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REVIEW

Current and Emerging Molecular Tests for Human Papillomavirus–Related Neoplasia in the Genomic Era

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Laboratory tests have a key role in preventing human papillomavirus (HPV)–driven carcinomas and in guiding therapeutic interventions. An understanding of the virology, immunology, and carcinogenesis of HPV is essential for choosing appropriate diagnostic test modalities and developing new and even more effective cancer prevention strategies. HPV infects basal epithelial cells on multiple surfaces and induces carcinoma primarily in the cervix and the oropharynx. HPV types are stratified as high risk or low risk based on their carcinogenic potential. During oncogenesis, HPV interferes with cell cycle regulation and incites DNA damage responses that thwart apoptosis and enable mutations to accumulate. Such mutations are an adverse effect of innate and adaptive antiviral immune responses that up-regulate DNA-editing enzymes, with natural selection of cells having a chromosomally integrated viral genome lacking expression of viral proteins targeted by the immune system. Infected cancers share a similar mutation signature, reflecting the effect of apolipoprotein B mRNA-editing catalytic polypeptide enzyme DNA-editing enzymes. It is feasible that genomic tests for characteristic mutations or methylation signatures, along with tests for dysregulated HPV gene expression, add value in predicting behavior of premalignant lesions. Furthermore, these tumor markers in cell-free DNA of plasma or body fluids may one day assist in early detection or monitoring cancer burden during treatment. *(J Mol Diagn 2017, 19: 366–377; http://dx.doi.org/10.1016/j.jmoldx.2017.01.006)*

Microbial Influence on Human Malignancies

Over a century ago, the pathologist Peyton Rous showed that cell-free tumor extracts containing the Rous sarcoma virus were able to transmit tumors between chickens. Several years later, he identified a papillomavirus as the cause of benign warts in rabbits. Despite his extraordinary work, microbe-mediated oncogenesis was considered a scientific curiosity until Epstein and Barr's 1967 identification of viral particles in human Burkitt lymphoma.[1] Since the discovery of the Epstein-Barr virus, six more viruses have been identified as the etiological agents of human malignancies, including hepatitis B virus, hepatitis C virus, human T-lymphotropic virus-1, human herpevirus 8, merkel cell polyomavirus, and human papillomavirus (HPV).[1] Current estimates link a viral etiology to 10% to 15% of all human malignancies.[2] In addition, the bacterium

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Helicobacter pylori and the parasites *Clonorchis sinensis* and *Schistosoma haemotobium* are associated with human malignancies.[3]

Despite significant progress, much remains to be learned about the mechanism by which microbes contribute to malignant transformation. In some instances, microbial-derived factors interact directly with specific cellular pathways to increase cellular proliferation, inhibit apoptosis, and induce accumulation of mutations within infected cells. Activation of the immune system can cause bystander damage to DNA through nonspecific toxic mediators, such as reactive oxygen species, and direct mutagenesis via up-regulation of DNA-editing enzymes such as apolipoprotein B mRNA-editing catalytic polypeptide enzymes (APOBECs).[1,4] Recent large-scale genome studies showed the effects of this family of enzymes by virtue of distinct mutation signatures in microbe-related cancers including HPV^+ neoplasms.[5]

In this review article, we examine recent updates in our understanding of HPV carcinogenesis and the implications of this knowledge on current and future diagnostic strategies to improve early detection and targeted interventions.

HPV Virology and Type Prevalence

HPV is a nonenveloped circular double-stranded DNA (dsDNA) virus in the papillomaviridae family that infects basal epithelial cells in the epidermis and mucosae.[6] There are now five genera, 49 species, and 202 HPV types recognized by the International Human Papillomavirus Reference Center.[6] Genera exhibit <60% sequence homology in the L1 gene encoding the major capsid protein, and are designated α, β, γ, μ, and ν.[6] Members of the α genus infect the genital mucosa, whereas members of all five genera infect the oral mucosa and skin.[7] Species exhibit 60% to 70% homology, and types exhibit >90% homology.[6]

High-risk HPV types typically refer to those types having a causal association with cervical cancer. Overlapping subsets of these high-risk HPV types are causally related to other lower anogenital tract cancers and head and neck cancers. The International Agency for Research on Cancer working group classifies HPV types as carcinogenic to humans, probably carcinogenic, possibly carcinogenic, or low risk. Commercial molecular tests described below can identify each high-risk type, including the two most oncogenic types, HPV 16 and 18.[8] Massive parallel sequencing is capable of distinguishing all HPV types, and emerging data suggest prognostic impact of HPV type in oropharyngeal carcinomas.[9–11]

A meta-analysis of 423 published studies reveals the percentage of each high-risk type in cervical carcinoma and premalignant lesions.[7] Some geographic variability in type prevalence is seen both in cervical and in head and neck cancers. For example, in North America, HPV is identified in 60%, 13%, and 19% of carcinomas arising in the oropharynx, oral cavity, and larynx, respectively, and global rates are similar; HPV16 is the most common type at all geographic locations.[12] The next most common types vary by geographic location and anatomical site (Table 1). Notable variations include identification of HPV18 in 25% of HPV^+ carcinomas in the oral cavity worldwide versus 7% in North America and up to 40% in Asia.[12] Furthermore, HPV types associated with laryngeal cancer in Central and South America differ from other geographic locations with higher rates of HPV58, 45, 51, 59, and 66 and minimal HPV18.[12] Laboratorians should note these geographic differences and choose diagnostic test strategies that are suited to their patient population.

HPV Gene Expression and Its Effects on Host Cells

HPV infection requires abrasion of the surface epithelium with exposure of the basement membrane, enabling viral binding to exposed heparan sulfate proteoglycans.[13] Furin bound to the basement membrane cleaves L2 on the viral capsid, exposing the major viral capsid protein L1, which is the target of prophylactic vaccines.[13,14] L1 binds to an unknown receptor on adjacent basal epithelial cells, resulting in viral entry via endocytosis in the first 2 to 4 hours, uncoating in the late endosome (8 to 12 hours), and release of viral dsDNA complexed with L2 into the cytosol.[13] Viral DNA enters the nucleus within 24 hours, with transcription of six early genes: E1, 2, 4, 5, 6, and 7.[13] E1 and E2 are required for viral episome replication to yield approximately 100 viral genomes per cell. Multiple copies of the viral genome facilitate localization of HPV in tissue sections by *in situ* hybridization with probes targeting viral DNA.[15] E2 also tethers viral DNA to the human genome with a predilection for fragile sites.[16,17] E5 is a membrane protein that activates the epidermal growth factor receptor signaling pathway, leading to cell proliferation.[17] Simultaneously, expression of E7 inhibits the retinoblastoma protein, RB1, leading to activation of elongation factor 2, promoting S phase and viral and cellular DNA replication.[17] RB1 inhibition up-regulates CDKN2A/p16, permitting us to exploit p16 immunohistochemistry as a surrogate marker for HPV infection.[18] Meanwhile, expression of E6 inhibits TP53 and BAK, allowing for mutation accumulation in infected cells that are now restricted in their ability to induce apoptosis in response to DNA damage.[17] E6 also induces the transcription of methyltransferases and methylases, resulting in epigenetic-mediated changes in gene expression that promote carcinogenesis. E6 and E7 mRNAs are targeted by *in situ* hybridization probes to localize HPV infection to specific cells in biopsy specimens.[19]

As infected epithelial cells mature, E4 binds to cytokeratins and helps orchestrate viral capsid assembly and intracellular trafficking of viral components.[14] The effects of

Table 1 HPV Types in Infected Cervical Carcinoma and in Head and Neck Carcinoma

HPV type		Prevalence globally or (in North America) [in Central and South America] {in Asia}*				
IARC group	Viral type	Cervix	Oropharynx	Oral cavity	Larynx	Various†
	Any type	~100	46 (60) [15]	24 (13) [33]	22 (19) [32]	Low
1: High-risk carcinogenic	16	56	88 (91) [99]	62 (80) [70]	61 (68) [78]	
	18	14	<1.5	25% (7) [23]{40}	7 (15) [20]	
	45	5			[10]	
	33	4	1.5		<1	
	58	4	<1.5		[20]	
	31	4		<4		
	52	3				
	35	2	<1.5			
	39	1				Low
	59	1				
	51	1			<1 [10]	
	56	1				
2A: probably carcinogenic	68	0.5				
2B: possibly carcinogenic	53	0.5				Low
	73	0.5				Low
	66	<0.5			[10]	Low
	67	<0.5				
	26	<0.5				
	30	<0.5				
	69	<0.5				Low
	70	<0.5				
	82	<0.5				
	85	<0.5				
	34/64	<0.5				
α Group: low risk	6	<0.5	<1.5	<4	6	
	11	<0.5		<4	<1	
	8					Low
	13					Low
	32					Low
β Group: low risk	38			<4		

*Data are given as percentage.
†Various head and neck carcinomas rarely harbor the types noted; coinfections account for >100% totals across types.
HPV, human papillomavirus; IARC, International Agency for Research on Cancer.

E4 are likely responsible for the diagnostic perinuclear halo observed in HPV-infected epithelial cells known as koilocytes.[14] With further cell maturation, the capsid proteins L1 and L2 are produced and inserted into newly formed viral capsids that exit the cell via exocytosis.[13]

There is no lytic phase of viral infection; instead, new virions enter adjacent basal epithelial cells via endocytosis and do so irrespective of prior cell infection.[13] This can expose the same cell to multiple waves of infection.[13] Although genomic analyses have identified multiple viral types in HPV^+ clonal neoplasms,[20] definitive proof of coinfection at the single-cell level is lacking, and published studies using microdissection and *in situ* hybridization localize viral types to distinct cellular populations in coinfected specimens.[21] HPV coinfection is present in 12% of cervical carcinomas and up to 39% of precursor lesions, and in 0.3%, 2%, and 1% of carcinomas in the oropharynx, oral cavity, and larynx, respectively.[7,12] In addition, certain HPV combinations occur more often than by chance alone (HPV11-HPV53, HPV31-HPV33, and HPV34-HPV42).[22] The burden of HPV16 viral DNA in precursor lesions correlates with a higher risk of cancer progression[23] and the same is likely true for other high-risk HPV types.

HPV Activation of the Innate Immune Response

HPV infection poorly elicits innate and adaptive immune responses.[24] Traumatic injury to epithelial cells during initial infection releases danger-associated molecular patterns that activate pattern recognition receptors on epithelial cells and leukocytes, leading to inflammation.[25] After healing, the virus spreads with minimal danger-associated molecular pattern production and inflammation.[14] Although the cytoplasm contains innate dsDNA sensors (eg, POLR, IFI16, MB21D1, TMEM173, DDX41, ZBP1), and endosomes harbor Toll-like receptors (Toll-like receptor 9), HPV dsDNA seems to be unrecognizable.[26] Instead,

HPV dsDNA may be either restricted to the nucleus, surrounded by viral capsid, or complexed to L2 proteins, making it unavailable for innate immune recognition.[13] In addition, the CGAS-STING complex is inhibited and expression of Toll-like receptor 9 is down-regulated by viral proteins.[24] In the meantime, the adaptive immune response is impeded by scant inflammation, infection restricted to epithelial cells that lie outside the basement membrane, and relatively low viral protein expression, all of which limits the utility of serological assays for viral diagnosis.[24] Serology is also insufficient for viral typing and poorly responsive to clearance of high-risk HPV from the body, whereas tests for viral nucleic acid are more informative of infection status.[27] Although viremia is lacking for intraepithelial lesions, detection of HPV DNA in plasma or serum is considered a promising biomarker for HPV^+ carcinomas.[28,29] It seems that invasive tumor cells quickly turn over, releasing genomic and viral DNA into the blood.[27,30]

Histopathological diagnosis of HPV^+ precursor lesions relies primarily on architectural distortion of the epithelium. Increased epithelial permeability is thought to expose epithelial cells and resident leukocytes to commensal bacteria and to proinflammatory molecules, resulting in inflammation and bystander damage to HPV-infected cells.[31] Viral uptake by infiltrating leukocytes activates intracellular dsDNA sensors, type 1 interferon production, antigen presentation, and activation of adaptive immunity.[14,24] Autocrine and paracrine effects of type 1 interferon α/β on infected epithelial cells up-regulates >300 genes that work in concert to degrade viral nucleic acid, inhibit protein translation, promote proteasomal protein degradation, inhibit viral exocytosis, and mutate viral genomes via APOBEC enzymes.[24,26]

Antiviral APOBEC Activity and Mutation Signatures Identifiable by Genomic Sequencing

APOBEC enzymes deaminate cytosine to uracil on single-stranded DNA molecules, resulting in single base substitutions.[32] The best characterized member of the APOBEC family is activation-induced cytidine deaminase, which mediates B-cell somatic hypermutation and has known off-target mutagenicity (eg, in *MYC* and *BCL6*).[32,33] Interferon β up-regulates the expression of at least two APOBEC enzymes (3A and 3G) that induce mutations in single-stranded DNA (both human and viral).[33,34] Single-stranded DNA is prevalent after viral-driven cellular proliferation that leads to reagent exhaustion and exposure of stalled DNA replication forks and transcription bubbles.[34–36] More important, most transcribed genes in infected cells are involved in cellular proliferation, establishing a dangerous situation in which the single-stranded DNA exposed to APOBEC mutagenesis is enriched for proto-oncogenes.[32,37] A multistep model of HPV oncogenesis is illustrated in Figure 1.

Mutation signatures are unique patterns of DNA damage that occur because of the action of a specific mutagen and subsequent host DNA repair response.[38] Even when the mutagen is no longer present, mutation signatures persist and reflect the mutagen's contribution to hit-and-run events culminating in cancer.[38] Mutation signatures are formidable in their capacity to provide mechanistic information on how a particular mutation might have occurred.

Virus-associated cancers, including HPV-infected malignancies, exhibit an APOBEC mutation signature characterized by deamination of cytosine to uracil in single-stranded DNA. This results in a C>T conversion if DNA replication occurs before base excision repair, or C>G and C>A conversions if repair occurs before DNA replication.[5,37,38] Recent analysis of head and neck squamous cell carcinoma identified mutation signatures mechanistically consistent with aging, smoking, UV light, and APOBEC activity.[4] The latter was linked to HPV presence in cancers of oropharyngeal origin.[12] Cervical cancer is nearly universally HPV positive, and mutation signatures point to age and interferon-stimulated APOBEC activity as the main mechanisms of oncogenesis.[4] The power of genomic assays to trace back to causative mutagens cannot be underestimated as a tool for epidemiology and for devising cancer prevention strategies.

Mutation analyses have the potential to identify actionable mutations that predict treatment response or that confer prognostic information.[39] However, given that surgical excision and radiation therapy typically obviate the need for targeted therapy, the clinical utility of sequencing HPV^+ carcinomas is limited. As the cost and convenience of performing next-generation sequencing in clinical laboratories continue to improve, it may become routine to sequence carcinomas at the time of diagnosis to identify patient-specific tumor mutations by which to track tumor burden and to predict recurrence, using either repeat biopsies or serial plasma DNA analysis. Likewise, it may become practical to sequence cervical precursor lesions to predict their aggressiveness or to devise customized surveillance plans. Given that current data are lacking, future research efforts should focus on identifying the sensitivity and specificity of genomic mutations to identify precursor lesions at high risk of progression to cancer.

Common recurrent mutations in cervical cancer and HPV^+ head and neck carcinoma are listed in Figure 1.[40,41] The genes that are frequently mutated in HPV^+ carcinomas are involved in cell signaling, cell growth, histone modification, chromatin remodeling, and DNA repair.[40,42] The most common mutations in HPV^+ carcinomas activate the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin cell signaling pathway to promote cell growth.[40,42] Some variants in the histocompatibility complex and in *B2M* involved in antigen presentation, likely thwart immune responses to viral epitopes and to tumor-derived neoantigens.[40,42]

Incorporating viral targets into next-generation sequencing panels could streamline the process of identifying viral and somatic tumor markers to be used to monitor tumor burden and to demonstrate efficacy of therapy.[39]

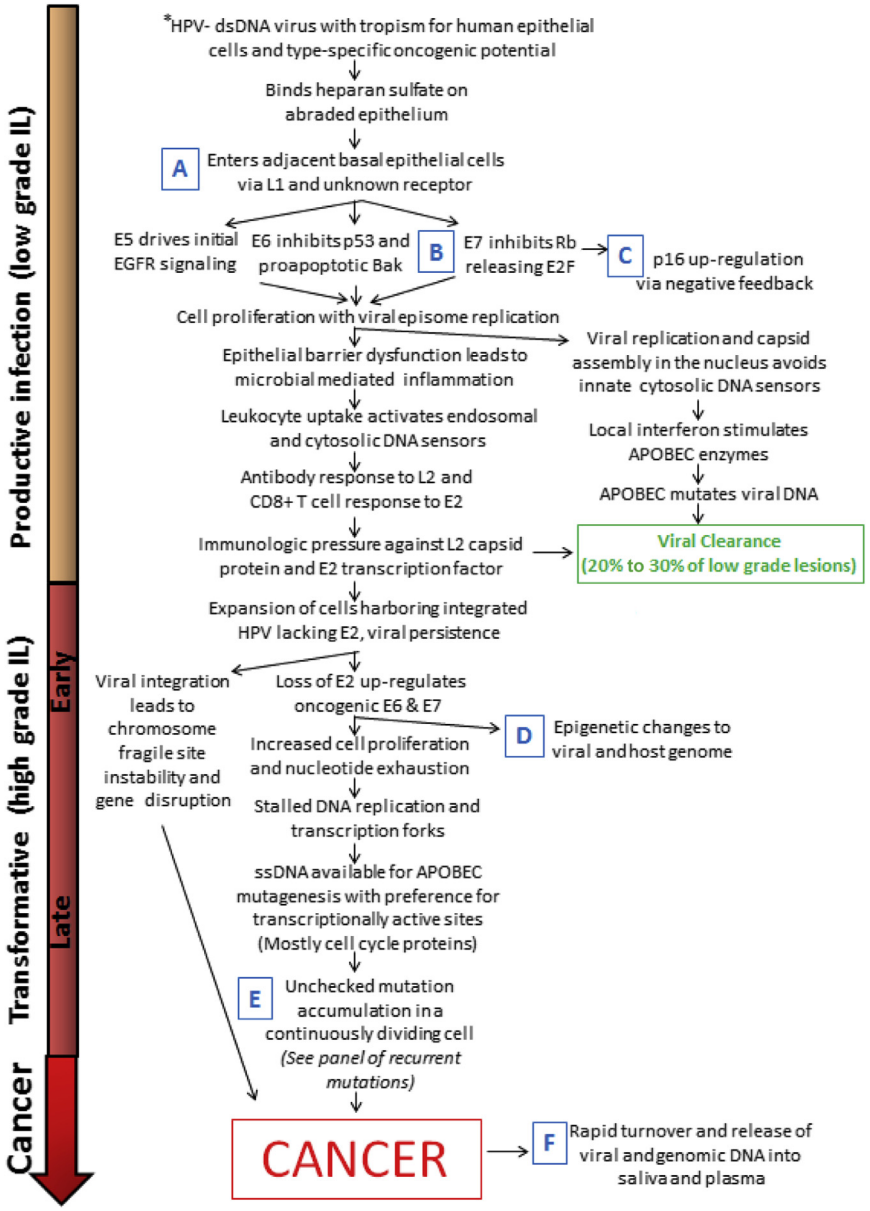

Figure 1 The pathobiology of HPV carcinogenesis and clinical assays to characterize disease status. **A:** Molecular tests that identify viral DNA include *in situ* hybridization (ISH), the Qiagen Hybrid Capture 2, Roche Cobas, and Hologic Cervista HPV assays. **B:** E6/E7 viral RNA is targeted by ISH and the Hologic Aptima HPV assay. **C:** The HPV surrogate CDKN2A/p16 is targeted by immunohistochemistry. **D:** The risk of progression of precursor lesions can be detected by promotor methylation panels. **E:** Druggable mutations in HPV$^+$ tumors are detected by massive parallel sequencing and RNA sequencing. **F:** Noninvasive measurements of tumor burden in plasma samples can be performed with massive parallel sequencing of tumor-specific human variants and by measuring viral load by real-time quantitative PCR. Recurrent mutations in HPV$^+$ cervical carcinomas are identified in genes encoding proteins involved in cell signaling [*PIK3CA* (25%), *APC* (10%), *NOTCH1* (10%), *PTEN* (8%)], histone modification [*MLL3* (20%), *MLL2* (18%), *EP300* (10%)], transcription regulation [*NFE2L2* (15%), *CREBBP* (12%) *SPEN* (12%)], chromatin remodeling [*ARID1A* (15%), *ATRX* (8%)], cell cycle control [*CDK12* (10%)], DNA repair [*TP53* (5%)], and the immune response [*HLA-B* (9%)]. Recurrent mutations in HPV$^+$ head and neck carcinoma are identified in genes encoding proteins involved in cell signaling [*PIK3CA* (56%), *FGFR3* (11%)], transcription regulation [*E2F1* (19%)], and the immune response [*TRAF3* (22%), *HLA-B* (11%)]. APOBEC, apolipoprotein B mRNA-editing catalytic peptide; Bak, Bcl2 homologous antagonist killer; dsDNA, double-stranded DNA; E2F, elongation factor 2; EGFR, epidermal growth factor receptor; IL, intraepithelial lesion; p53, tumor protein 53 (TP53); qPCR, real-time quantitative PCR; Rb1, retinoblastoma protein; ssDNA, single-stranded DNA.

Adaptive Immunity and Selection of Cells with Integrated Viral Genomes

The major components of the adaptive immune response to HPV include a CD8$^+$ cytotoxic T-cell response to E2 and B-cell–mediated production of antibodies to E2, E6, E7, L2, and the major capsid protein L1.[24] Antibodies to L1 inhibit virions from binding to the unidentified receptor on basal epithelial cells,[13,14] and thus current vaccines aim to induce L1 antibodies to levels 10-fold over the level seen with natural infection to prevent new infections.[14,24] CD8$^+$ T-cell responses to HPV antigens kill infected epithelial cells that synthesize enough target viral protein (eg, E2, E6) for intracellular processing and major histocompatibility complex class I antigen presentation.[14,24] This is the likely mechanism for viral clearance and lesion regression observed in some patients.[14]

Integration of the viral genome into host chromosomal DNA can disrupt viral gene expression patterns, leading to lack of immunogenic viral proteins and escape from adaptive immune control.[14,20] Integration events occur at a rate of approximately 36 per cervical carcinoma clone, or 1 to 16 per head and neck carcinoma clone.[43,44] HPV DNA *in situ* hybridization staining patterns can distinguish episomal (diffuse nuclear) versus integrated viral genomes (punctate nuclear).[15] Massive parallel sequencing can also be used to identify HPV insertion sites into the host genome.[45] The breakpoint in the circular HPV16 genome occurs most frequently in E1>L1>L2>E2 and the same pattern is likely true for other high-risk HPV types.[43] E2 disruption favors oncogenesis because E2 negatively regulates E6 and E7 transcription, so E2 disruption increases expression of the primary viral drivers of proliferation and cell survival to facilitate mutation

accumulation.[13,36] In addition, diminished E2 expression promotes evasion from cytotoxic T cells.[36]

Recent viral transcriptome analyses in HPV^+ tumors confirm low or absent E2 expression in most HPV^+ tumors and near universal high level expression of E7.[20] A subset of tumors overexpress E4 and E5, likely representing replication of nonintegrated virus.[20] There are variable levels of intact functional E6, although more commonly tumors produce truncated E6 transcripts encoding questionably functional, nonimmunogenic E6 protein that eludes the immune system while also thwarting apoptosis. In the meantime, cell growth is driven by E5 and E7, and antigen presentation is thwarted in host cells via E7-mediated inhibition of TAP1 required to load peptides onto major histocompatibility complex 1 molecules.[24,36]

Viral Integration into Host Chromosomes Affects Host Gene Structure and Expression

Genomic sequencing reveals that viral integration regions favor chromosomal fragile sites, so named because they are prone to breakage, rearrangement, or gene amplification.[16,43,44] These fragile sites often contain large genes (>800 kb) requiring prolonged time for transcription,[46] raising the possibility that under conditions of proliferative stress, gene transcription may not be completed before S phase, triggering the DNA damage response during G_2.[32,46] In the context of HPV infection, E7-driven cellular proliferation increases proliferative stress, and viral episomes tethered to chromatin take advantage of the DNA damage response to replicate viral genomes during G_2.[16,43,47] Viral replication in close proximity to a fragile site may increase risk of integration. Of HPV integration sites, 42% occur within 10 kb of a human gene copy number variant.[16,20]

Some HPV integration events occur at recurrent microhomology-dependent hot spots, and these hot spots are often in genes with a plausible role in tumorigenesis, such as the DNA repair factor *FHIT* (9% of integration events), the cell signaling protein *DLG2* (5%), the cell-cell interacting protein *SEMA3D* (5%), and transcriptional regulators: *POU5F1B* (10%), *KLF12* (8%), *KLF5* (7%), and *HMGA2* (8%).[43] Alternate genes without a known link to tumorigenesis include the low-density lipoprotein receptor *LRP1B* (6%) and collagen-hydroxylating enzyme *LEPREL1* (5%). In cancers other than cervical, HPV integration hot spots are reported in genes involved in cell signaling (*ERBB2/HER2*), DNA repair (*RAD51B*), DNA methylation (*MLL4*), and cell-cell interaction (*FN1*), and in long noncoding RNAs involved in MYC regulation (*PVT1*, LOC727677).[20]

Transcriptome analyses of HPV^+ versus HPV^- head and neck cancer identified 1897 genes with greater than twofold change and 597 genes with greater than fourfold change in transcript levels.[20] Indeed, expression patterns in HPV^+ head and neck cancer were more similar to HPV^+ cervical carcinoma than to HPV^- head and neck cancer, emphasizing the large and consistent impact of HPV and virtually eliminating the possibility that the virus is an innocent bystander in carcinogenesis. The most up-regulated gene in HPV^+ tumors is CDKN2A/p16 (10.6-fold; bolstering evidence for its use as an immunohistochemical surrogate for HPV infection), followed by genes involved in transcriptional regulation (*RANBP17, HLF, MYB, POU2AF1, MYCN*), cell proliferation (*CDKN2C*), and immune activation (*CD79A, TNFRSF17*).[20] Down-regulated genes include transcription factors (*FOXL2, HMGA2, HOXC13*) and cell signaling factors (*ROS1, FSTL3*). The net effect is to impede cell differentiation while promoting proliferation and immune evasion. RNA sequencing is a powerful new tool to simultaneously profile dysregulated human genes and viral transcripts as well as to detect somatic mutation in expressed genes.[48]

Epigenetic Modifications Reflect Viral Presence and Characterize High-Risk Precursor Lesions

HPV infection induces epigenetic changes in viral DNA as well as in the host genome.[49] Viral E6 and E7 both up-regulate the enzyme DNA methyltransferase 1, resulting in methylation of multiple genomic sites.[49] In addition, HPV infection up-regulates DNA methyltransferase and histone lysine demethylases, which function in concert to alter gene transcription and promote carcinogenesis.[49] For a comprehensive review of epigenetic changes associated with HPV, refer to Steenbergen et al.[49] In brief, methylation of E2 binding sites on the HPV genome interferes with E2-mediated inhibition of E6 and E7 expression.[49] Molecular assays examining selected methylated promoters (eg, *RUBCNL, JAM3, ANKRD18CP*, *TERT*) may add value beyond high-risk HPV presence to identify lesions with cervical intraepithelial neoplasia $\geq$2.[19] In head and neck carcinoma, an HPV-related methylation signature is prognostic.[50]

Vaccines and Immunotherapy

Understanding the pathobiology of HPV carcinogenesis has led to novel interventions to limit viral infection in exposed patients. Efficacious prophylactic vaccines work by eliciting protective neutralizing antibodies to the L1 protein of selected HPV types, and bivalent, quadrivalent, and nine-valent vaccines are available.[51] The nine-valent vaccine (Gardasil-9) blocks HPV types responsible for 90% of cervical carcinomas. Vaccination will likely help prevent HPV^+ head and neck carcinomas as well.[52] Despite their benefits, prophylactic vaccines are underutilized, do not target all pertinent HPV types, and lack efficacy against preestablished infections.[53] Several novel approaches to cancer therapy target viral E6 and E7 proteins, or PDL1 inhibition to promote T-cell activity.[14,20,54,55]

Table 2 Laboratory Tests for Human Papillomavirus–Related Neoplasia

Test	FDA approved	Technology	Advantages	Disadvantages
CDKN2A/p16 immunohistochemistry	No	Targeted antibody binds CDKN2A/p16, tagged secondary antibody, colorimetric	Rapid, inexpensive, readily interpreted by pathologists using practice guidelines, localizes an effect of oncogenic HPV to neoplastic cells	Does not detect virus, rather detects a non-specific effect of oncogenic virus, no genotyping, subjective interpretation
E6/E7 RNA *in situ* hybridization	No	Tagged DNA probes complementary to oncogenic E6/E7 RNA, colorimetric	Identifies viral transcripts not a surrogate marker; rapid, inexpensive, localize to neoplastic cells	No genotyping, varying RNA integrity, subjective interpretation
HPV DNA *in situ* hybridization	No	Cocktail of tagged DNA probes complementary to HPV DNA, colorimetric	May distinguish episomes from integrated virus, can genotype certain HPV types, rapid, inexpensive, localize to neoplastic cells	Less analytic sensitivity than above tests, potential cross-reactivity with low-risk HPV, subjective interpretation
qPCR or RT-qPCR	No	Extract nucleic acid, amplify conserved or type-specific gene segments, measure products during amplification compared to standards and endogenous control DNA/RNA	Sensitive detection of conserved regions across all HPV types, or of type-specific regions, quantify viral load in fixed tissue, cells, or body fluids	Difficult to distinguish virus in neoplastic versus non-neoplastic cells or extracellular
Qiagen Digene HC2 High-Risk HPV DNA assay	Yes	RNA probe hybridizes to viral DNA, primary antibody captures DNA-RNA hybrids, secondary labeled antibody detects DNA-RNA hybrids, chemiluminescent signal	Detects 13 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68)	No genotyping, no control for specimen adequacy, large specimen volume, potential cross-hybridization with low-risk types
Hologic Cervista HPV HR assay	Yes	Partially overlapping probes bind viral DNA, enzyme cleaves viral probe and product hybridizes to labeled probe, enzyme cleaves that probe, fluorescent signal	Detects 14 high-risk HPV types (same 13 as Qiagen HC2 plus type 66)	
Hologic Cervista HPV 16/18	Yes	Same as above but probes target HPV16 or HPV18 and cleavage products bind to differentially labeled probes	Genotypes HPV16 and 18	
Roche Cobas HPV test	Yes	Multiplex real-time PCR to amplify HPV L1 gene segment with labeled TaqMan probes with specificity for HPV16 only, HPV18 only, or 12 other high-risk HPV types	Primary screening for cervical neoplasia, detects 14 high-risk HPV types (same 13 as HC2 plus type 66), genotypes HPV16 and 18	
Hologic Aptima HPV	Yes	Probes on magnetic particles hybridize to E6/E7 mRNA, reverse transcribed to cDNA, T7 RNA polymerase transcription for amplification, chemiluminescence	High specificity for oncogenic viral types	

(*table continues*)

Table 2 (*continued*)

Test	FDA approved	Technology	Advantages	Disadvantages
Hologic Aptima HPV 16 18/45	Yes	Same as above except oligomer targets unspecified viral mRNA, chemiluminescent probes bind HPV16, 18, and 45, rapid light emission kinetics denote HPV16, whereas slow kinetics identify HPV18 or 45	Genotypes HPV16 and HPV18 and/or 45	
Massive parallel sequencing	No	Enrich for HPV genome and other gene regions of interest, add barcodes and adapters before sequencing, record signal as nucleotides are added during strand replication, call each nucleotide position, align reads to reference sequence, view aligned reads, interpret for viral presence and/or type and other sequence variants	Theoretically detects all HPV types; streamlined incorporation into tumor mutation sequencing panels	Expensive, long turn-around time, complex interpretation
Methylation panel	No	Bisulfite treatment of DNA followed by methylation-specific PCR or sequencing of recurrently methylated genes in high-risk precursor lesions of the cervix	Excellent specificity for precursor lesions likely to progress	Technically challenging, complex interpretation

FDA, Federal Drug Administration; HC2, Hybrid Capture II; HPV, human papillomavirus; qPCR, real-time quantitative PCR; RT-qPCR, quantitative RT-PCR.

Early Diagnosis of Pertinent Cervical Lesions and Triage of Patients with Atypical Squamous Cells

Prevention of cervical carcinoma is the primary goal of screening programs that continue to be refined as new evidence emerges. Current US guidelines for screening are summarized by the American College of Obstetricians and Gynecologists (*http://www.acog.org/Womens-Health/Cervical-Cancer-Screening*, last accessed January 5, 2017). Briefly, cytology alone every 3 years is recommended for women from age 21 to 29 years, because this is an age range in which HPV infection and clearance is common, rendering detection of HPV less informative.[24,56] Immune suppression increases viral persistence, warranting different screening strategies.[56] After the age of 29 years, HPV detection suggests persistent infection. Thus, women aged 30 to 65 years have the additional option for cotesting (cytology plus a high-risk HPV assay) every 5 years. Recent Federal Drug Administration (FDA) approval of a standalone high-risk HPV molecular assay (cobas HPV test from Roche) every 3 years is also an option for women >25 years.[57] Women with pathology of moderate-to-high grade cervical intraepithelial neoplasia (2 or 3) should receive continued screening for at least 20 years using cotesting, even after the age of 65 years.[56]

Another role for high-risk HPV testing is to stratify patients with a cervical cytology diagnosis of atypical squamous cells of undetermined significance into those at low risk versus the 40% at higher risk for cervical neoplasia in whom colposcopy is indicated for better endocervical sampling.[56,58,59] By detecting 96% of cervical intraepithelial neoplasia 3 or cancer, high-risk HPV screening limited colposcopy to 56% of women with atypical squamous cells of undetermined significance, saving costs and anguish while effectively identifying women who need intervention.[58]

HPV Laboratory Tests Validated for Use in Cervical Screening Programs

Only six HPV molecular tests are currently FDA approved for screening programs in cervical samplings[8] (Table 2). Use of non−FDA-approved screening tests is discouraged because of many complex analytic and clinical aspects that cannot be validated outside of large clinical trials, and

because of the medical and economic impact of suboptimal assay performance.

The Qiagen Digene HC2 high-risk HPV test detects 13 HPV types using probe hybridization to viral DNA in a 96-well microplate format.[60] The assay is FDA approved for atypical squamous cells of undetermined significance stratification and screening in combination with cytology in women age 30 years and over.

The Hologic Cervista HPV HR assay detects 14 HPV types, including the 13 detected by the Hybrid Capture 2 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) as well as type 66 using Invader probe technology.[61] The assay is FDA approved for essentially the same indications as the Qiagen Digene HC2 test. A separate genotyping assay, Cervista HPV 16/18 assay, specifically detects types 16 and 18 and is performed in reflex to a positive Cervista HPV HR assay to further risk stratify women referred for colposcopy and follow-up.[62]

The Roche cobas HPV test relies on real-time PCR to detect the same 14 high-risk HPV types and optionally to individually identify types HPV16 and HPV18.[63] The test is FDA approved for essentially the same indications as the Hologic Cervista assays and is additionally approved as a standalone primary screening tool for cervical neoplasia in women aged 25 to 64 years.[63,64]

The Hologic Aptima HPV assay uses transcription-mediated amplification of viral E6/E7 mRNA transcripts to detect HPV with oncogenic potential, and is capable of detecting the same 14 high-risk HPV types.[65,66] The assay is FDA approved for essentially the same indications as the Qiagen Digene HC2 test. A separate test, the Hologic Aptima 16 18/45 assay, specifically identifies these three common high-risk HPV types to further risk stratify women referred for colposcopy and follow-up.[67]

HPV Status in Oropharyngeal Carcinoma Affects Prognosis

There is no currently recommended screening strategy to identify patients at risk for developing HPV^+ head and neck carcinoma. However, oropharyngeal cancer has increased by >50% in the past several decades, driven in part by changing patterns in sexual behavior, particularly oral sex.[68–71] Males are more often affected and are usually younger non-smokers from a higher socioeconomic status than HPV^- head and neck cancer patients.[70] HPV^+ oropharyngeal cancer is more sensitive to chemoradiation and carries a better prognosis; however, a subset develop locoregional recurrence or distant metastases, resulting in clinician reluctance to deescalate treatment intensity.[72] Smoking history imparts a worse prognosis in affected cancer patients.[12,73–75] However, HPV16 appears to confer a better prognosis than other HPV types,[11] and studies are underway to evaluate how HPV typing and/or genomic tests could help stratify risk of progression to cancer, and in those who already have cancer to predict outcome in response to particular treatment regimens. Analysis of cell-free tumor DNA in blood or saliva may help identify metastasis or local tumor recurrence.[27,28]

Histochemical Methods for HPV Detection and Viral Typing on Paraffin-Embedded Tissue

Multiple approaches are available to detect and type HPV in tumor tissue. Although up-regulated in many tumors, immunohistochemistry targeting the host protein CDKN2A/p16 is a sensitive and specific surrogate marker for integrated HPV when positivity is interpreted as >70% of malignant epithelial cells.[18,20,76–78] Draft guidance from the College of American Pathologists recommends p16 immunohistochemistry as the first-line test to support management decisions in oropharyngeal cancer patients, and National Comprehensive Cancer Network guidelines support this strategy.[79] Unlike oropharyngeal carcinoma, other carcinomas of the head and neck (oral cavity, larynx, hypopharynx, nasopharynx, sinonasal) harbor HPV less frequently, and limited data suggest there may not be HPV-related prognostic differences. It remains controversial whether p16 immunostain is more informative than HPV status for response to particular therapy for oropharyngeal cancer, and clinical trials continue to gather evidence supporting best practices. In the lower anogenital tract, p16 immunostain may be helpful to distinguish HPV-related squamous intraepithelial neoplasia from mimics (atrophy, squamous metaplasia, or tangential cuts).[18] Testing for HPV and Epstein-Barr virus in squamous carcinoma of unknown primary can assist in determining the site of origin and in weighing radiotherapy options.[79]

Several tests are available to directly detect HPV or its gene products in paraffin-embedded tissue, including HPV E6/E7 mRNA *in situ* hybridization, HPV DNA *in situ* hybridization, E6/E7 immunohistochemistry, HPV E6/E7 RT-PCR, or HPV DNA Q-PCR.[19] Because HPV PCR does not localize viral DNA to cells of interest, this test method is best used in reflex to a positive morphology-based assay, such as p16 immunostain. HPV DNA *in situ* hybridization is limited by probe designs that typically cannot detect all pertinent high-risk types but when positive can help reveal episomal versus integrated staining patterns. Histochemical tests for E6 and E7 mRNA also perform well for localizing potentially oncogenic HPV because overexpression is characteristic of integrated (tumor-related) virus,[65,66] and parallel control probes help ensure that RNA is adequately preserved in the tissue. There is near universal high level E7 expression in HPV^+ tumors.[15,20] In contrast, E6 is often truncated and nonfunctional with variable expression undermining its diagnostic utility.[20]

HPV genotyping typically relies on primers, probes, or sequencing of type-specific sequences in the L1 gene or its RNA transcript.[6,8] Even among the high-risk types, data suggest that HPV typing in cervical precursor lesions adds value in predicting risk of progression.[77,78] In

oropharyngeal cancer, HPV16 seems to carry a better prognosis than the other high-risk types, indicating a potential future role for genotyping in clinical decision making.[11] HPV typing is feasible in paraffin-embedded tissue specimens.[9,45,80]

Progress in Developing Genomic Tests for HPV-Related Neoplasia

There is interest in using massive parallel sequencing to simultaneously identify virus, assess viral types and additional viral genomic variants, and detect relevant human gene mutations in tissue, cytology, or body fluid samples.[39] Genomic methods have recently been described.[9,10,45,80] Cell-free DNA assays for HPV DNA in plasma show promise as a noninvasive measure of HPV^{+} carcinoma burden, and work is underway to evaluate this approach to monitor treatment efficacy, to identify recurrence, and to enhance early cancer detection.[27,30] A combinatorial approach is envisioned in which plasma is analyzed for viral DNA and for mutations common in carcinoma (eg, in *PIK3CA*).[41] Sequencing panels permit broad and agnostic pathogen detection alongside human genomic characterization that seem important for exploring synergistic mechanisms of carcinogenesis involving infections (viruses, bacteria, fungi, parasites), host immune response polymorphisms, DNA damage markers of prior mutagen/microbe exposure, and DNA repair defects.[4] This ambitious work is currently in the experimental phase but holds great promise to transform laboratory diagnostics.

Summary

HPV-driven carcinogenesis imposes a great burden on human health. The interplay between viral propagation, the immune response, and genomic damage is multifaceted, and large-scale genomic studies are revealing fascinating insights into how a virus with a small eight-protein encoding genome can disrupt the biology of its host so dramatically. Prophylactic vaccination and screening are effective defenses to prevent a large fraction of HPV-driven carcinomas. Viral detection and DNA methylation analyses enable further stratification of infected patients at high risk of progression to carcinoma. It is imperative that molecular professionals choose analytic test strategies that are in line with current evidence and that suit the needs of their patient population. New diagnostic modalities, such as massive parallel sequencing, have the power to revolutionize HPV diagnostics and to identify changes in the human genome, such as mutation signatures reflecting the history of DNA damage and repair processes during clonal evolution. Molecular pathologists are well positioned to drive future improvements in test strategies to better guide therapeutic interventions and to help prevent progression of neoplasia.

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