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# Molecular Bases of Human Papillomavirus Pathogenesis in the Development of Cervical Cancer

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## 1. Introduction

Human papillomavirus (HPV) is a DNA virus which belongs to the newly named *Papillomaviridae* family (de Villiers et al., 2004). The papillomaviruses induce a variety of proliferative lesions in the skin and internal mucosa. Nearly half of the over 200 types of HPVs can infect the genital mucosa, producing benign epithelial lesions. However, a subset of these viruses is found in over 90% of malignant carcinomas of the genital tract, the majority of which contain the HPV16 and 18 types considered to be of "High Risk" (HR), as they help the progression into cervical cancer (Lorincz et al., 1992; Shah & Buscema, 1988).

The mechanisms through which HPVs induce cell transformation have been intensively investigated in the last few years. The most abundant viral transcripts in tumor and tumor cell lines are produced from the *E6* and *E7* Open Reading Frames (ORFs) that are known to be oncogenic. These two genes from HPV are necessary and sufficient to induce HPV-mediated transformation of murine cells (Yasumoto et al., 1986), transform human fibroblasts (Pirisi et al., 1987), and in cooperation with *ras* are capable of transforming baby rat kidney cells (Phelps et al., 1988) and primary human keratinocytes (Yoshida et al., 2008). The best evidence for the role of *E6* and *E7* proteins in malignancy comes from biochemical studies. *E6* and *E7* oncoproteins from the HR-HPV types have the ability to alter pathways involved in cell cycle control, interacting with and neutralizing the regulatory function of primarily two suppressor proteins, p53 and pRb respectively (Dyson et al., 1992; Werness et al., 1990). These effects produce genetic instability and accumulation of mutations that might contribute to the oncogenic process. Besides, several other target proteins for *E6* and *E7* oncoproteins have been identified and probably those interactions also promote cellular transformation. The latest oncogene identified from HPV was the *E5* from HPV16. The *E5* oncoprotein showed to be capable of increasing the half-life of Epidermal Growth Factor

(EGF) and Platelet-Derived Growth Factor (PDGF) receptors (Leechanachai et al., 1992). Contrary to *E6* and *E7*, the *E5* oncogene is lost in the late stages of cervical cancer due to viral integration to the cellular genome. However, the close association with growth factor receptors could suggest that *E5* has a key role in viral life cycle and may have an important function in the early development of the neoplasia.

The oncogenes mentioned above codify for oncoproteins that have developed a plethora of strategies resulting in the alteration of cell cycle and apoptosis control by targeting the same or different cellular pathways, favoring viral persistence and leading to a strong synergism in promoting cellular transformation. Several studies have been performed to elucidate the mechanisms through which these HPV oncogenes allow the cellular transformation and the generation of the cervical cancer. Recently, the development of genomic and proteomic techniques has promoted the research of cervical cancer progression due to HPV infection, allowing the identification of protein interaction networks and the discovery of biomarkers useful for diagnostic and therapeutic systems.

## 2. Organization and classification of human papillomaviruses

HPV is a non enveloped DNA virus with icosahedral capsid that surrounds one copy of circular double stranded DNA close to 8 kb long. HPV genome codes for eight proteins classified as early (E) and late (L) proteins expressed from polycistronic mRNA by a positive DNA strand. Between E and L coding sequences is a Long Control Region (LCR) that has low conservation among members of the *Papillomaviridae* family. This region promotes and enhances the viral gene expression and it does not include ORFs (de Villiers et al., 2004; Syrjänen & Syrjänen, 1999). The HPVs maintain high fidelity of their genome as they use the host cell proofreading polymerases, generating mutations at similar rates as the host DNA genome. HPVs involve different genotypes, defined as a complete cloned genome whose *L1* ORF most conserved segment has a DNA sequence at least 10% different from any other HPV type (Bernard, 2005; de Villiers et al., 2004; Myers et al., 1996). HPV subtypes may be found (differences between 2-10% of homology) like HPV55 that is a subtype of HPV44. The HPV variants are defined as those that show differences of less than 2% of homology in nucleotide sequence in the coding regions and 5% in the non-coding regions from the original isolate (prototype) (Bernard et al., 1994; Bernard, 2005; de Villiers et al., 2004). Now at days, more than 200 suggested types have been detected and 100 types have been completely sequenced, all of them registered at the German Cancer Research Center in Heidelberg, Germany (Bernard, 2005; de Villiers et al., 2004).

All HPV types are epitheliotropic and may be found in mucous tissue and/or in skin (de Villiers, 1989; Myers et al., 1996). Recently, the new taxonomy of papillomaviruses grouped them in 16 higher-order cluster groups (super-groups or major branches) named *genus* which share from 23 to 43% of identity. The genera are identified by Greek letters (alpha to pi) and divided in lower-order clusters named *species* that share 60-70% of identity within a genus and 71-89% within a species (de Villiers et al., 2004). Species are identified by numbers and group HPV types with common phylogenetical, biological and pathological properties (Bernard, 2005; de Villiers et al., 2004). HPVs are classified into five genera (alpha, beta, gamma, mu and nu) (Bernard et al., 2010). The alpha-papillomavirus genus contains all the clinically important HPVs associated with mucosal and genital lesions. While, the beta-

papillomavirus genus includes all cutaneous HPV types involved in *Epidermodysplasia verruciformis* or skin cancer in immunosuppressed subjects, the gamma-papillomavirus genus includes cutaneous HPV types distinguished by intracytoplasmic inclusion bodies in histological tests (Bernard, 2005; de Villiers, 1989; de Villiers et al., 2004).

There are a limited number of variants for each HPV type, some of which are highly divergent, especially those taken from ethnic groups that evolved without geographical contact. Thus, it seems that each HPV type was not transferred from other species, has been with the human race since its origins, evolved together and spread as human ethnic groups arose and populated the world (Bernard, 2005). The HPV16 variants can be divided into six geographically clustered phylogenetic groups, the European (E), African 1 and 2 (Af1 and Af2, respectively), Asian (As), Asian-American (AA) and North American (NA) (Yamada et al., 1997). Clinically speaking, there is an interesting concern on the biological activity of HPV variants because some of them are more frequently detected (94%) in high-grade cervical lesions than the HPV16 prototype (6%) (Zehbe et al., 1998) and being associated to the development of cervical lesions grade 2-3 (Xi et al., 2002). However, more epidemiological and laboratory studies need to be performed to confirm these observations. Bravo and Alonso suggested an alternative classification system based on the nucleotide sequence of the *E1* and *E2* ORFs. Also, they proposed that concatenated *E1-E2* proteins sequences can be incorporated as a suitable evolutionary standard for *Papillomaviridae* classification (Bravo & Alonso, 2007). Due to the increasing number of new sequences of Papillomaviruses (PVs), Bernard and coworkers proposed the expansion of the family *Papillomaviridae*. To accomplish this, the names of genera were extended to the end of the Greek alphabet (from *Alphapapillomavirus* to *Omegapapillomavirus*), followed by the prefix “*Dyo*” (i.e., “a second time”), omitting the designation from *Dyoalphapapillomavirus* to *Dyogammapapillomavirus* because the *Alpha*, *Beta* and *Gamma* genera include the most common and medically important HPVs. Also, they named the rest of PVs genera waiting for classification as *Rho* to *Omega*, assigning the terms “*Dyodelta-PVs*”, “*Dyoepsilon-PVs*” and so on, for the new PV genus (Bernard et al., 2010).

Phylogenetical analysis of the *E5*, *E6* and *E7* gene sequences have revealed that only *E6* and *E7* genes from the alpha genus are highly conserved (>75% and >65% homology, respectively). However, when only the HR-HPV types of the alpha genus are considered the homologies go up to 80% for *E6* and up to 70% for *E7* sequences. The less conserved viral sequences from the alpha genus are those from the *E5* gene, since the homology in this genus is only of 25% and 40% when only the HR-HPV types are considered. Few years ago, Bravo and Alonso (2004) described the evolutionary characteristics of the *E5* proteins and compared them with the *E6* and *E7* oncogenic proteins and with the structural proteins *L1* and *L2*. They showed that there is a clear pattern of divergence from late to early genes at the protein level, which increased in the progression  $L1 < L2 < E6 \approx E7 < E5$ . Among the *E5* proteins from the alpha genus it was clear that there was not an evident sequence similarity and that the evolutionary divergence between present proteins rises to 80% (Bravo & Alonso, 2004). The few common characteristics for the alpha genus *E5* proteins are the high hydrophobicity, the high Ile+Leu+Val content and the presence of transmembranal regions. Taking into consideration these characteristics of the *E5* protein, Bravo and Alonso (2004) identified 4 groups of *E5* proteins in the alpha genus (*E5* $\alpha$ , *E5* $\beta$ , *E5* $\gamma$  and *E5* $\delta$ ) and proposed that besides of the phylogenetic characteristics, the chemical part of the proteins have to be

taken into consideration to have a more specific classification, as it has been suggested that the protein chemistry is an important restriction for protein evolution (Babbitt & Gerlt, 1997).

On the other hand, Wang and coworkers (2010) showed that the Canine Papillomavirus type 2 (CPV2) encodes an E7 protein that has Ser substituted for Cys at the LXCXE motif, important in the E7 proteins from the alpha genus, to bind and degrade the pRb protein. This mutation in the CPV2 E7 protein abrogates pRb binding at this site; however a new domain with a binding site for pRb was identified at the C-terminal region of canine E7, and was able to degrade the pRb protein. At the same time, these researchers demonstrated that the HPV4 E7 protein also binds pRb in a similar way as CPV2 E7 protein and by screening of HPV genome sequences identified that the LXSXE motif of the CPV2 E7 protein was also present in the gamma HPV genus (Wang et al., 2010).

Altogether these data show that phylogenetic sequence analysis is important to trace the papillomaviruses evolution and define ancestors, but also showed that when chemical and biological characteristics of the oncogenic HPV proteins are integrated into the analysis, there is a better classification of HPVs within the genus, as subgroups were identified. However, more work needs to be done before this type of integrative analysis can be used to give some light between clinical manifestations and the development of cancer associated to HPV.

### 3. Viral cycle and HPV integration into the genome of host cell

During HPV infection, the different viral proteins are expressed sequentially (Middleton et al., 2003; Peh et al., 2002). The HPV infection starts in the basal cell layer of the cervix since these cells express the specific receptors for viral entry and also because are the only cells in the squamous epithelium capable of dividing. Molecules like heparan sulfate and glycosaminoglycans seem to mediate the attachment of the virions via interaction of the major capsid protein, L1, to the basal membrane of human keratinocytes. The internalization of some HPV types is a slow process mediated by clathrin-coated vesicles, or the caveolae-dependent route (Bousarghin et al., 2003; Horvath et al., 2010). Once the virus has penetrated the cell, disassembly of HPV particles occurs in cytoplasmic vesicles, followed by the delivery of the viral DNA into the nucleus mediated by L2 (Day et al., 2004). After infection, HPV genomes are established as autonomous replicating extra-chromosomal elements or episomes and start a low expression level of the *E6* and *E7* genes (Middleton et al., 2003; Moody & Laimins, 2010). The *E6* and *E7* gene expression is achieved by cellular transcription factors that interact with the LCR, a region where the E2 protein interacts to repress or to activate viral transcription. The promoter region from HPVs contains TATA boxes for binding cellular transcriptional factors like TFIID and an epithelial cell-specific enhancer, which holds binding sites for transcription factors like Sp-1 and AP-1, among others (Butz & Hoppe-Seyler, 1993; Doorbar, 2005; Middleton et al., 2003; Tan et al., 1994). The expression of E6 leads mainly to the ubiquitin-dependent proteolysis of p53 (Farthing & Vousden, 1994; Münger et al., 1992; Rapp & Chen, 1998), preventing cell growth inhibition in both undifferentiated and differentiated cells (Moody & Laimins, 2010) and the E7 expression to the liberation of the transcription factor E2F by sequestration of pRb (Münger et al., 1992), effects that promote cellular proliferation and genome instability throughout the infected tissue.



While the basal HPV infected cell migrates to upper layers and differentiates, the viral cycle continues with the E1, E2, E4 and E5 protein expression and viral DNA replication (Doorbar, 2005; Longworth & Laimins, 2004; Middleton et al., 2003; Peh et al., 2002). First, the E1 protein hexamerizes to recruit the cellular DNA polymerase and replication accessory proteins, while it displaces Histone H1 and functions as a helicase/ATPase to start the viral genome replication (Hughes & Romanos, 1993; Lambert, 1991; Swindle & Engler, 1998). It has been observed that the E1 and E2 expression is auto-regulated and activated by replication. The E2 protein modulates E1 expression and E6 splicing differentially regulates E1 and E2 expression (Hubert & Laimins, 2002). Once the viral replication has finished, E2 levels raise and repress the E6 and E7 expression by binding to the HPV early promoter as a dimmer and promoting the liberation of cellular transcriptional activator factors (Tan et al., 1994).

On the other hand, the E4 protein is translated from a spliced E1<sup>E4</sup> transcript to form a fusion protein that contains the first 5 amino acids (aa) from the E1 protein and the E4 ORF. In warts, E4 exists as multiple species that are formed from a combination of progressive proteolysis of the N-terminal residues, oligomerization, and phosphorylation. The species resulting from proteolysis are the 17 kDa species in the parabasal cell layer of epithelium and this is coincident with viral genome amplification. The E4 16, 11 and 10 kDa species are accumulated in superficial keratinocytes, where capsid proteins expression and assembly occurs (Doorbar et al., 1988; Middleton et al., 2003). It is known that E4 binds to zinc, cytoskeleton and cytokeratins (Roberts et al., 1994; Wang et al., 2004). Therefore, it is suggested that E4 may alter the normal keratinization process to benefit the viral cycle progress and generate a cytoskeleton collapse (Gaillard et al., 1992) inducing apoptosis through alteration of mitochondrial function (Raj et al., 2004) and favoring the viral particle liberation (Gaillard et al., 1992). However, in natural infections only a limited amount of keratin collapse has been observed (Doorbar et al., 1996). The E4 protein sequesters the CDK1/Cyc B1 complex (CDK, Cyclin dependent kinase and Cyc, Cyclin) onto the cytokeratin network, preventing their nuclear accumulation and therefore inducing inhibition of the G2/M transition of the cell cycle (Davy et al., 2002; Nakahara et al., 2002) and allowing viral and genomic DNA replication. The HPV16 E4 coding region possesses a splicing enhancer element required for the early viral mRNA splicing (Rush et al., 2005), especially for the expression of late viral transcripts of E1<sup>E2</sup>, E1, E4 and E5, thus regulating the viral DNA amplification (Wilson et al., 2005).

Other protein suggested to be expressed at the same time as E4 is E5, both of which may contribute to viral genome amplification (Doorbar, 2006; Syrjänen & Syrjänen, 1999). A polycistronic mRNA containing the E5 sequence is the most abundant transcript in HPV positive cervical carcinomas *in situ* (Stoler et al., 1992). E5 transcripts expression is very low in undifferentiated cells (Reagan & Laimins, 2008). The E5 mRNA and protein are mainly present in the lower third of the epithelium of Low Grade Squamous Intraepithelial Lesions (LSILs), and might contribute to neoplastic proliferation during the early stages of infection (Chang et al., 2001; Stoler et al., 1992), or to be essential for malignant transformation (Stoppler et al., 1996). The gene encoding the viral E5 protein is frequently disrupted or lost when viral DNA is integrated into human genome (Pater & Pater, 1985). Even though, E5 protein expression pattern in cervical epithelia has not been accomplished yet, it has been observed that E5 function is needed to maintain the cell proliferation stimuli driven by the

EGF Receptor (EGFR) signaling pathway at this stage (Syrjänen & Syrjänen, 1999). In this way, the E5 function complements the E7 and E6 functions, to generate and maintain the transformed phenotype (Stöppler et al., 1996).

A special characteristic of the viral cycle is that the last stages take place in terminally differentiated keratinocytes (Schiller et al., 2010). In this stage, viral particles are generated when the expression of capsid proteins (L1 and L2) starts by a promoter localized in the late region of the HPV genome. The L1 and L2 proteins polymerize into icosahedral capsids in a thermodynamic stable and spontaneous process that involves the binding of the HPV DNA to the N-terminal sequence of the L2 protein (Hagensee et al., 1993; Zhou et al., 1994). During their formation, HPV capsids require maturation (formation of intermolecular disulfide bonds in L1), event that leads to stability and virions liberation in the external surface of the squamous epithelium by cellular death (Buck et al., 2005; Syrjänen & Syrjänen, 1999). Up to now, it is still unknown if encapsidation of the viral genome takes place during or after capsid assembly (Conway & Meyers, 2009; Holmgren et al., 2005), but it has been suggested that L2 uses E2 to recruit the viral genome to the site of virion assembly (Holmgren et al., 2005).

During HPV productive infections that lead to cervical lesions, the viral genome is episomal with a great copy number that depends on differentiated cells. Throughout the HPV persistent infection, there is a gradual deregulation in the expression of E6 and E7 proteins that may lead to the development of low-grade cervical lesions, where one third of the epithelium is formed by E6/E7 expressing basal cells. The progression from High Grade Squamous Intraepithelial Lesions (HSILs) to cancer usually occurs in lesions that contain integrated copies of the viral genome. This event leads to an abortive infection (Farthing & Vousden, 1994; Münger et al., 2004; Peitsaro et al., 2002) and cells cannot longer produce new viral particles (Matlashewski, 2006). It has been observed that the viral integration occurs mainly between *E1-E2* ORFs, event that produces the loss of *E2* and *E4* genes expression. Thus, the loss of *E2* and *E4* genes generates a down-replication of the viral genome, G2 arrest, and E6 and E7 over-expression (Jeon & Lambert, 1995; Wilson et al., 2005). Thus, the integration of the HPV DNA in the host genome represents an important event in cervical carcinogenesis (Pett & Coleman, 2007), as this may cause cellular immortalization (Band et al., 1990; Jeon & Lambert, 1995; Münger et al., 1992; zur Hausen, 2000), reduction of cellular differentiation, cellular dysplasia and TNF- $\alpha$  non-responder cells (TNF, Tumor Necrosis Factor) (Syrjänen & Syrjänen, 1999; zur Hausen, 2000).

Besides increasing the transcript stability and protein expression of the viral E6 and E7 oncoproteins, integration of the viral genome may cause chromosomal rearrangements (Jeon & Lambert, 1995) and influences cancer progression through interaction with hTERT, p53 and pRB (Raibould et al., 2011). The integration of HPV sequences in the host genome occurs randomly, although in invasive genital carcinomas or cervical cancer-derived cell lines, HPV genome has been found frequently integrated near fragile sites, like translocation breakpoints (Koopman et al., 1999; Mammias et al., 2008), or modifying proto-oncogenes sequences either by rearrangement or amplification as has been observed for *myc* genes (Ocadiz et al., 1987; Sastre-Garau et al., 2000). Disruption or deregulation of defined critical cellular gene functions by insertional mutagenesis of HPV genome fragments has been hypothesized as the major promoting factor in the pathogenesis of HPV-associated cancers (Wentzensen et al., 2004). Further investigation on early integration events needs to be done to describe the mechanism by which viral infection is aborted and cell transformation is generated.

## 4. Role and function of HPV oncoproteins

### 4.1 E5 oncoprotein

#### 4.1.1 E5 biochemical properties

The E5 oncoproteins are small highly hydrophobic proteins important in the carcinogenic process and recently have been identified as potential oncogenes, although their role is not well understood (Bouvard et al., 1994). The E5 proteins from both animal and human sources can transform mammalian cells with different degrees of efficiency (Bouvard et al., 1994; Horwitz et al., 1989; Straight et al., 1993). Both BPV-1 (bovine papillomavirus) and HPV E5 proteins are transmembrane proteins localized in Golgi and endosomes, but they have also been found in plasmatic membrane and endoplasmic reticulum (ER) (Cartin & Alonso, 2003; Conrad et al., 1993; Disbrow et al., 2005; Lewis et al., 2008). The E5 oncoprotein from HPV16 has 83 aa residues with an estimated molecular weight of 10 kDa. The E5 protein presents three transmembranal helices and short hydrophilic regions at the C- and N-terminus (Alonso & Reed, 2002; Ullman et al., 1994; Yang et al., 2003), being the first hydrophobic region important for cellular localization of E5 (Fig. 1) (Cortese et al., 2010; Lewis et al., 2008). In HaCat cells (immortalized human keratinocytes) this region confers anchorage-independent growth and is associated with the capacity of these cells to invade extracellular matrix in organotypic “raft” assays (Barbaresi et al., 2010; Lewis et al., 2008). It has been demonstrated that E5 proteins contribute to cellular transformation by increasing the mitogenic signal from growth factor receptors to the nucleus (Leechanachai et al., 1992).

#### 4.1.2 E5 functional properties

A proposed mechanism for E5 transforming activity is through increasing the half-life of the tyrosine kinase-containing growth factor receptors like EGFR, the phosphorylation state of this receptor or both (Genther-Williams et al., 2005; Straight et al., 1993, 1995; Tomakidi et al., 2000). It has been also proposed that HPV E5 proteins interact with the 16 kDa subunit of the protonic ATPase, inhibiting the acidification of endosomes and retarding the receptor degradation (Andresson et al., 1995; Briggs et al., 2001; Conrad et al., 1993; Straight et al., 1995). At the same time, E5 induces a number of functional effects like causing alkalinization of endocellular pH, tyrosinase activation, melanin deposition and modulation of sensitivity to dopamine mimetic drugs as it has been shown in melanocytes that express E5 from HPV16 (Di Domenico et al., 2009). The second and third transmembranal domains cooperate to bind the protonic ATPase-16 kDa subunit (Adam et al., 2000; Rodriguez et al., 2000). However, some contradictory studies indicate that E5 proteins bind, but do not disturb the activity of the vacuolar protonic ATPase (Adam et al., 2000; Ashby et al., 2001; Rodriguez et al., 2000), and it has been shown that the final effect is due to a perturbation of the endocytic trafficking (S.L. Chen et al., 1996c). In contrast, it has been demonstrated that HPV16 E5 stimulates the EGFR-mediated signal transduction by inhibiting the interaction with c-Cbl and decreasing the receptor degradation pathway (B. Zhang et al., 2005a) (Fig. 2). Recently, it was shown that HPV16 E5 down-modulates the KGFR/FGFR2b (keratinocyte growth factor receptor) by interference of the endocytic receptor pathway and perturbing the differentiation process (Belleudi et al., 2011).



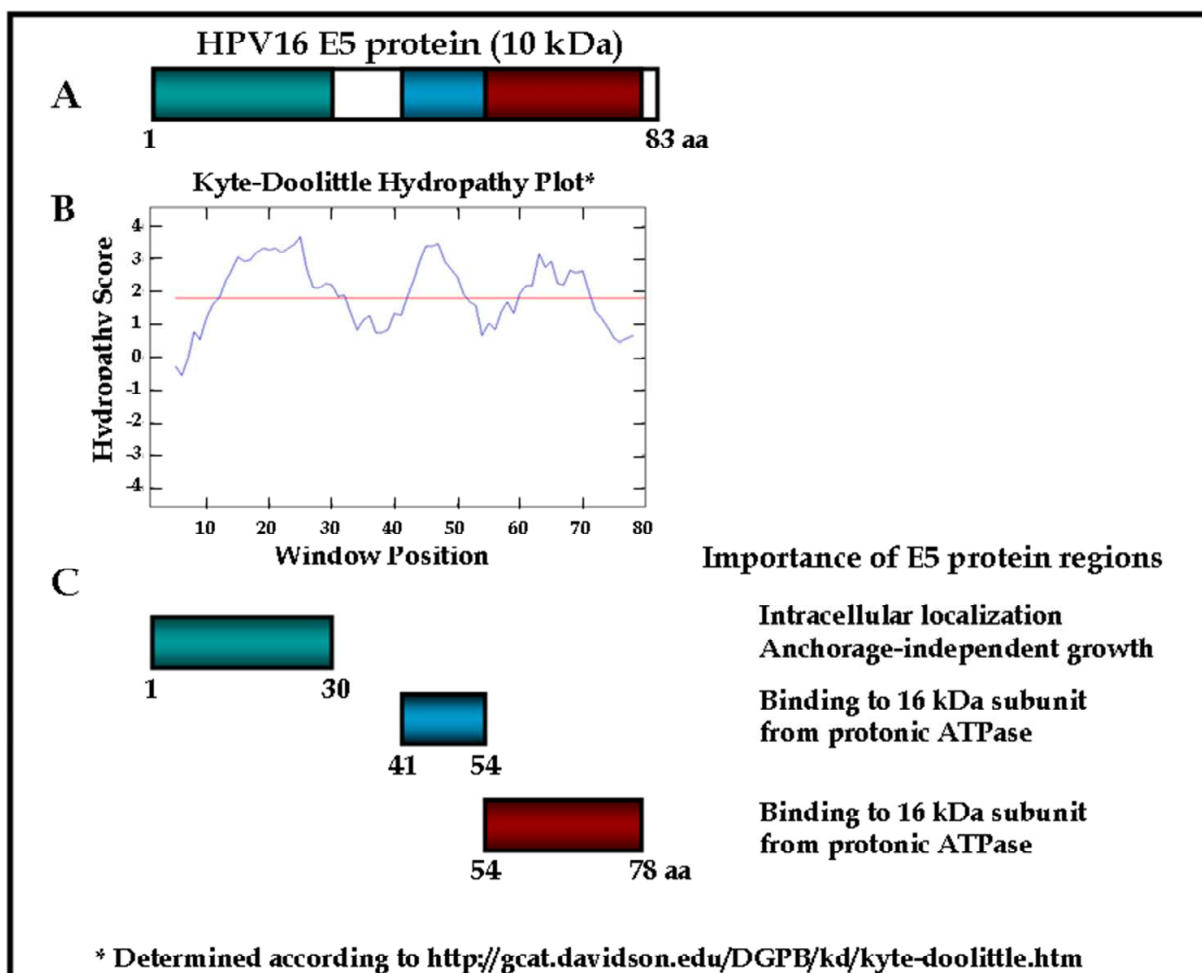


Fig. 1. Biochemical characteristics of the HPV16 E5 oncoprotein. (A) The E5 protein with 83 amino acid residues has an estimated molecular weight of 10 kDa. (B) E5 is divided in three hydrophobic regions as estimated by the Kyte-Doolittle Hydropathy Plot. (C) The first region (aa 1-30) is important for membrane localization and for anchorage-independent growth. The second (aa 41-54) and third (aa 54-78) hydrophobic regions contain the domains for binding to the protonic ATPase-16 kDa subunit.

The expression of E5 protein has been associated with the expression of other members of the EGFR family (i.e. ErbB1, ErbB4) as well as the activity of ErbB2 (Crusius et al., 1998) and components of the EGF signaling pathway, like MAPKs (ERK1/2) (Crusius et al., 1997; S.H. Kim et al., 2006). The activation of members of the MAPK pathway may lead to regulation of other set of genes implicated in growth, as was observed that HPV16 E5 favors the over-expression of transcriptional factors such as c-Fos and c-Jun that form the AP-1 complex (S.L. Chen et al., 1995, 1996a, 1996b), and stimulates transcription of genes involved in cell growth. During the cell cycle the E5 proteins from HPV11 and 16 have shown to be able to modulate cell proliferation, due to repression of *p21<sup>Waf1</sup>* gene expression through *c-jun* activation (Tsao et al., 1996). In addition, our laboratory has identified that HPV16 E5 increases the down-regulation of *p27<sup>Kip1</sup>* CDK inhibitor in an EGFR dependent pathway, allowing the cells to stay for longer time into the cell cycle due to an increment in the S-phase (Pedroza-Saavedra et al., 2010) (Fig. 2). Also, HPV16 E5 is able to activate other type of transcriptional factors like NF- $\kappa$ B and leading to COX-2 expression (S.H. Kim et al., 2009)

(Fig. 2). More recently, it has been suggested that HPV16 E5 protein can act independently of the EGFR through the PLC $\gamma$ 1 (Crusius et al., 1999) and on MAPKs (ERK1/2 and p38) activities, when the cells are placed under stress conditions (Crusius et al., 2000). On the other hand, E5 is also able to induce the expression of other type of receptors like the EP4 subtype of prostaglandin E2 receptor, which increases the colony-forming efficiency and the Vascular Endothelial Growth Factor (VEGF) secretion in cervical cancer cells, both of which are required for tumor growth, angiogenesis and metastasis (Oh et al., 2009).

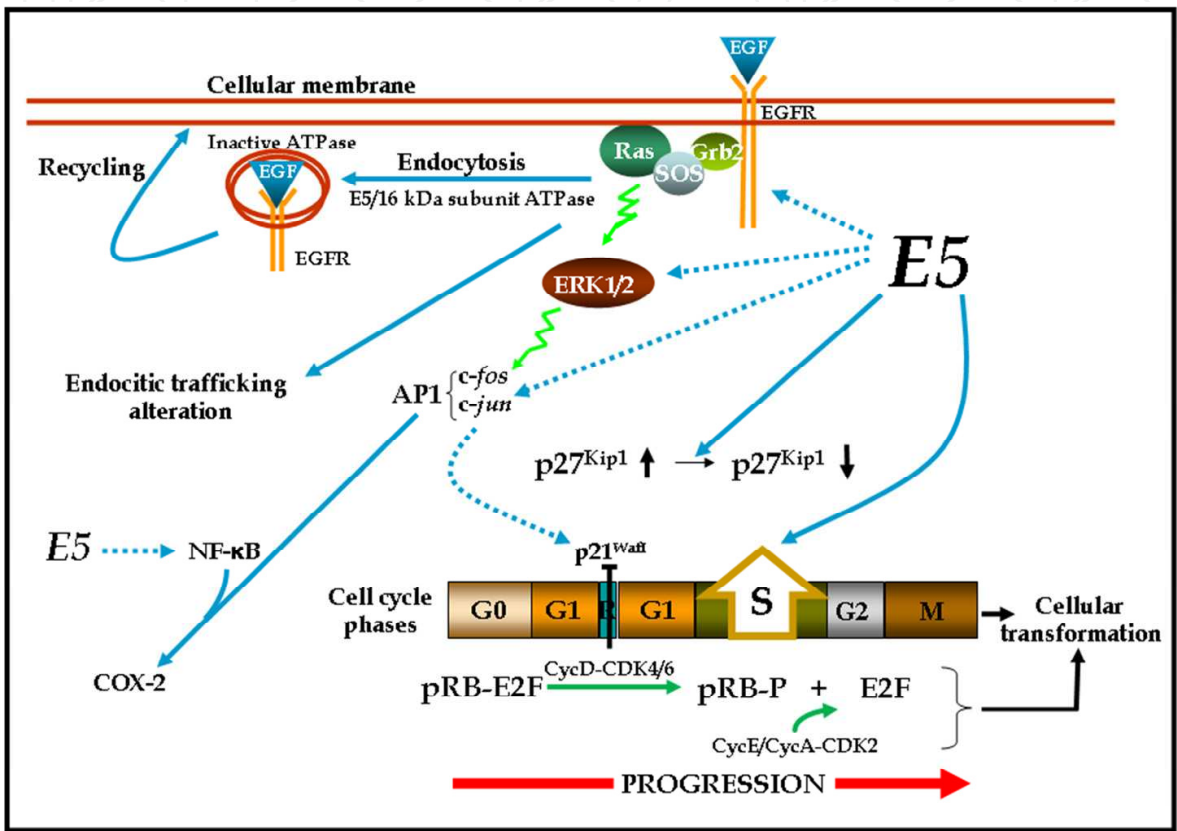


Fig. 2. Important functions of HPV16 E5 in the cell. E5 can modify the half-life of the EGFR through binding with the protonic ATPase-16 kDa subunit, avoiding acidification of endosomes and favoring the recycling of receptors and perturbing the endocytic trafficking. E5 targets molecular components of the MAPKs cascade that have incidence on the progression of the cell cycle. E5 decreases the level of the CDK inhibitor p21<sup>Waf1</sup> and modulates p27<sup>Kip1</sup> protein levels allowing deregulation of the cell cycle, by increasing the S-phase of the cell cycle and promoting cellular transformation.

Other important activity observed for E5 proteins is the ability to keep the major histocompatibility complex (MHC) class I in Golgi, preventing its transport to the cell surface. In the case of HPV16 E5, this blockage is rescued by treatment with interferon. Unlike BPV E5, HPV16 E5 does not affect the synthesis of HLA class I heavy chains, but rather interferes with the transporter associated with antigen processing (TAP). The absence of cell surface HLA class I molecules in E5 expressing cells may allow the HPV to establish an infection by avoiding immune clearance of virus-infected cells by CTLs (Araibi et al., 2004; Ashrafi et al., 2005; Marchetti et al., 2002, 2006). In this context, Gill and coworkers (1998) observed that women with increasingly severe HPV16-associated lesions have

decreasing T cell HPV16 E5-specific proliferative responses. At the same time, HPV16 E5 impairs TRAIL- and FasL-mediated apoptosis in HaCat cells possibly as a mechanism to escape immune surveillance that otherwise would be likely to detect infected cells at an early stage in the viral life cycle (Kabsch et al., 2004).

Finally, the close association of E5 with growth factors and their receptors, and the fact that the *E5* ORF is lost in cervical carcinoma cells, suggest that E5 may play a critical role in the genesis of cervical cancer as an initiator of the transformation process, as it has been shown to happen with the Ras oncoprotein in colorectal cancer (Gryfe et al., 1997; Takami et al., 1995). Additionally, these findings support the idea that the E5 protein could be an early predictor marker of cervical cancer (Gill et al., 1998; Hsieh et al., 2000; Kell et al., 1994).

## 4.2 E6 oncoprotein

### 4.2.1 E6 biochemical properties

E6 proteins from HPVs comprise molecules between 151 and 158 amino acids that are usually localized within the cell nucleus (Nomine et al., 2006; Rapp & Chen, 1998). It has been difficult to study E6 proteins because they are expressed at very low levels in HPV infected cells and are difficult to purify in a stable folded form as they tend to aggregate in complexes of misfolded molecules when over-expressed in bacteria (Nominé et al., 2001; Ristriani et al., 2002).

In general, the E6 proteins contain two Cys-Xaa<sub>2</sub>-Cys-Xaa<sub>29-30</sub>-Cys-Xaa<sub>2</sub>-Cys zinc binding domains located at the N- and C-terminal parts of the molecule. These domains are novel types of zinc-binding structures characterized by  $\alpha\beta\alpha\beta$  secondary structures separated by 29 to 30 residues (Ullman et al., 1996). The E6 C-terminal domain is a DNA binding site that consists of 3-stranded  $\beta$ -sheet (S1, S2 and S3) with two short helices (H1 and H2) packed on one of its sides, a peripheric zinc binding site that protrudes away from the  $\beta$  sheet and that involves the long loop (L2) connecting H1 and H2 with a short C-terminal helix (H3). E6 has a main hydrophobic core (conserved among HPVs) between the  $\beta$ -sheet and helices H1 and H2, that it is not exposed to water in the full-length E6 protein. This part of the E6 protein has a mostly positive charge potential, except for a neutral area at the top of H1 and H2 corresponding to the hydrophobic patch. Conserved amino acids are on the surface of the N- and C-terminal zinc binding motifs of E6 protein in all HPV species. Some may participate in generic functions shared by all HPVs E6 proteins like trans-activation, transformation, and protein-protein interactions, while others may have a structural role. In this sense, it seems that specific amino acids located at the hydrophilic sites in high- and low-risk HPVs, allow certain physicochemical characteristics which confer them the ability to recognize different subsets of cellular molecules. An example is the C-terminal region from HPV16 E6, which displays a strong positive charge at the nucleic acid binding site and the highest surface potential (Nominé et al., 2006).

The HPV16 E6 full-length protein is composed of 151 amino acids (Rapp & Chen, 1998), with a calculated molecular weight of 18 kDa and with a bimodal half-life in SiHa cells of 30 min and 4 h that seem to be related to E6 interactions with target proteins (Androphy et al., 1987). HPV16 E6 protein C-terminal region has an IEP of 10.6 (IEP, Isoelectric Point) and it is the part of the molecule necessary for binding to p53, while the N-terminal region contains the binding site for E6AP (Lxx $\phi$ Lsh motif) important for p53 degradation (Howie et al., 2009;

Thomas et al., 1999). These Lxx $\phi$ Lsh motifs (where xx is a dipeptide and one of the residues is Asp, Glu, Asn or Gln;  $\phi$  is a hydrophobic residue; s is a small amino acid; h is an amino acid capable of accepting hydrogen bonds) are Leucine-rich amphipathic helices and binding through these motifs is a conserved property of the E6 proteins from HPVs from the alpha genus. In this sense, the minimal segment still functional for p53 degradation comprises residues 1-142 and coordinates with two Zn<sup>+2</sup> ions (Beerheide et al., 1999; Lipari et al., 2001) by means of conserved cysteine residues 30, 33, 63 and 66 at the N-terminal and residues 103, 106, 136 and 139 at the C-terminal (Nominé et al., 2001), where the loss of the Zn<sup>+2</sup> at the C-terminal domain leads to loss of tertiary structure and protein aggregation (Y. Liu et al., 2009). Other proteins like E6AP, E6BP, IRF3, Paxilin and MCM7 are also able to bind E6 through these Lxx $\phi$ Lsh motifs, interaction that appears to regulate the activity of different subset of proteins (J.J. Chen et al., 1998; Elston et al., 1998; Tong & Howley, 1997).

Besides, the E6 proteins from oncogenic HPV types have a motif designated XT/SXV at their C-terminal motifs that mediates binding to specific domains known as PDZ (Howie et al., 2009). The PDZ domains are approximately 90 amino acid stretches. The PDZ proteins known to bind E6 are hDlg1 and 4, tumor suppressor proteins (Kiyono et al., 1997; S.S. Lee et al., 1997), MAGI-1, -2 and -3, Membrane Associated Guanylate kinase homolog proteins (Thomas et al., 2001), MUPP-1 a multi PDZ protein (S.S. Lee et al., 2000), hScrib (Nakagawa & Huibregtse 2000) and PTPN3, both tyrosine phosphatase proteins (Jing et al., 2007). These proteins bind to E6 by the PDZ domain (aa 141-151) (Pim et al., 2009; Storrs & Silverstein, 2007; Y. Zhang et al., 2007), acting as an adaptor to link the ubiquitin ligase to the target for ubiquitination by means of its C-terminal region (Pim et al., 2009). Moreover, differences in binding PDZs by different HPV E6 proteins have been associated with the pathogenicity of HPV types as a single amino acid substitution in this binding motif among HR-HPV E6 proteins has a profound effect upon its binding to PDZ bearing target proteins (Thomas et al., 2001).

E6 protein may be found full length and C-terminally truncated (E6\*) expressed from a subset of spliced transcripts, differing in number depending on the HPV type (HPV16 E6 presents 4 species, while HPV18 E6 only one). The E6\* protein of HPV18 has the first 44 amino acids of the full-length protein and thereafter is composed by 13 unique amino acids derived from E6 intronic sequences. One of these sequences results in an E6\* protein of 7 kDa able to bind to E6AP, but not p53. This interference with the p53-mediated E6 degradation by E6\* re-activates the p53-dependent growth arrest and apoptotic functions (Pim et al., 2009). In this sense, the presence of E6 over E6\* may lead to aggressive cancers as E6\* is present at low levels in Asian-American HPV16 variant, the one detected in the most aggressive cancers, and at high levels in African variant, together with higher levels of p53 (Filippova et al., 2009).

#### 4.2.2 E6 functional properties

The HR-HPV E6 proteins are distributed in the cytoplasm and the nucleus, and its expression leads to the transformation of NIH3T3 cells as well as the immortalization of human mammary epithelial cells (Holt et al., 1996). The first function associated to E6 protein was its anti-apoptotic activity due to the ability to mediate the p53 degradation through the E6/E6AP complex, allowing the regulation of expression of proteins controlling the cell cycle, being the most important the CDK inhibitor p21<sup>Waf1</sup> (Cooper et al., 2003). The p73 protein, homologue in structure and function to p53, also binds to E6 but its



degradation is not promoted by the oncoprotein (Das et al., 2003). However, in HPV18 transformed cells there is an abrogation of p53 degradation due to the Pitx2a protein that binds to E6 and interferes with the E6/E6AP complex, leading to the accumulation of functional p53 protein (Wei, 2005). In this case, the anti-apoptotic effect of E6 is carried out through the interference of the p53/PUMA/Bax cascade (M. Vogt et al., 2006).

On the other hand, E6 is able to transactivate the survivin promoter and along with E7, transactivate the c-IAP-2 promoter and confer resistance to apoptosis (Yuan et al., 2005). Isoforms of E6 regulate the extrinsic apoptosis in a concentration-dependent manner. When low levels of large (E6) and short E6 (E6\*) proteins are expressed, the cells become resistant to TNF. On the other hand, with moderate and high levels of expression, there is a sensitization to TNF aided by the E6 protein ability to form complexes with each other. In this sense, the cellular response to TNF depends on the ratio of the two E6 isoforms, being high levels of large E6 responsible for the low response to TNF and E6\* reverting the E6 effect (Filippova et al., 2009). The resistance to TNF-mediated apoptosis is related to high levels of E6 and low levels of E6\*. Other ways by which E6 protein regulates cell death pathways is by inhibiting the transactivation action of IRF3 (Ronco et al., 1998) and by inhibiting TLR9 transcription generating functional loss of TLR9-regulated pathways (Hasan et al., 2007).

In contrast, the capacity of E6/E6AP-mediated p53 degradation favors the accumulation of genetic alterations. A possible explanation is that E6 alters molecules implicated in DNA-repairing such as degradation of MGMT (Srivenugopal & Ali-Osman, 2002) and MCM7 proteins, producing chromosomal abnormalities (Kühne & Banks, 1998; Kukimoto et al., 1998). Also E6 from the HR-HPVs increases cellular telomerase activity via transcriptional activation of the telomerase catalytic subunit, hTERT (X. Liu et al., 2005) and degradation of the telomerase inhibitor NFX1-91 (Gewin et al., 2004), events that lead to reconstitution of telomerase activity allowing the immortalization process in the cells (J.P. Liu, 1999; Longworth & Laimins, 2004).

Recent studies have demonstrated that HR-HPV E6 proteins have a PDZ-binding motif, which probably mediates the disruption of signal transduction where PDZ proteins are implicated (Watson et al., 2003). It has been reported that E6 binds PDZ proteins and promotes their ubiquitination and further degradation as observed for hDlg (Grm & Banks, 2004), MUPP-1 (S.S. Lee et al., 2000; Massimi et al., 2004), h-SCRIB (Thomas et al., 2005), MAGI-1, -2 and -3 (Thomas et al., 2001, 2002). The MAGUK family protein MAGI-2 interacts with PTEN tumor suppressor (Wu et al., 2000), which inactivates IP<sub>3</sub>. Thus, PKB remains activated promoting cell survival and proliferation. Additionally, HPV E6 oncoprotein has been linked to the MAPK signaling pathway by increasing the levels of MAPK1/2, MEK 1/2, and BRAF specially the E6 variant in amino acid 83, promoting cell proliferation by another pathway (Chakrabarti et al., 2004).

Other molecules that bind E6 proteins (in some cases with its concomitant degradation) are the Rap GTPase-activating protein E6TP1 (Gao et al., 1999; L. Singh et al., 2003), the CDK inhibitor p16<sup>INK4a</sup> (Malanchi et al., 2004), the regulator of insulin signaling pathway tuberin (Lu et al., 2004), the regulator TRIP-Br1 for the E2F/DP1/pRb complex during cell-cycle (Gupta et al., 2003), the extracellular matrix Ca<sup>2+</sup>-binding protein fibulin-1 (Du et al., 2002) and the putative Ca<sup>2+</sup> binding protein E6BP (J.J. Chen et al., 1995). Viral oncoproteins induce mislocation of select PDZ proteins allowing disruption of tight junctions and cause polarity defects in epithelial cells. The development of human cancers is frequently associated with a

failure of epithelial cells to form tight junctions and to establish proper apicobasal polarity and this is probably another pathway that E6 follows to disrupt the cellular system (Latorre et al., 2005). On the other hand, E6 interacts and promotes the degradation of NHERF-1, a PDZ domain containing protein leading to the activation of the PI3K/AKT signaling pathway (Accardi et al., 2011), and promoting cellular proliferation also by this mechanism. Furthermore, E6 protein participates in the up-regulation of SGLT1 promoting the glucose intake in tumor cells (Leiprecht et al., 2011).

### 4.3 E7 oncoprotein

#### 4.3.1 E7 biochemical properties

The nucleotide sequence of *E7* oncogenes are highly conserved among different HPV types. The *E7* ORF encodes for a small acidic phosphoprotein of 98 amino acids. The predicted molecular weight of this *E7* protein is 11 kDa and 14 kDa when phosphorylated *in vitro*, characteristics that are observed in the *E7* protein from HPV16 (Armstrong & Roman, 1993). However, the electrophoretic mobility of HPV16 *E7* protein observed when analyzed by SDS-PAGE is approximately 17 kDa and this molecular weight is not affected by *in vitro* phosphorylation by CKII (Armstrong & Roman, 1992; Gage et al., 1990). Heck and coworkers (1992) showed that the anomalous behavior of the HPV16 *E7* protein resides within the amino-terminal residues that confer a net negative charge to the protein.

The *E7* proteins of different HPV types are highly similar among them and share 3 regions of homology with the Adenovirus E1A protein and with the SV40 large T antigen (Fig. 3) (Barbosa et al., 1990; Brokaw et al., 1994). The amino-terminal portion of *E7* protein contains the CR1 (Consensus Region 1) between amino acids 1-20 important for DNA synthesis and cellular transformation (Gulliver et al., 1997). The CR2 (aa 21-43) contains the binding site for pRb (aa 21-26), the phosphorylation sites for CKII (aa 31 and 32) and the site for binding the cyclin A-E2F complex (Barbosa et al., 1990; Gulliver et al., 1997; Münger et al., 1989). Although the C-terminal region of the HPV16 *E7* (aa 44-98) does not contain an extensive amino acid homology with the CR3 of E1A, it consists of a metal binding domain composed of two CXXC motifs separated by 29 amino acids (Fig. 3) (Brokaw et al., 1994; Phelps et al., 1988). The *E7* proteins from HPV16 and 18 are able to bind  $Zn^{+2}$  with the CXXC motifs, sites that are important for dimerization and intracellular stabilization of the molecule (Clemmens et al., 1995). This region also contains a low affinity pRb binding site and is involved in the disruption of the E2F/pRb1 complex (Braspenning et al., 1998).

Different molecular weights for *E7* have been reported and suggested that the negative charge and the N-terminal region of the protein are related to these changes. Pulse-chase experiments by Smotkin and Wettstein (1987) with HPV16 were unable to detect changes in the *E7* protein mobility in SDS-PAGE, suggesting that no other modifications were present. However, other groups have reported the presence of two *E7* species in HPV6, 16 and 18 (Gage et al., 1990; Greenfield et al., 1991; Sato et al., 1990; Selvey et al., 1994), but neither the origin or the biological significance have been explained. The presence of phosphorylated species of *E7* can explain some of the different molecular weights, although treatment of *E7* protein with alkaline phosphatase suggests the presence of other posttranslational modifications (Selvey et al., 1994). Differences in cellular localization also exist for the *E7* protein, some groups have reported this protein as cytoplasmic, but others have found it in nucleus (Kanda et al., 1991; Sato et al., 1990; Selvey et al., 1994).

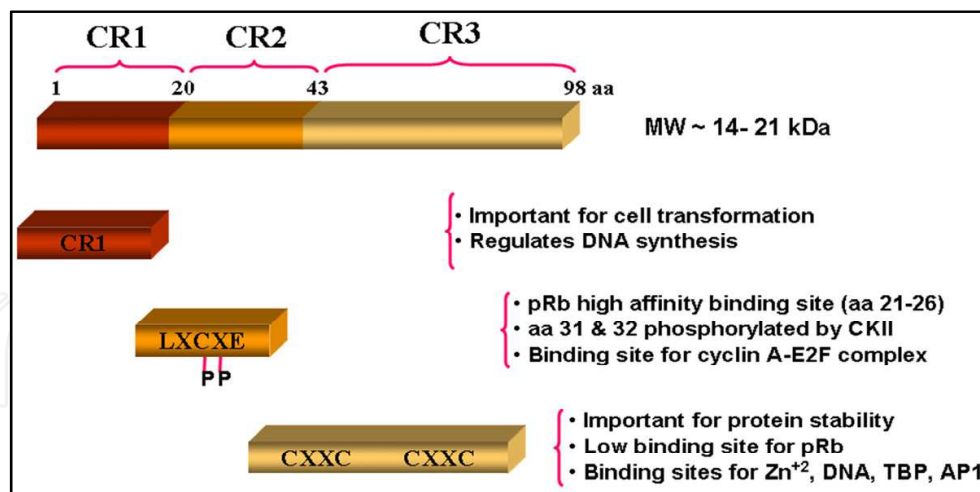


Fig. 3. Biochemical characteristics of HPV16 E7 protein. The HPV16 E7 protein has been reported with different electrophoretical molecular weights (from 14-21 kDa), differences that have been partially explained by the net negative charge of the protein. E7 shares 3 homology regions with the Adenovirus E1A protein and with the SV40 large T antigen denoted as CR1, 2 and 3. Mutational analysis of the E7 protein has shown that CR1 is important for cellular transformation; CR2 contains the high affinity binding site (LXCXE) for pRb and the phosphorylation sites for CKII (aa 31 and 32); CR3 contains two binding motifs (CXXC) for Zn<sup>+2</sup> important for the protein stability and DNA binding.

The HPV16 E7 is able to translocate through the nuclear pores via a non-classical Ran-dependent pathway, independent of the main cytosolic Kap beta import receptors (Angeline et al., 2003), and it possesses two NLSs and one NES domains to shuttle between nucleus and cytoplasm (Knapp et al., 2009). Recently, our group identified 3 isoforms of E7 from HPV16 present in Caski cells, E7a1 (17.5 kDa and IEP of 4.68), E7a (17 kDa and IEP of 6.18) and E7b (16 kDa and IEP of 6.96). The processing of the E7 protein was followed up by pulse-chase experiments and found that the first isoform synthesized was a 17 kDa protein and after approximately 1 h, it was processed to a faster moving band of 16 kDa with a short half-life (Valdovinos-Torres et al., 2008). A broad phosphorylated band of a calculated molecular weight of 17.5 kDa was also identified, and this could be the 17 kDa protein that due to the change in charge because phosphorylation is retarded in the SDS-PAGE. However, the lower molecular weight band of 16 kDa could not be explained by phosphorylation and it is suggested that an unidentified posttranslational modification could exist and generated the E7b isoform (Valdovinos-Torres et al., 2008). Until now, the only posttranslational modification identified for E7 has been the phosphorylation. However, immunofluorescence experiments in our laboratory have shown that a fraction of E7 is present in ER and Golgi apparatus (Fig. 4 and Valdovinos-Torres et al., 2008). This suggests that during the processing and transit of E7 through the different cell compartments, the protein could be posttranslationally modified.

According with the amino acid sequence of the HPV16 E7 protein, it contains putative posttranslational modification sites such as the Asp 29 that can be glycosylated, or modified by sulphation at Tyr 23 and 26, and phosphorylated in four other residues apart from the already known Ser residues 31 and 32. Until now, it is unknown if all these posttranslational modifications apply to E7 protein, but some of them could explain the different molecular weights found and their presence in different cell compartments.

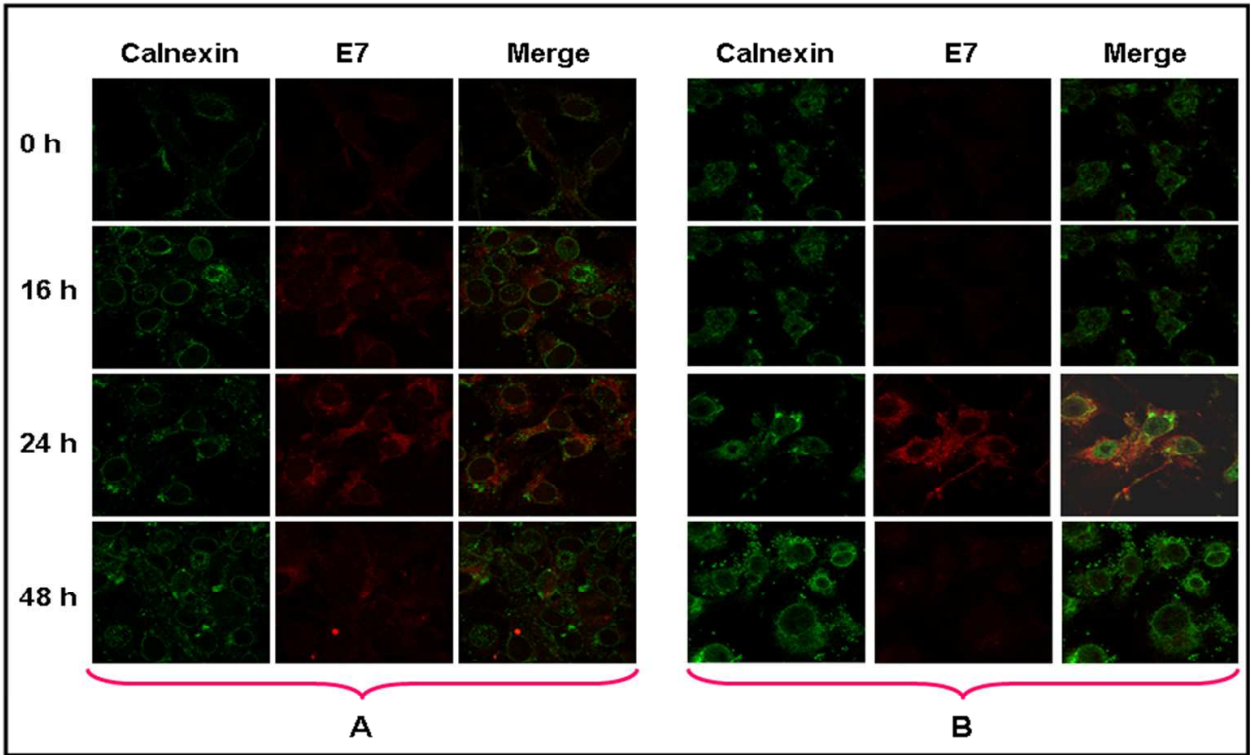


Fig. 4. Immunolocalization of HPV16 E7 protein. Cos-7 cells were transiently transfected with the pcDNA-E7 plasmid and chased for different times (0, 16, 24 and 48 h). Cells were fixed (4% p-formaldehyde) and permeabilized with 0.2% saponine. Cell preparations were tested with different anti-E7 antibodies produced in our laboratory (Valdovinos-Torres et al., 2008). **(A)** Cells were tested with D11 anti-E7 monoclonal antibody and with anti-calnexin polyclonal antibody as marker for ER. **(B)** Cells were tested with B4 anti-E7 monoclonal antibody and calnexin as in **A**. Secondary fluorescent antibodies were anti-rabbit IgG conjugated with Alexa 488 (green) or anti-mouse IgG conjugated with Alexa 594 (red). Images were taken at a magnification of 1000X using Confocal Microscope. The results showed that E7 protein is recognized by different antibodies in different cell compartments as the B4 monoclonal antibody recognized the E7 protein at the ER (co-localized with calnexin), while the D11 monoclonal antibody recognized a different isoform of E7 as no co-localization with calnexin was observed.

4.3.2 E7 functional properties

The E7 proteins are the major oncoproteins of HPVs that do not have known intrinsic enzymatic activities. Expression of HR-HPVs *E6* and *E7* genes in primary human keratinocytes is necessary for immortalization (Hawley-Nelson et al., 1989; Münger et al., 1989). The HPV16 *E7* gene encodes a multifunctional oncoprotein that can subvert multiple cellular regulatory pathways. Studies on mucosal HPV types showed that E7 deregulates the cell cycle mainly by binding to and promoting degradation of the tumor suppressor pRb protein (Caldeira et al., 2005; Collins et al., 2005), resulting in the dissociation of pRb from E2F transcription factors and the premature cell progression into the S-phase of the cell cycle. This activity is mediated by the LXCXE motif and the CR3 zinc binding domain of the E7 protein (Singh et al., 2005). However, E7 from HR-HPVs can also bind E2F1 and activates



E2F1-driven transcription independent of pRb (Hwang et al., 2002). It has been postulated that E7 binds and activates  $\mu$ -calpain (Darnell et al., 2007) to target pRb, that in turn is polyubiquitinated by cullin 2 (Huh et al., 2007) and degraded by the 26S proteasome through the interaction of E7 with the subunit 4 of the proteasome (Berezutskaya & Bagchi, 1997). In a similar way, E7 affects the pRb-related pocket proteins p107, p130 (Davies et al., 1993; B. Zhang et al., 2006) and the retinoblastoma protein associated factor p600 (Huh et al., 2005). However, cells try to defend themselves from the HPV infection through the expression or activation of molecules such as the transglutaminase 2 that inhibits pRb binding to HPV18 E7, by incorporating polyamine (J.H. Jeon et al., 2003) and PAI-2, and regulating ARF protein expression, which in turn protects pRb from the accelerated degradation mediated by E7 (Darnell et al., 2003). At the same time, the E7 oncoprotein is able to modulate the G2/M phase of the cell cycle by regulating the kinase of the histone H1 through a complex with the p107 protein, effect that allows progression of the viral cycle (Davies et al., 1993). It has also been shown that HPV16 E7 associates mainly with the cyclin A-E2F complex, or to cyclin E retaining their CDK2-associated kinase activity (Ghittoni et al., 2010; He et al., 2003). On the contrary, E7 inhibits the activity of CDK inhibitors p21<sup>Waf1</sup> (Funk et al., 1997; Jones et al., 1997), p27<sup>Kip1</sup> (Zerfass et al., 1996) and indirectly the p16<sup>INK4a</sup> (Giarrè et al., 2001). The regulation of all these cell cycle proteins by E7 may allow the activation of a subset of substrates important for the completion of the viral life cycle.

Apart from the already described E7-protein interactions, there are other groups of E7-target proteins that allow the modification of several different pathways such as metabolism, metastasis, transcription, apoptosis among others, and these subsets of E7-target proteins have been summarized in Table 1.

Finally, E7 has evolved to escape the immune-response by interfering with cytokines signaling pathways: abrogates the immune surveillance by binding to IRF-1 and preventing activation of the INF $\alpha$  and  $\beta$  promoters (Barnard & McMillan, 1999; Park et al., 2000), as well as represses the TGF- $\beta$ 2 promoter by releasing E2F from pRb (Lee et al., 2002b; Murvai et al., 2004). The cytotoxic response against HPV is also evaded by E7 through the down-regulation of TAP1, a key protein for peptide transportation from cytosol into the ER, reducing MHC I-dependent antigen presentation, impairing in this way a specific CTL response (Vambutas et al., 2001).

Cellular differentiation-dependent HPV life cycle has been difficult to study due to the lack of an *in vitro* culture system to differentiate epithelial cells efficiently. The recently development of xenographs and raft cultures have allowed the replication of HPV under *in vitro* conditions (Chow & Broker, 1997). And now, it is important that the activity of the oncogenic viral proteins be examined in their proper physiological contexts with complete viral genomes rather than relying on over-expression assays in different cell types.

## 5. Proteomics and genomics in the study of HPV

Recent advances in proteomics and genomics have allowed the study of modifications of profiles of cellular proteins and gene transcripts involved in a particular phenomenon compared to a normal status. In this way, Lee and coworkers (2004) identified a group of modulators regulated by E7 oncogene using proteomics and genomics technology. By using MALDI-TOF MS, 47 spots were identified in a HPV-negative cervical cancer cell line (C33A)

stably transfected with HPV16 E7. Proteins like disulfide isomerase A3, integrase interactor 1 protein, glutathione S-transferase P and *vav* proto-oncogene were down-regulated, whereas HSP60, Ku70 binding protein, 26S proteosome subunit were up-regulated. In the genomic approach using DNA microarrays the researchers showed that IL-12R $\beta$ , cytochrome c and TNF II were induced by E7 oncogene (K.A. Lee et al., 2004).

Activity Pathways subgroups	Proteins Modulated by HPV E7	References
1) Metabolism-related	Pyruvate kinase $\alpha$ -glucosidase	Zwerschke et al.,1999, 2000
2) Metastasis suppressors	Nm23-H1 Nm23-H2	Mileo et al., 2006
3) Transcription factors	TBP TAF-110 AP-1 family factors MPP2 SMAD3 MYC p48 from ISGF3 complex	Massimi et al., 1996 Mazzarelli et al., 1995 Antinore et al., 1996 Lüscher-Firzlauff et al., 1999 D.K. Lee et al., 2002b Y.W. Wang et al., 2007 Barnard & McMillan, 1999
4) Transcriptional coactivators	pCAF acetyltransferase FHL2 Skip IRF1 SRC-1 p300 TAF9	Avvakumov et al., 2003 Campo-Fernández et al., 2007 Prathapam et al., 2001 Park et al., 2000 Baldwin et al., 2006 Bernat et al., 2003 Enzenauer et al., 1998
5) Transcriptional repressors	E2F6	McLaughlin-Drubin et al., 2008
6) DNA modifying enzymes	DNMT1 BRG-1 from SW1/SNF complex	Burgers et al., 2007 D. Lee et al., 2002a
7) Histone-related	Mi2 $\beta$ from NURD complex	Brehm et al., 1999
8) Tumor suppressor	BRCA1 hTid1-TNF- $\alpha$ modulator	Y. Zhang et al., 2005b Schilling et al., 1998
9) Senescence regulators	DEK PML	Wise-Draper et al., 2005 Bischof et al., 2005
10) Anti-apoptotic	Mcl-1	Y.W. Cheng et al., 2008
11) Pro-apoptotic	PP2A Siva-1 IGFBP-3	Pim et al., 2005 Severino et al., 2007 Mannhardt et al., 2000
12) Miscellaneous	TAP-1 $\gamma$ -tubulin I $\kappa$ B kinase complex F-actin	Vambutas et al., 2001 Nguyen et al., 2007 Spitkovsky et al., 2002 Rey et al., 2000

Table 1. Subsets of E7-target proteins according to activity pathways.

On the other hand, Yim and coworkers (2004b) analyzed the genomic and proteomic expression patterns in 2 different HPV16 E6 transfected human carcinoma cell lines. They reported that among 1024 known genes and expressed sequence tags (ESTs) tested by cDNA microarray and by performing two dimensional gel electrophoresis and MALDI-TOF-MS, the authors found that only the genes and proteins of CDK5, Bak, and I-TRAF matched in both systems, the cDNA microarray and the proteomics. In addition, proteomic profiling of altered proteins by anti-cancer drugs on cervical cancer cells may contribute to provide the fundamental resources for investigation of disease-specific target proteins, elucidation of the novel mechanisms of action and development of new drugs (Yim & Park, 2006) as it has been analyzed with the 5-fluorouracil (5FU) drug (Yim et al., 2004a).

Other study with a genomic approach carried on by Garner-Hamrick and coworkers (2004), analyzed the mRNA from primary human keratinocytes infected with retroviruses that expressed the HPV18 E6 and E7 genes and used to generate probes for querying Affymetrix U95A microarrays, which contain >12,500 human gene sequences. The results showed that HPV18 E6/E7 expression significantly altered the expression of 1,381 genes. A large increase of transcripts associated with DNA and RNA metabolism was observed, with major increases noted for transcription factors, splicing factors and DNA replication elements, among others. Multiple genes associated with protein translation were down-regulated. In addition, major alterations were found in transcripts associated with the cell cycle and cell differentiation.

This review does not include an exhaustive search of all gene transcripts or proteins which expression pattern is altered in each report. In Tables 2 and 3 were summarized only those important genes or proteins that were strongly associated to the phenomenon studied.

HPV Gene	Research Focused on	Concluding Remarks	Reference
HPV16			
E6/E7	Anti-tumor immunity	Down-regulated: MCP-1, osteopontin and midkine.	Smahel et al., 2005b
E6/E7	Chromosome alterations	HPV16-negative tumors had loss at 18q12.1-23 but gain in HPV16-positive tumors.	Smeets et al., 2006
E6/E7	Chromosome amplifications	Duplication of chromosome 5q and 20q and macrodeletions in chromosomes 6q and 20q.	Ramirez et al., 2004
E6, E7	Chromosome amplifications	Genes (DNA metabolism) up-regulated: 6 in chromosome 20q and 25 in chromosome 5.	Klingelhu tz et al., 2005
E6/E7	Differentiation	Blocked or delayed differentiation. Alteration in TGFβ expression / TGFβ inducible genes.	Nees et al., 2000
E6/E7	Differentiation	Altered expression of 80 cellular genes: INF-responsive genes, NF-kB stimulated genes, cell cycle progression and DNA synthesis.	Nees et al., 2001

HPV Gene	Research Focused on	Concluding Remarks	Reference
E7	Effect of E7 on p53 stability	E7 can modulate normal turnover of p53. No transcriptional consequences on p53 targets.	Eichten et al., 2002
E6/E7	Episome integration	53 genes up-regulated: IFN modulators, EGFR, cytoskeleton proteins, and 32 genes down-regulated during episomal integration.	Alazawi et al., 2002
E6/E7	Episome loss and integration	Activation of antiviral response genes MX1, MX2, OAS1, TRIM22, GIP3 and IRF7.	Pett et al., 2006
E6	Gene expression	Up- or down-regulated genes: 85 in RKO and 70 in A549 cells. Genes and proteins matched: CDK5, Bak and I-TRAF.	Yim et al., 2004b
E6/E7	Gene expression	Expression of 13 genes from HPV16-positive cell lines is down-regulated.	Ruutu et al., 2005
E7	Host cell effectors interaction	E7 interacts with Siva-1 factor.	Severino et al., 2007
E6/E7	Immortalization	Over-expression of IGFBP-3 is a late event after E6/E7 expression in the infected cells.	Berger et al., 2002
E6/E7	Immortalization and progression	HPV immortalized EGFR null cells have elevated levels of mRNAs of p21 <sup>Waf1</sup> and insulin-like growth factor-binding protein-2 (IGFBP2).	Woodworth et al., 2000
E7	Immuno-surveillance	Induction of IL12R $\beta$ 1, cytochrome c and TNFR type II.	K.A. Lee et al., 2004
E6, E7	miRNA in carcinoma	Up-regulation of miR-363 and down-regulation of miR-181a, mR-218, and miR-29a.	Wald et al., 2011
E6/E7	Oncogenicity and metastases	Down-regulation of CKIs (p57 and p16) and up-regulation of S100 proteins (A6, A10) might be involved in the increase of oncogenicity.	Smahel et al., 2005a
E6/E7, E7	Regulation of cellular genes	54 up- or down-regulated genes involved in DNA damage, differentiation, signal transduction, immune response, cell-cycle.	Duffy et al., 2003
E7	Senescence	Associated genes: BRAK, DOC1 and IGFBP-3 but down-regulated in immortalization.	Schwarze et al., 2002
E6/E7	Tumor angiogenesis	Down-regulation thrombospondin-1, maspin and up-regulation of IL-8 and VEGF.	Toussaint et al., 2004
HPV 18			
E6	Effects of E6 gene knockdown	359 genes up- or down-regulated. Cell cycle: p21; apoptosis: CASP4, CASP6, IGFBP3; ubiquitin proteolysis pathway: UBE3A; differentiation: KRT4, KRT6E, KRT18; anti-oncogenes RECK, VEL.	Min et al., 2009



HPV Gene	Research Focused on	Concluding Remarks	Reference
E6/E7	E2-induced senescence	E2 induces senescence by up-regulating 10 genes (p21) and down-regulating 24.	Wells et al., 2003
E6/E7	Gene expression	Affects DNA synthesis genes: MCM, cdc6, cdc7, PCNA. Regulators: cdk 1-2, CycB, Wee1, CycE. Centrosome abnormalities: Bub1, TTK.	Garner et al., 2004

Table 2. Genomics in HPV research.

HPV Protein	Research Focused on	Concluding Remarks	Reference
HPV 16			
E6	Chemotherapeutic agents sensitization	Cdc2 is the most dramatically up-regulated protein.	Z.G. Liu et al., 2007
E5	Early cervical cancer markers related to E5	Both EGFR and TfR assays detected HSIL with very high accuracy (100% and 96.3%, respectively).	Keese et al., 2002
E6/E7****	Expression levels for protein features	This large-scale analysis provides a framework for understanding the cooperation between oncoproteins in HPV-driven carcinogenesis.	Merkley et al., 2009
E5 */**	Expression of membrane proteins	Decreased amount of calnexin and increased hsp70 expression, both associated to MHC-I processing.	Leykauf et al., 2004
E5/E6/E7	HPV proteins immunogenicity	E7 proteins of HR-HPV types are more reactive in cancer patients and discriminating between cancer and HSIL or LSIL patients.	Luevano et al., 2010
E7 */***	Immunosurveillance	36 proteins down-regulated and 11 up-regulated including Ku-70 binding protein involved in DNA metabolism and hsp60 KD protein 1.	K.A. Lee et al., 2004
E6 */***	Protein expression	In RKO and A539 cells: 26 up- and down-regulated proteins. Only three proteins matched with its corresponding gene: CDK5, Bak and I-TRAF.	Yim et al., 2004b
E7*/***	Proteins modulated by E7	Two down-regulated proteins: actin and leukocyte elastase inhibitor and 26 proteins up-regulated, amongst these: catalase and peroxiredoxin.	K.A. Lee et al., 2005
E6/E7 */***	Proteins modulated by E6, E7 or E6/E7	Annexin III, gp96, transaldolase 1, elongation factor 1, proteasome 26S were up-regulated and have been confirmed at the transcriptional level.	Ciotti et al., 2009

HPV Protein	Research Focused on	Concluding Remarks	Reference
E7 */***	Resistance to oxidative stress-induced apoptosis	E7 induces higher resistance to ROS-induced cell injury, probably via the modulation anti-oxidant enzymes, including catalase and peroxiredoxin.	Shim et al., 2005
HPV 18			
E6/E7 */***	Anti-cancer effect of 5-fluorouracil treatment	22 proteins up-regulated (CIDE-B, caspase-3, caspase-8, Apo-1/CD95 (Fas) and 12 proteins down-regulated (BUB3, c-myc protein, src substrate cortactin, transforming protein p21A, among others).	Yim & Park 2006; Yim et al., 2004a
E6/E7 */***	Anti-cancer effect of paclitaxel treatment	Paclitaxel showed anti- proliferative activity through the (DR)-mediated apoptotic pathway with TRAIL-dependent caspase-8 activation and the mitochondrial-mediated pathway with down-regulation of bcl-2 by cytochrome c release.	K.H. Lee et al., 2005; Yim & Park 2006

Used systems: \* 2-DE: two-dimensional electrophoresis; \*\* nanoESI-MS: nanoelectrospray ionization mass spectrometry; \*\*\* MALDI-TOF-MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry; \*\*\*\*2D-DIGE: two-dimensional difference gel electrophoresis.

Table 3. Proteomics in HPV research.

Working with genomics and proteomics is a great challenge as the following issues must be overcome: 1) The use of a standard genomic chip or an appropriate cell line has not been achieved, leading to results not easily comparable between different laboratories; 2) Many genes can be down-regulated or up-regulated during transcriptional profile analysis of a particular phenomenon, but this may not correspond necessarily at all with the proteomic profile; 3) Only a lesser number of proteins actually are modified as consequence of an alteration in the expression of the related gene; 4) Changes in gene expression may predict changes in protein expression, but not necessarily changes in functionality that in most cases is related to protein posttranslational modifications; 5) There are many human genes in data banks that codify for hypothetical proteins which activity or function are not yet known. Therefore, the expression of selected genes needs to be confirmed by means of other kind of procedures like real-time RT-PCR and the corresponding protein analysis by Western blot or in functional assays. In this regard, only two reports showed that some of the proteins studied with altered gene expression matched with the altered protein expression levels (Ciotti et al., 2009; Yim et al., 2004b). More remains to be done in genomics and proteomics, because once all the proteins modified in their expression have been characterized, the researchers need to investigate the way by which the proteins interact with each other, the sequence in which those interactions take place, and finally to establish the mechanism through which cervical cancer develops. This information is of great value when anti-cancer drugs are assayed to attack tumor cells, because the identification of the cellular targets of

the drug will help to design better molecules to induce specific malignant cell death. In the case of HPV, these studies are focused on the research of the proteins or genes that are altered by the presence of HR-HPV in the cell. In this sense, the identification of a biomarker to design chips would be useful in high-throughput screening test for uterine cervical cancer (Kim et al., 2006; Steinau et al., 2005), early diagnosis and prediction of response to therapy.

## 6. Conclusions

Intense work has been done to elucidate the molecular mechanisms through which the HPV E5, E6 and E7 oncoproteins generate cellular transformation. At the same time, it has become clear that the HPV oncoproteins use and modify different signal pathways, specially those related to cell growth, differentiation and apoptosis. The study of the transformation process associated to HPV has given some light about target proteins and disturbed mechanisms that could be considered for the design of drugs, which in the future would be specifically generated for the treatment of HPV associated cancers.

Early detection remains one of the most important issues in cervical cancer research. Therefore, intensive screening to search for biomarkers (genes and/or proteins) particularly sensitive to differences between early and late stage cancer patients is the main target of the new technology. The development of the genomics and proteomics in cervical cancer associated to HPV infection will help in the identification of most accurate biomarkers for an automated early detection of this kind of cancer, as this approach allows the correlation of changes in host gene expression with the biological functions of viral genes. Although proteomics and genomics have simplified the analysis of a great quantity of genes or proteins that are modulated by the presence of HPV oncoproteins in a cell, a major concern has always existed as to whether the discovered biomarkers and the derived multivariate models are truly associated with the disease process. There is still much to do regarding to how these genes or its codified proteins are interconnected and which of them are actually important to the carcinogenic process.

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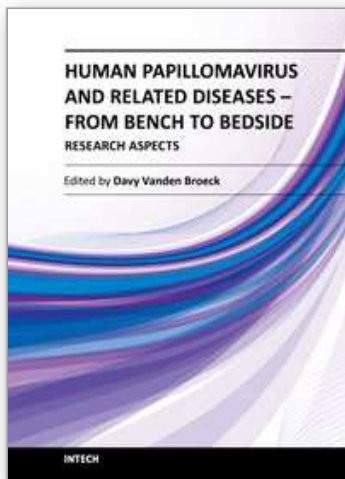


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## **Human Papillomavirus and Related Diseases - From Bench to Bedside - Research aspects**

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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on epidemiological and fundamental research aspects in the area of HPV, and it will update those working in this fast-progressing field with the latest information.

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