Human papillomaviruses in skin cancer and cervical cancer

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Abstract

The causal relationship between persistent genital infections with human papillomavirus (HPV) and development of cervical cancer is well established. In contrast, the significance of infections with cutaneous HPV for development of non-melanoma skin cancer (NMSC) is not well understood. We have evaluated whether seropositivity to cutaneous HPV is a marker for cutaneous HPV infection and used high throughput HPV serology to investigate the risk for developing NMSC in relation to seropositivity for cutaneous HPV infection and PCR techniques to investigate the risk for NMSC in relation to presence of HPV DNA in the skin. We have also investigated how different sexually transmitted infections interact with HPV in the aetiology of cervical cancer.

Two of our NMSC studies were hospital-based case-control studies where biopsies from skin tumours and healthy skin were analysed for presence of HPV DNA and serum samples for presence of antibodies to 14 different HPV types. The third NMSC study and the cervical cancer study were designed as prospective biobank-based case-control studies where biobanks were linked to cancer registries for identification of cancers that have occurred after donation of a serum sample. For patients with cervix cancer also formalin-fixed paraffin embedded tumour tissue was retrieved and tested for HPV DNA.

In the skin cancer studies, we found that both DNA and seropositivity to HPV of genus beta species 2 associated with an increased risk for development of squamous cell carcinoma (SCC) of the skin and that sun-exposure is a risk factor for cutaneous HPV infection. In the cervical cancer study we found in addition to the exposure to the oncogenic HPV type that is found in the cancer tissue, that history of Chlamydia trachomatis stood out among the different sexually transmitted infections as being associated with increased risk for cervical cancer, suggesting that it may acts as a co-factor to HPV in cervical carcinogenesis.

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Till Meja

Table of contents

Summary

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Papillomvirus (PV) är små virus som förmodligen infekterar alla däggdjur samt fåglar. De papillomvirus som infekterar människa kallas humant papillomvirus (HPV) och kan infektera antingen huden eller slemhinnor.

Att livmoderhalscancer orsakas av bestående infektion med HPV har varit känt länge. Man har länge också misstänkt att HPV-infektioner i huden kan orsaka icke-melanom hudcancer (NMSC) men orsakssambandet är inte bevisat. I gruppen NMSC ingår i huvudsak diagnoserna skivepitelcancer (SCC) och basalcellscancer (BCC). Vi har undersökt risken att utveckla NMSC om man har en HPV-infektion i huden tillsammans med andra kända riskfaktorer för hudcancer och om detektion av antikroppar mot HPV som infekterar huden sammanfaller med förekomst av HPV-DNA i huden. Vi har även tittat på hur sexuellt överförbara HPV-infektioner samverkar med andra faktorer, så som andra infektioner och rökning, i utvecklingen av livmoderhalscancer.

Två av studierna om NMSC är designade som sjukhusbaserade fall-kontroll-studier (individer som redan har en sjukdom jämförs med individer utan sjukdomen), där vävnad från tumör och frisk hud analyserats för förekomst av HPV-DNA och serumprov testats för förekomst av antikroppar mot 14 olika HPV-typer. En tredje NMSC-studie och en studie om livmoderhalscancer är båda designade som prospektiva (framåtblickande) fall-kontroll-studier där biobanker länkats till cancerregister för att identifiera individer med sjukdomen som lämnat prov till biobanken. Inom varje biobank har kontroller sedan valts efter matchning mot fallen (faktorer så som kön, ålder och tidpunkt för provtagning) och serumprov från både fall och kontroller samlats in och analyserats. Från fallen med livmoderhalscancer har även tumörvävnad testats för HPV-DNA.

Sammanfattningsvis fann vi att infektion i huden med HPV från genus beta species 2 innebar en ökad risk att utveckla SCC i huden samt att förhöjd exponering av huden för solljus var en riskfaktor för att få en HPV-infektion. För livmoderhalscancer fann vi att om DNA-test och antikroppstest var positivt för

samma HPV-typ ökade risken att utveckla livmoderhalscancer jämfört med om man bara testats positiv för antikroppar eller om HPV-typerna inte överensstämde. Att ha varit infekterad med Chlamydia trachomatis var också kopplat till livmoderhalscancer och bidrar troligen till risken.

List of papers

This thesis is based on the following papers:

I: Cutaneous Human Papillomaviruses Found in Sun-Exposed Skin: *Beta-papillomavirus* **Species 2 Predominates in Squamous Cell Carcinoma**

Ola Forslund, Thomas Iftner, Kristin Andersson, Bernt Lindelöf, Eva Hradil. Peter Nordin, Bo Stenquist, Reinhart Kirnbauer, Joakim Dillner and Ethel-Michele de Villiers for the Viraskin Study Group.

Journal of Infectious Diseases 2007:196, 876-883

II: Seroreactivity to Cutaneous Human Papillomaviruses among Patients with Non-melanoma Skin Cancer or Benign Skin Lesions

*K*ristin Andersson, Tim Waterboer, Reinhard Kirnbauer, Katharina Slupetzky, Thomas Iftner, Ethel-Michele de Villiers, Ola Forslund, Michael Pawlita and Joakim Dillner

Cancer Epidemiol Biomarkers Prev 2008:17 (1), 189-195

III: Prospective study of HPV seropositivity and non-melanoma skin cancer

Kristin Andersson, Kristina Michael, Tapio Luostarinen, Tim Waterboer, Randi Gislefoss, Timo Hakulinen, Ola Forslund, Michael Pawlita and Joakim Dillner

Submitted for publication

IV. Prospective seroepidemiological study of HPV and other risk factors in cervical cancer.

Kristin Andersson, Lisen Arnheim Dahlström, Tapio Luostarinen, , Steinar Thoresen, Helga Ögmundsdottir, Laufey Tryggvadóttir, Fredrik Wiklund, Gry B. Skare, Carina Eklund, Kia Sjölin, Egil Jellum, Pentti Koskela, Göran Wadell, Matti Lehtinen and Joakim Dillner

Manuscript

Abbreviations

AC	Adenocarcinoma
AK	Actinic keratosis
BCC	Basal cell carcinoma
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma in situ
CRPV	Cottontail rabbit papillomavirus
E	Early genes
E6-AP	E6-associated protein
EV	Epidermodysplasia verucciformis
GAG	Glycosaminoglycans
HPV	Human papillomavirus
HR	High risk
HSPG	Heparan sulfate proteoglycan
IARC	International agency for research on cancer
L	Late genes
LBC	Liquid based cytology
LCR	Long control region
LR	Low risk
NCR	Non-coding region
NMSC	Non-melanoma skin cancer
ORF	Open reading frame
ORI	Origin of replication
Pap	Papanicolaou staining
RRP	Recurrent respiratory papillomatosis
PV	Papillomavirus
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesions
TMC	Trans membrane channel-like
URR	Upstream regulatory region
UV	Ultraviolet
VLP	Virus like particle

Introduction

History

The papillomaviruses (PVs) are a taxonomic family of their own, *papillomaviridae*. They have a tropism for epithelial cells and are highly species-specific and probably occur in most mammals and birds (1). The first PV described was the cottontail rabbit papillomavirus (CRPV) which in 1933 was found to cause warts in cottontail rabbits (2) and a few years later also was found to induce malignant transformation (3, 4).

The carcinogenic potential of human papillomavirus (HPV) was first suggested in the 1950's, in patients with the rare hereditary disease epidermodysplasia verucciformis (EV) (5). In 1976 zur Hausen proposed that HPV can cause cervical cancer (6, 7), and was awarded with the Nobel prize in 2008. The two HPV types most commonly found in cervical cancer, HPV 16 and 18, were discovered short after the first proposal was made (8, 9). The first epidemiological studies on HPV and cervical cancer was published in 1987 (10) and since then the aetiological link has been established in studies from all over the world.

Morphology and genomic organisation

Papillomaviruses are non-enveloped virus with an icosahedral capsid about 60 nm in diameter that is composed of two capsid proteins, the major capsid protein L1 and the minor capsid protein L2 (9). The genome consists of circular and double-stranded DNA of about 8,000 base pairs which is associated to cellular histones to form a chromatin like complex (11) . The PV genome is divided into three regions based on functional properties, the early region (E) which contains up to six open reading frames (ORFs), E1, E2, E4, E5, E6 and E7, and encodes regulatory proteins involved in replication, translation and transformation. The late (L) region encodes for the two structural capsid proteins as mentioned earlier. The third region, located between the L1 and E6 ORFs (Figure 1), is non-coding and is known as the non-coding region (NCR), long control region (LCR) or upstream regulatory region (URR). This region contains the origin of replication and enhancer elements for regulation of gene expression (12).

Among the high-risk HPV types (the types known to induce cervical cancer) there are two important promoters controlling the gene expression. The early promoter is active both in the undifferentiated as well as in the differentiated cells whereas the late promoter is activated in differentiated cells (13, 14).

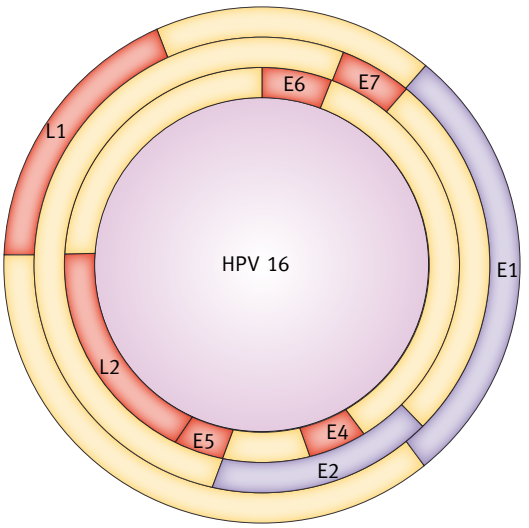


Figure 1. Schematic picture of the genomic organisation of HPV. URR is the upper regulatory region. Reprinted from Frazer IH, Prevention of cervical cancer through papillomavirus vaccination, in Nat Rev Immunol 2004;4:46-54 with permission from Nature Publishing Group.

Classification

Papillomaviruses are grouped into genus, species, types, subtypes and variants based on the DNA-sequence similarity of the L1 gene (1). PVs within a genus have less than 60% sequence similarity to PVs in other genus and between species within a genus the sequence similarity is between 60 and 70 % (Figure 2). If the sequence similarity is between 70 and 90 % the papillomaviruses are divided into types and with a sequence similarity between 90 and 98 % they are considered to be subtypes and between 98 and 99 % they are variants to a PV type (1).

Today, well above 100 different HPV types has been fully sequenced and characterized. They divide into two groups, the mucosal types that infect mucosa and the cutaneous types that infect skin. Mucosal types are mainly found in genus alpha whereas cutaneous types mainly are found in genus beta, gamma, mu and nu but also in the genus alpha (Figure 2).

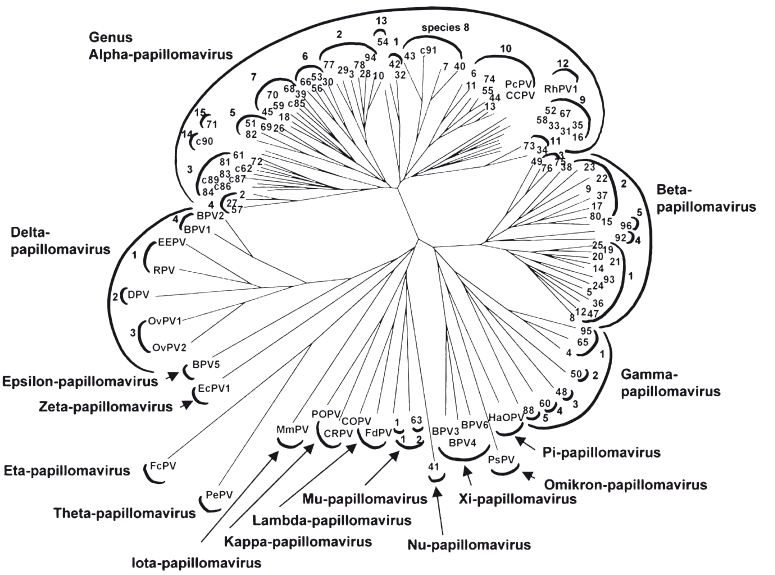


Figure 2. Phylogenetic organisation of the papillomaviruses. Reprinted from de Villiers EM et al. Classification of the papillomaviruses, in Virology 2004;324:17-27, with permission from Elsevier.

The mucosal types are further grouped as high risk (HR) or low risk (LR) types, where the HR types are the oncogenic types with the ability to cause cancer and LR types that can produce benign genital warts (condyloma acuminata) that rarely progress to malignant lesions even if left untreated (12). Differences between HR and LR types can at least partly be explained by differences in function of the oncoproteins E6 and E7. HR types have been found to immortalise cells in cell cultures containing primary baby rat kidney epithelial cells and keratinocytes (15, 16) as well as human keratinocytes (17). In phylogenetic analyses of the mucosal alpha types based on the whole genome or the early genes E1, E2, E6 and E7 the oncogenic HR types cluster together which they do not do if the phylogenetic tree is based on the late genes L1 and L2. This suggests

that there might be a common ancestor of all genital HR types (18).

Many efforts have been made to assess the risk of individual HPV types found in the genital tract and cervical cancer. In 2005 a meeting at the International agency for research on cancer (IARC) with the purpose to reassess the carcinogenicity of HPV concluded that the following types should be classified as carcinogenic; HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 (19).

The replicative cycle of HPV

HPV infect mucosal and cutaneous stratified squamous epithelial cells and its replicative cycle is closely linked to the differentiation of the epithelial. Entry occurs in the basal epithelial cells, the only cells in the epithelium that can divide, after which the cell first follows the normal procedure and leaves the basal layer. An uninfected cell would thereafter exit the cell cycle and start to differentiate but instead the HPV infected cells escape the cell cycle arrest resulting in continued cell division, a step that is crucial for the HPV since it relies on cellular enzymes to replicate its genome (20). It is thought that the virus gain access to the basal epithelial cells via micro-trauma in the skin or mucosal surface (Figure 3) (21, 22), but the virus is also detected in hair follicles and endocrine ducts (23).

The receptor or receptors mediating attachment and entry of HPV into the host cell is not yet entirely know. Heparan sulfate, probably together with a secondary receptor, has for a long time been reported as a requirement for HPV infection (24-26). Also cell surface glycosaminoglycans (GAGs) has been shown to possibly provide an initial binding that could be followed by a secondary receptor binding and entry (24). Lately it has been suggested that HPV initially binds to the basement membrane to mediate early changes to the viral capsid that are essential for infection, and it is most likely that the heparan sulfate proteoglycan (HSPG) dependent binding is the first of several essential steps that takes place on the basement membrane (21). The characteristics of binding may however differ between HPV types, also within a genus and species (27).

Virion entry into the host cell has been shown to be a very slow process mediated by the clathrin-dependent receptor-mediated endocytosis (28, 29). Follow-

ing entry into the cell, the virion is uncoated within the endosome (26), and the HPV genome is transported to the nucleus where the early promoter is activated (20). The viral replication occurring in the basal layers is considered to be non-productive, the virus establishes it self as a low-copy-number episome and replicates its genome using the host cell DNA replication machinery and is being passed on to the daughter cells (30). On average the viral genome is replicated once per cell cycle, but it appears that it can be either by replicating once per S phase or by random replication (31).

As the HPV infected basal cells starts to differentiate and migrate into the upper layer of the epithelium, the cell cycle still remains active mainly because of the activities of the E7 protein (32). The productive phase of the HPV replicative cycle occurs in the differentiated epithelial cells and starts with the activation of the late promoter. In the productive phase the viral DNA is amplified into high copy-numbers, the capsid proteins L1 and L2 are synthesised and finally the virion particles are assembled and released as the upper layer of the epithelium is shed (Figure 3) (33).

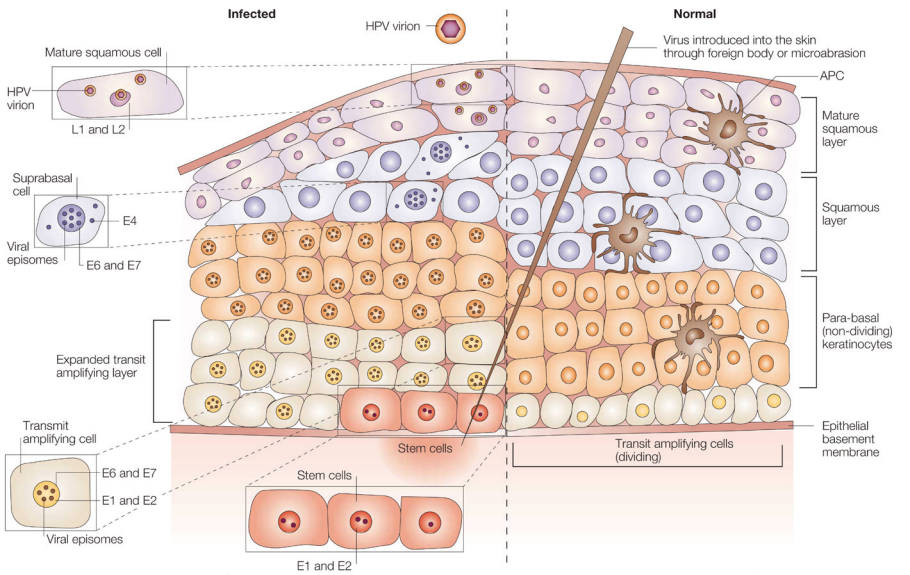


Figure 3. Schematic picture of HPV infected squamous cell epithelial. The viral proteins are expressed sequentially with differentiation as shown, and mature virions are produced only at the most superficial layers of the epithelium. Reprinted from Frazer IH, Prevention of cervical cancer through papillomavirus vaccination, in Nat Rev Immunol 2004;4:46-54 with permission from Nature Publishing Group.

The viral proteins

E1

The E1 protein is required for viral replication together with the E2 protein. E1 on its own binds weakly as a hexameric complex to the origin of replication (ORI) and possesses DNA dependent ATPase and DNA helicase activity (34). To enhance the binding affinity to the DNA sequence and for replication to occur it is necessary for E1 to form complex with E2 (35, 36). E1 is required for both initiation and elongation of replication and is believed to do so by recruiting cellular DNA polymerase to the viral replication origin (37, 38)

E2

The E2 protein is a multifunctional DNA binding protein with the DNA binding activity located at the C-terminal end of the protein and the N-terminal end has been shown to harbour the transacting activity of the protein (39). It is needed for pre-initiation of replication where it acts as a complex together with E1 to strengthen the binding affinity to the DNA strand (38). The E2 protein also both activates and represses transcription (40). For example can E2 repress the early promoter in HPV 16 and 18 which is likely to be important for the controlled expression of viral genes transcribed from the early promoter (40, 41).

E2 also has an important role in plasmid maintenance by attaching the viral genomes to the mitotic chromosomes, a process mediated by the cellular protein Brd4 (42, 43), but E2 of at least some HPV types associate with the mitotic spindle rather than the chromosomes (44). The E2 protein has also been shown to be able to induce apoptosis in cell cultures (45).

E4

The E4 ORF is located among the early genes but the protein is predominately found in differentiated cells where the late genes are expressed (46). The E4 ORF is overlapping with the E2 ORF (Figure 1), but since the proteins are expressed from different reading frames the proteins have entirely different amino acid sequences. Translation of the E4 protein occurs from the spliced transcript E1^E4, where a few codon from E1 are spliced to E4 (47). The E1^E4 protein

has been suggested to be important for activation of the late viral functions and DNA amplification in HPV 31 (48) and 18 (49) but this effect was not found in HPV 11 (50). This suggests that type-specific differences exists between E1^E4 proteins from different HPV types. Another function that has been suggested for the E1^E4 protein is to be important for the release of new virions by disturbing the cytokeratin matrix (51).

E5

The E5 protein is coded for by the mucosal high risk (HR) types but the low risk (LR) types either lack an E5 ORF and/or translation start codon for the E5 (52). The cutaneous HPV types do not encode E5 either (53), but for bovine papillomavirus 1 (BPV1) E5 is the major transforming protein (54, 55). E5 proteins coded by HR types also display weak oncogenic properties in tissue culture (56, 57), and have also been shown to have a role in modulation of late viral functions through activation of proliferation capacity in the differentiated cells (58). It has also been suggested the E5 protein is involved in establishment of persistent infection and can possibly inhibit apoptosis by affecting several cellular pathways involved in cell adhesion, cell motility and mitogenic signalling (59).

E6

E6 is an important oncoprotein among the HR HPV types. It is a zinc-binding protein produced early in infection (60) with the main function to inactivate the tumour suppressor protein p53 (61). To inactivate p53, E6 requires a cellular protein known as E6-associated protein (E6-AP) and together they form a p53-specific ubiquitin-protein ligase (62). HR E6 also has other functions, for example can they interact with PDZ domain containing proteins, an interaction that is thought to be necessary for induction of epithelial hyperplasia (63). This PDZ binding ability has not been found among LR or cutaneous HPV types (64).

E7

E7 was the first oncogene of HR HPVs that was identified and is predominantly found in the nucleus of the cell (65). Like the E6 protein it has zinc-binding properties (60) and its main function is to bind to and inactivate proteins of the retinoblastoma (pRb) family. Rb proteins are cell cycle regulators that

control transition from G_1 to S-phase negatively by binding to the E2F transcription factors, a process regulated by phosphorylation. When E7 binds to hypophosphorylated Rb the cell cycle control is hindered since E2Fs are released and transcription occurs (66). E7 also promotes the transcription of E2F by binding to histone deacetylases (HDACs), transcriptional co-repressors, which leads to increased E2F transcription and thereby S-phase replication (67, 68).

L1 and L2

L1 and L2 are the two capsid proteins of HPV. They are not expressed until late stage of the viral life cycle which occurs in the highly differentiated cells (69). The L1 ORF is highly conserved and is used to classify papillomaviruses. Five L1 monomeric proteins form a pentameric capsomer and 72 of those capsomers form the viral capsid (70). L1 proteins are known to self assemble into virus like particles (VLPs). The L2 protein is situated in the centre of the L1 capsomer and is not able to self assemble (71). The L2 protein binds to DNA and is thought to be important for viral assembly by introducing the viral genome into the viral particles (72), it is also responsible for the transport of the viral genome to the nucleus once the viral particle has been uncoated (73).

HPV-associated diseases

Mucosal infections

Genital infections

Genital HPV infections are mainly transmitted by skin-to-skin or mucosa-to-mucosa contact (74). Genital HPV infections are very common, particularly in young women in their first decade of sexual activity but most sexually active women have been infected with at least one genital HPV type at some time point (75). The vast majority of genital HPV infections are transient infections that are cleared within 1-2 years (76, 77).

Condyloma acuminata (genital warts) occur anywhere on the external genitalia and is a very common sexually transmitted infection. They are mostly caused by the LR HPV types 6 or 11, even if a minority of the lesions might be

co-infected with a HR type (78).

Cervical cancer is caused by persistent infection of at least one HR HPV type (79) and the most commonly detected HPV types in cervical cancers are HPV 16 and 18 (80, 81). Cervical cancer is the second most common cancer among women worldwide with an estimated global incidence of 493,000 new cases and 274,000 deaths in 2002. A majority of the cases, 83%, occur in developing countries (82). The majority of cervical cancers are squamous cell carcinomas (SCC), occurring at the transformation zone where columnar epithelium transforms into squamous epithelium, whereas adenocarcinomas (AC) occurring from glandular epithelium within the cervical canal, are less common (82). In areas where the incidence of cervical cancer is low the proportion of adenocarcinomas is generally higher than in areas with high incidence of cervical cancer (83). This is probably because cervical screening has little effect in reducing the risk of adenocarcinomas of the cervix (84). Cancer of the vulva and vagina are also HPV related. They are however much more rare than cervical cancer (82).

Development of cervical cancer follows four major steps; HPV transmission, viral persistence, progression of a clone of persistently infected cells to precancer and invasion. Reversed steps, such as clearance of HPV infection and more rarely regression of precancer to normality also occurs (Figure 4) (85). Both HR and LR types can cause persistent infections but virtually only the persistent HR infections progress into precancer lesions or cancer (52). The premalignant, non-invasive precancerous lesions are called cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL) according. The lesions are further histologically divided based on to what degree the epithelial cells have lost cytoplasmic maturation and exhibit cytological atypia. CIN1 corresponds to mild dysplasia, CIN2 to moderate dysplasia and CIN3 to severe dysplasia and carcinoma in situ (CIS). The Bethesda classification system, used in the USA, only has two classes, low-grade SIL (LSIL) and high-grade SIL (HSIL) where LSIL corresponds to CIN1 and HSIL to CIN2-3 (86). The classification atypical cells of undetermined significance (ASCUS) is also used and represents poorly visualised cells from LSIL, HSIL and other infectious or non-infectious processes (75). A two-year follow-up study found that women diagnosed with CIN1 did not have greater risk for development of CIN2 or 3 than women with normal cytology and that LSIL and HPV positive ASCUS were clinically equivalent (87).

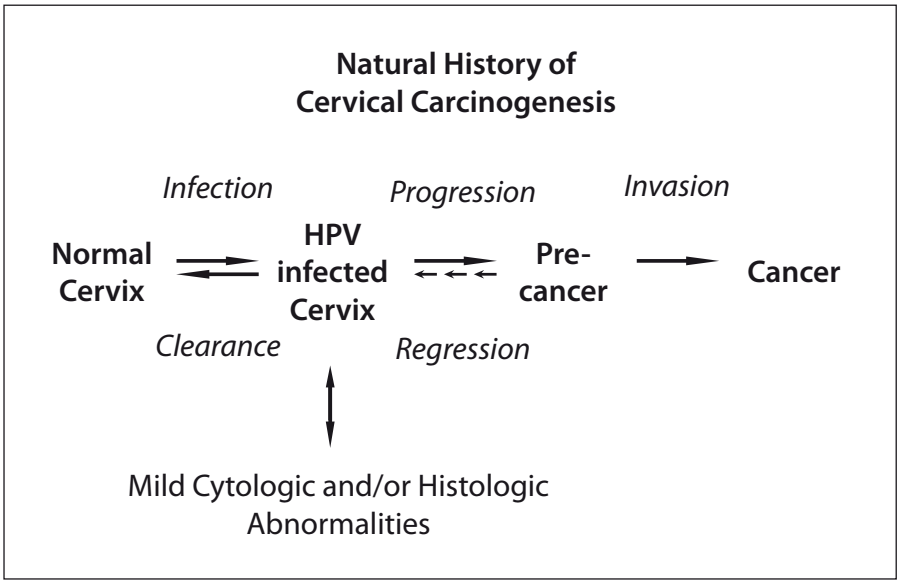


Figure 4. Schematic model over development of cervical cancer from HPV infection.

Even if HPV infection and lack of effective screening are the major risk factors for cervical cancer several co-factors have been found to contribute to the risk. For example other sexually transmitted infections such as herpes simplex virus type 2 (HSV-2) (88) and *Chlamydia trachomatis* (89-94) have been found to increase the risk even if the findings for HSV-2 are not consistent (95). *C. trachomatis* infections appears to influence whether an HPV infection becomes persistent or not (96). It is possible that infections with other sexually transmitted infections are not true co-factors but simply an indication of a higher risk behaviour that increases the exposure to HPV (97). Environmental factors such as smoking (98-100) also increase the risk for developing cervical cancer, as does multiparity (101, 102) and sexual behaviours such as age at first intercourse and lifetime number of sexual partners (103). Long-term use of hormonal contraceptives was found to be a risk factor in a meta-analysis addressing the issue (104). The risk for cervical cancer increased with increasing duration of contraceptive use and it was also suggested that the risk decreases after the use of hormonal contraceptives has stopped (104). Familial aggregation of cervical cancer has been found suggesting that genetics can be a risk factor (105-107) but some studies have also stated that familial aggregation due to shared environmental factors cannot be ruled out (107).

Conventional prevention of cervical cancer has so far been organised cytology-based cervical screening programs and this has lead to a reduction in both incidence and deaths related to cervical cancer. The screening has been based on Papanicolaou (Pap) staining of epithelial cells from the cervix in the expectation that detectable nuclear abnormality will be representative of histologically defined underlying lesions. Women with normal cytology continue with fixed-interval screening whereas women with abnormal cytology will be monitored through follow-up cytology or by referral to colposcopy and possibly treatment (108). As an initiative from the Europe Against Cancer Programme, screening programs are evaluated and revised guide lines published as European Guidelines for Quality Assurance in Cervical Cancer Screening (109). Despite the success of the cytology screening programs when it comes to decreasing incidence and mortality, cytology has limitations. A particular problem is the high false negative rate which has important public health implications (110). As cervical cancer is caused by HPV infections, HPV testing has been suggested to be included in screening. Even a single HPV test has been found to have a higher negative predictive value than a single cytology test, and if the HPV test is combined with a normal cytology test the negative predictive value is as high as 99% (108). HPV testing has so far been used for three main screening or management-related purposes: i) to complement the results from Pap smears in primary screening for detection of cervical cancer or precursor lesions among asymptomatic women, ii) in triage of women with abnormal Pap smears either as a complement to cytology or as a substitute for repeat smears or iii) as follow-up of treated cases for improved surveillance of recurrent cervical lesions, to permit more aggressive management of cases that are likely to recur because of HPV persistence (110).

Oral infections

Tobacco use and alcohol consumption have for long been well established as risk factors for development of SCC of the head and neck, in particular cancers of the oropharynx and base of tongue. A small proportion of the cases (15-20%) do however occur in non-smoking, non-drinking people which suggested the presence of other risk factor. It has been demonstrated in several studies that HR HPV is involved in development of SCC of the head and neck and that HPV 16 is the predominantly detected type (111, 112). It is also known that patients suffering from HPV positive head and neck cancer has better survival prognosis than patients with other head and neck cancers (113).

Recurrent respiratory papillomatosis (RRP) is a rare disease where papillomas occur anywhere in the respiratory system but most commonly in the larynx, of which the vast majority are caused by HPV 6 or 11. Despite the benign nature of the lesions, they cause a significant morbidity and sometimes even mortality because of the location in the respiratory tract and recurrence after surgical removal. Progression into malignancy is rare but does occur. The disease has a poorer prognosis if the lesions are extended to the lower airways. The incidence of RRP is estimated to about 2 per 100,000 in adults and 4 per 100,000 in children (78, 114).

Cutaneous infections

HPV is a very common infection in the skin and the type spectrum is considerable (115, 116). Sun-exposed areas of the skin, like the face and hand, have more HPV than non-sun-exposed parts, like the back or buttock, and most of the infections are asymptomatic irregardless of location (116).

Warts

Skin warts are benign lesions that predominantly occur on hands and feet, although they can arise in almost any location. They are mostly caused by HPV1, 2, 3, 4, 10, 41 and 57 (117, 118) and most of the lesions regress within two years even if some persist indefinitely. Butchers' warts is a kind of warts that are predominantly found in butchers and meat handlers and are caused by HPV 7 (119). Warts in toe webs have also been found to associate with HPV7 (120).

Psoriasis

Psoriasis is a non-contagious lifelong dermatological disease with genetic predisposition. It is a systemic disease but is characterised by an extensive keratinocyte proliferation (121).

The role, if any, of HPV in development of psoriasis is unclear. Several studies have found that primarily HPV5 but also HPV 36 and 38 are more common in patient suffering from psoriasis than in both healthy individuals and individuals suffering from other skin diseases (122-127). A causal role for HPV has however

not been supported (128) and antibodies to at least HPV 5 are also generated under other conditions with rapid keratinocyte growth (129). The question if psoriatic skin is more permissive for viral presence than normal skin is under discussion.

Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) was first described in 1922 by Lewandowsky and Lutz (130). It is a rare autosomal, recessive dermatological disease associated with a high risk for carcinoma of the skin and an abnormal susceptibility to a specific group of related HPV types, previously known as EV-types now grouped into the genus beta (Figure 2) (131). Among the genus beta types, particularly HPV 5 and 8 are found in SCC in EV patients and are considered to be high-risk types, but also HPV 14, 17, 20 and 47 have been found in SCC and suggested as high-risk types (132). EV patients have higher seroprevalences to most HPV types, particularly types from genus beta, compared to matched healthy controls, an effect not seen if compared to first degree relatives (133).

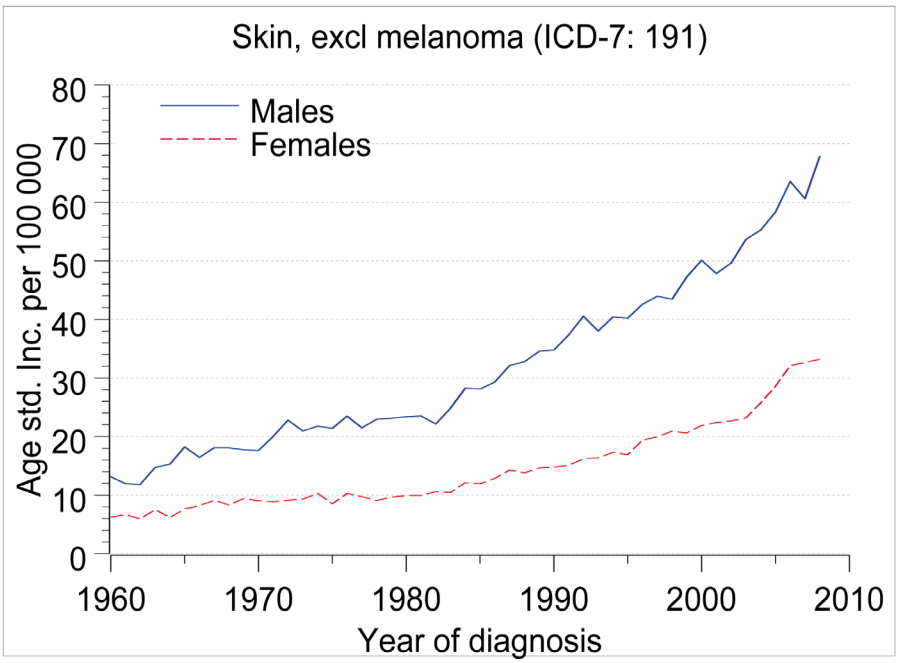
A first susceptibility locus for EV was mapped to chromosome 17 (EV1) at a region where also the p53 gene is located (134). At this locus two EV sensitive genes (EVER1 and EVER2) have been found and EV-associated mutations identified (135, 136). A second susceptibility locus (EV2) has been mapped to chromosome 2 (137). The EVER1 and EVER2 genes encode for integral membrane proteins and belong to the transmembrane channel-like (TMC) gene family and have also been labelled as TMC6 for EVER1, and TMC8 for EVER2 (138). Both the EVER1 and EVER2 proteins form complex and interact with the zinc transporter protein (ZnT-1) (139). Transcription factor activities induced by zinc and cytokines are inhibited by EVER and ZnT-1 proteins and the AP-1 transcription factor, a key transcription factor for HPV, is negatively regulated by the protein complex. HPV 16 E5, a functional protein found to be lacking in cutaneous HPV, was found to bind to EVER and ZnT-1 and inhibit their negative regulation of AP-1(139). This might explain not only why EV patients are susceptible to infection with cutaneous EV types but also how the genital HPV types bring about the high levels of free zinc and AP-1 activity that they need to express their viral genome (139).

Non-Melanoma Skin Cancer

An overwhelming majority of non melanoma skin cancers (NMSC) are basal cell carcinomas (BCC) or squamous cell carcinomas (SCC), where BCC is about 4 times as common as SCC. Over the last decade NMSC, BCC excluded, is one of the most rapidly increasing malignant tumours in Sweden. An average annual increase of 3.9 per cent is observed for men and 5.9 for women in the last 10-year period (140). In Figure 5 the increase in incidence over the last 50 years and the age specific distribution in Sweden 2008 can be seen. Almost all individuals diagnosed with NMSC are over 60 years of age.

BCC is the most common skin cancer among humans and is most often found on areas of the skin that are exposed to sunlight or ultraviolet (UV) radiation. It starts to develop from basal cells, small round cells found in the lower layer of the epidermis (Figure 6), and is a cancer form that grows slowly and only rarely metastasises (0.028-0.55%) (141). Tumour size can vary from only a few millimetres up to several centimetres in diameter. BCC was until 2003 not reported to the cancer registry in Sweden.

Most cutaneous SCCs, similar to BCC, occur on skin that is regularly exposed to sunlight or other ultraviolet radiation and are most often seen in middle-aged or elderly people. SCC develops from squamous cells (Figure 6) and can either occur de novo, in the absence of any precursor lesions, or in rare occasions from the sun-induced precancerous lesion actinic keratosis (AK). Multiple AKs is a riskfactor for development of SCC. Bowen's disease, another name for SCC in situ, is the earliest form of SCC where the cancer has not yet invaded surrounding tissue. Interstingly, in extragenital Bowen's disease, particularly of the hands, the genital HR type HPV 16 has frequently been detected (142, 143). Even if SCC generally is slow growing it is capable of locally infiltrative growth, spread to regional lymphnodes and distant metastasis (144).



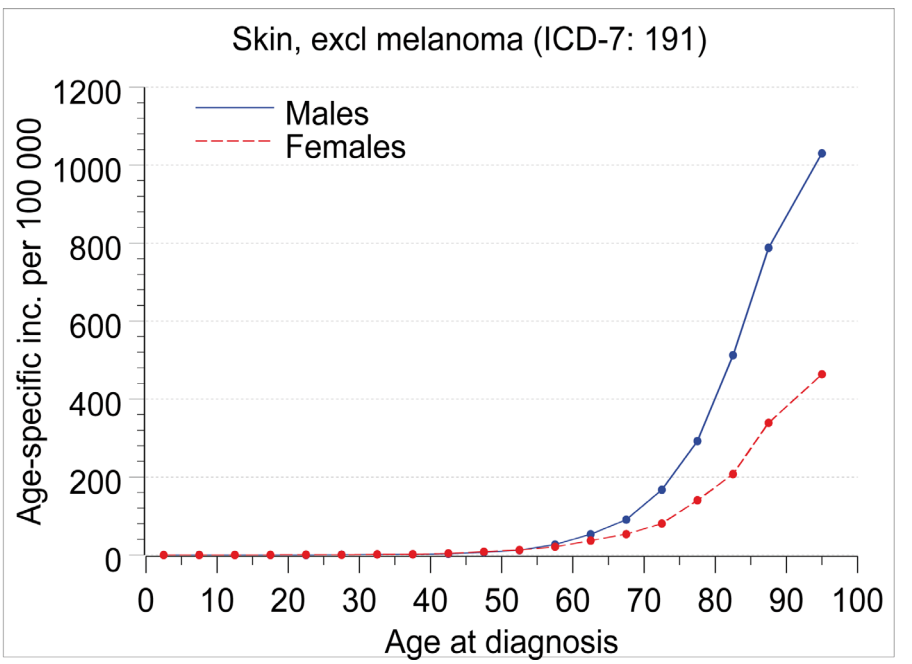


Figure 5. Age standardised incidence of NMSC per 100 000 and age-specific incidence of NMSC per 100 000 for males and females in Sweden 2008. Adapted from Cancer incidence in Sweden 2008 published by Socialstyrelsen 2009.

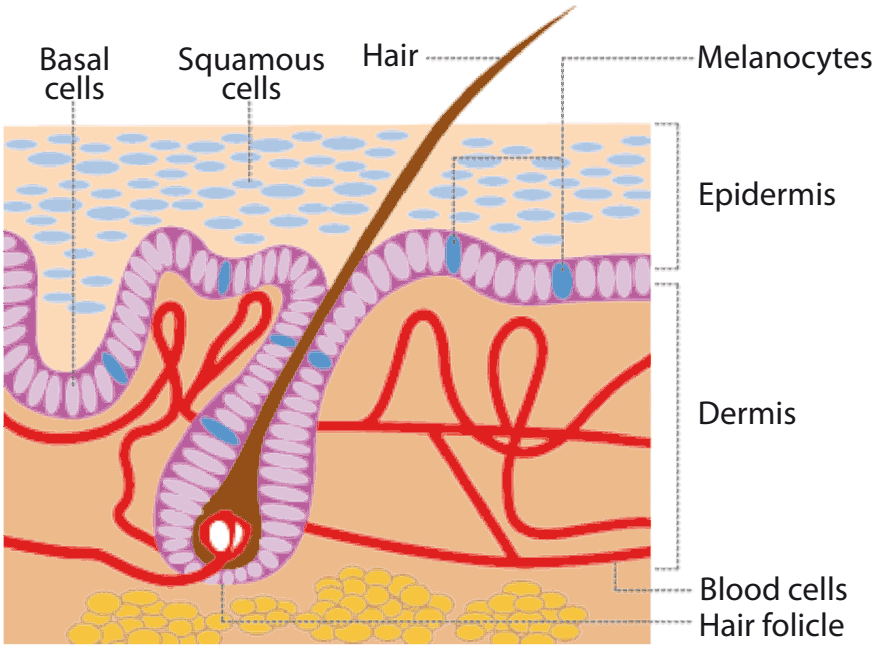


Figure 6. Schematic picture of cross section of the skin.

Risk factors for NMSC

The most well known riskfactor for NMSC is exposure to sunlight and it has been found that both UVA and UVB can cause damages that can lead to cancer (145). It is believed that the inflammatory response following sun-exposure plays a critical role in development of NMSC (146). Also fair skin, red or blond hair and blue eyes are well established risk factors for NMSC (147).

Epidemiological studies and incidence statistics, point at a difference in risk for NMSC for men and women (140, 146). It is however currently believed that this disparity mainly is caused by lifestyle choices, like men tending to be less likely to use sun protection and historically tend to have outdoor occupations (148).

Organ transplant recipients have to undergo a life long immunosupressive treatment to accomplish organ and patient survival. This treatment has been

foud to cause an increased risk for development of various cancers, with NMSC being the most common post-transplant malignancy (146). Solid organ transplant recipients have a well documented 65- to 100- fold increased risk of developing cutaneous SCC compared with the general population (149, 150). As mentioned before the general population is more likely to develop BCC but among transplant recipients SCC is the more common skin cancer (150, 151). HPV DNA is present in up to 90% of the skin lesions, particularly in SCC and AK, among immunosuppressed individuals which is higher than among immunocompetent individuals (152-160).

HPV AND NMSC

Many epidemiological studies have during the last decade investigated the relationship between cutaneous HPV infections and development of NMSC both in the general population and among immunosuppressed individuals. Based on the results from those studies an association between SCC with HPV, particularly from genus beta is suspected.

HPV DNA studies have found an association between beta HPV and SCC (155, 156, 161-163) or the precursor lesion actinic keratosis (AK) (164) but not BCC (162). HPV DNA viral load is higher in AK than in SCC, which might indicate a role of HPV in the early steps of tumour development (165). The prevalences detected in the different diagnoses vary a lot depending on type of sample, method used and immune status of the patients. Not only samples from cancer tissue but also surrounding tissue and tissue from healthy individuals have been found to frequently harbour HPV DNA of genus beta (115, 116, 156, 166) and HPV from genus beta tend to persist on healthy skin more than HPV from other genera (167). Persistent infection with HPV from genus beta has also been associated with an increased occurrence of actinic keratosis. This needs to be confirmed by additional studies to determine the possible association of beta papillomavirus persistence with SCC (164).

Serological studies have found an increased risk for SCC among subjects seropositive for antibodies to HPV types in genus beta (168-171), but lately also genus gamma has been implicated (171). Some individual HPV types have been found to have higher risk for development of SCC than others, for example HPV5 (169), 8 and 38 (168), whereas most often only genus beta has been sug-

gested to have an increased risk for SCC (169, 170). To evaluate the relevance of using antibody prevalence as a marker for HPV infection, serological results were compared to prevalence of HPV DNA in eyebrow hairs (170). Poor agreement between DNA and serology results was found, which might either indicate an assay problem or that not all cutaneous HPV infections are immunogenic, which is also true for genital HPV infections (172, 173). Another important issue is the time point for serum collection. When comparing antibody prevalence in plasma samples collected prior to diagnosis (incident cases) and after diagnosis (prevalent cases), it was found that the prevalent cases had much higher prevalence of beta HPV than both incident cases and controls (174). This might indicate that the antibody response observed in SCC patients after diagnosis is a consequence of the cancer disease. No differences in HPV seroprevalences have been found in studies comparing populations from different areas with different sun-exposure (175).

Several serology studies have used the multiplexed method where antibody detection is based on glutathione S-transfrease (GST) capture ELISA in combination with fluorescent bead based Luminex technology (GST-L1) (176-178). A major advantage of this method compared to traditional ELISA is the high through-put approach. In a single test it is possible to analyse up to 100 different antigens which allows for a broad spectrum of HPV types. With all analyses performed simultaneously the comparability of the results are ensured. Serology for cutaneous HPV is not yet as well validated as serology for mucosal HPV types. In contrast to genital HPV infections where no different serotypes have been reported within a genotype, the cutaneous type HPV 5 have been found to have at least three different serotypes (HPV5a, 5b and 5c) (123). More studies of natural history of antibodies (acquisition and loss) to cutaneous HPV infections would be of great interest for future development of methods and epidemiological studies. A new high though-put serology method, using pseudovirions as antigen has recently been developed (179).

Different markers of HPV infection have been used in the epidemiological studies, for example presence of HPV DNA in plucked eyebrow hairs, skin swabs and skin biopsies from tumour or healthy skin and presence of HPV antibodies in serum or plasma. A variety of PCR techniques have also been used and this has led to discrepancies in prevalences and type spectrums reported. To clarify which sampling method is most appropriate to use for cutaneous HPV, different methods have been compared (180). Samples obtained with less-invasive

techniques (plucked eyebrow hair and swab samples) were found to have poor specificity. If combined with analysis of antibody prevalence the specificity increased, and the combination of eyebrow hair + antibodies or even eyebrow hair + antibody + swab sample was recommended because of the less-invasive sample collection methods, even if both punch biopsies and shave biopsies performed better regarding both sensitivity and specificity, although never analysed together with antibody results which might have increased specificity and sensitivity even further (180). Results from plucked eyebrow hairs have been compared to biopsies from various parts of the body and it was found that eyebrow hairs only to some degree can serve as marker of beta HPV in epidemiological studies (181). The prevalence and multiplicity of HPV DNA in plucked hair is also dependent on the cellular DNA input (23). The method for collecting biopsies also influence the results since a lot of the HPV DNA is found on the surface of the skin and not within the actual tumour (182).

In support of the epidemiological findings of association between cutaneous HPV infections and risk for NMSC some cutaneous HPV types have been investigated for transforming properties. For example HPV38 E6 and E7 have both been found to have transforming activities (183-185), where as HPV10 and HPV20 E7 proteins was found not to have any transforming activities (184). HPV 8 E6 and E7 have been found to have transforming activities both in vitro and in vivo (186-189). Promoter activity in different HPV types has been found to be affected in different directions by UV-B irradiation. HPV8 was activated but HPV38 and 93 were inhibited and HPV92 and 96 were not affected at all in one study (190) and an other study investigating the non-coding region (NCR) promoter activity in HPV 5, 8, 9, 14, 23, 24, and 25 in primary human epithelial keratinocytes found that only HPV 5 and 8 were activated (191).

HPV VACCINES

Today there are two prophylactic vaccines against mucosal HPV available. They are both based on virus like particles (VLP) containing L1 proteins. VLPs are empty HPV particles without the viral genome. One of the vaccines, Gardasil, developed by Merck, comprises VLPs of four mucosal HPVs: HPV16, HPV18, HPV 6 and HPV11. The other vaccine, Cervarix, developed by GlaxoSmith-Kline (GSK) comprises VLPs of HPV16 and HPV18. Both vaccines are approved for use in many countries. The Swedish National Board of Health and Welfare decided that HPV vaccination should be included in the national

vaccination program, and administered to girls age 10-12 (192). Opportunistic vaccination has been available since 2006 and is subsidised for girls age 13-17. Today approximately 100 000 Swedish females have been vaccinated against cervical cancer, as well as a few males (excerpt from the Swedish HPV vaccination registry).

Both vaccines have shown over 90% efficacy against HPV16 and 18-related precancerous lesions in clinical trials and both vaccines were shown to be highly immunogenic in clinical trials, resulting in essentially 100% seroconversion (193). None of the vaccines have any therapeutic effect, which was also not expected. The vaccines are generally well tolerated; the proportion of women experiencing serious adverse events of any type was about the same in VLP vaccinated women and control subjects (193).

Present studies

Aims

Paper I

To determine the risk factors for HPV infection and if some species of HPV is associated with non-melanoma skin cancer (NMSC).

Paper II

To investigate if antibodies to cutaneous HPVs are associated with presence of HPV DNA and if seropositivity to HPV is associated with cutaneous lesions.

Paper III

To investigate if prediagnostic presence of HPV antibodies is a biomarker for increased risk for SCC or BCC and if persistent HPV seropositivity is a risk factor for NMSC.

Paper IV

To obtain unbiased and stable estimates of how different sexually transmitted infections interact in the aetiology of cervical cancer.

Material and methods

Paper I

This study was designed as a hospital based case-control study of NMSC, premalignant and benign skin lesions. Immunocompetent patients attending five different dermatology clinics, one in Austria and four in Sweden, with medical indications to surgically remove the skin lesion were included in the study. All patients answered questionnaires about skin type, previous sun burns, eye and hair colour and the level of sun exposure at the biopsy site was classifies by a dermatologist. Two punch biopsies with the superficial skin layer removed were collected from each patient, one from the lesion and one from healthy skin. In total, 349 patients were included, 82 diagnosed with squamous cell carcinoma (SCC), 126 with basal cell carcinoma (BCC), 49 with actinic keratosis (AK) and 92 with benign lesions. SCC and BCC diagnoses were histologically confirmed. The biopsies were extracted and the quality of the DNA checked at one laboratory, where after the samples were aliquoted and analysed for presence of HPV DNA with different PCR techniques and cloning for type detection in three different laboratories. To be scored as positive a sample had to be tested positive in two out of the three laboratories.

Paper II

The same patients as for paper I were included here, but for this study also serum samples were analysed. The patient material was extended to include in total 434 patients, 72 diagnosed with SCC, 160 with BCC, 81 with AK and 121 had benign skin lesions. The biopsies were tested for presence of HPV DNA using the same methods as in paper I. The serum samples were tested for presence of antibodies against the major capsid protein L1 for HPV 1, 5, 6, 8, 9, 10, 15, 16, 20, 24, 32, 36, 38 and 57 and the oncoproteins E6 and E7 for HPV 8 and 38. A multiplex serology assay, where antibody detection is based on glutathione S-transferase (GST) capture ELISA in combination with fluorescent bead based Luminex technology was used.

Paper III

This study was designed as a prospective biobank based nested case-control study. Two major serum banks, the Janus Biobank in Norway and the Southern Sweden Microbiology Biobank, were linked to the population based national cancer registry in the respective country to identify cases diagnosed with SCC and in Norway also BCC of the skin. To be included in the study the patient had to have donated a serum sample at least one month prior to diagnosis and if multiple samples were available all samples were collected. For each case one control was selected, alive and free of skin cancer at the time of the case's diagnosis, matched for age, sex, cohort, number of sampling occasions and time of follow-up. In the Janus Biobank cases and controls were also matched for county. From the Janus Biobank 497 cases diagnosed with SCC and 1990 cases diagnosed with BCC were included and from the Southern Sweden Microbiology Biobank 280 cases diagnosed with SCC were included. Altogether the study contained 9260 samples due to multiple sampling occasions for several cases and controls. For the Swedish cases and controls information about life-time cumulative UV exposure was based on history of residence during lifetime.

The serum samples were analysed for presence of antibodies to the L1 protein of HPV 1, 2, 3, 4, 5, 7, 8, 9, 10, 13, 15, 17, 20, 23, 24, 27, 32, 36, 38, 41, 48, 49, 50, 63, 65, 75, 76, 77, 92, 95, 96, 101 and 103. Except for the increased number of HPV types analysed the method was the same as used in paper II.

Paper IV

This study was also designed as a prospective biobank based case-control study. Four serum biobanks in four countries, Sweden, Norway, Finland and Iceland, were linked to respective cancer registry to identify cases diagnosed with invasive cervical cancer. Serum samples had to be donated at least one month prior to diagnosis and if several serum samples were available the oldest was chosen. For each case five controls were matched for sex, age at serum sampling, storage time and region. In total 543 cases were included, 216 from Norway, 169 from Finland, 103 from Iceland and 55 from Sweden. From the cases in Norway, Finland and Sweden formalin-fixed paraffin-embedded (FFPE) cancer tissue was also collected from the pathology departments were they were diagnosed.

The serum samples were tested with ELISA for antibodies against HPV 6, 16 and 18, Herpes simplex virus type 2 (HSV-2) and *Chlamydia trachomatis*. Cotinine levels were also measured as a measurement of exposure to tobacco smoke. The FFPE samples were tested for presence of HPV DNA using PCR and the HPV type detected either by reverse dot-blot hybridisation or a multiplex fluorescent bead based method.

Results and discussion

Paper I

The major findings was that presence of HPV of genus beta species 2 associates with increased risk for SCC (OR 4.40, 95% CI 1.92-10.1) and that sunlight exposure is a strong risk factor for HPV infection in the skin (OR 4.49, 95% CI 2.44-8.11 for lesions and OR 3.65, 95% CI 1.79-7.44 for healthy skin). Other studies have found either increasing levels of HPV at sun-exposed sites (116, 194) or no effect at all (156). A possible explanation for the strong association in this study might be the use of "stripped" biopsy material which has not been used in any other studies. This sampling method removes surface contaminations and makes it possible to detect HPV DNA found inside the tumour (182). The association between HPV from genus beta species 2 and SCC has been confirmed by one other study that also looked at biopsies from the lesion, although not "stripped" (161) and one serological study (171). Other studies have not confirmed the findings and instead found an association with genus beta species 1 (162, 169) or just genus beta (170). The divergence in results might depend on sample material used (serum, plasma, hair and formalin fixed tumour tissue) and that the viral copy numbers in the stripped biopsies that we used is generally rather low (182).

The possibility that the association found between HPV and SCC could be attributable to confounding via UV-exposure must be considered, but it is also possible that a carcinogenic effect of UV-exposure is mediated by causing an increase in HPV levels. This possibility has been supported by analyses of UV irradiation of HPV 38 E6 and E7 transgenic mice (185).

None of the self-reported factors (skin type, previous sunburns, hair and eye colour) associated with risk for HPV infection.

The PCR methods used in the study detect HPV types from all genera (153, 158, 195, 196) and 42 different HPV types or putative types were detected. A majority of the detected types, 37 types, belonged to the genus beta, only three to the genus gamma and two to the genus alpha. This is consistent with other

studies detecting HPV DNA in skin biopsies (156, 161).

Paper II

HPV seroprevalences were consistently higher among SCC patients than in BCC patients, $p<0.001$, even if there were only small difference in seroprevalences for individual HPV types between the diagnoses.

When comparing the HPV DNA results to the serological results for the same type poor agreement was found. For all HPV types, on average 20% of the DNA positive individuals were also seropositive for the same type. The HPV type with highest agreement was HPV 8 where 28% (11 out of 40 samples) of the DNA positive samples also were seropositive. This suggests that serology has a very low sensitivity for cutaneous HPV which has also been reported in a compaison of HPV DNA prevalence in eyebrow hair and HPV serology (170).

Seropositivity among subjects HPV DNA positive for any of the HPV types included in the serology assay was 64%, whereas among those without HPV DNA detected the corresponding seropositivity was only 34%. This suggests that even if the type specific antibody response to cutaneous HPV is low, there is a specificity for cutaneous HPV infection in general in the response.

Paper III

The main finding of paper III was that seropositivity to HPV in genus beta species 2 was associated with increased risk for SCC. This was observed in baseline samples (OR 1.3, 95% CI 1.1-1.7), if persistently seropositive (OR 1.5, 95% CI 1.0-2.3) as well as in samples taken more than 18 years prior to diagnosis (OR 1.8, 95% CI 1.1-2.8). The concern about reverse causality is highly relevant for HPV and skin cancer since it has been shown that seroprevalences increase in samples taken after diagnosis (174). However, the association we found that HPV beta 2 seropositive subjects have an increased risk for SCC also more than 18 years after the samples are taken is unlikely to be attributable to reverse causality.

We found no effects of UV-exposure in the Swedish material in the study. This does not mean that we can exclude confounding by UV light, even if information about life-time residential area and UV-exposure down to zip-code area was available. All individuals were enrolled from the same part of Sweden and variation in mean UV-exposure between the groups was low; 9938 mW/m^2, 10416 mW/m^2 and 10521 mW/m^2. Life-style factors like out-door activities could affect the total UV-exposure more than place of residece.

Paper IV

The risk of developing invasive cervical cancer was almost twice as high among women seropositive for HPV 16 (OR 2.4, 95%CI 1.9-30) than for seronegative women and HPV 18 seropositive women had a small increased risk (OR 1.4, 95% CI 1.0-1.8). The risks were increased only for cervical cancers that were positive for the same type of HPV DNA.

Both *Chlamydia trachomatis* (92-94) and HSV-2 have been reported to be risk factors for cervical cancer, but the data for HSV-2 is not consistent (88, 95). In the present study we found that history of *Chlamydia trachomatis* was cleary associated with an increased risk of cervical cancer (OR 2.0, 95% CI 1.7-2.5) and for all histological types investigated, SCC (OR 2.1, 95% CI 1.7-2.6), AC (OR 2.8, 95% CI 1.0-8.1) and ASC (OR 1.5, 95% CI 1.0-2.4). Earlier studies have only reported association for SCC (89, 93, 94). For HSV-2 only a weak, barely significant, association was observed that could possibly be due to residual confounding.

We also observed an antagonistic interaction between HPV 16 and 6. Thismeans that women seropositive for HPV 16 alone was at higher risk for developing cervical cancer than women who were seropositive for both types. These findings support the findings in earlier studies (199, 200).

Concluding remarks

Our findings suggest that HPV from genus beta species 2 is associated with development of SCC of the skin. The same conclusion was found in analyses of two different studies with different designs and different methods of analyses (paper I and paper III), one hospital-based case-control study using biopsies for HPVDNA testing and one prospective biobank-based case-control study where serum samples were analysed for HPV antibodies. Persistent seropositivity for HPV in genus beta species 2 was also found to associate with SCC of the skin and the risk was increased also for samples taken more than 18 years prior to diagnosis, which rules out reverse causality.

We found that UV-exposure is a risk factor for HPV infection, UV-exposure must always be considered as a possible confounder when analysing relation between HPV and skin cancer. It is difficult to measure or estimate the degree of UV-exposure in an individual and it is almost impossible to rule out residual confounding, even if UV-exposure is adjusted for. We could not identify any other risk factor for HPV infection based on answers to the questionnaires, but self-reported information generally has rather low validity.

Despite the possibility to detect HPV DNA from all genera the absolute majority of the detected types belong to genus beta.

Serological testing for cutaneous HPV was found to have low type-specificity. There appears to exist a non-type specific serological response (that is, a response related to cutaneous HPV in general) but has to be further investigated by studies of natural history of antibodies (acquisition and loss) in relation to cutaneous HPV infections. Patients diagnosed with SCC consistently had significantly higher seroprevalences than patients diagnosed with BCC.

We found that seropositivity for oncogenic HPV types other than the type actyally present in the tumour did not affect the cervical cancer risk. A history of *Chlamydia trachomatis* was a risk factor for cervical cancer both for SCC, AC and ASC. Our result expands earlier studies that have only found a risk in-

crease for SCC but not for AC, possibly because of insufficient statistical power. History of HPV6 had an antagonistic effect on risk for SCC and AC, where as history of HSV-2 infection had a weak, just barely significant, risk increase. The knowledge of which cofactors that are important for development of cervical cancer can be useful for evaluations of the effect of cervical pravention programs.

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