Human Papillomavirus-Related Diseases of the Female Lower Genital Tract: Oncogenic Aspects and Molecular Interaction

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ABSTRACT

The causal role of human papillomavirus (HPV) in all cancers of the uterine cervix has been firmly established biologically and epidemiologically. Most cancers of both the vulva and the vagina are also induced by HPV. Papillomaviruses are perfectly adapted to their natural host tissue, the differentiating epithelial cell of skin or mucosae, and exploit the cellular machinery for their own purposes. The infectious cycle is initiated once the infectious particles reach the basal layer of the epithelium, where they bind to and enter the cells. The critical molecules in the process of virus replication are the viral proteins E6 and E7, which interact with a number of cellular proteins. In experimental system these interactions have been shown to induce proliferation and eventually immortalization and malignant transformation of cells. Binding of E7 to pRb activates the E2F transcription factor, which then triggers the expression of proteins necessary for DNA replication. Unscheduled S-phase would normally lead to apoptosis by the action of p53. However, in HPV-infected cells, this process is counteracted by the viral E6 protein, which targets p53 for proteolytic degradation. Besides blocking p53 function in regulation of apoptosis, high-risk HPV proteins interact with both extrinsic and intrinsic apoptotic pathways. As an aberration of virus infection, constant activity of the viral proteins E6 and E7 leads to increasing genomic instability, accumulation of oncogene mutations, further loss of cell-growth control and ultimately cancer. The immune system uses innate and adaptive immunity to recognize and combat foreign agents that invade the body, but these methods are sometimes ineffective against human papillomavirus. HPV has several mechanisms for avoiding the immune system. Furthermore, HPV infections disrupt cytokine expression with the E6 and E7 oncoproteins, particularly targeting the expression of interferon genes. Approximately 10% of individuals develop a persistent infection, and it is this cohort who is at risk of cancer progression, with the development of high-grade precursor lesions and eventually invasive carcinoma.

Key words: human papillomavirus, early viral oncogenes, tumor suppressor genes, malignant transformation

Introduction

Genital Human papillomavirus (HPV) infections are the most common sexually transmitted infections among women1–3. The immune system effectively repels most HPV infections, and is associated with strong localized cell mediated immune responses. However, approximately ten percent of infected women develop a persistent infection, with the risk of developing high-grade precursor lesions and eventually invasive carcinoma4–6. The causal role of HPV in all cancers of the uterine cervix has been firmly established6–8. An estimated 40–50% of vulvar cancers and more than 80% of vulvar intraepithelial lesions have also been associated with HPV. In cancers of the vagina and their precursor lesions, HPV DNA was detected in 64% and 91% of cases9. Tumor formation is not an inevitable consequence of a viral infection; it rather reflects the multi-step nature of oncogenesis where each step constitutes an independent (reversible or irreversible) genetic change that cumulatively contributes to deregulation of the cell cycle, cell growth, and survival8,11,12.
**Human Papillomaviruses**

Papillomaviruses (PVs) are a large family of small double-stranded DNA viruses, which infect basal layer cells of stratified squamous epithelia (or cells with the potential for squamous maturation). PVs are considered primarily epitheliotropic, but corresponding viral genetic material has been retrieved from a large variety of tissues. In humans, DNA and mRNA from different HPVs have been detected not only in the blood of patients suffering from cervical cancer but also in healthy blood donors.

Papillomaviruses are classified into genera, species and types based on their viral genome heterogeneity. At present, about 170 types have been identified by sequences of the gene encoding the major capsid protein L1. HPVs can be classified into high or low-risk types depending upon their oncogenic potential. High-risk genotypes 16 and 18 have been associated with 70% of cervical carcinomas, and about 60% of HPV positive persistent infections of the cervix. Genotypes 6 and 11 have been linked to over 90% of genital warts.

**HPV genome organization**

Virus particles consist of about 7900 base-pairs (7.9 kbp) long circular DNA molecules wrapped into a protein shell. The HPV genome can be functionally divided into two regions: Upstream Regulatory Region (URR) and Open Reading Frames (ORFs). URR does not code for proteins but contains cis-elements required for regulation of gene expression, replication of the genome, and its packaging into virus particles. ORFs can be divided into the Early Region (E), necessary for the replication, cellular transformation, and control of viral transcription, and the Late Region (L) that codes for the capsid proteins comprising the outer coat of the virus.

Within the Early Regions (E) it is possible to distinguish at least six different genes with specific functions. E1 and E2 genes have an important role in basal DNA replication. During viral persistence, the immune system keeps the infection in this state. E2 participates in the regulation of LCR (low-copy repeats) transcription, and decreases the expression of E6 and E7. E4 gene codes for one family of small proteins involved in the transformation of the host cell by producing alterations of the mitotic signals and interacting with keratin. E5 decreases intercellular communication, isolates the transformed cells, interacts with the growth factor’s receptors, and encourages cellular proliferation. It also stimulates the expression of E6 and E7. E6 is oncopgenic, stimulating the growth and transformation of the host cell by inhibiting protein p53’s normal tumor-suppressor function. E7 also acts as oncogene, inducing cellular proliferation by inhibition of protein pRb. Within the Late Region (L), it is possible to distinguish the L1 gene, which codes for the major capsid protein and can form virus-like particles, and the L2, which codes for the minor capsid protein.

**Natural history of genital HPV infection**

Genital HPV infections are sexually transmitted infections with a lifetime risk of 50–85%, having a peak prevalence between ages 18 and 30. Compared with low transmission probabilities of other sexually transmitted infections such as human immunodeficiency virus (HIV) and herpes simplex virus type 2 (HSV-2), the transmission rate from male to female per intercourse for genital HPV has been estimated from 0.4 to 0.8.

Most of these infections clear spontaneously. 75% of infections clear within a year, and individuals with suboptimal immune responses may be at increased risk of persistent HPV infection and associated malignancy.

At present, about 30–40 genotypes of HPV can be isolated in the female genital tract; nevertheless only 12–15 genotypes are usually associated with the development of carcinoma.

**HPV life cycle**

HPVs are perfectly adapted to their natural host tissue, the differentiating epithelial cells of skin or mucosa, and are exploiting the cellular machinery for their own purposes. HPVs are undergoing a complete life cycle only in a fully differentiated squamous epithelium. These viruses infect the basal cell layer, where they establish their small double-stranded DNA genome as a circular extra-chromosomal element or episome in the nucleus of infected cells. Following entry into the suprabasal layer, the viral genome replicates and in the upper layers of epidermis complete viral particles are released. The existence of the viral genome in the infected cell is central to the life cycle of papillomaviruses and their associated pathologies. Maintenance of the viral genome requires the activity of E1 (the replicative helicase of papillomavirus) and E2, the two viral proteins necessary for replication of the HPV genome in conjunction with the host cell DNA replication machinery. As initiator protein E1 acts as both a DNA binding protein to recognize the viral origin of DNA replication, and subsequently a helicase to unwind the origin and the DNA ahead of the replication fork. In lesions containing HPV episomes, the viral E2 protein directly represses early gene expression as part of a mechanism to regulate copy number. Integration of viral DNA usually disrupts E2 expression, leading to the deregulated expression of early viral genes, including E6 and E7. The expression of viral gene products is closely regulated as the infected basal cell migrates towards the epithelial surface. Genome amplification, which is necessary for the production of infectious virions, is prevented until the levels of viral replication proteins rise, and depends on the co-expression of several viral proteins. Viral persistence leads to clonal progression of the persistently
infected epithelium. Events which are still not completely understood lead infected cells to malignant transformation.

**HPV DNA replication**

A viral infection of epidermal stem or transit amplifying cells can result in long term viral persistence, and the development of carcinogenesis over a significant amount of time then requires additional cooperating genetic hits\(^\text{38}\). The papillomavirus DNA replication depends entirely upon the cellular DNA synthesis machinery. The problem for the virus is that the necessary cellular DNA polymerases and replication factors are only available in dividing cells. However, the virus also replicates in non-dividing cells. To solve this problem, HPV encodes proteins that, in the context of the viral life cycle, reprogram cellular DNA synthesis in non-cycling cells, inhibit apoptosis, and delay the differentiation program of the infected keratinocyte, creating an environment permissive for viral DNA replication\(^\text{39}\). The precise details by which E1 complex that has unwinding activity, most likely a gin serves as a starting point for the assembly of a larger polymerase binds with high affinity to sites in the viral origin. As- interaction with E2, a transcription/replication factor that binds with high affinity to sites in the viral origin. Assembly of a ternary complex between E1, E2, and the origin serves as a starting point for the assembly of a larger E1 complex that has unwinding activity, most likely a double hexamer necessary for bidirectional unwinding. E1 interacts with DNA replication factors, including the polymerase α-primase and the single-stranded binding protein RPA (Replication Protein A), to promote viral DNA replication\(^\text{42}\).

**Inhibitors of HPV DNA replication**

Interaction between E2 protein and E1 helicase of human papillomaviruses is essential for the initiation of viral DNA replication. Research performed by Wang and colleagues\(^\text{43}\) led to the identification of the first small molecule inhibitors of HPV DNA replication. Characterization of their mechanism of action has shown that this class of inhibitors binds to E2 and prevents its interaction with the E1 helicase. These inhibitors defined a previously unrecognized small-molecule binding pocket on E2. This class of inhibitors was found to antagonize specifically the E1-E2 interaction in vivo and to inhibit HPV DNA replication in transiently infected cells. These results highlighted for the first time the potential of the E1-E2 interaction as a small molecule antiviral target for the treatment of HPV infections\(^\text{44}\). These inhibitors also provided a rare example of a class of small molecules that can antagonize a protein-protein interaction.

**Malignant transformation of the lower genital tract**

The female genital tract, a continuum of squamous epithelium from the vulva to the cervix, is commonly infected by human papillomavirus. The outcome of HPV infection depends on the immune response, the viral genotype (low-risk or high-risk/oncogenic) and the site of infection (the cervical squamo-columnar junction is more susceptible to HPV disease). The key role of HPV in most cancers of the female lower genital tract has been firmly established biologically and epidemiologically\(^\text{20,41,45-47}\).

The cervical cancer is marked by a premalignant phase of various grades of cervical intraepithelial neoplasia (CIN), which is a genetically unstable lesion and is characterized by a spectrum of histological abnormalities. HPV viral integration into the host genomic DNA is associated with progressive genetic instability, and these events play a fundamental role in the progression from low-grade (CIN1) to high-grade (CIN2/3) lesions, and eventually to invasive cervical cancer (ICC). In longitudinal natural history studies, the elapsed time from the detection of high-risk HPV to the development of CIN3 is 3–5 years\(^\text{45}\), and the progression to ICC takes a further 10–20 years\(^\text{47}\). Most cancers of the vulva and the vagina in younger women are also induced by oncogenic HPV types\(^\text{22,24}\). These cancers are preceded by both a high-grade vulvar intraepithelial neoplasia (VIN2/3) and vaginal intraepithelial neoplasia (VaIN2/3).

**Molecular basis of HPV-induced oncogenesis**

The HPV DNA usually exists as extrachromosomal plasmid, mostly as a monomeric circular molecule in benign cervical precursor lesions. However, in cervical cancer cells the HPV DNA is integrated into the host genome. During the HPV DNA integration, the viral genome breaks in the E1/E2 region. The break leads to the loss of E2, which encodes proteins including the one that inhibits the transcription of the E6 and E7 regions, resulting in the increased expression of E6 and E7 oncogenic proteins\(^\text{48,49}\). The proteins coded by these genes are multifunctional and interfere with important cell cycle regulatory proteins. Expression of viral oncogenes is tightly controlled in non-differentiated keratinocytes by at least two signaling cascades, one operative at the functional level and the other at the transcriptional level. Integration of the viral DNA could occur, resulting in increased expression of E6 and E7. Additionally, mutations or methylation of host DNA could occur, abrogating the transcriptional control of differentiation and viral gene expression; there is evidence for both of these mechanisms\(^\text{50,51}\). The oncoproteins E6 and E7 interact with many cellular proteins and change fundamental cellular.
functions like cell cycle regulation, telomere maintenance, susceptibility to apoptosis, intercellular adhesion, and regulation of the immune response. These effects are in accordance with the essential changes in cell physiology that are acquired during tumor development and that have been proposed by Hanahan & Weinberg. Evading the immune system surveillance has been recognized as an additional basic feature of malignant growth.

**Regulation of the cell cycle**

Maintenance of genetic integrity from one generation to the next requires accurate replication of chromosomess during the S-phase and their faithful segregation during mitosis. Protein p53 is known as the -genome’s guardian, and in case of DNA damage, p53 can provoke the arrest of cellular division to assure the time necessary for DNA repair.

If damage cannot be repaired, p53 is able to induce the programmed cellular death (apoptosis) and prevent the propagation of DNA damage in the subsequent generation of cells. The product of another tumor suppressor gene, pRb acts as a repressor of E2F transcription factors, E2F regulates various genes including those involved in the progression of the cell cycle (the G1-S transition). By binding E2F, pRb prevents the entry into the S-phase, providing the time necessary for checking genome integrity. Oncoproteins E6 and E7 cooperatively disrupt the functions of p53 and pRb, with profound changes in the cell cycle regulation. Furthermore, E6 and E7 proteins can directly provoke DNA mutations of the host cell genome, and they interact with the members of retinoblastoma protein family: pRb, p107, and p130 (also called «pocket proteins»). Most of the pRb functions are related to the repression of the E2F transcription factor. The E7 protein directly binds pRb and targets it for degradation through the ubiquitin-dependant pathway. Suppression of p53 function by E7 results in the activation of E2F and stimulation of the cell cycle progression. E7 is also capable of a direct interaction with E2F factors and chromatin modifiers, such as histone deacetylases (HDACs), what additionally affects the expression of S-phase genes. E7 protein interacts with cyclin dependent kinases (CDK) inhibitors like p21 and p27, while E6 inhibits p21 transcription by inactivating p53, E7 inhibits p21 functions by direct binding, thus contributing to sustained activity of CDK, such as CDK2. High-risk E7 also increases the expression of CDC25A phosphatase that promotes CDK2 activation. All these effects on cell proliferation are not only favourable for the HPV life cycle and replication, but they also contribute to the uncontrolled proliferation of infected cells. Besides disrupting cell cycle control, and allowing the cell division in the presence of DNA damage, E6 and E7 are capable of directly inducing DNA damage. Thus, in HPV infected cells a deleterious combination could be present: increased DNA damage and impaired response to DNA damage.

**Oncoprotein E6 functions**

HPV 16 E6 is a 151 amino acid protein with two zinc finger domains. E6 is one of the primary onco genes of the virus. E6 together with E7 causes immortalization of cells and plays an important role in malignant transformation. Oncoprotein E6 interacts with numerous cellular proteins. E6 targets p53 through recruitment of a cellular E3 ubiquitin ligase – E6 associated protein (E6AP). This trimeric complex leads to p53 degradation by ubiquitin-proteosomal pathway. Besides targeting it for degradation, E6 is capable of binding directly to p53, interfering with its DNA-binding activity. In addition, E6 protein blocks apoptosis, alters the transcription machinery, and disturbs intercellular interactions, a crucial step towards malignancy. Another important target for E6 is the group of PDZ proteins. The name is related to the first three members identified: PSD-95 (a post-synaptic density signalling protein), Dlg (the Drosophila disc large protein) and ZO1 (the zonula occludens 1 protein). High-risk E6 associates with PDZ proteins. These proteins play an important role in cell signalling, cell adhesion, and tight-junction integrity. Experimental evidence indicates that the interaction of E6 with PDZ proteins is necessary for the development of epithelial hyperplasia.

**Telomere maintenance**

While normal cells have finite numbers of doublings before they become senescent («Hayflick limit»), malignant cells acquire limitless replicative potential. The immortality of malignant cells is closely related with telomere maintenance. The majority of malignant cells achieve telomere maintenance by up-regulation of telomerase, the enzyme that adds hexanucleotide repeats to the 3' end of DNA strands in the telomere regions. Telomerase is a ribonucleoprotein complex containing three subunits: a catalytic subunit – human telomerase reverse transcriptase (hTERT), a RNA subunit and, a protein subunit (dyskerin). The expression of hTERT is proportional to telomerase activity in the cells. It has been shown that high-risk E6 protein activates transcription of hTERT. E6 interacts with Myc protein either alone or in complex with E6AP, and heterodimer Myc/Max binds to the hTERT promoter and activates its transcription. E6 also affects other hTERT activators including Sp1, which binds to the hTERT promoter and histone acetyltransferases that increase histone acetylation.
the hTERT promoter\textsuperscript{76,77}. E6 modulates activity of hTERT repressors as well. The HPV 16 E6/E6AP complex targets hTERT repressor X box-binding protein 1-91 (NFX1-91) for polyubiquitination and degradation. E6 affects the binding of two other hTERT repressors — upstream stimulating factors 1 and 2 (USF1 and USF2). Additionally, E6 directly associates with NFX123 that increases hTERT activity by several mechanisms including those on transcriptional and post-transcriptional level\textsuperscript{75}. A second mechanism of telomere maintenance is recombination-based and is termed alternative lengthening of telomeres (ALT) pathway. It has been suggested that the E7 protein affects telomere length through the ALT pathway\textsuperscript{79}. Thus, a cooperative effect between E6 and E7 could be achieved regarding telomere maintenance and cell immortalization. The E7 effect could be important in early cancer development while E6 might play a role in the later phases of oncogenesis\textsuperscript{60}. This is in accordance with the observation that high levels of hTERT expression are found in advanced cervical lesions and invasive carcinomas\textsuperscript{79}.

\textbf{Evading apoptosis}

HPV has developed numerous mechanisms that block host-mediated apoptosis. These mechanisms regulate the survival of infected cells thus facilitating the HPV replication cycle. Besides blocking p53 function in regulation of apoptosis, high-risk HPV proteins interact with both extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is triggered by various extracellular signals that activate «death receptors», the members of the tumor necrosis factor receptor (TNFR) family. After binding, the ligand death receptors form trimers and associate with adaptor molecules and initiator caspases. The result is the formation of the death inducing signalling complex (DISC). DISC activates caspase 8, which cleaves downstream caspases in the apoptotic pathway leading to cell death. High-risk E6 protein interacts with all components of the DISC complex. E6 binds to the death receptor TNFR-1 and blocks its association with adapter molecules\textsuperscript{80}. Furthermore, E6 can accelerate the degradation of some adapter molecules like FADD and the initiator caspase-8\textsuperscript{75,81}. The intrinsic apoptotic pathway is activated by various intracellular stressors (DNA damage, oxidative stress, and others) and includes mitochondrial permeability transition. Then pro-apoptotic signals dominate changes in mitochondrial membrane are initiated with the formation of pores and release of pro-apoptotic proteins. These proteins form an apoptotic signaling complex that results in cleavage of downstream caspases (like caspase-3 and caspase-7), leading to degradation of cellular components. The E6 protein interacts with intrinsic apoptotic pathway signaling by binding Bak, a pro-apoptotic member of Bcl-2 family. E6 binds Bak and induces its degradation through the ubiquitin-proteasome pathway\textsuperscript{82}. The HPV E6 protein is also capable to up-regulate the expression of inhibitors of apoptosis, such as survivin and the inhibitor of apoptosis protein 2 (IAP2). The studies of HPV E7 in regulation of apoptosis obtained variable results; both anti-apoptotic and pro-apoptotic effects have been found\textsuperscript{83}. HPV oncoproteins target a number of factors important for anoikis, a specific type of apoptosis that is induced by the loss of cell adhesion or by inappropriate cell adhesion\textsuperscript{84,85}. High-risk HPV proteins bind to or are associated with changes in expression levels of fibronectin, fibulin-1, focal adhesion kinase (FAK), and paxillin\textsuperscript{86}. These interactions contribute to the capability of HPV infected cells to become resistant to anoikis and grow in the absence of anchorage to the extracellular matrix and their neighbouring cells. Anchorage independent growth is considered to be a hallmark of malignant phenotypes.

\textbf{Escape from immune system surveillance}

The major lines of defense against various pathogens are natural mechanical barriers, innate and adoptive immunity. Dendritic cells (DC) are highly specialized antigen presenting cells (APC) that play important roles in innate immunity and provide a link between innate and adoptive immunity. Toll-like receptors (TLR) located in the membrane or inside the DC recognize typical molecular motifs of various pathogens called pathogen-associated molecular patterns (PAMPs). Langerhans cells are the main DC of the skin and mucosa, being important detectors at the site of HPV infection. Activated dendritic cells migrate to draining lymph nodes, mature during the migration to highly effective APC and present antigens to naïve T lymphocytes, thereby initiating cell-mediated responses. The activated effector (cytotoxic) cells target infected cells at the site of infection\textsuperscript{4}. Indeed, in case of HPV infection in the majority of cases the virus is cleared by cell-mediated mechanisms that are clinically associated with complete remission. However, the time necessary for clearance ranges from months to years suggesting a delay in immune response. 10–20\% of infected persons do not manage to clear the HPV infection and develop persistent infection that is associated with the risk of high-grade cervical lesions and invasive carcinomas\textsuperscript{86,87}. HPV has developed several mechanisms for evading the immune surveillance. The majority of these mechanisms contribute to evading of innate immunity that delays the adoptive immune response. Some of these mechanisms are related with the characteristics of the viral site of infection and some are related to the effects of viral oncoproteins. HPV does not have a lytic phase, and thereby does not cause cell injury that would initiate inflammation and/or immune response. There is no virologic stage during HPV infection. Therefore both locally and systemically there is no favourable situation for contact between HPV and the immune system. Hasan and colleagues\textsuperscript{88} have shown that high risk E6 and E7 proteins inhibit TLR9 transcription leading to impaired activation of the innate immune response. Additionally, high-risk proteins interact with interferon regulatory factors (IRF) required for the expression of type I interferons: E6 binds IRF-3 while E7 interacts with IRF-1\textsuperscript{89,90}. Microarray analysis has shown that high-risk proteins down-regulate the expression of IFN-inducible genes, in-
clusing signal transducer and activator of transcription (STAT1)38. One of the possible mechanisms that underlie this phenomenon is a direct interaction of HPV 16 E7 with p48-48-dna binding component of the interferon-
stimulated gene factor 3 (ISGF3) transcription complexes, thus blocking the translocation of this complex to the nucleus92. Furthermore, HPV proteins interact with the proximal components of interferon-inducible pathways. E6 binds and inhibits the function of tyrosin ki-
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Conclusions

The association between persistent HPV infection and the risk of malignant transformation of the lower fe-

male genital tract is well established. HPV E6 and E7 oncoproteins are critical molecules in the process of ma-
lignant tumor formation. Interacting with various cellular proteins, E6 and E7 influence fundamental cellular functions like cell cycle regulation, telomere maintenance, susceptibility to apoptosis, intercellular adhesion, and regulation of immune responses. High-risk E6 and E7 cooperatively disrupt p53 and pRb functions with pro-
found changes in the cell cycle regulation. Uncontrolled cell proliferation leads to increased risk of genetic insta-
-bility; the generator of mutant phenotypes that will con-
tribute to conferring other abnormalities and possible advantages for tumor growth. Furthermore, oncoprote-
ins E6 and E7 are capable of directly provoking DNA damage. It usually takes decades for cancer to arise. Thus, cervical carcinogenesis is a multifactorial process involving genetic, environmental, hormonal, and immunological factors in addition to HPV infection.

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Bolesti donjeg dijela ženskog spolnog sustava povezane s humanim papilomavirusom – onkogeni aspekti i molekularne interakcije

Sažetak

Uzročna veza infekcije humanim papilomavirusom (HPV) i karcinoma vrata maternice potvrđena je biološki i epidemiološki. Većina karcinoma vulve i vagine također su inducirani HPV-om. Infektivni ciklus započinje kada virusne čestice dospiju na bazalni sloj epitela gdje se vezu i ulaze u stanice. Ključne molekule u procesu virusne replikacije su virusni proteini E6 i E7. HPV tipovi visokog rizika blokiraju funkciju p53 protein a te djeluju na ekstrinzične i intrinzične puteve apoptoze. HPV koristi brojne mehanizme za izbjegavanje humanog imunološkog sustava i mijenja ekspresiju citokina putem E6 i E7 onkoproteina. Prosječno 10% inficiranih osoba razvije perzistentnu infekciju i upravo je ovo rizična skupina za razvoj prekursorskih lezija visokog rizika i invazivnog karcinoma.