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A goal is not always meant to be reached,
it often serves simply as something to aim at
Lee Jun-Fan (1940-1973)

ABSTRACT

Aaro Turunen

Involvement of human papillomaviruses, herpes simplex virus and Epstein-Barr virus in head and neck carcinomas.

University of Turku, Faculty of Medicine, Institute of Dentistry, Department of Oral and Maxillofacial Pathology and Radiology and Institute of Biomedicine, Virology. Finnish Doctoral program for Oral Sciences (FINDOS-Turku)

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Human papillomaviruses (HPV) are common oncogenic DNA viruses that replicate in differentiating epithelial cells. HPVs cause a significant proportion of squamous cell carcinomas of the head and neck (HNSCC), particularly of oropharyngeal subsites, the prevalence of which has been increasing. HNSCC patients are often afflicted by common Herpes simplex type 1 (HSV-1) infections during radiotherapy. In addition, Epstein-Barr virus, another herpesvirus, is a causative factor in nasopharyngeal carcinomas but its influence on carcinomas in other anatomical subsites of the head and neck is unknown.

This work aims to describe the effect of differentiation-related calcium signaling on HPV E6 and E2 expression and cell proliferation in HPV-positive and -negative gingival cells and hypopharyngeal carcinoma cells. As cells from these areas can be exposed to HSV-1 *in vivo*, the next aim was to determine what effect HSV-1 infection and irradiation has on the viability and apoptotic gene expression of cultured cells. Lastly, the prevalence of EBV in head and neck carcinomas with previously known HPV and HSV-1 status was studied using *in situ* hybridization and immunohistochemistry in order to determine what role HSV-1 and EBV play in HPV-positive or -negative HNSCCs.

The results show that calcium signaling increases E6 oncogene expression in gingival and hypopharyngeal carcinoma cells, potentially in order to resist differentiation and subsequent cell death. Alone, HSV-1 infection or irradiation caused cytotoxic effects. However, the combined effects increased radiation resistance and halved the apoptotic caspase 3 signaling after irradiation compared to uninfected cells. Simultaneously, the relative HPV16 E6 and E7 expression in HPV16-positive carcinoma cells increased. Moreover, the viability of gingival cells was increased, whilst similarly treated skin cells did not display such effects. EBV was present in 21%, whereas HSV-1 was present in a minority of HNSCCs, 47% of which were also HPV-positive. Therefore it is possible these viruses could also coinfect cells *in vivo* and affect their radiation responses via the inhibition of apoptotic signaling, potentially acting as cocarcinogens. Lastly, the presence of coinfections leads to poorer prognoses in HNSCC. Thus the effects of herpesviruses coinfecting HPV-positive tissues need further study, particularly in patients undergoing radiation therapy.

Keywords: human papillomavirus, Herpes simplex virus type 1, Epstein-Barr virus, head and neck carcinoma, irradiation, oncogenes, HPV, HSV, EBV, oral cancer, prevalence, cell culture

TIIVISTELMÄ

Aaro Turunen

Ihmisen papilloomavirus, herpes simplex -virus ja Epstein-Barrin virusinfektiot pään ja kaulan syövissä.
Turun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Suupatologian ja -radiologian osasto ja Lääketieteellinen tiedekunta, Virusoppi.
Suun terveystieteiden tohtoriohjelma FINDOS-Turku
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Pään ja kaulan syöpien esiintyvyys on lisääntymässä niin Pohjoismaissa kuin muuallakin länsimaissa. Osa pään ja kaulan alueen syövistä on ihmisen papilloomavirusten (HPV) ja erityisesti HPV16 genotyypin aiheuttamia. Myös Herpes simplex -virus, tyyppi 1 (HSV-1) on yleinen pään ja kaulan alueen virus, jota esiintyy usein etenkin syöpää sairastavilla- ja leukakirurgisilla potilailla suussa. HSV-1:tä ei kuitenkaan pidetä itsenäisenä pään ja kaulan alueen syöpien vaaratekijänä, mutta sen vaikutusta syöpäsolujen säteilyvasteeseen ei ole juurikaan tutkittu. Epstein-Barrin virus (EBV) infektoi puolestaan lähes kaikkia aikuisia latenttina infektiona B-lymfosyyteissä. EBV aiheuttaa nenänielun karsinoomaa, mutta sen merkitys muissa pään ja kaulan syövissä on epäselvä, koska syöpäkudoksissa on myös B-lymfosyyttejä, jotka PCR-pohjaisessa diagnostiikassa antavat vääriä positiivisia tuloksia, kun määritetään EBV:n yhteyttä ko. syöpään. EBV:n esiintyvyydestä itse syöpäsoluissa on vain vähän tutkittua tietoa.
Tässä tutkimuksessa selvitettiin ikenen epiteelisoluissa ilmentyvän HPV16:n syöpägeenin E6-vastetta solun erilaistumissignalointiin. Lisäksi HSV-1:n aiheuttamaa epiteelisolun vastetta säteilytykseen tutkittiin ihosoluissa, ikenen soluissa ja HPV16-positiivisissa syöpäsoluissa. Lopuksi selvitettiin HPV:n, HSV-1:n ja EBV:n esiintyvyyttä pään ja kaulan syöpänäytteissä useilla eri menetelmillä ja näitä tuloksia verrattiin syövän tautispesifiseen ennusteeseen.

Tutkimukset osoittivat, että erilaistumissignalointi lisää HPV16:n E6-syöpägeenin ilmentymistä, joka puolestaan saattaisi pitkittää solun jakaantumista ennen sen lopullista erilaistumista. Ihosoluissa HSV-1-infektio ja säteily yhdessä ja erikseen aiheuttivat solukuolemaa. HPV16-positiivisissa syöpäsoluissa ne lisäsivät yksinään solukuoleman ja siihen liittyvien mRNA-molekyylien ja proteiinien ilmentymistä. HSV-1:n ja säteilyn yhdistelmä kuitenkin heikensi näiden erikseen aiheuttamaa solukuolemaa ja siihen liittyvää signalointia ikenen soluissa ja erityisesti HPV16-positiivisissa syöpäsoluissa. EBV havaittiin 21 %:ssa kaikista pään ja kaulan alueen syöpänäytteistä. EBV oli todettavissa itse syöpäsoluissa myös osassa suun ja kurkunpään syövistä, joista 47 % oli myös HPV-positiivisia. HSV-1 havaittiin vain muutamassa suun ja suunielun syövässä. Useiden virusten koinfektion havaittiin liittyvän tilastollisesti huonompaan taudin ennusteeseen. Tulokset antavat aihetta jatkotutkimuksille virusten ja säteilyn yhteisvaikutuksesta pään ja kaulan syöpien synnyssä ja hoidossa.

Avainsanat: ihmisen papilloomavirus, Herpes simplex tyyppi 1, Epstein-Barrin virus, pään ja kaulan syöpä, säteily, onkogeenit, HPV, HSV, EBV, suusyöpä, esiintyvyys, soluviljely

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ABBREVIATIONS

BER	Base excision repair
CAF	Cancer associated fibroblasts
CBCT	Cone-beam computer tomography
CDK	Cyclin-dependent kinase
DDR	DNA damage response
DSB	Double-strand break
dsDNA	Double-stranded DNA
DSS	Disease-specific survival
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
E6AP	E6-associated protein
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin-embedded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HNSCC	Head and neck squamous cell carcinoma
HPSCC	Hypopharyngeal squamous cell carcinoma
HPV	Human papillomavirus
HSV-1	Herpes simplex virus type 1
hTERT	Human telomerase reverse transcriptase
IHC	Immunohistochemistry
ISH	In situ hybridization
LarSCC	Laryngeal squamous cell carcinoma
LCR	Long control region
LMP	EBV latent membrane protein
MDM2	Mouse double minute 2 homolog
MOI	Multiplicity of infection
miRNA	Micro-RNA
MMP	Matrix Metalloproteinase
NFκB	Nuclear factor kappa B

NHEJ	Non-homologous end joining
NPC	Nasopharyngeal carcinoma
OPSCC	Oropharyngeal squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse-transcriptase PCR
ROS	Reactive oxygen species
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

LIST OF ORIGINAL PUBLICATIONS

The list of original publications, referred to in the thesis by the Roman numerals (I-IV).

I. Turunen A, Syrjänen S. 2014. *Extracellular calcium regulates keratinocyte proliferation and HPV 16 E6 RNA expression in vitro.* APMIS 122(9):781-9.

II. Turunen A, Hukkanen V, Nygårdas M, Kulmala J, Syrjänen S. 2014 *The combined effects of irradiation and herpes simplex virus type 1 infection on an immortal gingival cell line.* Virology Journal 8(11):125

III. Turunen A, Hukkanen V, Kulmala J, Syrjänen S. 2016. *HSV-1 infection Modulates the Radioresponse of a HPV16-positive Head and Neck Cancer Cell Line.* Anticancer Research 36(2):565-74.

IV. Turunen A, Rautava J, Grénman R, Syrjänen K, Syrjänen S. 2017. *Epstein-Barr virus (EBV)-encoded small RNAs (EBERs) associated with poor prognosis of head and neck carcinomas.* Oncotarget 8(16):27328-27338.

1. INTRODUCTION

The human upper airways are lined with mucosa which protects against infectious agents, moisturizes the inhaled air, enables eating and speech and rapidly heals in cases of tissue damage. The mucosal surfaces are covered by epithelia, consisting of terminally differentiated and differentiating keratinocytes and proliferating basal and parabasal cells underneath. Rapidly proliferating cells are at risk for acquired mutations during cell division, despite the genome being well-protected against such incidents. If the mutations are especially harmful, the cell either stops division and suicides, activating programmed cell death, apoptosis, or is eliminated by the immune system. This prevents cells with genomic changes from persisting and acquiring further DNA damage. However, some of the mutated cells may survive and acquire a growth advantage, starting their path toward malignancy. In the head and neck region, this heterogenic group of carcinomas is known as head and neck cancer (HNSCC).

The exposure of epithelial cells to carcinogens is of main importance in head and neck carcinogenesis. Tobacco and alcohol are the main etiologic agents, explaining approximately 80% of HNSCC. In addition, viruses, bacteria and yeasts can act as biological carcinogens directly via expression of their specific oncogenes or indirectly via chronic inflammation which promotes carcinogenesis. Lastly, exposure to ionizing radiation from medical and natural sources is an important cause of carcinogenesis.

The most important viral etiology for HNSCC is infection with human papillomaviruses (HPVs). Approximately 20% of oral and laryngeal cancers and 50-70% of oropharyngeal cancers are HPV-related. The prevalence of HPV-positivity in HNSCC is globally variable, however. The reservoir of HPV in the head and neck region is unknown but possible sites include tonsillar crypts and the gingival pocket epithelium. HPVs exert their carcinogenic effects by chronically infecting the basal keratinocytes and their oncogenes, E6 and E7 especially, forcing the infected cell to divide. Simultaneously, the capacity of the cell to enter apoptosis is blocked, which leads to its genomic instability, making the cell more susceptible to further carcinogenic mutations.

Herpes simplex viruses (HSV) are common and cause recurrent labial herpes infections (i.e. cold sores) in otherwise healthy individuals. HSV is also often shed to the saliva without any clinical symptoms. HSV-1 is the most prevalent virus type in the oral region and is often detected in the saliva of HNSCC patients as it reactivates more readily in these patients. HSV-1 can inhibit apoptotic mechanisms and cause genomic instability, but has not been shown to be an independent risk factor for HNSCC. This is mainly because the target cell is killed due to lytic infection in almost all cases. The role of coinfections of different herpesviruses with each other and/or HPV has not been studied in detail.

Epstein-Barr virus (EBV), another member of the herpesvirus family, is a well-recognized oncovirus, infecting almost everyone by adulthood. It remains latent in the B-lymphocytes and circulates in the bloodstream. EBV is able to infect the epithelial cells of the head and neck region, particularly in the nasopharyngeal area, where it can cause nasopharyngeal carcinoma. It has also been found in other HNSCCs such as oral, oropharyngeal or laryngeal carcinomas, but its significance in these diseases remains obscure. Because EBV may be carried over to the inflamed carcinoma tissue via B-lymphocytes, EBV detection in a carcinoma sample might not signify infected carcinoma cells. Therefore the significance of EBV detection in HNSCCs is unclear. Moreover, although reported, the effect of EBV coinfection of HPV-positive cells is not understood.

The present thesis aims to determine if extracellular calcium, irradiation and/or HSV-1 infection affect the apoptosis-related gene expression or cell viability of HPV16-positive and -negative cells of the head and neck area as single or combined exposures and if these lead to differing outcomes. In addition, the effects of EBV presence as a coinfection with HPV or HSV-1 on HNSCC outcomes was analyzed in HNSCC patient samples using *in situ* hybridization and immunohistochemistry. These studies reveal whether coinfections with these viruses might play a role in HNSCC development or prognosis.

2. REVIEW OF THE LITERATURE

2.1 Head and neck cancer

Head and neck cancers comprise a diverse group of mostly squamous cell carcinomas (head and neck squamous cell carcinoma, HNSCC) affecting different anatomical subsites of the upper aerodigestive tract. According to the anatomic localization, HNSCC are categorized as carcinomas of the oral cavity, pharynx (including oropharynx, nasopharynx and hypopharynx) and larynx **(Figure 1)**. Sinonasal carcinomas are also classified as HNSCCs but arise from the pseudostratified ciliated epithelium of the sinonasal passages, and as such differ from the squamous epithelium-derived carcinomas that comprise the majority of HNSCC. Malignancies of the lymphoid tissues, sarcomas and melanomas are different disease entities and thus included in their own respective cancer classes. This discussion will limit itself to HNSCC derived from squamous epithelia (with the exception of the incidence ratings shown below).

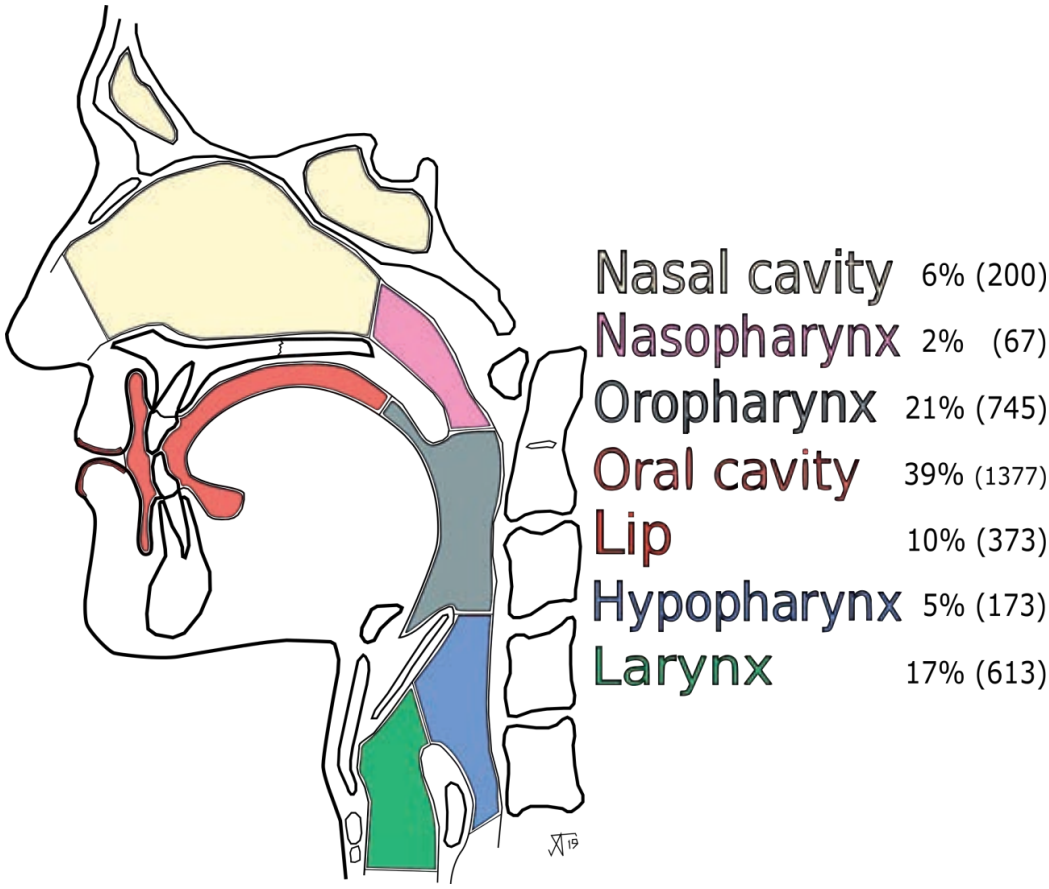


Figure 1. The incidence of HNSCC in Finland in 2010-2014.

Incidence percentage (from all HNSCC) and numbers derived from the NORDCAN database (http://www-dep.iarc.fr/NORDCAN/FI/frame.asp, accessed 20.11.2017). Percentages are calculated from the incidence figures (per 100 000) for males and females combined and derived from the database.

2.1.1 Incidence and prevalence worldwide

HNSCCs are a global burden with an annual mortality of around 370 000 patients and with more than 680 000 new annual cases (Ferlay et al. 2015, Ndiaye et al. 2014). Therefore HNSCCs comprise the sixth most common cancer type worldwide, being among the most common cancers of the developing countries (Joshi et al. 2014). Worldwide, sinonasal carcinomas are also less common, with an average global incidence rate of 7.5 per one million (Youlden et al. 2013). Furthermore, despite the fact that smoking confers an increased risk for these carcinomas, a pronounced risk from exposure to other environmental carcinogens such as wood dust is also observed (Franchi et al. 2011).

2.1.2 Risk factors for HNSCC

The classical major risk factors for HNSCCs are smoking and alcohol consumption (Hashibe et al. 2007) (**Figure 2**). Other known risk factors include the use of smokeless tobacco (Luo et al. 2007) and poor oral hygiene (Ahrens et al. 2014), exposure to certain chemicals such as formaldehyde and betel quid chewing habits, which predominate in Asia, especially in India (Chen et al. 2014). Dietary risk factors include consumption of meat and starch-rich foods, whereas fruit and vegetables are claimed to confer a protective effect (Bravi et al. 2012, Edefonti et al. 2012). Still, the effect of diet on HNSCC incidence as a whole is likely of limited clinical significance (Barasch & Litaker, 2011)**.** Spontaneous DNA damage from hydrolysis, reactive oxygen species (ROS) formed in cell metabolism, ionizing radiation from varying sources (discussed separately in 2.2) and DNA replication errors are possibly responsible for sporadic HNSCC in patients without any known risk factors. However, the carcinoma risk for individuals is also affected by their genetic predisposition, as exemplified in patients with Fanconi anemia, which is a DNA repair disorder leading to genomic instability and susceptibility to cancer (Sasaki & Tonomura 1973)**.** As the etiology of HNSCCs is variable, novel yet unknown causes for HNSCC are likely to be discovered in the future.

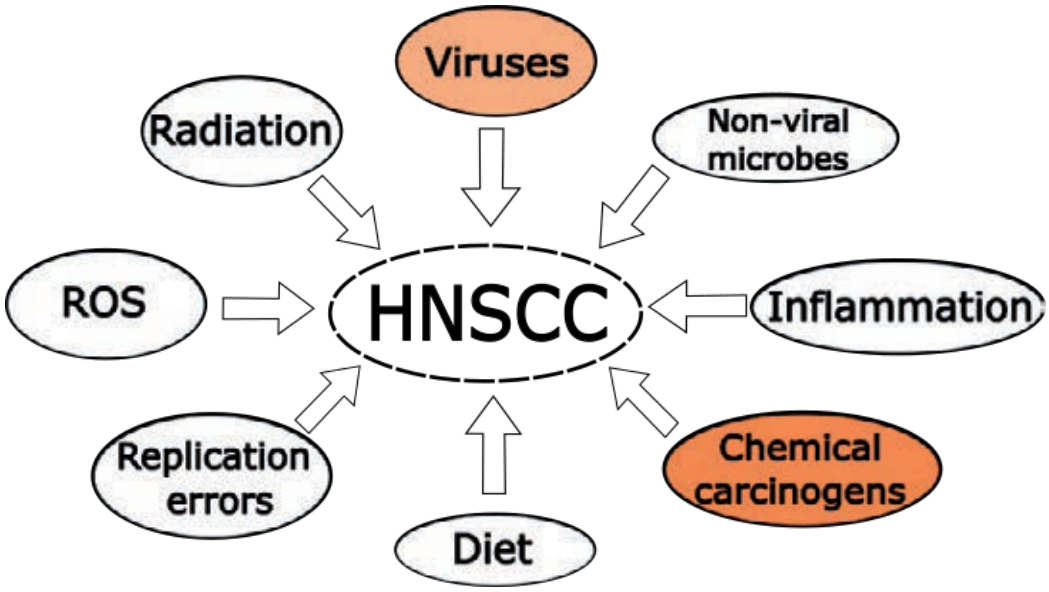


Figure 2: Factors influencing the development of HNSCC.

The most important risk factors are chemicals, such as tobacco-related nitrosamines or acetaldehyde. The second most important risk factors are viral infections with HPV and Epstein-Barr virus. Non-viral microbes such as bacteria and yeasts have been linked to HNSCC indirectly in patients with poor oral hygiene. Other contributing factors are intrinsic errors in DNA replication, metabolic pathways, extrinsic radiation or chronic inflammation (Brennan et al. 2017).

With a decrease in smoking prevalence, HNSCCs due to the classical risk factors have been in decline in first-world countries. This is particularly evident in the reduction in lip and larynx carcinomas (Chaturvedi et al. 2008, Engholm et al. 2010). In Nordic countries, the incidence of tongue cancer has continued to rise from the 1960s (Annertz et al. 2012). This same trend has been present in the USA (Myers et al. 2000). In addition, according to the NORDCAN database, which catalogs the 50 most significant cancers reported in the Nordic countries, the incidence of oral cavity and oropharyngeal cancers is clearly on the rise in Finland, with 10-year annual incidence rises of 1.25% and 9.9%, respectively (Engholm et al. 2010), and is seen in **Figure 3**. A similar trend is apparent in a recent German cohort, signifying that the factors affecting the etiology of these carcinomas are still not completely known (Tinhofer et al. 2015). The prevalence of oncogenic human papillomavirus- (HPV) associated HNSCC has significantly increased, but nevertheless the observed increases in incidence are not seemingly only attributable to HPV.

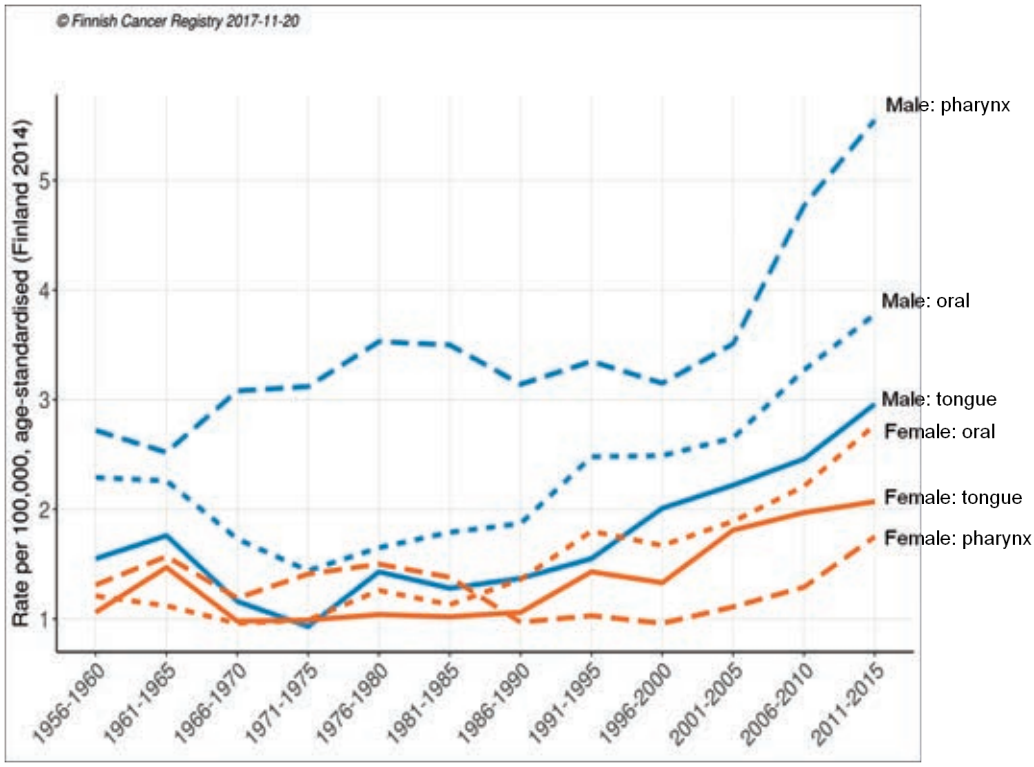


Figure 3: HNSCC incidence trends in Finland

Incidence ratings of tongue (straight lines), pharyngeal (dashed lines, long dashes) and non-tongue oral (dashed lines, short dashes) HNSCCs in female (orange) and male (blue) patients in Finland from 1956 to 2014. Increases in each category of HNSCCs are seen. Data from the Finnish Cancer Registry website, (https://tilastot.syoparekisteri.fi/syovat, updated 23.09.2016 accessed 19.11.2017). Licensed under Creative Commons BY 4.0.

2.1.3 The importance of anatomic localization

The specific risk factors and prognosis for HNSCC vary with the anatomical subsite (**Figure 1**). Lip carcinomas are associated with UV light and smoking and the 5-year survival is nearly 90% (Han et al. 2016). Oral carcinomas are associated with tobacco smoking, alcohol consumption and the use of betel quid or smokeless tobacco. To a lesser extent, oral squamous cell carcinomas (SCCs) are associated with HPV infections (Ndiaye et al. 2014). The 5-year disease-free survival of HPV-attributed advanced oral SCC is over 50% but whether HPV positivity confers a survival advantage remains unclear (Lai et al. 2017). Nasopharyngeal carcinoma arises most often due to infection with EBV. The prognosis ranges from 93% to 48.8% in early-stage compared to advanced carcinomas (Kang et al. 2017). Oropharyngeal carcinomas, which include tonsillar and base of the tongue carcinomas, are predominantly associated with HPV. Dahlstrom et al. (2016) reported survival ratings of between 94% and 69% between T1 and T4 carcinomas. The prognosis is better in the never-smoker group, with disease-specific survival ratings of up to 99% having been reported. Smoking decreases this to 80% and further to 76% in HPV-negative HNSCCs (Broglie et al. 2017)**.** Hypopharyngeal SCCs are less often HPV positive and have a poorer outcome. For example, 86% and 31% 3-year survival ratings were detected for HPV-positive and -negative hypopharyngeal carcinomas, respectively in a recent Swedish cohort (Dalianis et al. 2015). Laryngeal carcinomas are most often related to smoking, although a subset of them are associated with HPV infection. The disease is of average prognosis, with a 5-year DSS of 62% (Finnish Cancer Registry database, accessed 11.2017).

2.1.4 Molecular pathology

The "Hallmarks of cancer" theory first proposed by Hanahan and Weinberg in 2000 and updated in 2011 is a widely accepted theory on the properties of cancer cells that make them tumorigenic compared to their normal counterparts (**Figure 4**). Despite the wide acceptance of this view, called the somatic mutation theory, some authors criticize the focus on the single cancer cell. Sonnenschein and Soto (2013) emphasized that cancer is always a tissue-specific disease that is dependent on the interactions between the different cell types present in the tissue. In addition, cells in a benign tumor already demonstrate a significant number of these attributes. Therefore, HNSCC development requires both the epithelium and its interactions with the underlying altered stroma. This requires the mutation of specific genes and the deregulation of several pathways which are only now beginning to be characterized. The following sections will describe current knowledge of head and neck carcinoma development.

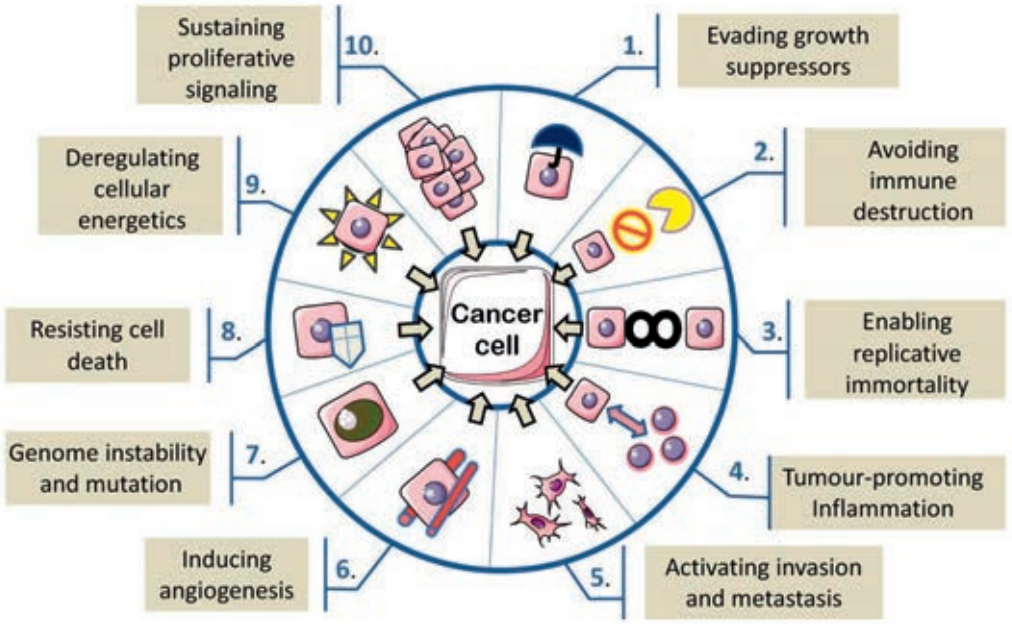


Figure 4. **The Hallmarks of cancer** (Modified from Hanahan & Weinberg 2011)

1: Evading growth suppressors - Immunity from extra- and intracellular signaling that suppresses cell cycle progression such as differentiation-related signaling.

2: Avoiding immune destruction - Immunity from detection by cytotoxic lymphocytes and resistance to apoptosis-inducing signals such as the FAS pathway.

3: Enabling replicative immortality - Ability to divide indefinitely, via telomerase expression.

4: Tumor-promoting inflammation - Growth stimulation from inflammatory mediators.

5: Activating invasion and metastasis - Ability to degrade the extracellular matrix and later to enter the vascular or lymphatic structures and metastasize into different organs.

6: Inducing angiogenesis - A tumor rarely grows past ~500 µm without novel vessel formation.

7: Genome instability and mutation - Loss of DNA repair mechanisms adds to the mutation rate.

8: Resisting cell death - Resistance to apoptotic mechanisms. Key pathways p53, pRb and caspase 8 and 9 are often inhibited and oncogenes such as Bcl-2 overexpressed.

9: Deregulating cellular energetics - Cancer cells use anaerobic glycolysis to maintain energy levels even in the low oxygen concentrations associated with radiation resistance, for example.

10: Sustaining proliferative signaling - Upregulation in proliferative gene expression.

2.1.5 DNA damage in cancer

DNA damage happens constantly due to extrinsic exposure to radiation, reactive molecules or the intrinsic generation of DNA-damaging agents such as ROS formed during mitochondrial oxidative energy production (Czarnecka et al. 2010). A large proportion of mutations can be attributed to spontaneous depurination or deamination reactions or mistakes in replication during cell division. Worse yet, a break in one or both DNA strands can form. These are usually caused by ionizing radiation, which is an important extrinsic cause of DNA damage. Fortunately, the bulk of the human genome is comprised of intron sequences whose mutations affect the phenotype of the cell less often. Because thousands of mutations happen in the human cell daily, the presence of DNA repair mechanisms is imperative for the survival of the genome. Diseases in which these repair mechanisms malfunction result in high probabilities of cancer (Hashimoto et al. 2016).

2.1.6 DNA damage repair

The most important mechanisms in cellular DNA repair include the base excision repair (BER) pathway in which an aberrant nucleotide is excised and a correct one is inserted. This is utilized in the repair of common damage caused by ROS and other reactive molecules (Clancy 2008). The worst damage sustained by DNA is a double-strand break (DSB). This is usually caused by high-energy ionizing radiation (γ-ray or X-ray irradiation) or UV, but may also happen during DNA replication (Shee et al. 2013). Radiation-induced DNA double-strand breaks (DSB) induce the DNA damage response initiated by the MRN complex, formed from the RAD50, MRE11 and Nibrin proteins, which senses DNA damage and is able to bind the damaged strands in order to phosphorylate the ATM kinase, which phosphorylates p53 and the checkpoint kinases CHK1 and CHK2. This leads to cell cycle arrest via the p21 in order to induce the DNA repair pathways until the damage has been repaired. The non-homologous end-joining (NHEJ) and homologous recombination (HR) DNA repair pathways are then employed, depending on the phase of the cell cycle in which the lesion has to be repaired (Branzei & Foiani 2008). NHEJ uses the remains of the partially single-stranded DNA at the break to join the ends, whereas the more often used and error-free HR pathway utilizes the homologous chromosome as a template for repair (Valerie & Povirk 2003). Should the damage prove too severe for repair, the cell either senesces (i.e. stops dividing permanently) or activates apoptosis via the mitochondrial pathway and caspase 3 activation (Maier et al. 2016). This is countered by NFκB, activated by exposure to ionizing radiation and able to induce antiapoptotic gene expression (Ahmed and Li 2008). Carcinoma development may result from the malfunction of these protective mechanisms that allow unrepaired DNA damage to accumulate.

2.2 Effects of radiation on tissues

Humans are exposed to radiation from various sources, natural and medical being the most important. Ionizing radiation consists of either high-energy electromagnetic (photon-) radiation such as ultraviolet or X-ray radiation or particle (nuclear-) radiation such as alpha or beta radiation, which carries higher radiation energies. Ionization affects human tissues in two ways: Firstly, it can directly affect the molecules of the cell and break molecular bonds; secondly, water molecules can become ROS upon radiolysis. These form "oxidative stress", causing increased damage to cells (Azzam et al. 2012). Mitochondria occupy a substantial portion of the cellular cytoplasm, and as radiation strikes the mitochondria it leads to increased ROS production and contributes to genomic instability (Dayal et al. 2009), increasing radiation toxicity after therapeutic irradiation (Nishida 2014). As oxygen is radiosensitizing and ROS mediates large parts of clinical effects of radiation, hypoxic tissues such as are present in HNSCCs are less susceptible to radiation therapy (Pettersen et al. 2015). Massive doses of irradiation lead to necrosis, as detected during early attempts at therapeutic irradiation (McGurk & Goodger 2000). The effects of irradiation are not confined to the exposed cells but to nearby nonirradiated cells as well via the bystander effect. This is caused by radiation-induced signaling molecules such as exosomes and microRNAs, which diffuse through gap junctions to influence the entire vicinity of the irradiated cells (reviewed by Yahyapour et al. 2018)**.** These pathways function to protect the cell from day-to-day exposures but, upon aberrant expression in diseases such as HNSCC, may adversely affect their treatment.

2.2.1 Ionizing radiation in the imaging of the head and neck region

It is said that one malignant tumor develops for every two million exposures of panoramic radiograph or two bitewings. In Finland, up to 2.3 million intraoral radiographs and 400 000 panoramic radiographs are taken annually (Suutari 2016). However, as the effective dose is used to compare the whole-body risk of cancer development in a population, the entrance dose in the path of the primary beam itself is of interest for better understanding the local mucosal irradiation dose. The entrance dose of intraoral imaging was 1.15-2.8 mGy in a recent study (Hart et al. 2012).

Certain clinical occasions necessitate consecutive intraoral radiographs. For example, endodontic treatment might begin with a panoramic tomograph (PTG, 19-75 uSv) and two bitewing examinations (2-20 uSv). After this stage additional intraoral X-rays (1-10 uSv) or at times cone-beam computer tomography (CBCT, 27-674 uSv, Suomalainen et al. 2009) are used to confirm a diagnosis. If a dental infection is diagnosed that requires root canal treatment the root canal is navigated with a file whose position is verified radiographically (1-10 uSv). Before filling the root canal, the position of the filling cone is verified and a postoperative radiograph is taken (2-20 uSv). This accounts for a total of five direct

exposures, one panoramic exposure, a possible CBCT and one indirect (i.e. the bitewing from the other side) exposure in this possible clinical scenario whose total radiation exposure can be calculated**:**

19-75 uSv (PTG) + 1-10 uSv (intraoral) x5 + 27-674 uSv (CBCT)
=51-799 uSv effective dose in total.

In a recent study by Granlund et al. (2016), the oral mucosa received the highest absorbed doses from both intraoral and panoramic radiography. The organ dose was reported as 41 uGy for one intraoral radiograph and 348-2151 uGy for panoramic tomographs. Therefore the above calculation may not represent the actual energy transfer to the oral mucosa. Using absorbed doses, the result would be up to 2356 uGy absorbed into the oral mucosa, without a CBCT examination. CBCT would further increase the value significantly, by 915-17581 uGy (Ludlow & Ivanovic 2008). In addition, metallic implants and fillings are able to potentiate the radiation intensity locally (Beyzadeoglu et al. 2006). This signifies that the oral mucosa can be exposed to biologically significant amounts of radiation even during routine dental treatments.

2.3 HNSCC specific mutations

As DNA damage may lead to carcinogenesis via several different mutations, finding specific driver mutations for HNSCCs has proved more problematic than originally thought. This is because HNSCCs are a more heterogeneous disease than can be appreciated from their histologic appearance. Only recently has next-generation sequencing (NGS) revealed novel and already-known genes to be associated with HNSCC. After considerable effort, the strongest evidence for established cancer genes in HNSCC to date has been found for the following genes (in italics, their protein products in parentheses): *TP53* (p53), the pRb family of genes (*RB1*, *RBL1* [p107], *RBL2* [p130]), *Casp8* (Caspase 8), *CDKN2A* (p16INK4a), *CCND1* (cyclin D1), *EGFR* (epidermal growth factor receptor), *PI3KCA* (PI3-Kinase, p110), its inhibitory gene *PTEN*, *NOTCH1* (Notch1) and *SMAD4* (Stransky et al. 2011, Tan et al. 2013, Martin et al. 2014). It has been postulated that HNSCCs present with at least three different patterns of gene expression: HPV-driven, HPV-negative with and HPV-negative without copy number alterations (Leemans et al. 2018). In addition, a possibly inflammation-related subtype has been described (Brennan et al. 2017). The mutational pattern is also related to the age of the patient (Meucci et al. 2016). HPV-positive carcinomas display more wild-type *TP53* and *pRb* as HPV itself inactivates these gene products in HPV-driven carcinomas (Stransky et al. 2011). Interestingly, if an HPV-positive carcinoma recurs or metastasizes, the gene expression profile more closely matches that of HPV-negative HNSCCs with *TERT and TP53* mutations (Morris et al. 2017). Moreover, for example, the frequency of FGFR3 mutations has been shown to be significantly higher in HPV-positive than in -negative HNSCCs, likely due to the different etiology of these carcinomas (Lawrence et al. 2015). HPV-positive carcinomas also

tend to harbor mutations in PIK3CA in >55% of cases and inactivations in PTEN in one third (Lechner et al. 2013). However, no mutation has been found to be specifically associated with HNSCC, as they may also be found in other carcinomas. Further, none of these detected mutations are present in all HNSCCs (Leemans et al. 2011). Therefore, a proportion of HNSCCs develop through yet unknown mechanisms which require further study.

2.3.1 Key tumor suppressors p53 and pRb

The tumor suppressor protein p53 is a transcriptional activator that is activated on detection of DNA damage caused by radiation, for example. This activation is via the ataxia telangiectasia mutated (ATM) DNA damage sensor pathway leading to p53 activation (Smith et al. 2010). Activated p53 promotes the transcription of several genes, CDKN1A in particular, leading to the synthesis of cyclin-dependent kinase inhibitor p21 which halts the cell cycle in order to repair DNA damage (Xiong et al. 1993). If it is unrepairable, p53 activates the apoptosis via the mitochondrial route (**Figure 5**). *TP53* mutation, present in 60-100% of HNSCCs, is regarded as an early event in carcinogenesis (Boyle et al. 1993) and is associated with a poorer prognosis (Poeta et al. 2007). p53 function may be lost not only due to mutation, but also via MDM2 overexpression (**Figure 5**) or viral interactions (Kang et al. 2015).

The retinoblastoma protein (pRb) binds to the E2F transcription factor to prevent S-phase progression in the G1/S restriction checkpoint (Vélez-Cruz & Johnson 2017). **Figure 5** displays the simplified pRb pathway. Normally as the cell cycle progresses, cyclin D1/CDK4/6 complex phosphorylates pRb, releasing E2F that leads to S-phase progression. If this pathway is mutated, uncontrolled cell replication may ensue. Cell cycle progression is prevented by the action of the p16INK4a (p16) protein via a feedback loop from the *CDKN2A* gene products p16 and p14 which inhibit the cyclin/CDK complex and p53 degradation, respectively. This leads to cell cycle arrest and senescence. The loss of p16 is a frequent observation in HNSCC (Wu et al. 1999), whereas deletions in *CDKN2A* have been detected in up to 55% of HNSCC (Lechner et al. 2013).

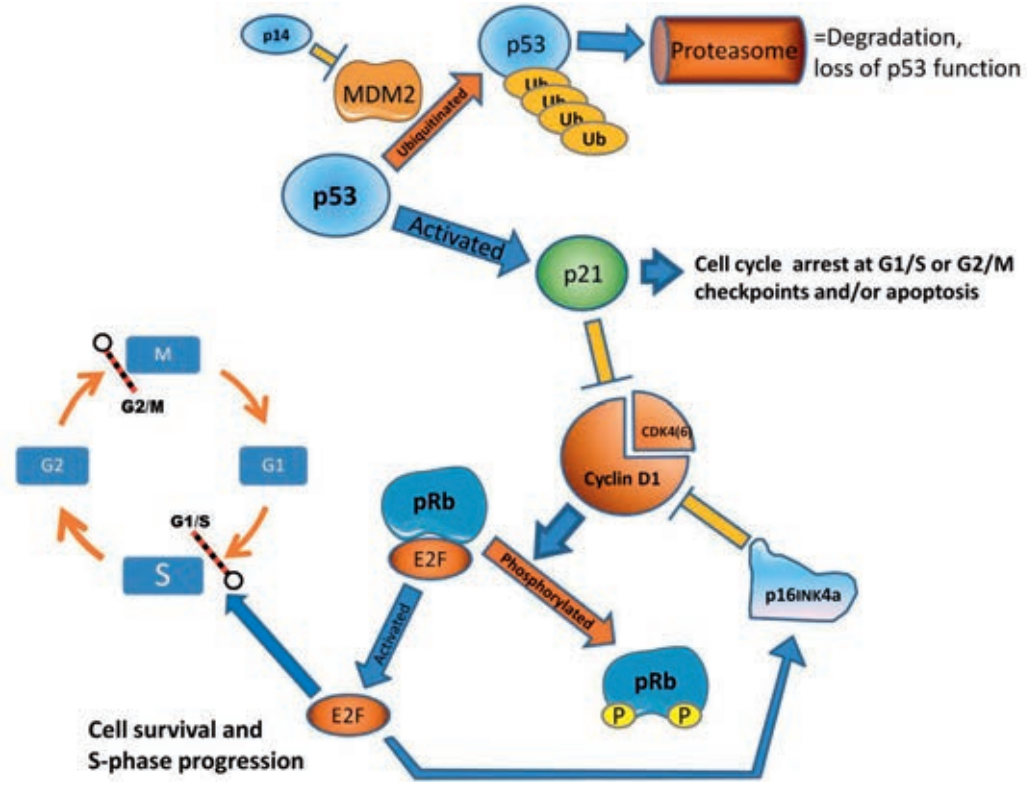


Figure 5. Function of the p53 and pRb tumor suppressor proteins.

Activated p21 mediates the p53 response to DNA damage caused e.g. by irradiation. It accomplishes this by the inhibition of CDK proteins which would enable the progression of the cell cycle when interacting with cyclins. Upon release from pRb control, E2F transcription factor family proteins transactivate their target genes which are involved in DNA replication and cell cycle progression. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.4 Apoptosis and cell death

Cell death due to genomic alterations or immune clearance is mediated by apoptosis, and apoptosis-related gene expression is crucial in cell transformation. This also applies to cancer cell survival after nonsurgical treatments (Wang & Scadden 2015). Apoptosis is not the only modality of cell death, however. Necrosis generally encompasses cell death due to catastrophic damage and leads to inflammation as cellular contents are spilled into the surrounding tissue (Rock & Kono 2008). This does not happen with apoptosis and most of the other pathway-mediated forms of cell death, in which the dying cell is absorbed by its neighbors or shed off the body. However, even necrosis is partly mediated by protein pathways such as RIP-1, drawing a fine line between caspase-mediated apoptotic signaling and certain forms of necrosis. Necroptosis further complicates the issue, being a pathway-mediated reaction of cell death leading to necrotic outcomes via necroptotic signaling such as mediated via RIP1 and RIP3 kinases (Patil et al. 2015). Senescence can also be considered a form of cell death, as terminally differentiated surface cells of the mucosae or skin shed off. This is also, in part, mediated by p16 signaling (Takahashi et al. 2006). Lastly, autophagy is a cell stress reaction in which the cell absorbs its own organelles to survive increased cell stress.

Apoptosis is driven by caspase proteins. Caspases are a group of cysteine aspartic proteinases that are crucial to the programmed cell death pathways. Each caspase effects its own parts in the activation of the caspase cascade that leads to apoptosis and cell death (Evan & Vousden 2001). The so-called extrinsic pathway starts from extrinsic signaling, i.e. from the cell surface, such as the activation of the FAS receptor from interaction with the FAS ligand from, for example, a cytotoxic T-cell. This pathway utilizes caspase 8 for activation. The intrinsic pathway starts as the mitochondrial membrane potential is disrupted and cytochrome C is released from the inside. This leads to apoptosome formation, which activates caspase 9. Both these pathways converge into caspase 3 and 7 activation and caspase 3, the downstream effector caspase, effects the cell destruction in an organized manner (**Figure 6**).

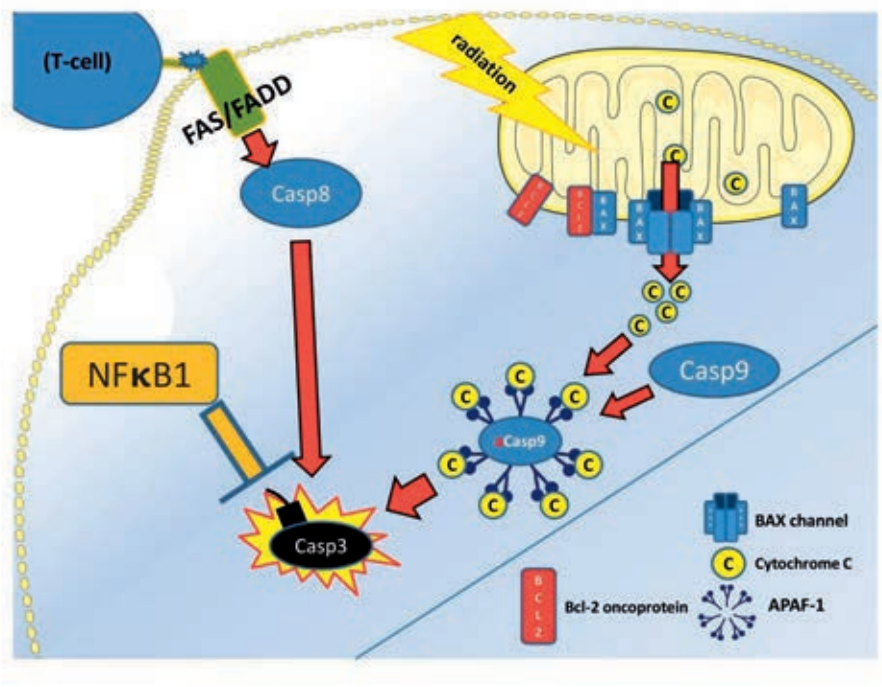


Figure 6: The caspase cascades in apoptosis induction. The extrinsic pathway utilizes caspase 8 as the effector, leading to "executioner-caspase 3" activation. This is caused via, for example, T-cell induction or NK-cell mediated FAS-signaling (Zhu et al. 2016). The intrinsic pathway or mitochondrial pathway is induced from mitochondrial damage or loss of membrane potential, for example. The released cytochrome c oligomerizes with caspase 9 and the Apaf-1 protein to form the apoptosome (seen in the figure) which activates caspase 3. Caspase 3 cleaves its specific substrates such as DNA and actin. Bcl-2 oncoprotein is localized in the mitochondria and Bcl-2/BAX interaction controls the rate of apoptosis. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.5 The field cancerization theory

The head and neck mucosa are seldom exposed to carcinogens in a local and site-specific manner. Rather, large areas are exposed simultaneously. For example, tobacco smoke pervades the entire upper aerodigestive tract, and alcoholic drinks expose several anatomical areas to acetaldehyde, the first carcinogenic metabolite of ethanol. As early as in 1953, Slaughter proposed a model for field cancerization (Slaughter et al. 1953). This is supported by the following observations:

HNSCC has a high rate of recurrence after successful curative treatment (Kim et al. 2015). This is postulated to be due to persistent genetic alterations in the clinically normal mucosae surrounding the original malignancy (Tabatabaeifar et al. 2017). It has been reported that premalignant changes such as aneuploidy persist for over a 7 cm distance from the original carcinoma, although the mucosal surface can be morphologically normal (Califano et al. 1996, Lydiatt et al. 1998). The underlying mechanism is thought to result from the mutation of a specific local stem cell. This would seem plausible, since as a stem cell population gains a selective growth advantage, outgrowing its nonmutated counterparts, it forms a heterogeneous area of premalignant foci that can conquer adjacent areas of tissue, forming fertile ground for future carcinogenic changes. These observations are outlined in **Figure 7.** For virally induced carcinomas it is thought that the carcinogenic exposure of the surrounding epithelia is lower, as the virus-infected cells reside in only a limited area of the epithelium, more readily ablated after treatment. Moreover, the number of genetic alterations is smaller. Therefore, it is plausible that these cancers display lower recurrence rates and better survival after treatment (O'Rorke et al. 2012).

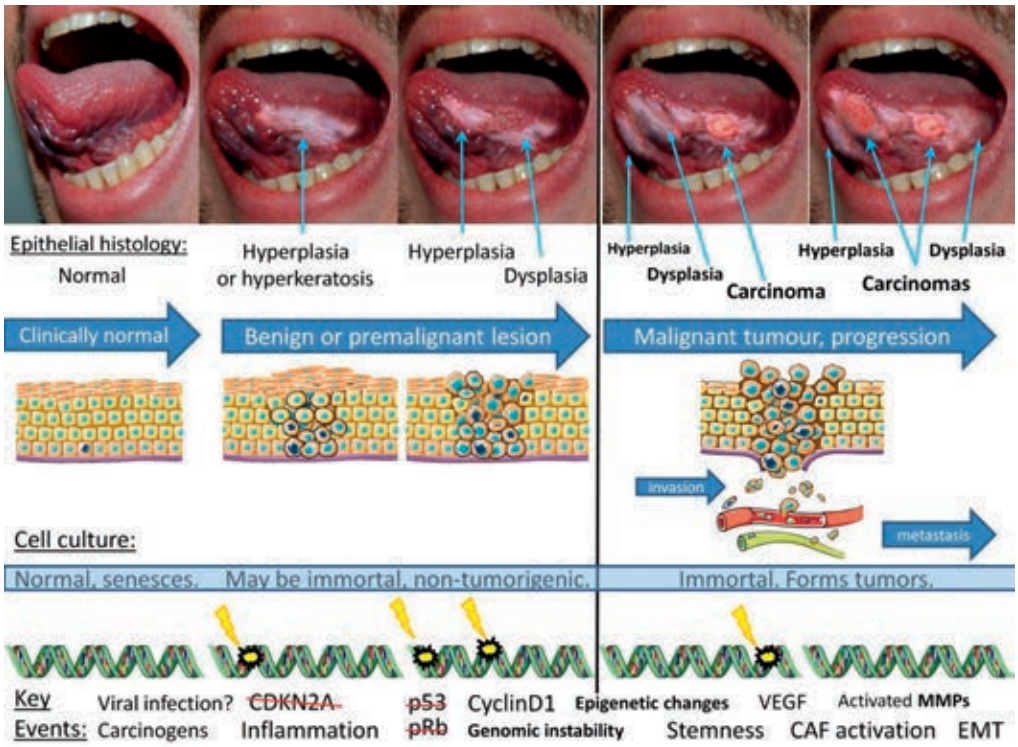


Figure 7: Progression of potentially malignant disorders of the oral mucosa toward invasive carcinoma. To the left, the clinical image and a presentation of the histology of normal tongue epithelium are shown. The normal cells senesce after a short time in culture, (Rheinwald et al. 2002). Moving to the right: during the progression, clinically oral potentially malignant disorders may be visible as a leukoplakia or erythroplakia. Histologically epithelial dysplasia may be present. Cultured cells become gradually immortal. Possible losses of gene functions are shown as a red dash over the gene name. A viral carcinogen may be a key event in carcinogenesis. Telomerase expression enables replicative immortality. The black vertical line denotes irreversible transformation. Premalignant, dysplastic epithelium may regress or cease progression. Epigenetic changes include gene inactivation via methylation, acetylation and microRNAs. Cancer cell stemness is detected by a rise in stemness markers. VEGF=Vascular Endothelial Growth Factor. CAF=carcinoma associated fibroblasts support cancer growth by intercellular signaling. MMP=Matrix metalloproteinases enable invasion. EMT=Epithelial-mesenchymal transition in which cells acquire an invasive phenotype. Figure layout modified with permission from (Forastiere et al. 2001), Copyright Massachusetts Medical Society. Photographs are used and edited with the permission of the patient. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.6 Treatment modalities in HNSCC

Head and neck cancers are not novel diseases: there is evidence of HNSCC-associated bone destruction in ancient skull findings (Capasso 2004). The mainstay of the treatment from the 18th century onward has been surgery and from the 20th century, surgery combined with radiotherapy or chemoradiotherapy (McGurk & Goodger 2000). Radiotherapeutic methods have developed from conformal radiotherapy to intensity-modulated radiotherapeutic approaches, allowing higher doses to the tumor whilst minimizing the exposure of normal tissues. Chemotherapeutic adjuvant treatment regimes use cisplatin- or 5-fluorouracil-based cytotoxic chemotherapeutics, or EGFR inhibitor therapy with monoclonal antibodies such as cetuximab, the first targeted therapy showing benefit in HNSCC. The blockade of EGFR improves locoregional control in HNSCC (Bonner et al. 2006) in HPV-positive cases as well (Pogorzelski et al. 2014). These, where applicable, mainly function to increase the effectiveness of radiotherapy as a curative treatment (Mendenhall et al. 2010). Still, chemotherapy has proven to be beneficial adjunct treatment of unresectable cancers, as is often the case with nasopharyngeal carcinomas. However, the treatments have remained fundamentally similar for decades and survival rates have improved only in small fractions. In addition, radioresistance has been implicated in treatment failure in many HNSCCs (Linge et al. 2016).

2.7 Viruses and head and neck cancer

It is estimated that 12% of the 14 million total new cancers in 2012 were attributed to infections with oncogenic viruses (Plummer et al. 2016). Overall, the viruses described as direct biological carcinogens related to HNSCC are EBV, and the high-risk HPV types (HR-HPVs) (Plummer et al. 2016). These share several features: they cause the infected cell to express viral oncogenes, causing proliferation and avoidance of apoptotic cell death. This is accomplished via upregulation of oncogene expression and downregulation of tumor suppressor gene expression. In addition, the infection generally does not result in the death of the host cell, allowing the virus to remain latently or chronically infecting the host. The difference between latent and chronic viral infections is that latently infected cells do not produce infectious virions. Chronic infections, such as Epstein-Barr virus and HPV, lead to longstanding virus production at a low rate. The patient is often free of symptoms. Both these patterns of long-term virus persistence may lead toward malignant transformation of the infected cell. However, from the point of view of the viruses, cancer formation can be considered an "accident," since the purpose of viral oncogene expression is to enhance and facilitate the production of progeny viruses, not to kill the host organism.

2.8 Human papillomaviruses (HPV)

2.8.1 Overview

HPVs are small, nonenveloped double-stranded DNA viruses with a genome of approximately 8 kb (**Figure 8**). High-risk HPVs (HR-HPV) are major human carcinogens. As the main cause of cervical carcinoma, HPVs cause over half a million new annual cases worldwide. HPV-related anogenital tract carcinomas also include SCCs of the external genitalia and anus of both sexes, with cervical carcinomas being the most important. HR-HPV types 16 and 18 are the most important, causing a vast majority of malignancies such as 70.8% of all cervical carcinomas (de Martel et al. 2017).

2.8.2 Prevalence and incidence in the head and neck area

After the first descriptions of HPV-related changes in HNSCCs and their later detection using molecular techniques in laryngeal and oral carcinomas (Syrjänen & Syrjänen 1981, Syrjänen et al. 1982, Syrjänen et al. 1983), HPVs have been detected in several different malignancies of the head and neck. Today, more conservative estimates attribute around 38 000 cases (~7%) of HNSCCs in the world to HPV each year. Of these, 29 000 (30.8%) are oropharyngeal, 4400 (2.2%) oral and 3800 (2.4%) laryngeal carcinomas (de Martel et al. 2017). The attributable fraction of HPV in HNSCCs is confirmed by DNA and E6/E7 mRNA expression detection by PCR and qRT-PCR combined, as PCR-only leads to the overestimation of this fraction (Ndiaye et al. 2014). HPV prevalence has increased during the last decade (Chaturvedi et al. 2008, Näsman et al. 2009, Marur et al. 2010, Chaturvedi et al. 2011). The increasing incidence of HPV-related cancers is proposed as a major reason for increases in HNSCC incidence in general. This is best exemplified in tonsillar carcinomas examined by Näsman et al. (2009), where the prevalence of HPV-positive cases in Stockholm increased from 23% in the 1970s to 57% in the 1990s and finally to 93% in 2007. Similar trends in oropharyngeal cancer are evident in studies from the USA (Javadi et al. 2017), Brazil, other Nordic countries (Simard et al. 2014) and the UK (Melchers et al. 2015). Furthermore, a recent assessment of tonsillar carcinomas in a worldwide cohort showed a prevalence of 47% for HPV DNA (Castellsague et al. 2016). The same report showed a rise in oropharyngeal carcinoma prevalence from 10.8% in 1990 to 34% in 2012. For comparison, a recent analysis of nonmalignant tonsils revealed a low prevalence of HPV (1%) in a Finnish cohort (Ilmarinen et al. 2017). Thus, rises in HPV-related oropharyngeal carcinoma ratings are now an international cause for public health concern if these trends continue (Gillison et al. 2015), and the number of cases worldwide has been reported to be up from 22 000 in 2008 (estimated by de Martel et al. 2012) to 29 000 in 2012 (de Martel et al. 2017). Although oral tongue carcinomas are also becoming more prevalent, the role of HPV seems to be of less importance (Simard et al. 2014). Nevertheless, HPV is also a risk factor for oral SCC (Gheit et al. 2017). This is supported by the fact that HPV infection is also a risk factor for oral leukoplakias and dysplastic lesions (Syrjänen et al. 2011). Moreover, as oral SCCs outnumber

oropharyngeal SCCs by a margin, even small attributable fractions can still mean a large number of patients. Multiple HPV types have been found in the oral cavity. In adults, HPV is detected in 8.8% of oral rinse samples (Orosco et al. 2016), which invariably also contain oropharyngeal secretions and a large amount of DNA from other microbes. In healthy young women and men of the Finnish Family HPV study cohort, the point prevalence of oral HPV varied from 15% to 24% and from 15% to 31% during the 6-year follow-up (Rautava et al. 2012, Kero et al. 2012). The samples were collected with a brush from the oral cavity mucosa, likely leading to more sensitive analysis.

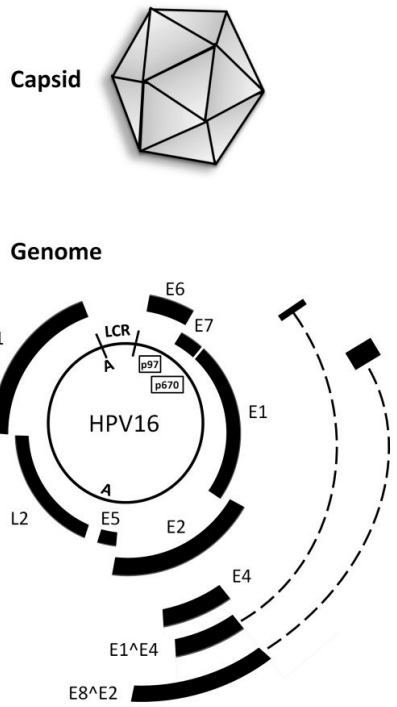


Figure 8: HPV Virion and genomic organization. The capsids of all HPV types are non-enveloped icosahedral protein capsids formed from the L1 and L2 proteins. HPV16 genome, depicted here, is a circular dsDNA molecule. Transcription of HPV genes is regulated by the binding of viral and cellular transcription factors into its largely noncoding sequence, the long control region (LCR, also known as upstream regulatory region URR) where the binding sites necessary for replication and transcription reside. Transcription is initiated from the early promoter p97 or late promoter p670 for example, and terminated in either of the polyA signals (shown as ”A”). HPV E1^E4 and E8^E2 are fusion products that are transcribed from several locations (depicted with a dashed line). The genome is transcribed in one direction from one DNA strand (Graham 2010) using leaky scanning (Stacey et al. 2000).

2.8.3 HPV Entry

HPVs infect the undifferentiated basal keratinocytes of squamous epithelia via small breaks in the epithelial integrity (**Figure 9**). Although mucosal HPV has been considered a sexually transmitted disease, HPV can be also transmitted via salivary contact, digital transmission or breast-feeding (for review see Syrjänen 2011). Transmission to the newborn is also possible through the birth canal or vertically, across the placenta from an infected mother (Rombaldi et al. 2008, Koskimaa et al. 2012). As HPV requires access to the basal membrane, tissues with easy access through the epithelium are susceptible to HPV. Classically, the transformation zone in the cervix, where the stratified epithelium transforms into endocervical columnar epithelium, is a well-known site for HPV, with an increased probability for microtrauma during intercourse facilitating viral entry. Hypothetically, targets in the head and neck region would be junctions between the salivary gland ducts and oral epithelium, the bottom of the gingival sulcus or periodontal pocket and tonsillar crypts. However, the evidence is so far scanty at best (Hormia et al. 2005, Begum et al. 2005, Morbini et al. 2015). Similarly to the cervical transformation zone, epithelial junctions are also found in the oral cavity in the mobile tongue and oropharynx (the tonsillar crypts, most importantly). According to the current concept, in wounded epithelia, HPV can attach to the basement membrane components of the extracellular matrix (ECM), specifically the heparan sulfate molecules, of which Syndecan-1 is the most important (Shafti-Keramat et al. 2003). During healing, the laterally growing basal cells come into contact with the virus, which is endocytosed via the clathrin- or caveolin-mediated endocytic pathways, dependent on the HPV type (Sapp & Day 2009). Then, HPV L2 is cleaved by furin protease to expose its active site, leading to lysosomal exit, and the HPV particle is transported via the microtubule network to the nucleus, where it releases the genome. The mechanisms of nuclear entry are not yet well-known but may involve nuclear breakdown during mitosis or other mechanisms of entry such as karyopherin-mediated import (Schiller et al. 2010, Day & Schelhaas 2014). At first, the genome replicates until it reaches 10-50 copies per cell (McBride, 2017). It then lies dormant, expressing only its early genes, unless the cell progresses further in the differentiation regime and the expression pattern begins to favor the late gene expression and capsid formation.

2.8.4 Keratinocyte cell cycle and HPV in differentiating epithelium

Keratinocytes normally follow a tightly regulated differentiation pattern, where the basal compartment houses the stem cells that divide and form a replicating compartment of basal cells. As the cell divides, the daughter cell moves upwards in the epithelium, ceases mitosis and begins to differentiate. As the cell moves further upward in the layers, local paracrine factors such as an increasing gradient of intercellular calcium, sensed by the calcium-sensing receptor (CasR), and vitamin D, signal the cell to eventually start cornification (Bikle et al. 2016), lose its nucleus and synthesize differentiation-related cytokeratins 1 and 10 and involucrin that form the cornified envelope. In addition, ceramide and other fatty acids important

for the mucosal (or skin) protective barrier formation are secreted in the extracellular compartment. The end result of the terminally differentiated cell is senescence and shedding from the mucosal, or skin surface (Gandarillas & Freije 2014). This happens constantly and rapidly *in vivo*: for example, complete epithelial replacement by new cells takes 21 days in the floor of the mouth, 14 days in the buccal mucosa, and ca. 27 days in the labial skin (Squier & Kremer, 2001). It has long been known that HPVs hijack this program to their own ends, expressing their L1 and L2 genes only in terminally differentiating cells. This property may aid in avoiding the dendritic/Langerhans cells of the epithelium by expressing immunologically more detectable proteins last in the viral life cycle. HPV particles form in the keratinocyte nuclei of the suprabasal epithelial compartments. The virus is then released, likely through a passive process, as the cornified cells are shed and begin to degrade (Graham 2017). The life cycle of HPVs in differentiating epithelia is shown in **Figure 9.**

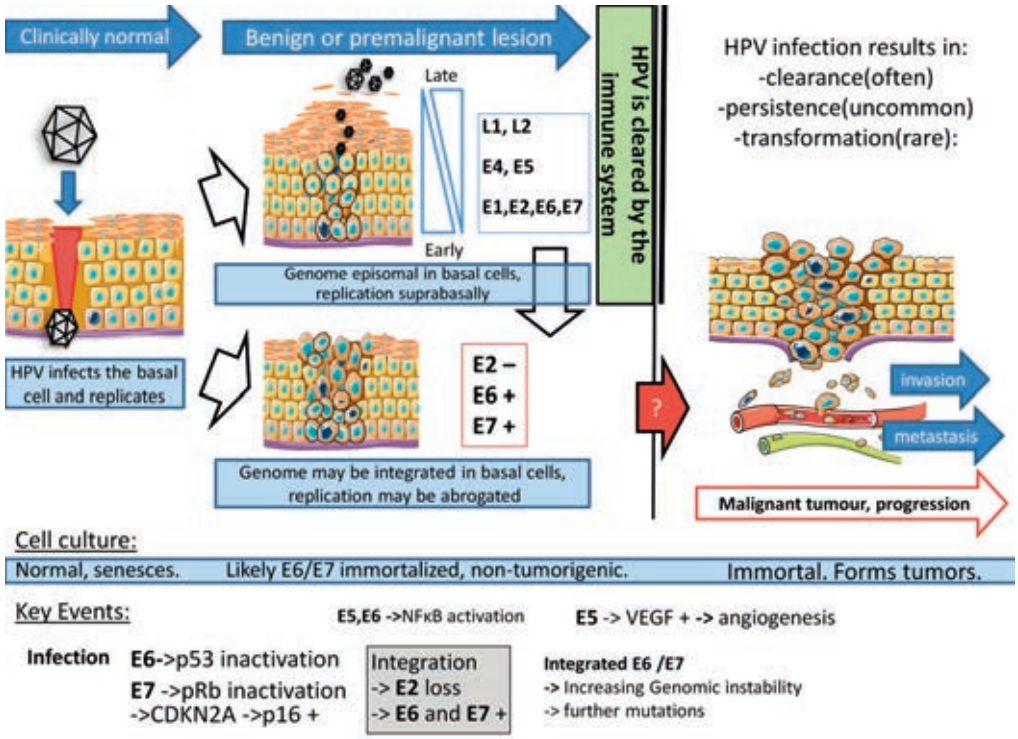


Figure 9. The life cycle and potential carcinogenic progression of HPV infections. First, HPV enters the basal cell, likely via epithelial microtrauma. It then internalizes, replicates its genome and persists as an episome. As the basal cell divides and the daughter cells differentiate, HPV sequentially expresses its early to late genes, completing its life cycle as the host cells of the epithelial surface are nearing terminal differentiation followed by shedding and virus release. If the HPV genome integrates, this usually happens in the E2 area. This may cause a loss of E2 regulatory functions and deregulated E6 and E7 expression. The HPV genome was integrated in 43% of oropharyngeal carcinoma patients in a recent study, although episomal genomes may also lead to carcinogenesis (Vojtechova et al. 2016). In select patients, persistent infection of the host cell may eventually lead to transformation. However, most often the infection is cleared by the immune system in 1-2 years. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.8.5 HPV and carcinogenesis in head and neck region

HPV has been shown to play a significant role in HNSCC, particularly in oropharyngeal carcinomas (Mehanna et al. 2013)**.** The relation of different HPV types implicated in the development of most common pathologic lesions in the head and neck area is depicted in **Table 1.** It is now known that not only alpha papillomaviruses (the so-called "mucosal types") are found in the head and neck region. Beta and gamma HPVs have also been detected (**Table 1**). Low-risk HPVs of the beta and gamma subtypes have recently been described in a minority of verrucous lesions of the oral mucosa in a Finnish cohort (Kerge et al. 2018). The function of HPV genes is listed in **Table 2.** Overall, HPV-encoded E1 and E2 replicate its genome, where E1 orchestrates DNA replication and E2 regulates this process, inhibiting E6 and E7 transcription. E2 is able to induce apoptosis via caspase 8 and p53 (Desaintes et al. 1999). The main carcinogenic functions of HPVs can be attributed to their E6 and E7 oncoproteins. Moreover, E5 is an oncogene but its main role is in the early steps of oncogenesis where E5 facilitates viral entry and exit, controls viral genome maintenance and amplification, and aids in immune evasion. However, E5 is also implicated in cell adhesion and migration, crucial components of HNSCC invasiveness (Kivi et al. 2008). Mutation in HPV E8 increases HPV transcription and replication (Straub et al. 2015). Therefore, E8^E2 functions to inhibit the viral life cycle, likely to avoid immune recognition. The HPV E1^E4 fusion protein is the most highly expressed protein during HPV-productive infection (Wang et al. 2011) and functions to amplify the genome and facilitates genome packaging to virions by interacting with the cytoskeleton (Doorbar et al. 1991). Late genes L1 and L2 form the capsid, where L2 plays a crucial role later in infecting the next target cell.

Table 1. Different genotypes of HPV and the lesions they cause in the head and neck area. SCC=Squamous cell carcinoma

HPV Genus	Category	HPV Type	Associated diseases in the head and neck region	References
Alpha-PV	Low-Risk	HPV6	Papilloma Condyloma Lichen planus Laryngeal papillomatosis SCC	Mattila 2012 Weiss 2015 Kreimer 2005
	Low-Risk	HPV11	Papilloma Condyloma Lichen planus Laryngeal papillomatosis SCC	Mattila 2012 Kreimer 2005
	Low-Risk	HPV13 HPV32	Focal epithelial hyperplasia	Henke 1989 Pfister 1983
	High-Risk	HPV16	Papilloma Leukoplakia Lichen planus SCC Verrucous carcinoma	Syrjänen 2011 Brandsma 1986
	High-Risk	HPV31	Leukoplakia SCC	Abogunrin 2014
	High-Risk	HPV33	Laryngeal papillomatosis Leukoplakia SCC	Kreimer 2005 Arndt 1997
	High-Risk	HPV18	Papilloma Leukoplakia Lichen planus SCC	Campisi 2004 Syrjänen 2011
Beta-PV	Low-Risk	HPV5	Oral SCC Laryngeal SCC	Agalliu 2016
	Low-Risk	HPV8	Cutaneous SCC	Efird 2011
Gamma-PV	Low-Risk	γ11,γ12 -types	Oral SCC Larynx SCC	Agalliu 2016

Table 2: HPV genes with special reference to the development of carcinomas of the head and neck region

Gene/Protein	Function	Mechanism	References
E1	DNA replication Episome maintenance Induces the DNA damage response	DNA helicase Interacts with ATM kinase	Fradet-Turcotte 2011
E2	Transcription activator Regulates gene expression Induces apoptosis Potentially oncogenic	Negative regulation of E6 and E7 Recruits E1 to the DNA Chromosomal instability	Bellanger 2011
E4 (E4^E1)	Replication of viral DNA Regulates viral transcription G2 arrest Cell cytokeratin network remodeling Virion release	Facilitates E1 nuclear localization, stabilizes E2 Inhibits multiple host kinases via SRPK1 Activates MAPK pathway Associates with cytokeratins	Doorbar 2013 Prescott 2014 Egawa 2017
E5	Antiapoptotic Decreases radiation sensitivity Increases proliferative signaling Immune evasion Angiogenesis	Stabilisation of EGFR Downregulates tumor suppressor p21 TGF-β down- and NF-κB upregulation VEGF stimulation	Tsai and Chen 2003 Zhang 2002 De Freitas 2017
E6	Antiapoptotic Replicative immortality Immune evasion Increase in angiogenesis Genome instability	P53 degradation via E6-AP hTERT and NFκB activation Interleukin and MMP upregulation Interferon inhibition	Mittal and Banks 2017 Hong and Laimins 2017 Scheffner 1990
E7	Antiapoptotic Increased invasion Genome instability Immune evasion	pRb degradation and E2F activation E-cadherin downregulation Interferon inhibition	Mittal and Banks 2017 Münger 1989
E8 (E8^E2)	Inhibits viral transcription and replication	Truncated E2^E8 fusion protein interacts with histone deacetylases and represses E2 activation of transcription.	Stubenrauch 2000
L1	Structural protein	Self-assembles into icosahedral capsids	Buck 2013
L2	Minor capsid protein HPV entry	Facilitates endosome escape after furin cleavage	Wang and Roden 2013

2.8.6 Molecular pathways to carcinogenesis

The progression of HPV infection toward malignancy is a long and stepwise process, with alterations in the cell cycle considered to be the most important mechanism (zur Hausen 2002). E6 and E7 are the main HPV oncoproteins that disrupt cellular tumor suppressor pathways p53 and pRb, respectively. p53 and pRb normally prevent S-phase entry after differentiation-related signaling, trigger apoptosis on detection of DNA damage, and protect the cell against progression toward malignant phenotype (seen in **Figure 4**). The main functions of E6 and E7 are outlined in **Figure 10**. The E6 protein of HR-HPVs interacts with the cellular E6-AP ubiquitin ligase to target the p53 tumor suppressor protein for proteasomal degradation (Scheffner 1993). It also represses p53 transcriptional activity (Zimmermann et al. 1999). This in turn leads to a decline in p21 expression, negating the inhibitory effects of p21 on the cyclin/CDK function. Loss of p53 also leads to genetic instability and inhibits DNA repair. E6 also activates telomerase (hTERT) to immortalize the cell and inhibits innate immunity via NFkappaB - related signaling.

HPV E7 interacts with several host proteins. For example, E7 directly binds the pRb (Dyson 1992) and targets it for proteasomal degradation (Boyer 1996), activating S-phase progression via E2F. p21 is also inhibited by E7, signifying cooperation between HPV oncogenes (Funk et al. 1997). E2F upregulates p16 that inhibits the cyclin D1/Cdk4 complex which in HPV-positive cells is unable to inhibit cell proliferation as E7 degrades pRb (Khleif et al. 1996). Thus, overexpression of p16 has been used as a surrogate marker for HPV infections.

The transforming potential of HPV oncogenes with either high-risk or low-risk E6 and E7 has been shown (Woodworth et al. 1989). High-risk E6 contains a PDZ-binding domain that enables interaction (and ubiquitin-mediated degradation) with several substrates contradictory to that of the low-risk E6 proteins (Ganti et al. 2015). High-risk E7 binds p21 efficiently, whereas low-risk E7 proteins only present with low levels of p21 inhibition. As E6 causes its inhibition, p53 pathway mutations are uncommon in HPV-driven cancers (Westra et al. 2008), and an otherwise often mutated CDKN2A/p16 pathway is made redundant due to the release of E2F from E7-phosphorylated pRb (as seen in **Figure 10**) (Lechner et al. 2013). Activation of the PI3K pathway has been suggested to be sufficient for tumor progression in p53 and pRb-deficient states such as are caused by HPV (Martin et al. 2014, **Table 2).** The pathway activation has been recently linked to APOBEC proteins, upregulated by HPV, which increase genomic instability (Vieira et al. 2014). Taken together, both E6 and E7 are promising targets to treat HPV-associated carcinomas (Hoppe-Seyler et al. 2017). However, HPV E2 also has potential oncogenic functions. It interacts with several cellular proteins and has been postulated to increase abnormal mitoses leading to aneuploidy, a premalignant trait for cells (Bellanger et al. 2011).

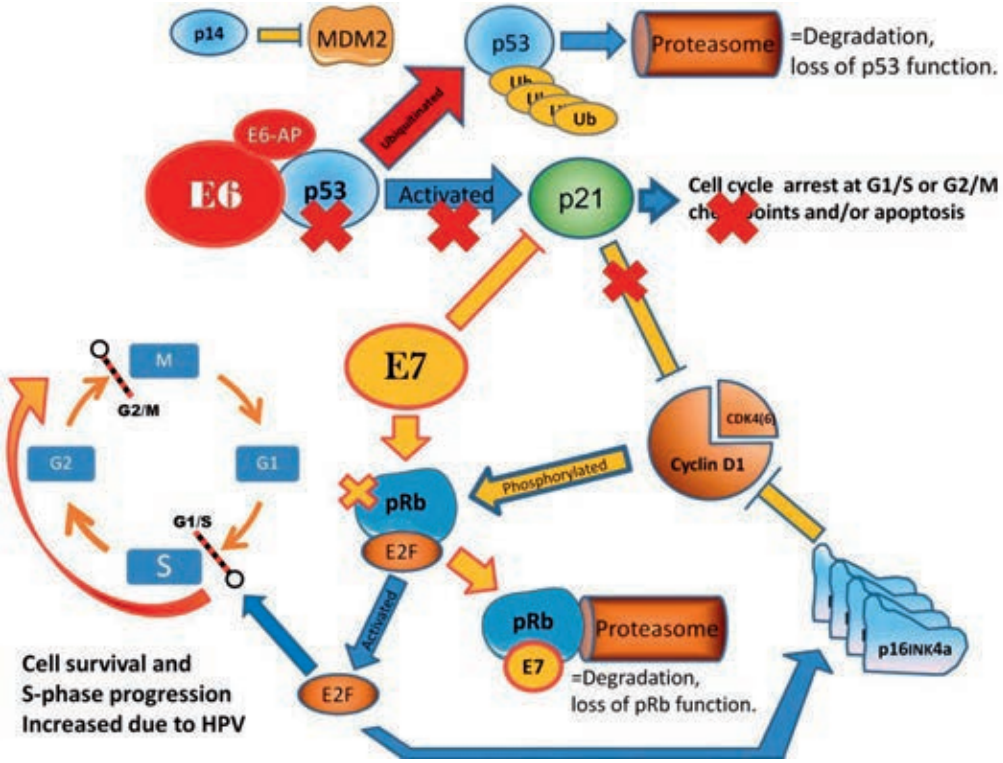


Figure 10. The effects of high-risk HPV E6 and E7 oncoproteins on pathways related to cell cycle progression. These functions enable HPV persistence and viral replication but expose the cell to further carcinogenic changes. Note the increase in p16 expression due to E7. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.8.7 Effect of HPV on cellular radioresponse

HPV-attributed HNSCCs are more readily treatable by irradiation (Ang et al. 2010). As p53 is wild type in most HPV-induced tumors, low levels of residual p53 expression in HPV-positive carcinomas could result in cell death after radiation (Kimple et al. 2013), explaining the better radiation responses. HPV-negative carcinomas usually harbor p53 mutations that prevent such a response. HPV16 E6, E7 and E5 proteins increase the PI3K/AKT pathway signaling that regulates several genes important for radiation resistance via the mTOR and MAP pathways (Zhang et al. 2015). The HPV16 E5 protein sensitizes the cell to EGF signaling, which increases proliferation (DiMaio & Mattoon 2001). High EGFR expression has been linked with activation of PI3K-Akt and RAS, leading to radioresistance (Zimmermann et al. 2006), whereas E5 also has potential interactions with the antiapoptotic Bcl-2 (Auvinen et al. 2004) and

may prevent apoptotic cell death via the PI3K-Akt pathway. Therefore, E5 may play a role in radioresistance as well. Lastly, Vermeer and colleagues (2013) showed that CD47, a tumor surface self-marker critical for immune clearance, was downregulated after irradiation of HPV-positive tumor cells, leading to better tumor immune clearance in mice. Therefore, the effects of immunity, which HPV alters, are also of importance for the outcomes of the radiated cell.

2.8.8 Specific treatments for HPV-driven HNSCC

HPV-positive HNSCCs often present with large cystic lymph nodes in the neck, signifying a late-stage disease (Lajer & Von Buchwald 2010). Nevertheless, HPV-positive HNSCCs, particularly those of the oropharynx, have been shown to retain a better prognosis (Ang et al. 2010). Therefore, de-escalation of treatment aggressiveness has been proposed. The 8th edition of the TNM classification (2017) has reclassified HPV-positive HNSCCs, detected using p16 staining. The novel classification downstages HPV-positive oropharyngeal carcinomas that may lead to de-escalated treatment tailoring to individual patients (Würdemann et al. 2017). The evidence supporting this practice is, however, still controversial (Wu et al. 2016). Despite this, de-escalation protocols have been planned with enthusiasm, and seemingly excellent disease control rates using deintensified chemoradiotherapy have been published (Chera et al. 2018). HPV-positive HNSCCs have also shown superior responses to chemoradiotherapy using an EGFR inhibitor (Cetuximab) when compared to the standard chemotherapeutic agent, cisplatin (Huang et al. 2016). Furthermore, modern immunotherapeutic approaches using programmed death-1 (PD-1) antibodies have shown benefit in treatment of recurrent disease (Saleh et al. 2018). Despite these facts, in select patient subgroups there is also evidence to the contrary. HPV-positive late-stage cancers may display an increased risk for distant metastases and, therefore, grim prospects for survival (Lee et al. 2012). Moreover, the effect of coinfections between other viruses and HPV could theoretically play a role in carcinoma invasiveness, but has not been studied in detail.

2.9 Herpes simplex virus type 1 (HSV-1)

Herpes simplex virus type 1 (HSV-1) is one of the most common herpesviruses, infecting 50% of the population in the developed and 90% of the population in the developing world at some point in their lives (Whitley & Roizman 2001). Herpesviruses are large viruses composed of a lipid bilayer, the envelope, covering the proteinaceous tegument and nucleocapsid that harbors its linear dsDNA genome (**Figure 11**). Attached to the envelope are nonglycosylated and glycoprotein spikes which function in the first steps of the infection to facilitate cell entry. The tegument contains viral proteins, microRNAs and exosomes that aid in the first stages of viral genome internalization and replication and the avoidance of apoptosis and innate immunity reactions, such as the viral protein 16 (VP16/α-TIF) and virion host shutoff (vhs) protein for HSV-1. The icosahedral nucleocapsid encloses the double-stranded DNA genome of 152 kb that encodes over 80 genes.

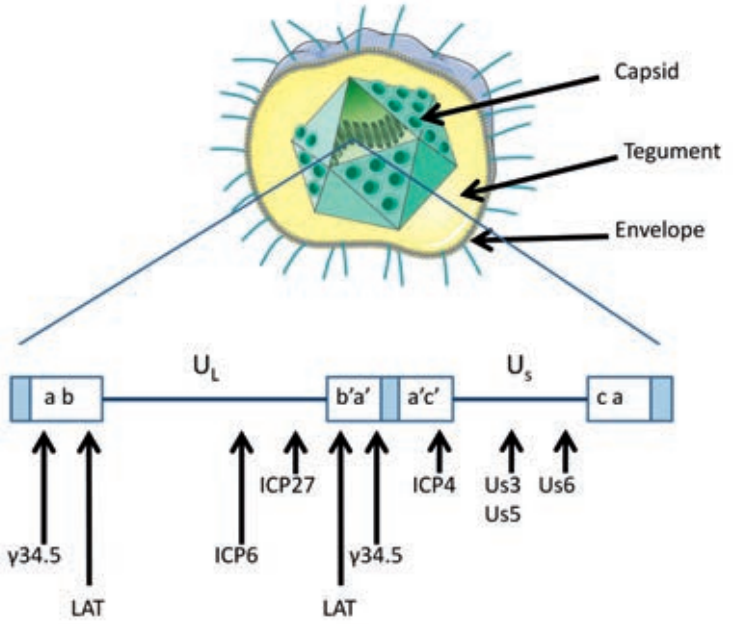


Figure 11: HSV-1 virus particle and its genome.

The HSV-1 capsid encloses its linear dsDNA genome. The HSV-1 genome consists of unique long and short segments Ul and Us, respectively. The segments are flanked by inverted repeat regions named ab and b'a' for long and a'c' and ca for short segments. The "pac" signals HSV uses in delivering its genome into new capsids are shown as blue rectangles. The localizations of key genes involved in apoptotic cell death are shown as arrows. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.9.1 HSV entry

Herpesviruses infect new hosts primarily via mucosal secretions, i.e. oral or genital fluids. As the epithelial barrier mechanisms are powerful in preventing pathogen entry, herpesviruses likely require a breach in the epithelium, such as a skin abrasion on the fingertip or a gingivitis lesion in the gums, to enter the basal keratinocyte layer (**Figure 12**). Infecting keratinocytes, the first target cell HSV-1 encounters in the body, the virus attaches to the heparan sulfate glycoproteins (HSPG), mainly with its surface glycoproteins C (gC) and B (gB) (Campadelli-Fiume et al. 1990**,** Herold et al. 1994). It uses these glycoproteins to react with its glycoprotein D (gD) which facilitates interaction with the herpes virus entry mediator (HVEM) and Nectin-1 (Thier et al. 2017). Then, it uses surface proteins gB and gH-gL in order to fuse its envelope with the cell surface membrane. HSV-1 is also able to use additional entry mechanisms such as endocytosis to enter some cell types (Nicola et al. 2003). The end result is release of the contents of the viral particle such as the tegument and capsid into the cytoplasm. The tegument proteins αTIF and virion host shutoff (vhs) activate viral transcription and shut down host cell defenses (Strand & Leib 2004). Concurrently with HSV-1 particle ingress, exosomally transported viral and cellular factors such as STING may function to suppress cellular innate immunity (Kalamvoki et al. 2014). The viral DNA is transported through the cytoplasm encased in its capsid via cellular microtubular transport until it comes into contact with the outer nuclear membrane. The contents of the capsid are interestingly under several atmospheres of pressure, and upon contact with the nuclear pore complex are "injected" into the nucleus at high pressure (Bauer et al. 2013). Subsequently, immediate-early gene transcription can proceed (Rahn et al. 2011).

2.9.2 HSV replication

The entire replicative life cycle of HSV-1 takes less than 24 hours (Lehman & Boehmer 1999). The tegument, upon release into the cytoplasm, initiates viral gene transcription via VP16, suppresses host responses via γ34.5 and vhs (virion host shutoff) proteins and inhibits apoptotic cell death with Us3 and ICP4 (Peri et al. 2011). On entering the nucleus, the viral DNA circularizes (Strang & Stow 2005). First, viral protein VP16 induces the expression of viral α (immediate-early) genes in order to commence β (early) gene expression in a process that lasts around 4 hours. β genes then induce DNA synthesis via the viral DNA polymerase. Then, γ gene expression results in capsid formation in the nucleus and finally, the formation of new infectious virions (Lehman & Boehmer 1999).

The DNA polymerase protein complex is necessary for viral replication and is a target for anti-HSV medications such as acyclovir (Schaffer et al. 1971). The viral capsids that form in the nucleus are packaged with the unit-size viral DNA. This capsid envelops at the inner and then fuses at the outer nuclear membrane, crossing into the cytoplasm where virion maturation can occur. This proceeds by the addition of the proteinaceous tegument and finally the viral envelope, which is gained as the particle

fuses with the outer cell membrane at locations now enriched with virus-specific outer membrane glycoproteins (Mettenleiter et al. 2009). Thus, part of the viral architecture is formed from components of the host cell. The release of infectious virions results in the death of the host cell. The mechanism by which HSV-1 infection kills cells is not completely clear; however, shutoff of cellular gene expression via the early ICP27 and late vhs (virion host shutoff, UL41) proteins, apoptosis and necroptosis play significant roles, controlled by viral gene expression (Yu & He 2016).

2.9.3 Establishment of latency

After HSV-1 infects the epithelium and undergoes a period of lytic cycles, it infects and retreats to the nearby sensory neurons. It then travels to the neuronal nucleus in its respective ganglion where it resides in latency, reactivating only occasionally to cause herpetic lesions and, more often, asymptomatic shedding. The mechanisms that govern the establishment of latency are not well-known, but the LAT gene expression plays a major role. HSV-1 can also become latent in organotypic keratinocyte cultures, where LAT mRNA and HSV-1 DNA are present, but no cytopathic effects or replication are detected (Syrjänen et al. 1996).

2.9.4 Reactivation and shedding

HSV-1 reactivation is mediated by sensing cell stress, changes in metabolic parameters or trauma (Vink et al. 2017**,** Bloom 2016). The molecular pathways governing reactivation are only beginning to be characterized, although inhibition of the PI3K/AKT pathway and calcium signaling are important in the induction of reactivation (Suzich & Cliffe 2018). This reactivation may or may not coincide with recrudescent, blister-forming infections such as "cold sores." Still, it is known that asymptomatic shedding of the virus is far more common than symptomatic replication, and the asymptomatic presence of HSV in saliva, for example, is considered a major transmission route. It has been reported that at least 70% of saliva samples from asymptomatic patients contained HSV-1 DNA at least once a month (Miller & Danaher 2008). A daily sample has an up to 7.6% chance of being HSV-1 positive, with viral loads between 100 and 2.8x10(6) per ml of saliva (Miller & Danaher 2008). Recently, oral HSV-1 DNA was found in 11.8% of 304 young Finnish mothers (Mäki et al. 2015), while 12.9% their spouses were HSV-1 DNA-positive at some point in their 6-year follow-up (Mäki et al. 2017). Asymptomatic shedding into the saliva has been detected in 84.6% of patients visiting an oral surgery clinic, which shows the effect of anxiety and postoperative states on HSV replication (Hyland et al. 2007). Moreover, HSV-1 has been detected in the gingival biofilms of patients with periodontal disease (Nishiyama et al. 2008, Ling et al. 2004), underlining the importance of HSV-1 in clinical patient care. Recently, HSV-1 has also been demonstrated in the ganglions of the parasympathetic system of the head and neck area (Lee et al. 2015),

signifying that HSVs do not seem to require the trigeminal nerve root ganglia specifically, and others can support the infection as well. Furthermore, it is not known how UV light affects the sensory neurons to reactivate HSV-1 and how UV-protecting creams can prevent such occurrences.

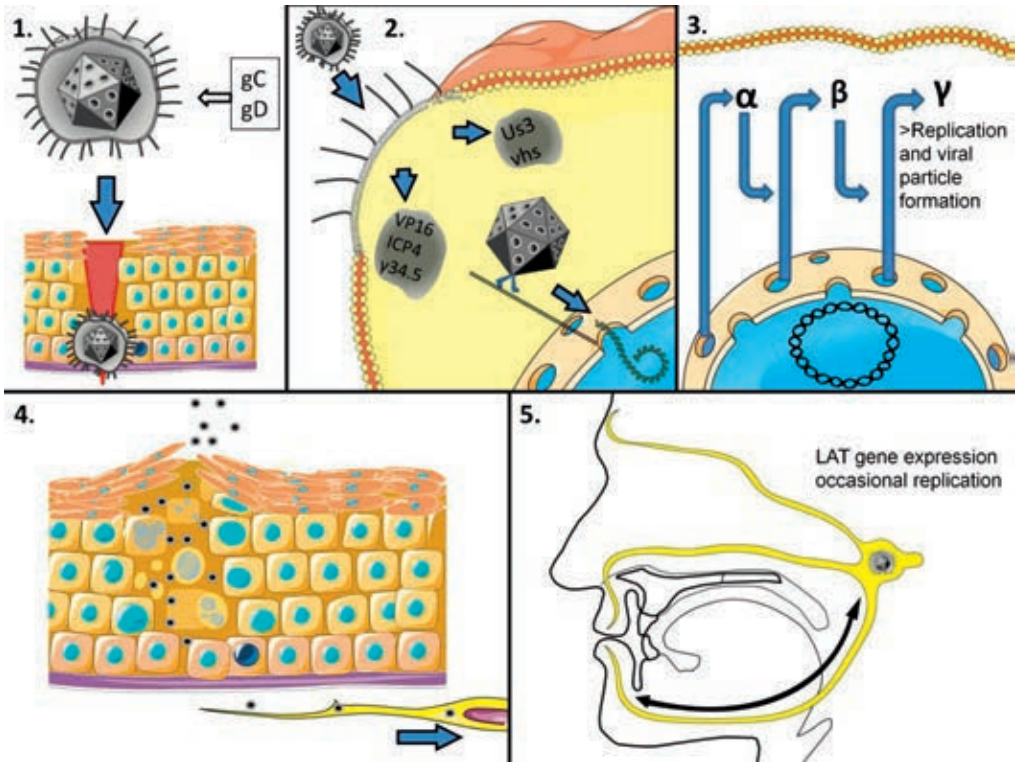


Figure 12: The life cycle of HSV-1

1. HSV-1 enters the basal cell compartment via small openings in the epithelial barrier such as microtrauma.

2. HSV-1 fuses with the cell outer membrane or the membrane of an endosome, releasing the tegument and capsid into the cell. The capsid travels via microtubules using the dynein motor protein until it reaches the nucleus.

3. HSV-1 genes are expressed in a sequential fashion, where alpha (immediate-early) genes induce beta (early), which induce DNA replication and subsequently, gamma (late) gene expression.

4. The productive infection results in cell destruction which clinically manifests as an epithelial blister. The virus then exits the body into the surface. HSV-1 may also enter the sensory neurons and travel (arrow) to their nucleus where it is able to remain latent for the life of the host.

5. The trigeminal nerve (yellow) ganglion hosts the virus in a latent state where it occasionally replicates and travels back to the mucosal surfaces in order to spread to new hosts (arrow).

2.9.5 HSV-1 and apoptosis

In HSV-1 infected cells, apoptosis is prevented, facilitating efficient viral replication. This downregulates many pathways such as the p53 pathway and inhibits caspase activation to allow viral persistence and replication. The antiapoptotic features of HSV-1 genes are outlined in **Table 3**.

Table 3. Antiapoptotic gene products of HSV-1 and their function. The cell line of the original observation is depicted as follows: Vero cells are African green monkey kidney cells, Jurkat cells are an immortalized human T-lymphocyte cell line, HEp2 Human HeLa derivative contaminant cervical carcinoma cell line (HPV18+), C1300 are mouse neuroblastoma cells. [1]=likely an indirect effect on the pathway, [2]=not known, [3]=also expressed early in the infection, so-called ”leaky-late gene.” R1=Ribonucleotide reductase large subunit 1. grB Granzyme B mediates cytotoxic T-lymphocyte cell killing. The FAS ligand is the inducer of the extrinsic apoptotic pathway leading to caspase 8 activation. BAD is a Bcl2 inhibitor. CI-MPR is a cation-independent mannose 6-phosphate receptor. Its inhibition blocks apoptosis and may result in radiotherapy resistance in HNSCCs (Jamieson et al. 2003).

***Gene* (Protein)**	**Timing / Function**	**Potential mechanism**	**Cell Line**	**References**
Ul54(ICP27)	α / Antiapoptotic	Caspase 3 inhibition[1]	HEp2 (HeLa)	Aubert et al. 1999
ICP4(ICP4)	α / Antiapoptotic	Caspase 3 inhibition[1]	HEp2 (HeLa)	Galvan et al. 1999
ICP6(R1)	β / Antiapoptotic	Caspase-8 inhibition	HeLa	Dufour et al. 2011
Us6(gD)	γ / Antiapoptotic	CI-MPR inhibition	SK-N-SH, HeLa, Vero	Zhou and Roizman 2002
Us3(Us3)	β / Antiapoptotic	BAD blockade	Rabbit skin	Leopardi and Van Sant 1997
Us5(gJ)	N[2] / Antiapoptotic	Blockade of FAS ligand and grB	Jurkat, Vero	Jerome et al. 2001
γ34.5 (γ34.5)	γ[3]/ Prevents shutoff of protein synthesis	PP1α dephosphorylates eIF2α	SK-N-SH	Gross and Roizman 1997
LAT	Latency / antiapoptotic	AKT stabilization blocks BAD and caspase 9	C1300	Perng et al. 2000 Liu and Cohen 2015

2.9.6 Generation of DNA instability and effect on radiation responses

HSV-1 interacts with DNA repair mechanisms, particularly the MRN protein complex of the Ataxia telangiectasia mutated (ATM*)* pathway essential in cellular responses to radiation (De Braekeleer et al 1992, Balasubramanian et al. 2010). Moreover, HSV-1 DNA synthesis preferentially uses the homologous recombination DNA repair pathways and interferes with the Fanconi anemia DNA repair pathway used in NHEJ-type DNA repair (Karttunen et al. 2014) after radiation damage. HSV-1 infections also result in chromosomal instability of the infected cell, which has led to *in vitro* embryo fibroblast transformation (Larsson et al. 1992), for example. Chromosomal instability, on the other hand, is a byproduct of radiation damage and thus, these two different mechanisms of DNA damage might hypothetically function together to increase radiation damage in infected cells. Induction of the key transcription factor NFkappaB1 is a known result of both irradiation and HSV-1 infection and prevents apoptosis in HSV-1-infected cells (Goodkin et al. 2003). Accordingly, inhibitors have been proposed to prevent the oncogenic functions of NFkappaB1 (Luo et al. 2005). However, it is not known whether NFkappaB1 induction after HSV-1 infection might be altered in the context of simultaneous radiation damage to the infected cell.

2.9.7 HSV-1 in carcinomas other than HNSCC

HSV-1 replicates readily in immunosuppressed individuals such as cancer patients, especially during chemotherapy. HSV reactivation in the oral cavity causes pain and difficulty in eating and swallowing that further complicates the cancer treatment (Elad et al. 2017). A dreaded complication of HSV infection is encephalitis, which may fatally complicate treatment, of cerebral malignancies in particular (Koudriavtseva et al. 2010). Therefore many patients undergoing cancer chemotherapy are prescribed acyclovir prophylaxis during treatment (Glenny et al. 2009). After HPV was confirmed as a causative factor in cervical carcinoma, HSV was concluded to be noncarcinogenic in cervical cancer by itself, mainly based on immunological data (Lehtinen et al. 2002). However, HSV-2 has been epidemiologically associated with cervical carcinoma development after adjustment for confounding factors, and it has been hypothesized that HSV infections might act as cofactors with oncogenic HPV types to increase carcinoma risk (Smith et al. 2002). Due to its cytolytic activity, genetically engineered HSVs are currently being researched as oncolytic therapies, where the neurovirulence gene (γ34.5) has been deleted to prevent HSV-1 lytic effects outside of the tumor it infects. However, although already approved for clinical use, more studies are needed on the effects of recombinant HSVs in HNSCC treatment.

2.9.8 HSV-1 and head and neck cancer

Older studies clearly show the carcinogenic potential of HSV-1 in animal models (Wentz et al. 1981). Although HSV-1 exposure itself is unable to lead to carcinogenesis, for example Hirsch and coworkers observed oral SCC formation in rats exposed to Swedish “snus” (smokeless tobacco) and HSV-1 in combination (Hirsch et al. 1984). Serological evidence links HSV-1 to increased risk for oropharyngeal carcinomas, interestingly only for patients with other risk factors such as HPV infection or heavy smoking (Starr et al. 2001). In addition, antibodies to HSV-1 were progressively higher in patients with increasing stages of their oral carcinomas (Shillitoe et al. 1986) and in patients with normal oral mucosa compared to those with premalignant or malignant lesions (Jain 2016). These findings signify that HSV-1 may further damage the cells in progression toward malignancy. Lastly, HSV-1 mucositis has been shown to complicate up to half of patients receiving radiotherapy for head and neck cancer (Nicolatou-Galitis et al. 2006), but its potential effects on the treatment are unknown.

2.10 Epstein-Barr virus (EBV)

Most (95-98.5%) of Finnish adults are infected by Epstein-Barr virus (EBV) (Puhakka et al. 2015). In the Western world, EBV typically causes infectious mononucleosis and some cancers such as Burkitt’s lymphoma and nasopharyngeal carcinoma, where EBV is regarded as a causative factor in the majority of carcinomas (Young & Dawson 2014). Although EBV is implicated in the development of several lymphoma types (Kim et al. 2009), this discussion will focus on epithelial carcinoma development. EBV is, along with other herpesviruses, a double-stranded DNA virus, containing a 171656-171764 kb genome (EBV types 1 and 2, respectively (Palser et al., 2015)). Like most herpesviruses, EBV expresses over 100 genes which all function to enable highly efficient spread, persistence in latency and evasion of immunity for the virus **(Figure 13).**

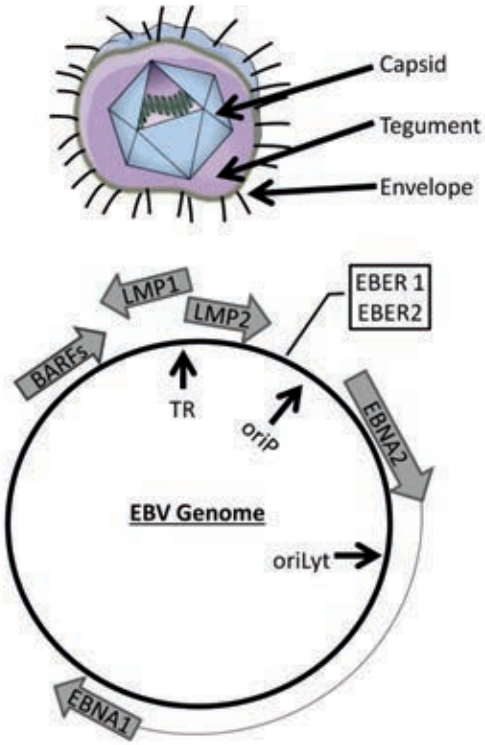


Figure 13: Epstein-Barr virus particle and genome. The viral particle is that of a typical herpesvirus: an icosahedral capsid surrounded by organized tegument proteins within a lipid bilayer envelope with its surface glycoproteins. Compared to the alphaherpesvirus HSV-1, EBV -a gammaherpesvirus, is highly similar in size and structure. Two origins of replication are shown: Origin of plasmid replication oriP and origin of lytic replication oriLyt. Latent membrane proteins (LMPs) are transcribed from both sides of the terminal repeat (TR) region which is the location where the genome circularizes upon latency (Young & Rickinson, 2004). Latent nuclear antigens (EBNAs 1, 2, 3a/b/c and LP), present in the nuclei of latently infected cells, are transcribed from their own promoters and are spliced from a single long transcript, represented by the line between the arrows. The short EBERs 1 and 2 are transcribed in all stages of EBV infection and latency (Herbert & Pimienta, 2016). They interact with host proteins such as the ribosomal proteins and are implicated in EBV-related transformation. Produced using Servier Medical Art (Servier 2019), licensed under a CC BY 3.0

2.10.1 Epstein-Barr virus infection and latency

EBV first infects the epithelial cells in the upper respiratory tract, mainly in the oropharynx and tonsils (Sixbey 1984), which it reaches using shared saliva as an infection medium, spread by kissing, shared utensils, bottles, etc. The entry receptor in epithelial cells is integrin αvβ6, to which EBV surface glycoprotein gH and gL bind, allowing the virus to fuse with the cell membrane with the aid of gB and release its contents inside the cell. In lymphocytes, EBV is endocytosed instead (Möhl 2016). During acute infection, which clinically manifests as infectious mononucleosis, EBV spreads to the B-lymphocytes throughout the body and after the disease has passed, remains latent in the resting memory B-lymphocytes for the lifetime of the individual (Henle et al. 1968**,** Lieberman, 2013).

EBV has also been detected in the saliva and oral epithelia of otherwise healthy persons (Oosterveer et al. 1993, Mao & Smith 1993)**,** which signifies its likely persistence and/or occasional replication in the epithelia as well. EBV is thought to reach the oral epithelium via plasma cells that enter the lytic viral cycle (Young & Rickinson 2004). EBV is carried over to the epithelial site by B-lymphocytes in which EBV then replicates lytically and infects the epithelium from a basal direction. Epithelial infection is most apparent in immunocompromized patients, where EBV causes bilateral hairy leukoplakias of the tongue (Greenspan et al. 1984).

EBV has three forms of latency in its host cell. Type III latency, the so-called “growth pattern,” is utilized first during EBV infection in B-lymphocytes where a majority of EBV genes are expressed and the virus spreads rapidly as it drives its host cell to proliferate. This aggressive pattern, however, leads to immune response and host cell death via cytotoxic T lymphocytes. Therefore, EBV switches its gene expression into the less immunogenic type II – the “default” pattern, in which LMP-1, EBERs and EBNA1 are expressed. This pattern leads the infected cell to enter the resting phase and there EBV can activate its stealthy latency patterns I (EBNA1 and EBV-miRNAs) and 0 (EBV-miRNAs only), expressed to persist indefinitely in the human host (Dheekollu et al. 2016). The type I latency pattern is used in proliferative B-lymphocytes such as those of Burkitt's lymphoma whereas the most common latency type in epithelial – such as gastric and nasopharyngeal carcinoma cells – is type II.

2.10.2 EBV in epithelial cells

EBV was discovered over three decades ago in oral hairy leukoplakia lesions (Greenspan et al. 1985). Despite this, the epithelial life cycle of EBV is poorly characterized. Infected B-lymphocytes transport the virus to the apical surface of the mucosal epithelium. There, EBV enters the basal cell after release from the B-lymphocytes, likely mediated by endocytosis (Nanbo et al. 2016). EBV then establishes latency and spreads as the infected cells start to differentiate (Temple et al. 2014). Recent *in vitro* studies

performed by laser-capture microdissection and high-sensitivity qRT-PCR have demonstrated EBER transcripts without lytic gene expression in basal epithelial cells, suggesting EBV latency (Nawandar et al. 2015). EBV genome replication and immediate-early gene expression are triggered in the suprabasal layer during cell differentiation (Nawandar et al. 2015). In addition, epithelial cell-cell contacts are needed for persistent EBV infection in the epithelium *in vitro,* linking EBV infections to differentiating epithelia (Chang et al. 1999). However, although latent EBV infections in putative epithelial cell reservoirs have not been conclusively proven in nonmalignant epithelia *in vivo* (Hutt-Fletcher 2016), the expression of LMP-1 has recently been linked to the lytic reactivation of EBV in cultured epithelial cells (Nawandar et al. 2017). Lastly, EBV has also been detected in the normal tongue epithelium (Walling et al. 2001, Frangou et al. 2005), normal base-of-tongue (Jiang et al. 2015) and tonsillar and adenoid epithelia (Endo et al. 2001), thus it is clear that the effects of EBV on head and neck epithelia are only beginning to be characterized.

2.10.3 EBV and carcinogenesis

EBV is one of the most intensively studied oncogenic viruses because its ubiquity causes a high disease burden to society. In 2014 Khan and Hashim calculated that approximately 143 000 people died that year globally due to EBV-related malignancies (1.8% of global cancer deaths in 2010) and 92% of these deaths were attributed to epithelial, nasopharyngeal and gastric cancers (over 132 000 cases), whereas EBV-related lymphomas were less prevalent (Khan & Hashim 2014). However, because of its ubiquity and the geographical distribution of NPCs (i.e. more common in Asian countries), EBV infection alone is thought not to suffice for development of carcinoma, and additional cofactors are likely needed (Huang et al. 2013).

In order to replicate and persist, EBV encodes several proteins that lead to cell cycle dysregulation, apoptosis avoidance and stimulation of growth receptor pathways (Kang & Kieff 2015) (summarized in **Table 4)**. LMP-1 is considered the main oncoprotein of EBV and is expressed in the majority of EBV-linked malignancies (Chen et al. 2015). LMP-1 functions to prevent p53-mediated apoptotic cell death, being itself upregulated by p53 expression (Wang et al. 2017). LMPs 1 and 2A lead to calcium-mediated signaling via PKC and activate NF-κB, for example. These properties may immortalize the infected cell and compromise DNA repair (Wang et al. 2017). EBNA (Epstein-Barr virus nuclear antigen) proteins enable latency and EBV DNA replication (Ambinder et al. 1991). They also inhibit p53, increasing the malignant potential of the infected cell (Sivachandran et al. 2008).

EBERs (EBV-encoded small RNAs) are small RNAs, present in all EBV infected cells. They affect a multitude of host cell proteins, with the intention of enabling EBV persistence while preventing apoptosis and detection by the immune system (Ahmed et al. 2014). BARTs (BamHI-A rightward

transcripts) are a family of microRNAs that also have a variety of roles within the cell that are only recently being characterized. BHRF1 (BamHI fragment H rightward open reading frame 1) encodes a Bcl-2 protein analog that localizes at the mitochondrion to block the intrinsic apoptotic pathway (Dawson et al. 1995, Khanim et al. 1997).

Each EBV latency pattern leads to different outcomes. For reasons not well-known, epithelial malignancies such as nasopharyngeal carcinomas display EBV LAT II pattern–type expression in which LMPs confer apoptosis avoidance and cell immortalization functions and EBNAs cause cell cycle entry. It has been shown *in vitro*, that EBV causes normal oral keratinocytes grown in organotypic cultures to invade the underlying collagen network (Nawandar et al. 2015). LMP-1, LMP-2A and EBNA2 genes seem to be the most important oncogenes in the development of nasopharyngeal carcinoma (Liu et al. 2006). EBNAs are proteins that affect gene expression in the nucleus (Kang and Kieff 2015). EBNA2 activates the expression of c-myc and it has been shown *in vitro* that reducing EBNA2 and LMP1 expression with Cidofovir seems to increase the radiosensitivity of epithelial cells through the activation of caspases 9 and 3 and downregulation of Bcl-2 (Abdulkarim et al. 2003). In addition, EBV-encoded miRNAs are able to modulate resistance to apoptosis and immune evasion in latency (reviewed by Flór & Blom, 2016).

Table 4: EBV main oncogenes related to HNSCC

Gene (molecule)	Timing / Function	Potential mechanism	References
LMP-1 (protein)	Latency Antiapoptotic Enables immortality Increases mutation rate DNA repair suppression Radiation resistance	Activates NFκB by CD40 mimicry, leading to hTERT and Bcl-2 activation. Chromosomal instability	Chen 2015 Liu 2006
LMP-2A (protein)	Latency Antiapoptotic Immune evasion	Activates PI3K-Akt and NFκB pathways, leading to p53 degradation and c-myc oncogene upregulation. Mdm-2 activation Suppression of IL-6	Stewart 2004
EBNA1 (protein)	Latency Cell cycle entry Antiapoptotic Regulates gene expression DNA repair inhibition	P53 degradation via interaction with PML proteins.	Sivachandran 2008
EBNA2 (protein)	Latency Cell cycle entry Antiapoptotic Regulates gene expression	Activates the expression of c-myc	Abdulkarim 2003
EBERs (small noncoding RNA)	Antiapoptotic Cancer-related inflammation	Disables innate immunity-related signaling such as interferons via TLR3. NFκB activation	Flór and Blom, 2016 Tsao 2015
BARTs (micro-RNAs)	Antiapoptotic 22 different miRNAs	Present in exosomes. p53 pathway inhibition Bcl-2 pathway alterations	Flór and Blom, 2016 Kang & Kieff 2015
BHRF1 (micro-RNAs and protein)	Antiapoptotic 3 different miRNAs	Bcl-2 homologue, prevents cytochrome C release.	Dawson 1995

2.10.4 EBV and radioresponse

Irradiation causes oxidative stress via ROS that is known to lead to ATM-DNA repair pathway activation and p53 activation. p53 activation is said to be necessary for EBV reactivation *in vitro,* suggesting a link between EBV reactivation and radiation responses (Huang et al. 2013). The same group also observed EBV reactivation after H_2O_2 treatment of NPC, linking EBV lytic activation with ROS increase and activation of DNA damage-signaling pathways. EBV reactivation causes increased expression of EBV oncogenes such as BARF1 and genomic instability, contributing to carcinogenesis (Hu et al. 2017). EBNA1, LMP1 and EBERs are associated with oxidative stress, and it has even been proposed that EBV-positive tumors are ROS-driven (Hu et al. 2017). In their recent review, Hu et al. (2017) emphasized that cancer stem cells (CSC), the most radiation-resistant carcinoma cell type, have lower levels of ROS after irradiation and this may be caused by enhanced NFκB and HIF-1 (hypoxia-induced factor) expression in EBV-positive cells. These factors contribute to the possible inhibition of radiation-induced cell death by LMP-1 that may confer CSC-like characteristics, potentially enhanced by the expression of antiapoptotic EBERs, BHRF, EBNA1 and BARTs (**Table 4,** Yang et al. 2013).

2.10.5 EBV in head and neck cancer

EBV is a leading cause of NPC (Guidry et al. 2017). EBV DNA is also found circulating in the bloodstream of NPC patients (Leung et al. 2003). In addition, EBV DNA has been detected in 0% to 100% of oropharyngeal carcinomas and its epidemiology in other HNSCC is unclear at best (Guidry et al. 2017). Therefore, the role of EBV in the carcinogenesis of these non-nasopharyngeal tumors is unclear, because carcinoma tissue always contains B-lymphocytes which may harbor EBV DNA and lead to confusing results. EBV has been detected in oral premalignant disorders in up to 77.8% of cases (Shamaa et al. 2008). Horiuchi et al. (1995) detected EBV in 5.3% of leukoplakias and 40% of carcinoma *in situ* lesions of the oral cavity with PCR, but only in single premalignant cases with *in situ* hybridization (ISH). Moreover, 27% of oral cancers were positive for EBV-*in situ* hybridization in their study. EBER expression seems to be rare in oral cavity carcinomas, although LMP-1 has been detected more frequently, especially in carcinomas of the lateral border of the tongue. Although LMP-1 is not reliable as a diagnostic marker for EBV, this has cautiously been reported to associate with oral tongue cancer (Gonzalez-Moles et al. 2002).

2.11 Viral coinfections

2.11.1 HSV-1 coinfecting tissues with HPV

HSV-1 and HPV are ubiquitous in the human head and neck region. Their coinfections are not uncommon, although fairly scarce in the younger Finnish population (Mäki et al. 2015). These coinfections have been reported in dental abscesses, commonly encountered by dentists worldwide (Ferreira et al. 2011). Thus, it is plausible that these pathogens as coinfections might affect the development of HNSCC and/or even the treatment responses. So far, the presence of HPVs, HPV16 in particular, is well-established as the etiologic agent of a subgroup of HNSCCs. However, HPV16 is also the most prevalent HPV genotype in normal oral mucosa. Hyland and coworkers (2007) detected HSV-1 in up to 84.6% of patients before oral surgery. In addition, HNSCC patients undergoing radiation treatment develop oral ulcerative mucositis. HSV-1 has been detected in up to 48.2% of these patients (Nicolatou-Galitis et al. 2006). The ulcers also healed after valacyclovir antiviral medication and recurred after quitting the antivirals, proving the presence of HSV-1 as a causative factor in many oral mucositis cases (Nicolatou-Galitis et al. 2006). Therefore, as HPV-positive HNSCCs are often treated with radiation therapies, coinfection with HSV-1 may be a common occurrence, reported to be present in 52% of OSCCs (Jalouli et al. 2015). Meurman (2010) reviewed data from 1990 to 2009 and concluded that seropositivity to HSV-1 was associated with oral cancer. It is known that as many as 62% of tonsillar carcinomas harbor high-risk HPV (Herberhold et al. 2017). Interestingly, up to 82% of tonsillar carcinoma patients have been found to be seropositive to HSV-1 (Starr et al. 2001) and the authors

observed that HSV-1 seropositivity was associated with a two-fold increase in tonsillar carcinoma risk among individuals with HR-HPV DNA in either tumor tissue or oral scraping samples, but not in HPV DNA-negative patients. Mechanistically, HSV-1 increases genomic instability (Deng et al. 2014), as do HR-HPVs, which could augment these effects in coinfected tissues. To date, HSV-1 is thought to kill all infected keratinocytes. Despite these findings, abortive HSV infections could still lead to cellular damage (Barreca & O'Hare 2006). In addition, chronic replication in tissues adds to the ROS burden of cells, which can function as a cofactor in carcinogenesis (Williams et al. 2011). Exosomal HSV-1 signaling also influences cells next to those infected in ways that are only starting to be discovered (Kalamvoki & Deschamps 2016), therefore HSV-1 must be studied further as a cofactor in HPV-mediated carcinogenesis.

2.11.2 EBV coinfecting tissues with HPV

In vitro, EBV has been proven to enter HPV-infected cells, where HPV increases the capacity of the cell to sustain the EBV genome in the infected cell (Makielski et al. 2016). Interestingly, HPV has been detected in oral hairy leukoplakia caused by EBV(reviewed by Greenspan et al. 2016). HPV + EBV coinfections proved the most common coinfection type in all corners of the world in a recent study, with a 21% overall prevalence in OSCC (Jalouli et al. 2012). EBV has also been found coinfecting HPV-infected cervical cancer cells, where its presence was a significant predictor of the integration of HPV types 16 and 18 (Kahla et al., 2012). Moreover, recent studies have suggested coinfection by EBV and HPV in tonsillar and base-of-tongue (oropharyngeal) carcinomas, where the target cells of HPV and EBV are also present in greater volumes. This presents a suitable environment for the viruses to spread into the same cells (Jalouli et al. 2012, Polz-Gruszka et al. 2015).

Due to the prevalence of EBV and HPV in certain carcinomas, the occurrence of coinfections is plausible (Shi et al. 2016). Truly, EBV/HPV coinfections have been detected in oral carcinomas (Jiang et al. 2015), cervical precursor lesions and carcinomas (Lattario et al. 2008, Khenchouche et al. 2013), breast cancers (Glenn et al. 2012), middle ear squamous cell carcinomas (Surono et al. 2018), nasopharyngeal carcinomas (Rassekh et al. 1998, Punwaney et al. 1999), and esophageal carcinomas (Kunzmann et al. 2017). Curiously, EBV increased the likelihood of integrated HPV16 genomes seven-fold in cervical carcinomas (Szostek et al. 2009). EBV was also present in over 10% of esophageal carcinoma cases in combination with HPV16 or HSV-1, increasing the pathological grade (Zhang et al. 2011). Jiang (2015) showed that EBV/HPV coinfections were detectable in a subset of oral carcinomas. Taken together, these results show that EBV and HPV are found coinfecting HNSCCs, where their coinfection could affect the carcinoma cells. However, these associations require further study, as the prevalence of coinfections is low and the ratings vary greatly between countries.

2.11.3 HSV-1 coinfecting tissues with EBV

HSV/EBV coinfections have been detected in up to 17% of periodontal pockets in patients with periodontitis (Kazi et al. 2017). Reports on the effects of multiple herpesvirus infections in HSNCCs are scanty, although coinfections with multiple herpesviruses are prevalent in the general otherwise healthy populace, in particular those from lower socioeconomic groups (Delaney et al. 2015). No coinfections for EBV/HSV-1 in Polish HNSCCs were detected although HPV/EBV coinfections were fairly common (15%) (Polz et al. 2011, Polz-Gruszka et al. 2015). Mixed results have been found when attempting to analyze HPV or herpesviral prevalence ratings in different countries: for example, OSCC patients from the UK displayed significantly higher numbers of HSV-1 (55%) and EBV (80%) than patients from Yemen (HSV-1 10% and EBV 20%). Furthermore, 11% of 155 OSCC patients from industrialized countries displayed HSV-1/EBV coinfections (Jalouli et al. 2012).

Mechanistically HSV-1 and EBV coinfections are possible in HNSCCs: EBV might facilitate the replication of HSV-1 via tegument protein BNRF1 (Lu et al. 2016). On the other hand, HSV-1 cellular entry and Us3 protein activates EBV via the PKA-CREB pathway, and thus HSV-1 may facilitate EBV reactivation (Wu et al. 2012). Therefore HSV-1 and EBV have been detected in HNSCCs as coinfections, although the results of coinfections between these viruses are mostly unknown.

3. AIMS OF THE STUDY

The main aim of this work was to clarify if HSV-1 infection alters the radiation response of HPV-positive cells *in vitro* and, if so, whether the infection of HPV-positive or -negative HNSCCs by herpesviruses HSV-1 or EBV influences the survival of HNSCC patients. Because these infections concern differentiating epithelia, the effect of differentiation-related signaling on HPV gene expression was studied first.

The specific aims of the study were to:

1) Identify HPV16 E6 and E2 gene expression in head and neck keratinocytes or carcinoma cells, induced to differentiate.

2) Determine the effects of HSV-1 on oral keratinocytes with and without exposure to radiation.

3) Determine the radioresponse on head and neck cancer cells infected with HPV16 alone or with a coinfection of HSV-1.

4) Estimate the prevalence and possible prognostic influence of EBV in non-nasopharyngeal HNSCCs with special reference to the use of radiation therapy in their treatment.

4. MATERIALS AND METHODS

4.1 Cell lines (I-III)

Table 5 presents the cell lines used. All cell lines used had undergone routine testing for mycoplasma contamination before experimentation. Same-passage cells were used for replicate studies where possible.

Cell line	Origin	Cell type	Genome	Culture medium	Supplements	Reference	Acquired from
HMK	Human gingiva	Spontaneously immortalized, nontumori-genic	Hypo-tetraploid	KSFM 0.09mM calcium	BPE 20-30 µg/ml and EGF 0.2ng/l	Mäkelä 1999	Original author
IHGK	Human third molar gingiva	Immortalized with HPV16 E6/E7, nontumori-genic	Aneuploid	KSFM 0.045mM calcium	BPE 20-30 µg/ml and EGF 0.2ng/l	Oda 1996	Original author via professor Salo
UD-SCC-2	Hypopha-ryngeal SCC	Well-differentiated squamous cell carcinoma, tumorigenic	Aneuploid, 600 copies of HPV16 integrated and episomal	DMEM	10% FCS	Balló 1999	Prof. Kunz-Schughart, University of Regens-burg, Germany
HaCat	Human skin, melanoma periphery	Differentiates normally, nontumori-genic	P53 mutated, hypo-tetraploid	DMEM	10% FCS	Boukamp 1988	CLS Cell Lines Service GmbH, Eppel-heimer, Germany
Vero	African green monkey kidney	Epithelial, nontumori-genic	Diploid	RPMI	5% FCS and gentamycin	Yasumura 1963	ATCC (Bethes da, MD, USA)

4.2 Exposure to calcium (I)

After passaging in their own respective culture medium until ~70% confluent, the cells were plated and allowed to attach for 48 hours until the medium containing 0mM (i.e. no calcium), 0.045mM, 0.09mM, 1.8mM, 3mM, 4mM, 5mM or 6mM was added. The concentrations were achieved by adding CaCl solution to the original growth medium without calcium (KSFM, Keratinocyte serum-free medium, Gibco, Grand Island, NY, USA).

4.3 Preparation of growth curves (I)

After calcium exposure, the cells were cultured for 3, 6 and 9 days and trypsinized free at the time points. D-MEM with 10% FCS was added to prevent further trypsin activity. The suspension was then centrifuged in 10 000g for 5 minutes, resuspended and calculated using Bürker hemocytometers. The cell number was calculated and averaged from two triplicate high-power fields. These were then used to prepare the growth curves seen in (I).

4.4 HSV-1 infections and quantification (II,III)

HSV infections were performed by adding the infection medium to the culture wells for 1 hour. After this attachment period, the cells were washed with PBS and the medium was replaced. Control cells were mock-infected with only the medium. HSV-1 quantification was performed using plaque titration assays on confluent B-Vero cell monolayers as per standard protocol (Nygårdas et al. 2011)**.** The plates were incubated for 1 hour with the dilution series of medium samples. After 3 days the plates were fixed with 4% methanol at 4˚C, stained using crystal violet and read using an inverted microscope. To detect HSV-1 replication in HMK cells, immunostaining for glycoprotein gC was also used as described previously (Ziegler et al. 1988) (II).

4.5 Viability assays (II,III)

Quantification of cell viability was done using the CellTiter-Glo ATP assay (Promega, Madison, Wisconsin, USA). This method analyses the presence of cellular ATP using a luminescence reaction with a firefly luciferin and luciferase enzyme. In the experiments presented herein, 24-well plates instead of 96-well plates were used due to their larger cell content per well (depicted in **Figure 14**). In addition to the samples, medium only-controls were included as quadruplicates.

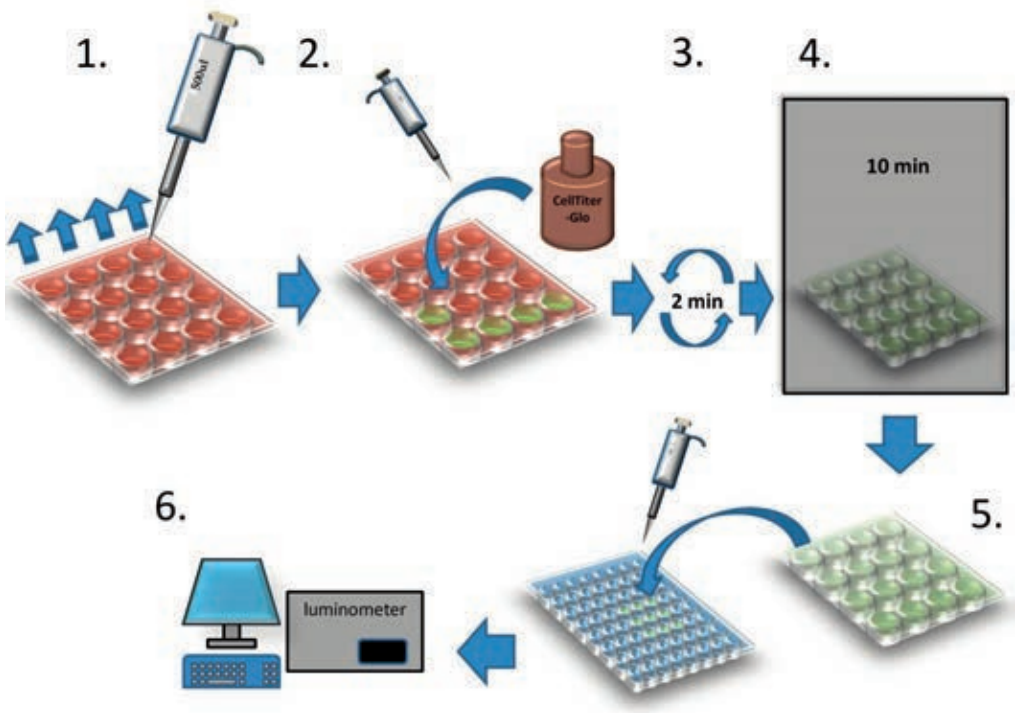


Figure 14: CellTiter-Glo assay for determining cell viability (original publications II,III). 1. Half of growth medium is removed. **2.**Removed medium is replaced with CellTiter-Glo reagent. **3.**The plates are shaken in an orbital shaker for two minutes. **4.**Then the luminescent reaction continues in the dark. **5.**After 10 minutes, 200ul from each well is transferred into an 96-well plate in a minimum of duplicates. **6.**The result is then read using a luminometer.

4.6 Quantitative reverse-transcriptase PCR (I-III)

The response of HPV16 E2 and E6 to extracellular calcium in IHGK and UD-SCC-2 cells (I) was analyzed using TaqMan. NFkB1, Bcl-2 and caspases 3, 8 and 9 were similarly studied in HSV-1 infected HMK and UD-SCC-2 cells exposed to 2 Gy radiation (II-III). Total RNA was extracted using the TRIZOL reagent (Life Technologies, Invitrogen Corporation, Carlsbad, CA, USA). The First-strand cDNA Synthesis Kit was then used (Applied Biosystems, Foster City, CA, US) with total RNA as a template. Real-time RT-PCR (TaqMan) reactions were performed using TaqMan Universal PCR MasterMix and TaqMan® Gene Expression assays (Applied Biosystems, described in detail in their respective original publications I-III). The custom primers and probes for HPV16 (original publications I and III) were constructed by Applied Biosystems custom TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA, USA). The custom primers and probes for HPV-1 E6 and E2 cDNAs were constructed according to Peitsaro et al. (2002), and Kari et al. (2007, Applied Biosystems, III) for E7. In

addition to HSV plaque titration assays, to further determine viral replication, the Rotorgene qRT-PCR method was used to quantify the cellular HSV-1 ICP27 and VP16 mRNA expression, described in detail in original publication II and by Broberg et al. (2003) and Nygårdas et al (2011). A list of primers and probes used in the current thesis is given in **Table 6.**

Table 6: Primers and probes used in the PCR analyses.

The method and original publication the primers/probes were used in are shown in parentheses.

E6 (TaqMan, I,III) probe	CAGGAGCGACCCAGAAAGTTACCACAGTT
E6 forward primer	GAGAACTGCAATGTTTCAGGACC
E6 reverse primer	TGTATAGTTGTTTGCAGCTCTGTGC
E2 (TaqMan, I) probe	CACCCCGCCGCGACCCATA
E2 forward primer	AACGAAGTATCCTCTCCTGAAATTATTAG
E2 reverse primer	CCAAGGCGACGGCTTTG
E7 (TaqMan, III) probe	CCAGCTGGACAAGCAGAACCGGAC
E7 forward primer	CAGCTCAGAGGAGGAGGATGAA
E7 reverse primer	CACACTTGCAACAAAAGGTTACAATATT
EBV (Luminex assay IV) probe	GGAAACCAGGGAGGCAAATCTA
EBV Forward primer	GACTGTGTGCAGCTTTGACGAT
EBV Reverse primer	CAGCCCCTTCCACCATAGGT
HSV-1 (Luminex assay, IV) probe	CAGTTATCCTTAAGGTCTCT
HSV-1 Forward primer	ATCACGGTAGCCCGGCCGTGTGACA
HSV-1 Reverse primer	CATACCGGAACGCACCACACAA

4.7 Luminex-multiplex assay for EBV and HSV-1 (IV)

The presence of both high- and low-risk HPV and HSV-1 in the tumors had already been reported earlier by Rautava (2012). EBV DNA was detected, similarly to HSV-1, using a Luminex xMAP-based method (Paaso et al. 2011, Original publication IV).

4.6 Immunocytochemistry (III)

The cells were scraped free from the plates using a sterile technique in PBS. The suspension was gently vortexed and diluted to achieve an estimated 20 000 cells / 250ul PBS which was drawn gently through a sterile .23G needle to avoid cell clustering and then applied to glass silanized coverslips. After drying, fixation was performed using 10% formalin for 10 minutes. The fixed slides were washed twice with PBS and stored at -20C. The antibody dilutions were verified beforehand using a test series of control UD-SCC-2 cells, fixed and stained using the same protocol. The specific staining conditions are presented in original publications II-IV and **Table 7**. The staining positivity percentage was calculated manually.

Table 7: **Antibodies and staining protocols for immunohisto/-cytochemical stains.**

Mc=Microwave 5min in citrate buffer, Mt=Microwave 5min in TrisEDTA (pH 9), H_2O_2 = hydrogen peroxide 2.5%, SB = serum blocking.

Antibody	Dilution / Clonality Source	Pretreatments	Visualisation	Original publication
Casp 3 (cleaved)	1:200 Polyclonal Biocare Medical (CA/USA)	Mc, H_2O_2	Dako LSAB kit	III
Ki67	1:150 Monoclonal Dako (Glostrup, Denmark)	Mc, H_2O_2	Dako LSAB kit	III
Bcl-2	1:100 Monoclonal Dako	Mt, H_2O_2	Dako LSAB kit	III

p16INK4a	Ready to use Monoclonal (Ventana, Tucson, AZ, USA)	Mt, H_2O_2	CINtec Kit	III
Cyclin E	1:20 Monoclonal Leica Novocastra (Wezlar,Germany)	Mc, H_2O_2	Dako LSAB kit	III
MDM-2	1:100 Monoclonal Leica Novocastra	Mc, Sb	Dako LSAB kit	III
HSV-1	1:100 Polyclonal Biogenex (Fremont, CA,USA)	Mc, H_2O_2	Dako LSAB kit	III
HSV-1 gC	1:1000 Polyclonal Dako	H_2O_2	HRPO-labelled antibodies, IPS (Ziegler 1988).	II

4.7 Immunohistochemistry (IV)

The formalin fixed paraffin-embedded (FFPE) samples were stained using routine laboratory immunohistochemical staining protocols in an automated tissue stainer (Dako TekMate®, Dako, Glostrup, Denmark) with a compatible LMP-1 staining kit (Dako). Counterstaining was performed using Mayer's Hematoxylin. The samples were analyzed visually. Particular attention was paid to the localization of the staining and its pattern.

4.8. Clinical data of cancer patients from the 1988-2009 cohort studied in (IV)

For EBER-ISH and LMP-1 staining, the FFPE blocks were acquired from the department of Pathology, Turku University Hospital, and patient survival data were collected from the Turku University Hospital records (Rautava et al. 2012).

4.9 In situ hybridization for detection of EBV infection (IV)

The presence of EBV RNA transcripts EBER-1 and EBER2 was examined with the "Epstein-Barr Virus (EBER) PNA Probe/Fluorescein and PNA-ISH Detection Kit" (Dako, Glostrup, Denmark) according to the manufacturer's instructions on the FFPE cancer samples (IV). Samples were counterstained with Eosin.

4.10 Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows with SPSS advanced statistical package, Version 19, Armonk, NY: IBM Corp. Released 2010). Levene's T-testing was utilized to detect intergroup differences (I). Univariate general linear model (GLM) was employed to detect the effects of calcium on gene expression (I). Mann-Whitney U-testing was used to analyze for differences in viability and gene expression ratings (II, III). Univariate GLM was used to determine whether irradiation had a general effect on HSV-1 VP16 expression (II, III). The X^2-test was used for categorical variables with the likelihood ratio (LR) or Fisher's exact test (IV). Odds ratios (OR) were calculated using the exact method and with 95% confidence intervals. Disease-specific survival (DSS) was analyzed using univariate survival analysis for outcome measures, using the Kaplan-Meier method and Mantel-Cox log-rank statistics. Analyses were performed two-sided. P-values of <0.05 were considered significant in all studies.

4.11 Ethical considerations

The use of human samples was approved by the Ethical Committee of the Hospital District of Varsinais-Suomi, Finland (4/2009) (IV).

5. RESULTS AND DISCUSSION

5.1. Proliferation and HPV gene expression in head and neck cell lines exposed to increasing concentrations of extracellular calcium (I).

As the viral life cycle of HPVs is linked to the epithelial differentiation of the target cell, the effects of HPV16 E6 and E2 were first studied in order to clarify the oncogene expression changes according to the differentiation-related signaling of keratinocytes. HPV16 E6 and E7-immortalized IHGK cells and HPV16-positive UD-SCC-2 cells were cultured and exposed to increasing concentrations of extracellular calcium of the medium as outlined in the Methods (4.1-4.2). It was hypothesized that as the epithelial differentiation proceeded, the HPV early gene expression would be downregulated concomitantly with upregulation of late gene expression, as described previously (Yasumoto 1989). Interestingly, as the calcium concentration rose, IHGK cells gained a growth advantage, proliferating well in up to 1.8mM calcium, considerably over the so-called "calcium switch" where keratinocytes start to differentiate when exposed to over 1mM calcium (Hennings et al. 1980). This contrasted with the HPV-negative oral keratinocytes (HMK) that ceased to proliferate at 1.8mM calcium and died, likely via senescence, by days 6 and 3 of the 9-day experiment at 3mM and 4mM of calcium. Although senescence was not analyzed *per se*, the cells begun detaching from the culture flasks displaying polygonal, terminal differentiation-related morphology. UD-SCC-2 cells reacted similarly: proliferation slowed after 1.8mM calcium and halted after 3mM. Higher concentrations resulted in cell death. E6 and E2 were similarly upregulated in high-calcium concentrations (**Figure 15**, E2 not shown). Preliminary studies using DMEM and KSFM from 0mM to 6mM calcium also showed an almost linear upregulation in E6 expression from 2mM concentration upward (data not shown). This work was the first to describe the increased expression of E6 and E2 due to calcium. This likely resulted in resistance to differentiation-related senescence and enables proliferation in the presence of differentiation-inducing signals (I). Confluence may be sufficient to trigger the differentiation program in keratinocytes (Kolly et al. 2005). In the present study, most of the cells reached confluence by the end of the study at day 9. Despite this, involucrin expression, a marker for terminal differentiation, was upregulated only in cells grown in a high-calcium medium. It has been reported that transforming HPV16 infection utilizes E6 in order to suppress Notch1 via p53 inhibition. This enables keratinocyte proliferation at high cell densities. It appeared that E6 plays a dominant role in nullifying the effects of cell-cell contact-related signaling and its inducing effect on keratinocyte differentiation. Therefore, elevated E6 is likely to play a significant part in resistance to differentiation and, by extension, the progression of high-risk HPV lesions toward malignant transformation.

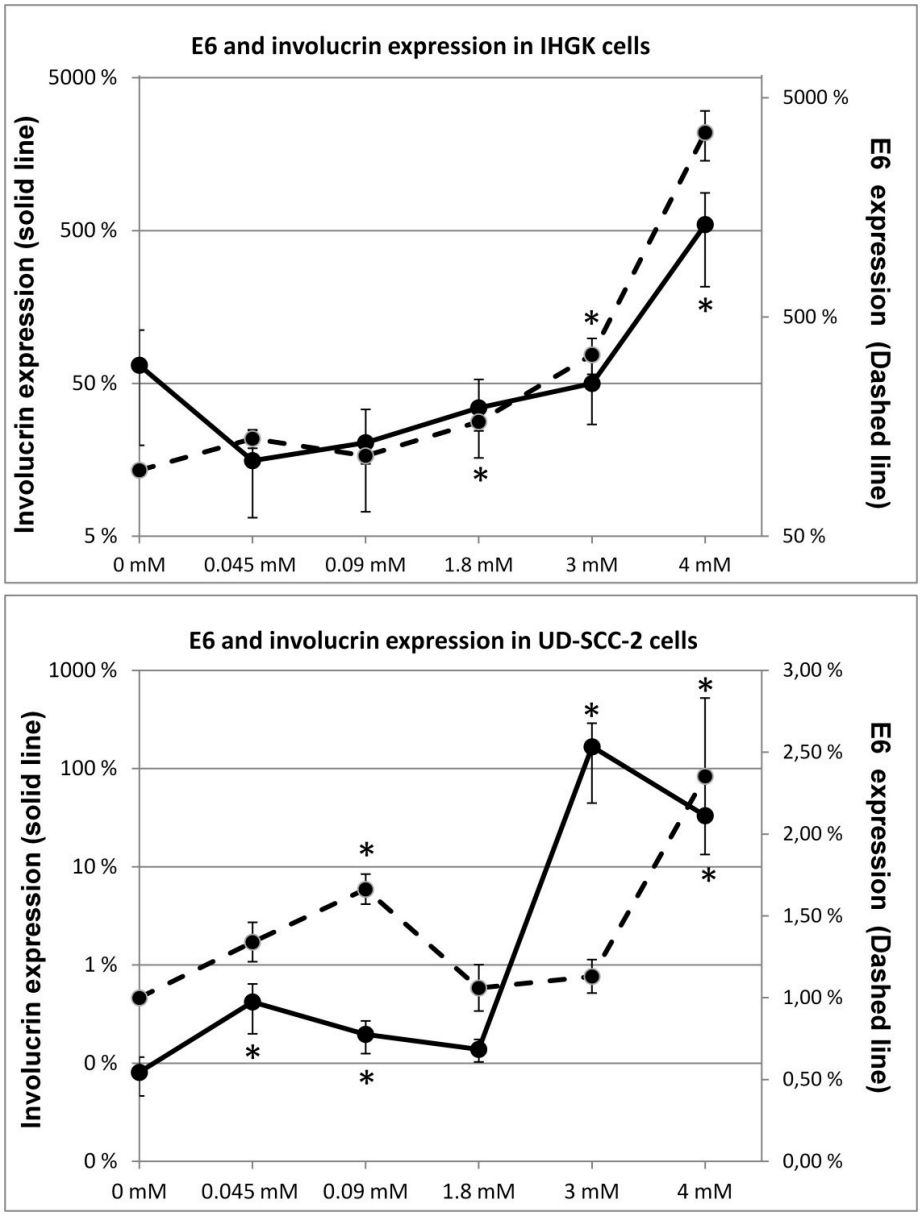
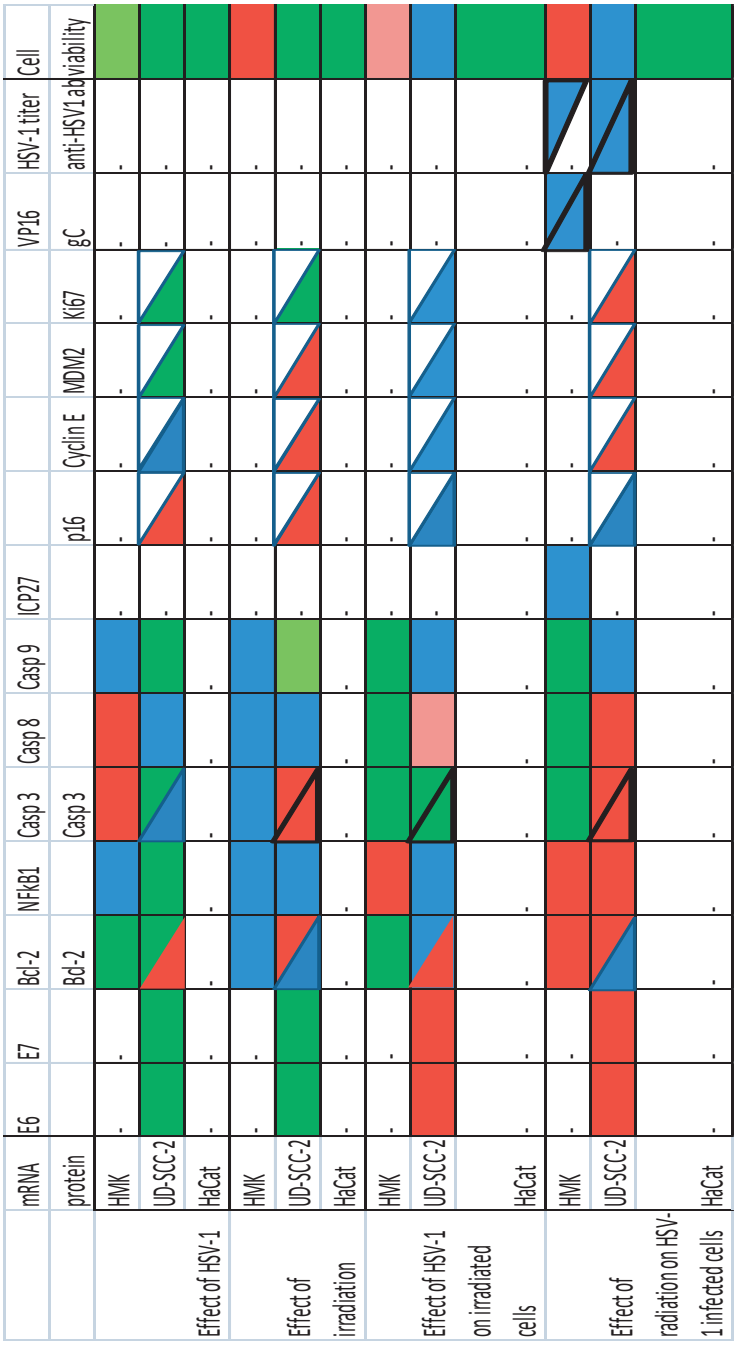


Figure 15: HPV16 E6 and involucrin expression in different calcium concentrations (l). The expression levels of E6 or involucrin, a differentiation marker, in different medium calcium concentrations for monolayer-cultured UD-SCC-2 cells and IHGK cells at day 9 after exposure to calcium are presented. * = $p<0.05$ compared to result without calcium. Dashed line = E6 expression. Solid line = Involucrin expression.

5.2 Effects of HSV-1 and irradiation on gingival keratinocytes

HMK cells were infected at a low MOI (0.0001-0.00001) corresponding to 20 to 2 pfu at day 0 per culture plate in order to introduce a progressive HSV-1 infection to the cultures that would enable detection of the effects of a low-grade HSV-1 infection (i.e. cells still viable in 6 days whereas a high MOI would likely destroy the cells) (II). In most published experiments concerning HSV-1, the viral stock is used at several-fold higher concentrations than used in our studies. This introduces HSV-related exosomes to the cells that could alter their responses (Kalamwoki & Deschamps 2016). Therefore, one advantage of our studies is that due to the use of low MOI, smaller amounts of soluble contaminants from the virus stock are added to the cell culture. The infection progressed during the experiment and practically all the cells infected by the higher MOI were infected by 144 hours of analysis, as was proven by the HSV-1 gC staining (II). The lower MOI resulted in around 50% positively staining cells. However, because gC is a late gene, the total number of cells infected was likely higher, as more recently infected cells would express only early genes. Irradiated HMK cells displayed transiently elevated NFkappaB1 and Bcl-2 mRNA expression, consistent with the literature, but most expression level alterations faded during 6 days in culture. Caspase 8 is blocked by HSV-1 ribonucleotide reductase but functions actively in determining the apoptotic or necroptotic death of the infected cell (Guo et al. 2015). Caspase 9 can be blocked by the PI3K-Akt pathway, activated by HSV-1 (Liu & Cohen 2015). In the presence of HSV-1 infection 6 days after irradiation, HSV-1 combined with irradiation significantly lowered the expression of caspases 3, 8 and 9 and coincided with a relative upregulation of Bcl-2 and NFkB1 compared to nonirradiated HSV-1 infected HMK cells (compare these effects with the pathway seen in **Figure 6**). Simultaneously, cell viability was significantly higher than in nonirradiated cultures. Interestingly, downregulation in caspases was only seen in irradiated HSV-1 infected cultures. Therefore HSV-1 might confer radioresistance to infected human gingiva cells and expose them to further DNA damage after irradiation, should the cell resist apoptosis due to infection. **Table 8** compares the means of the results from HMK, UD-SCC-2 and HaCat cells (II,III) with the results from their respective untreated control cells.

Table 8: Expression of mRNA (qRT-PCR) and protein (IHC) in oral keratinocytes, skin cells or HPV16-positive HNSCC cells caused by HSV-1 infection and/or irradiation (II and III). Green signifies downregulation/decrease in level (immunohistochemical detection of >5% change from untreated). Red signifies upregulation or increase. Blue represents no change. White represents unavailable data. Light red or green signifies up or downregulation trends that are not statistically significant but may be of interest. The upper row of triangles contain mRNA and the lower row (colored triangles) contain the immunocytochemical data. If only mRNA expression data is available, a solid square is used.



HSV-1 seemingly functioned to decrease apoptotic gene expression in these cells after irradiation. At the same time, after HSV-1 infection, nonirradiated cells displayed lower Bcl-2 and higher caspase 3 and 8 expression. NFkappaB1 and caspase 9 were not altered. The Bcl-2 oncogene has been previously shown to be rescued from downregulation in HSV-1-infected cells exposed to the cytotoxic agent cisplatin, putatively via ICP27 and ICP4 (Zachos et al. 2001). Here it was shown that HSV-1 infection resulted in higher Bcl-2 levels in irradiated cells after 144 hours, which might increase cell survival. In contrast, uninfected cells displayed non-significant changes due to irradiation. These cells were genetically abnormal **(Table 5)** and so may have alterations in their responses to viruses: further experimentation was therefore performed on HMK cells after determining that some viability increases can also be noted after irradiation in the absence of HSV. UV-inactivated HSV-1 virus stock was used to infect the cells. These cells did not display lytic HSV-1 effects but the highest viability ratings in the series were observed. In addition, it seemed that these inactive viral particles likely enabled the cells to increase their radioresistance (II). Moreover, inactive viruses may trigger the keratinocytes' innate immunity responses, leading to the NFkappaB1 upregulation that is known to increase resistance to apoptosis and radiation (Veeraraghavan et al. 2011). In the present study, NFkappaB1 was upregulated by HSV-1 infection and/or irradiation, although 144 hours after irradiation, the effect was still present only in cells irradiated and infected with 0.0001 MOI. Inactivated HSV-1 has been shown to transform cells *in vitro* (Michútová et al. 2017). As HSV-1 can be inactivated by other common factors such as snuff via its antiapoptotic proteins ICP4 and -27 (Larsson et al. 1992), the effects observed here due to UV-inactivated HSV-1 could theoretically happen *in vivo* as well. In the future, additional studies concerning bystander reactions to epithelial HSV-1 infection could reveal whether the epithelial cells surrounding the HSV-infected cells are affected. These results could explain the previous results of HSV-related cell transformation *in vitro* where, although HSV-1 is known to increase genomic instability and mutation rate (Shillitoe et al. 1993), transformation by HSV-1 *in vivo* seems to be rare. Lastly, as HSV-1 has been only weakly linked to lip carcinomas in smokers (Blomqvist et al. 1991) it seems plausible that complete carcinogenic change is unlikely without cofactors.

It has been noted that radiographic exposure, even panoramic radiography (Waingade and Medikeri 2012), is sufficient to bring about genomic alterations in oral cells. Should these alterations remain long-term, these alterations may push the cells further toward malignant change, especially if the patient suffers from premalignant conditions in their oral mucosa that likely contain aneuploid cells (Islam et al. 2010), as used in the present study. As asymptomatic HSV-1 infection is a distinct possibility in these patients (Jain 2016), the findings presented here further emphasize the rational use of X-rays in clinical practice. It can be concluded that for immortal oral epithelial cells, HSV-1 will most likely not lead to carcinogenesis alone, but when combined with exposure to irradiation, may function to increase cell

survival, evasion of apoptosis and therefore potential carcinogenic change, the clinical relevance of which needs further study.

5.3 Effect of HSV-1 and irradiation on skin keratinocytes

The effects of HSV-1 and irradiation were studied in the HaCat monolayers as control cells to the head and neck mucosal keratinocytes. HSV-1 caused infections similarly in HaCat cells as in HMK cells (II). HSV-1 did, however, significantly lower the viability ratings of HaCat cells, whether irradiated or not, and radiation exposure also caused viability losses (**Figure 16)**. This is in contrast to HMK cells, in which even higher viability was observed, particularly after combined exposures (**Table 8**).

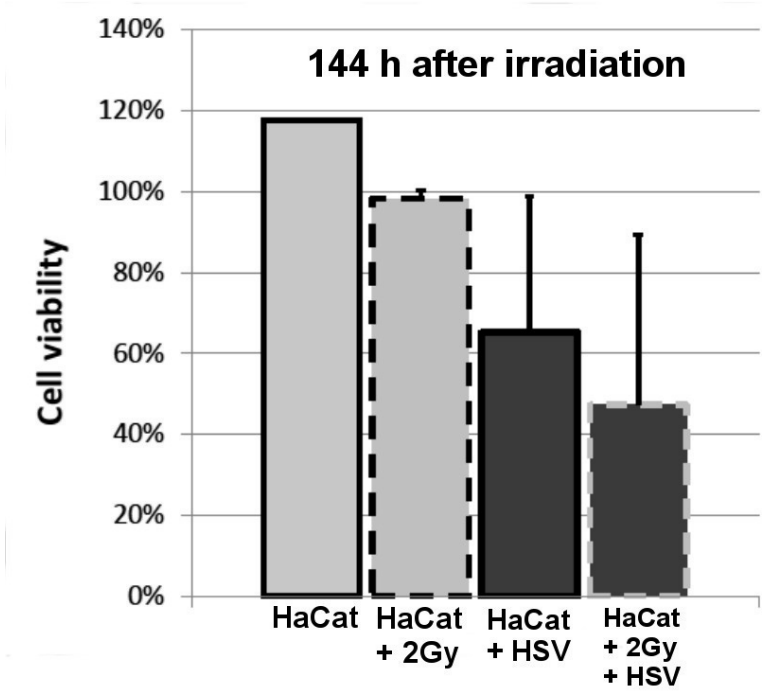


Figure 16. Viability of HaCat cells, infected (+HSV) or mock-infected with 0.0001 MOI HSV-1, 144 hours after irradiation with 2Gy (+ 2Gy). Error bars are standard error margins from triplicate analyses of a minimum of triplicate cultures.

HaCat cells seem to display more "normal" responses to increasing cytotoxic insults, such as HSV-1 and radiation, when compared to the other cell lines from the head and neck studied. Cell viability is lowered by HSV-1 or irradiation and further by a combination of these. Radiation with 2Gy has been proven to lower HaCat cell viability (Assad et al. 2018) as well as infection with a low-MOI HSV-1 (Megyeri et al. 2009), supporting our findings. Therefore it can be concluded that skin keratinocytes, the most common

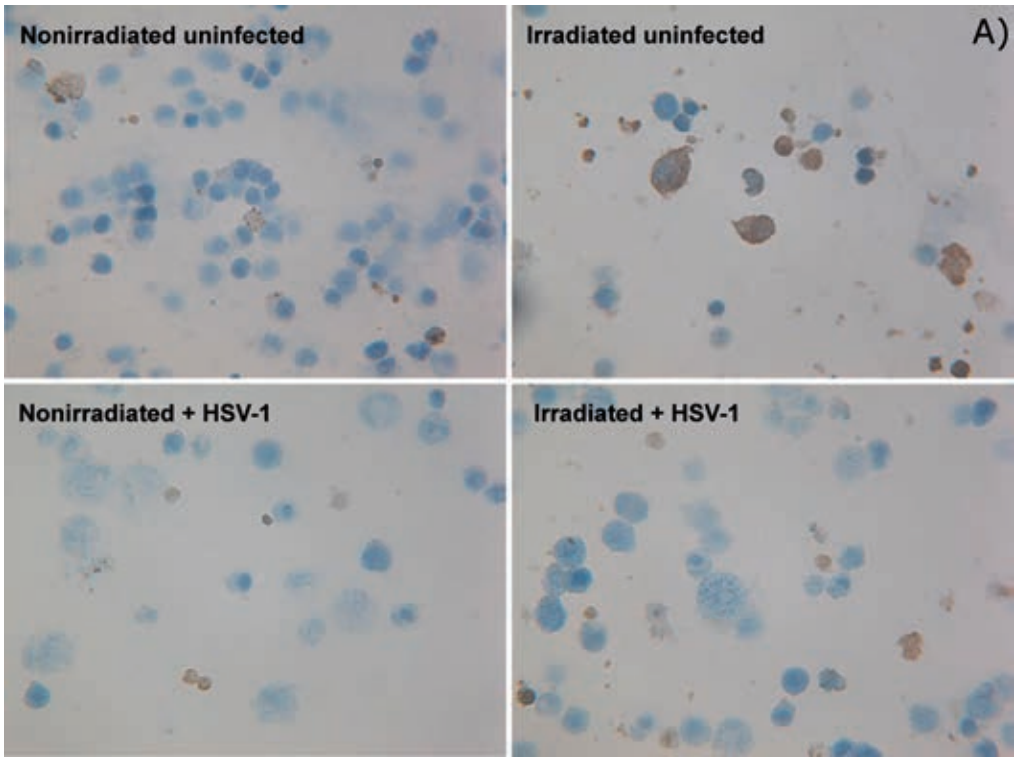
targets for HSV-1, harbor properties that make them permissible for HSV-1-induced cytopathic effects, thus enhancing viral spread.

5.4 Effect of HSV-1 and irradiation on HPV16 positive HNSCC cells

UD-SCC-2 cells are hypopharyngeal carcinoma cells harboring 600 integrated HPV16 genome copies per cell. The cells are sensitive to radiation and 2Gy lowers the survival of UD-SCC-2 cells to around 10% (Sørensen et al. 2013). Therefore the cell line was chosen for the experiments. In the present work, 2Gy of irradiation lowered the viability to 75%. These differences likely relate to the irradiation procedure, with our cultures being irradiated with a faster protocol (3Gy/min compared to 0.58Gy/min) and analyzed with a viability (ATP) assay rather than a clonogenic assay, as cells might retain more of their viability but still lose efficient growth of new colonies after irradiation. Disregarding these differences in methods, it is certain that UD-SCC-2 cells, like most HPV-positive carcinoma cell lines, display sensitivity toward radiation, and this corroborates with clinical experience gained from treatment of these HPV-positive tumors (Lindquist et al. 2007). HPV-positive cells are known for their E6-mediated loss of p53 (Scheffner 1993). Kimple et al. (2013) showed that HPV-positive carcinomas contain low amounts of residual p53 which are sufficient to cause apoptosis via caspase 3 after irradiation with 2 Gy, similar to the present study, which could explain the mechanism of radiosensitivity in these cells. Interestingly, in this study, the UD-SCC-2 E6 and E7 mRNA levels were both downregulated 144 hours after irradiation, coinciding with increased apoptosis. This could mean that radiation-mediated lowering of E6 levels allows the residual p53 levels, as postulated by Kimple, to elevate sufficiently to trigger apoptosis via caspase 3. More studies need to be performed to validate this finding.

Meyers and coworkers (2003) reported that HSV-1-infected HPV-positive tissue (raft) cultures sustained a lytic infection that did not destroy the culture, instead leading to cyclical replication of HSV-1 in the epithelium. HPV infection prevented the complete destruction of the epithelium, likely via the expression of its oncogenes. The UD-SCC-2 cells described here, although cultured as a monolayer, enabled relatively higher HPV-E6 and E7 oncogene expression in HSV-1 infected cells compared to uninfected irradiated cells (III, **Table 8**). Moreover, after combined exposure to irradiation and HSV-1, the UD-SCC-2 cells presented with a lower expression of apoptotic proteins than after irradiation or infection separately. Caspase 3 showed the most striking results, as the cultures irradiated with 2Gy and infected with HSV-1 displayed less cells staining positive for activated caspase 3 than those with either treatment alone. In addition, the mRNA expression coincided with the results of the immunocytologic analysis (**Figure 17)**. Simultaneously, the number of cells staining positive for Cyclin E was highest in the irradiated and infected cultures. MDM2 staining was lowest in HSV-1 infected cells although the combined exposure lead to higher levels than in control cultures (IV). These results support the Meyers'

(2003) conclusions, suggesting HPV oncogenes might aid HSV-positive cells to resist apoptosis and perhaps could increase the possibility of abortive HSV infections leading to malignant change.



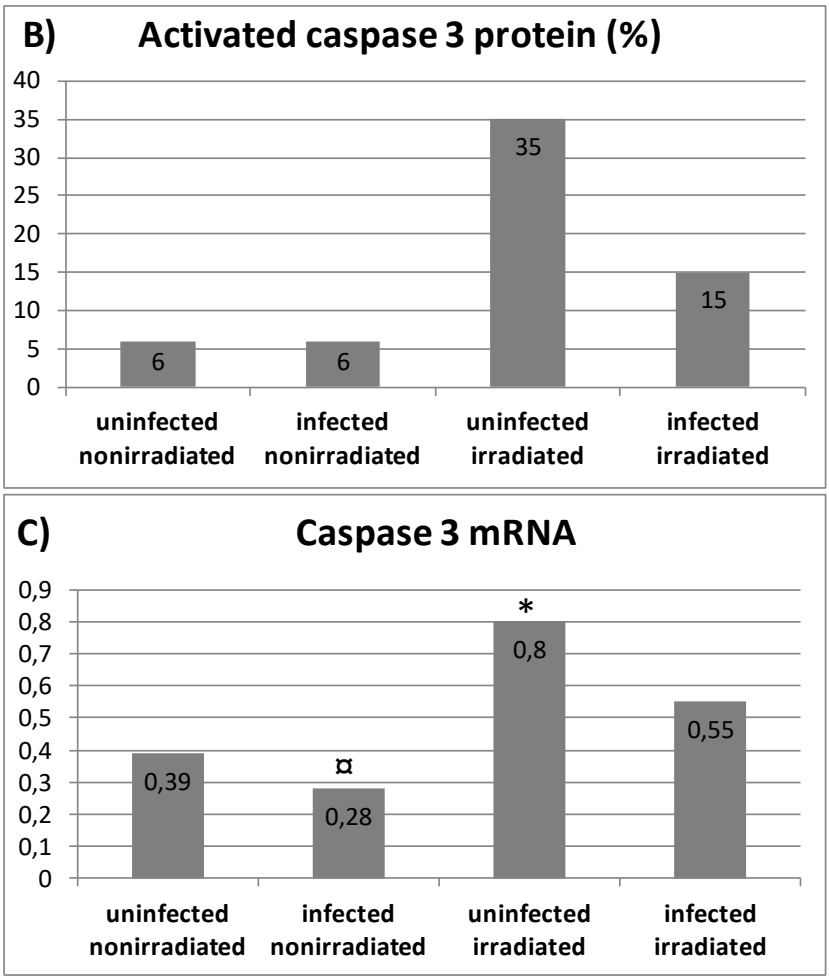


Figure 17.

A) The effect of irradiation with 2Gy and 0.0001 MOI HSV-1 on activated caspase 3 protein expression in monolayer cultures UD-SCC-2 cells infected with HSV-1. Results from 144 h postirradiation (III). Irradiation leads to increases in activated caspase 3, prevented in HSV-1-infected cultures. The photomicrograph is modified from Original publication III with permission. **B) Quantification of activated caspase 3, described as a percentage of positive cells in different groups, seen in A). C) Quantification of caspase 3 mRNA expression by qRT-PCR in HSV-1 infected cells after irradiation.** Upregulation in caspase 3 mRNA expression is seen in uninfected irradiated cells ($p<0.05$; *=compared to control, ¤= compared to infected and irradiated cells).

5.5 Effect of irradiation on HSV-1 replication

Irradiation has been postulated to inactivate HSV-1, and ionizing radiation itself is able to deactivate viruses, herpesviruses included (Henderson et al. 1978). It has also been shown that inactivated HSV-1 has oncogenic potential (Duff & Rapp 1973, Henderson et al. 1978). This could imply that the increases in viability and downregulation of apoptotic gene expression observed in irradiated and HSV-1-infected HMK and UD-SCC-2 cells result from inactivation of HSV-1 due to irradiation. However, this study revealed that at 2 Gy intensity, high-energy X-rays are not sufficient to significantly reduce HSV-1 spread and infectivity in a monolayer cell culture of HMK or UD-SCC-2 cells (Original publications II and III). Although inactivated viruses were also capable of increasing viability in irradiated HMK cells, it seems that virus inactivation due to irradiation is not the sole mechanism able to increase the viability of HSV-1-infected cells (II).

5.6 EBV DNA detection in head and neck carcinomas (IV)

EBV has been detected in several HNSCC types from different anatomical locations. Here, we analyzed 73 HNSCCs with PCR where Epstein-Barr virus DNA was detected in 80% of lipSCCs, 66% of OSCCs, 88% of NPCs, 63% of OPSCCs, 78% of HPSCCs and 63% of LarSCCs. EBV DNA was therefore present in a majority of all HNSCC samples. The presence of EBV DNA in HNSCCs has been studied previously, with varying results: EBV was present in 59.5%, 95%, 79.5%, 64% and 50% of OSCCs, NPCs, OPSCCs, HPSCCs and LarSCCs respectively in a Japanese cohort (Deng et al. 2014), similar to our findings. Therefore, EBV is a common occurrence in PCR analyses of HNSCCs, although with varying prevalence rates worldwide. These alterations are likely due to differences in patient demographics, as detection methods (PCR or nested PCR) are fairly consistent in the literature, but also susceptible to contamination, which could increase the number of positive samples reported worldwide.

The prognosis was not altered by the presence of EBV DNA in the HNSCC samples analyzed (IV). This result was partially supported by the recent results of Foltyn et al. (2017) who described EBV DNA presence in 52.7% of oral and laryngeal cancer samples with nested PCR. The histological grade of oral carcinomas, size and overall number of deaths was also smaller in EBV DNA-positive than -negative cases (IV). This might seemingly contrast with the main observation of worse outcomes after EBER-1 RNA detection in tumor cells (discussed below). However, as stated previously, in PCR-positive ISH (in tumor cells) negative cases, the EBV signals are likely detected from lymphocytes. With the presence of tumor-infiltrating lymphocytes, the overall survival of patients is improved significantly in HNSCCs (de Ruiter et al. 2017). However, additional B-lymphocytes increase the probability of EBV DNA-positive cells in the tissue and hence, higher probability of EBV detection. This might lead to the observed better prognostic parameters in EBV DNA-positive HNSCCs.

5.7 EBER-1 RNA and LMP-1 detection

Using ISH for EBER-1 in HNSCC patient samples (n=73), overall 21% were positive for EBER-1 with ISH (IV). These tumors presented with a significantly lower DSS than their EBER-negative counterparts (Kaplan-Meier analysis, p=0.012). Unsurprisingly, NPCs presented with intense staining in all but one case, in which staining was not uniform, although still present. EBER-1 expression was found in a subpopulation of cancer cells in eight cases, of which one was NPC, two tonsillar, one hypopharyngeal, two laryngeal and two tongue carcinomas (Original publication IV). This is in contrast to the majority of carcinomas being positive with PCR, as discussed in the previous chapter. 74% of ISH-positive carcinoma samples were positive with PCR. Recently it was suspected that false positive findings were common in the EBER-ISH of oral tongue carcinomas, as PCR revealed no EBV genomes and immunohistochemistry for the EBNA-1 protein was negative (Wilms et al. 2017). In contrast, our patients presented with several simultaneously EBER-1 (ISH), -DNA (PCR) and LMP-1 (IHC) positive cases, two oral (**Figure 18**), one laryngeal and one tonsillar carcinoma. Due to the positive findings in several different analyses, these are certainly true positives. This study therefore showed that EBV can be found in non-nasopharyngeal HNSCCs, and lead to worse DSS. This is likely explained by the inclusion of additional head and neck areas in this study. Interestingly, Jiang and coworkers (2015) detected a similar discrepancy between samples presenting PCR negativity toward EBV, although expressing EBER-1 RNA. These samples were laser-capture microdissected from locations of EBER-1 ISH positivity and further qRT-PCR revealed these cells to be EBER-1 RNA-positive as well. Therefore, nonuniform ISH staining is most likely not a marker for false positivity and could result in underreporting of EBV detection. In conclusion, the presence of EBV-ISH signals in carcinomas must be confirmed by PCR and protein expression where possible. For comparison, for a head and neck carcinoma to belong to the HPV-attributable fraction, the detection of HPV DNA alone is concurrently considered insufficient and additional validation is required such as E6/E7 mRNA or p16-IHC. Similarly, further research into EBV-specific markers and their use in clinical cohorts will also clarify what attributable role, if any, EBV plays in non-nasopharyngeal HNSCCs worldwide.

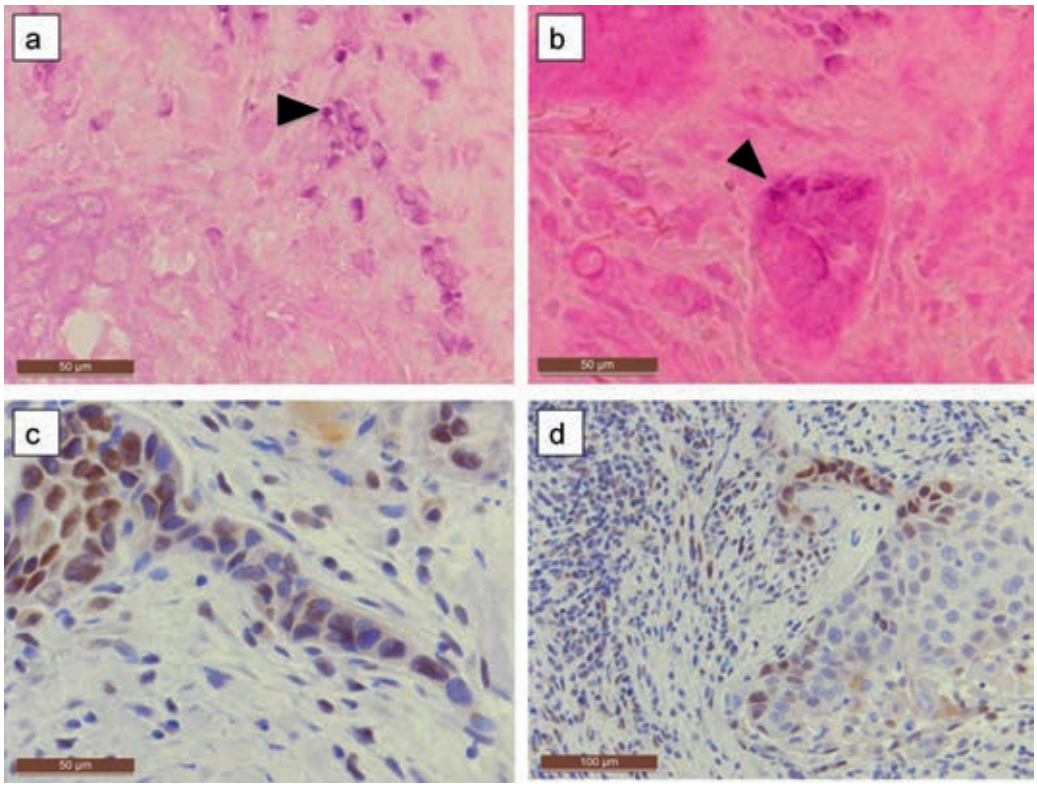


Figure 18: Carcinoma of the oral tongue expressing EBER-1 and LMP-1. EBER-1 expression (purple, a and b, arrowheads) is restricted to tumor islands infiltrating connective tissue. Eosin is used as background staining (a, b). LMP-1 (brown staining, c and d) is simultaneously expressed in a number of carcinoma cells, counterstained with hematoxylin (blue). This carcinoma was also positive for HPV 6. Original magnification a-c 200x, d100x. Original image published by Turunen et al. Oncotarget 2017, distributed under the terms of the Creative Commons Attribution 3.0 License (CC-BY) (IV).

Recently, She and coworkers (2017) performed a meta-analysis of EBV detected in oral squamous cell carcinomas (OSCCs) compared to normal oral tissues. They concluded after an extensive review that the OR between EBV and OSCC risk was 5.03 and therefore EBV could increase the risk for oral carcinomas. However, despite trying to control confounders by only including tissue samples, most of these results were also based on PCR analysis of whole-tissue samples that are no doubt inflamed significantly more than the normal tissues used as controls due to the likely ulceration and subsequent inflammation of oral tumors. Out of 13 included articles, two used LMP-1 immunohistochemistry (Shamaa et al. 2008, Kis et al. 2009) but Kis and coworkers found no positive samples with LMP-1,

whereas Shamaa and coworkers detected LMP-1 in heterogenous OSCCs and oral premalignancies. Two original publications used EBER-1 *in situ* hybridization (Kikuchi et al. 2016, Shimakage et al. 2002), Kikuchi et al. found a high prevalence of LMP-1 and EBER-1 expression in severe dysplasias (72% and 94.4% respectively) but less in OSCC (38.7% and 34.7%, respectively). In our study, LMP-1 staining was detected in carcinoma cells of 85% of all carcinomas (n=62, original publication IV) and EBER-1 in 21%, as stated above. LMP-1 was also positive in all EBER-1 ISH-positive carcinomas and in lymphocytes in 42% of samples. Statistical analysis revealed no statistically significant effect of LMP-1 expression on prognosis, grade or stage on any HNSCC type analyzed. Interestingly, it was more common to detect LMP-1 in the invasion front of the carcinoma, whether in lymphocytes (29% of carcinomas positive) or carcinoma cells themselves (48% of carcinomas analyzed). **Figure 18d** demonstrates one example of the localization of LMP-1 staining in which cells invading from the larger tumor bulk express LMP-1 whereas those in the tissue otherwise do not. LMP-1 can contribute to cell survival by the activation of NFkB1 and induction of cancer stem cell-like properties and is linked to EMT of carcinoma cells (Kang & Kieff 2015). All of these functions could result in LMP-1-expressing cells acquiring a more invasive phenotype, explaining their presence in the invasive front of these carcinomas, histologically important in the prognosis of HNSCC (Almangush et al. 2018). However, the LMP-1 expression pattern was not statistically associated with the DSS of the patients in this work (IV). This may result from the low number of patient cases, and the intriguing observation of LMP-1 localization clearly requires further study.

EBER-1 has been linked to increasing levels of epithelial abnormality: Jiang and coworkers detected an increasing likelihood of EBER-1 expression with worsening dysplasia grades until OSCC, compared with normal oral epithelium (Jiang et al. 2012). Their later analyses further confirmed this, as EBER-1 was detected in 27% of base-of-tongue and 37% of tonsillar carcinomas compared to 7% and 15% in their normal tissue counterparts (Jiang et al. 2015). It is interesting to note that this study also detected expression of both EBER-1 and LMP-1 only in a subpopulation of carcinoma cells, similarly as observed. Altogether these data suggest that firstly, LMP-1 immunohistochemical detection only may be unreliable as a marker for EBV-attributable carcinomas, as positivity ratings vary significantly. Second, EBER-1 is likely a more reliable marker for EBV-related disease, but this too seems in need of further study. At any rate, the anatomical detection of EBV oncogene expression seems mandatory for the proper analysis of relationship between EBV and non-nasopharyngeal HNSCCs.

5.8 LMP-1 in nonmalignant tissues

Our study also showed LMP-1 expression in the dysplastic epithelia next to head and neck carcinomas **(Figure 19).** This interesting finding needs further analysis, as EBV carries a potential for further malignant changes of the epithelium, and one reason for local HNSCC recurrences is premalignant

epithelium surrounding the carcinoma. EBV has been detected in oral epithelial premalignant lesions (Shamaa et al. 2008) and its presence was more frequent in dysplastic tissues than in normal epithelium (Jiang et al. 2012). Hypothetically, EBV infection of a dysplastic epithelium could also function as a late step in carcinogenesis, initiated by carcinogens such as tobacco smoke. This could set the epithelial cells further down the carcinogenic pathway, and therefore the analysis of EBV in premalignant tissues would be of interest in the future.

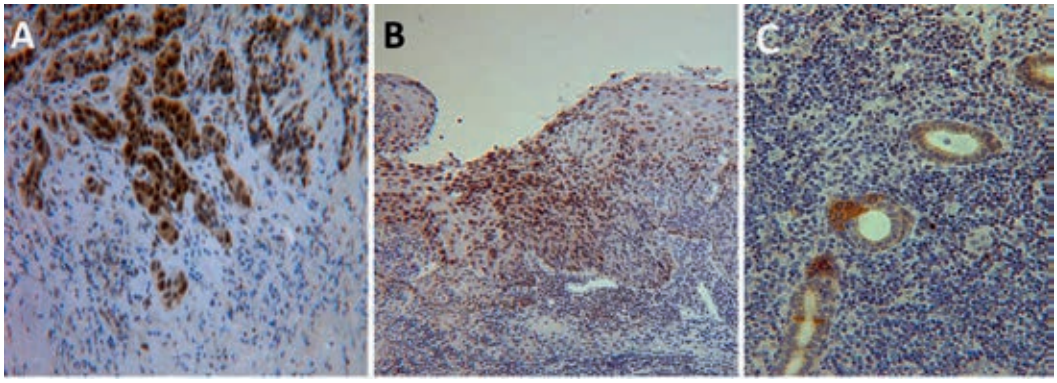


Figure 19. EBV LMP-1 staining. A) Positive EBV LMP-1 staining is seen here in invasive buds of a tongue carcinoma also harboring HPV6. B) In this area of severely dysplastic epithelium, a zone of LMP-1 staining is seen. Occasional positively staining lymphocytes can also be easily detected. C) LMP-1 staining seen in otherwise normal salivary duct epithelium in the vicinity of an invasive carcinoma. Positivity to LMP-1 stains brown, cell nuclei blue (Hematoxylin staining).

5.9 LMP-1 did not affect HNSCC prognosis

Contrary to recent meta-analysis by Chen and coworkers (2015), and despite the fact that LMP1 is a powerful oncogene which provides significant radioresistance to NPC cells (Yang et al. 2014), we did not establish LMP-1 as a prognostic parameter of HNSCC or with an SCC histological grade (Original publication IV). NPCs were also unaffected by LMP-1 expression patterns. This explanation may either be technical, since LMP-1 staining was not completely in agreement with EBER-1 expression and the sample size did not allow for sufficiently powerful statistical analysis, or LMP-1 expression does not always reveal the true carcinogenic potential of EBV infections, as EBV has oncogenic potential unrelated to LMP-1 expression. Yoshizaki and coauthors postulated that LMP-1 expression in cancer stem cells may remain undetected in histological examinations and could play a role in cancer progression. As 50% of advanced NPCs may not express LMP-1, it remains to be determined whether LMP-1 expression in carcinoma cell subpopulations affects HNSCC in a significant way (Yoshizaki et al. 2018).

5.10 HPV detection in head and neck carcinomas

HPV types were detected in 73 HNSCCs using Luminex-PCR (IV). Overall, high-risk and low-risk HPV were prevalent (**Table 8**, IV). As HPV DNA detection has recently been rigorously evaluated in a worldwide patient cohort (Castellsague et al. 2016), and a high-quality meta-analysis has previously been performed from worldwide data (Ndiaye et al. 2014), a closer examination of this data was therefore of interest (**Table 9**).

Table 9. HPV prevalence Original publication IV compared to the ratings reported by Castellsague et al. (2016) and from the meta-analysis by Ndiaye et al. (2014) that reports all HPV types combined. Results from Northern Europe are shown from Ndiaye et al. (2014) and when unavailable, pooled results from Europe are shown as (E). n/a = data unavailable.

	Original publication IV			**Castellsague 2016**			**Ndiaye 2014**	
	HR-HPV	LR-HPV	n	HR-HPV	LR-HPV	n	HPV (any type)	n
LipSCC	80%	80%	n=5	4.8%	2.3%	n=42	0-4% (E)	n=50
OSCC	52%	43%	n=24	7.4%	0%-1.4%	n=1222	14%	n=494
OPSCC	47%	11%	n=19	24.9%	0%-2.1%	n=1090	57%	n=890
HPSCC	55%	22%	n=9	3.9%	0%	n=127	22% (E)	n=173
LarSCC	50%	0%	n=8	5.7%	0%-1.9%	n=1042	12%	n=362
NPC	38%	13%	n=8	7.9%	0%	n=101	n/a	n/a

High rates of HPV were found in our study. The results are most in agreement in oropharyngeal carcinomas and least in carcinomas of the lip and larynx, where the number of cases was also the lowest. PCR and Multimetrix-HPV genotyping data was used here, whereas SPF-10 PCR and a DNA enzyme immunoassay with further E6 mRNA detection were used by the Castellsague group and various PCR techniques in the meta-analysis by Ndiaye. These methods, utilized on samples from around the world, may lead to differences in results. In addition, Castellsague et al. gathered FFPE blocks from different locations, whereas the results observed in (IV) were from frozen samples. This could allow for better detection rates; however, the differences observed are not necessarily purely technical in nature. Although contamination of PCR products is always a possibility, the frequent inclusion of negative

controls in each 9 wells of plates analyzed makes this unlikely. Another explanation, as recently reported by Anantharaman and colleagues (2017) is that HPV detection rates show wide geographical heterogeneity, ranging from 60% in oropharyngeal SCCs from the US to 4% in their Brazilian counterparts, for example. HPV incidence has risen in Finland, and in the analysis by Castellsague and coworkers, Northern Europe showed the highest HPV DNA-prevalence (11.6%) in oral cavity carcinomas. The meta-analysis by Ndiaye and coworkers showed pooled HPV DNA positivity from Northern Europe (Ndiaye et al. 2014) and their results are more in line with the ones observed here (IV). Up to 31.3% of oral HPV positivity was reported in healthy Finnish subjects previously from the same hospital district, using the same method as used here (Kero et al. 2016). Therefore our hospital district area may demonstrate comparatively high oral HPV positivity compared to worldwide data, but its effect on HNSCC requires further study.

The description of "oral HPV" is a constant problem in the literature. The oral cavity contains many subsites, such as the gingiva, palate or floor of the mouth, all of them different tissues, but most studies pool all subsites together under OSCC. Worse yet, many include base-of-tongue (OPSCC) or even oropharyngeal sites such as the tonsils in "oral cancer," making pooled analyses such as meta-analyses difficult to interpret. Lastly, laryngeal carcinomas from Europe presented with around 30% HPV DNA prevalence in a recent meta-analysis of 179 studies (Gama et al. 2016), also more in line with our study. Therefore, our results may be explained by the differences in Finnish HPV prevalence ratings than those averaged from worldwide analyses.

In the current study, LR-HPVs 6 and 11 were detected in the same dataset (IV), being present in 24% of all HNSCCs taken together (**Table 9**). Low-risk HPV infections have been generally considered insignificant to HNSCC development and progression and have been detected in up to 17.9% of oral carcinomas (reviewed by Miller and White as early as in 1996). Despite these views, low-risk HPVs have been associated with malignant transformation of cells *in vitro* (Schmitt et al. 1994). LR-HPV types 6 and 11, especially, have been linked with the transformation of laryngeal papillomas (Lindeberg et al. 1989). More recently, LR-HPVs were associated with poor DSS and OS in oral cavity carcinoma patients in a large Taiwanese cohort, which also showed increases in LR-HPV prevalence (Lee et al. 2013) In the future, it also seems of interest to analyze the effect of LR-HPVs on HNSCC treatment responses.

5.11 HSV-1 in head and neck carcinomas

HSV-1 was detected in only a few oral and oropharyngeal carcinomas (4-11%) (IV). Therefore, the impact of HSV-1 on HNSCC treatment likely affects only a minority of patients, although potentially leading to radiation resistance. One possibility is that as conventional PCR has been shown to potentially miss 2/3 of HSV-1 infections in specific settings, our results may underestimate HSV-1 prevalence and future research might further benefit from qRT-PCT analyses (Aggarwal et al. 2014). However, the overall high percentage of oral and oropharyngeal SCC cases compared to all HNSCCs signifies that despite being in the minority, HSV infections may yet affect a number of patients with HNSCC.

As stated previously, HNSCCs are treated with radiotherapy, given as 2 Gy fractions to patients often on a daily basis that continues for several weeks. No effect on HSV-1 replication was noted in our cell culture studies after 2 Gy of X-ray irradiation, as stated previously. Also, 2 Gy X-ray irradiation may enhance HSV-1 replication if it coincides with the temporal switch from beta to gamma gene expression (Advani et al. 2011). Therefore, HSV replication is not likely fully prevented by 2 Gy of irradiation. As HSV might confer protection from apoptosis via, for example, ICP4 and Us3 gene product, as demonstrated by Galvan and Leopardi (Galvan et al. 1999, Leopardi et al. 1997) whether this might alter the clinical properties of HSV-1-infection in settings of irradiation requires further study. In addition, a recent observation linked the mannose-6-phosphate pathway, downregulated by HSV-1 gD (Zhou & Roizman 2002), with inhibition of the insulin-like growth factor 2 signaling, leading to poor radioresponse in HNSCC patients (Jamieson et al. 2003), further suggesting the possibility that HSV-1 alters these responses *in vivo* as well.

The oncogenic potential of HSV-1 has been reviewed by Metgud and colleagues (2012). To summarize, the carcinogenic properties of HSV-1 are not comprehensively known, but include abortive infections, hit and run-type mutagenesis of the host cell genome and cocarcinogenesis with other viruses such as HPV or EBV. These could explain the small minority of HSV-1--positive patients who we observed to suffer from treatment resistance (IV). This hypothesis however suffers from the fact that these few patients in our study also had coinfections with either EBV, HPV or both (see below). Nevertheless, high-dose X-ray irradiation is able to inactivate HSV-1 as well as EBV, and therefore shut down their proapoptotic features (Henderson et al. 1978). Theoretically this could then allow the antiapoptotic cellular effects of HSV-1 and innate immunity reactions triggered by the virus to persist. As the number of HSV-1 -positive HNSCC patients is low and coinfections with other viruses common, further research into the potential prognostic effects of HSV-1 as single or coinfections in HNSCC is certainly challenging, although of the utmost importance.

5.12 Coinfections in HNSCC

Several synergetic effects between herpesviruses and HPVs have been discovered in cell cultures. The EBV oncogenes LMP-1, LMP-2A, EBNA1 and BARF1 (already discussed in the literature review) result in inactivation of the key tumor suppressor genes p53 and pRb, which interestingly are the targets for HPV E6 and E7 oncoproteins as well. As early as in 1998, HPV was linked to EBV-positive NPCs in 52.9% of patients of Western origin (Rassekh et al. 1998) where EBV was present in 88.2% of NPCs, much like in our study, where 87.5% were EBV-ISH-positive and 38% coinfected. Recently, EBV/HPV coinfections were not detected in a Finnish NPC cohort, and EBV or HPV individually led to better disease outcomes (Ruuskanen et al. 2019). When their results are considered, the observed lower survival ratings presented herein most likely result from non-nasopharyngeal HNSCC patients in which the worst DSS was found due to EBV/HPV coinfections. In a recent review of EBV/HPV coinfections, Shi and coworkers stated that the probability of a patient to be infected with both viruses in many different carcinomas of the body is increasing (Shi et al. 2016). In addition, EBV and HPV have been detected with PCR in 46% of NPCs (Tyan et al. 1993), similarly as in our study (38%). The more recent analysis done by Deng and coworkers (2014) revealed that approximately 22% of Japanese HNSCC patients had a coinfection with HPV/EBV of a total of 209 patients detected by PCR. However, only 10% of NPCs and no other HNSCC types were positive for both viruses using ISH like in our study, where 3/8 NPCs (38%) were positive for HPV types 16 or 16 and 11 (IV). Our results differ from those of Deng in the finding that HPV (DNA-positive) coinfections were present in overall 47% of all EBV-ISH (in carcinoma cells) positive patients (n=7/15, **Table 10**). Of these, HPV16 was by far the most prevalent, but types 6, 11, 31 and 56 were also found. The result does not contradict previous findings, as patient ethnicity and geographical location are known to affect NPC pathogenesis. A majority of HPV-positive NPCs are detected in Caucasian patients, whereas HPV is less common in Far Eastern patients (Punwaney et al. 1999, Robinson et al. 2013).

Table 10. EBER-1 positive carcinomas and their association with HPV (Original publication IV). EBV-*in situ* hybridisation (ISH detected in carcinoma cells) results and the HPV types present, as detected with Luminex PCR-assay, are shown. Coinfections with multiple HPV types were also found in select carcinomas.

	EBV-ISH positive	HPV types present
NPC (n=8)	7	HPV16 (n=2) HPV11&HPV16 (n=1)
OPSCC (n=19)	2	0
OSCC (n=24)	2	HPV6 (n=1)
LipSCC (n=5)	0	0
HPSCC (n=9)	1	HPV16 (n=1)
LarSCC (n=8)	3	HPV16 (n=1) HPV31&HPV56 (n=1)

Jiang and coworkers (2015) detected HPV/EBV coinfections in 25% of tonsillar carcinomas. They also detected HPV/EBV coinfections in 20% of base-of-tongue carcinomas using the same method, and concluded that the increased tumorigenic potential of these coinfections warranted further study. None of our patients with oropharyngeal carcinomas had HPV/EBV (EBV in carcinoma cells) coinfections. This could be due to geographical variation or differences in patient characteristics as stated above (Original publication IV). Our patient group had four cases in which HPV/EBV coinfections were detected in anatomically neighboring areas. This signifies that although uncommon, HPV/EBV coinfections are distributed widely in the head and neck area, with the possible exception of lip carcinomas, as no EBER-1 expression was detected there. Although EBV-DNA was present in up to 80% of all HNSCCs studied, the number of cases was small and in need of future larger analyses. Overall, the conclusion of Jiang and coworkers was further supported, and it appears that EBV research needs to widen its focus away from NPCs to include other HNSCCs. In addition, contrary to what is occasionally reported in the literature, HPV and EBV infections are most likely not mutually exclusive, as coinfections between these viruses were found (reviewed by Shi et al. 2016). Lastly, our study (IV) detected no EBV, HPV or HSV-1 in only 11% of patient samples. This interesting fact implies that HNSCC studies must consider viral infections in their interpretation, as a majority of HNSCC tissues seem to contain at least one if not several viruses.

We described carcinomas that were EBER-1 or LMP-1-positive in only certain areas of the tumor tissues. Interestingly, this finding was also reported previously (Jiang et al. 2015). They also reported increased invasiveness of HPV/EBV coinfected cell cultures and attributed this change to LMP-2 and E6/E7-oncogene coexpression. Importantly, the recent report by Cantalupo and coworkers (2017) analyzed 22 different human cancers from 3052 patients using next-generation sequencing for genome, exome and RNA libraries. They showed that EBV genomes were present in only a subset of tumor cells in many suspected EBV-driven carcinomas. Furthermore, HPVs were present in around 20% of HNSCCs. However, the presence and active association in the form of LMP-1 alongside EBNA- and EBER expression in a fraction of HNSCCs was interesting. Moreover, HSV-1 was detected in a minority of HNSCCs. This corroborates our findings (IV), where EBER-1 and LMP-1 were clearly detected by staining only in a subset of invading carcinoma cells, and paves the way for future research.

In HNSCC patients studied in (IV), if EBV DNA was found with HPV DNA using PCR, the survival time was highest, followed by virus-negative cancers (up to 90 months). However, a troubling finding was that DSS was significantly lower if combined HPV DNA-positivity and EBER-ISH expression was found in carcinoma cells ($p=0.003$). HPV/HSV-1 coinfection (PCR detection) had a similar effect ($p=0.016$). EBER-ISH-positivity in carcinoma cells led to a lower survival time ($p=0.012$) and detection together with LR-HPV or HR-HPV DNA, led to the shortest survival times (9 and 19 months, respectively). Tumor inflammation contains B-lymphocytes leading to EBV DNA presence and has been linked with favorable prognosis in HNSCC (De Ruiter et al. 2017). This could explain why different EBV detection methods led to different survival, observed here. Despite this hypothesis, it is not known which property is the cause: either certain HNSCCs harbor alterations that not only make them more susceptible to infections by viruses but also altering their clinical behavior, leading to worse treatment responses, or these herpesviruses in question are the cause for the development and possibly different clinical behavior of these tumors. It remains a priority to analyze larger numbers of HNSCCs to confirm these findings and determine what property of carcinomas coinfected with HPV/EBV or HPV/HSV-1 could cause their clinical behavior to differ from other similar HNSCCs.

EBV leaves lasting procarcinogenic effects on the epigenome of cells, even several passages after the virus has been lost from the culture (Queen et al. 2013). This interesting finding signifies that even if the infection itself is transient, EBV might still contribute to the oncogenic process via imprinting alterations on the epigenetic profile of the cell, suggesting a "hit and run" form of carcinogenesis (Queen et al. 2013). As all herpesviruses share the same epigenetic traits, these effects may be present in HSV-1-infected cells as well, reviewed by Galloway and Dougall as early as in 1983 (Galloway & Dougall 1983). Herpesvirus-induced transformation could be indistinguishable from other causes of

transformation, leaving no trace of the previous exposure, as reported *in vitro* for HSV-1 (Hwang & Shillitoe 1990, Bauer et al. 1992). The controversial but plausible "hit and run" mechanism was later reviewed by Veronika (Veronika et al. 2018). Disappointingly however, no conclusive studies on these potential mechanisms have been conducted for over two decades, and the data are as yet inconclusive.

Lastly, one would consider the oral environment in carcinogenesis and cancer progression. Interestingly lactoferrin, an antimicrobial protein present in saliva, has proven efficacy against EBV and HSV-1, and its levels have been reportedly downregulated in NPCs caused by EBV (Zheng et al. 2012). Radiotherapy could therefore predispose to persistent infections via hyposalivation and potentially increase the risk for cellular damage by EBV and HSV-1. This could be even further complicated, as HSNCC patients have difficulties in maintaining oral hygiene, increasing the prevalence of dental infections that may further increase HPV, HSV-1 and EBV replication as well (Chen et al. 2009). This could hypothetically lead to the observed nonmonoclonal detection of EBV should the carcinoma tissue be infected after disease initiation (Shah & Mehta 2016). In addition, HPV, EBV and HSV-1 are all modifiers of the immune system (Schmiedel & Mandelboim 2017) and, due to their potential in establishing life-long latency (Ermel et al. 2018), are implicated in oncogenesis via affecting the immune microenvironment of the head and neck, the further discussion of which is out of the scope of this thesis. Therefore the combined effects of HPV, HSV-1 and EBV may form fertile ground for cells to thrive and resist apoptosis, despite potentially oncogenic damage that would trigger apoptosis in the altered cells in uninfected situations (Alibek et al. 2014).

5.13 Limitations of the studies

Original publication I: Firstly, a high-calcium concentration might cause technical cell culture problems. The 5mM and 6mM calcium concentration in KSFM resulted in loss of cell cultures due to cell death. Moreover, from 3mM calcium upwards, the formation of calcium precipitations was observed. This may have affected the total amount of free calcium in the experimental medium, making the concentration of available free calcium in the medium difficult to estimate.

Original publications II and III: Utilization of ATP assays for studies in cell death involving HSV-1 requires the consideration of a key point: HSV-1 infection has been shown to increase the ATP content of the infected cell, which might be speculated to lead to falsely high viability ratings. Incidentally, the data from Peri et al. (2011) answers these concerns, as no significant increase in the viability of cells infected with different wt-HSV strains (KOS and F) at late infection time points was seen. Therefore, the amount of additional ATP generated by the HSV-1 infection is not sufficient to cause an upward bias in CellTiter-Glo results. One main issue with the cell culture experiments is that monolayer cultures lack the signaling present in 3D cocultures. In the future it would be useful to study these effects on 3D-

culture models to determine if increased differentiation of cells and signaling from the connective tissue fibroblasts affect these findings.

The "EBV-study" (Original publication IV) was limited due to HPV localization not also being studied using *in situ* hybridization, which would have allowed HPV/EBV coinfections to be colocalized. As the main aim was to detect EBV in carcinoma cells, we wanted first of all to see whether the null hypothesis held or was rejected. In addition, insufficient samples were available from the original work (Rautava et al. 2012) for analysis of the complete set. The comparison between these two works is therefore unsuitable statistically. Epstein-Barr virus has two subtypes which may possess different transformative properties. These two were not clearly differentiated by the EBER-1 probes. In addition, the EBV's viral load was not studied, although it may have an effect on tumor properties. Lastly, low case numbers and a lack of complete information on confounding factors such as tobacco and alcohol consumption have led to a need for novel studies on the subject.

6. CONCLUSIONS

These studies have revealed previously uncharacterized ways in which HPV and herpesviruses HSV-1 and EBV might influence cell death in infected cells *in vitro* and *in vivo*. All these viruses share similar target cells, the keratinocytes of the head and neck mucosa, which, when transformed, are responsible for HNSCC. As normal keratinocytes grow as differentiating stratified epithelia, differentiation-inducing signaling is of paramount importance to the behavior of these cells and the life cycle of the viruses infecting them. For HPV16, it was shown how HPV oncogene E6 expression closely follows differentiation-inducing calcium signaling. It is already known that this same signaling would be triggered by HSV-1 in order to infect the keratinocyte and express its proteins (Cheshenko et al. 2013), whereas EBV requires calcium signaling to transform cells (Chami et al. 2006). Understanding of these mechanisms is key in developing novel therapies against these viruses. As described previously, these differentiating head and neck keratinocytes are exposed to irradiation from several medical sources. During the treatment of HNSCC, surrounding areas of keratinocytes are exposed to irradiation. These studies revealed how skin cells behave differently to immortal aneuploid (i.e. premalignant) oral keratinocytes where, contrary to skin cells, an increase in viability was found, especially in the presence of HSV-1 infection. Further evidence was found on observing the downregulation of apoptotic gene expression in these irradiated and HSV-1 infected cells. HPV16-positive HNSCC cells also, despite already well-characterized sensitivity to irradiation, showed reduced apoptotic gene expression and increased HPV16 oncogene expression after irradiation in the presence of HSV-1. EBV was also detected infecting HNSCC cells in patients with non-nasopharyngeal carcinomas in which HPV and HSV-1 are commonly found, and its detection was a sign of poor prognosis. Furthermore, EBV, HSV-1 and HPV were then detected coinfecting a minority of HNSCC patients, leading again to a poor prognosis and shorter survival time in the context of radiotherapy, a previously undetected effect in Finnish HNSCC patients which necessitates further studies. Lastly, as these studies suggest, benign as well as malignant head and neck keratinocytes are exposed to several different viruses that may have profound cell type-dependent effects on their behavior, particularly after irradiation. It is therefore of the utmost importance to consider these potential effects when designing future studies on HNSCC development and treatment.

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