



Universiteit
Leiden
The Netherlands

Biological properties of the oncoproteins E6 and E7 from mucosal and cutaneous HPV types

Dong, W.L.

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1. Introduction

The Papillomaviruses (PVs) are carcinogenic DNA tumour viruses that are classified as the *Papillomaviridae* family. These viruses are widespread in nature and infect higher vertebrates (e.g. human, rabbit, horse, dog, sheep, deer, elk, bovine and primates) but have also been found in reptiles and birds and induce papillomas or warts, which can progress into a malignancy. There are over 300 different PV types, including possibly 200 different human papilloma viruses (HPVs), and it is likely that more types will be found. The HPVs can be further subdivided based upon their tissue-tropism into mucosatropic and cutaneotropic HPVs.

An association between human papillomavirus and cervical cancer was first proposed in the 1976 (zur Hausen, 1976). Since then, intensive studies on PVs have led to an extensive characterisation of HPVs, which confirmed the role of some mucosal HPV types called high risk types as a causative agent in human carcinogenesis, particularly cancers of the mucosa of the genital tract. All of the currently known so-called “high-risk” HPV types belong to the genus Alpha of the phylogenetic tree.

Much of the data available on HPVs has been obtained from studies done on mucosal types and still little is known of the types that infect the skin and their possible involvement in carcinogenesis. Only recently, epidemiological studies have shown the presence and recurrence of cutaneous HPV types of the genus Beta in skin tumours and functional studies have started to elucidate their molecular mechanisms in cellular transformation.

This general introduction will give a comprehensive review of what is known about the mucosal and cutaneous HPV types and their role in carcinogenesis.

2. Papillomaviridae

Papilloma viruses constitute a very heterogeneous group of viruses, which were traditionally grouped with the polyomaviruses in the family of Papovaviridae. In the middle of the 1980s it became apparent that polyoma- and papillomaviruses have different genome sizes (+/- 5kb vs. 8 kb), different transcriptional strategies and poor homology between proteins of the 2 viruses. But it wasn't until 2004 that the family *Papillomaviridae* was formally recognized by the International Committee on taxonomy of Viruses (ICTV) (De Villiers *et al.*, 2004).

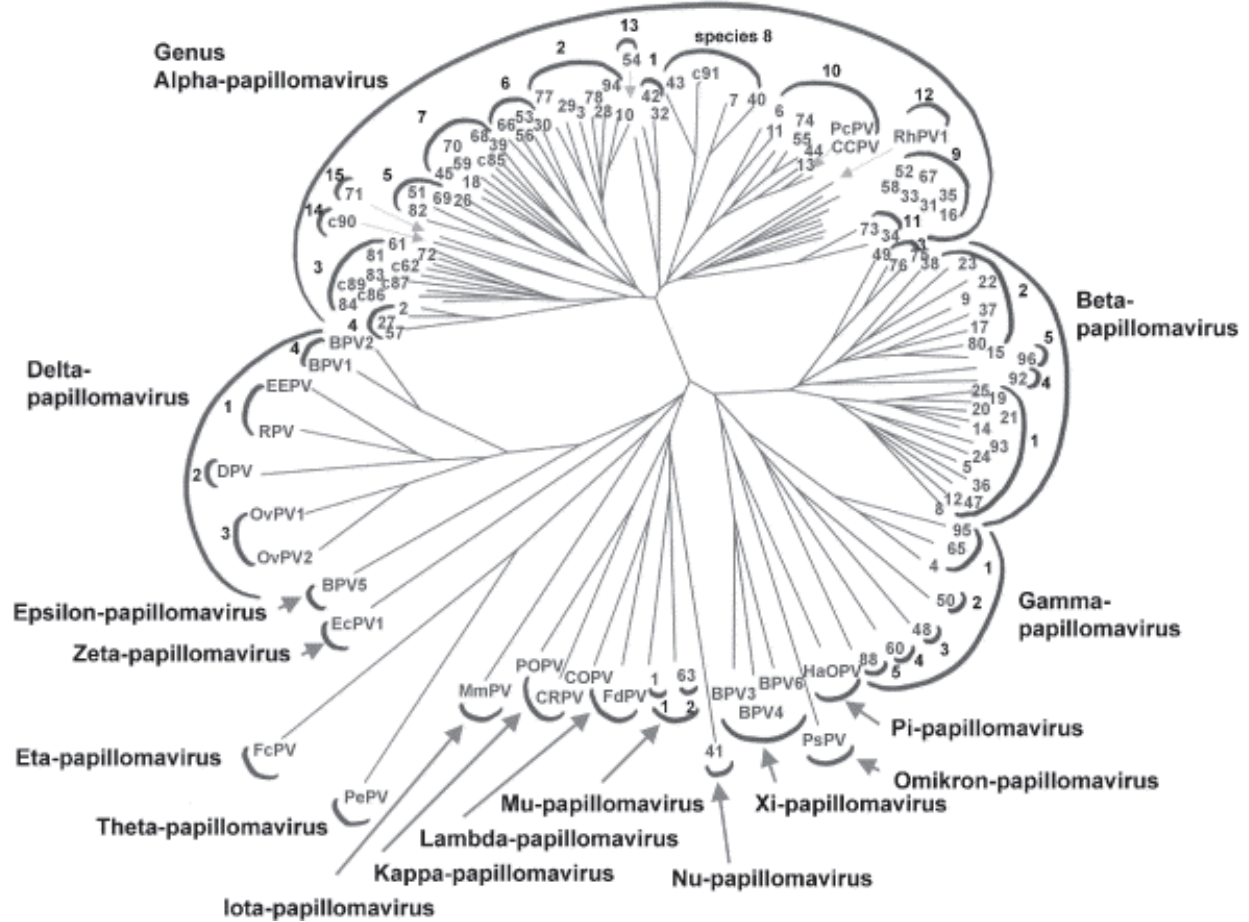


Figure 1. Phylogenetic tree containing the sequences of 118 papillomavirus types. The L1 ORF sequences were used in a modified version of the Phylip version 3.572 and based on a weighted version of the neighbour-joining analysis. The tree was constructed using the Treeview program of the University of Glasgow. The numbers at the ends of each of the branches identify an HPV type; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. The outermost semicircular symbols identify papillomavirus genera, e.g. the genus alpha-papillomavirus. The number at the inner semicircular symbol refers to papillomavirus species. For instance, the HPV types 7, 40, 43, and 91 together form the HPV species 8 in the genus alpha-papillomavirus. *Reprinted from Classification of papillomaviruses. de Villiers et al., 2004, with permission from Elsevier.*

PVs have a very similar genomic organization and any pair of two PVs contains at least 5 homologous genes, although the nucleotide sequence may diverge by more than 50% (Bernard, 2006). Phylogenetic algorithms that compare the homologies have led to the hypothesis that PVs normally evolve with their respective host species and do not cross host species, do not recombine and have maintained their basic genomic organization for a period exceeding 100 million years. Further sequence comparisons have laid the foundation for PV taxonomy and the formation of a phylogenetic tree for the different PV types identified up to date. The current phylogenetic tree (Figure 1) of PVs has been designed based on the

nucleotide sequence of their respective L1 capsid protein. Two HPV genomes are considered to be separate types when the nucleotide sequence of their L1 genes has more than 10% difference (De Villiers *et al.*, 2004). However, as more HPV types will be identified in mammals and possibly other vertebrates in the future it is clear that this tree will need refinement in the future.

3. Papillomavirus research, a history

The viral aetiology of common warts in humans was first indicated in the turn of the 20th century by Ciuffo, who reported the transmission of common warts using intradermal inoculation of cell free filtrates (Ciuffo, 1907). The first papillomavirus was described in 1933, when Richard Shope identified the cottontail rabbit papillomavirus (CRPV) as the etiologic agent responsible for cutaneous papillomatosis in the cottontail rabbit (Shope, 1933). Following the demonstration that papillomas in domestic rabbit could undergo malignant conversion (Rous and Beard, 1934; Rous and Beard, 1935). Strauss *et al.* investigated the nature of the infectious agent responsible for the induction of warts in 1949 and were the first ones to detect the viral particles in human warts by electron microscopy (Strauss *et al.*, 1949). Another important step in PV research was made by Crawford and Crawford who characterized the physical properties of the viral DNA (Crawford and Crawford, 1963). However, the characterization of the viral life cycle and the natural history of virus infection were and still are hampered by the difficulty of amplifying the viruses in *in vitro* cell culture systems.

An association between human papillomavirus infection and cervical cancer was first proposed in 1976 by H. zur Hausen (zur Hausen, 1976), but was disputed due to the failure of regular skin warts to progress to malignancies. These inconsistencies were overcome by reports of the heterogeneity of HPVs (Gissman *et al.*, 1977) and by reports that sera from skin warts failed to react with virus particles from genital warts. In the beginning of the 1980s novel HPVs were identified in cervical cancer biopsies (Boshart *et al.*, 1984; Dürst *et al.*, 1983) using hybridization protocols described by Law *et al.* (Law *et al.*, 1979). Transforming activity in cell culture was first demonstrated for the whole HPV genome (Dürst *et al.*, 1987) with the subsequent identification of the importance of the products of the early genes, E6 and E7, for maintaining the transformed phenotype.

The characterization of the E6 and E7 protein facilitated the understanding of the role of tumour suppressor proteins in cell cycle and apoptosis control, such as p53 and pRb. In the last few decades the papillomavirus field has evolved rapidly, leading to confirmation the role of HPV in ano-genital cancers and identification of the high risk HPV types involved.

Despite the association between HPV and ano-genital cancers, it has been long disputed whether HPV also plays a role in skin carcinogenesis. Only recently did the association between HPV and skin cancer become more widely accepted when it was shown that there was a high prevalence of HPV in NMSC. However, due to the presence of a multitude of HPV types in skin lesion and the presence of these HPV types in healthy skin, the identification of a potential high risk cutaneous type has been difficult (Harwood *et al.*, 2000; 2004; Pfister, 2003). Up to date several potential high risk HPV skin types have been identified using biochemical studies to address the *in vitro* properties of E6 and E7 (Caldeira *et al.*, 2003; Akgul *et al.*, 2003, 2005). These studies have shown that some cutaneous HPV types have the potential to transform cells, but the exact mechanism of transformation appears to be different and more studies will be required to fully understand the role of HPV in skin carcinogenesis.

4. Human papillomaviruses

So far, 92 HPV types have been fully sequenced, of which 60 belong to the alpha genera and the other 32 belong to the beta and gamma genera. However, more than 120 putative novel types belonging to the beta and gamma genera have been partially characterized (zur Hausen, 2000). HPVs are strictly epitheliotropic and

Table 1. Some examples of identified HPV genotypes and their origin.

HPV GENOTYPES	ORIGINATED FROM
Cutaneous HPV GENOTYPES	
1	plantar warts
2,4,26,27,29	common warts
3,10,28	flat warts
5,8,47	benign and malignant EV lesions
9,12,14,15,17,19-25, 50	EV lesions
38	melanoma; malignant cutaneous lesions
41, 48	cutaneous squamous cell carcinomas
49	flat warts under immuno-suppression
75-77	common warts in renal allograft recipient
Mucosal HPV GENOTYPES	
6,11	genital warts, laryngeal papillomas
13	oral focal epithelial hyperplasia's
30	laryngeal carcinomas
32	oral focal epithelial hyperplasia's; oral papillomas
16,18,31,33,35,39,45,51,52,56, 58,59	anogenital intraepithelial neoplasia and cancer
34,40,42-44,53-55,61,62,64,67-69,71,74	anogenital intraepithelial neoplasia
72,73	oral papillomas (HIV patients); anogenital intraepithelial neoplasia's

EV= *Epidermodysplasia verruciformis*; HIV= human immunodeficiency virus

infect epithelial cells of the skin or of the anogenital and oropharyngeal mucosa. Based on their tissue-tropism HPVs can be divided into cutaneous or mucosal HPV types. Examples of several HPV genotypes and their associated lesions and tissue-tropism are given in table 1.

The mucosatropic papillomaviruses make up most of the alpha genera, whereas the beta and gamma genera consist of cutaneous HPV types. The beta group consists of so-called EV-HPVs, which were first isolated from patients with Epidermodysplasia verruciformis (EV), a rare hereditary disease, and the gamma PV types consist mostly of PVs which are phylogenetically related to the beta HPV types.

4.1. Mucosal HPVs and cancer

Studies on the natural history of HPV have revealed that they are the most common sexually transmitted infections in most populations. A large and consistent group of studies have given evidence that HPV infections is the main aetiological factor for development of lesions or cancers of the anogenital tract, including the cervix, vagina, anal canal and vulva penis (International Agency for Research on Cancer, 1995). Cancer of the uterine cervix is considered the second most common cancer worldwide in women and is responsible for approximately 200 000 deaths per year with an approximate 500 000 new cases found every year worldwide (Parkin *et al.*, 2001).

Biological and epidemiological data from the mucosal HPV types have allowed the division of these types into two groups; the so-called low-risk HPV types, like HPV6 and 11, which are rarely associated with cancers and the high-risk HPV types, e.g. HPV16 or 18, which are preferentially associated with benign and malignant anogenital lesions (reviewed in zur Hausen, 1997). Within the high-risk

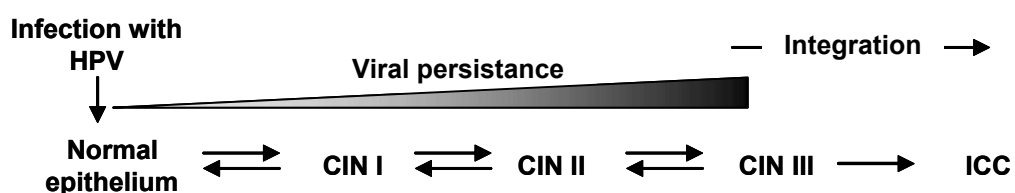


Figure 2. Different stages of conversion from HPV infection to ICC

Upon infection with the virus, the site of infection can develop into a benign wart or lesion. Viral persistence can lead to cervical intraepithelial neoplasia (CIN) in the cervix; these can however regress again due to clearance of the viral infection and defective cells by the cellular defence mechanism and the immune system. However, it is also possible that the lesion will develop into an invasive cervical carcinoma (ICC).

family, HPV16 and HPV18 are the most commonly detected genotypes in cervical lesions and are responsible for approximately 50% and 25% of the cervical cancer cases worldwide, respectively (Matsukura *et al.*, 1995; Zehbe *et al.*, 1996 and 1997). Penile, vulvar, anal and peri-anal carcinomas have been analysed only to a certain extent but it is conceivable that high risk mucosal HPVs also play a central role in the pathogenesis of these cancers (Melbye and Frisch, 1998). In addition, HPV infection of the upper airway appears to be associated with a subset of head and neck squamous cell carcinomas (HNSCC) (Gillison *et al.*, 2000).

The low-risk types are mainly associated with genital warts or condylomas, but also with papillomas in the oral cavity or the larynx. Since the histology of the oral mucosa resembles the one of the anogenital tract it is not surprising that we find both high and low-risk HPV types in oral lesions. Accordingly, oral squamous cell carcinoma, condyloma, verruca, and focal epithelial hyperplasia (FEH) are associated with HPV infection. Interestingly, some HPV types, like HPV32 or 13, appear to be confined to the oral cavity and not to the genital tract.

Whereas most infections will result in a spontaneous clearance without any clinical manifestations a small fraction of the infected individuals will retain the virus and develop lesions that will progress to pre-invasive or invasive tumours. Persistent HPV infection with a high-risk virus is essential and observed in cervical intraepithelial neoplasia (CIN) II and III stages (Ho *et al.*, 1995; Koutsky *et al.*, 1992). There is sufficient morphological and epidemiological consensus for the assumption that CIN III is a dynamic disease in, which some cases regress spontaneously, whereas others progress to invasive cervical carcinoma (ICC) (Gustafsson and Adami 1989; Ostor 1993; Moreno *et al.* 1995). The rate of progression of the disease from CIN III to ICC varies from approximately 12 to 69%, according to various reported studies. It can be concluded that tumour formation is not an inevitable consequence of viral infection; it rather reflects the multi-step nature of carcinogenesis where each step constitutes an independent (reversible or irreversible) genetic change that cumulatively contributes to deregulation of cell cycle, cell growth and survival. HPV infections represent one of these steps and a cancer might only develop if the other steps also occur in the same cell (Figure 2).

4.2. Non melanoma skin cancer and cutaneous HPVs

Non-melanoma skin cancer (NMSC) is the most common cancer in the Caucasian adult populations (Pisani *et al.*, 2002) which outnumbers all other cancers and comprises two main histological types; basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The incidence of NMSC is rising continuously across the globe mainly due to the aging of the population and the migration of people with

sun-sensitive skin to subtropical regions. In the United States, approximately one million new NMSC cases are reported every year and increased NMSC incidence has been observed in other regions such as Europe and Australia (Greenlee *et al.*, 2000; Kiviat, 1999; Marks, 1995). Although NMSC has a good prognosis and is not normally associated with mortality the increased incidence of other invasive cancers and cancer mortality in individuals with a history of NMSC have been reported (Frisch and Melbye, 1995; Kahn *et al.*, 1998; Levi *et al.*, 1998; Levi *et al.*, 1997). In addition, the economic burden is increasing, as progressively more patients require treatment and costly operations.

Risk factors for NMSC development include UV exposure, fair skin, fair eyes, advancing age, impairment of the immune system (Pfister, 2003) and although still under heavy debate, more evidence is rising for a possible causal relationship between beta, but not gamma, HPV types and NMSC

The first evidence for a possible involvement of HPV in human skin cancer was demonstrated in patients with a rare hereditary disease called *Epidermodysplasia verruciformis* (EV). These patients develop warts which have a high-risk of progressing into SCC and HPV DNA is found in nearly all SCC. Additional evidences strongly supporting the association of cutaneotropic HPV types with NMSC in non-EV patients came from studies using very sensitive polymerase chain reaction (PCR) methods, which demonstrated the presence of HPV DNA in a high frequency in skin lesions from renal transplant patients under immunosuppressive treatment. The same results can be found in other patients with immunological disorders, like psoriasis and transplant patients, who also have an increased incidence of HPV infection and progression to NMSC.

However, although there is a high incidence of cutaneous HPV DNA in skin lesions, no difference in prevalence or in spectrum of HPV types was observed in healthy skin (Harwood *et al.*, 2004). In addition, although HPV DNA can be detected in NMSC, the very low copy number (far less than one HPV DNA copy per cell) in NMSC biopsies (Pfister, 2003) and *in situ* hybridization techniques indicate that only a minority of the tumour cells contain viral DNA. This could suggest that, unlike in lesions related with mucosal HPV infections, HPV DNA and viral gene expression might not be necessary to maintain the malignant phenotype in cutaneous lesions.

This data however is compatible with the hypothesis that cutaneotropic HPV types are possibly more important for tumour initiation than for its progression, the so-called hit-and-run mechanism. Evidence supporting this hypothesis is found in studies on BPV4, where BPV DNA is detected in pre-malignant warts, but no viral DNA was usually detected in tumour material (Campo *et al.*, 1985). In addition, the malignant conversion occurred only in animals that were exposed to mutagens and/or immunosuppressive agents (Campo *et al.*, 1994). This supports the

assumption that there is a special need for activators and additional risk factors in skin lesions. As the majority of NMSC occurs mainly on sun-exposed body sites, UV radiation, and especially UV-B, is considered the major environmental risk-factor in the pathogenesis of these tumours (Ananthaswamy *et al.*, 1997; Armstrong and Kricger, 2001; Preston and Stern, 1992). Interestingly, some cutaneous HPV types are strongly activated by UV light through UV induced p53 accumulation (Purdie *et al.*, 1999; Akgul *et al.*, 2005) and it is possible that in turn expression of the viral genes could inhibit the cellular defence mechanism against UV induced cellular damage.

4.2.1. The *Epidermodysplasia verruciformis* human papillomavirus types

The *Epidermodysplasia verruciformis* or EV HPV types comprise the largest and most variable group in the cutaneotropic HPV group. They were first isolated in cancer-prone patients suffering from a rare autosomal recessive genetic disorder called *Epidermodysplasia verruciformis* (EV), characterized by a defect in cell-mediated immunity leading to abnormal susceptibility to widespread and persistent HPV infection of the skin (Orth *et al.*, 1978; Orth *et al.*, 1979). Due to their abnormal susceptibility to HPV infection, EV patients develop disseminated pityriasis versicolor-like lesions and flat warts (Jablonska *et al.*, 1972; Majewski *et al.*, 1997; Orth *et al.*, 1979). These skin lesions differ from normal warts and appear as red, brown or achromic plaques. They arise early in life and represent a risk for SCC development. Indeed, in approximately 30–60% of cases, HPV lesions develop into multifocal SCC at sun-exposed regions. HPV types 5 or 8 are the most frequently detected in skin lesions of EV patients, but types 14, 17, 20 and 47 are also occasionally found.

EV was recently linked to mutations in either of two related genes EVER1 and EVER2 (also termed TMC6 and TMC8, respectively) (Ramos *et al.*, 2002). The function of the proteins encoded by these genes is still unknown, but analysis of their primary structure has revealed that they are likely to be integral membrane proteins (Ramos *et al.*, 2002), which are likely to be involved in controlling the immune response to viral infections and their lack may favour HPV persistence. The hypothesis that impairment of the immune system favours the development of EV HPV-associated diseases is also supported by other recent findings. After haematopoietic stem-cell transplantation, individuals with a rare primary deficiency of T-cell- and B-cell-mediated immunity are prone to chronic HPV infection and to develop skin lesions similar to those found in EV patients (Laffort *et al.*, 2004). Immuno-suppressed organ transplant recipients have a 50–100-fold increased risk of developing NMSC compared to the general population. NMSCs occur 10–20

years earlier in immuno-suppressed individuals than in immuno-competent ones (Boyle *et al.*, 1984; Kiviat, 1999; Walder *et al.*, 1976; Walder *et al.*, 1971). The cumulative incidence of skin cancer in patients under immunosuppressive treatment for 10–25 years is approximately 30–40%. The link with immune status strongly supports the role in NMSC of an infectious agent such as the cutaneous human papillomaviruses (HPVs), in particular those belonging to genus beta of the HPV phylogenetic tree (de Villiers *et al.*, 2004).

Up to date the genome of 25 EV HPV types have been fully sequenced, namely 5, 8, 9, 12, 14, 15, 17, 19–25, 36–38, 47, 49, 75, 76, 80, 92, 93, 96, but it is certain that many more EV types exist and new, previously uncharacterized EV HPV types continue to be reported. Several epidemiological studies have tried, in the past 10–15 years, to assess the prevalence of different EV HPV types in specific cutaneous lesions and their possible role in the development of non melanoma skin cancer (NMSC) in non EV patients. However, the progress has been hampered by the high heterogeneity of EV HPV types, the presence of more than one EV HPV type in a skin lesion and the lack of a universal protocol for their detection. In fact, independent investigations have used different HPV typing methods and obtained inconsistent results with a wide spectrum of HPV types detected within a skin lesion.

4.2.2. Prevalence of HPV types in NMSC of non-EV individuals

EV HPV DNA can be found in healthy skin of immuno-compromised or immuno-competent individuals (Antonsson *et al.*, 2000; Astori *et al.*, 1998; Berkhout *et al.*, 2000; de Jong-Tieben *et al.*, 2000; Forslund *et al.*, 1999; Harwood *et al.*, 2004; Stark *et al.*, 1994). A wide variety of skin HPV types was found in skin swabs from five different sites of renal transplant recipients, dialysis patients and healthy individuals (Antonsson *et al.*, 2000), but no difference in prevalence or in spectrum of HPV types was observed in healthy skin from sun-exposed and non-exposed sites (Harwood *et al.*, 2004). These findings show that the EV HPV types are highly ubiquitous in healthy skin. However, comparative studies of EV HPV DNA prevalence in healthy skin of individuals with or without NMSC have shown that history of NMSC is significantly associated with EV HPV types, but not with other cutaneous HPV types that are normally responsible for benign skin lesions (Harwood *et al.*, 2004). Boxman and colleagues have shown that EV HPV DNA can be detected in plucked eyebrow hair, suggesting that hair follicles are a natural reservoir of the virus. However, the prevalence of EV HPV DNA in plucked eyebrow hairs in patients with solar keratoses (Boxman *et al.*, 1997; 1999; 2000; 2001) or SCC (Struijk *et al.*, 2003) is higher than in healthy individuals. and DNA from

several EV HPV types was found in 70–80% of NMSC in immuno-suppressed patients (Arends *et al.*, 1997; Berkhout *et al.*, 2000; de Villiers *et al.*, 1997; Ferrandiz *et al.*, 1998; Harwood *et al.*, 2000; Hopfl *et al.*, 1997; Meyer *et al.*, 2003; Shamanin *et al.*, 1996; Stark *et al.*, 1994; Stockfleth *et al.*, 2004; Tieben *et al.*, 1994).

Several studies have convincingly proved that the DNA of EV HPV types is also present in NMSCs of immuno-competent individuals, although at a lower rate than in immuno-suppressed patients (up to approximately 50%) (Arends *et al.*, 1997; Astori *et al.*, 1998; Biliris *et al.*, 2000; Boxman *et al.*, 2000; Caldeira *et al.*, 2003; Ferrandiz *et al.*, 1998; Forslund *et al.*, 1999; Harwood *et al.*, 2000; Iftner *et al.*, 2003; Meyer *et al.*, 2003; Pfister and Ter Schegget, 1997; Shamanin *et al.*, 1996; Stockfleth *et al.*, 2004). In immuno-suppressed patients often more than one EV HPV type was found in the same skin lesion and a few epidemiological studies based on serological tests found that antibodies against the major capsid protein L1 of EV HPV8 are more prevalent in immuno-compromised patients, who have high-risk of skin cancer, than in the general population (Stark *et al.*, 1998). In addition, seroreactivity to L1 virus-like particles of EV-HPV types 8 and/or 38 is significantly increased in immuno-competent individuals with SCC (Feltkamp *et al.*, 2003; Masini *et al.*, 2003; Stark *et al.*, 1998).

4.2.3. Psoriasis and EV HPV types

A few recent studies have shown that EV HPV DNA can be detected in up to 90% of skin samples from patients with psoriasis, a T-cell-mediated immunological disorder characterized by epidermal proliferation (Favre *et al.*, 1998; Mahe *et al.*, 2003; Weissenborn *et al.*, 1999; and reviewed in Pfister, 2003). The most commonly detected types are HPV5 and HPV36, but other members of the genus beta have also been found, *e.g.*, HPV38. DNA of genital HPV types was detected only sporadically in these skin specimens. In addition, the cutaneous HPV1, which is exclusively associated with benign skin lesions, was clearly less prevalent than the EV HPV types in psoriatic skin (Favre *et al.*, 1998).

Although, serological data have confirmed the association between HPV5 and psoriasis (Favre *et al.*, 1998), the role of the EV HPV types in the pathology of psoriasis is still uncertain. In fact, it is not yet clear whether the HPV is directly involved in the disease or is merely taking advantage of the increased keratinocyte proliferation that occurs in psoriatic skin to efficiently complete its life cycle.

Psoriasis is treated by photo-chemotherapy, which consists of administration of the photosensitising compound psolaren followed by UVA radiation (PUVA). However, long term treatment represents a risk factor for the development SCC

(Bruynzeel *et al.*, 1991; Chuang *et al.*, 1992; Forman *et al.*, 1989; Lindelof *et al.*, 1991; Stern and Lange, 1988; Stern *et al.*, 1979). The precise mechanisms involved in SCC development remain to be elucidated, but it is possible that PUVA treatment may favour EV HPV infection, either positively influencing HPV activities (*e.g.* viral transcription and/or replication) and/or inducing local immune suppression with consequent persistence of the virus (Harwood *et al.*, 1998; Wolf *et al.*, 2004). A recent study has shown that the prevalence of EV HPV types in plucked hairs is higher in patients with psoriasis treated with PUVA than in patients without a history of PUVA exposure (Wolf *et al.*, 2004). These findings provide support for the involvement of EV HPV types in increasing the risk of skin cancer development in PUVA-treated patients.

5. Biological properties of HPV

Papillomavirus particles have a diameter of 52-55 nm (Figure 3) and are composed of two structural proteins, L1 and L2, 55 and 70 kDa in size, respectively. They are arranged as 72 subunits called capsomers. The particles contain a single molecule of double-stranded circular DNA, which constitutes about 12% of the virion by weight and is associated with normal or modified cellular histones to form a chromatin like complex.

5.1. Genome organization and function of the viral proteins

All PVs are approximately 8 kb in size and consist of covalently closed double-stranded DNA, with a highly conserved organization among all known PVs.

The PV genome contains a 400-850 bp long noncoding region (long control region, LCR, or upstream regulatory region, URR), which contains all regulatory

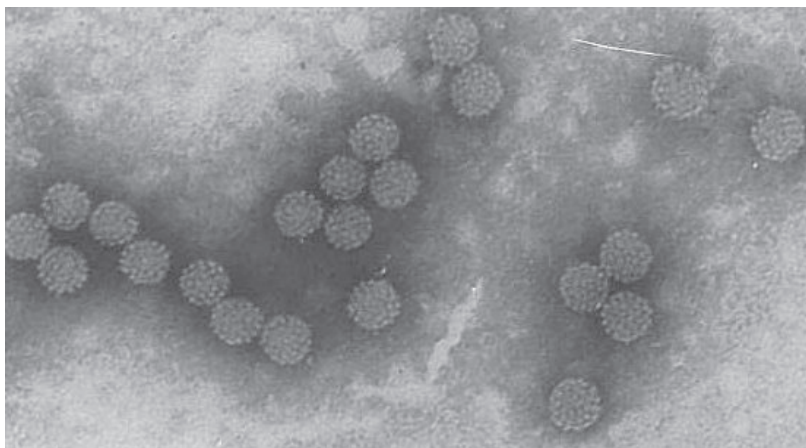


Figure 3. Electron micrograph of purified papillomavirus

elements for viral transcription and replication. The coding region can be divided in early and late regions, which, in case of HPV16 comprise six early (E) and two late (L) genes (reviewed in Tommasino, 2001) (Figure 4). The transcription of these genes occurs uni-directionally from one or more promoters located in the URR or in the E6 and E7 genes.

The proteins encoded by the early genes function primarily in viral episomal replication and in cellular transformation, whereas the late genes encode the viral capsid proteins.

5.1.1. The upstream regulatory region and gene expression control

The promoters and the sites for transcriptional initiation are located in the E6/E7 region and the URR. The intensity and use of these promoters however, is regulated by elements in and around the URR (Figure 5). The transcriptional termination is regulated by two signals, located between the E2/E5 and the L2 gene and one downstream of the L1 gene. However, not much is yet known about the regulation of the transcriptional termination and the early-late switch of transcription of HPVs. Most studies of the URR and viral gene expression control were performed on alpha PV viruses and not much is known yet about the gene expression control in cutaneous and animal PVs. Therefore, the make-up and the regulation of Beta, Gamma and animal PVs might be different.

All Alpha HPVs have a conserved structure of the early promoter, the E6 promoter or p97 (for HPV16), that initiates transcription of the E6/E7 polycistronic mRNA. The principal factor responsible for activation of this promoter is Sp1 (Apt *et al.*, 1996). Several other cellular transcription factors like NF-1, AP1, KRF-1, Oct-1 and YY-1, bind the central region of the URR of HPV16 and can modulate the promoter activity (Chan *et al.*, 1989; Gloss *et al.*, 1989; Offord and Beard, 1990;

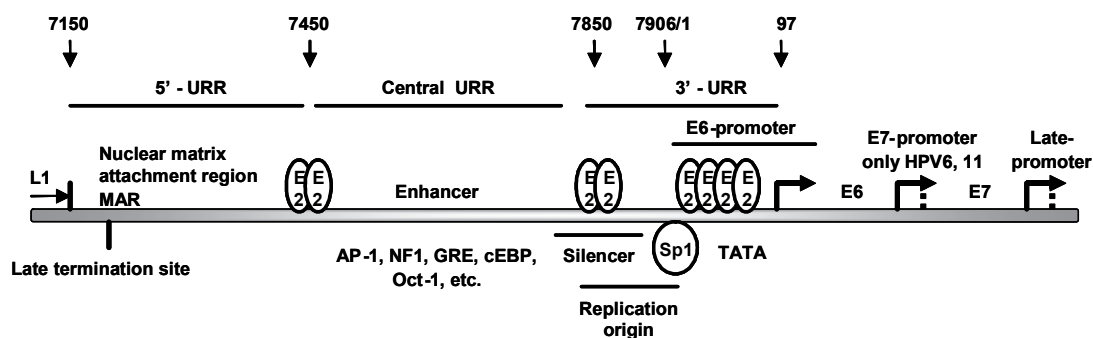


Figure 5. Cis responsive elements in the upstream regulatory region, the E6 and E7 genes of alpha papillomaviruses. The number indicates the genomic positions in HPV16. Data support that this model applies to HPV-6, 11, 16, 18 and 31, but has to be modified for certain cutaneous and animal PVs.

Mack and Laimins, 1991).

The URR is considered a hypervariabel region of the HPV genome and therefore, natural polymorphisms of the URR are detected frequently (Chan *et al.*, 1992, Zehbe and Tommasino, 1999). As the URR contains all elements necessary for viral transcriptional regulation, sequence variations in this region might have an impact on the carcinogenic potential of the virus. Indeed it has been shown that nucleotide changes in 3' end of the URR can greatly increase the activity of the URR (Veress *et al.*, 1999).

Recently it has been shown that the URR of some cutaneotropic HPV type can be activated by UVB irradiation. This activation might be dependent on the specific activation of p53 by UV irradiation as it was shown that HPV 77 contains a p53 consensus binding region to which p53 can bind specifically. In functional studies exposure of cells with HPV 5, 8 or 77 to UV leads to an increase of p53 and a p53-dependent increase of the promoter activity (Purdie *et al.*, 1999; Akgul *et al.*, 2005).

The viral early proteins E1 and E2 are also involved in the transcriptional regulation of the early genes. They have shown to be able to act as a transcriptional silencer or enhancer. E2 can bind to four specific *cis* elements in the URR of HPV16 (Cripe *et al.*, 1987) and inhibit the binding of other cellular factors like SP-1. However, depending on the E2 concentration, the association of E2 with these sites can activate or repress the expression of the E6 and E7 (Dostatni *et al.*, 1991; Tan *et al.*, 1994; Bouvard *et al.*, 1994).

A 110 bp T and A rich segment between the enhancer and the E6 promoter are able to bind the E1 protein and functions as replication origin. This segment however has also been shown to act as a transcriptional silencer of the E6 promoter, regulated by the factors YY1 and CDP (Bauknecht *et al.*, 1992; Ai *et al.*, 1999). The level of these repressors is found to be high in undifferentiated cells and decrease during differentiation, while the concentration of the enhancers is increased. Therefore, it can be concluded that the silencers and enhancers work sequentially and lead to a reduction of HPV proteins in undifferentiated cells and stimulate HPV gene expression upon differentiation (Ai *et al.*, 1999, 2000).

5.1.2. The early proteins

The proteins expressed early in the viral life-cycle are necessary for the initiation of viral protein expression, viral DNA replication and cell cycle proliferation even in presence of anti-proliferative factors.

The **E6** and **E7** genes play an important role in bypassing the cellular arrest signals, such as differentiation and immortalization of primary keratinocytes, to

allow completion of the viral DNA replication. HPV16 E7 for example has been shown to play a critical role in the DNA replicative stage of the viral life cycle by delaying the onset of differentiation (Flores *et al.*, 2000).

In addition, these viral proteins can associate and alter, or completely neutralize, the normal functions of several cellular proteins (Scheffner *et al.*, 1990; Davies *et al.*, 1993) leading to deregulation of cell cycle, apoptosis and ultimately to transformation. In the early 1990's it was shown that E6 and E7 expression can lead to transformation of human cells and many other studies since then have contributed to the characterization of the individual properties of these proteins and how they affect the life of the infected cell. The properties and the involvement of these two proteins in human malignancies will be discussed in more detail in chapter 6.

The early proteins **E1** and **E2** play an important role in the initiation and regulation of viral DNA synthesis and gene expression. They can form a stable complex and bind to the replication origin of the HPV genome, where it recruits cellular factors essential for DNA replication (Yang *et al.*, 1991; Sedman and Stenlund, 1995). E1 has a cyclin-binding RXL motif and associates with cyclin E and A and is phosphorylated by cyclin E or A-associated kinases. Mutation of E1 phosphorylation sites results in a reduction of HPV DNA replication, supporting the idea that the E1/cyclin association plays an important role in viral DNA replication (reviewed in Tommasino, 2001).

Moreover, it has been shown that E1 has an ATPase and helicase activities (Hughes *et al.*, 1993; Lee *et al.*, 1999). It unwinds the origin of DNA replication (*ori*) (Seo *et al.*, 1993) and associates with human DNA polymerase α primase *in vitro* (Park *et al.*, 1994).

The role of **E4** and **E5** in the viral life-cycle is not fully understood yet. In cutaneous HPV induced lesions, E4 is present at very high levels and several E4-isoforms have been detected. It has been shown that this protein associates with and disrupts the cytoplasmic keratin network (Doorbar *et al.*, 1991). Doorbar *et al.* (2000) have also reported that the E4 protein of HPV16 associates with a putative RNA helicase, suggesting a role for E4 in the productive phase of the infection establishing a favourable condition for viral maturation.

Initial studies on bovine papillomavirus (BPV) have shown that E5 is a potent oncoprotein. It has been shown that HPV16 E5 is also able to induce cellular transformation, although with less efficiency than BPV E5, and can activate growth factor receptors such as epidermal growth factor (EGFR) (Martin *et al.*, 1989) and platelet derived growth factor receptor (PDGFR) (Petti *et al.*, 1991). Interestingly, most of the cutaneous HPVs do not possess an E5 open reading frame (ORF).

Since the integration of viral DNA, which occurs in tumour cells, results in a loss of E5 gene, it is probable that it is involved in early events during the multi-step

process of cervical carcinogenesis, and that its function is no longer required after the establishment of the transformed phenotype.

5.1.3. The late proteins L1 and L2

L1 and L2 are the major and minor capsid proteins, respectively. The capsid comprises 360 molecules of the major capsid protein L1, arranged as 72 pentamers. The number of L2 molecules per capsid has been estimated between 12 and 36 (Okun et al., 2001). The expression of these late genes occurs only in the terminally differentiated keratinocytes and it is regulated at both transcriptional and post transcriptional level (Tan et al., 1995; Zhao et al., 1996; Sokolowski et al., 1999).

5.2. The viral life cycle

Viral infection occurs through a disturbed epithelial barrier, like a site of wounding. Figure 6 shows an overview of the HPV life cycle. Only the basal, replicating keratinocytes are infected by HPVs (Olson, 1987). Infection of these actively proliferating cells results in a delay of differentiation and an expansion of

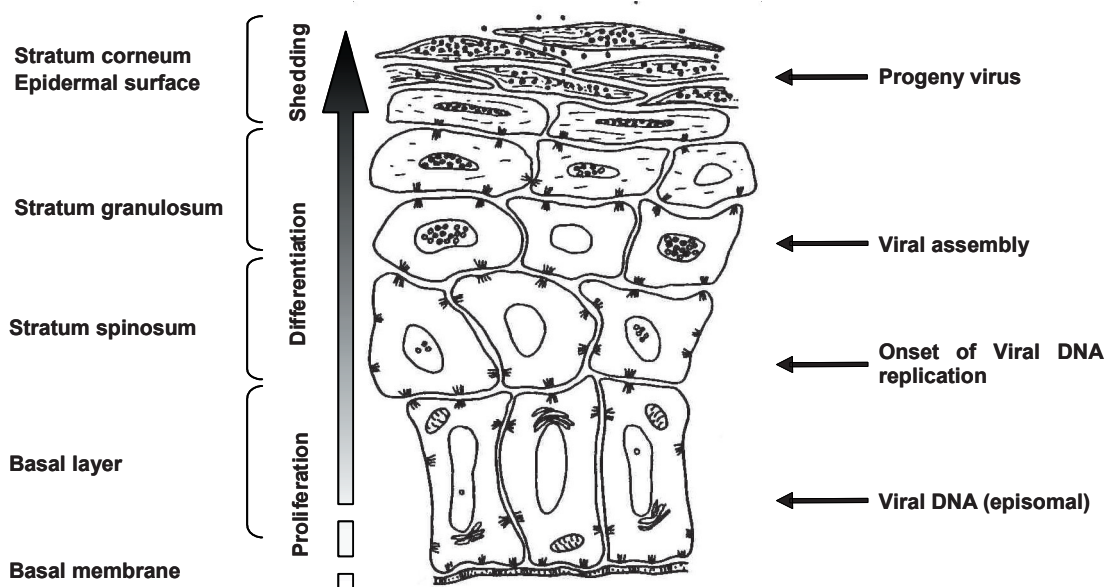


Figure 6. Viral life cycle in the epidermis. Infected basal cells migrate into the stratum spinosum and stratum granulosum still proliferating in order to replicate the viral genome. At the late stage of keratinocytes differentiation L1 and L2 are produced and viral assembly occurs. Progeny viruses are released upon normal shedding of epithelial cells.

the infected clone, due to the expression of the early proteins E6 and E7. During this phase, also called the non-productive stage, host cellular factors control transcription of early genes and viral DNA replication, while synthesis of structural proteins remains restricted and no viral virions are produced. Papillomaviruses modify the normal differentiation of the infected epithelium resulting in continued proliferation in the suprabasal layers (*stratum spinosum* and *stratum granulosum*) where replication of viral DNA occurs.

Since the HPV genome does not encode for the enzymes required during DNA synthesis, the virus is completely dependent on the host replication machinery. As the DNA polymerases are usually only active in the S-phase of the cell cycle, the papillomaviruses encoded proteins (E6, E7 and probably E5) ensure continued growth and division of the cells after they have left the basal layers. Although the reason is not entirely clear, the productive stage of the viral lifecycle where the late genes are expressed and virions are assembled only occurs in the suprabasal differentiated keratinocytes. (Stoler *et al.*, 1990; Byrne, 1997; Howley, 1996; Laimins, 1993; Meyers and Laimins, 1996). Several findings indicate that differentiating keratinocytes produce differentiation specific factors that upregulate the viral late gene transcription (Collier *et al.*, 1998). This absolute requirement for terminal differentiation of squamous epithelial cells for the expression of the late viral functions is responsible for the lack of success in propagating HPVs in culture.

The mature viruses are released into the environment by shedding of the surface cell layers without the need for a lytic viral cycle for the dissemination of progeny. This mode of infection guarantees that a single basal cell, originally infected by HPV, may lead to the emergence of a field of virus producing cells at the surface of the developing lesion.

6. E6 and E7 play a key role in cellular transformation

First indications that the E6 and E7 proteins were involved in the cervical cancer came from studies that showed the presence and expression of these two viral genes in cancer cells and cervical cancer tissue samples.

In pre-malignant HPV infected lesions, the viral DNAs exist as extra chromosomal plasmids, mostly as monomeric circular molecules (Lambert, 1991). However, in the majority, if not all cancers, the viral DNA is usually integrated into the host cell genome. This integration appears to be a random event with regard to sites of host chromosomal integration, but most often results in disruption of the viral E2 open reading frame and the loss of E2 expression (Schwarz *et al.*, 1985, Yee *et al.*, 1985; Matsukura *et al.*, 1986; Smotkin and Wettstein, 1986; Baker *et al.*, 1987; Schneider-Maunoury *et al.*, 1987). Since E2 is a regulator of the E6/E7

promoter, the loss of E2 results in deregulation of the expression of the E6 and E7 genes, which are consistently found expressed in HPV-associated cervical cancers (Schwarz *et al.*, 1985; Smotkin and Wettstein, 1986; Baker *et al.*, 1987).

The first direct evidence for the transforming activity of the viral genes was obtained from studies performed in NIH3T3 cells (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986) and later it was shown that HPV16 E6 and E7 are able to immortalise primary human cells, including primary human keratinocytes, the natural host of the virus (Mansur and Androphy, 1993). However, E6 and E7 from low-risk viruses (1, 6 and 11) failed in immortalisation experiments (Woodworth *et al.*, 1989; Barbosa *et al.*, 1991), providing a direct correlation between the *in vitro* properties and the potential carcinogenicity of the HPV. Transgenic mice co-expressing the E6 and E7 genes from the high risk HPV type 16 exhibit epidermal hyperplasia and various tumours (Lambert *et al.*, 1993). Additional studies in human cells showed that expression of high-risk E6 and E7 proteins is required for the maintenance of the transformed phenotype (Yoshinouchi *et al.*, 2003; Yamato *et al.*, 2005). Consistent with this fact, inhibition of transcription of E6 or E7 genes in cervical carcinoma cell lines leads to a decrease in proliferation state and reversion of the malignant phenotype (Crook *et al.*, 1989; von Knebel-Doerberitz *et al.*, 1992).

In the last 10-15 years, biochemical studies have provided further support to the carcinogenic functions of E6 and E7. Both viral oncoproteins are able to form stable complexes with cellular proteins and alter, or completely neutralise, their normal functions. These events lead to the loss of control of cell cycle checkpoints, of apoptosis and differentiation and eventually to transformation of the infected cell.

6.1. The E6 protein and p53

The HPV E6 proteins are small proteins of around 150 amino acids and contain a highly conserved motif consisting of four Cys-X-X-Cys motifs, which permit the formation of two zinc fingers and are believed to be important for E6 activity (Figure 7) (Kanda *et al.*, 1991; Sherman and Schlegel, 1996).

The most significant property of mucosal high-risk E6 proteins was initially identified by Werness *et al.*, 1990. HPV16 E6 binds to (Werness *et al.*, 1990) and directs the degradation of the tumour-suppressor protein p53, a key protein in preserving the genome integrity and is found mutated in 50% of all the cancers (Scheffner *et al.*, 1993), via the cellular ubiquitin proteolysis system (Figure 8) (Scheffner *et al.*, 1990). This E6-mediated p53 ubiquitination requires another protein, named E6AP (E6 associated protein), an E3 ligase (Huibregtse *et al.*, 1991; reviewed in Vousden, 2000).

P53 function is controlled through several mechanisms, one of the most effective being regulation of protein stability. Central to this process is MDM2, an E3 ligase that targets both p53 and itself for ubiquitination and proteasome mediated degradation (Vousden and Van de Woude, 2000). MDM2 is also a transcriptional target of p53, creating a negative feedback loop where p53 activates expression of MDM2, which keeps p53 levels low during normal growth and development. Activation of p53 occurs upon cellular stresses, such as DNA damage, oncogene activation, telomere erosion and hypoxia. It is mediated, at least in part, by inhibition of MDM2 and rapid stabilization of the p53 protein by post-translational modifications.

E6 proteins from high-risk types have been shown to bind p53 with much higher affinity than low-risk HPVs and only the high-risk proteins are able to bind E6AP and direct the degradation of p53 (Hoppe Seyler and Scheffner, 1997). Thus, the E6 protein from the high-risk HPV type 16, by binding and targeting p53 for degradation, prevents cell cycle arrest and apoptosis in stressed cells favouring accumulation of DNA damages and cellular transformation (Gu *et al.*, 1994).

However, several studies on the E6 proteins from Beta HPV types, *eg.* 5, 8 and 38, have shown that they are unable to target p53 for degradation, but are still able to inhibit apoptosis and promote cellular proliferation in presence of p53 (Steger and Pfister, 1992; Jackson *et al.* 2000; Caldeira *et al.*, 2003). Interestingly,

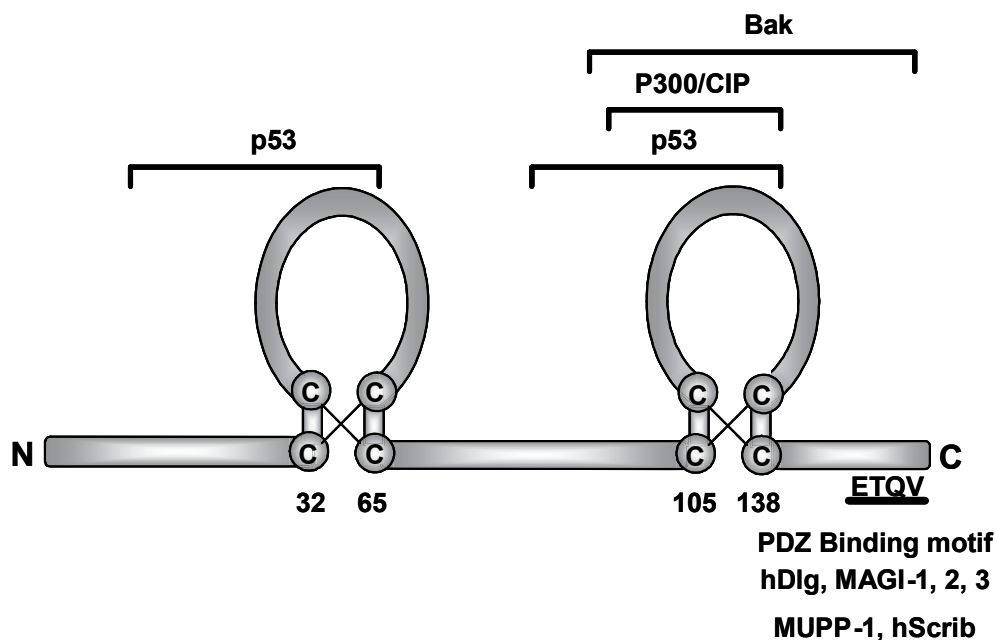


Figure 7. A schematic diagram of the HPV-18 E6 protein and regions involved in interactions with some of its known cellular targets.

treatment with UV irradiation leads to an increase of p53 levels, but not apoptosis nor activation of p53 responsive apoptotic genes (Jackson and Storey, 1999). It has also been shown that the LCR of some cutaneous HPV types (5, 8 and 77) are stimulated by UVB irradiation (Akgul *et al.*, 2005; Purdie *et al.*, 1999). In the case of HPV77 this activation of the viral promoter is mediated through a p53 consensus binding region (Purdie *et al.*, 1999). This indicates that Beta HPV types have developed alternative mechanisms to inactivate p53 or overcome the anti-proliferative effects of p53 and induce transformation in presence of this tumour suppressor protein.

6.2. E6 and other cellular targets

Although it can be assumed that the degradation of p53 contributes strongly to the carcinogenic potential of the high-risk HPVs, several studies have shown that mutants of HPV16 E6 defective in their ability to induce degradation of p53 can still immortalise primary cells (Liu *et al.*, 1999). Many cellular factors that associate

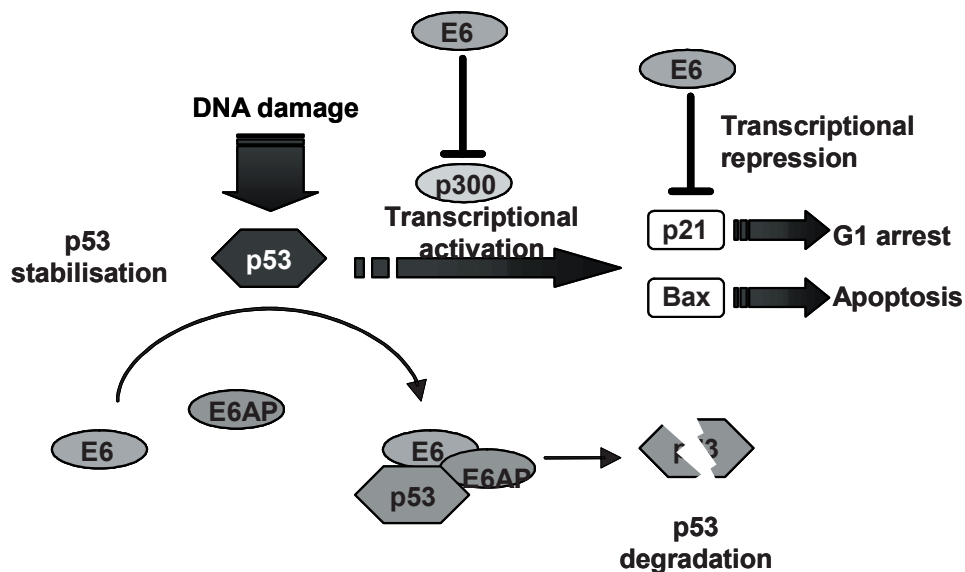


Figure 8. p53 pathways targeted by HPV16 E6. When cells are exposed to DNA damaging agents, e.g. UV irradiation or X-rays, the half-life of p53 can be greatly increased by post-translational modification. p53 then can activate the transcription of the cyclin dependent kinase (CDK) inhibitor p21WAF1/CIP1, leading to a G1 arrest and the DNA repair before replication, or can activate the transcription of the pro-apoptotic genes, e.g. Bax, leading to apoptosis. HPV16 E6 can neutralize p53 activity through two distinct mechanisms, (i) association with and the subsequent degradation of p53 through E6AP and (ii) Inhibition of transcription of p53 gene by binding the co-adaptor protein p300. In addition to inhibiting p53, E6 can also transcriptionally downregulate p21 directly. Thus, cells expressing HPV16 E6 proteins are resistant to the cell cycle arrest or apoptosis.

with E6 have been identified (Figure 7), but the biological significance of these E6 associations and their role in the viral life cycle and malignant conversion are not entirely understood yet.

Some of the E6 interacting proteins are involved in cell-cell contact and cell polarity, *e.g.* PDZ proteins, the mammalian homologue of the *Drosophila* disc large tumour suppressor (hDLG) protein (Kiyono *et al.* 1997). Studies in *Drosophila* show that mutations in this tumour suppressor result in relaxation of cell/cell contact, and neoplastic transformation. In addition, E6 physically interacts with the focal adhesion protein paxillin (Tong and Howley 1997), a cytoplasmic protein involved in actin organisation, attachment of cells to the extra cellular matrix (Wood *et al.* 1994), and transduction of signals from the plasma membrane. Deregulation of paxillin results in disruption of the actin cytoskeleton, suggesting that this interaction may play a vital role in carcinogenesis. Indeed, the protein levels of paxillin and the focal adhesion kinase (FAK) are upregulated in cervical carcinoma cell lines and in cells immortalized by 'high-risk' HPV (McCormack *et al.* 1997).

Other targets of E6 are involved in apoptosis. The E6 proteins from both cutaneous and mucosal HPV types are able to interact and induce degradation of the pro-apoptotic protein Bak (Thomas and Bank, 1999; Jackson *et al.*, 2000). Bak is upregulated in cells treated with UVB and down-regulation of Bak by cutaneous HPV types prevented apoptosis, which can lead to accumulation of DNA mutations and transformation. In addition, HPV type 16 E6 is able to induce degradation of the FADD death effector and inhibit the Fas apoptotic signalling pathway (Filippova *et al.*, 2004).

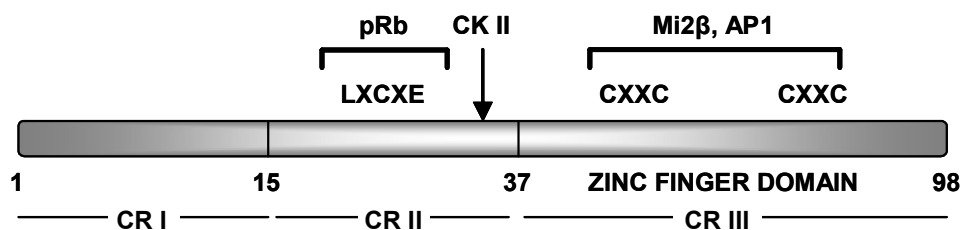


Figure 9. A schematic diagram of HPV16 E7 protein. The CR2 domain is involved in pRb degradation and mutations in this region lead to a strong reduction in the transformation ability of the protein (Banks *et al.*, 1990; Phelps *et al.*, 1992). The pRb binding site (LXCXE) mediates the association of E7 with pRb (Dyson *et al.*, 1989) and is located in CR2 at position 22-26. The CKII phosphorylation site is located at position 31-32. Between the LXCXE and the CKII site, a small motif of 3 aa is responsible for pRb degradation (Giarrè *et al.*, 2001). CR3 contains two CXXC motifs involved in zinc binding, which is involved in the dimerisation of the protein (Braspenning *et al.*, 1998). The function of the CR1 region is not entirely clear yet. The numbers indicate the position of each conserved region.

6.3. The E7 protein and pRb

The E7 proteins are small acidic proteins of around 100 amino acids that is structurally and functionally related to other tumour viruses proteins like adenovirus E1A and SV40 large T. Based on its homology with the viral oncoproteins, E7 can be divided in three conserved regions (CR), known as CR1, CR2 and CR3 (Moran and Mathews, 1987) as depicted in Figure 9.

The most well studied property of E7 is its ability to bind to the unphosphorylated form of the retinoblastoma protein (Dyson *et al.*, 1989; Munger *et al.*, 1989), a cell cycle regulator that plays a key role in controlling the “restriction point” (R) in G1/S transition (Woolard and Nurse, 1995). This binding is mediated by the LXCXE motif in CR2, which is highly conserved in other viral and cellular proteins associating with pRb, like E1A, SV40 large T, cyclin D1 and HDAC1 (histone deacetylase).

Both high risk and low risk HPV types can associate with pRb. Studies on the E7 proteins from low-risk HPV types 6 and 11, which are rarely associated with malignant lesions, have demonstrated that a reduced affinity for pRb correlates with a lack of *in vitro* transforming activity (Storey *et al.*, 1988; Munger *et al.*, 1989). However, strong affinity does not necessarily correlate with the ability to

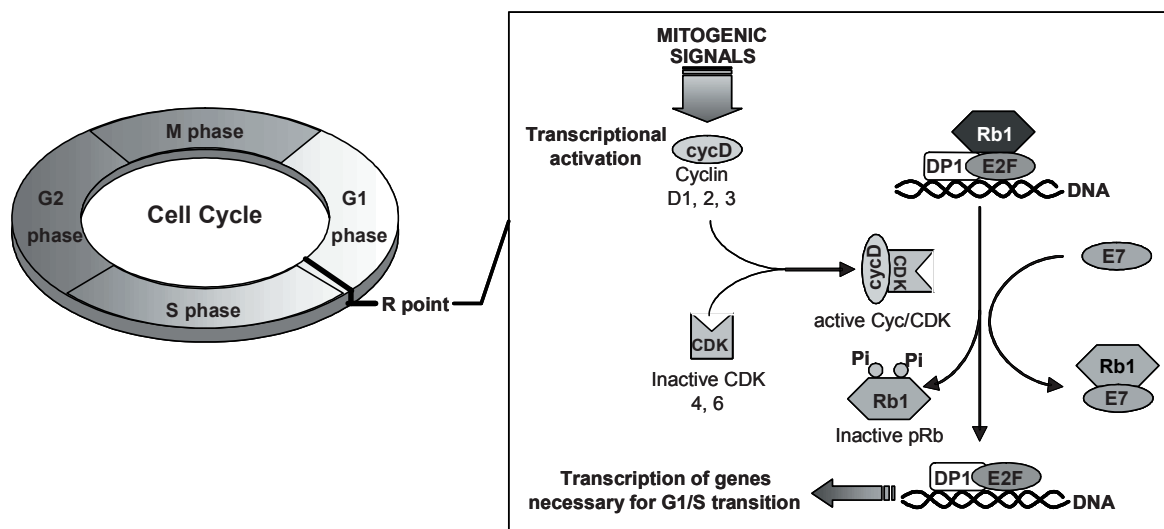


Figure 10. Deregulation of the restriction point (R) by HPV16 E7. In quiescent cells, pRb is present in a hypo-phosphorylated form and is associated with E2F molecules, thereby inhibiting their activity. When quiescent cells are exposed to mitogenic signals, genes encoding the G1 specific D-type cyclins (D1, D2 and D3) are activated. These then associate with cyclin dependent kinases (CDK) 4 or 6, which after transportation into the nucleus are able to phosphorylate pRb in mid G1 phase. This leads to a dissociation of pRb and a release of the DP1/E2F complexes. This complex is then able to regulate the transcription of several genes necessary for cell cycle progression through the restriction (R) point. The HPV16 E7 protein by inactivating pRb is able to mimic pRb phosphorylation and promote S phase entry in absence of mitogenic signals

induce cellular transformation (Ciccolini *et al.* 1994; Caldeira *et al.*, 2000). Indeed, the E7 protein from the low risk HPV type 1 can associate strongly with pRb, but fails to induce degradation and transformation (Alunni-Fabbroni *et al.*, 2000). Studies on the E7 protein from high risk HPV types has shown that degradation of pRb leads to the release of active E2Fs, which are transcription factors that stimulate S-phase entry and progression of the cell cycle (Brehm and Kouzarides, 1999, reviewed in Tommasino and Jansen-Dürr, 1997) (Figure 10). It can therefore be assumed that the ability to induce degradation of pRb is a key feature of high risk HPV types. Indeed, the E7 protein of the high risk mucosal HPV type 16 or cutaneous HPV type 38 have shown to be able to bind and degrade pRb and induce transformation (Giarre *et al.*, 2001; Caldeira *et al.*, 2003).

6.4. E7 and other cellular targets

Besides the ability to interact with pRb, E7 of the high-risk type HPV16 is able to bind E2F1 directly and activate transcription from different E2F regulated promoters (Hiraiwa *et al.*, 1996; Hwang *et al.*, 2002). Therefore, it appears that E7 can relieve repression of E2F1 through pRb and direct binding and activation of E2F1 leading to an increased transcription of cell cycle promoting factors. In addition to this ability to induce cell cycle progression, E7 has been shown to interact with insulin-like growth factor binding protein-3 (IGFBP-3). This p53-inducible gene, over-expressed in senescent cells, can suppress cell proliferation and induce apoptosis. E7 binds to and triggers proteolytic cleavage of IGFBP-3, hence overriding senescence (Mannhardt *et al.* 2000).

HPV16 E7 can also efficiently inactivate cell cycle inhibitors from the CIP/KIP family like p27^{kip1} or p21^{cip1/waf1} (Funk *et al.*, 1997; Jones *et al.*, 1997; Ruesch and Laimins, 1997; Zerfass Thome *et al.*, 1996).

A role for E7 has also been proposed in the invasion of underlying tissue in malignant tumours. The cutaneous HPV type 8 and the mucosal HPV type 16 E7 can induce expression of metalloproteinase MT-1 a protein involved in degrading extracellular matrixes and often found at sites of tumour invasion (Smola-Hess *et al.*, 2005). Increased MT-1 MMP levels might play a role in the HPV life cycle by promoting proliferation and contribute to the invasive phenotype of malignant cells.

Brehm *et al.* (1999) have shown that HPV16 E7 is able to associate with a histone deacetylase, Mi2 β , via its zinc finger domain and that this activity is an important parameter for the growth promoting activity of the HPV16 E7 by modulating chromatin activation pathways (Brehm *et al.*, 1999). The E7 gene product is also capable of interacting with several transcription factors like AP1, the TATA box binding protein (TBP) or c-jun (reviewed in zur Hausen, 2000).

The mechanisms and the biological significance of interaction of E7 with the latter proteins are yet to be elucidated, but regarding the role of many of these proteins in cell proliferation and cellular protein homeostasis it can be deduced that a deregulation of these proteins results in increased immortalisation and carcinogenicity in presence of E7.

6.5. E6 and E7 act in concert

Although E6 or E7 of high risk mucosal and cutaneous HPV types by themselves can extend the lifespan of cells, there is a significant extension of proliferative capacity and an increase of the likelihood of establishing immortalised cell lines by the combination of E6 and E7 (Halbert *et al.*, 1991; Tommasino and Jansen Dürr, 1997, Caldeira *et al.*, 2003). The expression of E7 of HPV 16 alone has been shown to strongly sensitize keratinocytes to apoptosis and during the normal viral replicative cycle, by causing a deregulated E2F activity (Stoppler *et al.*, 1998). E7 can induce p53 expression in post G1 crisis ('extended life span') leading to a p53 dependent apoptosis or cell cycle arrest (Demers *et al.* 1994). If this were left unperturbed, an infected cell would die even before there is viral replication. Hence, the ability of E6 to modulate p53 protein levels may be an integral event during productive infection and a necessity for carcinogenesis (Figure 11).

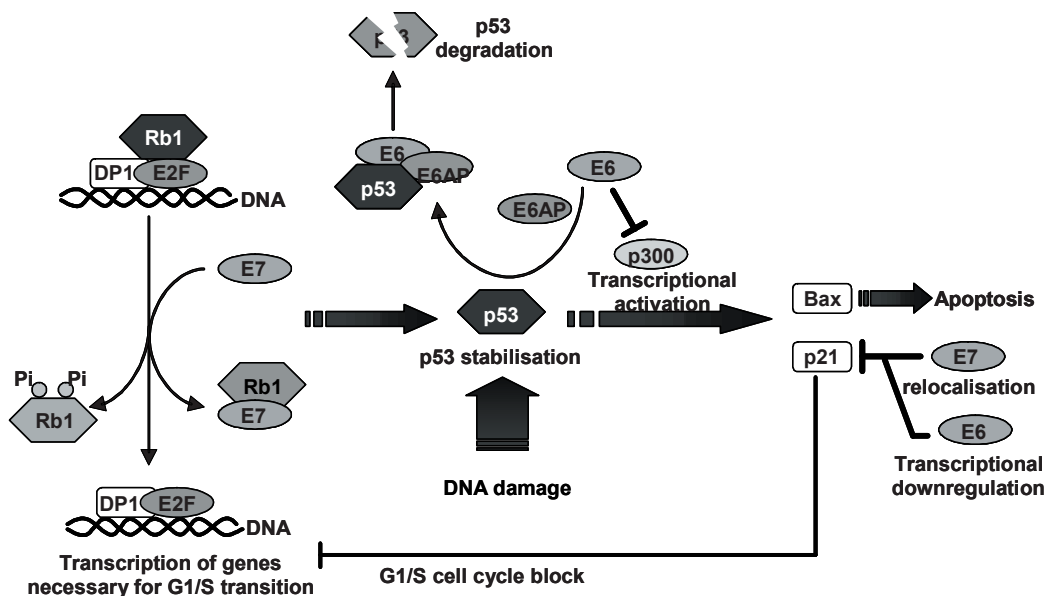


Figure 11. Co-operation of E6 and E7 of high-risk mucosal HPV types

E7 causes a deregulation of the cell cycle by targeting and inactivating pRb, this unscheduled proliferation however leads to a stabilization and accumulation of p53, which can induce apoptosis or cell cycle arrest. E6 blocking p53 function prevents the cell cycle arrest and apoptosis induced by p53.

However, the mechanisms of p53 modulation and inhibition appear to be different for mucosal and cutaneous HPV types. Whereas expression of E6 from the high risk mucosal HPV types leads to rapid degradation of p53, the expression of the E6 from the high risk cutaneous HPV types induces stabilization of p53 (Jackson *et al.*, 2000; Caldeira *et al.*, 2003). This accumulation of p53 does not induce cellular arrest nor apoptosis, indicating that cutaneous HPV types have developed an alternative mechanism of preventing p53 mediated cell cycle arrest or apoptosis. More studies will be required to elucidate the role of the viral oncogenes E6 and E7 in transformation of the skin and the importance of p53 in this process.

7. Concluding remarks and outline of thesis

Through epidemiological and biochemical studies in the past three decades the role of mucosal HPV types in human cancer has been confirmed and the molecular mechanisms underlying the transformation process have been elucidated. However, although the research on the cutaneous HPV types is progressing fast, many questions remain to be answered regarding their potential role in human skin carcinogenesis. Epidemiological studies have clearly demonstrated that specific cutaneous HPV types (the EV HPV types) are commonly and consistently found in NMSC. However, due to the high heterogeneity and the presence of multiple HPV types in skin lesions it is still difficult to confirm the role of potential high-risk cutaneous types in skin carcinogenesis. The identification of high-risk cutaneous HPV types will require both epidemiological studies, to map the distribution of these types in human skin cancer, and biochemical studies, to elucidate their mechanism and role in skin carcinogenesis.

In this thesis we present data and development of a system to test the efficacy of the viral oncogenes to facilitate identification of potential high-risk HPV types and on the identification and analysis of the carcinogenic potential and mechanism employed “high-risk” cutaneous HPV type to transform cells.

Biochemical studies on the mucosal HPV types have shown that immortalisation of human cells of various origins can be efficiently achieved with E6 and E7 oncogenes. These oncoproteins promote this activity by association and inactivation of the cellular protein p53 and pRb, respectively. Therefore, analysis of these proteins and their ability to interact with the cellular proteins is a valid tool to predict the potential carcinogenicity of specific HPV types. **Chapter 2** covers the development and use of *in vitro* systems to evaluate and quantify the ability of E7 to associate pRb.

However, although binding of the cellular proteins by the viral proteins is an important event we show in **Chapter 3** that the E7 protein from the mucosal HPV type 32, despite its high affinity for pRb, does not promote degradation of the cellular protein and therefore is unable to induce cellular proliferation. This data shows that high affinity of E7 for pRb doesn't always correlate with *in vivo* carcinogenicity, but that other biological properties are also important for carcinogenicity.

In a previous study (Caldeira *et al.*, 2003) it was shown that E6 and E7 from the cutaneous HPV type 38 are sufficient to corrupt the cell cycle and senescence programs in primary cells, inducing active and long-lasting proliferation of primary human keratinocytes, the natural host of HPVs. However, an *in vitro* system does not reflect the complexity of an *in vivo* situation. In **Chapter 4** we present data of an *in vivo* model in which we show that expression of the viral genes in mouse skin induces cellular proliferation, hyperplasia, and dysplasia in the epidermis. Keratinocytes in the epidermis of these animals showed a deregulation of the cell cycle checkpoints and did not react to UVB induced DNA damage. In addition, expression of the E6 and E7 proteins from HPV38 led to an attenuation of the keratinocytes in the skin towards tumour development upon stimulation by carcinogens.

HPV38 E7 has been shown to bind and target pRb for degradation (Caldeira *et al.*, 2003). However, although E7 activity is sufficient in some cases for immortalization and transformation of keratinocytes it is clear that this procedure is more efficient in context of co-expression of the E6 gene to inactivate p53. Based on the *in vitro* and *in vivo* data on HPV38, this type could be considered a potential high-risk HPV type. The mechanism of transformation however, remained unclear. In **Chapter 5** we show for the first time that an EV HPV types, HPV38, can alter the functions of p53. However, the mechanism of p53 inactivation mediated by HPV38 differs substantially from that mediated by HPV16. In fact, while HPV16 E6 promotes p53 degradation via the ubiquitin pathway, our data demonstrate that HPV38 E6 and E7 induce stabilisation of a wild-type p53, which in turn selectively activates transcription of $\Delta Np73$. This in turn inhibits the capacity of p53 to induce the transcription of genes involved in growth suppression and apoptosis leading to inhibition of p53 function and promotion of cell survival.

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