (early genes, late genes, and an upstream regulatory region) separated by two polyadenylation (pA) sites (early pA and

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late pA). The upstream regulatory region, usually referred to as the long control region (LCR) (but also referred to as the noncoding region [NCR] or upstream regulatory region [URR]), covers about 10% of the genome and contains the origin of replication, multiple transcription binding sites, and the early and late open reading frames (ORFs). The early region comprises just over 50% of the genome and carries 6 ORFs, i.e., E1, E2, E4, E5, E6, and E7. Early genes E1 and E2 are involved in initiation of viral DNA replication and regulation of early transcription. The E4 protein is expressed during productive infection and is thought to facilitate virus assembly and release of virions from infected cells. The E5, E6, and E7 genes are not universally present in all HPV types and function to modify the cellular environment to support completion of virus replication. The E5 gene is notably absent in the beta-HPVs (3). Presence of the E5 gene is associated with oncogenic potential, but sequence analysis has revealed that the E5 proteins produced by different HPV types are varied and distantly related (3). In high-risk HPV types, the E5 proteins have been shown to interact with a variety of cellular targets, with subsequent effects that may contribute to transformation (3). These effects include overexpression of epidermal growth factor receptors and a variety of proto-oncogenes, evasion of immune detection, and inhibition of apoptosis. E5 proteins are weakly transforming when expressed alone but have been shown to enhance the transforming ability of E6 and E7. The major transforming activity of high-risk HPVs is due to the E6 and E7 genes, which interfere with regulators of the host cell cycle and control of transcription (4). HPV101, HPV103, and HPV108 are unusual in that they do not have an E6 gene, similar to some animal papillomaviruses (5). The late region occupies almost 40% of the genome, and late genes L1 and L2 encode the major and minor viral capsid proteins used in the construction of new viruses.

Papillomavirus taxonomy has been deliberated over the past several decades and continues to be reviewed The present classification system was accepted in 2003, and there have been proposals for future amendments to harmonize the common naming usage in the scientific community with official groupings recognized by the International Committee on Taxonomy of Viruses (ICTV) (5, 6). The current approach to papillomavirus taxonomy does not follow the classic rules for nomenclature, since there is not a practical culture or serological system. Papillomavirus classification is based on the sequence of a 291-bp segment in a highly conserved region of the L1 ORF gene spanned by primers MY09 and MY11 (5, 6).

Papillomavirus genera (previously called "supergroups" or "major branches") share more than 23% but less than 43% nucleotide sequence identity in full-length sequence genomes and less than 60% nucleotide sequence identity in the L1 ORF. Papillomavirus genera are designated by a Greek letter (e.g., alphapapillomaviruses, betapapillomaviruses, etc.). There are currently 39 genera in the family *Papillomaviridae*. The HPVs are contained in five of those genera (alphapapillomaviruses, betapapillomaviruses, gammapapillomaviruses, mupapillomaviruses, and nupapillomaviruses) (Fig. 1). The non-human papillomavirus types that have been characterized to date are assigned across all of the different genera, including alphapapillomaviruses and betapapillomaviruses, leaving only the gammapapillomavirus, mupapillomavirus, and nupapillomavirus genera to solely contain only HPV types.

Papillomavirus clusters below the level of genus have been designated species in common usage, supplanting the previously used terms "groups," "subgroups," or "minor branches" (7). Species within a genus share between 60% and 70% L1 ORF nucleotide sequence identity. Phylogenetic groups of papillomaviruses at the level of species are designated by the Greek letter associated with the genus combined with a number, e.g., alpha-9 (Fig. 1).

Strains within an HPV species are commonly described as "types" and are designated HPV followed by a number (e.g., HPV16). New papillomavirus types are assigned when the completely characterized full-length genome has been deposited at the International HPV Reference Center at the Karolinska Institutet, Stockholm, Sweden, and the L1 ORF sequence is verified as differing by more than 10% (less than 90% similarity) from the closest known HPV type. Currently, 201 papillomavirus types isolated from humans (HPV1 to HPV205) have been completely sequenced and deposited. HPV46, -55, -64, and -79 were described prior to foundation of the reference center but did not meet the criteria as unique HPV types, so they were reclassified and their numbers were left vacant.

Common usage suggests that differences between 2% and 10% homology define a subtype and differences less than 2% define a variant. ICTV papillomavirus nomenclature, if accepted, would leave the current designation of genera but would raise the present papillomavirus types to species and the present species to subgenera.

The vast majority of HPV types cause benign warts of the skin or genital region (Table 1) (8–13). Some HPV types have the potential to cause lesions that progress to cancer. Alphapapillomaviruses are categorized as high-risk or low-risk according to the likelihood that an infection by the HPV type can lead to the development of cancer. In 2012, the International Agency for Research on Cancer (IARC) categorized HPVs as group 1 (carcinogenic to humans) carcinogens, group 2A (probably carcinogenic to humans) carcinogens, and group 2B (possibly carcinogenic to humans) carcinogens (Fig. 1). The 12 HPVs in IARC group 1 (i.e., HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59) are considered high-pressure liquid chromatography risk. There is less evidence for including HPV68 as high risk, so it is classified in group 2A as probably carcinogenic. Ninety-six percent of cervical cancers are attributable to one of these 13 HPV types (groups 1 and 2A) (14). There are other alphapapillomaviruses, i.e., HPV26, HPV30, HPV34, HPV53, HPV66, HPV67, HPV 69, HPV70, HPV73, HPV82, HPV85, and HPV97, that have been associated with rare cases of cervical cancer and are considered group 2B probable carcinogens. Because the number of cases due to the group 2B HPV types is smaller, carcinogenicity is somewhat more difficult to assess. However, studies have shown that markers of HPV-induced carcinogenesis, such as E6 mRNA, increased expression of p16, and decreased expression of cyclin D1, p53, and Rb, are similar in cervical cancers among all group 1, 2A, and 2B carcinogens (14). Including the 2.6% of cervical cancers due to the group 2B HPV types with the 96% due to group 1 and 2a types brings the total to 98.7% of all HPV-positive cervical cancers. Additional data show that HPV68, HPV26, HPV66, HPV67, HPV73, and HPV82, although rare, are significantly more common in cases of cervical cancer than in women with normal cervical cytology, and upgrading the carcinogen classification could be considered (14, 15).

HPVs are highly tissue tropic, and infection is limited to strat-

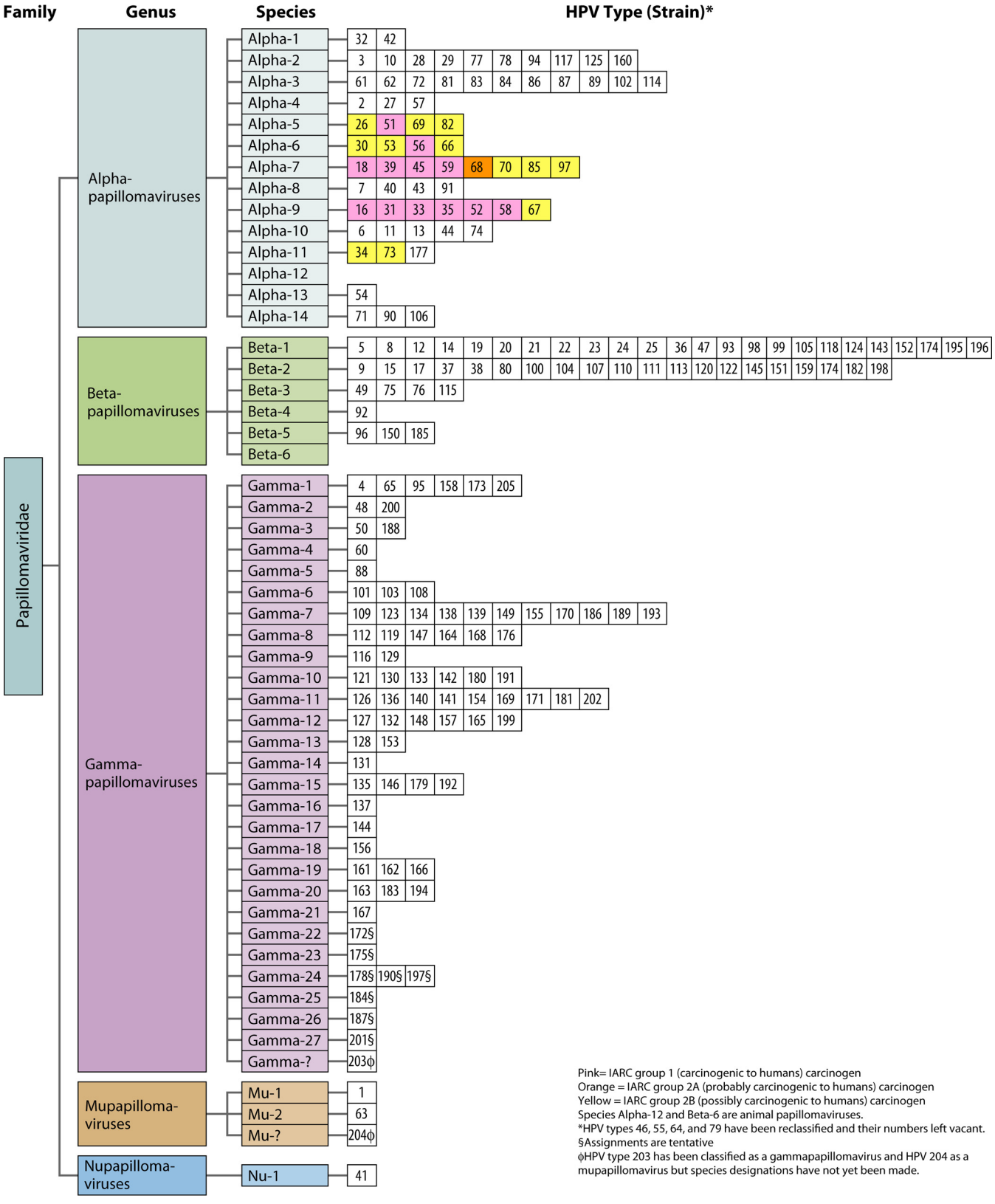

FIG 1 HPV classification based on the nucleotide sequence of the capsid protein L1 gene.

TABLE 1 Disease and most frequently associated HPV types[a]

Disease	Most frequently associated HPV type(s)[b]
Nongenital skin disease	
Benign	
Common warts	1, 2, 4, 7; occasionally other types, especially in immunosuppressed patients (e.g., HPV75 to -77)
Palmoplantar warts	1, 2, 4
Flat plane warts	3, 10; less frequently 26 to 29, 41
Ungual warts	1, 2, 4, 27, 57
Butcher's warts	2, 7
Epidermodysplasia verruciformis	5, 8
Malignant	
Ungual squamous cell carcinoma	16, 18
Epidermodysplasia verruciformis	5, 8
Oral mucosal disease	
Benign	
Respiratory papillomatosis	6, 11
Laryngeal papilloma (recurrent respiratory papillomatosis)	6, 11
Oral squamous cell papillomas	6, 11
Oral focal epithelial hyperplasia of Heck	13, 32
Potentially malignant	
Oral lichen planus	6, 11, 16
Oral leukoplakia	6, 11, 16
Oral erythroplakia	6, 11, 16
Malignant	
Oropharyngeal carcinoma, squamous cell	16
Ocular mucosal disease	
Benign	
Conjunctival papillomas	6, 11
Malignant	
Squamous cell carcinoma of conjunctiva	16
Anogenital disease	
Benign	
Condyloma acuminate	6, 11
Anogenital warts	6, 11, 40, 42, 43, 44, 54, 61, 72, 81, 89
Giant condylomata (Buschke-Löwenstein tumors)	6
Potentially malignant	
Bowenoid papulosis	16, 55
Malignant	
Carcinoma of vulva	16, 18
Carcinoma of vagina	16, 18
Squamous cell carcinoma of cervix	Group 1 (carcinogenic to humans), 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59; group 2A (probably carcinogenic to humans), 68; group 2B (possibly carcinogenic to humans), 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97
Adenocarcinoma of cervix	16, 18, 45
Squamous cell carcinoma of anus	16, 18
Carcinoma of penis	16, 18, 6, 11

[a] Data are from references 8, 10, 11, 12, 13, and 164.
[b] Data are combined from review references but not meant to be all-inclusive.

ified epithelium at either cutaneous or mucosal surfaces, usually at specific body sites. HPVs in the alphapapillomavirus genus cause both mucosal and cutaneous lesions. Beta-, gamma-, mu-, and nu-HPVs cause cutaneous lesions. HPV infection can follow either of two pathways: productive or transforming (nonproductive or abortive). Recent studies suggest that productive infection is supported by the normal basal epithelial stem cells found at the ectocervix but that nonproductive infection is promoted in the specialized stem cell populations at the transformation zone (16). Productive infection can be produced by either low- or high-risk HPV types. The initial steps are the same regardless of the pathway. It is thought that the virions enter primitive basal epithelial cells by a complex binding mechanism which appears to be preceded by some degree of trauma in most cases. Virions initially bind to heparan sulfate proteoglycans on the surface of basal epithelial cells or basement membrane, which exposes a furin/proprotein convertase cleavage site at the amino terminus of L2 (16, 17). It is also thought that HPV entry requires interaction with a

secondary receptor, which has not yet been fully characterized (17). It is believed that host antibodies to the virus coat protein L1 play a major neutralizing role and are effective while the virions still reside on the basement membrane or cell surfaces (18). If infection continues, the virus is taken into the basal cell through clathrin-, caveolin-, lipid raft-, flotillin-, cholesterol-, and dynamin-independent endocytosis (17). The viral DNA is transported to the host cell nucleus in complex with the L2 minor capsid protein. An initial round of genome amplification occurs independent of the host cell cycle, and copy numbers reach about 100 nuclear episomes per cell (18). The infectious process is slow, taking 12 to 24 h for initiation of transcription. The virus is maintained in low copy numbers in the nuclear episome phase, with minimal expression of early proteins E1 and E2, until the host keratinocyte begins to divide. In productive infection, the expression of viral genes necessary for replication is controlled, with replication of the viral genome occurring about once per cell cycle. When the E2 protein reaches high levels, expression of the E6 and E7 genes is turned off and a read-through into the late region of the genome occurs, resulting in expression of HPV late genes L1 and L2 (19). The epithelial cells become increasingly differentiated as they reach the upper layers of the epithelium, and completion of the viral life cycle depends entirely on the mitotic activity of the host cell to supply DNA polymerases and other necessary replication factors. Virus assembly occurs in nondividing, terminally differentiated keratinocytes of the superficial layers. In productive infections, the HPV genome is propagated in episomal form, and this is a polyclonal proliferative process with viral copies increasing about two to four logs (16). Eventually, most of the virus-containing keratinocytes desquamate and are removed from the body by natural processes. HPV virions can be released as a result of degeneration of the infected exfoliated host cells. In productive infection, HPV DNA can therefore be measured inside as well as outside host cells (20). This has important implications for laboratory testing. The time from infection to release of infective virions can vary from weeks to months. HPV can persist in the environment and remain infectious for at least several days without a host (21). Productive infections produce huge amounts of virions (with HPV DNA inside) but never lead to cancer because desquamating cells never divide and are eliminated from the body. Productive HPV infections are transient, and HPV becomes undetectable after several months.

In the nonproductive (transforming) pathway, the ordered expression of genes does not occur and the normal HPV life cycle cannot be completed, resulting in a nonproductive or abortive infection (17). The highest risk for cancer development occurs as a result of prolonged persistent infection over many years. The nature of the replicative cycle of high-risk HPV types and a battery of immune evasion and subversion mechanisms allow the viral genome to be maintained at an almost constant copy number over many cell divisions (22). In this process, the viral genomes replicate once along with host cellular DNA during the S phase of the cell cycle and are divided equally between the two resulting daughter cells (16). Rarely in the process of persistent episomal viral replication, the HPV DNA randomly linearizes and integrates into the host cell genome. HPV integration occurs randomly at many sites in the human genome, with a tendency to prefer genomic fragile sites (23, 24). The break in the viral genome usually occurs in the E2 ORF and causes deletion or disruption of the E2 gene while retaining the E6 and E7 oncogenes and the LCR. Integration results in downregulation of E2-mediated transcriptional repression and leads to overexpression of the E6 and E7 oncogenes as well as activation of human telomerase.

In vitro studies have shown that integration of HPV DNA occurs during long-term culture and is preceded by a rapid decrease in episomal HPV DNA (25). During the period of integration, some cells contain a mixture of integrated HPV DNA and episomal HPV DNA at a reduced load. These cells express lower levels of E2 and show partial inhibition of transcriptional activity from the integrated DNA, likely because of suppression by E2 proteins from the episomal forms. Uncontrolled high-level expression of E6 and E7 oncogenes as a result of integration is not observed until E2-expressing episomes are completely lost.

In vivo, a significant proportion of invasive tumors have been noted to contain integrated HPV forms, but in some studies integrated forms have not been consistently detected (24, 26). There does appear to be a consistent association between increasing episomal viral loads and severity of lesions (27). Because of these data, newer models of HPV carcinogenesis suggest that higher loads of episomal HPV could result in increased expression of viral oncogenes. Studies further suggest that integration occurs relatively late in the progression of high-grade lesions and is a consequence of an overall destabilization of chromosomal integrity in replicating epithelial cells that express the viral E6 and E7 oncogenes (23). Further studies that evaluate the transcriptional activity of integrated forms in the presence and absence of episomal forms will contribute to our understanding of the role of integration and which integrated forms contribute to transformation in cancerous lesions (23, 24).

The E6 and E7 proteins in high-risk HPV types are functionally different from those in low-risk types. In high-risk types they interfere with regulation of the cell cycle, apoptosis, and maintenance of chromosomal stability, which triggers proliferation of infected basal and suprabasal cells, allowing expansion of the lesion. Extensive studies have shown that the effects of E6 and E7 are attributed to the inactivation of two host cell tumor suppressor proteins, p53 and retinoblastoma proteins (pRb). Binding of the viral E7 protein to pRb causes release of E2F and other proteins that serve as signals for the cell cycle to progress. As long as the E7 protein stays attached to pRb, uncontrolled cell proliferation will continue. The HPV E6 protein is a ubiquitin ligase and contributes to oncogenesis by attaching ubiquitin molecules to p53, thereby making it inactive and subject to proteosomal degradation. The normal function of p53 is to stop cell division and repair damaged DNA so that damaged cells do not reproduce. When p53 is inactive, cells with changes in the DNA, such as integrated viral DNA, are not repaired. This destabilizes the cell and further increases the risk of malignant transformation. HPV E6 protein also activates telomerase, which synthesizes telomere repeat sequences and maintains a repeated cell cycle that continues to produce infected cells. The disruption of the normal p53 and pRb/E2F cell cycle regulatory mechanisms leads to the emergence of clonal cell populations that form a lesion. Evan at this stage, lesions frequently regress, and HPV becomes undetectable by molecular tests within 1 to 2 years. In only a small portion (10 to 20%) of infected individuals, lesions progress to develop virus-associated cancers and precancers (18). Molecular biology studies have firmly established a causal relationship between persistent infection with high-risk HPV genotypes and several types of cancer (Table 2) (28). The types that have been classified as carcinogenic

TABLE 2 Cancers attributable to HPV in the United States[a]

Anatomic site	Cases attributable to HPV		Annual no. of cases attributable to HPV	
	%	Range (%)	Avg	Range
Cervix	96	95–97	11,500	11,400–11,600
Vulva	51	37–65	1,600	1,200–2,000
Vagina	64	43–82	500	300–600
Penis	36	26–47	400	300–500
Anus	93	86–97	2,900 women 1,600 men	2,700–3,000 1,400–1,600
Oropharynx	63	50–75	1,500 women 5,900 men	1,200–1,800 4,700–7,000

[a] Adapted from reference 28.

to humans can differ by an order of magnitude in risk for development of cancer and additional factors may increase the risk of developing cancer following a high-risk HPV infection. Two HPV types, HPV16 and HPV18, are responsible for about 70% of all cervical cancer cases worldwide (29). HPV also causes anal cancer, with about 85% of all cases caused by HPV16 (30). HPV16 and -18 have also been found to cause close to half of vaginal, vulvar, and penile cancers (30). In the United States, more than half of the cancers diagnosed in the oropharynx are linked to HPV16 (31).

OROPHARYNGEAL CANCER

Squamous cell carcinomas of the head and neck (HNSCC), including the oral cavity, pharynx, and larynx, have historically been diagnosed in older individuals with a history of tobacco use and/or heavy alcohol consumption. The nitrosamines and benz-(*a*)-pyrene carcinogens found in tobacco produce the types of guanine nucleoside changes found in the p53 mutations seen in HNSCC. There is sufficient evidence that smokeless tobacco, smoking tobacco, and betel quid with and without tobacco are carcinogenic agents. The effects of alcohol are somewhat less well defined, but it is known that the ethanol in alcoholic beverages inhibits production of the p53 protein, and there is sufficient evidence that alcohol is a carcinogenic agent. A diet poor in fiber and vitamins, poor dentition, and polymorphisms in the genes involved in the metabolism of tobacco and alcohol carcinogens and DNA repair may also affect the higher risk of development of oral cavity cancers in some individuals (32). HNSCC tumors typically present late, are aggressive, do not respond well to high doses of radiation/chemotherapy, and are associated with high mortality (35 to 38% overall survival) (33, 34).

As early as 1983, histopathologic features consistent with HPV were identified in the subset of HNSCC occurring specifically in the oropharynx (middle part of the throat, including the soft palate, the base of the tongue, tonsil, uvula, and Waldeyer's ring) (35). Subsequent studies and stringent meta-analyses have confirmed that oropharyngeal squamous cell carcinomas (OPSCC) constitute a distinct molecular, pathologic, and clinical entity attributable to chronic HPV infection and minimally associated with tobacco or alcohol use (36, 37). The tonsil is most commonly affected. HPV may rarely be present in association with cancer in the oral cavity (~3 to 18.5%), larynx (~7%) nasopharynx (7.4%), paranasal sinus (5.6%), and hypopharynx (~0 to 16.7%) (36–38). HPV has also been detected in esophageal tissue and is associated with a 3-fold-greater chance of esophageal squamous cell carcinoma (39).

The distinction of HPV-positive OPSCC from tobacco-related HNSCC is clinically important. Despite the fact that HPV-positive HNSCC are more likely to be detected as late-stage cancers, patients with HPV-containing tumors have a more favorable prognosis, with a better response to surgical or nonsurgical treatment and overall survival about three times greater (80 to 85%) than for those with HPV-negative tumors (33). A subset of HPV-positive HNSCC carry a risk for poor prognosis and are associated with smoking, advanced nodal stage, epidermal growth factor receptor (EGFR) overexpression, and chromosomal instability (34, 40).

The incidence of HNSCC has been decreasing over the past several decades, and this is suspected to be partly due to a reduction in tobacco smoking. In contrast, the incidence of HPV-associated oropharyngeal cancer has surged over the past 2 decades or so, especially among men. In 2012, the rate of oropharyngeal cancer among men was reported to be about four times that among women (28). It has been estimated that by 2020, HPV will cause more oropharyngeal cancers than cervical cancers in the United States (41). Several studies indicate that oral HPV is likely to be sexually acquired (42, 43). OPSCC is more common in younger men who do not smoke or consume alcohol but have a history of sexual activity that could expose them to oral infection by HPV, including a higher numbers of sex partners, lack of condom use, and higher oral sex exposure (43). Posttransplant immune suppression and HIV infection also confer a higher risk of oral HPV infection (43).

Compiled studies have shown that most OPSCC are mediated by HPV16 (76 to 95%), followed by HPV18 (1 to 8%) with HPV33, -35, -56, -58, and -67 found infrequently (37, 44, 45). In 2009, the IARC recognized HPV16 as a causal agent in OPSCC (46). Genome-wide sequencing studies of HPV16 in OPSCC tissues have shown that HPV nucleic acid is episomal in most cases and was integrated into the human genome in only 15.4% (47). This is in contrast to the case for cervical cancer, where HPV is almost always integrated and episomal HPV is not observed. An additional difference is that although there is high expression of E6 and E7 oncogenes in OPSCC, integration of HPV nucleic acid sequences, when it occurs, does not involve disruption of early gene E2 (47). The process of carcinogenesis without disruption of E2 needs further investigation.

The HPV viral load in OPSCC tissues appears to vary widely, but copy numbers are generally lower than in the cervix (48). Tonsillar tissues have been most widely studied, with HPV copy numbers of 154 to 13,467,920 E6/50 ng DNA (38). Nontonsillar tissues have lower viral loads (38).

Tumor suppressor protein p16 is lost in most (54 to 82%) cases of HNSCC but is upregulated in HPV-related tumors as a result of transcription of the E7 viral oncogene (35). The presence of p16 has been shown to be an effective marker of transcriptionally active HPV infection (33). Based on both HPV viral load and p16 expression, Weinberger et al. (49) classified head and neck cancers into three groups: class I, HPV negative, p16 low; class II, HPV positive (median, 3.6 copies), p16 low; and class III, HPV positive (median, 46 copies), p16 high. Using this classification, patients with class III tumors were found to have a significantly increased 5-year survival, an increased disease-free survival rate, and a decreased rate of local tumor recurrence (49). The authors postu-

lated that the better outcomes may be due to the absence of field cancerization or enhanced sensitivity to radiation/chemotherapy-induced apoptosis because of intact p53 in HPV-associated cancers (49).

Since oral and oropharyngeal cancers are often detected in later stages, substantial efforts have been made toward early detection. A number of oral lesion detection systems have been introduced and are based on autofluorescence or tissue reflectance (e.g., the Dentlight Oral Exam Light kit, Microlux DL, Orascoptic DK, Sapphire Plus, Trimira Identafi 3000, and ViziLite-Blue and VELscope). The ability of these systems to discriminate between cancerous/precancerous lesions and benign mucosal lesions has been limited. The U.S. Preventive Services Task Force (USPSTF) oral cancer screening guidelines state that there is insufficient evidence to recommend for or against routine screening of asymptomatic adults for oral cancer (50). The evidence-based guidelines provided by the American Dental Association determined that screening using a conventional oral and tactile examination may result in detection of oral cancers at earlier stages, but there was insufficient evidence to determine the effect of screening on disease-specific morbidity and mortality (51). The guideline did not support the use of adjunct systems for detection of lesions because of limited and conflicting evidence.

Because some parts of the oropharynx are deep inside the neck and are difficult to visualize, a comprehensive exam in a symptomatic individual may include the use of long-handled mirrors or endoscopy. Some HPV-associated lesions are very small or are located deep in the tissue crypts and are hidden from clinical detection. There are currently no standardized screening methods for detection of oral cancer outside conventional visual and tactile examination that would facilitate early diagnosis. Testing saliva for the presence of HPV was thought to be promising for early detection of disease but was ultimately found to have insufficient sensitivity and specificity. An evaluation of detection of HPV in saliva rinses by PCR did not reveal statistically significant differences between patients with oropharyngeal carcinoma and age- and sex-matched controls, and the authors were unable to support the detection of HPV as a diagnostic method (52–54). However, detection of HPV16 DNA in saliva rinses and plasma after primary treatment may allow for early detection of recurrence in patients with known HPV16-positive OPSCC (55). Quest Diagnostics offers OraRisk HPV, a laboratory-developed PCR-based test to detect high- or low-risk HPV types in saliva rinses. Further research is needed to define the populations that may benefit from tests to detect HPV and to provide evidence regarding the clinical value of these tests.

Diagnosis of oropharyngeal cancer can be done only by microscopic examination of tissues or exfoliated cells. Tissues are typically collected by excisional biopsy. Specimens for exfoliative cytology are easy to collect, but this method does not detect all cancers, and an incisional biopsy may still be needed. Fine-needle aspiration (FNA) biopsy may be done in patients already know to have oropharyngeal cancer to determine if the cancer has spread to the lymph nodes in the neck.

The histopathologic terminology and descriptions used for HPV-related OPSCC have been variable. Lesions develop in the absence of premalignant changes in the surface squamous epithelium and typically form sheets and rounded lobules with distinct borders (56). Lesions may contain infiltrating lymphocytes, but there is little inflammatory response in the surrounding stroma (56, 57). Tumor cells tend to be immature, nonkeratinizing, and basaloid (with a small amount of cytoplasm), with a uniform nuclear appearance and a high mitotic rate (56, 57). Comedo necrosis is also a characteristic histopathologic feature (57). Grading and differentiation have not been clinically useful since OPSCC tumors are often described as "poorly differentiated," but that designation loses descriptive power since the epithelium from which the tumors arise is also poorly differentiated (56, 57).

Because HPV status has biological significance in OPSCC, tumors are often described by the presence of HPV and/or molecular changes associated with HPV instead of relying on specific histologic features. The National Comprehensive Cancer Network and the College of American Pathologists have suggested routine testing for HPV in all OPSCC (35). It is important to highlight that testing should be restricted to specimens from the oropharynx, since the association of HPV with tumors outside the oropharynx is low. Reasons to test include the following.

1. Prognostic indicator: HPV-positive tumors are a completely distinct epidemiological, biological, and clinical subset of tumors that are associated with better clinical outcomes.
2. Tumor localization: to aid in localization of the site of primary tumor origin for patients who present with metastatic disease (58).
3. Distant metastases: the rate of distant metastasis in both HPV-negative and HPV-positive tumors is <5% (59). For HPV-negative tumors, metastases tend to occur in the lung following bilateral bulky adenopathy. For HPV-positive tumors, metastases tend to occur in brain, liver, and spine and are not preceded by bulky disease or advanced disease.

HPV testing in OPSCC may also have a future role in patient management, as trials to determine if OPSCC can be treated less aggressively than smoking-related tumors (e.g., if radiotherapy can be directed more specifically to the oropharynx rather than irradiating a wider range of head and neck structures) are ongoing. Other future uses could potentially include the assessment of response to treatment and monitoring for recurrence of disease following treatment.

While testing is suggested, there are no recommendations as to the types of test(s) or test combinations that should be used to identify HPV-associated OPSCC. Several types of tests with various strengths and weaknesses have been used.

Although consensus HPV DNA PCR tests and type-specific PCR tests have been approved by the U.S. Food and Drug Administration (FDA) for other types of specimens, there are no FDA-approved molecular tests for OPSCC screening in the United States. Any of the commercially available tests approved for cervical cytology could be validated by a clinical laboratory to detect HPV in oropharyngeal biopsy specimens. Case-control studies have found that HPV DNA can be found widely in head and neck squamous dysplasia, squamous cell carcinoma, other types of cancer, papillomas and other benign lesions, and some normal tissues (60). A recent comparison of the Roche cobas 4800 HPV real-time PCR test (Roche Molecular Systems, Inc., Pleasanton, CA) with standard methods of *in situ* hybridization (ISH) for high-risk HPV and immunohistochemistry (IHC) for p16 on HNSCC specimens demonstrated a sensitivity of 100% and a specificity of 86% for the cobas system (61). Because of the concern that detection of HPV

DNA may not be specific to the tumor, it was thought that quantitative HPV DNA tests or tests that assess HPV transcriptional activity may have more pathophysiologic relevance.

Detection of E6/E7 mRNA is generally considered to be the standard to indicate transcriptionally active, clinically relevant oncologic HPV (34, 62–65). Extraction of RNA and amplification of E6/E7 mRNA by PCR can be applied to fresh or frozen tissues but remain technically challenging and are unreliable for formalin-fixed and paraffin-embedded tissues. Reliable immunohistochemical probes for E6 and E7 proteins are not available (56).

Numerous other markers of cell proliferation and biological activity, such as Ki67, proliferating cell nuclear antigen, overexpression of epidermal growth factor receptor, p53, and others, have been studied, but none have been consistently reliable. HPV DNA ISH and p16 IHC have emerged as the most useful tests. HPV DNA ISH has been found to be highly specific but not entirely sensitive, and p16 IHC as a surrogate marker of transcriptionally active HPV infection has been found to be highly sensitive but not entirely specific. DNA ISH assays for high-risk HPV allow for direct visualization of the virus in the nuclei of tumor cells in the tissue, which better demonstrates HPV as a causal agent. DNA probes that hybridize to type-specific HPV DNA sequences are available. Mixtures of type-specific probes in a single reaction mixture to cover an extended range of HPV types are also available. These probe cocktails help to overcome the potential limitation of ISH assays that detect only HPV16, since about 10% of OPSCC are associated with high-risk subtypes of HPV other than HPV16. Other probes bind to a consensus sequence shared by multiple HPV types. Some improvements in ISH assays, such as the use of nonfluorescent chromogens and changes in signal amplification steps, have increased sensitivity and allow the assays to be a more practical tool for diagnostic purposes (56).

Tumor suppressor protein p16 (also known as p16INK4a, MTS1, and CDKN2) is lost in 54 to 82% of HNSCC but is upregulated in HPV-related tumors as a result of transcription of the E6, E7, and/or E5 viral oncogene (35). Since there will be some p16 immunohistochemical staining in virtually any squamous cell carcinoma, it is important to consider a specimen to be positive only when the staining is nuclear plus cytoplasmic, strong and generally diffuse, and present in at least 50% of tumor cells (66). When defined in this way, p16 immunohistochemical staining alone is sufficiently sensitive and specific and is the most useful prognostic indicator for patients with known OPSCC, regardless of HPV status (56, 62). Outcomes for $p16^+$, HPV^- OPSCC are not significantly different from those for $p16^+$, HPV^+ tumors and are significantly better than those for p16-negative tumors (62). Clearly designating HPV status, however, will be important as targeted therapies become available (67). Stepwise algorithms that supplement highly sensitive p16 staining with more specific HPV-specific ISH or PCR are indicated in some situations. Specific indications include focal or weak p16 staining, p16-negative OPSCC with typical HPV histologic morphology, and p16-positive OPSSCC that does not demonstrate typical HPV histologic morphology (56). For lymph node metastases, p16 immunohistochemical staining may substitute for HPV testing in patients with a known primary OPSCC or an oropharyngeal mass (56).

ANAL CANCER

Squamous cell carcinoma of the anal canal is clinically and histologically similar to squamous cell carcinoma of the cervix. Lesions arise in the squamous epithelium at the junction between the rectal columnar epithelium and squamous cells of the anal canal, similar to the transitional zone within the cervix. The most common HPV types that are linked to anal cancer include HPV16, HPV6, HPV42, HPV18, HPV11, HPV31, and HPV52 (68–70). About 85% of anal cancer and precancerous lesions are caused by HPV16 (30). Some cancers other than squamous cell carcinoma are categorized as anal cancers because of their location but are not associated with HPV. These include cloacogenic carcinoma, adenocarcinoma, basal cell carcinoma, and malignant melanoma (71, 72). Anal cancer has a very low incidence in the general population (28, 68, 69, 73). The rate of anal cancer among females is higher than that among males (28, 73). The incidence of anal cancer in HIV-infected individuals has increased significantly over the past 2 decades, especially among men who have sex with men (MSM) (74, 75). Age at diagnosis is generally greater than 50 years. Risk factors are largely related to sexual behavior, with acquisition of infection often due to contact of fingers or external assist devices with infected fluids. Autoinoculation from cervix to anus or anus to cervix in the same woman is though to occur frequently (76). Histories of anal warts, multiple sexual partners, and smoking (which increases risk of nonclearing HPV infection) also place individuals at increased risk (77). Immune-suppressed individuals, including patients taking immune-suppressive medications, transplant recipients, and patients with HIV infection, have higher rates of persistent HPV infection and are at higher risk of developing anal cancer. HIV-infected individuals have a 20- to 30-times-higher risk of developing anal cancer than the general population and tend to present at a younger age, with a mean age at diagnosis of 42 years (69). Among HIV-infected individuals, receptive anal intercourse is the most prominent risk factor, and HIV-infected MSM have a 3-times-greater risk than HIV-infected women and twice the risk of both HIV-infected men without a history of anal receptive intercourse and HIV-negative MSM (69). Independent risk factors in HIV-infected individuals include a $CD4^+$ cell count of <200 cells/mm^3, infection with multiple HPV types, history of anal warts, non-Caucasian ethnicity, smoking, or history of cervical dysplasia or cancer in women (68, 69, 76).

HPV infection of the anal canal is frequent in women and in HIV-infected MSM, with cumulative rates ranging from about 70% to greater than 95% (69, 76). Most infections are mixed with multiple HPV types. Compiled studies in MSM show a median of 5 types, with a range of 0 to 18 types and an average of 3 high-risk types (69). Anal HPV is frequently cleared, with persistent infection occurring in few individuals, most commonly in those who are infected with HIV. Several studies have reported that the presence of multiple HPV types is associated with an increased likelihood of development of high-grade lesions (69).

Anal cancer has many features in common with cervical cancer, and natural history studies have shown that high-grade anal intraepithelial neoplasia (AIN) is a precursor to invasive anal cancer. Because of the success of the cervical cancer screening program, many experts have advocated for routine screening to detect precancerous anal lesions in high-risk individuals. There are currently no formal recommendations in the United States for routine screening in the general population or for any subgroup. It is recommended that sexually active women have a digital anorectal exam to check for lesions and other abnormalities during regular gynecological visits. Men are not regularly screened for anal HPV-related abnormalities, and some studies indicate that screening

TABLE 3 Cytology and histology terminology for HPV-associated preinvasive squamous lesions of the lower anogenital tract, including vulva, vagina, cervix, penis, perianus, and anus[a]

Dysplasia findings	Bethesda 2001 cytology terms	2012 LAST histology terms
No epithelial abnormalities or benign cellular changes	Negative for intraepithelial lesions or malignancy (reactive cellular changes associated with inflammation or radiation and infectious organisms such as *Trichomonas vaginalis*, *Candida* species, or cellular changes associated with herpes simplex virus [HSV]may be noted in a comment)	Normal
Atypia, squamous epithelial cells; the squamous cells do not appear completely normal, but doctors are uncertain about what the cell changes mean; sometimes the changes are related to an HPV infection, but they can also be caused by other factors; ASC-H lesions may be at higher risk of being precancerous than ASC-US lesions	Atypical squamous cells (ASC): ASC-US (unspecified significance), ASC-H (cannot exclude HSIL)	Atypia
Koilocytosis, mild dysplasia, mild abnormalities caused by HPV infection	Low-grade squamous intraepithelial lesion (LSIL)	LSIL (formerly intraepithelial neoplasia [IN-1])
Moderate dysplasia, severe dysplasia, carcinoma *in situ*, suspicious; more severe abnormalities that have a higher likelihood of progressing to cancer if left untreated	High-grade squamous intraepithelial lesion (HSIL)	HSIL/IN-2, IN-3 includes carcinoma *in situ*; when the diagnosis is IN-2 or when the differential diagnosis is between precancer (IN-2 or IN-3) and a mimic of precancer (atrophy, reparative epithelial changes, etc.), perform p16 immunostain to upgrade or downgrade; if negative, classify as LSIL and if positive, classify as HSIL
Invasive squamous cell carcinoma (cervical cancer)	Squamous cell carcinoma	Squamous cell carcinoma
Atypia, glandular epithelial cells	Atypical glandular cells (AGC); endocervical adenocarcinoma *in situ* AGC, favor neoplastic	NA[b]

[a] Adapted from reference 105 with permission from Elsevier.
[b] NA, not applicable.

seems warranted for high-risk populations, particularly MSM, but further studies are needed (69, 78). Recommendations from the New York State Department of Public Health AIDS Institute for annual digital anorectal exams and cytologic screening for HIV-positive MSM, any patient with a history of anogenital condyloma, and women with abnormal cervical and/or vulvar histology have been in place since 2007 (79).

Cytologic screening for precancerous anal lesions can be done using Papanicolaou-stained smears. Anal specimens for cytology are generally obtained by blindly inserting a moistened Dacron swab or cytobrush 5 to 6 cm into the anal canal and rotating the device to sample the mucosa. Since it is important to sample the transition zone, a proctoscope or anoscope may be used to directly visualize the sampling site. Conventional smears or liquid-based cytology can be used. Inflammatory cells, mucus, blood, fecal material, bacteria drying artifacts, and thick preparations can obscure evaluation of the exfoliated anal cells, and liquid-based cytologic preparations have been shown to increase diagnostic yield (80). The number of cells needed to provide an adequate smear is not precisely known, but Bethesda system guidelines recommend a minimum of 2,000 to 3,000 unobscured nucleated squamous cells for conventional smears. Equivalent liquid-based specimens would contain 1 to 2 nucleated squamous cells per high-power field for ThinPrep (Hologic, Marlborough, MA) and 3 to 6 nucleated squamous cells per high-power field for SurePath (BD Diagnostics-TriPath, Burlington, NC). Anucleate squamous cells are not considered to be adequate for diagnosis.

The criteria used to evaluate anal cytology smears are analogous to those for cervical cytology. Precancerous lesions caused by HPV infections are classified using Bethesda nomenclature according to the severity of the cellular abnormality. Bethesda categories for anal cytology include negative for dysplasia, atypical squamous cells of undetermined significance (ASC-US), and atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion (ASC-H); anal squamous intraepithelial lesions include the categories of low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) (Table 3). Although similar to cervical cytology, there are some differences in anal cytology. The major differences are that koilocytic dysplasia associated with HPV may not be as pronounced in the anal canal, even in low-grade lesions, although binucleation and multinucleation of epithelial cells may be prominent, and cytoplasmic keratinization may be more widespread than in cervical lesions. Another difference is that there is a higher incidence and lower specificity of ASC-US in the anal canal (81, 82). The incidence of ASC-US in cervical lesions ranges from about 1 to 10.4%, but it is 14 to 18% in HIV-positive MSM and 12% in HIV-MSM (81, 82). Similar to cervical cytology, there is considerable interobserver variation in interpretation of anal cytology smears.

Anal cytology is generally considered to be operationally similar to cervical cytology. The performance characteristics of anal cytology depend on the population being studied, HIV status, sample size, HPV prevalence, and whether or not ASC-US are

included as abnormal. Sensitivity estimates for anal cytology range from 42 to 93%, and specificity ranges from 16 to 98% (83, 84). One large study in 401 HIV-positive MSM found that when ASC-US were used as a threshold for abnormal, cytology detected histologically confirmed intra-anal AIN 2+ with 84% sensitivity, 39% specificity, 31% positive predictive value (PPV), and 88% negative predictive value (NPV) (81). Elevating the threshold to HSIL resulted in 21% sensitivity, 91% specificity, 45% PPV, and 78% NPV (81). At present, anal cytology seems to be useful for screening of high-risk individuals, including HIV-positive patients and MSM (83, 85). The optimum screening frequency also needs to be established. Preliminary data suggest that annual screening of HIV-positive MSM and biennial screening of HIV-negative MSM appear to be cost-effective (83).

Direct evidence that establishes the efficacy and cost-effectiveness of the treatment of dysplastic precursor lesions for the prevention of anal cancer is also needed to help inform the decision process for adopting a screening program. Data are limited, but since disease is often widespread throughout the mucosa in HIV patients, recurrences requiring retreatment arise frequently with many ablative treatment techniques, and definitive surgical resection carries a high risk of complications.

Testing for HPV has limited utility for anal cancer screening because of the high prevalence of HPV and presence of multiple HPV types in the anal canals of women and HIV-infected MSM. Data from a large ($n = 334$) study of HIV-infected men showed that amplification of HPV DNA for 13 high-risk HPV types as a primary test has a sensitivity of 96%, specificity of 33%, PPV of 37%, and NPV of 95% for associated high-grade anal disease on histopathology (86). When detection was limited to the five most common high-risk HPV types (HPV16, HPV18, HPV31, HPV33, and HPV45), sensitivity dropped to 81% but specificity increased to 58%, with a PPV of 44% and NPV of 89%. Detection of RNA for the same 5 HPV types was slightly more specific (65%), but the PPV and NPV were similar to those for the DNA tests. When DNA amplification was restricted to only HPV16 and HPV18, sensitivity was significantly reduced (62%) and specificity was increased (77%), with a corresponding PPV of 53% and NPV of 83%. Primary HPV testing may be more useful in HIV-negative MSM because the presence of oncogenic HPV, especially HPV16, is highly specific for high-grade anal intraepithelial neoplasia on biopsy (83). When used as an adjunct test for HIV-infected men with anal ASC-US, HPV DNA amplification was found to have a high sensitivity of 100% but low specificity of only 17.5% for corresponding high-grade lesions on histopathology (81).

When cytologic abnormalities are identified, high-resolution anoscopy is the standard procedure used to obtain tissue biopsy specimens for histologic evaluation. Biopsy specimens are evaluated for the presence of abnormal squamous epithelial lesions using criteria comparable to that used for cervical lesions. In older nomenclature, precancerous anal lesions were termed anal intraepithelial neoplasia (AIN) and were categorized as grade 1, 2, or 3 based roughly on the distribution of the abnormalities within the epithelium, which corresponded to mild, moderate, and severe disease. AIN 1 describes abnormalities that are confined to the lower third of the epithelium. In AIN 2, abnormalities extend to the middle of the epithelium, and in AIN 3, they extend to the top third of the epithelium. AIN 3 is also called carcinoma *in situ* and can develop into invasive anal cancer if not treated. Since histologic evaluation of AIN is somewhat subjective with significant interobserver reproducibility, the Lower Anogenital Squamous Terminology Standardization (LAST) Project was convened by the College of American Pathologists (CAP) and the American Society for Colposcopy and Cervical Pathology (ASCCP) in 2012 to develop standardized histology terminology to describe preinvasive squamous epithelial lesions in all locations of the lower anogenital tract (87). The recommended terminology is low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) and may include subgrading using IN terminology with or without specifying the anatomic location (e.g., for an IN 1 lesion, specify AIN 1 for anus or CIN 1 for cervix, etc.) (Table 3). Discrepancies between anal cytology and histopathology findings are common, and the severity of disease detected by cytology may not correlate with the severity of disease found on biopsy. Discrepancies can be due to sampling errors and/or interpretation errors for the anal cytology specimen, the biopsy specimen, or both. Accurate interpretation is important, since HSIL is treated and LSIL is followed with annual high-resolution anoscopy. Invasive squamous cell carcinoma is not usually a diagnostic difficulty and is defined by a proliferation of squamous epithelial cells that penetrate the basement membrane and invade the underlying stroma, often with an associated inflammatory response and desmoplastic reaction.

Several biomarkers (e.g., Ki67, ProExC, p16, and others) have been investigated as possible means to improve the accuracy of histologic diagnosis. Based on a review of evidence, the LAST Project work group determined that no biomarker could replace hematoxylin and eosin (H&E) histopathology as the primary diagnostic tool but that the operating characteristics of p16 IHC have been well documented and that it is useful as an adjunct to standard histopathology in certain situations (87). Because interobserver variability of IN 2 (under the old terminology) is high and the biologic behavior is variable, p16 IHC is indicated to clarify a diagnosis of a high-grade lesion when considering an interpretation of IN 2. Diffuse, strong p16 staining in the area that meets the H&E morphological criteria for a preinvasive lesion supports categorization of the lesion as HSIL. Absence of p16 staining or presence of minimal patchy staining supports downgrading the interpretation to LSIL. Pathology reports should note when p16 IHC is used. Recommendations stress that p16 IHC should not be used to assess biopsy specimens with morphological interpretations of negative, IN 1, or IN 3, since the long-term natural history of p16-positive CIN 1 and p16-negative CIN 3 lesions is not fully known. An additional recommendation is to use p16 IHC when a potential high-grade lesion cannot be differentiated from a benign condition that can mimic a high-grade lesion, such as atrophy, reparative epithelial changes, reactive squamous metaplasia, or tangential sectioning. Other circumstances in which p16 IHC is indicated include resolution of IN 2 or IN3 cases when there is a professional disagreement on histologic specimen interpretation. p16 IHC is also indicated for biopsy specimens interpreted as ≤IN 1 in patients with prior cytology specimens interpreted as HSIL, ASC-H, ASC-US/HPV16$^+$, or atypical glandular cells, to help ensure that high-grade disease is not missed.

CERVICAL CANCER

Numerous studies have unequivocally established that virtually all cervical cancers are caused by high-risk human papillomavirus (HPV) infections. HPV infections are common, and it is estimated

that approximately 75% to 80% of sexually active individuals will become infected in their lifetime (88, 89). Most women become infected shortly after becoming sexually active, and the highest prevalence is seen in women under age 25. Common risk factors for HPV infection in young women include earlier age of onset of sexual activity, two or more sexual partners in the previous year, and coinfection with *Chlamydia trachomatis*, herpes simplex virus, or bacterial vaginosis (89, 90). Cross-sectional studies indicate a second peak of infection in women aged 35 to 54 years, which corresponds to the age of highest incidence of cervical cancer. The incidence drops off rapidly after age 55.

Of the 12 IARC class 1 carcinogenic HPV types, HPV16 is the most frequently detected in cervical cancer, followed by HPV18. Together, HPV16 and HPV18 are responsible for about 70% of cervical squamous cell carcinomas worldwide, with percentages varying from 65% in South America and Central America to 76% in North America (91). The next most frequently detected HPV types in cervical cancer are also the same worldwide and include HPV31, -33, -35, -45, -52, and -58, with the slight exception of Europe, where HPV56 was the eighth most common type instead of HPV52 (91). The prevalence of HPV58 and HPV52 in cervical cancer was notably higher in Asia and accounted for 5.6% and 3.8% of cases, respectively (91). Worldwide, HPV types other than these eight are individually associated with no more than 2% of cervical cancer cases (91). HPV infection with a high-risk type is necessary for development of cervical cancer, but cofactors such as smoking, immune suppression, long-term oral contraceptive use, chronic inflammation, and having given birth two or more times may increase the risk (92, 93).

An important observation emerged when a large number of HSIL and cervical cancer cases (7,094 HSIL and 9,494 cervical cancer) were studied (91). It was found that the HPV types most frequently associated with cervical cancer, i.e., HPV16, HPV18, and HPV45, were not the most prevalent types in HSIL. HPV31, -33, -35, -39, -52, -56, -58, -68, and -73 were each 2- to 3-fold more prevalent in HSIL than in cervical cancer, and HPV6, -11, -51, -66, -70, and -82 were 5- to 10-fold more prevalent in HSIL than in cervical cancer (91).

Acquisition of HPV is generally asymptomatic and results in an infection of variable duration. HPV infections in adolescents and young and middle-aged women are usually transient, at least when duration is measured by how long the virus can be detected in cervical cytology specimens using molecular tests. Up to half of adolescents and young to middle-aged women who develop an incident infection will clear the infection within 6 months, and 70 to 90% will clear within 12 to 30 months (89, 94). Large-cohort studies with follow-up of up to 15 years have shown that once cleared, as measured by one or two negative HPV molecular tests, there is a very low risk of risk of subsequent carcinoma (76). During the duration of a transient infection, dysplasia may or may not develop and resolve on its own.

Persistence of HPV within cervical epithelial cells is necessary for the development of high-grade cervical disease and cancer (95). It is generally estimated that it takes several years to decades from the time of an initial HPV infection until development of a high-grade lesion and eventually formation of a tumor (96, 97). On average, a CIN 3+ lesion is 9.4 years only when detected (97). This is because in the beginning of the disease process, the population of infected cells is not morphologically different enough from normal cells to be detected on cytology screening. Each infected cell contains a fixed amount of HPV DNA (1 to 500 copies), and the doubling time of the clonal population in a persistent infection is about 286 days (97). Infections with a greater number of infected cells will have a higher corresponding viral load and a greater probability for abnormal cytology (98). The minimum viral load associated with visual detection of abnormal cytology (ASC-US$^+$) is 6.5 HPV copies/cell (98). Some women with fewer HPV DNA copies per clonal (cancer) cell will take more time to be detected by the commercially available molecular tests, since they use a fixed cutoff (98). It has been estimated that it takes an additional 23.5 years for a CIN 3+ lesion to progress to cancer (96). Mean high-risk HPV16, -18, -31, and -33 viral loads of 1.9×10^6 copies/million cells have been associated with normal cervical cytology and not significantly associated with persistence or the subsequent development of cervical lesions (99).

Current Cervical Cancer Screening Guidelines

The long natural history from HPV infection to development of cervical cancer provides the opportunity to screen asymptomatic women to allow for detection of the process in the early stages and treat preinvasive lesions before they become cancerous. The most common screening test is liquid-based cytology, in which epithelial cells are collected using a cervical sampling broom, brush, or spatula, processed into a thin layer on a glass microscope slide, stained with Papanicolaou stain, and read using a microscope. The premalignant phases are interpreted using the 2001 Bethesda system (Table 3). Atypical squamous cells (ASC) are the most common abnormal finding in exfoliated cervical cells. The Bethesda system divides this category into two groups, atypical squamous cells of undetermined significance (ASC-US), in which the cells do not appear completely normal but there is uncertainty about the significance of the changes, and atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion (ASC-H), in which the significance of the changes is uncertain but there may be a higher risk of being precancerous than for ASC-US. Low-grade squamous intraepithelial lesions (LSIL) are considered an early change or mild abnormality caused by HPV infection. High-grade squamous intraepithelial lesions (HSIL) are a more severe abnormality that includes both moderate to severe dysplasia and carcinoma *in situ*. HSIL lesions are more likely to progress to cancer if left untreated. In accordance with the Bethesda classification, cytology can be read as unsatisfactory (UNSAT) for the following reasons: obscuring blood, obscuring inflammation, poor fixation, cytolysis, or inadequate cellularity (defined for liquid-based cytology as <5,000 cells visualized). The Bethesda system also allows for description of glandular cell abnormalities which are categorized as atypical glandular cells (AGC), which do not appear normal but for which is uncertainty about what the cell changes mean. Adenocarcinoma *in situ* (AIS) indicates that precancerous cells are found only in cervical glandular tissue.

Longstanding programs since the 1950s using annual screening with Papanicolaou-stained cervical cytology smears have been successful, and in developed countries most cases of cervical cancer occur in women who have not had regular cervical cytology screening. Concerns about cytology screening are that it is subjective and has significant interlaboratory variation (100). Women with abnormal cytology findings are referred to colposcopy to visually examine the cervix and obtain biopsy specimens for histologic examination. Premalignant cervical histologic changes represent a spectrum of abnormalities that are categorized accord-

TABLE 4 Summary of current (2012) cervical cancer screening guidelines for average-risk women from the American Cancer Society (ACS), American Society for Colposcopy and Cervical Pathology (ASCCP), American Society for Clinical Pathology (ASCP), U.S. Preventive Services Task Force (USPSTF), and American College of Obstetricians and Gynecologists (ACOG) with interim guidance from the Society of Gynecologic Oncology and ACOG[a]

Age (yr)	Screening recommendations
21	Initiation of screening
21–29	Cytology every 3 yr, or primary HPV testing can be considered starting at age 25 every 3 yr[b]; if primary HPV testing is positive, test for HPV16 and HPV18 and refer to colposcopy if positive or reflex to cotesting if negative
30–65	Cytology every 3 yr and HPV testing for triage of ASC-US, or HPV cotesting every 5 yr[c] and test for HPV16 and HPV18 if normal cytology but HPV positive, or primary HPV screening every 3 yr[b] as indicated above
Discontinuation of screening	Women age >65 who have 3 or more consecutive negative cytology tests or two consecutive negative cotests within 10 yr with the most recent test performed within 5 yr; women of any age who have a total hysterectomy and have no history of cervical cancer or precancer should not be screened

[a] Summarized from references 102, 103, and 104. (Adapted from reference 105 with permission from Elsevier.)
[b] Interim guidance (182).
[c] HPV cotesting is the preferred screening method according to ACOG guidelines and is a grade A recommendation (as is cytology every 3 years) by USPSTF. ACS designates cotesting as a strong recommendation but as the preferred method a weak recommendation.

ing to LAST 2012 consensus terminology as low-grade squamous intraepithelial lesions (LSIL) or high-grade squamous intraepithelial lesions (HSIL) and may include subgrades ranging from mild cervical intraepithelial neoplasia (CIN 1) to moderate dysplasia (CIN 2) to severe dysplasia/carcinoma *in situ* (CIN 3) (87) (Table 3). Histology findings of CIN 2 are confirmed with p16 immunostaining as recommended by the 2012 LAST guidelines, with cases lacking p16 staining considered to be <CIN 2 (87). Discrepancies between cytology and histopathology are common and can be due to sample differences or to cytology and/or histopathology interpretation errors.

Cervical cancer screening guidelines have been reevaluated frequently over the past 2 decades as new testing technologies became available and the epidemiology and natural course of development of cervical cancer were better understood. Guidelines also take into consideration the feasibility, acceptability, sustainability, and cost-effectiveness of the recommended strategies (101). The most recognized guidelines have been developed independently by the American Congress of Obstetricians and Gynecologists (ACOG), the U.S. Preventive Services Task Force (USPSTF), and the American Cancer Society (ACS) in partnership with the American Society for Colposcopy and Cervical Pathology (ASCCP) and the American Society for Clinical Pathology (ASCP) (102–104). Prior to 2012 there were considerable differences among the guidelines from these societies (105). In 2012, the guidelines were reevaluated, and the current guidelines do not have any major differences (Table 4). There are minor differences in the strength of the preference for HPV cotesting. There is consensus that women of any age who have had their uterus and cervix removed and have no history of cervical cancer or a high-grade precancerous lesion should not be screened. All normal-risk women with a cervix should begin cervical cancer screening at age 21 even if initiation of vaginal intercourse or other behavioral risk factors occurred at a younger age. Between the ages of 21 and 29 years, women should be screened using either liquid-based or conventional cytology every 3 years. Because HPV infections are common and typically self-resolving in this age group, HPV testing should not be used unless it is needed following an abnormal cytology smear. For women aged 30 to 65 years, screening can be done using cytology alone every 3 years or cytology plus simultaneous HPV testing every 5 years. HPV cotesting every 5 years is preferred for this age group by ACOG and ACS/ASCCP/ASCP. The USPSTF does not indicate a screening strategy preference but considers cotesting every 5 years as a reasonable alternative for women who prefer an extended screening interval. Criticisms of cotesting, in addition to expense, are that this strategy combines a relatively insensitive test with a sensitive test and results in the need for a complex set of algorithms for management of patients (106). All guidelines support the use of HPV DNA testing as a follow-up to mildly abnormal Pap test results and HPV genotyping for women who are cotested and have normal Pap smear results but test positive for high-risk HPV.

The guidelines support discontinuation of screening in women older than 65 years if they have had adequate screening with normal results. Adequate screening is considered to be three consecutive normal cytology results or two consecutive negative cotest results within the previous 10 years, with the most recent test performed within the past 5 years. Women who have had abnormal screening results should continue to be screened. Women treated for cervical cancer or high-grade lesions remain at risk, and routine age-based screening is recommended for at least 20 years, even if it extends beyond age 65. The age at which cervical cancer screening should be terminated has come under recent review because of new evidence for the effectiveness of continued screening after age 65 (107). Several studies have revealed cases of cervical cancer in women over age 65 who have had previous negative screening according to existing guidelines (107). It appears that the inferred lack of risk is sustained during the first few years after adequate negative screening but wanes significantly with time (107). There is mounting evidence supporting latency and reactivation of HPV infection in older women as possible explanations for new HPV detection at older ages (107, 108). Further evaluation of benefits and risks will help determine if extended screening beyond age 65 is warranted (107).

A quadrivalent vaccine (HPV4) (Gardasil; Merck and Co., Inc.) that is directed against two oncogenic types (HPV16 and -18) and two nononcogenic types (HPV6 and -11) was licensed by the FDA in 2006 for use in adolescent and young adult women aged 9 through 26 years and in 2009 for use in adolescent and young adult men aged 9 through 26 years. A bivalent HPV vaccine (HPV2) (Cervarix; GlaxoSmithKline) directed against HPV16 and -18 was licensed in 2009 for use in adolescents and young women aged 10 through 25 years. The Advisory Committee on Immunization Practices (ACIP) currently recommends routine vaccination of all

adolescents at 11 or 12 years of age using either HPV4 or HPV2 for females and only HPV4 for males (109). Catch-up vaccination using the routine dosing intervals can be given at age 13 through 18 years if there was no previous vaccination. Because the duration of protection provided by the vaccine is not known and vaccinated women remain at risk for infection with high-risk HPV types not covered by the vaccine, women who have been vaccinated against HPV should continue to be screened following the recommendations for their age group.

Women who were exposed *in utero* to the synthetic estrogen drug diethylstilbestrol (DES) are infected with human immunodeficiency virus (HIV), are organ transplant recipients, or are immune suppressed from other causes are at high risk for cervical cancer and should be screened more frequently. Recommendations for women who were exposed to DES *in utero* have been in place since 1978. These recommendations indicate that annual screening should be done using standard cervical cytology along with a four-quadrant vaginal Papanicolaou smear to allow for detection of clear-cell adenocarcinoma (105). Annual cytology screening should continue even after hysterectomy in this population. Women with HIV infection should be screened using cytology twice in the first year after diagnosis and then annually after that if results are normal (110). Women who were diagnosed with HIV before age 21 should not begin screening until age 21. The utility of HPV testing for women with HIV has not been determined.

COMMERCIALLY AVAILABLE TEST SYSTEMS FOR HPV DETECTION IN THE CLINICAL LABORATORY

Efforts in clinical diagnostic laboratories have focused largely on enhancing the sensitivity of cervical cytology screening smears to reduce false-negative diagnoses and improving the specificity of molecular tests to detect high-risk HPV to reduce unnecessary colposcopic examinations. It was anticipated that additional *in situ* molecular or immunocytochemical tests on cytology smears with ambiguous results, such as ASC-US, ASC-H, and AGC, would allow more definitive diagnosis and perhaps serve as an alternative to the molecular tests that have become routinely used. However, studies that evaluated *in situ* hybridization to detect high-risk HPV in liquid cytology specimens from patients with cytologic diagnoses of ASC-US or greater have concluded that the test lacks sufficient sensitivity and negative predictive value to be useful in conjunction with cytology smears (87, 111–113). Similarly, studies that evaluated immunostaining for p16 tumor suppressor protein as a surrogate biomarker for the presence of high-risk HPV in cytology smears that had subsequent CIN 2+ or CIN 3+ biopsy results have shown that performance characteristics were not sufficient to add value as a triage test (87, 114–116). Other biomarkers, such Ki-67 to detect deregulated cell cycle proliferation, dual staining for Ki-67 plus p16, and BD ProEx C (Becton, Dickinson and Company, Franklin Lakes, NJ) to detect aberrant S-phase induction, genes with aberrant DNA methylation, and chromosomal aberrations in the telomerase RNA gene have not proven useful, or there is insufficient evidence related to the clinical value of the test (117–123). Further, the leading medical professional societies have not recommended these tests in their cervical cancer screening guidelines.

The use of immunohistochemical detection of p16, Ki-67, and HPV DNA ISH has also been explored as an adjunctive aid with cervical biopsy specimens that are difficult to interpret (87). Extensive review of the literature by the LAST Project concluded that only p16 had sufficient evidence on which to make recommendations (87). Those recommendations, as well as the use of p16 immunostaining in OPSCC, are described above.

HPV Molecular Detection Methods

At this time, there are at least 193 distinct molecular tests that are commercially available on the global market for the detection of HPV in cervical specimens (124). Of the 193 available tests, 110 (57%) have a least one publication in peer-reviewed literature, but only 69 (35.7%) ae supported by published analytical and/or clinical evaluation (124). Several of the available molecular tests have been approved by the FDA for use in the United States or clinically validated using the Meijer criteria for use in Europe and Canada (Table 5) (125, 126). There are important differences among these tests, which will be delineated below. Most of these tests generate a pooled result and are designed to detect nucleic acids of the 12 IARC HPV group 1 carcinogens (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, and 59). Some tests also detect HPV66 and -68, which were included in this group in previously published classifications. Genotyping tests that distinguish individual HPV types, typically HPV16 and HPV18, are also available for further triage of women with a positive pooled result.

HPV tests are approved by the FDA for follow-up testing of women who have abnormal cervical cytology results to determine if a referral to colposcopy is needed and for cervical cancer screening to assess the presence or absence of high-risk HPV in combination with cervical cytology in women over age 30. In March 2014, the cobas HPV test (Roche Molecular Systems, Inc., Pleasanton, CA) was FDA approved with an additional indication as a stand-alone cervical cancer screening test for women age 25 years and older.

To simplify testing algorithms, HPV tests were FDA approved to use the same collection devices that are used to collect cervical cells for cytology testing so that a single sample can be processed for both cytology and HPV tests. This introduces a bias when HPV tests and cytology are compared, because HPV tests and cytology are performed on different fractions of the same sample (127, 128). HPV tests, as approved by the FDA, are performed on a fixed volume of random sample from the cytology collection vial. These samples can contain one or all of the three possible fractions in which HPV can be detected, namely, (i) free virions, (ii) desquamating cells with new virions from the productive HPV-induced pathway, and (iii) dividing cells containing HPV DNA from the transforming HPV-induced pathway. Cytology examines only cells (fractions 2 and 3). In transient infections, HPV tests performed on random samples from the vial mainly measure HPV nucleic acid from free virions (the largest fraction since it is necessary to infect someone new to keep the life cycle going). Because HPV tests are performed on the whole vial (3 fractions) and cytology is performed only on cells (2 fractions), there is nothing to see in the corresponding cytology sample because virions are not visible with a light microscope. When HPV tests are performed using an enriched cellular fraction obtained after centrifugation rather than a random sample from the whole vial, the sensitivity of cytology is improved (128, 129). When HPV tests performed only on enriched cellular fractions are compared with the Hybrid Capture 2 HPV DNA test (HC2; Qiagen, Germantown, MD), which is performed on a random sample from the whole vial, the specificity for detection of CIN 2+ of the HC2 test for a same sensitivity of

TABLE 5 Summary of molecular diagnostic tests FDA-approved in the United States (FDA-approved) and clinically validated in Europe for HPV detection and genotyping[a]

Assay type	FDA approval, yr	Meets Meijer criteria (125)	Test	Manufacturer	Chemistry	Instrumentation	Target	Internal control	Test medium	Analytical sensitivity
DNA-based assays	1999 (replaced HC1, which was approved in 1995); in 2003 expanded use to include cotesting indication	Standard comparator test	Digene Hybrid Capture 2 high-risk HPV DNA test	Qiagen, Redwood City, CA	DNA-probe hybridization with chemiluminescent signal amplification in a microtiter plate	Manual or semiautomated Rapid Capture system	Multigene probes; 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) as a pooled result	None	PreservCyt or Qiagen specimen transport Medium	1,000–5,000 copies/reaction
	No	Standard comparator test	GP5+/6+-EIA	NA[b]	Target amplification with detection of digoxigenin-labeled PCR products with solution hybridization to specific HPV group- and type-specific probes using the PCR ELISA Dig Detection kit (Boehringer Mannheim, Germany) biotin-labeled probes	Standard thermocycler and colorimetric microplate reader	L1; 14 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)	None	Cytobrush in phosphate-buffered saline	50 copies/reaction of HPV16
	2009	Partially validated	Cervista HPV HR and Genfind DNA extraction kit	Hologic Gen-Probe, Inc., San Diego, CA, USA	Invader signal amplification	Manual or semiautomated on the Cervista high-throughput automation system	L1, E6, E7 genes; 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) as a pooled result	Intrinsic control, human histone 2 gene	PreservCyt	1,250–2,500 copies/reaction for types 16, 18, 31, 45, 52, and 56; 2,500–5,000 copies/reaction for types 33, 39, 51, 58, 59, 66, and 68; 5,000–7,500 copies/reaction for type 35
	2011	Yes	cobas HPV test	Roche Molecular Systems, Inc., Pleasanton, CA, USA	Multiplex real-time PCR and nucleic acid hybridization	Automated on the cobas 4800 system	L1 gene; 14 high-risk HPV types; identifies HPV16 and HPV18 and concurrently detects high-risk types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 as a pooled result	Intrinsic control, β-globin	Cobas PCR collection medium or PreservCyt	150 copies/ml for type 45; 300 copies/ml for types 31, 33, 39, 51, and 59; 600 copies/ml for types 16, 18, 35, and 58; 1,200 copies/ml for types 55, 66, and 65; 2,400 copies/ml for type 52
	No	Yes	PapilloCheck HPV	Greiner Bio-One GmbH, Frickenhausen, Germany	Low-density microarray-PCR amplification of the E1 gene by a group of new E1-specific primers, followed by hybridization to a DNA chip with immobilized HPV oligoprobes	Different manual or automated extraction methods; thermocycler; hybridized signals are visualized on the CheckScanner with CheckReport software	E1 ORF; separate identification of 18 high-risk/probable-risk and 6 low-risk HPV types (6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44/55, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82)	Intrinsic control, ADAT1 (tRNA-specific adenosine desaminase 1)	PreservCyt (Hologic, Bedford, MA USA), Surepath, (BD, Franklin Lakes, NJ, USA), STM (Qiagen, Gaithersburg, MD, USA), biopsy tissues	30–750 copies/reaction
	No	Yes	Abbott real-time high-risk HPV test	Abbott Molecular GmbH & Co. KG, Wiesbaden, Germany	Multiplex real-time PCR with 14 type-specific probes	Automation with m2000 System (extraction, m2000sp, m24; amplification and detection: m2000rt)	Separately identifies HPV16 and HPV18 while concurrently detecting the 12 remaining high-risk types as a pooled result (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68)	Intrinsic control, β-globin	PreservCyt solution (Hologic, Inc.). SurePath preservative fluid (TriPath Imaging), Abbott Cervi-Collect specimen collection kit	Types 16, 18, 35, 39, 45, 51, 59, 66, and 68 can be detected at 500 copies/assay, types 31, 33, 52, and 56 at 2,000 copies/assay, and type 58 at 5,000 copies/assay

	No	Yes	BD Onclarity	Becton, Dickinson and Company, Sparks, MD, USA	Real-time PCR and fluorescently labeled detector probes	BD Viper LT system	E6/E7 DNA; six discrete high-pressure liquid chromatography-risk HPV genotypes (16, 18, 31, 45, 51, and 52) with the remaining eight high-risk genotypes reported in three small groups (33 and 58, 35, 39, and 68, and 56, 59, and 66)	Intrinsic control, β-globin	BD Onclarity HPV cervical brush collection kit, BD SurePath preservative fluid, and PreservCyt solution	Type 16 can be detected at 137 copies/ml (Onclarity cervical brush diluent), 1,584 copies/ml (SurePath preservative fluid), and 1.835 copies/ml (PreservCyt); type 18 can be detected at 51copies/ml (Onclarity cervical brush diluent), 915 copies/ml (SurePath preservative fluid), and 1,786 copies/ml (PreservCyt); limits of detection for other HPV types can be found in the package insert
	No	Yes	HPV-Risk	Self-Screen BV, Amsterdam, The Netherlands	Multiplex real-time PCR with detection by hydrolysis probes	Different automated DNA extraction procedures and real-time PCR platforms (e.g., Life Technologies and Bio-Rad)	E7 DNA; 15 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51,52, 56, 58, 59, 66, 67, and 68); individual results for HPV16 and HPV18 and a pooled result for non-HPV16/HPV18 high-risk HPV types	Intrinsic control, β-globin	Physician-collected cervical scrapings in SurePath preservative fluid; self-collected vaginal brush (VibaBrush (Rovers Medical Devices, Oss, The Netherlands), and cervicovaginal lavage specimens (Delphi Screener; Delphi Biosciences, Scherpenzeel, The Netherlands); formalin-fixed, paraffin-embedded tissue samples	460 copies/reaction for types 16,18, and 56; 4,600 copies/reaction for types 31, 33, 35, 39, 45, 51, 59, and 68; 46,000 copies/reaction for types 52, 58, 66, and 67
E6/E7 mRNA-based assay	2012	Yes	Aptima HPV assay	Hologic Gen-Probe, Inc., San Diego, CA	Target capture; transcription-mediated amplification; hybridization protection assay	Fully automated on the Tigris DTS system or Panther system	E6/E7 viral mRNA; 14 high-risk HPV types (16,18,31,33,35,39, 45,51,52,56,58, 59,66, and 68) as a pooled result	Extrinsic control; noninfectious RNA transcript	Aptima cervical specimen collection or Preserv Cyt	24–488 copies/reaction
HPV genotyping assays	2011	NA	cobas HPV test	Roche Molecular Systems, Inc., Pleasanton, CA	See entry above	See entry above	See entry above	See entry above	See entry above	See entry above
	2009	NA	Cervista HPV16/18	Hologic Gen-Probe, Inc., San Diego, CA	Invader signal amplification	Manual or semiautomated on the Cervista high-throughput automation system	Detects and differentiates HPV16 and HPV18	Intrinsic control, human histone 2 gene	PreservCyt or Qiagen specimen transport Medium	625–1,250 copies/reaction
	2012 (Tigris instrument); 2013 (Panther instrument)	NA	Aptima HPV 16 18/45 genotype assay	Hologic Gen-Probe, Inc., San Diego, CA	Target capture; transcription-mediated amplification; hybridization protection assay	Fully automated Tigris DTS System or Panther System	E6/E7 viral mRNA of HPV16, -18, and -45; differentiates HPV16 from HPV18 and HPV45 but does not differentiate between HPV18 and HPV45	Extrinsic control, noninfectious RNA transcript	Aptima cervical specimen collection or Preserv Cyt	57.3 copies/reaction for HPV16; 84.8 copies/reaction for HPV18; 60 copies/reaction for HPV45

[a] Adapted from reference 105 with permission from Elsevier.
[b] NA, not applicable.

96% is only 69.2%, and for a specificity of 95% it is only 78.3% sensitive (128).

Broom-type or cytobrush/spatula collection devices in ThinPrep Pap test PreservCyt Solution have been FDA approved for use with the currently available tests. SurePath preservative fluid (Becton, Dickinson and Company, Franklin Lakes, NJ) is approved for liquid cytology and for use with the ProbeTec CT/GC Q^x amplified DNA assays (Becton, Dickinson and Company, Franklin Lakes, NJ) for *Chlamydia* and *Neisseria gonorrhoeae*, but not for HPV testing in the United States. The FDA, in conjunction with the manufacturer, issued a warning letter in June 2012 indicating that the HC2 HPV DNA test using SurePath specimens can produce an unacceptably high number of false-negative results. In a large study at John Hopkins Hospital, only 84% of HSIL cases tested positive, while 9% tested equivocal and 7% tested negative, by HC2 from the SurePath vial (130). Similarly, one of two cases of squamous cell carcinoma tested positive and the other tested equivocal (130). The concern is that false-negative HPV results can occur due to cross-linking between proteins and nucleic acids generated over time by the formaldehyde present in the SurePath preservative fluid (130, 131). Chemical linkages can be counteracted to some extent by boiling the sample to inactivate the formaldehyde in the sample preparation process before nucleic acid extraction. False-negative results can occur when SurePath samples are not boiled, but it is important to note in addition that cancers with a small number of HPV DNA copies per cancer cell can be missed even if the sample is boiled if the lower viral loads are below the HC2 cutoff (97). Clinical validation of protocols that involve boiling and also using the cell-enriched fraction generates fewer HPV-positive samples (free virions are not measured) than when performing the same HPV test on the whole vial, which are then called false negative (128). Proper clinical validation, however, shows that the presence of fewer HPV-positive samples results in increased clinical sensitivity for detection of CIN 2+ (128). Some laboratories use specimens collected in SurePath as an off-label laboratory-developed test (LDT) with a boiling step in the procedure and have done validation studies to show that the test produces reliable results in their hands that are acceptable under the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88).

Versions of the Amplicor HPV test and the cobas HPV test (both manufactured by Roche, Basel, Switzerland) are registered for use in Canada, the European Union, and other countries that accept CE Mark certification. The Amplicor HPV test is also approved in Japan. These tests are approved for use with both PreservCyt (Hologic) and SurePath (BD) liquid-based cytology media.

Even tests with high analytical sensitivity of about 10 to 400 HPV DNA copies per reaction are not sensitive enough to detect all cancers (97). For example, if one million cells are sampled and 1 in 2,000 cells is abnormal (as in ASC-US/HSIL), that generates 500 cells containing a fixed amount of HPV DNA (e.g., 1 HPV16 copy/cell) in 10 ml (SurePath) or 20 ml (ThinPrep) of fluid in the collection vial. DNA is extracted from 2 to 4 ml of the vial, equaling 100 cells (100 HPV16 copies) in a 100-μl extraction volume, of which 25 to 50 μl is used in the reaction (PCR). The lower limit can easily be reached when few clonal (abnormal) cells are present in the sample.

DNA-Based Tests: Signal Amplification

HC2 HPV DNA test. The Hybrid Capture 2 (HC2) HPV DNA test was developed by Digene Corporation (Gaithersburg, MD) and is now marketed by Qiagen (Germantown, MD). The HC2 test was FDA approved in 1999 and replaced the original Hybrid Capture (HC1) tube-format assay, which was approved in 1995. The HC2 test was initially approved for reflex testing of patients with ASC-US cytology results and was expanded to include cotesting in conjunction with routine cytology testing for women over age 30 in March 2003. The HC2 HPV DNA test is a microtiter format nucleic acid hybridization assay with signal amplification. Acceptable specimens for the HC2 test include cervical specimens collected using the HC2 DNA collection device or HC cervical sampler (cervical broom) with samples transported in either Qiagen specimen transport medium or Cytyc PreservCyt ThinPrep Pap test solution. Specimens in Qiagen specimen transport medium can be stored at room temperature for up to 2 weeks, refrigerated at 2 to 8°C for an additional week, or frozen at −20°C for up to 3 months prior to testing. PreservCyt solution specimens can be stored for up to 3 months at temperatures of 2 to 30°C but cannot be frozen. There must be at least 4 ml of PreservCyt solution for use in the HC2 test. The specimen is treated to release and denature target DNA, and a mixture of multigene RNA probes specific for high-risk HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68 is added. The probes combine with high-risk HPV DNA, if present, and the resultant DNA-RNA hybrids are captured onto wells of a microtiter plate that are coated with monoclonal antibodies to DNA-RNA hybrids. A second monoclonal antibody conjugated to alkaline phosphatase is added, which binds to the captured hybrids in multiples. The alkaline phosphatase dephosphorylates a chemiluminescent substrate which produces light. The alkaline phosphatase acts on many copies of substrate, resulting in an amplified target/signal level. The emitted light is measured in relative light units (RLU) on a luminometer. An RLU measurement equal to or greater than the cutoff value indicates the presence of high-risk HPV DNA but does not distinguish the specific HPV genotype present. An RLU measurement less than the cutoff value indicates either absence of high-risk HPV DNA or high-risk HPV DNA levels below the limit of detection of the test.

The test can be performed manually or by using the semiautomated Rapid Capture system (RCS). The RCS is a benchtop pipetting and microplate handling station that allows for a partial reduction in hands-on time and processing up to 352 specimens in an 8-h shift.

The Hybrid Capture HPV test was the only test available until 2009, and HC2 at a cutoff of one relative light unit (RLU) is often used as the comparator for new diagnostic HPV tests. The test has a sensitivity of 0.2 to 1 pg/ml, equivalent to 1,000 to 5,000 genome copies of HPV. It is problematic to compare a new diagnostic HPV test to an existing test with a fixed cutoff of detection, considering that cervical cancers are clonal and do not always have the same number of HPV DNA copies per cancer cell. This approach will not allow improvement in the clinical sensitivity of new HPV tests because it will always be necessary to wait until the cutoff of 1 RLU is reached, which will never occur for some cases.

The HC2 test has been widely studied worldwide to determine the clinical sensitivity for detection of HSIL and cervical cancer. Large cross-sectional clinical screening studies have demonstrated

that the HC2 test showed high sensitivity for detection of histologically confirmed high-grade lesions and that testing for high-risk HPV identified more women with high-grade lesions than cervical cytology (132, 133). Sensitivities for detection of CIN 2 or greater ranged from 84.9% to 100% and specificities from 69.5% to 95.8% (133). When both HPV DNA detection and cervical cytology were performed, the sensitivity but not the specificity of the combination was generally somewhat improved compared with that of HPV testing used alone. The negative predictive values were high and ranged between 0.988 and 0.999 to 1.000 (132, 133). Meta-analyses of longitudinal studies have shown that the duration of low risk after a negative HC2 result is at least up to 6 years (134).

One limitation of the probe technology used in the HC2 test is that despite the presence of base pair mismatches, there has been cross-reactivity of the probe mixture with nontargeted noncarcinogenic and possibly carcinogenic HPV6, -11, -26, -30, -40, -42, -53, -54, -61, -67, -70 to -73, -81, -83, -84, -87, and -91 (135, 136). In addition to cross-reactivity, HC2 was found in one study to have a 5% false-positive rate when no HPV DNA is detected in the same specimen by PCR-based tests (137). The rate is actually probably slightly higher when the HPV test is performed not on the whole vial but on the cell-enriched fraction (128). Data from studies performed in several different countries using different molecular tests as comparators indicate that the HC2 test has a false-negative rate of about 5 to 12% in patients with cervical cancer (131, 138–144). Since the HC2 test does not contain an internal control, it is not possible to determine the adequacy of the specimen or the presence of potentially interfering substances.

Cervista HPV HR test. The Cervista HPV HR test (Third Wave Technologies, Madison, WI [now Hologic/Gen-Probe, San Diego, CA]) was FDA approved in 2009. This tests uses proprietary Invader signal amplification chemistry to detect HPV DNAs from 14 high-risk types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68), including the same 13 types detected by the HC2 test plus HPV66. The test does not identify the individual HPV type. Since the HC2 test reportedly cross-reacts with HPV66, the impact of the additional target when performance characteristics are compared should be marginal. In one observational study, HPV66 was detected in 1 of 93 CIN 2+ cases and HPV53, which is also detected by HC2, was detected in 2 of the 93 CIN 2+ cases (97). The test is performed manually, beginning with extraction of DNA from cervical specimens collected in PreservCyt solution using the Genfind DNA extraction kit (Hologic/Gen-Probe, San Diego, CA). The subsequent Invader assay uses two simultaneous isothermal reaction performed in a single tube. The primary reaction uses three mixtures of sequence-specific probes that target the L1, E6, and E7 genes and detect four groups of high-risk HPV based on phylogenetic relatedness (A5/A6, HPV51, -55, and -66; A7, HPV18, -39, -45, -59, and -68; A9, HPV16, -31, -33, -35, -52, and -58). A secondary reaction produces a fluorescent signal. If any one of the probes generates a signal that is greater than the cutoff value, the result is considered positive. The primary reaction mixtures also include oligonucleotides that bind to the human histone 2 gene (HIST2H2BE). Bound HIST2H2BE probe is detected using a distinct fluorophore and serves as an internal control to reduce the possibility of false-negative results due to insufficient sample. External positive and negative controls are also included to ensure quality in each run. The analytical sensitivity of the test depends on the HPV type and ranges from 1,250 to 2,500 copies per reaction for HPV16, -18, -31, -45, -52, and -56, from 2,500 to 5,000 copies per reaction for HPV33, -39, -51, -58, -59, -66, and -68, and from 5,000 to 7,500 copies per reaction for HPV35 (145).

To increase productivity, the DNA extraction and HPV detection steps can be automated on the Cervista high-throughput automation (HTA) system. The HTA system can accommodate batches of 24, 48, 96, 192, or 288 specimens, with results available in 8.5 h for 96 specimens and the operator able to walk away after 2 h 50 min. Throughput for 192 samples is 12.25 h, with the operator able to walk away after 6 h 35 min. The Cervista medium-throughput automation (MTA) system for smaller to midsized laboratories accommodates 24 samples in 5 h 40 min, 48 samples in 6 h 6 min, and 96 samples in 7 h 44 min, but is not available in the United States.

Advantages of the Cervista HPV HR test compared to the HC2 test include reduced sample volume (2 ml versus 4 ml) and the presence of an internal control (146). The analytical sensitivity of the Cervista HPV HR test varies somewhat depending on HPV type, with limits of detection of 1,250 to 2,500 copies per reaction for HPV16, -18, -31, -45, -52, and -56, 2,500 to 5,000 copies per reaction for HPV33, -39, -51, -58, -59, -66, and -68, and 5,000 to 7,500 copies per reaction for HPV35. There is potential cross-reactivity in the Cervista HPV HR test with some low-risk HPV types, such as HPV67, -70, -73, -84, and -91, but the degree of cross-reactivity is considered to be significantly less than that seen with the HC2 test (146–148).

A number of clinical trials have shown that the Cervista HPV HR test is at least comparable to HC2 in the ability to accurately detect high-risk HPV and has the advantage of significantly lower cross-reactivity to other HPV types (136, 146–151). Discordant results are seen with specimens that test HC2 negative but are positive with all three probe pools in the Cervista HPV HR test (136, 152). The majority of these "triple positives" are not confirmed when tested using PCR-based HPV tests. Researchers recommend that triple-positive Cervista results should be confirmed by another test and, if not confirmed, reported as indeterminate (136). Alternatively, validating an increase in the second cutoff from 1.93 to 5.0 could result in improved specificity without affecting the sensitivity of the test (152).

DNA-Based Tests: Target Amplification

cobas 4800 HPV test. The cobas 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, CA) was approved by the FDA in 2011 and has been available in the European market since 2009. The test uses multiplex real-time PCR and nucleic acid hybridization with four different fluorescent reporter probes that concurrently detect the L1 gene of HPV16 and HPV18 as individual reactions and HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 as a pooled result. Beta-globin is included as an extraction and amplification control. Positive and negative controls are also included to validate each run. The test is automated using the cobas 4800 system, with manual involvement needed only to load and unload the microwell plate. The system consists of two separate instruments, with the cobas z 480 instrument for automated nucleic acid extraction and the cobas x 480 analyzer for PCR amplification and detection reactions in a single tube. The accompanying software allows for testing for all 14 targeted high-risk HPV types as a pooled result or for pooled testing plus separate genotyping for HPV16 and HPV18. The system is designed to process up to 280

samples in 1 day and can be interfaced to laboratory information systems. Cervical specimens collected in PreservCyt solution may be stored at 2 to 30°C for up to 6 months prior to testing. Cervical specimens collected in cobas PCR cell collection medium (Roche Molecular Systems, Inc., Pleasanton, CA) can be held at 2°C to 30°C and are stable for 90 days.

The analytical sensitivity of the cobas 4800 HPV test at the clinical cutoff is 150 copies/ml for HPV45, 300 copies/ml for HPV31, -33, -39, -51, and -59, 600 copies/ml for HPV16, -18, -35, and -58, 1,200 copies/ml for HPV56, -66, and -65, and 2,400 copies/ml for HPV52. Validation studies comparing the cobas 4800 test to HC2 indicate that the cobas 4800 test has clinical sensitivity comparable to that of HC2 and improved specificity due to a lower level of cross-reactivity with low-risk HPV genotypes (153–155). The two false-positive results in one study occurred in women who had mixed infection with several low-risk/possibly carcinogenic HPV types (155). False-negative results can also occur, since the L1 gene is lost upon integration into the human genome in a substantial proportion of cancers and HPV tests, such as the cobas 4800, that detect only L1 can give false-negative results in these cases (156).

E6/E7 mRNA PCR (Reverse Transcriptase PCR)

Detection of HPV E6/E7 oncogene mRNA in cervical cells is an alternative to detection of HPV DNA. HPV DNA may be detected in transient infections, but there is very little expression of E6/E7 mRNA. It has been noted that although there may be little expression of E6/E7 mRNA, HPV tests based on nucleic acid sequence-based amplification (NASBA) also detect DNA, albeit with less efficiency, which is abundantly available in transient infections (157). In persistent infections, however, there is overexpression of E6/E7 mRNA, and the infection is less likely to regress. Detection of overexpression of E6/E7 mRNA may be more directly associated with disease progression. Since referral for colposcopic examination is recommended for women who test positive for high-risk HPV and have ASC-US cytology results, the increased specificity of the E6/E7 mRNA tests has the potential to reduce the number of referrals (158). The only commercially available FDA-approved tests that detect E6/E7 mRNA are the Aptima HPV assay and the APTIMA HPV16 18/45 genotype assay (Hologic Gen-Probe, Inc., San Diego, CA), which were FDA approved in late 2012. The Aptima HPV assay allows for the detection of E6/E7 mRNA transcripts of 14 high-risk HPV types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) but does not discriminate between the HPV types. A noninfectious RNA transcript serves as an extrinsic process control.

Cervical specimens can be collected either in PreservCyt solution or in an Aptima cervical specimen collection and transport kit, which contains a medium that lyses the cells to release the mRNA and protect it from degradation. Specimens collected in an Aptima collection and transport kit can be stored at 2 to 30°C for up to 60 days or frozen at −20°C or colder for up to 24 months before testing. For specimens collected in PreservCyt solution, a 1-ml aliquot must be transferred to an Aptima specimen transport tube either before or after processing for cytology. Before transfer, PreservCyt liquid Pap specimens should be stored at 2 to 8°C, with no more than 30 days at temperatures up to 30°C. Once a specimen collected in PreservCyt liquid has been transferred to an Aptima specimen transfer tube, it may be stored at 2 to 30°C for up to 60 days prior to testing.

The Aptima assay is performed in a single tube and uses complementary oligomers to isolate the HPV mRNA target onto magnetic microparticles. The target mRNA is amplified using transcription-mediated amplification (TMA), and the amplification products are detected by a hybridization protection assay (HPA) using chemiluminescent labels. The processes are monitored by use of an internal control. The test is fully automated on either the Panther system or the Tigris DTS system. The technologist hands-on time to load specimens and reagents for either instrument system is about 45 min, and results are generated in about 4 h. The capacity on the Panther instrument is up to 275 tests in 8 h and up to 750 tests in 16 h. The Tigirs instrument can process approximately 450 samples in 8 h and up to 1,000 samples in about 13.5 h.

An advantage of the Aptima HPV test is that it does not show cross-reactivity with any tested low-risk HPV types (HPV6, -11, -42, -43, -44, -53, -61, -71, and -81) or with normal flora or opportunistic organisms that may be found in cervical samples (159). The Aptima test shows high analytical sensitivity, with a limit of detection of 24 to 488 copies per reaction (159).

FDA approval was based on data from the Clinical Evaluation of Aptima HPV RNA (CLEAR) trial (159). In this evaluation of more than 11,000 women, the detection of E6/E7 mRNA by the Aptima HPV RNA test was as sensitive for detecting CIN 2 and CIN 3 as detection of HPV DNA by the HC2 test (159). Of note is that the Aptima HPV RNA test showed statistically significantly higher specificity for high-grade disease than the HC2 test (159). Subsequent clinical studies have likewise shown that detecting E6/E7 mRNA has similar sensitivity but significantly higher specificity than tests that detect HPV DNA (115, 158–169).

HIGH-RISK HPV DETECTION AS A FIRST-LINE TEST IN PRIMARY CERVICAL CANCER SCREENING

Studies have allowed us to have a fairly comprehensive understanding of the natural history of HPV infection, which permits awareness of the importance of the role of HPV testing in cervical cancer screening and detection. In the United States, cervical cytology serves as the primary screening test, and HPV DNA or RNA testing is used as a cotest or reflex test to triage patients with normal cytology. Internationally, the trend is toward reversing this sequence. Some countries, including China, India, Sweden, the Netherlands, and Australia, are beginning to establish programs with HPV testing as the primary screen and cervical cytology used to triage patients who are HPV positive. This trend is based on the fact that numerous studies using different cervical cancer screening algorithms have systematically concluded that HPV DNA testing is more sensitive than cytology for identifying women with cervical precancer (170–176). Furthermore, longitudinal data from these studies have implied a reduced incidence of invasive cervical cancer by showing over time a reduced cumulative incidence of ≥CIN 3 among women who tested negative for HPV at baseline compared with those who had normal cytology. In these studies, however, the various testing strategies were not applied to each patient, and disease was often verified in different ways and at different intervals for the HPV and cytology arms of the study. Because of the design, these studies can result in overestimates of sensitivity and often provide biased data that cannot be corrected by statistical methods. This makes it difficult to determine if the test(s) is actually better or if more disease was detected because more patients were referred to colposcopy.

Other trials have recently been reported that were designed specifically to investigate the performance of HPV molecular testing as a stand-alone primary screening test to detect cervical precancer. In these studies, all patients had both an HPV molecular test and cytology performed at baseline. The design of these studies allows performance characteristics to be calculated for any combination of HPV and cytology test results for the entire study population without verification bias. All of the studies, however, carry the same general bias in that HPV tests and cytology are performed on different fractions of the sample. In the United States, the Addressing the Need for Advanced HPV Diagnostics (ATHENA) trial was a multicenter prospective cohort study that compared the cobas HPV test (Roche Molecular Systems, Inc., Pleasanton, CA) and liquid cytology as stand-alone screening tests in women 25 years of age and older (177–179). All study participants had both the cobas HPV test, which concurrently detects 14 high-risk types and distinguishes HPV16 and HPV18, and cytology performed at baseline. All patients with ≥ASC-US cytology, those with negative cytology but a positive test result for high-risk HPV DNA, and a randomly selected subset of patients with negative cytology and HPV results were asked to proceed to colposcopy. All colposcopy patients who did not have histology of ≥CIN 2 were invited to continue in a 3-year longitudinal study with annual cervical screening using cytology and HPV DNA testing as before. At year three, all patients were invited to colposcopy/biopsy so that the determination of true disease status would not be biased by false-negative normal cytology. The 3-year data were analyzed to compare three screening strategies: (i) cytology alone with HPV DNA testing performed for women with ASC-US, (ii) a hybrid strategy using cytology for women 25 to 29 years old and cotesting using both cobas HPV DNA and cytology for women 30 years or older, and (iii) cobas HPV DNA testing alone, with women who test HPV DNA negative rescreened in 3 years, those who are HPV16/18 positive referred to colposcopy and those with other genotypes triaged with cytology, and those with ASC-US or greater referred to colposcopy and those with normal cytology rescreened with both tests at 1 year (179). Of the three screening strategies, the highest sensitivity was obtained when HPV DNA testing was used alone and the screening population was women age 25 years or older. Compared to cytology, testing for HPV DNA alone provided a 28.3% increase in sensitivity for detecting CIN 3+ in women 25 years of age or older and a 24.3% increase in women 30 years of age or older. Cytology alone had higher specificity than either of the other two screening strategies. This is not unexpected, since HPV testing on a random fraction from the vial mainly detects women having sex, and most of these women do not have colposcopic abnormalities, which decreases specificity. Sensitivity is also decreased because the cutoff for detection in HPV tests is increased (less sensitive test) to minimize the rate of detection of women having sex without having disease. This results not only in longer times to detection for the women with CIN 3+ but also in the inability to detect a larger fraction of CIN 3+ cases that do not have enough HPV per cancer cell. It was observed that using HPV DNA testing alone resulted in a significant increase in the number of women referred to colposcopy compared to cytology alone, but the number of colposcopies needed to detect a single case was the same as with the cotesting strategy.

Based on ATHENA data, in April 2014, FDA approved the cobas HPV test as a primary screening test. The approved algorithm includes that women who test negative for high-risk HPV can be returned to triennial screening unless medical history or other risk factors indicate otherwise. Women who test positive for HPV16 and/or -18 should be referred to colposcopy. Women who test high-risk HPV positive but not for HPV16 or -18 should be evaluated by cervical cytology, and the patient should be referred to colposcopy if cytology shows ASC-US or higher. If cytology is normal, the patient may return to routine triennial screening.

A similarly designed study in Canada, the Canadian Cervical Cancer Screening Trial (CCCaST), was done to evaluate the performance of the Digene HC2 HPV test (Qiagen, Redwood City, CA) and cytology as stand-alone screening tests for women aged 30 to 69 (180). Combined data from the first round of screening for 9,959 women showed that HPV testing had significantly higher sensitivity than cytology (97.4% versus 56.4%) as a stand-alone test but had lower specificity (94.3% versus 97.3%) (PPV, 7.0) and resulted in the most referrals to colposcopy (6.1% versus 2.9%). Using HPV as the primary test and triaging all positive results by cytology emerged as the algorithm with the highest PPV (21.4) and referred only 1.1% of patients to colposcopy. Of all of the screening approaches, HPV plus cytology cotesting had the lowest PPV (5.1), triggered more colposcopies, and required more screening tests. Negative predictive values were higher than 99% for all screening strategies. Raising the HC2 cutoff from ≥1 pg HPV DNA/ml as recommended by the manufacturer to ≥2 pg HPV DNA/ml resulted in a better PPV.

A very large trial was performed at Kaiser Permanente Northern California (KPNC) in a routine clinical practice in which cotesting with cytology and HPV at 3-year intervals was the standard of care (181). The trial included more than 1 million women who were HPV negative and had normal cytology at entry. The most recent analysis was focused on providing further information on the utility of HPV primary screening by specifically looking at future cancer risks after negative screening results (181). The analysis found that a negative HPV test alone provides a very high 3-year assurance against CIN 3 or cancer that is either better than or similar to that with the current guideline intervals of 3 years for cytology and 5 years for cytology plus HPV cotest.

An evaluation of six HPV tests in residual liquid-based cervical cytology specimens was done in a population of women undergoing routine screening (165). The evaluation included some commercially available tests and some tests still in development. DNA-based tests included HC2 (Qiagen GmbH, Hilden, Germany), the cobas 4800 HPV test (Roche Molecular Diagnostics, Pleasanton, CA, USA), the Abbott RealTime high-risk HPV assay (Abbott Molecular GmbH & Co. KG, Wiesbaden, Germany), and the BD HPV test (BD Diagnostics, Sparks, MD, USA), and RNA-based tests included PreTect HPV-Proofer (NorChip, Klokkarstua, Norway) and APTIMA (Gen-Probe Inc., San Diego, CA, USA). All of these tests, with the exception of PreTect HPV-Proofer, produced high PPV and specificity values for high-grade cytology lesions, and the results of that study support their use for primary screening. The APTIMA test showed similar high sensitivity but better specificity than the DNA tests. Longitudinal data that demonstrate a long-term low-risk period following a negative test are available for some of the DNA-based tests that have been in use for longer periods of time (93, 134). Similar data have been generated for the Aptima HPV RNA test, which supports its use as primary screening test (166). Clinical trials and studies in health care settings in which patient recall and follow-up are structured provide important information about the performance of HPV as a screening

test. The performance of these tests in the opportunistic screening environments in the United States and some regions of Canada will be further revealed when primary testing becomes more widely adopted.

The Society of Gynecologic Oncology (SGO) and the American Society for Colposcopy and Cervical Pathology (ASCCP) issued an Interim Guidance Report in January 2015 after reviewing 11 English-language studies published since November 2011 as well as several key reports published prior to that date, all from studies that were designed to evaluate detection of HPV as a primary test performed for cervical cancer screening (182) (Table 4). The Interim Guidance Report recommends considering primary HPV testing for women starting at age 25 and continuing every 3 years as long as tests remain negative. Women between ages 21 and 25 continue to be screened using cytology alone every 3 years, as recommended by existing guidelines. Women with a positive primary HPV test result should be tested for HPV16 and HPV18 and referred to colposcopy if found to be positive. Women who are HPV positive but not for HPV16 and/or -18 should be followed by reflex cytology testing and referred to colposcopy if ASC-US or higher or followed up in 12 months if cytology is normal.

The utility of HPV as a primary test in immunocompromised patients has been the focus of several studies. It is well documented that, although effective treatment helps to somewhat reduce the risk, high-risk HPV can be detected with greater frequency in cervical specimens of HIV-infected women and persistence of infection is more prevalent in this population than in HIV-uninfected women (183, 184). These factors contribute to the finding that HIV-infected women are three to five times more likely than HIV-uninfected women to develop cervical dysplasia (183). HPV prevalence in HIV-infected populations is more than three times the estimated prevalence of 12% found in the general population and ranges from about 31% to 57%, with higher percentages in Africa than in North America or Western Europe (185). A pilot study conducted in Jos, Nigeria, evaluated a cohort of 97 HIV-infected women age 21 to 49 with normal cytology (186). High-risk HPV was detected by the Digene HC2 assay in almost half (44.9%) of the cohort, with a significant number of the positive patients being less than 30 years of age, and most were on successful antiretroviral therapy. The authors note a rising number of cancers in HIV-infected women less than 30 years of age and raise a concern about normal cytology and unrecognized high-risk HPV infection when cytology is used as the primary screening test. A follow-up to that study will determine whether precancerous lesions were missed on cytology smears in these women. A prospective longitudinal study of a large cohort of 652 HIV-infected women primarily of African origin but living in Belgium and with normal cervical cytology had similar findings (185). The prevalence of high-risk HPV infection at first screen was 42.8%, with the majority of positive results occurring in women under age 30 and in those with CD4 counts less than 200 cells/μl but with prevalence remaining higher than in the general population across each age and CD4 count group. The rate of acquisition of new high-risk HPV infection over the median 66-month follow-up was high at 13.4 per 100 woman-year, compared to a rate of 5 per 100 woman-year in HIV-negative women with normal cytology. Since early in the course of HPV infection, high-risk types ultimately causative of cervical cancer can be present in an individual with normal cytology and since HIV infection predisposes to HPV persistence, it is likely that HPV primary testing could be especially of benefit in preventing cervical cancer in HIV-infected women.

THE ROLE OF HPV GENOTYPING

HPV genotyping is the amplification of a single genotype of HPV by targeting a type-specific DNA sequence. It has been well established that that the majority of invasive cervical cancers worldwide are associated with HPV16 and/or -18. Some large trials suggest that identification of individual HPV genotypes, particularly HPV16 and -18, can identify a subset of women who have a markedly greater risk of having cervical lesions of grade CIN 2 or worse (153, 187–192). These trials found that HPV16 provided the biggest risk stratification, whereas the additional benefit of testing for HPV18 and HPV45 was limited. The observed lower risk for HPV18 may be because high-grade cervical lesions associated with HPV18 remain clinically unapparent for a longer period of time. For women with CIN 3+, the viral doubling time for HPV16 is 289.0 days and that for HPV18 is 408.5 days (97). This means that women with CIN 3+ due to HPV18 need to wait longer before the cutoff of the HPV test is reached. The type-specific viral load is lower in HPV18 CIN 3+ (less HPV18 detected per cancer cell) than in HPV16 CIN 3+. Most HPV18 CIN 3+ cases do not reach the HC2 detection limit, which results in older clones (more transformed) upon detection and worse prognosis. To be useful, the risk stratification provided by HPV genotyping must affect clinical management, and even with the notable risk stratification offered by genotyping for HPV16 with limited added value of HPV18 and HPV45, the risk among women testing negative for these genotypes but positive for other high-risk genotypes was not low enough to justify delaying colposcopy (189).

The prevalence of HPV16 and HPV18, along with the nontargeted but closely related HPV31, -33, and -45, is rapidly decreasing in populations who are being vaccinated, and HPV type replacement (with HPV51, -52, -53, -56, and -58) is already occurring (193–198). This creates the need for HPV genotyping tests that detect additional types besides HPV16 and -18.

Although calculation of risk is commonly used, HPV measurements can also be used to identify which HPV-driven process is ongoing for each HPV type present. HPV genotyping works very well if it is used to categorize an HPV infection as transient, productive, or clonal and not for risk stratification (97). Only clonal progressive processes lead to cancer. Women without these progressive clonal processes can be followed less aggressively. Because of this, the use of genotyping in the cervical cancer screening algorithm is potentially attractive. Practical issues that need to be considered include whether genotyping should be limited or universal, and if it is limited, the population that should be tested needs to be defined. Also considered is whether or not testing should be done as part of initial screening or as a reflex test.

The FDA has approved genotyping tests for two uses. The first is for women 21 years of age and older who have ASC-US cervical cytology results. Genotyping results in this population can be used to guide management along with other information, including cytology results, other risk factors, and professional guidelines. The second approved application is to evaluate women 30 years of age and older for the presence or absence of HPV16 and/or HPV18 if they have tested positive for high-risk HPV. Current recommendations in consensus guidelines include the use of HPV genotyping for women 30 years of age or older who test positive for high-risk HPV but have normal corresponding cervical cytol-

ogy (103, 104). It is recommended that women in this population who are found to be positive for HPV16 and/or HPV18 should be referred to colposcopy, while women who test negative for genotypes 16 and/or 18 may have repeat cytology and high-risk HPV testing in 12 months (103, 104). Interim guidelines also include the use of genotyping for HPV16 and HPV18 to triage women who test positive for HPV when it is used as a primary stand-alone screening test to determine which patients should be referred for immediate colposcopy (182).

Because of the clinical significance of HPV16 and HPV18, several HPV genotyping tests have become commercially available in recent years to identify these HPV types in cervical specimens. The cobas HPV test (Roche Molecular Systems, Pleasanton, CA) is a real-time PCR test that is automated on the cobas 4800 system and detects 14 high-risk HPV types as a group with integrated separate results for HPV16 and HPV18. The characteristics of this test are described above (Table 5). Two other tests are made by manufacturers of high-risk HPV screening tests and are meant to serve as reflex tests that specifically detect HPV16 and HPV18 or HPV18/45. The Hologic Cervista HPV16/18 test (Hologic Gen-Probe, Inc., San Diego, CA) was FDA approved in 2009 and uses the same Invader chemistry as the corresponding high-risk HPV screening test described above, but it includes oligonucleotide probes in the primary reaction that bind specifically to target sequences of HPV16 and HPV18. The specimen collection and storage requirements are the same as those for the Cervista HPV HR test. The analytical sensitivity of the HPV16/18 genotyping test is 625 to 1,250 copies per reaction for both types (199). A limitation of the Cervista HPV16/18 test is cross-reactivity with high levels of high-risk HPV31. The APTIMA HPV16 18/45 genotype assay (Hologic Gen-Probe, Inc., San Diego, CA) was FDA approved in late 2012 for use with the Tigris DTS System and in 2013 for use with the Panther system to identify RNA from HPV16, -18, and/or -45. The other approved genotyping tests do not include HPV45, but the APTIMA HPV16 18/45 genotype assay was designed to detect the HPV types most commonly associated with invasive cervical cancer as well as adenocarcinoma. Although it is not common, HPV45 is the third most common HPV type in invasive cervical cancer in some series and is associated with 12% of adenocarcinomas (200). Further rationale for including HPV45 comes from data suggesting that while the incidence of cervical cancer has been decreasing over the last several decades, the prevalence of adenocarcinoma has risen approximately 32% (201). The analytical sensitivity of the APTIMA HPV16 18/45 genotype assay reported by the manufacturer is 57.3 copies per reaction for HPV16, 84.8 copies per reaction for HPV18, and 60.0 copies per reaction for HPV45.

HPV SURVEILLANCE FOLLOWING COLPOSCOPY TREATMENT

The finding of high-grade cervical lesions with histology grade HSIL or higher is a generally accepted threshold for treatment. Treatment may include ablative or excisional procedures to remove or destroy the abnormal cells by conization, cryocauterization, laser ablation, or loop electrocautery excision procedure (LEEP). Data from clinical trials and meta-analyses have shown that treatment is very effective (202, 203). The largest study with the longest follow-up was the Costa Rica Natural History Study, which monitored 347 treated (mainly with LEEP) women for 7 years and found a clearance rate of 97.2% (203). Treated women still have a 2.8-fold-higher risk of developing high-grade lesions than the normal population for up to 20 years after treatment (204). The success of treatment has been traditionally monitored by repeat colposcopy and cytology. Current U.S. guidelines recommend that women continue to undergo routine age-based screening for 20 years after treatment, even if it requires that screening continue past age 65 years and even if the treatment included total hysterectomy (102, 103).

Because colposcopic detection and cervical cytology are subjective and comparatively insensitive, HPV testing is emerging as an important marker of recurrent/residual disease. Meta-analyses have shown that HPV testing alone is more sensitive than cytology alone for detection of residual/recurrent disease and that a combination of HPV and cytology further increases sensitivity (203, 205). These analyses have also confirmed that the absence of HPV is an accurate indicator of a low risk for residual/recurrent disease. The negative predictive value of HPV testing could approach 100%, and women who test HPV negative at 6 to 18 months following treatment can be safely returned to routine screening (202, 203, 206).

Although somewhat limited by length of follow-up, it appears that HPV detected posttreatment does not necessarily result in CIN 2+ disease (207). Close follow-up (i.e., every 6 months) is recommended (203). Persistent HPV infection with a minimum duration of 3 years was necessary before correlation to a diagnosis of posttreatment CIN 2+ was observed in the Costa Rica Natural History Study (203). HPV16 was more highly associated with risk of persistent infection (41-fold-higher risk) than other HPV types (203). Serial measures are needed to fully elucidate the time from treatment to detection, but available data show that the median time between treatment and diagnosis of recurrent HPV infection was 6 years (203).

If the HPV type causing the infection prior to treatment is known, determination of the HPV type detected following treatment may be helpful in determining if the posttreatment infection represents a recurrent infection or new infection (208). A new infection is indicated if the HPV type detected is different from the type present pretreatment. For new infections acquired posttreatment in the Costa Rica Natural History Study, no cases of disease were identified in the follow-up period (203).

FUTURE CHALLENGES

Current cervical cancer screening strategies using cytology with or without HPV testing have been effective, but with ongoing emergence of new information there is a continued need to frequently evaluate guidelines, with focus on improving the efficiency and effectiveness of screening, better management of patients, and preventing increased costs. The importance of HPV testing has been recognized, and its role in cervical screening is shifting from cytology alone to cytology plus HPV cotesting and now to the recognition of a new screening paradigm in which HPV testing can function alone as the primary screening test. Beyond cytologic examination and testing for the presence of high-risk HPV, there is a continuing need to find viral and/or host markers of disease progression that will help distinguish clinically insignificant, self-limited HPV infections from precancerous infections. There is also a need for evidence-based clinical practice guidelines for immunocompromised populations and anal, oropharyngeal, and other HPV-related conditions.

Given the general age of onset of cervical HPV-related precancerous conditions, it may be several years before the impact of

vaccination is known. Since vaccination is directed against high-risk HPV16 and -18 and does not protect against the other high-risk HPV types that cause approximately 30% of cervical cancers, it is possible that high-risk genotypes not covered by the vaccine will predominate in cervical cancers in the future. To aid in monitoring the possibility that high-risk genotypes not covered by the vaccine can predominate in cervical cancer in the future, there is a need for high-throughput broad-spectrum genotype assays. Technologies such as PCR followed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry can provide cost-effective HPV genotyping in large-scale epidemiological studies (197, 198, 209). Further, protection afforded by vaccination will not be absolute, the duration of protection is not known, and older women currently not covered by vaccination will continue to be at risk. As the impact of vaccination unfolds and presumably precancerous cervical lesions become rare, the screening paradigm will need to be adjusted. While there have not been definitive studies, it is possible that vaccination may also reduce the risk of other HPV-related health concerns, including cancers of the oropharynx and anus.

High-risk HPV DNA has been found in some unconventional HPV cancers, such as breast, prostate, colorectal, and lung cancers, and a causative role has been implied (210–213). Although known to be associated with human cancers, the potential role of high-risk HPV in the carcinogenic steps of these cancers is controversial. The burden of proof is high, and rigorous studies will be required to prove causation.

Advancements will undeniably continue to be made. New tests, new markers, and new evidence will improve our ability to differentiate insignificant HPV infection from precancerous and cancerous disease. The fundamental goal is to have a simple, robust and cost-effective system that will offer better care for patients.

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