The Roles of High-Risk HPV Oncoproteins E6/E7 ~~roles and functions~~ in Cervical Cancer.

Abstract:

Human Papillomavirus (HPV) is the most common cause of cervical cancer in the world. Constitutive expression of oncoproteins by ~~the~~ high-risk human papillomavirus, namely ~~the~~ HPV 16 E6 and E7 oncoproteins are important for malignant transformation in infected keratinocytes. In particular, E6 and E7 bind and inactivate ~~the~~ host cellular tumor suppressor proteins, p53, and pRB, respectively. These events subsequently delay ~~the~~ hosts' cellular differentiation and further induce proliferation in suprabasal keratinocytes, thus enabling HPV replication. One member of the pocket protein family, p130 appears to be an important target for E7 in promoting S-phase entry. Herein, we review ~~the~~ studies ~~of~~ on HPV 16/18 early proteins associated with high risk and the mechanism by which they abrogate the host cell cycle.

Keywords: human papillomavirus type 16, E6, E7, cell cycle

Introduction: Human papillomaviruses

Human papillomaviruses (HPVs) belong to the *Papovaviridae* family; they are double-stranded DNA, non-enveloped viruses that have been strongly implicated in the development of lesions ranging from skin or genital warts to cancer (Graham, 2010). Approximately 291 million women are infected by genital HPV types worldwide. Nearly 500,000 new cases of cervical cancer are diagnosed and over 270,000 women die from this disease around the world (Prati *et al*., 2018). The 8-kbp HPV DNA molecule contains ~~the~~ early and late genes clustered in separate regions of the HPV genome (WHO, 2007). Early genes (E1, E2, E4, E5, E6 and E7) code for regulatory proteins involved in viral DNA replication, transcription control and cellular transformation contributing to the transformation and immortalization potential of HPV, whereas late genes encode the major viral capsid protein (L1) and a minor capsid protein (L2) (Yeo-The., 2018).

HPVs can be divided into two subclasses (high-risk and low-risk) based on their oncogenicity. The high-risk (HR) types include HPV16 or 18; they are associated with 80% to 95% of the world's cases of cervical cancer (V.Bouvard, 2009; Mausumi Bharadwaj, 2018), while HPV6 and 11 are classified as benign wart-causing, low risk (LR) types with very limited tendency to malignant progression (Stanley, 2010). It appears that both high-risk and low-risk HPV-encoded E6 and E7 proteins show some ability to disrupt the normal control of cell proliferation, however, only ~~the E6 and E7~~ those encoded by high-risk types are able to contribute to cell transformation. It is well-established that cell division follows a series of complex events that constitute the cell cycle. Actively dividing cells ~~pass~~ progress through distinct stages of DNA synthesis (S-phase) and mitotic division (M-phase), and these activities are separated by gap phases (G1 and G2). The main control of the cell cycle involves a family of kinases, known as cyclin-dependent kinases (CDKs) which control the passage through both S and M phases (Tomaic, 2016).

HPV gene expression is polycistronic, initiating from multiple promoters (Graham, 2010). HPV DNA replication, which is required for the establishment and preservation of the viral episome, is achieved through association with the host DNA replication machinery. In many HPV-induced carcinomas, the episomal HPV DNA genome is integrated into the host cell DNA in a manner that disrupts the viral E2 gene, which normally acts to repress E6 and E7 expression, while leaving the viral E6 and E7 genes intact (Kennedy., 2014). While the expression of HPV E6 and E7 is not sufficient to induce cell transformation, ample evidence indicates that the continued expression of E6 and E7 is required for the continued viability and growth of HPV-transformed cancer cells. This requirement reflects the ability of E6 and E7 to serve as potent inhibitors of cellular tumour suppressor pathways. E6 induces degradation of the cellular tumour suppressor p53, thus preventing the activation of downstream effectors that induce cell cycle arrest and apoptosis (Chen, 2016). On the other hand, E7 functions to destabilize the retinoblastoma (Rb) protein, thus inhibits the formation of the Rb/E2F complex, leading to the disruption of cell cycle control and inactivates its tumour suppressor pathway.

HPV 16 and HPV18

HPV16 and HPV18 are the most prominent strains of high-risk (HR) type HPV, often associated with additional infections such as lichen sclerosis. ~~HPV16 and HPV18 the most prevalent types in cervical cancer (Meng *et al*., 2019) are~~ Not only are they both strongly linked to cervical cancer, they are also associated with vaginal, vulval, anal and penile cancers, and benign genital tumors (Otter *et al*., 2019). HPV16 is a small non-enveloped virus containing double-stranded DNA as its genetic material. Its genome consists of seven functional coding regions: (1) E1/E2 encode proteins that control the function of E6 and E7 genes, (ii) E4 encodes a protein whose precise function is largely unknown, but speculated to control virus release from the cell, (iii) E5 encodes

a hydrophobic protein which enhances immortalization of the cell, (iv) E6 encodes a protein that inhibits negative regulators of the cell cycle and further inhibit p53, a transcription factor for apoptosis, (v) E7 encodes for a viral protein that binds to retinoblastoma tumor suppressor proteins thereby permitting the cell to progress through the cell cycle in the absence of normal mitogenic signals, (vi) L1/L2 encode for structural proteins for late viral function and formation of complete virus particles, and (viii) LCR (long control region) lies between E and L genes and is necessary for normal virus replication and control of viral gene expression (Figure 1).

Replication of HPV16 is initiated with the replication of viral DNA independent of the host cellular chromosomes. ~~In this~~ During the process of cell division, one daughter cell moves apart from the basal lamina to undergo differentiation. Infection in the basal lamina can persist for years due to the restriction of virion production to differentiated cells only. E_1 and E_2 proteins are supposed to be the recognition factors and regulators for early viral transcription. The icosahedral capsid for virion generation is formed by L_1 and L_2 (Ryu, 2017). The three major HPV oncoproteins which have gained importance in ~~relation to~~ the development of cervical cancer are E_5, E_6, and E_7. These proteins are associated with cervical cancer studied both *in vitro* and *in vivo* (Narisawa-Saito & Kiyono, 2007). The E_6 and E_7 proteins are important for the inhibition of tumor suppressor genes, namely p53 and pRb (Tsai & Chen, 2003). As far as E_5 is concerned, it is known to possess weak oncogenic properties which result in increased activity of epidermal growth factor receptor (EGFR), thereby inhibiting the major histocompatibility complex (MHC) expression (Tsai & Chen, 2003). The most crucial step in initiating carcinogenesis is the integration of viral RNA into the host genome.

Cervical cancer can also be defined as cancer that originates at the opening of the womb which progressively migrates ~~to~~ throughout the cervix (Müller-Schiffmann *et al.*, 2006). HPV16 is known

to cause cervical cancer and accounts for the majority of cervical carcinomas and lesions (Herdman *et al.*, 2006; Müller-Schiffmann *et al.*, 2006). The two proteins ~~which play a~~ significant ~~role~~ in the onset of its malignancy are E_6 and E_7 proteins and their expressions have been observed in cell lines (Herdman *et al.*, 2006). IgG and IgA antibodies are associated with HPV16 and are found in cervical intraepithelial neoplasia (CIN) at a ratio of about 50-75%. Very few antibodies are detected in patients who have a precancerous cervical lesion.

E6 and E7 oncoproteins

HPV E6 is 150 amino acids residues ~~and its~~ with an approximate size ~~is~~ of ~18 kDa. The E6 protein consists of two N- and C-terminal zinc-binding domains E6N and E6C (Nomine *et al.*,2003). HR HPV E6 binds to ~~the~~ E6-associated protein (E6-AP), a cellular ubiquitin-ligase, and targets p53 for degradation (Martinez-Zapien *et al*., 2016). E6 also activates expression of hTERT, the catalytic subunit of telomerase: Activation of cellular telomerase (synthetic of telomerase) result~~sing~~ in maintenance of telomere length. Telomerase is an enzyme that is responsible for extending telomeric ends with the addition of hexamer repeats. Other than redirecting E6AP substrate specificity to ubiquitinate p53, the E6/E6AP complex also ubiquitinates other cellular proteins, whose ~~the~~ degradation ~~of which~~ contributes to E6-induced cellular immortalization or transformation (ref). NFX1-91 transcriptionally represses telomerase reverse transcriptase (TERT) expression (Gewin *et al.*, 2004). E6:E6-AP complex 16 has also been reported to target E6TP1 (E6 targeting protein 1), MCM7 (minichromosome maintenance 7) and Bak (Bcl-2 homologous antagonist/killer) for degradation (Munger *et al.*, 2004; Wise-Draper & Wells, 2008). Bak is a pro-apoptotic protein that is proteasomally degraded by HPV E6 (M. Thomas & Banks, 1999). MCM7 plays a role in ensuring that DNA replication occurs only once per cycle. Therefore, the interaction

of HPV E6 with MCM7 may result in over-duplication of chromosomes, contributing to genomic instability (Kukimoto *et al.*, 1998).

The E6:E6-AP complex also targets NFX1-91 for degradation, enhances telomerase activity and increases cellular life-span (Gewin *et al.*, 2004). ~~Besides, an~~ Apart from the activation of the NFκB pathway through NFX1-91 mechanism, there is evidence that NFX1-91 can function as a dual regulator, not only a transcriptional repressor, but also a transcriptional activator, when bound to DNA (Xu *et al*., 2010).

~~The~~ High-risk E6 proteins also differ from those encoded by ~~the~~ low-risk HPV types in having a C-terminal PDZ-binding domain. High-risk E6 proteins bind and stimulate the degradation of several cellular targets that contain PDZ motifs, such as hDlg (human homologue of Dlg) and hSrib (human homologue of the Drosophila scribble protein), which are thought to be involved in the regulation of cell growth and attachment (Zeithlet *et al*., 2014)

HR HPV E6 also binds directly to PDZ [PSD-95 (post-synaptic density-95 protein), Dlg (*Drosophila* discs-large protein), and ZO1 (zonula occludens 1 protein)] domain-containing proteins via its C-terminal domain which is highly conserved amongst HR HPV types (Glaunsinger *et al.*, 2000; Nakagawa & Huibregtse, 2000; M. Thomas *et al.*, 2001) . Low-risk (LR) HPV does not possess the PDZ binding motif (Pim *et al.*, 2000). PDZ proteins are necessary for cell-cell adhesion and are implicated in cell signaling (van Ham & Hendriks, 2003). Binding of PDZ by HPV E6 is an important feature in progression to carcinogenesis.

HPV E6 mutants that can no longer bind to PDZ ~~are~~ have been shown to be (i) deficient in E6-induced transformation in rodent cells, and (ii) display reduc~~edion of~~ tumor development in transgenic mouse models (James *et al.*, 2006; Kiyono *et al.*, 1997). There are other proteins that

have been discovered to interact with HPV E6. Paxillin has been reported to bind to HR HPV E6, while both LR and HR HPV E6 bind to p300, MCM7, and Bak. HR HPV E6 has a higher affinity for p300, MCM7 and Bak (Kukimoto *et al.*, 1998; Patel *et al.*, 1999; M. Thomas & Banks, 1999; M. C. Thomas & Chiang, 2005; Tong & Howley, 1997; Zimmermann *et al.*, 2000). p300 functions as a transcriptional coactivator and a histone acetyltransferase (Iyer *et al.*, 2004). Binding of p300 by HPV E6 prevents acetylation of p53 at p300-dependent sites, thus down-modulating p53-mediated expression (Zimmermann *et al.*, 2000)

E7 is a small nucleoprotein, ~100 amino acids residues in size. It can be divided into three regions: conserved region 1 (CR1, amino acids 2-15), CR2 (amino acids 16-38), and CR3 the C-terminal zinc-binding region (amino acids 39-98) containing two Cys-X-X-Cys motifs. CR1 and CR2 are conserved with adenovirus E1A and SV40 large T antigen. The zinc-binding C-terminal domain of E7 oncoprotein is proposed to be involved in homodimerization comprised of a unique β1β2α1β3α2 fold (Gage *et al.*, 1990; Jewers *et al.*, 1992; Munger *et al.*, 2004; Munger *et al.*, 2001) . Both HR and LR HPV E7 proteins bind to pRb family members through their LXCXE binding motif (N. Dyson *et al.*, 1989).

It can bind to zinc and is phosphorylated by casein kinase II. It can also bind to cellular regulatory protein product of retinoblastoma tumour suppressor gene (pRb) and related proteins p107 and p130. E7 bind preferentially to the hypo (de-) phosphorylated form of pRb resulting in its functional inactivation via the release of E2F transcription factor. This permits the cell to enter into the S phase of the cell cycle resulting in cell proliferation. It also interacts with cyclin dependent kinase inhibitors (CDKI) like p21 and p27 which helps the cell in overcoming TGF-β associated growth arrest. (Zhu *et al.*, 1995)

HR HPV destabilizes all pRb family members and this is a critical event that drives cellular transformation (Berezutskaya *et al.*, 1997; Boyer *et al.*, 1996; Davies *et al.*, 1993; Gonzalez *et al.*, 2001; Halbert *et al.*, 1991; Helt & Galloway, 2001). The main contributing factor that results in enhanced binding of HR HPV E7 to pRb and its ability to target pRb for degradation is an aspartic acid versus glycine residue ~~in~~ HPV 18 vs.

The molecular basis for the transformation ability of HR HPV E7 has been mapped to the amino-terminal half of the E7 protein (Heck *et al.*, 1992; Phelps *et al.*, 1992). The amino-terminal halves of HR HPV E7 proteins contain consensus recognition sequences for casein kinase II (CK II;(Barbosa *et al.*, 1990; Firzlaff *et al.*, 1989; Massimi & Banks, 2000). There are two serine residues in E7 that are specifically phosphorylated by CKII. When these sites are mutated, the transforming ability of HPV E7 decreases (Barbosa *et al.*, 1990).

E7 motif Pocket protein

pRb together with p107 and p130 make up the Rb family ~~of~~ tumor suppressor of pocket proteins. The three members share a well conserved small pocket region, which consists of A and B domains that are separated by a flexible spacer region (Classon & Dyson, 2001). These ~~A and B~~ domains each represent a single cyclin fold domain (Lee *et al*., 1998) and interact such that the small pocket is self-sufficient to form a transcriptional repressor on its own. The small pocket is the minimal fragment of pRB that is capable of interacting with viral oncoproteins, such as E1A and TAg (Hu *et al*., 1990). In addition to the viral proteins, a number of cellular proteins are reported to contain an LxCxE-like motif that allows them to interact with pRB, p107 and p130 (Dick, 2007). The

LxCxE domain is composed ~~with~~ of highly conserved amino acid residues where the X indicates any amino acids while L is leucine, C is cycsteine and E is glutamate.

The LXCXE motif and C-terminal domain which is present in HR HPV E7 protein synergistically form a high affinity bivalent interaction lead both anchors the E7 protein to pRB and dissociates the associated E2F transcription factor (Munger *et al*., 1989)**.** The LXCXE binding site is not required for the interaction of the pocket protein with E2F; mutations affecting LXCXE domain prevent Rb binding to ~~the~~ cellular proteins such as HDAC1 and 2 whereas Rb interaction with E2F persists intact (Dahiya *et al*. 2000)**.** Insertions in the B domain of their small pockets contain p107 and p130 proteins. Interestingly, there are some features that are present in p107 and p130 and not in pRB**.** In the case of p130, this insert is subject to regulatory phosphorylation to maintain protein stability (Litovchick *et al*., 2004). Furthermore, p107 and p130 contain longer spacer regions than pRB, and their spacers allow them to interact stably with ~~cyclin dependent kinase~~ CDK complexes (Lacy &Whyte, 1997). Lastly, p107 and p130 contain an N-terminal region that serves to inhibit ~~cyclin dependent kinases~~ CDKs (Woo *et al*., 1997).

HR HPV E7 proteins have a number of cellular binding partners other than the pocket proteins. HR HPV E7 has been reported to interact with cyclin A/cyclin dependent kinase (Cdk) 2, cyclin E/Cdk2, P300/CBP-associated factor (PCAF), TATA box-binding protein (TBP), histone deacetylases (HDAC), E2F transcription factor 1 (E2F1), cyclin-dependent kinase-interacting protein 1 ($p21^{CIP1}$) and cyclin-dependent kinase inhibitor 1B ($p27^{KIP1}$) via its C-terminus (Dell & Gaston, 2001; Munger *et al.*, 2004; Wise-Draper & Wells, 2008). Retinoblastoma protein (pRb) is a substrate of both cyclin A/Cdk2 and cyclin E/Cdk2 complexes. The interactions of HPV E7 with these complexes ultimately result in the reduction of Rb-associated transcriptional repression (McIntyre *et al.*, 1996; Tommasino *et al.*, 1993). For instance, binding of HPV E7 to HDAC is

indirect and is mediated by Mi2β (Brehm *et al.*, 1999). In addition, binding of E7 to $p21^{CIP1}$ and $p27^{KIP1}$ perturbs cell cycle inhibition (Funk *et al.*, 1997; Zerfass-Thome *et al.*, 1996). In differentiating cells, E7 binding to HDAC contributes to enhanced E2F-mediated transcription and increased proliferation (Hebner & Laimins, 2006)

Cell cycle

Cell cycle control is regulated by the activity of cyclin-dependent kinases (CDKs). The activity of CDKs (CDK1, CDK2, CDK4, CDK6) is regulated by the abundance of their activating partner cyclins (cyclins A, B, D, and E, respectively), phosphorylation by various kinases and interaction with CDK inhibitory proteins (CDKIs; (Beijersbergen & Bernards, 1996; Sherr, 1995); Figure 1.5). Two classes of mammalian cyclin-dependent kinase inhibitors have been described: the CIP/KIP family, comprised of p21, p27 and p57, and the INK4 family, comprised of p15, p16, p18 and p19 (Sherr & Roberts, 1995). Generally, CDKs, cyclins and CDK inhibitors function within several pathways, including the $p16^{INK4A}$, cyclin D1- CDK4/6-pRb-E2F and $p21^{WAFI}$-$p27^{KIPI-}$cyclin E-CDK2 pathways (Kim & Zhao, 2005). The INK4 molecules specifically inhibit cyclin D complexes by interaction with CDK4 and CDK6 components. The KIP family affects cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by binding to both the cyclin and CDK subunits. Alteration in CDKs, CDKIs, and cyclins can lead to uncontrolled proliferation and may contribute to malignancy of the uterine cervix (Sherr & Roberts, 1995).

In many cancers and cancer cell lines, the mechanisms that control growth and differentiation are disrupted or at least impaired (Sherr, 1996) In a dividing cell, mitogenic stimulation leads to synthesis and assembly of cyclin D/CDK4 complexes, which contribute to the phosphorylation and consequent inactivation of pRB, increased expression of cyclin E and sequestration of CDK2 inhibitors of the CIP/KIP family. Cyclin E/CDK2 continues to inactivate pRB and also phosphorylate substrates important for DNA synthesis and S phase entry. Cyclin A-CDK2 is assembled during S phase and remains active throughout the G2 phase (Sherr, 1993; Sherr & Roberts, 1999)

HPV16 E7 (16E7) affects these cyclin-CDK complexes not only by deregulating the transcriptional levels of cyclin E due to loss of E2F-mediated repression; but also by affecting the post-transcriptional levels of cyclin E (Botz *et al.*, 1996; Martin *et al.*, 1998; Zerfass *et al.*, 1995) . HPV16 E7 protein has been shown to bind to cyclinA-CDH\K2 in a cell cycle-dependent manner, with maximal binding in the S and G2 phases (Tommasino *et al.*, 1993). Cyclin A-CDK2 interacts with and phosphorylates E2F, leading to loss of its DNA binding capability. Hence, the interaction of HPV16 E7 with cyclin A/CDK2 prevents the inactivation of E2F and thereby permits the cell to bypass normal checkpoints with consequent loss of DNA replication fidelity, thus explaining the increased number of chromosomal abnormalities (A. E. White *et al.*, 1994). The key targets of these viral oncoproteins are the products of two tumour suppressor genes, *pRB,* and *p53*.

i. HPV replication

HPV replication is completely dependent on the host replication machinery. Viral infection occurs through a disturbed epithelial barrier, such as a wounding site. Infection of these actively proliferating cells results in a delay of differentiation and

subsequently, expansion of the infected clone, due to the expression of the E6 and E7 proteins. The early proteins E1 and E2 play an important role in the initiation and regulation of viral DNA synthesis and gene expression (Reinson et al., 2015). E1 is the viral DNA helicase and E2 is a transcription and segregation factor. The E1 replication mechanism depends on the same cellular proteins that are used for host DNA replication during the S phase (Reinsson et al., 2015). The role of E4 and E5 in the HPV life cycle is not fully understood yet. It has been shown that E4 protein associates with and disrupts the cytoplasmic keratin network (Doorbar et al., 1991) whereas E5 of the HPV 16 are able to induce cellular transformation (Maufort et al., 2010). However, E6 and E7 have been well-studied and clearly shown to be important contributors to cervical cancer.

ii. E6 binds to p53 in cell cycle

HPV16 E6 proteins are confined to the nuclear matrix and non-nuclear membrane fraction. Their major function is in zinc binding. The E6 protein favours abnormal cell growth by the process of binding and rapidly targeting the p53 protein (Müller-Schiffmann *et al.*, 2006). In HPV-infected cells, the p53 protein degrades by the ubiquitin pathway and the cell is allowed to replicate without the p53-associated negative growth control, including Mdm-2proto-oncogenes (Lace *et al.*, 2008). The E6-E6 AP complex specifically target ubiquitin-targeted substrates that cause p53 disruption and p53 mediated apoptosis (Figure 2) (Incassati *et al.*, 2006)

iii. E7 binds to a pocket protein family

Mutational analysis revealed that E7 protein plays a major role in transformation. This protein binds to the B pocket of the pRb of the host. pRb is a tumour suppressor gene, initially discovered as the main mutant allele of Rb present in the germline of an individual which is linked to a predisposition of the retinal tumour (Zhang *et al.*, 2006). The pRb plays a role in growth control by regulating cell cycle at G0/G1 via inhibition of various transcription factors, namely the E2F1 and cMyc proteins. Both cMyc and E2F1 proteins activate some of the essential genes required for DNA synthesis. This role played by pRb ensures the reduction of essential transcriptions factors that are related to normal control and cell division (N. J. Dyson, 2016). HR HPV E7 protein has a high binding affinity for pRb protein (Darnell *et al.*, 2007). Binding of E7 to pRb (Figure 2) causes inactivation of the latter, interferes with the cell cycle control mechanism, hence contributing to the development of malignancy.

More striking evidence reported by several studies is that E7 possesses and has the capability of overcoming the repair mechanism and G1 arrest of p53 dependant DNA damage. In precancerous lesions, the loss of cell cycle control favours and allows DNA-damaged cells to enter normal cell division, initiating cancerous or tumorigenic mutations. Evidence of mutations is well supported by a recent study which showed that deregulated expression of E2F in rat fibroblasts leads to S phase entry in the cell cycle and apoptosis in p53 (Smith *et al.*, 2007). Consequently, it can be summarized that the interference of DNA damage response by HR HPV oncogenes most probably occurs at two points: firstly, the E6 protein targets the p53 protein for degradation, thus

inhibiting transcriptional activation of kinase inhibitors; secondly, expression of E7 proteins can result in the constitutive inactivation of pRb and hence the deregulation of E2F1 transcription factor (Smith *et al.*, 2007).

E6/E7 role in malignancy progression and related therapeutic strategies

HPV E6 promotes p53 degradation and activates telomerase, but the multifaceted oncoprotein has numerous other functions which can also inhibit p53 activation through both degradation-independent and degradation-dependent (Wallace & Galloway, 2015). E6 preferentially targets MAGI-1, a major degradation target of both HPV-16 and HPV-18 within the nucleus at membrane sites. Its binding causes a loss of tight-junction integrity, as determined by mislocalization of the tight-junction protein ZO-1MAGI-1. (Kranjec & Banks, 2011). We demonstrate a striking increase in the number of potential PDZ targets bound by each E6 PBM as cancer-causing potential increases, and show that the HPV-16 and HPV-18 PBMs have the most flexibility in their PDZ target selection. Furthermore, the specific interaction with hScrib correlates directly with increased oncogenic potential. In contrast, hDlg is bound equally well by all the HPV E6 PBMs analysed, indicating that this is an evolutionarily conserved interaction, and was most likely one of the original E6 PBM target proteins that was important for the occupation of a potential new niche. Finally, we present evidence that the cell junction components ZO-2 and β-2 syntrophin are novel PDZ domain–containing targets of a subset of high-risk HPV types (Miranda Thomas *et al.*, 2016). ZFN16-E7-S2 and ZFN18-E7-S2 could effectively induce disruption of *E7* oncogenes and lead to type-specific and efficient growth inhibition and apoptosis of HPV-positive cells also repressed xenograft formation *in vivo* could be used as novel therapeutic agents

311 for the treatment of HPV-related cervical cancer (Ma *et al.*, 2019). HPV E7 interaction with
312 retinoblastoma family proteins alone ~~don't~~ do not account for high risk E7-specific cellular
313 immortalization and transformation activities. High risk genus alpha HPV E7 is known to interact
314 with the cellular non-receptor protein tyrosine phosphatase PTPN14 and degrade it via
315 proteasome-mediated degradation using ubiquitin ligase UBR4. This explains retinoblastoma
316 independent transforming activity of high risk HPV E7 (E. A. White *et al.*, 2016).

317 High risk HPV E6 and E7 utilise Ub-proteasome system (UPS) through the use of E1,E2
318 and E3 ubiquitin (Ub) ligases and deubiquitinases (DUBs) (Poirson *et al.*, 2017). The expression
319 of HPV 16 E6 and E7 either alone or together promotes keratinocyte proliferation at high cell
320 densities, through the combined inactivation of p53 and Notch 1. These are another measure of
321 these oncoproteins of stopping differentiation and promoting proliferation by targeting key gene
322 drivers thus promoting persistence of infected keratinocytes in the basal and parabasal layers
323 (Kranjec *et al.*, 2017). HPV-16 mediated downregulation of Host miRNA (Hsa-miR-139-3)
324 promotes oncogenesis in head and neck cancer (HNC) and cervical cancer (Sannigrahi *et al.*,
325 2017).

326 Lead in/intro to inhibitors?

327 Identification of thiadiazolidinedione compounds that bind to pRb with mid-high
328 nanomolar dissociation constants, are competitive with the binding of viral oncoproteins
329 containing an LxCxE motif, and are selectively cytotoxic in HPV-positive cells alone and in mice.
330 These inhibitors provide a promising scaffold for the development of therapies to treat HPV-
331 mediated pathologies (Fera *et al.*, 2012). Anti-cervical cancer potential of selected p300 inhibitor
332 C646 was demonstrated and a novel mechanism which was proposed in this study accompanied
333 by the accumulation of p53 protein by transcription of HPV E6-E7 lead to cell proliferation was

suppressed, glucose metabolism was disrupted and apoptosis was induced via the intrinsic pathway (He *et al.*, 2017)**.** Dual sgRNA-guided CRISPR is a highly specific method for reversing the malignant phenotype of cervical cancer cells in deletion of the E6 and E7 genes provides us a new therapy to treat HPV18 infection (Yu *et al.*, 2017)**.** In silico pipeline to identify small-molecule inhibitors of the E6-E6AP interaction preventing the formation of the E6-E6AP complex is one of the main strategies to inhibit the viability and proliferation of infected cells. the top-three compounds were selected, and their stability in the E6 docked complex and their effect in the inhibition of the E6-E6AP interaction was corroborated by molecular dynamics simulation which represent a new starting point in the development of anti-HPV drugs (Ricci-López *et al.*, 2019)**.** Inhibitions of targeting the viral oncogenes E6/E7 can be an important therapeutic strategy to treat HPV High risk cancer cause with high specificity. Targeting the CD71(+) subpopulation in cervical cancer cells with siRNAs or CRISPR/Cas9 may provide new insights for the development of novel therapeutic approaches for treating cervical cancer (Leung *et al.*, 2019)**.** (CRISPR/Cas system), a widely used genome editing tool in many organisms, to target HPV16-E7 DNA in HPV positive cell lines, we showed for the first time that the HPV16-E7 single-guide RNA (sgRNA) guided CRISPR/Cas system could disrupt HPV16-E7 DNA at specific sites, inducing apoptosis and growth inhibition in HPV positive SiHa and Caski cells, but not in HPV negative C33A and HEK293 cells. disruption of E7 DNA directly leads to downregulation of E7 protein and upregulation of tumor suppressor protein pRb. Therefore, the results suggest that HPV16-E7 gRNA guided CRISPR/Cas system might be used as a therapeutic strategy for the treatment of cervical cancer (Hu *et al.*, 2014)**.** When immunotherapy was combined with immune checkpoint blockade, an increased level of anti-tumor activity against large tumors was observed. When Ad5 [E1-, E2b-]-E6/E7 immunotherapy was combined with anti-PD-1 antibody, we observed

357 $CD8^+$ TILs at the same level but a reduction in tumor PD-L1 expression on tumor cells and reduced
358 PD-1^+ TILs providing a mechanism by which combination therapy favors a tumor clearance state
359 and a rationale for pairing antigen-specific vaccines with checkpoint inhibitors in future clinical
360 trials (Rice *et al.*, 2015). Tanshinone IIA (Tan IIA) possess as inhibitor proliferation against human
361 cervical cancer in-vivo and in-vitro study. Tan IIA was found to (i) downregulate expression of
362 HPV E6 and E7 genes and modulate associated proteins E6AP and E2F1, (ii) cause S phase cell
363 cycle arrest, (iii) induce accumulation of p53 and alter expression of p53-dependent targets, (iv)
364 modulate pRb and related proteins, and (v) cause p53-mediated apoptosis by moderating Bcl2,
365 Bax, caspase-3, and PARP cleavage expressions. As an agreement with the in-vivo study
366 (Munagala *et al.*, 2015). HPV-16 E6/E7 expressing tissues are highly susceptible to cancer
367 progression upon carcinogenic exposure, which can be prevented by mTOR inhibition with
368 rapamycin. These finding support that mTOR inhibition may represent a promising therapeutic
369 option to prevent HPV-associated human malignancies in high risk patient populations, as well as
370 cancer recurrence and appearance of second primaries in prior treated HPV+ SCC patients
371 (Callejas-Valera *et al.*, 2016). Alpha-linolenic acid (ALA), an omega-3 fatty acid has demonstrated
372 anticancer activity by regulating the growth of cervical cancer cells. This is achieved by reducing
373 cell migration, reducing expression of phosphorylated p38, pERK1/2, c-JUN, NFkB, and COX2
374 proteins. In addition, it also reduces expression of onco-proteins E6 and E7 and restore p53 and
375 pRB (Deshpande *et al.*, 2016). Transduction of HPV16 oncogene E6/E7 into mouse embryonic
376 fibroblasts (MEFs) up-regulated O-GIcNAc transferase (OGT) MRNA and protein, elevated level
377 of O-linked GIcNAcylation (O-GIcNAc) This proves that by targeting aberrant O-GIcNAc,
378 effective therapeutics can be developed against anogenital cancers (Zeng *et al.*, 2016). The newly
379 developed cyclin dependent kinase 9 (CDK9) inhibitor FIT-039 can work against cervical

neoplasma induced by HPV infection. This is achieved by inhibiting expression of E6 &E7 and restoring tumor suppressors p53 and pRB in HPV+ cervical cancer. FIT-039 also passed the toxicity test and showed potent anti HPV-activity (Ajiro *et al.*, 2018). Another emerging therapeutic approach is use of loaded polyion complex micelles that acts as delivery system of siRNA treatment (si16E6/E7 and si18E6/E7 to target SiHa and HeLa tumors and supress the cancer progression (Nishida *et al.*, 2016)**.**

Delays in differentiation

The life cycle of the HPV virus is closely linked to keratinocyte differentiation. HPVs initially infect proliferating basal cells of the squamous epithelium while virus production is associated with the terminally-differentiated layer. This activity of HPV16 E7 was shown to delay the induction of keratinocyte differentiation markers involucrin (IVL) and keratin 10 (Jones *et al.*, 1997). p130, in co-operation with p107 and pRb, appears to be involved mostly in terminal differentiation processes (induction of involucrin and probably at later stages). Predominant activity of p130 during G0 phase(Cobrinik *et al.*, 1993) suggest a progressive transition from G1 to G0 as in vivo keratinocytes withdraw from the cell cycle and move towards terminal differentiation (Paramio *et al.*, 1998).

High-risk and low-risk HPV E7s decrease p130 stability and they both also share the capability to decrease or delay the differentiation. These are important for the productive stage of the HPV life cycle because the virus must either delay differentiation or induce differentiated cells to create an environment supportive of the amplification of viral DNA (Zhang *et al.*, 2006). The cellular DNA replication machinery is reactivated by the E7 oncogene in differentiating keratinocyte to provide a cellular environment that is permissive for the replication of the viral genome (Longworth &

Laimins, 2004). This occurs by perturbing the keratinocyte differentiation program and inducing the host DNA replication machinery (Flores *et al.*, 2000). It was found that the ability of HPV16 to reprogram suprabasal cells to support DNA synthesis correlates with the ability of E7 to bind pocket proteins but not its ability to induce their degradation. In contrast, the ability of HPV16 to perturb differentiation correlated with both E7 binding to and degradation of pocket proteins (Collins *et al.*, 2005)

E7-expressing keratinocytes exhibited delayed cellular differentiation and elevated cdk2 kinase activity despite high levels of $p21^{Cip1}$ and association of $p21^{Cip1}$ with cdk2. This shows that the HPV E7 protein can interact with $p21^{Cip1}$ and abrogate $p21^{Cip1}$-mediated inhibition of cyclin A and E-associated kinase activities, thus explaining the ability of E7 to allow for cellular DNA synthesis in differentiated keratinocytes (Jones *et al.*, 1997). The downregulation of endogenous IVL expression by HPV16 E7 may not be caused by a direct and specific effect of E7 on the IVL promoter compared with HPV16 E6 (Gyongyosi *et al.*, 2012). Recent work on Tyrosine-protein phosphatase non-receptor type 14 (PTPN14) and HR HPV E7 led to the discovery that the loss of PTPN14 from human keratinocytes resulted in a prominent downregulation of epithelial differentiation program (Hatterschide *et al*., 2019). PTPN14 is targeted independent of RB1 and shows early promise as a regulator of keratinocyte differentiation. However, the mechanism by which PTPN14 degradation can limit epithelial differentiation is yet to be understood.

Conclusion

The studies of HPV E6 and E7 oncoproteins continue to display potential future usage in the management of cervical cancer. Subsequent scientific investigations will probably yield new diagnostic and prognostic tools for cervical cancer, provide insights into its underlying biology and contribute to the development of novel management strategies. This review identifies the key technologies, understanding the molecular mechanisms for HPV E6/E7 and development of new therapeutic strategies against cervical cancer.

Conflict of Interests

The authors have no conflict of interest.

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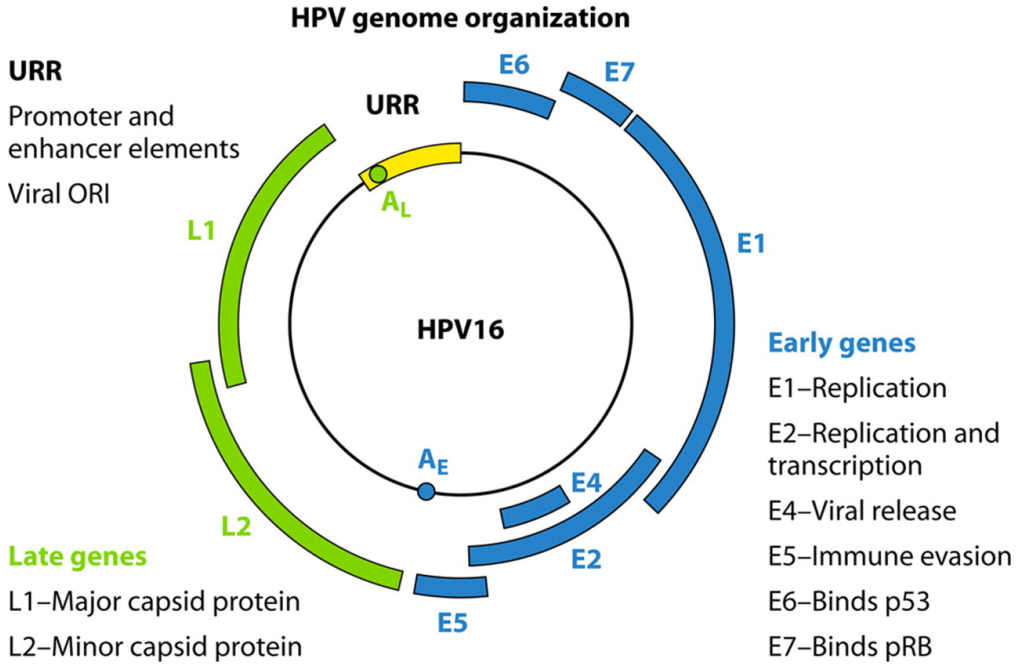
HPV genome organization
URR
Promoter and
enhancer elements
Viral ORI
URR
E6
E7
E1
L1
A_L
HPV16
A_E
E4
L2
E2
E5
Late genes
L1–Major capsid protein
L2–Minor capsid protein
Early genes
E1–Replication
E2–Replication and
transcription
E4–Viral release
E5–Immune evasion
E6–Binds p53
E7–Binds pRB

Figure 1. Schematic presentation of the HPV 16 genome.

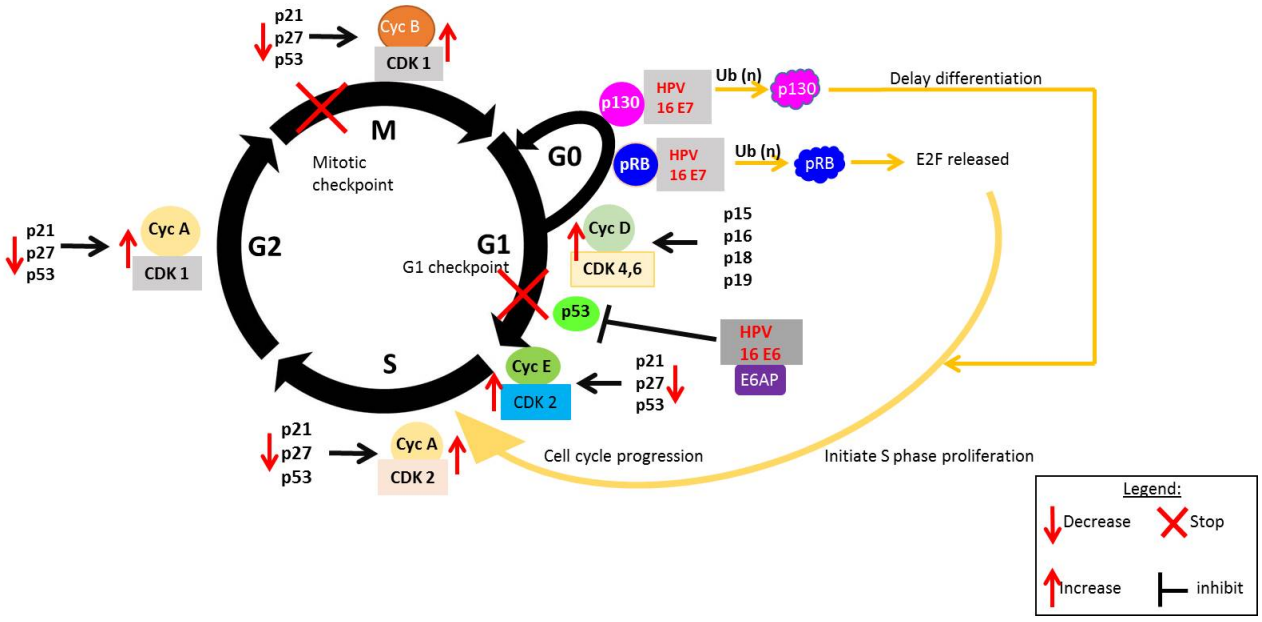

Figure 2: **Regulation of cell cycle by HPV 16**. Oncogenic high risk HPV E6 binds to p53 while E7 binds to pRB and p130. These interactions alter the host cell cycle causing uncontrolled mechanism of cyclin dependent kinases (CDKs) and their regulatory cyclins (A, B, D and E).

HPV E6 and E7 oncoproteins abrogate cell cycle checkpoints at the G1/S and G2/M boundaries. E6 binds and degrades p53, inducing apoptosis and liberate G1 arrest. E7 releases E2F transcription factor by binding to pRB, causing acceleration into S-phase. Cell differentiation is delayed due to binding of E7 to p130. This ultimately promotes cell cycle progression thus leading to cells transformation.