LET SLEEPING DOGS LIE – UNLEASHING THE TRANSFORMING POWER OF DORMANT HPV

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ABSTRACT

The rate of infection with human papillomavirus (HPV) worldwide is about 80% of the adult population. Anogenital HPV infections are usually transient and cause no lasting damage. In about 15-20% of the cases, however, the HPV infection may persist and in about 1% it may cause transformation of the infected epithelium that might subsequently progress to overt cervical cancer. The hallmark of the malignant transformation is the integration of the viral genome into the host cell genome, resulting in upregulation of the transcription of the viral oncoproteins E6 and E7. The latter are expressed early in the course of the infection, interacting with the major regulators of the cell cycle progression so as to retain the host cell in a state favourable for the replication of the viral genome. Among the crucial cellular partners in the neoplastic transformation of HPV-infected cells are tumour-suppressor proteins such as p53 and pRb, products of protooncogenes such as p21/WAF1, chromatin structure modifiers such as HMGA1, and controllers of the cellular senescence such as the telomerase complex. The high-risk types of HPV seem to have developed mechanisms capable of evading or disabling virtually any defence. Nature has put in place against cancerous transformation, which could account for the high incidence of cervical dysplasia and cervical cancer despite the efforts the modern medicine and healthcare puts into screening programmes, prevention and therapy. The role of the deregulation of the expression of each of these groups of participants in the pathogenesis of activation of persistent dormant infection is reviewed and their impact on the risk for progression to higher grades of cervical intraepithelial neoplasia (CIN) and development of cervical cancer is assessed.


Keywords: HMGa, HPV, cervical cancer, E6/E7 oncoproteins

HPV basics

Papillomaviruses are small non-enveloped viruses with a circular DNA genome. They are highly host-specific and tissue-specific, with explicit affinity for epithelial cells. Approximately 130 human papillomavirus (HPV) types have been identified so far, of which >70 infect specifically the genital and anal mucosae. Anogenital infection with HPV, especially with types 6 and 11, may manifest as exophytic cauliflower-like lesions known as condylomata acuminate. In healthy people, the lesions as well as the underlying HPV infection are usually cleared within several months without lasting damage, though they may recur. In a minority of cases (about 1%), however, anogenital HPV infection may persist for extended periods of time, causing lasting changes in the infected epithelium. In about 0.08% of all cases, anogenital cancer and specifically cervical cancer may develop.

The most strongly associated with cervical cancer HPV types are HPV 16 and HPV 18 (found in about 70% of the cases), followed by types 31, 33 and 45 (10). Other “high-risk” HPV types have been identified (35, 39, 51, 52, 54, 56, 58, 59 and 66), but these are less frequently discovered in cervical cancers (12). There are also a number of HPV types that are considered to be associated with low risk for neoplastic growth, namely, the already mentioned types 6 and 11, and others such as types 34, 40, 42, 43 and 44 (14).

The HPV genome is double-stranded, about 8 kb long, contains 9 open reading frames and is comprised of a long control region (LCR) containing cis-responsive elements required for the regulation of gene expression and DNA replication, an early region coding for proteins involved in the regulation of viral DNA transcription and replication (E1, E2), a gene coding for a protein enhancing the activity of host epidermal growth factor (E5); two genes implicated in the control of host cell proliferation so as to expand the population of infected cells (E6 and E7); a gene responsible for the correct virion assembly (E4) and a late region containing two genes which code for the capsid proteins L1 and L2. Alternative RNA splicing is employed so as to extend the coding capacity of the viral genome. For example, the genes coding for the E6 and E7 viral proteins have partially overlapping reading frames (78). E6 and E7 viral oncogenes of high-risk types of HPV are transcribed from a common promoter situated at the E6-proximal end of the LCR, stimulated by a keratinocyte-specific enhancer located within the LCR (19, 62).
HPV life cycle and virus-host interactions

My life has been full of terrible misfortunes – most of which never happened.

Michel de Montaigne (1533-1592)

It is believed that HPV infects the epithelium through micro-abrasions via receptor-mediated endocytosis. After partial uncoating, the viral genome is transported to the nucleus and establishes itself at a copy number between 10-200 viral genomes per cell. The infection stimulates the production of new epithelial cells, which, in turn, brings about their more rapid terminal differentiation, producing a hyperkeratotic state. After the newly produced viral particles are assembled, the resulting virions are sloughed off the skin or mucosa surface together with the upper keratinized layer and released into the environment, ready to launch a new infection cycle.

Most immunocompetent individuals infected with HPV do not develop any short-term or long-term clinical signs or symptoms after HPV infection as the virus is cleared out by the host’s immune system relatively rapidly. Certain types of HPV, however, cause lasting morphological changes in the epithelium of the lower genital tract and the anus. The persistent genital HPV infection may result in a prolonged phase of pre-invasive disease, collectively referred to as cervical intraepithelial neoplasia (CIN), characterized by increased nuclear and cellular atypia, increased mitotic rate and disorganization in the architectonics of the epithelium. The severity of CIN depends on the proportion of the relative thickness of the epithelium showing differentiated and undifferentiated cells. It is usually categorized into grades I, II and III which represent the pathologic continuum from mild to severe epithelial dysplasia, with CIN III (over 2/3 of the thickness of the epithelium showing dysplastic features) being a collective term for severe dysplasia as well as carcinoma in situ. The invasive carcinoma of the uterine cervix is thought to arise from CIN in the vast majority of cases. Only about 25-30% of the more dysplastic lesions, however, progress further to overt cervical carcinoma. Differentiation grade of the tumor is usually expressed as G1 (differentiated), G2 (moderately to poorly differentiated) and G3 (undifferentiated). Whereas most of the precursor lesions are readily curable (excision, chemical, cryogenic or laser ablation, cone biopsy, etc.), the prognosis for invasive carcinoma is generally poor even in modern clinical settings.

Molecular bases of the HPV oncogenic potential

In the infected cells, HPV DNA may exist either in episomal form or integrated into the host’s genome. The sole presence of HPV DNA does not mean that cancerous transformation is imminent, merely that the risk that such a transformation may occur at some point is increased. An independent factor that may act as a risk modifier is smoking, as the risk for progression to high-grade cervical intraepithelial dysplasia and cervical cancer seems to be higher for smokers (28, 64). Oral contraceptive (OC) use and presence of drug addictions were once considered to be independent modifiers of the risk for cervical dysplasia and cervical carcinoma, but recently these have been ruled out as independent factors, as research indicated that the increased prevalence of persistent HPV infections in OC users and drug addicted patients was generally related to behaviour specificities in those groups (more likely to engage in high-risk sex practices and/or to have multiple partners) (63, 64, 65).

The root of the oncogenic properties of HPV is in its ability to tamper with the cell cycle of the host cell. It is considered that the potential for carcinogenesis of the HPV virus is unleashed only after the viral DNA becomes integrated into the host cell’s genome. Generally, in the integrated state, only a subset of the viral genes – the E6 and E7 oncoproteins, are consistently expressed (9, 60). E6 and E7 are early viral proteins that are expressed at low level and serve to evade the host’s mechanisms for dealing with damaged cells, so that the HPV-infected cells would survive and continue to divide, producing more viral particles (21). The integration of the viral DNA into the genome of the host cell is usually accompanied by disruption of the E2 gene, and, sometimes, of the E1 gene as well. The E2, and, probably, the E1 protein too, are transcriptional repressors of the expression of E6 and E7 (56), therefore, loss of expression of E2 and/or E1 causes relaxation of the control over the transcription of E6 and E7, which in turn results in prolific expression of the relevant oncoproteins. The latter exert their pro-carcinogenic action via direct interaction with some of the major regulators of the cell cycle.

The main cellular targets of HPV oncoproteins are the p53 and retinoblastoma proteins (pRb) (6, 40, 47). They belong to the tumour-suppressor type of cell cycle regulators and act to prevent cells, whose DNA has been damaged or altered, from dividing. Thus when inactivated by HPV oncoproteins, immortalization of the affected cells may be effectively caused.

E7 and pRb

The protein product of the RB1 gene is a nuclear phosphoprotein that prevents cells from entering S phase of the cell cycle (20, 37, 77). The RB1 protein is regulated via a phosphorylation/ dephosphorylation mechanism during cell proliferation and differentiation, in G0/G1 cells virtually all the pRb protein being hypophosphorylated, while in S and G2 phase the protein is massively phosphorylated (13). Presumably, phosphorylation of pRb renders it unable to interact with proteins that regulate the progress into the S phase of the cell cycle, among which are the E2F family of transcription factors (Rb-E2F pathway). The unphosphorylated pRB-E2F complex imposes transcriptional repression onto the promoters of its downstream protooncogenes such as c-Myc, c-Jun and c-Fos. The E7 protein of HPV is capable of direct interaction with pRB, with a preference for the hypophosphorylated form of pRB. Thus, pRB is functionally inactivated, leaving the E2F proteins free to activate the expression of their downstream genes, and, consequently, the cell progresses to the S phase of the cell cycle (25, 37). E7 proteins from high-risk HPV (types 16, 18, etc.) bind more tightly to pRb than E7 proteins
of low-risk HPV types (e.g., HPV types 6 and 11) (48, 50). Aberrant activation of Rb-E2F pathway by E6/E7 of HPV type 16 has another adverse pro-carcinogenic consequence as well, namely, it is believed to promote genomic instability by decreasing the nucleotide levels in the newly transformed cells (11, 48) (see below).

**E6 and p53**
The p53 protein basically prevents a cell from completing the cell cycle if its DNA is damaged or if the cell has suffered other types of damage. When the damage is repairable, p53 is activated in a dose-dependent manner via different signalling pathways and causes cell cycle arrest until the damage is repaired. If the damage is deemed so serious or so extensive that it cannot be repaired, p53 reroutes the cell to the programmed cell death (apoptosis) pathway (57, 59). More than 50% of all human cancers harbour loss-of-function mutations in the TP53 gene, allowing cells with damaged or altered DNA to continue dividing instead of dying via the programmed cell death route. The E6 HPV oncoprotein binds to a cellular protein known as E6-associated protein which possesses ubiquitin-ligase activity. The resulting complex, in turn, induces the degradation of p53 via the ubiquitin-dependent proteolytic pathway (58, 73), avoiding clearance of the infected cells via apoptosis. Remarkably, as with E7 and pRb, there is a differentiation in the interaction between p53 and E6 in its complexed state between high-risk and low-risk types of HPV. Namely, rendering p53-dependent apoptosis pathway inoperative is more typical of the E6 of the HPV with high oncogenic potential (types 16, 18, etc.) than of low-risk HPV types (such as 6 and 12) (30, 32).

**E6, E7 and miRNAs**
Infection with high-risk HPV types may result in aberrant expression of cellular miRNAs. A large number of the miRNA-coding genes are downstream targets of the transcription factors p53, E2F, c-Myc, etc., therefore, E6- and E7-induced changes in the levels of these proteins would modulate the expression of the relevant miRNAs. As of now, it is known that the E6 and E7 oncoproteins deregulate the expression of several miRNA clusters, among which prominent is the mir-34 microRNA precursor family. In normal cells, the mir-34 microRNAs target the silent information regulator 1 (SIRT1) gene. This produces an increase in the rate of acetylation of p53 in response to damage, effectively resulting in its activation and expression of its downstream effectors, and, finally, in apoptosis (69, 75). SIRT1 is also known to suppress the transcription activity of some proto-oncogenes, such as c-Jun (31). Apparently, the decline in the level of expression of mir-34a is an early event in the process of transformation of infected cells, as it could be observed not only in overt cervical cancer but also in early-grade precancerous lesions. The reduction of mir-34a expression is attributed specifically to the expression of viral E6 (44, 72). It is believed that miRNAs influence the expression of papillomavirus genes as well by targeting viral RNA transcripts (79), therefore deregulation of miRNA expression is probably an important early-acting pro-carcinogenic mechanism.

By disabling two of the major cell cycle regulators – pRb and p53, the oncoproteins from high-risk types of HPV can indeed cause immortalization of the infected cells, but, as in all cancers, this is no 100% risk that the cell would eventually become malignant. For the latter to happen, additional alterations are needed, among which prominent are: activation of cellular proto-oncogenes; promotion of genomic instability; suppression of DNA repair and telomerase activation.

**E6, E7 and other related cell cycle regulators**
Except for their more or less direct targets – pRb and p53, several lines of evidence suggest that E6 and E7 target other cellular proteins as well, augmenting the oncogenic potential of the integrated viral genome (47, 49). E6 is known to bind to and induce degradation of pro-apoptotic proteins other than p53, such as Bak and procaspase 8 (27, 39, 68), resulting in sustained proliferation of infected cells.

E7 has too been found to cooperate with the major positive regulators of cell proliferation, such as the p21 (ras-type) proteins. Human p21 proteins are coded by three genes, termed H-ras, K-ras, and N-ras, respectively. The product is a 21-kDa protein, which, in cancer cells, may become constitutively activated, promoting cellular proliferation. E7 from HPV-16 has been found to interact with p21, blocking the p21-mediated inhibition of cyclin-dependent kinases such as CDK2 (29, 76). CDK2 is the catalytic partner of cyclin E. In normal dividing cells, cyclin E regulates the G1/S transition, the level of cyclin E protein correlating with how fast the cell passes through the G1 phase. Overexpression of cyclin E is a common finding in high-grade CIN and cervical cancer (29, 66). The E7 oncoprotein of high-risk HPV types acts to upregulate the expression of cyclin E, albeit indirectly, possibly via E7-pRb – mediated relaxation of the control over the E2F-type factors.

**E6, E7 and chromatin structure regulators such as high-mobility group A proteins**
High-mobility group A (HMG) proteins are a family of regulatory factors that play a crucial role in the maintaining and the remodelling of the chromatin architecture. HMG are considered master regulators of gene expression, though their participation in transcription control is seldom direct, rather, they act as chromatin structure modifiers, modulating the binding of transcription factors onto the promoters of their target genes; altering the topology of DNA so that distal enhancers could stimulate the expression of the target genes, or displacing transcriptional repressors from transcription initiation sites (7, 8, 16, 54). The high levels of expression of HMG proteins are typical of the undifferentiated state (embryonic cells, tumour cells) while in normal somatic cells the level of expression is usually very low (15, 26, 36). Up-regulation of the expression of the HMG genes is a common finding in all types of human cancers (26, 61), effectively reverting the chromatin of the cancerous cell to the hyperplastic state typical of undifferentiated cells and rendering the DNA
E6, E7 and genomic instability
Integration of the viral genome usually brings about significant chromosomal instability in the infected cells (23, 52). Genomic instability is an important mechanism in neoplastic growth, allowing the cancer cell to amplify loci containing genes with pro-carcinogenic potential (such as the telomere and telomere loci, allowing the cancer cell to amplify loci containing genes with instability is an important mechanism in neoplastic growth, chromosomal instability in the infected cells (23, 52). Genomic E6, E7 and genomic instability can be accomplished by activation of its transcription by some of the downstream participants of the pRb-E2F pathway (e.g., c-Myc, c-Jun, etc.), transcriptional repression of the latter being already eliminated via the E7-pRb interaction (17, 36, 67, 74); or via rearrangements in the HMGAl genes which abolish their dependence on the repressive action of the relevant tumour suppressor miRNAs (33, 43, 51). It is also believed that HMGAl bound to DNA facilitates the expression of E6 and E7 by generating topological DNA structures that enable the viral enhancer in the LCR to upregulate the transcription of its target genes (3, 8). In any case, upregulation of HMGAl is characteristic of more advanced dysplastic states and overt cervical cancer and is generally seen in moderately differentiated and in poorly differentiated tumours (differentiation grades ≥ G2) (46, 55).

E6, E7 and regulation of telomerase expression
Telomere attrition is a mechanism for control over the number of divisions that a cell may undergo before entering replicative senescence and, ultimately, dying. Telomerase activity is virtually nonexistent in most normal somatic cells, but may be significantly elevated in tumour cells, allowing for unrestricted proliferation. It has been demonstrated that E6 of high-risk HPV types such as type 16 activates the telomerase activity in epithelial cells, presumably early in the transformation process, sometimes even before immortalization occurs (41). The majority of advanced cervical carcinomas exhibit amplification of 3q, which, among others, contains the gene coding for the RNA component of the human telomerase gene (hTERC) (34). Gain of 3q is considered to be a reliable predictor of advancing from CINI/II to CINIII and subsequent cervical cancer (35). The telomerase positive rate is increasing steadily in cervical epithelium with progression from normal tissue through the phases of CIN to overt cervical cancer, reaching a threshold value of 60-70% in CIN II and 95-100% in invasive carcinoma (4, 5), which may serve as a potential marker for identifying patients at high risk of progressing into higher-grade CIN or overt cancer. E6 of HPV-16 increases the transcription of hTERT by binding to its promoter and causing its constitutive activation (70, 71).

Conclusions
Untutored courage is useless in the face of educated bullets.
George S. Patton (1885-1945)
The oncogenic potential of high-risk HPV types is deployed after the viral genome becomes integrated into the host cell genome, breaching the transcriptional repression of the E6 and E7 oncogenes typical of the presence of the virus in episomal form. E6 and E7 oncogenes from high-risk types of HPV promptly target the major tumour-suppressor proteins of the host cell, but they may also interact, albeit not always directly, with the protein products of genes coding for positive regulators of cell proliferation. E6 and E7 oncogenes can also modulate the expression of various miRNAs, ensuring expression of viral proteins and avoidance of p53-associated apoptosis of the transformed cell. High-risk HPVs are also capable of inducing chromosomal instability in the host cells via impaired centrosome duplication and overexpression of chromatin modifier proteins such as HMGAl. The latter also maintain the chromatin of the cell in a hyperplastic state, resulting in ectopic expression of proteins typical of undifferentiated cells and suppressing DNA repair. Finally, the capacity for triggering replicative senescence in the transformed cells by telomere attrition may be irreversibly lost in the transition from intraepithelial dysplasia to invasive carcinoma. Apparently, high-risk HPVs use every trick in the book in order to survive, having developed effective mechanisms to circumvent, evade or disable practically every possible line of defence of the cell against neoplastic transformation, which could account for the disturbingly high incidence of high-grade cervical dysplasia and cervical cancer despite the efforts the modern medicine and healthcare puts into screening programmes, prevention and therapy. Further research is needed in order to devise a strategy for prevention of the adverse outcomes of persistent HPV infections that would be resourceful enough to allow for early detection of signs of viral genome integration and safe and adequate intervention, as well as flexible enough to accommodate the needs of the individual patient.
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