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CLINICAL IMPLICATIONS OF HUMAN PAPILLOMAVIRUS INFECTION

Elizabeth A. Sisk and Erle S. Robertson

University of Michigan Medical School, Ann Arbor, MI

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1. ABSTRACT

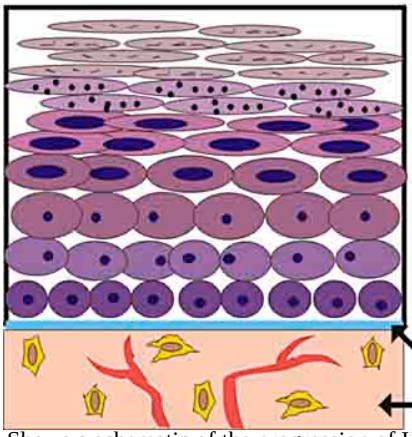
Human papillomaviruses (HPV) are small DNA viruses associated with specific mucosal and epithelial lesions ranging from benign proliferative lesions to invasive carcinomas. Over 100 types of HPV have been identified, some of which are associated with benign lesions (low risk types) and others are associated with malignancies (high risk types). While the genome consists of 6 early genes and 2 late genes, the E6 and E7 genes have been most studied because they interact with p53 and Rb, respectively, thus contributing to the ability of HPV to mediate oncogenesis. Cervical carcinoma is the most common and most studied HPV-related malignancy. These lesions are thought to be originated from persistent high-risk type HPV infections which progress to well characterized precursor lesions and finally to carcinoma. This same HPV related progression has also been observed in other anogenital malignancies including anal, penile and vulvar carcinomas. Although the evidence is not as conclusive, HPV also likely plays a role in the development of a subset of squamous cell carcinomas of the head and neck as well as other cutaneous malignancies. While HPV infection is common, the progression to malignancy is relatively rare indicating a potential role for immune protection against persistent infection. This is supported by the fact that HPV infection and related malignancies are common in the immunosuppressed population. Thus, efforts have been placed on development of HPV vaccines to prevent and treat these common and diverse groups of HPV related malignancies.

2. INTRODUCTION

Human papillomaviruses (HPV) are small double-stranded DNA viruses approximately 8Kbp in size. The group is comprised of over 100 different types, many of which are linked to specific epithelial lesions. All identified types appear to be strictly epitheliotropic and infect the cutaneous or mucosal epithelia of the anogenital tract or upper respiratory tract (1). Persistent HPV infections are associated with benign proliferative lesions such as common warts, genital condylomata, and recurrent laryngeal papillomatosis as well as malignant lesions such as squamous cell carcinomas of the cervix, skin, and the region of the head and neck (2). The majority of benign lesions are associated with types 6 and 11, and these are considered "low risk" types (2, 3). In contrast only a limited number of types, including 16, 18, 31, 33, 39, 45, 52, 58 and 69, are routinely found in carcinoma biopsy specimens and are considered to be "high risk" types (1,2, 4). HPV 16 is by far the most prevalent type in HPV-linked carcinomas, and over 90% of cervical malignancies contain HPV types 16, 18, 31, 33 or 45 (4,5).

3. HPV ENCODED LATENT GENES AND THEIR RELATED FUNCTIONS

The HPV genome is comprised of 6 early genes (E1, E2, E4, E5, E6 and E7), 2 late genes (L1 and L2), and an upstream regulatory region (URR) that contains promoters and elements involved in DNA replication and transcription. E1 and E2 proteins are essential for DNA replication and permissive infection. Following infection of the basal or epithelial stem cells, E1 and E2 are expressed and bind to the URR (4). E1 facilitates E2 binding to URR and E2 binding leads to repression of transcription of E6 and E7, which may initiate switching to late mRNA synthesis resulting in production of capsid proteins (6,7,8). Upon epithelial differentiation, the capsid proteins, L1 and L2, are translated and virus particles are produced (Figure 1). Therefore, differentiation of the



Epithelial Differentiation	HPV Gene Expression
Stratum corneum	Mature virions shed
Granular layers	Mature virions
Upper spinous layers	Virion assembly L1 and L2
Lower spinous layers	E1, E2, E4, E5 Differentiation-dependent E6 & E7
Transit amplifying cells	E1, E2, ?E5
Basal Cells	Primary infection E1, E2, ?E5

Figure 1. Shows a schematic of the progression of HPV infection of epithelium. Note that the primary infection usually occurs at the basal stem cells with establishment of replication and immediate early protein expression. The differentiation of these cells lead to the expression of the E6 and E7 proteins, which is differentiation dependent. Viral assembly takes place with the expression of the L1 and L2 proteins in the upper spinous layers and eventual production and shedding of mature virions from the stratum corneum.

infected basal epithelial cells appears to be required for virion production (4). Transient replication of HPV genomes only requires E1 and E2, but stable maintenance in normal keratinocytes also requires E6 and E7 expression (9,10). In malignant cells, the E1/E2 ratio may be changed by integration into the host genome, and the repression of E6 and E7 transcription is lost (11).

While the function of the E4 protein remains largely unknown, high-risk HPV types code for at least 3 proteins with growth stimulating and transforming properties (E5, E6, and E7). The contribution of the E5 protein remains poorly understood. E5 is a transmembrane protein whose biological actions appear to be through upregulation of cellular growth factor receptors such as epidermal growth factor receptor and platelet derived growth factor receptor (2). It may be involved in the early expansion of an infected cell clone while not being essential in maintaining the malignant phenotype (1).

Both E6 and E7 are responsible for tumor progression and cell immortalization and the ability of HPV to immortalize cells has been demonstrated to depend entirely on E6 and E7 (12, 13, 14). In addition, the maintenance of the malignant phenotype requires E6 as well as E7 expression (5). E6 binds to the cellular protein p53 mediated by the E6-associated protein and promotes p53 degradation via a ubiquitin-dependent pathway (14). p53 normally arrests the cell cycle at G1 to allow for repair of damaged DNA or programs the cell for apoptosis. Thus the cell cycle may be propagated by the specific effects of the E6 protein. The E6 protein of high risk types avidly binds to p53, while E6 of the low risk types binds only weakly and cell cycling is not increased (14, 15, 16). The E6 protein has other less well-characterized effects on infected cells, which are independent of p53 degradation. In fact, E6 mutants, which have lost the ability to bind to p53, still retain the capacity to transform cells (15). Some possibilities include cytoplasmic sequestration of p53, binding other host-cell proteins such as p300/CBP and the proapoptotic protein Bak, chromosomal destabilization, and enhancement of integration of foreign DNA into the host genome (1, 15). Thus while the E6 protein disrupts p53 activity, its effect on cellular function and stability is likely complex.

Similar to E6, the E7 protein promotes the G1/S transition of the cell cycle. E7 has been demonstrated to bind to the retinoblastoma (Rb) protein (17) leading to phosphorylation of Rb and enhanced degradation by ubiquitination. Phosphorylation leads to the release of transcription factors of the E2F family and activation of transcription of genes regulating cell proliferation (1). E7 has also been shown to inactivate the cyclin-dependent kinase inhibitors p21 and p27 required for regulation of the progression of cell cycle (18, 19, 20). In addition, the effects of the E7 protein may also be due to altered regulation of cyclin E, enhancement of integration of foreign DNA into host-cell DNA, increased mutagenesis, and enhanced mutagenicity of chemical carcinogens by poorly understood mechanisms (1). The cooperative interaction of E6 and E7 proteins leads to substantially enhanced immortalization efficiency. Thus, a model of carcinogenesis has been proposed whereby infection by a high risk type HPV leads to modification of host cell genes and proteins which may modulate viral gene expression, followed by viral integration and/or viral intragenomic modifications, all of which may increase the likelihood of additional mutations of host cell genes affecting differentiation or angiogenesis (1, 5).

4. CLINICAL MANIFESTATIONS OF HPV INFECTIONS

Several benign conditions ranging from common cutaneous warts to laryngeal papillomatosis have well known associations with HPV. Cutaneous infections are characterized by hyperplasia and hyperkeratosis. Common warts and plantar warts are associated with types 1, 2 and 4,

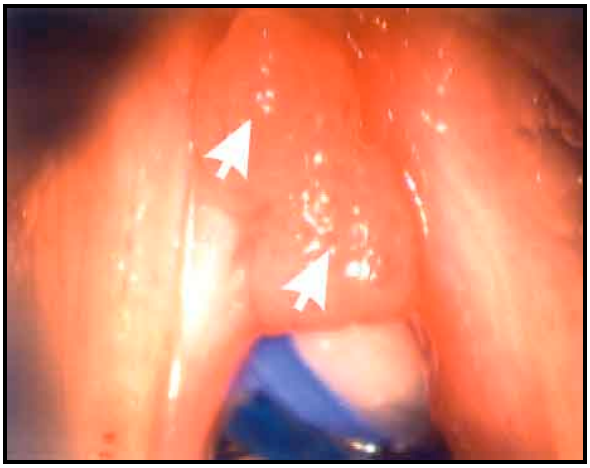


Figure 2. Shows a photograph of benign respiratory tract papillomas. Note that the papillomas originate from the true vocal cords (arrow). This is usually remedied surgically as it can leads to reduced speech capabilities, hoarseness and eventually airway obstruction.

while types 3, 10, 28 and 49 are associated with flat warts (7). Several HPV types are also mucosotropic. Genital warts are also known as venereal warts or condyloma acuminatum and are associated with HPV types 6 and 11 (7, 21). These are found in 1% of sexually active people although the prevalence of HPV DNA is considerably higher (22). Genital warts may be very difficult to eradicate with recurrence rates of 60% after treatment with keratinolytic agents (21). Recurrent respiratory papillomatosis is the most common benign laryngeal neoplasm (23). The infection is acquired in children when passing through the birth canal of mothers with genital HPV infections or in adults from oral-genital sexual activity. These lesions are commonly located on the vocal cords, and although benign, can grow to obstruct the airway necessitating their removal (Figure 2). However, recurrence is common with a mean of 4.4 surgical procedures per year required (24). The lesions may recur due to residual viral particles in normal appearing tissue, or due to viral migration to healthy sites (25, 26). While surgical excision remains the main treatment, intralesional injection of a potent antiviral cytosine nucleotide analog, cidofovir, has shown promise in a group of patients with severe recurrent papillomas (27). Respiratory papillomatosis is usually associated with HPV types 6 and 11, but one study found the presence of types 16, 31, 33, 35, and 39 as well (23). Two-thirds of lesions will spontaneously regress by adolescence. Progression to malignancy is rare, but when reported was found to be associated with a change in HPV type to the "high-risk" HPV type 16 (28).

5. HPV ASSOCIATED CERVICAL CARCINOMAS

HPV is also associated with multiple malignancies of the cutaneous or mucosal epithelium; of which the best studied is cervical carcinoma. Cervical carcinoma is the second most common malignancy affecting women. Cervical cancer is much more common than carcinomas at other anogenital sites presumably because the disease arises from the transformation zone on the cervix between the mature epithelium of the exocervix and the columnar epithelium of the endocervix. This zone is immature, hormonally responsive, and more susceptible to viral infection (2). Nearly 100% of cervical squamous cell carcinomas (SCC) and more than 70% of cervical adenocarcinomas are associated with HPV DNA (29). Large studies have shown that the risk of developing cervical cancer is strongly associated with the persistence of high-risk HPV types independent of other factors including chemicals, hormones, and other viral or bacterial infections (30). The "high risk" types 16, 18, 31, 33, 35, 39, 45, 50, 51, 53, 55, 56, 58, 59, 64 and 68 have been detected in carcinomas. However, the most common by far are HPV 16 and 18 with 50% of SCC's harboring type 16 and 50% of adenocarcinomas harboring type 18 (29). Specific HPV types do not appear to correlate with prognosis in cervical carcinomas (21).

Risk factors for HPV infection include increased numbers of sexual partners and young age of first sexual intercourse (31). HPV infects the basal epithelial cells of the cervical mucosa through sexual transmission. Viral replication and maturation proceeds during migration towards the mucosal surface and is tightly linked to epithelial differentiation. The late promoter, which regulates capsid protein production, is turned on only in partially differentiated cells (2). However, to maintain an infection, the virus needs to stimulate G1 to S-phase progression in the face of a cell programmed to terminally differentiate. Thus HPV must uncouple G1 to S-phase progression from differentiation. As cells are differentiating, DNA is amplified in the granular layer, late gene transcription occurs near the top of the epithelium and viral particles are assembled in the cornified layer (Figure 1) (2). Infection may be transient if the virus undergoes the full replication cycle at which point it is transmissible to others. These infections then disappear likely as a result of the host immune system. Viral persistence is common in a large proportion of infected individuals, but persistence decreases with time in the majority. A persistent HPV infection with abnormal cervical cytology is a strong marker for progressive cervical disease and may be related to certain high-risk types, oral contraceptive use, herpes simplex virus infection, or pregnancy (31).

6. HPV AND SQUAMOUS INTRAEPITHELIAL LESIONS

HPV plays an interesting role in development of cervical carcinomas given that they develop from well-characterized precursor lesions (Figure 3). While asymptomatic infection may occur in 30-60% of young women, only 10% of these will go on to develop cervical dysplasia and a much smaller percentage will develop invasive carcinoma (30). Low-grade squamous intraepithelial lesions (LSIL) consist of clonal expansion of basal epithelial cells, disorganized growth pattern and mild atypia. These often contain multiple "high risk" or "low risk" HPV types in high copy number (21, 29, 30), but

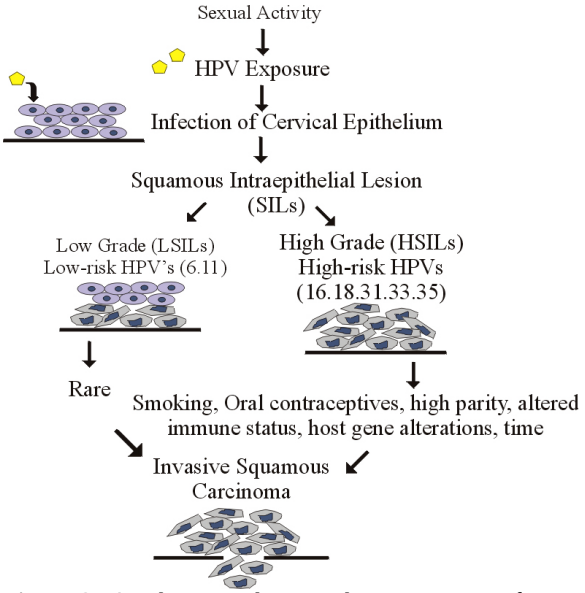


Figure 3. A schematic showing the progression of HPV associated pre-invasive lesions in the cervix to the development of invasive carcinoma. Increased sexual activity and number of partners results in increased risk of HPV exposure. The initial infection of the cervical epithelium by HPV leads to the development of squamous epithelial lesions that can progress to low grade or high-grade squamous intraepithelial lesions dependent on the subtype of HPV. High-grade lesions exacerbated by smoking and other genetic alterations can result in invasive squamous cell carcinomas. It should be noted that although rare the low-grade squamous intraepithelial lesions may also develop into invasive squamous carcinoma.

HPV E6 and E7 expression is weak or undetectable (32). Histologically LSIL's containing low risk HPV types cannot be differentiated from those containing high-risk oncogenic types. Interestingly, most of these lesions undergo spontaneous regression. Of the 15-25%, which progress to high-grade lesions, those with HPV 16 show the highest rate of progression (30). In contrast to LSIL, high-grade squamous intraepithelial lesions (HSIL) show marked cellular atypia, and harbor only high-risk types reflecting a selection process for the oncogenic HPV types (21). Specifically, most HSIL's contain type 16 with only 1% containing type 18 and 19% containing types 31, 33, or 35 (21). They also show high expression of the HPV oncogenes E6 and E7 (33). This may be due to viral integration disrupting the E2 gene or to changes in cellular transcriptional regulators. E6 and E7 expression leads to further cellular proliferation and dedifferentiation, and regression of high-grade lesions is rare.

7. HPV AND INVASIVE CERVICAL CARCINOMAS

Many HSIL's are precursor lesions to invasive cervical carcinomas. The HPV copy number decreases from LSIL's to HSIL's to invasive disease, perhaps representing a change from the acute productive infectious form of the disease to decreased virion production and more subtle dysregulation of the host cell (21). Interestingly, although HPV type 18 is rarely found in HSIL's, this type accounts for 20% of squamous cell carcinomas and up to 50% of adenocarcinomas (21). It is thought that HPV 18 may be more efficient in transformation and immortalization than some other oncogenic types and while rare in HSIL's, they have a greater incidence of progressing to cervical carcinoma. Invasive cervical carcinomas continue to have high levels of E6 and E7 expression, but also show accumulation of additional genetic defects (29). Integration occurs in at least two-thirds of invasive lesions while HPV is episomal in most LSIL's and HSIL's (2). This disrupts not only viral genes, but also cellular oncogenes or tumor suppressors leading to uncontrolled growth and the clinically invasive lesion (34). Integration of HPV DNA often inactivates the E2 transcriptional regulator that normally represses E6 and E7 expression and linearizes the DNA so the E6 and E7 genes are expressed from viral promoters (34). In addition, integration affects the host genome by integrating near fragile sites or oncogenes, deletion of cellular sequences, or altered expression of cellular genes such as N-myc and Jun-B (34). Two chromosomal regions, 8q24 and 12q14-15, are recurrently affected by HPV 16 and 18 integrations. These regions are also affected in other malignancies including lymphomas, sarcomas, renal cell carcinomas, and melanomas (34). Thus, it is postulated that E6 and E7 expression play a role in early transformation and maintenance of the phenotype since they are expressed in the HSIL stage, and allow for other genetic changes that lead to the invasiveness of the tumor cells (21). While HPV infection is the major risk factor leading to cervical carcinoma, other risk factors, which may interact with HPV in a multifactoral fashion, include coinfection with genital HSV, *Chlamydia* infection, smoking, and long-term use of oral contraceptives (31).

8. HPV AND OTHER ANOGENITAL MALIGNANCIES

HPV is also associated with other anogenital tract malignancies in addition to cervical carcinoma. Other sites include the vulva, vagina, penis, and anal canal. There is a much greater diversity of different HPV types in cervical LSIL's than in vulvar, vaginal, or penile lesions. While type16 is routinely detected at all lower genital tract sites, other "high risk" types are found in the cervix but rare at the vulva, vagina, or penis (21). Just as in the cervix, precursor lesions in the vulva, vagina and penis range from low-grade to high-grade SIL's and virtually 100% contain HPV DNA. Also copy number decreases as lesions progress from LSIL's (condylomas) to HSIL's and invasive disease, and types 6 and 11 are rarely found in HSIL's and invasive disease. However, unlike cervical carcinoma, vulvar carcinomas show two distinct histologic forms. One evolves from high grade SIL and is usually HPV positive, and the other is highly keratinizing, not related to sexual transmission, and is HPV negative (21).

Squamous cell carcinoma of the penis is uncommon, accounting for less than 1% of male cancers in developed countries. Penile cancers also develop from preneoplastic lesions. There is some evidence linking invasive penile cancer to HPV although an exact causal relationship is

difficult to establish since the prevalence of penile carcinoma is so low. Penile swabs from healthy men in western Europe reveal a prevalence of HPV DNA of 8-11% (22). The prevalence of HPV DNA in penile swabs is 5 times higher in countries with a high incidence of penile cancer such as Colombia than in those with low incidence (35). HPV DNA has been detected in 15-80% of invasive penile carcinoma (36). In addition, several studies have described an increased incidence of cervical carcinoma in the partners of men with penile cancer supporting an infectious etiology (36). As in cervical lesions, there is a high rate of spontaneous regression of early penile HPV-induced lesions. A persistent high-risk infection along with phimosis or immunocompromised state is probably necessary for progression of hyperplasia or low-grade lesions to higher-grade lesions or carcinoma (22). As in vulvar carcinoma, the rate of HPV infection in penile carcinoma depends on the histological type. The highest rate occurs in the basaloid type of squamous cell carcinoma while HPV infection is rare in the keratinizing or verrucous forms (22). While evidence links HPV infection to penile cancer, incidence of this cancer is declining without decline in the incidence of HPV infection. This suggests that other factors such as personal hygiene also contribute to this cancer (22).

It is now apparent that anal cancer is associated with HPV infection that is sexually transmitted. The fact that anal carcinoma is strongly associated with increased number of sexual partners, history of anal warts, genital warts, or cervical neoplasia supports HPV as playing a causal role (37). As in cervical carcinoma, anal cancer may arise from precursor lesions and dysplasia. 50% of homosexual men and 20% of non-homosexual patients with anal cancer report a history of anal warts (38). HPV has been detected in 88% of anal cancer specimens and 73% of these were type 16 (39). The incidence of HPV-associated anal cancer has been increasing, perhaps associated with an increase in the number of immunocompromised patients (40). In fact, a study of 174 patients found prior history of dysplasia and HIV infection to be risk factors for high-grade dysplasia and cancer (41).

9. HPV AND HEAD AND NECK SQUAMOUS CELL CARCINOMAS

In addition to anogenital lesions, there has been increasing evidence for the role of HPV in the development of squamous cell carcinomas of the head and neck (SCCHN). While the majority of these lesions are associated with tobacco and alcohol consumption, a small proportion of cases occur in patients without exposure to these substances. The route of transmission is suspected to be oral-genital sexual contact although this is not firmly established (42). Several studies have shown that individuals with higher numbers of sexual partners or history of genital warts had increased risk of SCCHN (43). In a review of published reports, the overall prevalence of HPV in head and neck tumors was found to be 34.5% (44), but may be as high as 47% (45). The most common tumor site in which HPV was detected was the oral cavity, followed by the pharynx and the larynx (44). More recently these findings were confirmed in a large study of 253 SCCHN tumor samples. They detected HPV DNA in 25% of specimens, and poor tumor grade and oropharyngeal site independently increased the probability of HPV presence (46). Others also found a significant predilection for tonsillar site (47, 48). HPV has also been detected in nasopharyngeal carcinomas (49), in cell lines derived from a variety of head and neck carcinomas (50), and in inverted papillomas that have progressed to SCC (51). Precancerous lesions (52, 53) and metastatic lymph nodes (54, 55) have also been shown to contain DNA of the same HPV type as the primary tumor, supporting the involvement of HPV in the development of SCCHN. Most commonly the "high risk" HPV types 16 and 18 are involved (42, 44), however, as many as 14 other types have been isolated from oral lesions (54, 56).

There is evidence to support the idea that HPV positive SCCHN is typically a biologically distinct subset among SCCHN. HPV detection, especially in the tonsillar region, may be correlated with poorly differentiated tumors or basaloid morphology (42, 46). As stated above, there is a similar correlation between the presence of HPV and tumor grade in vulvar and penile carcinoma with basaloid histopathology found in young patients with HPV positive tumors and well-differentiated squamous histology found in older patients with HPV negative tumors. In addition, while 45% of SCCHN contain p53 mutations (57), mutations may be infrequent in the HPV positive subset (46). HPV may be able to functionally create a p53 mutation in this group through the effects of the E6 protein. Next, survival appears to be statistically improved in patients with HPV positive tumors compared to those with HPV negative tumors (45, 46, 48). Finally, patients with HPV positive tumors may be more likely to be nonsmokers, but there is also evidence to support an interaction between HPV and tobacco-related carcinogens (42). Thus, it appears SCCHN is a multifactoral disease and while HPV plays a role in its development, its causal relationship is not as consistent as is seen in cervical carcinoma.

10. HPV AND ASSOCIATED NON-MELANOMA CUTANEOUS MALIGNANCIES

HPV has also been implicated in the development of non-melanoma cutaneous malignancies. These include basal cell carcinomas and squamous cell carcinomas, and are by far the most prevalent malignancies in fair skinned populations worldwide (58, 59). A role for HPV has been suspected in patients with epidermodysplasia verruciformis, a rare condition characterized by multiple wide spread skin warts and the development of cutaneous squamous cell carcinomas on sun exposed sites in 30-60% of patients by the fourth decade. HPV 5 and 8 are found in over 90% of these tumors (60). However, the mechanism of transformation by these HPV types remains unknown since these are generally considered "low risk" types and do not demonstrate immortalization properties or the ability for E6 and E7 to interact with p53 and Rb, respectively (59). These HPVs may act as cocarcinogens with ultraviolet light. In addition, immunosuppressed patients have an association between HPV infection and development of cutaneous squamous cell cancers. Renal transplant patients have a 50-100 fold increase of cutaneous squamous cell carcinoma, and clinical and histological features indirectly

support the progression of viral warts to dysplastic squamous lesions to invasive squamous cell carcinoma (59). A wide variety of "cutaneous" types are more common than the "mucosal" types discussed above, and thus detection requires altered methodology (61). HPV has been detected in over 90% of *in situ* and invasive squamous cell carcinomas in this transplant population, and 73% of these were either type 10, 20, 23, or 38 (62). Mixed HPV infection is also common. A similar course may be seen in patients with HIV infection. The role of HPV in cutaneous malignancies in the general population is less well studied. Several small studies have identified HPV DNA in approximately 40% of lesions, but again, detection has been limited by lack of knowledge of HPV types found in cutaneous lesions (59). In immunocompetent as well as immunosuppressed groups, detection of "high risk" types common to anogenital or head and neck lesions is less common, supporting the notion that HPV in precursor cutaneous lesions may act as a co-carcinogen with ultraviolet light. UV light may act directly to effect the HPV URR or indirectly effect HPV via inducing p53 mutations or effecting cytokine release (58, 61).

11. HPV AND THE IMMUNOSUPPRESSED

It is worth reiterating that HPV infections are common in immunosuppressed individuals as was discussed briefly in terms of cutaneous malignancies above. The incidence of HPV associated lesions is likely to increase given the increasing numbers of organ transplant procedures and the increased life expectancy of patients with HIV as more effective antiviral medications are available. In addition to cutaneous lesions, consistent associations between HIV infection and pre-invasive cervical SIL have been shown (63). HIV-infected patients are 4 times more likely to be infected with HPV than uninfected women, and these infections are more likely to be persistent (63). Also, HIV patients are 2-6 times more likely to have anal HPV infection and twice as likely to have progression to high grade lesions or carcinoma in a given time period as HIV negative patients (37). The risk is inversely proportional to the CD4 lymphocyte count suggesting loss of immune surveillance to HPV or to tumors themselves. These findings emphasize the need for heightened surveillance for early detection and prevention in this population.

12. CONCLUDING REMARKS

The above discussion illustrates the diversity of neoplasms linked to HPV, many of which are very common in the general population. For this reason some believe that development of an HPV vaccine could prevent more cancer than any other type of intervention (31). One difficulty in development of a vaccine is that there are multiple "high risk" types and immune reactions are type specific (64). Currently prophylactic vaccines based on the use of virus-like particles containing the L1 and L2 capsid proteins are under clinical trials for HPV 6/11 associated with genital condylomatas and for HPV 16 (64). Therapeutic vaccines designed to treat individuals already infected are also being developed. The first type contains E1 and E2 proteins and theoretically would induce cellular immunity to E1 or E2 and limit virus growth. The second type is designed for patients with existing cervical carcinoma and hopes to induce cytotoxic lymphocytes to recognize tumor associated antigens and destroy those cells expressing them (31). The antigens are generally the E6 or E7 proteins, which are constitutively expressed in these tumors. Therapeutic vaccines may also be based on genetic modifications of tumor cells containing suicide genes or genes for immunostimulatory factors such as interleukin-2, or they may rely on the use of dendritic cells. While these hold much promise, final determination of the efficacy of any of these efforts remains to be investigated.

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Send correspondence to: Dr Erle Robertson, University of Michigan Comprehensive Cancer and Geriatrics Center, Microbiology and Immunology and Otolaryngology/Head and Neck Surgery, 3217 CCGC Building, University of Michigan Medical School, Ann Arbor, MI 48109-0934. Tel: 734-647-7296; Fax: 734-647-9647; Email: esrobert@umich.edu