Chapter 7

Summary, General conclusions and Future perspectives

- HPV16 variants and their role in the development of cervical cancer worldwide
- Comparative functional analysis of the E6 and E7 of several beta HPV types
- Future perspectives
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HPV16 variants and their role in the development of cervical cancer worldwide

The high risk (HR) mucosal HPV type 16 is the most frequently detected HPV type in pre-malignant and malignant cervical lesions. However, only a small minority of these HPV16 infections persist and will lead to pre-malignant and malignant cervical lesions. This scenario suggests that additional host- and virus- related risk factors may contribute to cervical carcinogenesis. Candidate virus-related risk factors include HPV intratype variants, which have been suggested to influence viral persistence and the establishment of cervical lesions.

The aims of this thesis were to (i) characterize the HPV16 variants from geographical / ethnical diverse populations of women, (ii) determine the risk of HPV16 persistence and cervical cancer in relation to specific HPV16 variants, and (iii) evaluate whether HPV16 variant oncogenicity may vary according to the geographic / ethnic origin of the host.

Due to the unique selection and large number of cervical samples from different countries that were available in the IARC biobank, we were able to extend the HPV16 variant descriptions in the literature, as described in **Chapter 2.** The E6 and LCR variants of the cervical samples were analyzed by PCR amplification of respective viral regions and direct sequencing of the PCR product. This resulted in E6 and LCR sequence data of 953 cervical samples, with which a phylogenetic tree was constructed. This phylogenetic tree segregated in four major branches which form the lineages, as reported previously [4,9,12]. In addition, these major branches were divided into more defined sublineages:

(1) European-Asian (EAS), including the European (EUR) and Asian (As) lineages
(2) AA/NA, including the North-American (NA), Asian-American-1 (AA1) and Asian-American-2 (AA2) lineages
(3) AFR1, including the African-1a (AFR1a) and African-1b (AFR1b) lineages

(4) AFR2, including the African-2a (AFR2a) and African-2b (AFR2b) lineages

All viral isolates that classified in each of the lineages showed a specific core pattern of single nucleotide polymorphisms (SNPs). These core patterns of SNPs, and the SNPs that could distinguish the different lineages from each other were also described in Chapter 2.

Subsequently, the distribution of the HPV16 variant lineages per geographical region was shown. Samples with the EUR lineage appeared to be distributed around the world. The other lineages were less spread and mainly present in specific geographical regions, e.g. As and AFR variants predominated in samples from Eastern Asia and Africa, respectively, and the AA and NA predominated in samples from South America and North Africa, respectively.

This extended classification of the HPV16 variants facilitated the case-control study described in **Chapter 3.** In this study HPV16-positive normal cervical samples (controls, number = 400) and cervical cancer cases (number = 1121) of women from different countries were analyzed for HPV16 E6 variants. After the sequencing analysis, the viral isolates were classified in variant lineages according to the classification described in Chapter 2. The differences in distribution of cervical cancer cases and controls per HPV16 major variant lineage (e.g. EUR, As, AFR, and AA/NA) were examined with the Fisher's exact test. The distribution of these variant lineages around the world was confirmed to be highly geographically / ethnically specific. There were basically no significant differences in distribution of the lineages among cervical cancer cases and controls in any country or geographical region.

The EUR lineage can be divided into two sublineages, EUR-350T and EUR-350G, based on the absence or presence of the most common individual SNP in E6, T350G. This polymorphic position does not define phylogenetic sub-lineages, however it is suspected to influence HPV16 persistence and risk of cervical cancer [8,13]. The stratification of EUR lineage isolates into EUR-350T

and EUR-350G showed statistically significant differences in cervical cancer risk across different geographical regions. EUR-350G isolates were significantly underrepresented in cervical cancer in East Asia (OR = 0.02 *versus* EUR-350T; 95% CI = 0.00-0.37) and Europe/Central Asia (OR = 0.42; 95% CI = 0.27-0.64), whereas this was the opposite in South/Central America (OR = 4.69; 95% CI = 2.07-10.66).

In **Chapter 4** and **Chapter 5** two follow-up studies on the risk of HPV16 persistence associated to HPV16 variants are described. The studies of Chapter 4 and 5 were performed in France and Denmark, respectively. In both countries variants from the EUR lineage were predominantly detected. Therefore, we stratified the isolates of the EUR lineage into EUR-350T and EUR-350G and determined if these sublineages could influence HPV16 persistence. In the general population of Denmark there was a significantly higher risk of HPV16 persistence, found in women with and without development of ≥CIN3 combined, when women were infected with EUR-350T compared to EUR-350G (OR = 2.06; 95% CI = 1.04-4.26). In the general population of France we observed only a trend towards an increased risk of HPV16 persistence when women were infected with EUR-350T (OR = 1.6; 95% CI = 0.8-3.4).

In conclusion, the distribution of HPV16 variant lineages around the world, and their relative risks for cervical cancer, appear to be population dependent.

In three of the studies, the EUR-350T sublineage showed a more aggressive behavior in European population's e.g. a higher risk of HPV16 persistence in France and Denmark, and a higher risk of cervical cancer in Italy, Poland and Mongolia, compared to the EUR-350G sublineage. However, in South/Central America a higher risk of cervical cancer development was associated with the EUR-350G, rather than the EUR-350T variant. This suggests that the different HPV16 variants could alter the immunogenic properties of the virus, a phenomenon which apparently depends on the host-

specific factors. Host genetic factors, such as HLA-haplotypes, that differ by population, could play a role in the association between a particular HPV16 variant and cervical cancer development [2,5,7,13]. These findings further provide evidence for the importance of HPV natural variations in the persistence of the viral infection and development of cervical diseases. However, the use of variants as a predictive marker of disease remains doubtful.

Comparative functional analysis of the E6 and E7 of several beta HPV types

The beta HPV types, which infect the skin, appear to be involved in skin carcinogenesis together with UV-irradiation. However, since the beta HPV types are often detected on the skin of the normal population, their possible role as co-factor in skin carcinogenesis is still under debate.

In the study described in **Chapter 6,** a functional comparative study on six beta HPV types was performed, with the aim to predict their potential oncogenicity in human. In this study the biological properties of the E6 and E7 oncoproteins of the beta HPV types 14, 22, 23, 24, 36, and 49 were characterised. Their ability to immortalize human primary keratinocytes (HPK) and interfere with cellular pathways crucial for cellular transformation were examined.

It has previously been shown that the E6 and E7 of HPV38 can immortalize HPK [3]. Here, we found that in addition to HPV38 E6/E7, also HPV49 E6/E7 can efficiently immortalize HPKs. The immortalization efficiency of these two beta HPV types is tightly correlated with their ability to activate hTERT. In addition, the E6 and E7 of HPV 38 and 49 are both able to inactivate pRb by inducing its hyperphosphorylation. This leads to the constitutive activation of E2F-1 and deregulation of cell cycle control.

Both beta HPV types are also able to inactivate p53, although by different mechanisms. It was already reported that HPV38 E6/E7 expression in keratinocytes leads to hyperphosphorylation of p53, thereby activating ΔNp73α,

an inhibitor of p53 [1]. On the contrary, we showed that HPV49 E6/E7 is able to degrade p53 in an E6AP-dependent manner, similar to the most carcinogenic mucosal HPV type 16. Even thought the degradation of p53 by HPV49 E6 is weaker than for HPV16 E6, this is the first study highlighting similarities between a beta HPV type and HPV16.

All together, these results suggest HPV38 and 49 as high risk beta HPV types for skin carcinogenesis, and the other studied beta HPV types as low risk types.

Future perspectives

HR-HPV detection is getting implemented in the screening programs and, compared to cytology; it improves detection of clinically relevant cervical lesions substantially. However, many HR-HPV infections are transient and do not give rise to high-grade disease. Consequently, there is a need for triage markers to distinguish HR-HPV positive women with persistent infections, who are in need of close surveillance. Characterisation of the oncogenic potential of HPV intratype variants was a logic continuation of efforts to improve the ability of screening tests to identify HPV infections likely to develop into cervical cancer. However, the presented studies showed that the clinical application for HPV16 variants remains doubtfull.

In Chapter 2 specific SNPs to determine HPV16 variant lineages were identified. This finding can lead to fast, cheap, and reliable methods to detect the HPV16 variant lineages, e.g. by reverse hybridization techniques [10]. These tests would facilitate future research on HPV16 variants. Future research could include epidemiological studies with a larger sample size per country including knowledge on the ethnical origin of the women. These larger studies could also take in account host genetic factors, e.g. HLA-haplotype polymorphisms, to determine if specific host genetic factors together with a certain HPV16 variant lineage or individual SNP could be responsible for the differences in cervical cancer development in the different populations. Such studies could be

complemented with full genome sequencing of the HPV genomes to find out whether viral variables of different viral genomic regions could play a role as well.

These studies are important to understand the genetic, molecular and eventually biological basis of differences in the carcinogenicity of these variants. They can guide selection of variants for further *in vitro* functional studies, e.g. examine the biological properties of E6 with and without T350G (L83V) transfected in human primary kerationcytes of donors from different ethnical groups. This may help to unravel the biological and / or immunological interactions between the virus and host.

Regarding the study on the beta HPV types, our findings provide additional support for the role of these HPV types in skin cancer. Especially HPV38 and 49 showed to be able to immortalize and transform human primary keratinocytes. Therefore, it is important that large epidemiological studies focus on the detection of these two types e.g. in normal sun-exposed skin and not sun-exposed skin of the normal population, NMSC samples and / or pre-malignant skin lesions, etc.

On the basis of functional studies, mRNA microarray analyses are ongoing to understand the differences between the E6 and E7 of HPV16, 38 and 49 expressed in primary keratinocytes. This will give more insight in the mechanisms that HPV38 and 49 *versus* HPV16 use for the cellular immortalization and transformation.

In addition, studies already have been done on skin cancer development in transgenic mice expressing HPV38 E6/E7 [6,11]. Development of transgenic mice expressing HPV49 E6/E7 will also be important to evaluate the contribution of this HPV type in skin carcinogenesis *in vivo*. The mice can be treated with UV-irradiation to appreciate the involvement of HPV49 under conditions that mimic the sun-exposed skin in humans.

All together, this research can lead to better tools to predict the clinical outcome of HPV infections, and can give hints to pathways disturbed in other cancers or by other viruses.

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