Chapter 1

General introduction

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Human papillomaviruses

Human papillomaviruses (HPVs) represent a heterogenous group of viruses from the Papillomaviridae virus family. They are double-stranded circular DNA viruses that infect epithelial cells. Papillomaviruses are very common and have been isolated from human, mammals and birds. More than 120 different HPV types have been isolated and fully sequenced so far [12,33].

The genomic sequence of the viral open reading frame encoding for the major capsid protein L1 is well conserved and has been used to generate the HPV phylogenetic tree, in which HPV sub-groups have been divided in genera. A novel HPV type is assigned on the basis of a difference in the L1 sequence of at least 10% with the already known HPV types. HPV genomes displaying differences in the L1 gene between 2-10% are classified as sub-types [21].

The HPV phylogenetic tree is mainly divided in three large genera: the alpha genus, the beta genus, and the gamma genus (Figure 1). Other genera include a small number of HPV types that cause benign warts and papillomas as well as some non-human papillomaviruses [12,33]. The genus alpha mainly includes HPV types that infect the mucosa of the genital and upper aero-digestive tracts, also called mucosal HPV types. The mucosal HPV types are divided in low-risk (LR) HPV types that are moslty associated with benign genital warts and high-risk (HR) HPV types that are the etiological agents of cervical cancer and a subset of other human cancers [12,148]. The beta genus includes mainly HPV types that infect the skin, also called beta cutaneous HPV types, and are suspected to play a role in skin carcinogenesis together with ultra-violet (UV) irradiation. Beta HPV types are described in detail in the paragraph ‘beta human papillomavirus types’.

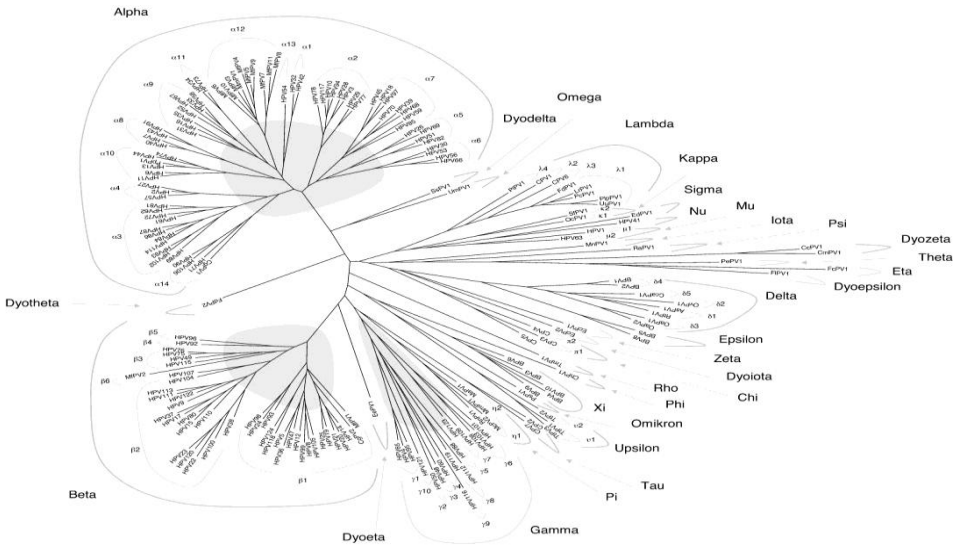

Figure 1. Papillomavirus phylogenetic tree. Bernard *et al*. 2010 [12].

Human papillomavirus genomic organization

The HPV genome is about 7900 base pairs long and is subdived in three regions: (i) the early region containing genes expressed in the early phase of the viral life cycle; (ii) the late region containing two genes encoding structural capsid proteins; and (iii) the regulatory region called “the long control region (LCR)” or, “upstream regulatory region (URR)”, which carries several regulatory *cis* elements for viral replication and transcription (Figure 2) [116].

The early genes E1 and E2 are important for viral replication, and are involved in the transcriptional regulation of the other early genes [111]. The role of E4 and E5 is not yet fully understood, although E4 appears to be involved in destabilizing the cytokeratin network and E5 acts as an oncoprotein that mediates mitogenic signals of growth factors, such as the epidermal growth factor [96]. In contrast to the mucosal HPV types, the beta cutaneous HPV types lack the E5 gene in their genome [46]. E6 and E7 are the major oncoproteins involved in HPV induced carcinogenesis; their functions are described in paragraph ‘Transforming abilities of the E6 and E7 oncoproteins’. The late

genes, L1 and L2 encode for the major and minor viral coat proteins, respectively [46]. These late proteins are assembled to form the icosahedral capsids of the virus [74]. The LCR contains the promoter and regulatory elements involved in viral DNA replication and transcription. In addition to LCR, there is a second non-coding region (NCR) that is very small and highly variable. The function of this NCR is not yet clear.

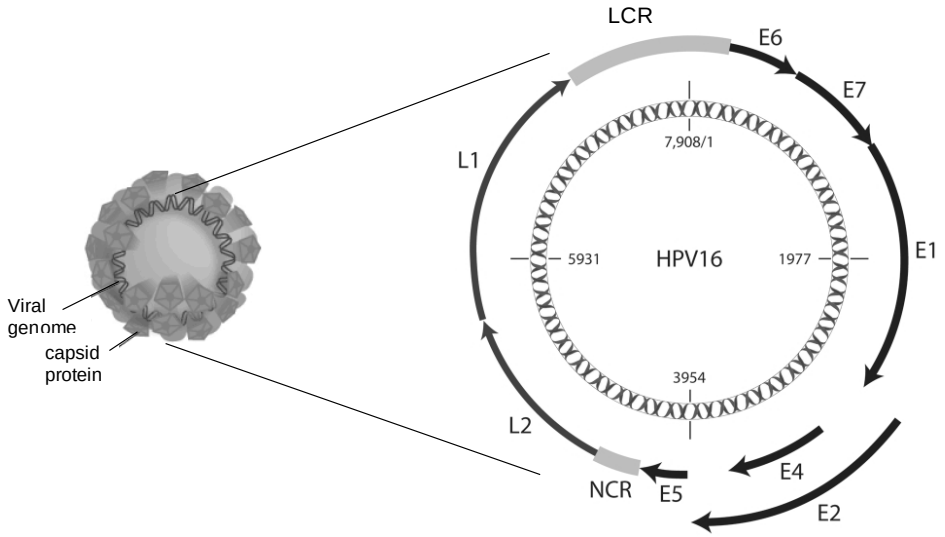

Figure 2. Schematic representation of the HPV16 viral structure and genomic organization. Early genes are drawn with solid black lines (E1, E2, E4, E5, E6 and E7) and late genes in blue lines (L1 and L2). Long control region (LCR) and the non-coding region (NCR) are drawn in gray. The viral genome and positions within the DNA sequence are displayed by the helix and numbers, respectively. Figure and legend are adapted from Smith *et al*. 2011 and The 2008 Nobel Prize in Physiology or Medicine – Press release [116,151].

Human papillomavirus replication

HPVs infect cells of the basal layer of the epithelium, which get exposed as a result of microwounds in the mucosal tissue or in the skin [36]. Particularly the transformation zone in the cervix, where the squamous epithelium from the cervix and the columnar epithelium from the endocervix meet, seems highly susceptible for HPV infection [86]. The virus specifically targets basal cells,

because the viral life cycle depends on epithelial differentiation and maturation[36,40,80,84]. After infection the HPV genome is maintained extra-chromosomally in low copy numbers [36,80,84]. Expression of the viral genes is tightly regulated by the cellular differentiation programme and migration of infected cells towards the epithelial surface. Expression of E6 and E7 is necessary to drive the cells through S-phase, which contribute to viral genome replication. Viral genome amplification requires the co-expression of several viral proteins, which is needed to produce infectious virions. The new viruses are not released by a lytic-infection, but get released when the cells reach the epithelial surface. How these different events occur depends on the infecting HPV type and the transformed state of the infected cell [36,80,84].

Human papillomavirus and carcinogenesis

The possible involvement of infectious agents in skin and genital warts has been proposed since long time [149]. Several studies showed that warts on the hands or genitals could be transmitted upon inoculation. Extracts from warts were inoculated on scarified sites of the arms, hands or genitalia where, after a certain period, similar warts appeared (reviewed in Rowson *et al*. 1967) [104,149]. These phenomena strongly suggested that an infectious agent was responsible for the transmission of the disease. Indeed, electron microscopy demonstrated the presence of viral particles in these lesions [122].

The first noted carcinogenic potential of papillomaviruses was in cottontail rabbits, and domestic rabbits, infected with the cottontail rabbit papilloma virus (CTPV). When wart extracts from the cottontail rabbit were inoculated in domestic rabbits, the papillomas that were formed were highly susceptible to malignant progression. In the cottontail rabbit the papillomas mostly regressed and rarely underwent malignant changes [101-103].

The initial suggestion for a role of HPV in cervical cancer was made by zur Hausen in 1976, who was awarded with a Nobel Prize in 2008 [150]. Epidemiological studies showed that HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56,

58 and 59 are associated with cervical cancer [16,83,117,142]. These types are classified as oncogenic or HR-HPV types and infection with one of these types is necessary for the development of cervical cancer [46,84,149].

HPV16 and 18 are the most frequently found HPV types in cervical cancers worldwide, and therefore very well studied [31]. HPV16 is mainly associated to squamous cell carcinoma (SCC) of the cervix and HPV18 with adenocarcinoma of the cervix [25,117]. In addition, it is shown that HR-HPV infections, mainly with HPV16, also play a direct role in other human cancers, e.g. vulvar, vagina, penile and other anogenital cancers and a subset of head and neck cancers [46,84,149,120,142].

From human papillomavirus infection to cancer

HPV infections are very common in young women and the life-time risk to contact HPV is about 80% [9]. However, the majority of the infections are cleared by the immune system within 6-12 months [88,105]. Only a small percentage of the infections persist and lead to cervical intraepithelial neoplasia (CIN); non-invasive premalignant lesions, which can be classified histologically on the basis of progressive atypia of the cells [56]. These CIN lesions can still regress to normal epithelia, which is usually associated with viral clearance [87,127]. Alternatively, the lesion can progress to a high-grade CIN lesion and subsequently to invasive cervical cancer (ICC) (Figure 3) [57].

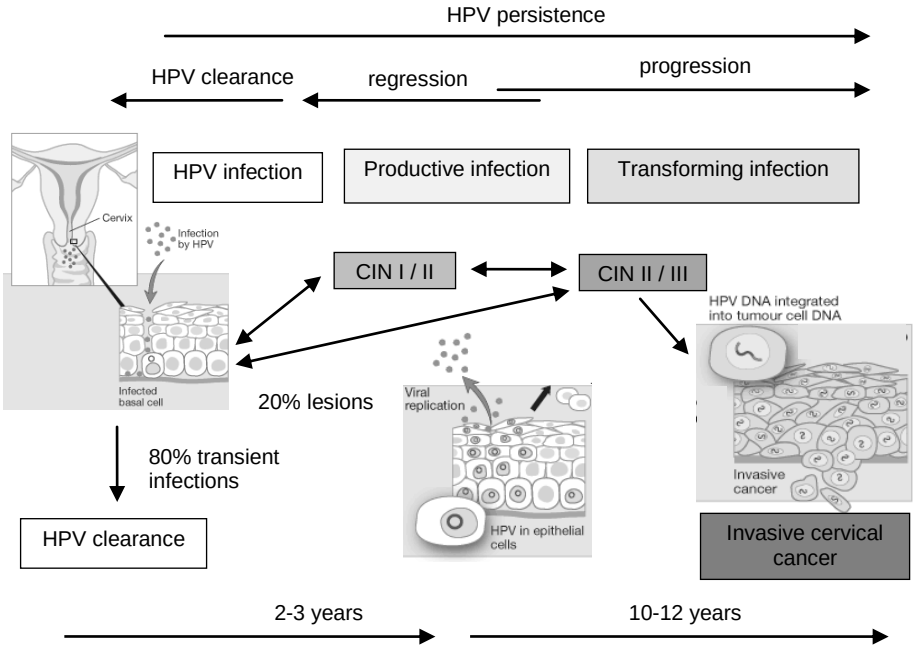

Figure 3. Model of cervical carcinogenesis, adapted from Snijders *et al*. 2009 and The 2008 Nobel Prize in Physiology or Medicine – Press release [118,151]. CIN: Cervical Intraepithelial Neoplasia. CIN I: mild dysplasia, CIN II: moderate dysplasia, CIN III: severe dysplasia / carcinoma in situ.

Upon progression of the lesion, the viral DNA often gets integrated in the host genome. Overall, the frequency of HPV DNA integration increases with the severity of the lesion. Viral DNA integration is found in the majority (~62%) of the cervical cancer cases, however this frequency is markedly different for various HR-HPV types [134]. HPV DNA integration in the host genome results in the disruption of several viral genes, with preservation of only the E6 and E7 genes, which are actively transcribed. The HPV E2 protein negatively regulates the transcription of the E6 and E7 genes. Therefore, HPV DNA integration is normally associated with a deregulation of E6 and E7 expression. However, HPV DNA integration is not required for the development of cervical cancer [93,137]. There is rising evidence that also episomal HPV DNA efficiently express E6 and E7; hereby the negative transcriptional function of E2 can be inactivated by alternative mechanisms, i.e. methylation E2 *cis* elements in the LCR [14,79].

Epidemiological studies have shown that, in addition to HPV infections, other factors may contribute to cervical carcinogenesis, such as host genetic factors, tobacco smoking, high parity, long-term use of hormonal contraceptives, immunosupression, co-infection with HIV (human immunodeficiency virus), and eventually other cofactors [54,84,135]. In addition, viral genetic factors can contribute to carcinogenesis [54,84,135], as described in the paragraph 'Human papillomavirus intratype variants'.

Transforming abilities of the HPV E6 and E7 oncoproteins

The oncogenic activity of mucosal HR-HPV types is mainly caused by the biological properties of their viral oncoproteins, E6 and E7. E6 and E7 are small proteins disrupting important cellular pathways, p53 and pRb pathways, respectively, involved in apoptosis and cell cycle regulation (Figure 4) [86]. HPVs disrupt these pathways principally to facilitate productive viral replication. However, as side effect, they also promote chromomal instability facilitating cellular transformation.

The best characterized activity of HR-HPV E6 is to induce degradation of the product of the tumour suppressor gene p53, which plays a key role in apoptosis and genomic stability. p53 is activated in response to DNA damage and other cellular stresses [19]. Normally, little DNA damage leads to cell cycle arrest and DNA repair, while larger and unrepairable DNA damage leads to apoptosis [7,59,68,71]. In both ways p53 prevents cell division with DNA damage, which can lead to cellular transformation. E6 interacts with p53 via a complex with the ubiquitin-protein ligase E6AP (E6-Associated Protein), leading to the ubiquitination of p53 and its degradation by the proteasomes [10,61,107,108]. This leads to the abolishment of cell cycle arrest and apoptosis, thereby allowing cell division when DNA damage is present [7,124]. Interestingly, the degradation of p53 is specific for mucosal HR-HPV types[55].

Another function of E6 is to activate the transcription of human Telomerase Reverse Transcriptase (hTERT), the enzyme of the telomerase

complex [64]. Somatic cells have very little or no telomerase activity. The telomeres shorten during normal cell division, leading to replicative senescence and aging [72,113]. HR-HPV E6 can activate hTERT in an E6AP-dependent manner [45,62,73]. This may contribute to high levels of hTERT, and subsequently to elongation of the telomeres and indefinite cellular proliferation [64]. However, only a subset of CIN3 lesions display elevated hTERT levels and telomerase activity, suggesting that also other factors contribute to hTERT activation in these lesions[119].

The HR-HPV E7 is mainly known to degrade the product of the retinoblastoma tumour suppressor gene (pRb) and the related pocket proteins p107 and p130. All three proteins negatively regulate the cell cycle. In normal cells, pRb is unphosphorylated in the G1-phase, and binds and represses the function of the transcription factor E2F-1, preventing progression to the S-phase. Upon mitogenic stimuli pRb gets phosphorylated by several cyclin-dependent kinases (CDKs) leading to the release of E2F-1, which in turn activates the transcription of genes encoding for essential proteins for cell cycle progression [37,121,140]. In HR-HPV-infected cells, E7 forms a complex with unphosphorylated pRb leading to its degradation via the proteasome. Upon pRb degradation E2F-1 is constitutively activated, inducing premature cells to enter the S-phase, leading to cell cycle deregulation [18,28,82,141].

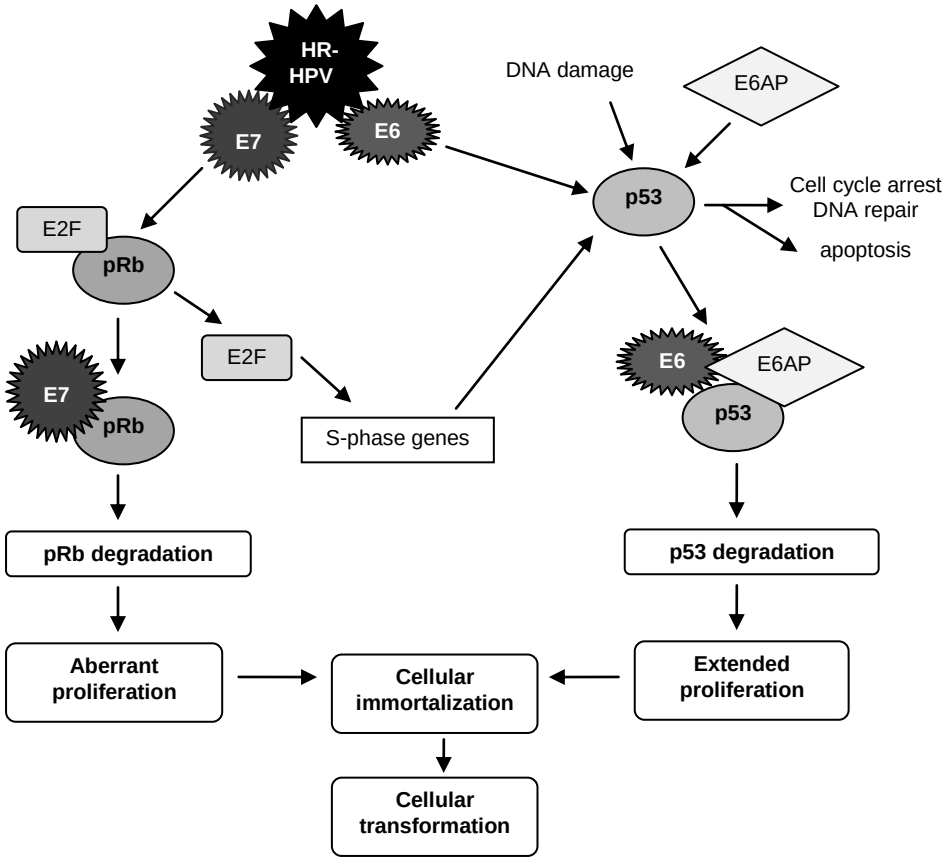

Figure 4. Schematic overview of E6 and E7 interference with the p53 and pRb pathways, involved in apoptosis and cell cycle control, respectively.

In addition to these HR-HPV E6 and E7 target proteins, many more exists, including proteins inducing genomic instability, malignant transformation, and escape of the immune surveillance (reviewed by Ganguly *et al.* [43]) [38,66,141].

Taken together, E6 and E7 are essential for HPV-induced increase in cell proliferation, cellular immortalization, accumulation of mutations in the host cell DNA, and finally malignant transformation of the cells. Consequently, it is been shown that HPV16 E6 and E7 together are able to immortalise primary human keratinocytes, the natural cellular host of the virus, *in vitro* [52,81,125].

Cervical cancer screening and prevention

Cervical cancer is worldwide the third most common cancer in women [41]. The mortality : incidence ratio is 52%, and the fast majority of the cervical cancer related deaths occur in developing countries [41]. This urges for effective screening programs to detect premalignant cervical lesions in order to decrease the morbidity and mortality of the disease [123]. The cytology-based Pap-test is the routine screening method that has been successfully used for several decades in many high-resource countries [92,110,138,148]. However, data from organized cytology-based screening programs indicate that, despite the significant decline in the occurrence of cervical cancer, this test is far from optimal given a sensitivity for ≥CIN2 that generally does not exceed 65%. Therefore, its application does not prevent pre-malignant and malignant disease in many of the screened women [24,39]. This is most likely due to the subjective nature of the Pap-test that may result in false-negative diagnoses. As a consequence, the success of cytology-based screening programs largely depends on the frequency of repeating the Pap-test, which in turn is a significant financial burden in health care systems [34].

To improve cervical cancer screening programs, HR-HPV DNA testing has been examined, either or not combined with cytology [75]. Given its much higher sensitivity for ≥CIN2 (i.e. about 95%) several recent randomized controlled trials have shown that HR-HPV testing provides a better protection against cervical cancer than cytology [5,99,100]. Therefore, HPV testing is expected to become an important primary cervical screening tool in the near future.

Human papillomavirus vaccination

In addition to effective screening programs, prophylactic vaccines have been recently developed. There are currently two HPV vaccines available on the market: i.e. (i) the quadrivalent vaccine from Gardasil™ (GlaxoSmithKline) against HR-HPV16 and 18, and LR-HPV6 and 11; and (ii) the bivalent vaccine from Cervarix™ (MSD) against HPV16 and 18 [1,2,23,63]. Both vaccines consist of

HPV L1 capsid proteins that have self-assembled into viral-like particles (VLPs). The VLPs are generated by different expression systems for both vaccines, and they contain different adjuvants [23]. VLPs do not contain viral DNA and are therefore not infectious. However, they efficiently induce high levels of neutralizing antibodies which prevent new HPV infections [23,131]. Nevertheless, these vaccines have no therapeutic effect and therefore will not influence existing HPV infections.

The vaccines are administered in three doses by intramuscular injection, and are primarily effective in prepubertal women who are naïve for HPV16, 18 and HPV6 and 11. It has been shown in phase II and III clinical trials that both vaccines are efficient in preventing HPV infections and HPV-associated anogenital and cervical pre-malignant lesions [8,44,48,49,131,132]. In addition, the HPV vaccines are safe, and show mild and only transient adverse effects [8,44,48,49,129,131-133]. The vaccines are type-specific; however some cross-protection is shown, particularly with Cervarix™. This occurs with HPV type 31, and 33 that are antigenetically closely related to HPV16, and HPV45, which is closely related to HPV18 [15,91].

However, HPV vaccination does not rule out cervical cancer screening, since: (i) non-targeted HPV types different from HPV16 and 18 can also cause cervical (pre)malignant disease and therefore the vaccine will only prevent about 70% of cervical cancers; (ii) the participation rate of vaccination will never reach 100%, leaving unvaccinated women unprotected; and (iii) it will take between 15 and 30 years until the effect of preventive vaccination will show its effect on the incidence of cervical cancer. However, the screening programs could be adapted in a vaccine era [130]. To further improve cervical cancer prevention, second generation vaccines are investigated, which could cover all HR-HPV types, be globally implemented, and eventually have some therapeutic effects [67,70,78,126].

Human papillomavirus intratype variants

HPV variants have been defined as HPVs that vary by less than 2% from the reference viral prototype in the nucleotide sequences of coding regions and less than 5% in the non-coding regions [12,21]. HPV variants are shown to cluster in lineages. A recent proposal to define major variant lineages is by approximately 1.0% difference between HPV genomes of the same type, with differences of 0.5-0.9% designated as sub-lineages [20,29,30].

The first complete nucleotide sequence of HPV16 was cloned, from a German woman suffering from invasive cervical cancer, by Seedorf *et al*. in 1985[112]. Subsequently, naturally occurring variants of the HPV16 genome have been observed throughout the genome, which led to revision of the reference HPV16 prototype sequence by Myers *et al.* in 1995 [85]. Currently, this revised HPV16 sequence is frequently used as reference prototype for HPV16 variant studies.

The first study done on HPV16 variants with different ethnical populations was performed by Ho *et al.* in 1993 [58]. They showed that HPV16 variants in LCR robustly segregate into a phylogenetic tree with five major variant lineages: European, Asian-American, Asian and 2 African lineages; African-1 and -2 [27,58,139,145]. Lineage names derive from the geographical origin of the populations in which they were first detected. Since different variant lineages are found in different human races, Chen *et a*l. suggested a co-evolution of the virus with the human [27].

Several epidemiological studies suggested that HPV16 variants, e.g. variant lineages and also individual SNPs, can influence HPV persistence and clinical outcome of the cervical disease [13,26,47,69,97,109,114,143,144,147]. However, there are also indications that the distribution and persistence of HPV variants could be related to the racial composition of a population [144]. In addition to the epidemiological studies, several *in vitro* functional studies have been performed on the HPV16 variants. Some of these studies on E6 suggest that several E6-target pathways are slightly differently regulated by the different variants [6,98,146].

Beta human papillomavirus types

In addition to mucosal HPV types, also the beta cutaneous HPV types appear to be involved in carcinogenesis. Beta HPV types infect the skin and are suspected to play a role in the development of non-melanoma skin cancer (NMSC) together with UV-radiation [17,94,95]. Beta HPVs were first isolated from patients suffering from a rare autosomal recessive genetic disorder called Epidermodysplasia Verruciformis (EV). EV patients have an increased susceptibility for beta HPVs and often develop NMSC in sun exposed areas[90,94]. In addition, the higher incidence of NMSC in renal transplant recipients and immuno-compromised individuals suggests a possible role for beta HPVs in cancer development [11,17,51,94,136]. However, due to the high frequency by which cutaneous HPV DNA is found in the skin and in plucked eyebrows of healthy individuals, and the inconclusive epidemiological data, the role of these viruses in carcinogenesis is still under debate [32,50,53,65,76,89].

On the other hand, it has been shown by functional studies that beta HPV types, e.g. HPV5, 8 and 38, have several transforming properties *in vitro* and *in vivo* [3,4,22,35,42,115,128]. For example, HPV38 E6 and E7 oncoproteins can alter p53 functions, inactivate pRb and lead to the overexpression of hTERT, which are all key events occurring in cancer cells [3,22,42]. These studies also highlighted that the mechanisms of beta HPV types to alter these pathways differ from the ones of the mucosal HPV types. Beta HPV types also displayed transforming activities in transgenic mouse models, e.g. HPV8 and 38 E6 and E7 [35,60,77,106,128]. Interestingly, a recent study showed that chronic skin UV irradiation of HPV38 E6/E7 transgenic mice promoted the formation of skin lesions that resembled the SCC-precursor lesions in humans, actinic keratosis and subsequently SCC, closely mimicking the scenario observed in humans [125]. In contrast, wild-type mice developed neither actinic keratosis nor SCC when exposed to the same dose of UV [125]. This study demonstrated the existence of a synergy between UV exposure and cutaneous beta HPV in the induction of pre-malignant and malignant skin lesions, supporting the role of the latter in the carcinogenic process.

Aims and outline of the thesis

In this thesis we have investigated how the potential oncogenicity of HPVs may be influenced by intra-type variations or by different genotypes of the same genus. Regarding the first topic, several studies on HPV16 intratype variants were performed (Chapters 2 - 5). For the second topic, a functional comparative analysis of several beta HPV types was performed (Chapter 6).

As described above, mucosal HR-HPV infections are very common in sexually active women, but only few will persist and lead to the development of cervical diseases. Additional risk factors appear to contribute to the establishment of persistent infections and the development of cervical lesions. In this thesis we mainly focussed on intratype variants of HPV16, the most common HR-HPV type, as a virus-related risk factor for viral persistence and cervical cancer development.

The International Agency for Research on Cancer (IARC) Biobank contains a large number of HPV16-positive cervical samples from women with and without cervical cancer from different countries worldwide (Figure 5). This Biobank was used to:

1) Characterize the HPV16 variants from geographical / ethnical diverse populations of women
2) Determine the risk of HPV16 persistence in relation to HPV16 variant status
3) Determine cervical cancer risks of different HPV16 variants
4) Evaluate whether the oncogenicity of HPV16 variants varies according to the ethnic / geographic origin of the host

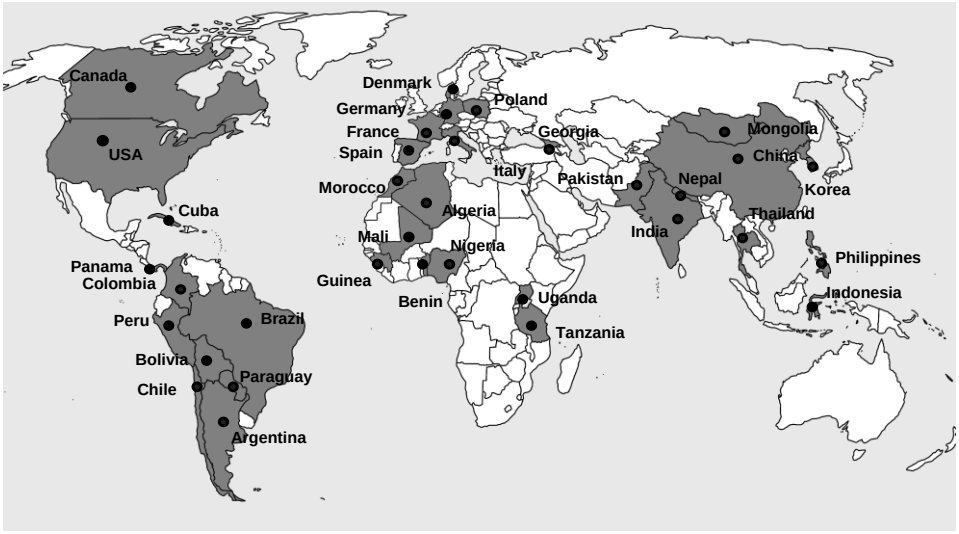

Figure 5. Schematic overview of countries from where cervical samples were available in the IARC biobank and that were used in the described studies.

In **Chapter 2** a descriptive study of the HPV16 E6 and LCR variants around the world was performed. This study consisted of the analysis of HPV16 E6 and LCR variants of a large number of HPV16-positive viral isolates from 24 countries worldwide. With these variants a phylogenetic analysis was performed, which identified nine sub-lineages; European, Asian, African-1a, African-1b, African-2a, African-2b, Asian-American-1, Asian-American-2 and North-American. All isolates belonging to each of the lineages showed a core pattern of single nucleotide polymorphisms (SNPs). These SNPs can be used in the future for a faster determination of variant lineages, and could guide selection of potentially important regions in the HPV16 E6 and / or LCR for functional studies.

In **Chapter 3** a worldwide case-control study is described, showing the cervical cancer risk related to HPV16 variants. Accordingly, we determined if the cervical cancer risk could vary in different populations. The classification of the HPV16 variant lineages of Chapter 2 was used to classify the samples,

analyzed for HPV16 E6 variants, in lineages. The differences in distribution of cervical cancer cases and controls per HPV16 major variant lineage were examined. This distribution was confirmed to be highly geographically / ethnically-specific and there were basically no significant differences in distribution of the lineages among cervical cancer cases and controls in any country or geographical region. However, when the samples of the European lineages were sub-classified according to the presence or absence of the individual variant T350G in E6, EUR-350T or EUR-350G, respectively, different risks associated in different populations were observed.

In both **Chapter 4**, and **Chapter 5**, population based follow-up studies for viral persistence in relation to HPV16 variant lineages are described. The studies were conducted in France and Denmark, respectively. Both studies focused on women infected with HPV16 having normal cervical cytology. At the follow-up visit HPV16 clearance or persistence was defined. HPV16 variants were analyzed, by PCR amplification and direct sequencing of the PCR product, and risk of persistence was determined. In addition, the study in Denmark focused not only on persistence, but also progression to ≥CIN3 in relation to the HPV16 variants was analyzed. Both studies showed that women infected with the EUR-350T variant have an increased risk of viral persistence.

Chapter 6 of this thesis focused on several beta HPV types. These viruses are suspected to play a role in the development of NMSC together with UV-irradiation. A functional *in vitro* study was performed with the aim to predict the potential oncogenicity of several beta HPV types in human. Therefore, in Chapter 6, the biological properties of the E6 and E7 oncoproteins of the beta HPV types 14, 22, 23, 24, 36, 38 and 49 were characterized. We evaluated their ability to immortalize human primary keratinocytes, and to interfere with cellular pathways crucial for cellular transformation, e.g. the p53 –and pRb pathways.

Among the studied HPV types, only HPV38 and 49 E6 and E7 could efficiently immortalize and transform primary keratinocytes. Interestingly,

HPV49 shares some characteristics with the most carcinogenic mucosal HPV type 16. This suggests that HPV38 and 49 could be classified as high risk- and the others as low risk -beta HPV types based on our *in vitro* study. This could give important hints for epidemiological studies on beta HPV types.

Finally, the results described in Chapter 2 to 6 are summarized and evaluated in **Chapter 7**. Also the future perspectives are discussed in this chapter.

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