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Human papillomaviruses: research priorities for the next decade

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Abstract

Human papillomaviruses are the causative agents of cervical, anal as well as many oropharyngeal cancers. While prophylactic vaccines have been developed, uptake is low in the US and other Western countries, and access is limited in less developed countries. A number of areas are emerging as critical for future study. These include investigation of the mechanisms regulating infection and progression to cancer at both cervical and oropharyngeal sites as these appear to be distinct. HPV-induced cancers also may be susceptible to immune therapy, revealing opportunities for treating advanced cervical disease and reducing the morbidity of treatments for oropharyngeal cancers. We believe these areas are critical focal points for HPV cancer research in the next decade.

Keywords

progression; DNA damage; epigenetics; integration; vaccines; therapeutic vaccines

HPV-infection and cancer

Human papillomaviruses are small DNA viruses that infect epithelial tissues at numerous anatomic sites. Over 200 types of HPVs have been identified and about one quarter of these infect epithelia in the genital tract. These genital HPVs are further grouped into low-risk and high-risk types by their association with anogenital cancers. High-risk human papillomaviruses are the causative agents of cervical and other anogenital cancers as well as many oropharyngeal cancers [1, 2]. Cervical cancer is the third most common cause of cancer-related death in women worldwide [1]. Over 12,000 cases of cervical cancer were diagnosed in the U.S. in 2011, as well as approximately 2,300 new cases of oropharyngeal cancer in women and 10,500 cases in men [3]. In Western countries, the number of HPV-induced oral cancers is increasing rapidly and deaths by these cancers will likely surpass those due to cervical cancer. Prophylactic vaccines have been developed that prevent initial infection by high-risk types, however, less than 60% of adolescent females and 40% of adolescent males in the US have received at least one dose of the vaccine [4, 5]. Importantly,

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these vaccines have no effect on existing HPV infections indicating a need to continue to develop therapeutics to treat these lesions. Enhanced public health efforts are needed to increase the uptake of HPV vaccines in the U.S. as well as development of single dose vaccines that would be highly beneficial in less developed countries where the incidence of cervical cancer is very high. Going forward, research efforts can be most effective when divided into three emerging areas as follows: 1). Understanding the mechanisms by which productive HPV infections progress to cancers, especially how the virus interacts with the host chromatin remodeling, DNA repair, and differentiation pathways; 2). Investigation of HPV infections and cancers of the oropharynx as they appear to be distinct from those of the anogenital tract; and 3) examination of the effects of immunomodulation in HPV infections and development of immune therapeutics to treat existing HPV infections.

HPV life cycle

Prior to discussing in detail these three areas for future study, it is important to review the HPV viral life cycle. All human papillomaviruses infect stratified epithelial cells and link their productive life cycles to differentiation [6]. HPVs infect cells in the basal layers of stratified epithelia that become exposed through microwounds and establish their genomes as nuclear episomes at about 100 copies per cell. In productive infections, viral genomes are maintained as episomes at constant copy number and replicated along with cellular DNA. This indicates that mechanisms exist for proper segregation and stable viral genome replication as infections can last from one or two years to several decades. In normal differentiating epithelia, cells become arrested in G0 as they leave the basal layer, however, HPV positive cells remain active in the cell cycle to allow for entry into S/G2 phases in suprabasal layers for productive viral replication in a process referred to as amplification (Figure 1). The E6 and E7 viral oncoproteins target p53 and Rb respectively and are responsible for maintaining differentiating cells active in the cell cycle. The E1 and E2 proteins mediate viral replication and act to recruit cellular replication factors to viral replication origins. Amplification of high-risk HPV genomes and late gene expression have recently been shown to be dependent upon activation of the ataxia-telangiectasia mutated (ATM) DNA repair pathway [7, 8], but how this facilitates viral functions is still not clear. Dependence on DNA damage pathways has also been shown for the life cycles of other DNA viruses, suggesting that it is a common viral strategy for productive genome replication [9]. The interplay between DNA repair, chromosome partitioning, and chromatin remodeling pathways has been shown to be important in regulating replication of a number of human viruses including HPVs.

Progression to cancer

While most sexually active adults acquire HPV infections in the genital tract during their lifetime, very few infections progress to cancer. Most genital HPV infections resolve in one to two years, but a small number of women become persistently infected and are at high risk of developing cervical cancer. HPV types 16 and 18 are the most common viral types found in cervical cancers and are associated with these persistent infections. What makes these types especially oncogenic is still not fully understood and further investigation of how

productive HPV infections persist and in some cases progress to cancer remains a critical area for study.

While the oncogenic activities of E6 and E7 are necessary for the development of cancers, they are not sufficient, as most infections fail to progress. Additional interactions with host tumor suppressors and oncogenes as part of a multiple-hit process are likely important along with the appearance of mutations in cellular genes. The Cancer Genome Atlas (TCGA) has identified a number of mutated genes in cervical cancers, though none are found in more than 35% of cases [10]. This suggests either that there are multiple pathways to cancer or that complex interconnected networks are involved. The most prominent changes occur in members of the PI3K pathway, chromatin remodeling factors, and cell cycle regulators. It will be important to determine how alterations in these pathways contribute to the development of cervical cancer and it is likely that these factors have similar effects in other cancers as well.

In productive HPV infections, the process of differentiation is only slightly altered from that seen in normal epithelia [7], viral genomes are maintained as episomes, and expression of the E6 and E7 oncogenes is low. In contrast, in cervical cancers, many of these processes are inhibited or altered. In particular, viral genomes are often not maintained as episomes but are integrated into host chromosomes and there is a lack of cellular differentiation (Figure 1). Cellular differentiation is controlled by transcription factors such as KLF-4 and p63 whose levels are increased in high-risk HPV positive cells by transcriptional and post-transcriptional mechanisms. There is a need to examine the full complement of transcription factors and differentiation markers that are altered in HPV infections to understand how differentiation is lost in cancers. It is equally important to investigate the factors that contribute to integration. Host proteins have been identified that control HPV episome maintenance and segregation such as Bromodomain-containing protein 4 (Brd4), Structural maintenance of chromosomes protein 1 (SMC1), and CCCTC-binding factor (CTCF) and it is possible their activities are altered during progression to cervical cancer [11, 12]. Integration is seen in many, but not all, HPV positive cervical cancers and leads to loss of E2-mediated repression of early viral gene expression including that of E6 and E7. The increased expression of viral oncoproteins may be critical to progression as it leads to further decreases in p53 and Rb levels, which are only slightly reduced in productive lesions. Cervical cancers are highly aneuploid with frequent mutations, indicating that DNA damage repair pathways have been inhibited or circumvented. The ability of E7 to induce centriole duplication may also play a role in causing aneuploidy [13]. Although DNA repair pathways are induced early in infection to promote viral replication, it is possible that the same pathways are suppressed in HPV cancer cells. Whether such suppression is a necessary step in progression and how this occurs are areas that need to be addressed.

Gene expression is controlled in part by post-translational modification of histones bound to promoter regions, which regulates the accessibility of transcription factors as well as basal transcriptional machinery. These modifications include acetylation and methylation as well as phosphorylation, ubiquitination, and sumoylation. Many histone modifying enzymes such as the histone deacetylases (HDACs), sirtuins and histone acetyltransferases (HATs) also regulate the expression of tumor suppressors, oncogenes and growth factors. Epigenetic

changes in chromatin can also provide important contributions to progression to cancer. Epigenetic control of gene expression is often altered in cancers through mutation or dysregulated expression of histone modifiers [14, 15]. Not surprisingly, viruses, such as HPV, also affect the expression and localization of chromatin modifiers to directly modulate viral replication as well as expression of viral and cellular proteins that are critical for the viral life cycle. The different stages of the viral life cycle are correlated with changes in the compaction of chromatin around viral promoters and this is mediated by the recruitment of chromatin modifiers to viral genomes [16, 17]. E7 regulates the expression and activity of several chromatin modifiers including DNA methyltransferase Dnmt1, histone deacetylases (HDACs), as well as sirtuins such as SIRT1 [18–22]. The expression of p16, which is critical for proliferation of HPV positive cells, is regulated by E7 by targeting the histone demethylase KDM6B [18]. HPV infected cells become dependent upon the activity of these factors for continued survival during cervical cancer progression [22, 23]. SIRT1 is a histone deacetylase that also targets non-histone proteins including DNA damage factors and helps recruit them to viral genomes. It also deacetylates histone H1K26, which is associated with host DNA damage repair [24]. SIRT1 has been shown to deacetylate H1K26 on the HPV31 genome, which may facilitate viral replication, suggesting an important linkage between epigenetic modifications and DNA repair that is necessary for HPV replication [21]. SIRT1 activity is also critical for progression as cancer cells become dependent on its activity for survival [22]. Whether this dependence is mediated by host or viral targets of SIRT1 is unclear. Epigenetic control of HPV expression may also contribute to integration of genomes or enhanced expression of E6 and E7 in cancers that maintain episomal copies of HPV genomes [25]. Methylation of the HPV genome is also seen during progression, though what role it plays is not fully understood. The expression of E6 and E7 can be down regulated through methylation of E2 binding sites (E2BSs) that regulate early promoter expression [26]. In cells with episomal genomes, methylation of HPV DNA at regions around E2BSs is low while it is increased significantly in carcinomas containing integrated copies, which could account for integration or increased expression of E6 and E7 [27, 28]. At the same time other studies report higher levels of methylation in different regions of the upstream regulatory region in cervical cancers suggesting a lack of consensus about which sequences in the URR are critical [29] In contrast, methylation of the L1 region of the HPV genome is consistently observed with decreased levels of methylation seen upon progression to cancers [30–32]. How epigenetic modifications of viral and cellular proteins along with alterations to HPV genomes influence integration and progression is a critical area for further study.

HPV-induced oropharyngeal cancers

An emerging area of high clinical significance is the role of HPV in the development of oropharyngeal cancers. In the United States, over 60% of oropharyngeal cancers, including those of the tonsils and tongue base, are HPV-positive and the preponderance of these occur in men [33]. The number of these HPV-positive cancers is increasing at a dramatic rate such that they will likely surpass the number of cases of cervical cancer. Over 90% of HPV-positive oropharyngeal cancers are caused by HPV 16 with the remaining associated with HPV 31, 33, and 35. In contrast, HPV 16 is associated with about 50% of cervical cancers while HPV 18 is found in about 20%. Surprisingly, HPV 18 is rarely detected in

oropharyngeal cancers [33]. Unlike cancers of the genital tract where the various stages of precancerous lesions can readily be isolated and examined, identification of corresponding lesions in the oral cavity has not yet been achieved. In addition, the pathways important for viral pathogenesis in oropharyngeal epithelia have not been identified. HPV cancers in the oropharynx arise from cells in the tonsillar crypts while in the cervix they usually initiate in the transformation zone. The oropharyngeal epithelium is located adjacent to lymphoid tissues that play critical roles in regulating immune surveillance of oral pathogens. HPV infections occur most frequently in the palatine and lingual tonsils, which have distinctly different architectures than cervical epithelium. The palatine tonsils consist of stratified non-keratinized epithelia associated with 10 to 30 underlying branched tubular crypts. The reticulated epithelium of these crypts contain discontinuous basal lamina with abundant intraepithelial blood vessels that allow for transit of immune cells as well as early metastasis of oropharyngeal carcinoma cells to lymph nodes in the neck. The lingual tonsils are similar in structure but contain a single crypt. Studies from TCGA have identified cellular mutations in HPV-positive oropharyngeal cancers but, similar to the cervix, none are found at frequencies greater than 35% [2]. The most prominent are mutations in the PI3K and notch pathways along with less frequent mutations in DNA damage factors along with the pluripotency factors KLF-4 and SOX2 that control differentiation [2, 33, 34]. In HPV negative cancers, p53 is frequently mutated but this is not seen in HPV positive lesions likely due to the reduced levels induced by E6/E6AP action. This provides support for the critical role of p53 in the development of oropharyngeal cancers. HPV-positive oropharyngeal cancers are treated with either a combination of radiation and chemotherapy, or surgery followed by radiation. The side effects of these treatments can be severe. It will therefore be important to use the information we gain from tissue culture models to identify therapeutics that can either clear these lesions or reduce the long-term morbidity associated with therapy. It remains to be demonstrated that the current tissue culture models that support HPV replication in genital epithelia are useful to study viral effects in tonsillar epithelia or whether new systems are needed. At the same it will be important to develop effective diagnostics for HPV-induced precancerous lesions of the oropharynx. Currently there are no screening tests that identify precursor lesions reliably, in part due to inaccessibility of the site of infection, and diagnosis that is based on presentation of a palpable cancer during examination.

Immunomodulation of HPV infections

Most HPV infections in the genital tract of immunocompetent women are cleared in less than two years, presumably through activation of the immune response [35]. A small percentage of individuals become persistently infected and these lesions have a high chance of progression to cervical intraepithelial neoplasia (CIN) grade 2/3. A subset of CIN2/3 lesions can also spontaneously regress through presumed activation of an immune response, indicating that immune stimulation can be effective therapeutic treatment for premalignant lesions [36]. Treatment for CIN2/3 lesions typically involves therapeutic excision of the infected transformation zone in the cervix and no other effective treatments are commonly in use. Recent studies using DNA vaccines against HPV E6 and E7 suggest that activation of an immune response in patients with high-grade CIN lesions can lead to regression [37].

Intramuscular injection of plasmids expressing E6 and E7 proteins dramatically reduced viral DNA in approximately 40% of recipients and regression of HPV lesions in a significant number of patients with cervical precancers [33]. This therapeutic vaccine elicited both T-cell and antibody responses to the viral oncoproteins. Further refinement of this methodology or development of complementary approaches hold promise for the treatment of HPV precancers particularly those of the anal and oropharyngeal epithelium where the side effects and morbidity of standard treatments is significant. These methods can also reduce the number of women requiring surgery for HPV positive precancers of the cervix While HPV positive oral cancers are highly responsive to a combination of chemotherapy and radiation, there are significant side effects that could potentially be reduced by using lower dosages when used in combination with therapeutic vaccines. Stimulation of the immune responses by DNA or other vaccine regimens may provide important therapeutic advances in the treatment of HPV-induced cancers and this remains an area high importance. It is still, however, unclear if all high-grade HPV lesions can be effectively treated with immunotherapy or if only a subset are responsive and this needs to be determined.

Concluding Remarks

While prophylactic vaccines have been developed that target high-risk HPV types, rates of uptake remain low. At the same time important basic scientific work needs to be done to understand the factors that control progression from infection to cancer. Identifying the factors that control progression, including perturbation of signaling growth and differentiation pathways as well as epigenetic changes in chromatin dynamics, will likely provide significant insights into mechanisms of progression for other cancers as well. HPV-induced oropharyngeal cancers are on the rise yet no tissue culture system exists to facilitate study of the life cycle in this cell type and it will be critical to investigate the processes regulating the HPV life cycle in oropharyngeal epithelia. Finally, immune therapy holds great promise for the treatment of existing HPV positive lesions including oropharyngeal lesions and further efforts in this area need to be encouraged (see outstanding questions). We feel that investigation of these areas is of high importance for HPV research in the next decade.

Acknowledgments

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Box 1

Culture models to study the differentiation-dependent life cycle of HPVs

Organotypic raft cultures duplicate epithelial differentiation in vitro and this system has been used to replicate the complete HPV life cycle including the production of HPV virions. In the raft system, human keratinocytes are first grown as submerged monolayer cultures and then transferred onto a collagen plug, which contains fibroblast feeders. The collagen/cell matrix is then placed onto a wire grid that is maintained at an air-liquid interface. The cells are thus exposed to air and subsequent feeding occurs from underneath by diffusion of media through the collagen. Cultivation of epithelial cells by this method induces differentiation that occurs over a period of two weeks. Raft cultures were first used to synthesize HPV 31 and 18 virions from cell lines that maintain viral episomes and subsequently this technique has been used to grow a variety of other high and low risk HPV types in culture. Recently, extensive use has been made of a simpler system for epithelial differentiation that involves growth of cells in high calcium media for up to 96 hours to induce full differentiation, genome amplification along with late gene expression. In this system, cells differentiate coordinately as a function of time, which allows for the rapid isolation of uniformly differentiated populations of cells for analysis. Suspension in methylcellulose provides a third method in which the differentiation dependent HPV life cycle can be studied. Alternative systems, such epithelial spheroids, may be useful to study HPV effects in tonsillar epithelia which appears to be distinct from genital epithelia.

Box 2

Prophylactic Vaccines

Two prophylactic vaccines, Gardasil and Cervarix, were approved by the FDA for use starting in 2006 and prevent initial infection by high-risk human papillomaviruses types HPV 16 and 18. These two types are responsible for approximately 70% of cases of cervical cancer and these vaccines are highly effective in preventing the development of these cancers. In addition, one of these vaccines, Gardasil also targets two low risk types, HPV 6 and 11, which are responsible for the majority of genital warts. Recently, a new vaccine has approved that blocks infections by the nine major high-risk types that account for over 98% of cases of cervical cancer. These vaccines have been approved for use in both girls and boys with the recommended ages of 9 to 16. Since these vaccines prevent initial infection by HPVs, it is desirable, though not necessary, that they be administered prior to the onset of sexual activity. The vaccine consists of viral-like particles that assemble spontaneously after expression of the major capsid protein, L1, in yeast or insect cells. Expression of L1 in these cells results in the formation of viral like particles approximately 50 nanometers in size that resemble HPV virions but lack viral DNAs. These vaccines generate a conformational antibody response that is highly effective in blocking infections. The recommended dosage consists of three injections administered on separate visits to the doctor and it does not appear that booster shots are needed to maintain efficacy. Uptake of the vaccine in the US is low while in Australia, where the government provides the vaccine free, approximately 70% of young girls have received all three doses. Vaccination of males along with females should be encouraged as this would reduce rates of oropharyngeal cancers as well as cancers of the genital tract.

Outstanding questions

1. What are the key mechanisms controlling the progression from an HPV infected cell to a cancer? How do the cellular mutations identified by the Cancer Genome Atlas contribute to progression?
2. Can the differences between cervical infection with HPVs and infection of tissues of the oropharynx be identified using existing tissue culture methods or are new techniques required?
3. Can therapeutic HPV DNA vaccines be improved to increase the frequency of regression of CIN2/3 lesions in patients and to reduce the morbidity of treatment of HPV-positive oropharnygeal cancers?

Trends Box

1. The HPV viral life cycle is dependent on activation of DNA repair pathways as well as epigenetic regulation of viral DNAs and proteins.
2. The number of HPV positive oropharyngeal cancers in the US is rapidly increasing and may soon exceed those of cervical cancers. HPV-16 is the primary HPV type found in these cancers.
3. Therapeutic DNA vaccines have shown efficacy in treating precancerous HPV positive cervical lesions.

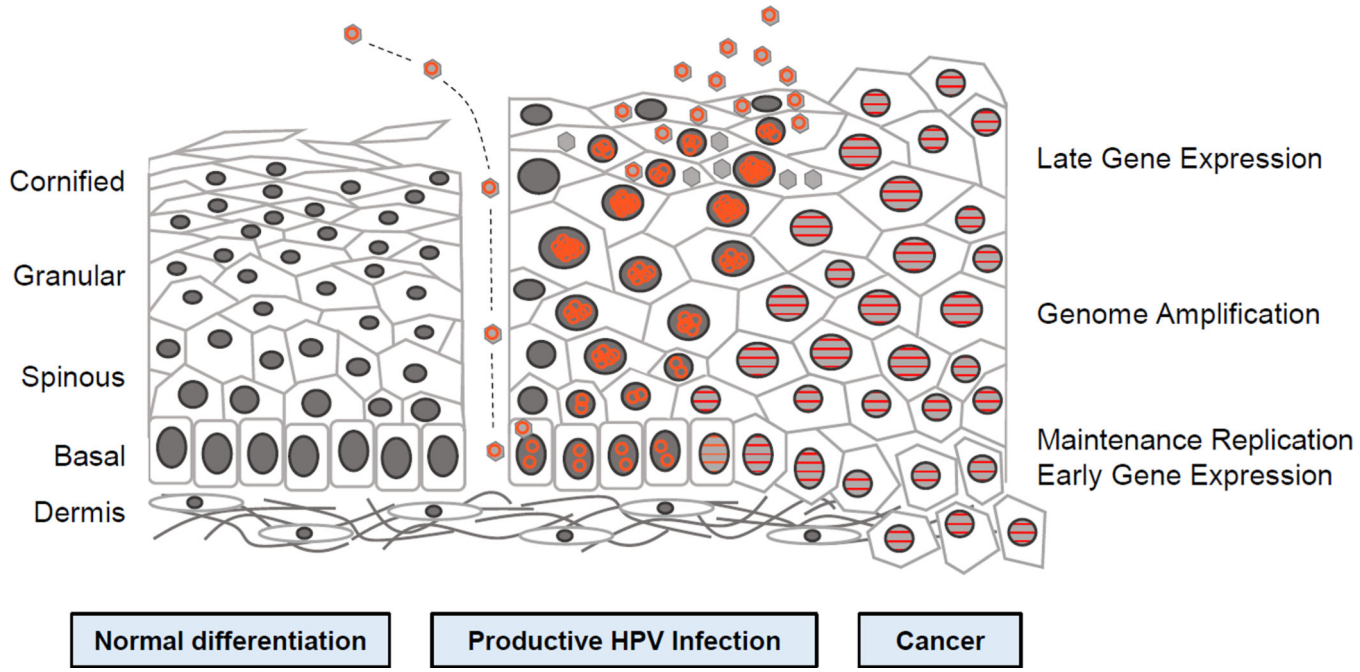

Figure 1. HPV Life Cycle and Cancer

Cartoon depicting normal stratified cervical epithelium (left), HPV infected epithelium (center), and HPV induced cancer (right). Epithelial layers are indicated on the far left and HPV life cycle stages are indicated on the far right. Episomal genomes are shown as orange circles and integrated genomes shown as orange stripes. Left: Normal keratinocyte differentiation. Basal cells divide and daughter cells migrate upward, beginning the differentiation program. As differentiation proceeds, cells exit the cell cycle. Fully keratinized squames slough off from the apical surface. Middle: Productive HPV Infection: HPV virions gain access to basal cells via microwounds. The viral genomes migrate to the nucleus, where they are maintained at approximately100 copies/cell. As daughter cells begin differentiation, viral genomes are amplified. Cell nuclei are retained and chromatin is activated to support viral DNA replication. Right: Cancer. Viral genomes often integrate into the host genome and E6/E7 expression is increased, leading to enhanced proliferation and accumulation of cellular mutations. Cellular differentiation is lost and cancerous cells invade into the dermal layer along with neighboring tissues.